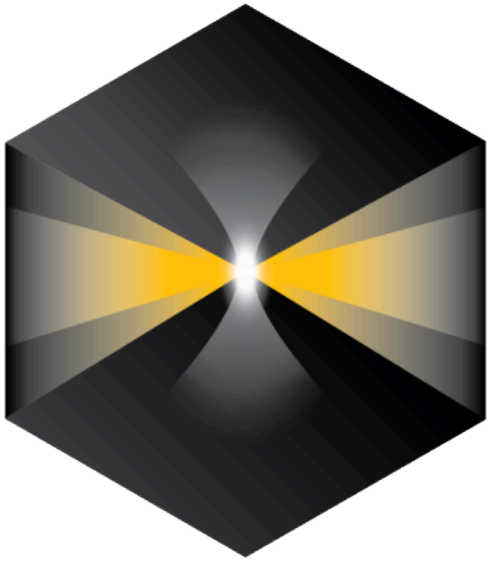

SlideBook

User's Guide

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New in SlideBook

SlideBook 2022 was released in the fall of 2022 and represented a significant upgrade over SlideBook 6. SlideBook 2025 and now 2026 represent further, incremental, improvements over the SlideBook 2022.0 and 2022.1 releases. The descriptions below pertain to new functionality of SlideBook over SlideBook 6 rather than additional features in SlideBook 2026.0 over 2025.

.sldy File Format
SlideBook 2022 introduced the .sldy file format, which is an open-source file format designed for very large data sets (>100GB) produced by modern microscope systems. The .sldy file is a distributed file format that points to a directory where each captured z-stack is stored in an independent file (by channel) alongside metadata files that describe the capture. The distributed nature makes many image manipulation operations, such as removing a channel from an image or removing a selection of time points, nearly instantaneous. Images, masks, and histograms are stored as python-compatible .numpy arrays, and the metadata is stored in the open-source YAML format, enabling users with coding experience to process images directly in Python. The default .sld file format is still available and appropriate for many users.

.sldyz Lossless Compression
SlideBook supports .sldyz compression of the new .sldy file format with the ZStandard compression algorithm. Zstandard is a new lossless algorithm developed at Facebook Labs that works to compress our very large files with a balance of speed and compression ratios that far outperforms many of the standard lossless algorithms. Each .numpy image file is compressed individually, so the structure remains the same, but the overall size of the directory is smaller. Compressed files can be opened natively in SlideBook with no need to
decompress them. More information can be found here: https://engineering.fb.com/2016/08/31/core-data/smaller-and-faster-data-compression-with-zstandard/

3D Capture Status
Volumetric projection during 4D capture is now supported across all instruments where dynamics can be best observed while an experiment is under way by looking at more than a single plane at a time, such as Lattice LightSheet, Marianas LightSheet, and VIVO Multiphoton.

Multipoint & Montage Tile Viewer
The Multipoint viewer allows for examination of multiple XYZ positions as they are being acquired and after they are acquired the user may review all the XYZ positions in a particular capture**.**
The Montage Tile viewer allows to preview raw montage captures to assess and give overview over each tile location before using the 2D/3D montage.

Multiwell Interface
The multiwell interface has been redesigned and incorporated into the focus window to allow for easier alignment and selection of wells and points for imaging. The selected points are seamlessly integrated into the normal SlideBook workflow, thus reducing the amount of time, mouse-clicks, and learning curve to perform multiwell imaging. There are several pre-set well plate layouts, and users can also define custom well plates of varying sizes up to 384 wells.

Large Data Montaging
SlideBook 2022 introduced a new interface and method for stitching 2D, 3D, and 4D montage images.

- It allows for better handling of large datasets in a constrained memory environment
- Users have the ability to test alignments on a smaller portion of the overall image
- Users can limit the alignment to a smaller Z-range in 3D and 4D datasets for
- It has a variety of user-definable outputs, such as whether or not to output to a new slide, or if the entire dataset will be stitched or subsampled.
- Users can also control how much processing power is dedicated to montaging by limiting the number of CPU threads as well as set the job to work in the background, freeing up SlideBook resources for other operations.

Focus Surface
Focus Surface allows for users to correct for non-planar deformities by selecting multiple points of focus and interpolating a surface across them to automatically adjust focus as the system moves between points, wells, or tiles in a montage. This is especially helpful in situations where tissue may be warped and undulating from fixing and dehydrating, or a multiwell plate is deformed due to imperfections in the plastic.

Redesigned GUI for VIVO Multiphoton
SlideBook 2022 introduced a completely redesigned interface for multiphoton systems. Designed specifically to assemble all the frequent controls and status displays into a single window to minimize user interface interaction during in vivo experiments. The console features an advanced, intuitive tool for adjusting laser power delivery for different depths that incorporates dynamic signal feedback.

All-New GUI for Cleared Tissue LightSheet
SlideBook 2022 introduced the Cleared Tissue LightSheet, with an all-new graphical user interface custom designed for the instrument. It is extremely intuitive and feature rich, with options such as 2D and 3D prescan, ROI selection, automated pattern generation, and axial
chromatic aberration correction.

User-Selectable Application Look
The color scheme and application appearance of SlideBook can be selected from among dozens of options.

Volume Rendering Improvements
The 3D and 4D Volume View now support a user-specified bounding box (cutaway view) and a story board interface where multiple perspectives can be assembled into a single movie with seamless transitions. These views also now support user-selectable colors and can display up to eight channels including masks. The bounding box in the 3D/4D Volume View allows for cut-away viewing of projected volumes in all three dimensions and selection of smaller portions of the image for other operations, such as PSF calculations and montaging. Users are also able to change the transparency/opacity of portions outside the bounding box, a feature not always readily available in other applications. SlideBook 2022 also introduced an all-new way to make movies, called Story Board, from 3D and 4D datasets utilizing keyframes and custom framerates. The movie templates can be saved and re-used for multiple datasets, and with the ability to use spline interpolation, users can render beautiful and smoothly animated movies quickly from large datasets.

LUT by Depth
SlideBook can now link to ImageJ lookup tables and display 3D volumes with different colors depending on depth. The same LUTs can be applied to other captures using the Pseudocolor Mode and are set and remembered on a per-filter basis.

Additional MATLAB & Python Interoperability
Through the Union & Synergy modules, hierarchical and conditional capture is now possible for user-supplied MATLAB or Python programs to control experimental workflow, such as which cells should be imaged at higher resolution or whether an live cell imaging experiment should continue or stop (because, for instance, an anticipated event has already been detected).

CUDA Acceleration
NVIDIA CUDA leverages parallel processing on the GPU to greatly speed up operations by executing many commands at the same time. Operations that have been CUDA-optimized since SlideBook 2022 include:

- Deconvolution (all modalities: No Neighbors, Nearest Neighbors, Constrained Iterative, Big Data, MLS Decon)
- MLS Localized Cross-correlation
- MLS POI Registration
- Adaptive Optics
- Software-based autofocus
- 3D montage
- Image deskew and rotations
- Max-intensity projections

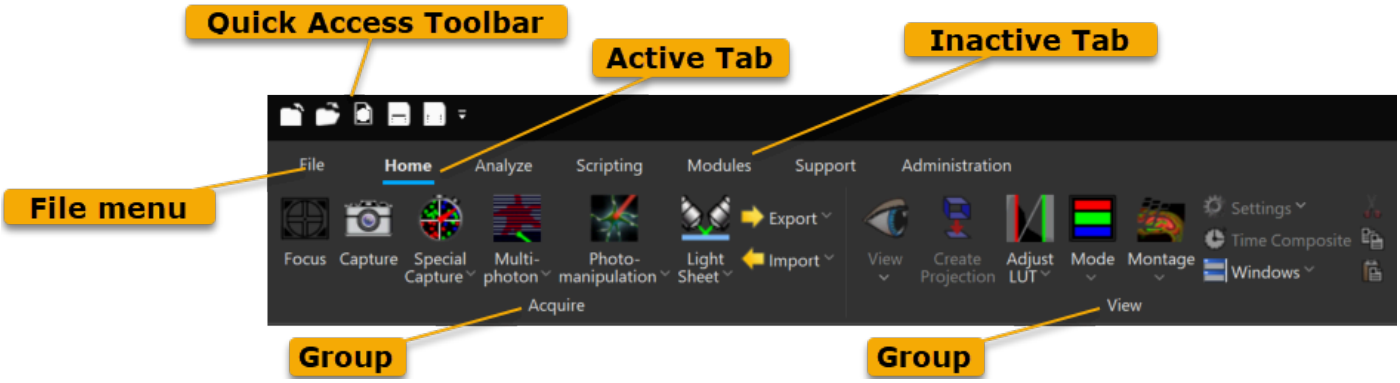
Getting Started with SlideBook

A series of tutorials have been provided to aid in getting familiar with the tools provided in SlideBook. These tutorials walk you through basic operations provided in the software. Alternatively, each user interface window has a help page associated with it that diagrams each function and advises in how to use the options available.

For tutorials and user interface pages, the following typographic conventions are used:

> Menu commands, dialog fields, and other permanent interface items are written in bold italics and follow the order of menu navigation. For instance, choosing "Open" from the "File" menu is written as ***File > Open***.
>
> **NOTES** are written in bold and capitals.
>
> A stylized font is used for user-defined fields or hot key shortcuts such as **file names** or **text entries** or press **Delete**.
>
> References to other sections of the help are highlighted such as Focus Controls Window.

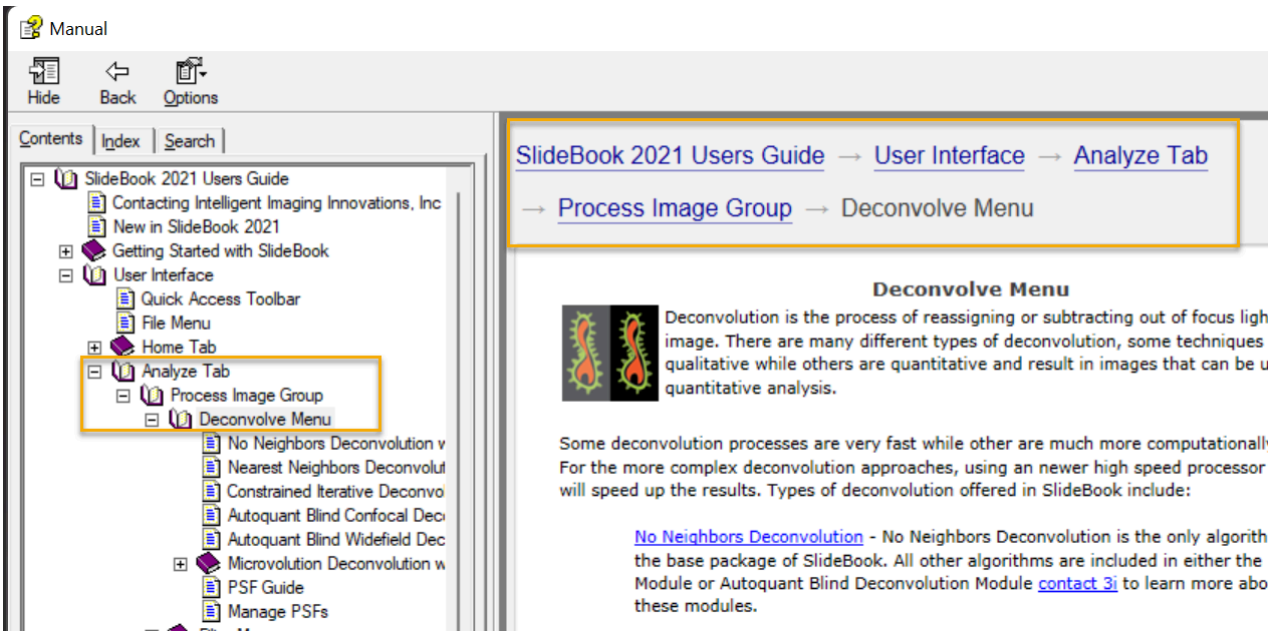
SlideBook is organized using a graphical menu and icon system. There is a customizable ***Quick Access toolbar*** across the top of the application with the rest of the program functionality being found in a tabbed system. The tabs are designed to group functionalities and improve workflow while minimizing the number of clicks required to accomplish tasks.



Terminology used in the manual refers to location by tab and group and often includes an image of the icon. A main portion of this help system is the User Interface section. User interface pages are organized in a structure that mimics the actual user interface:

Tab > Group > Menu/Icon > Function

When you are on a specific user interface page of the help system, the location of the page can be found in the organizational tree on the left side of the Help pane, or across the top of the window.



Tabs are the largest organizational feature of SlideBook. Tabs available include:

File - execute normal file functions such as open, close, create and save **.sld**
Home - control hardware to set up and acquire images, import and export images, view images using basic and advanced techniques, and access region tools and annotations.
Analyze - deconvolve and filter data, perform mask generation and analysis, graph, particle track, smooth curve analysis and analyze using other techniques
Scripting - execute, design, and edit existing scripts
Modules - access purchased analysis modules such as FRET, Ratio, FLIM, and Stereology
Support - easily update software and key, backup configuration, contact 3i technical support
Administration - resource for system administrators that includes setup, maintenance, and configuration tools

In the Home tab, you will find two main hardware control icons to open ***Focus*** control and ***Capture*** control dialogs. These two dialogs are all you need to set up anything from a simple 2D single channel image to a 3D multi-channel capture that visits multiple x,y locations over time (6D capture).

Focus controls allow you to arrange the sample and examine the specimen interactively.

Capture controls allow you to set up parameters for automated image capture.

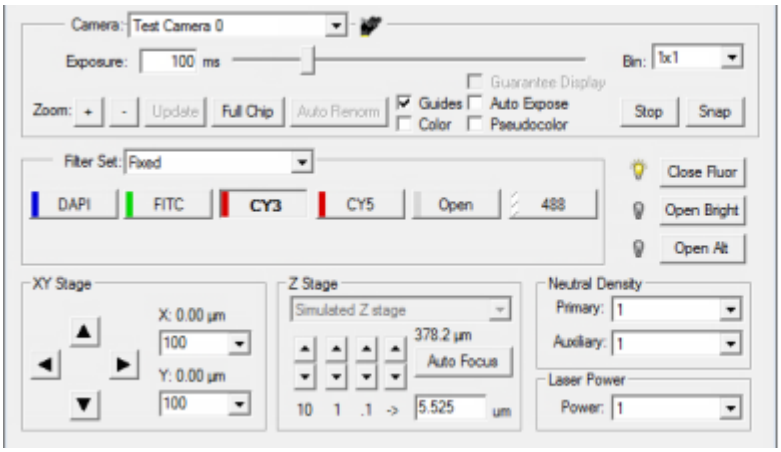
Introduction to Focus Controls

Focus controls are accessed from the ***Focus*** icon in the Home tab, or by pressing **Ctrl + F**.

The Focus Controls window is used to help arrange and view the specimen before setting up an acquisition. You can use the Focus Controls to center the specimen relative to the camera, check fluorescence levels, take a 2D single channel snapshot, adjust camera parameters, or set up a point list for multi-point visiting experiments.

The lower portion of the window is static while the top portion of the window has different tabs for specific functionality. The upper portion of the window changes with regards to control modules available. We will first examine the lower portion of the window as it is used intermittently with tabs of the top portion.

Lower portion of Focus Controls



Adjust exposure time, binning, and appearance of live image window.

"Snap" a 2D single channel image.

Select filter sets for viewing.

Open and close shutters.

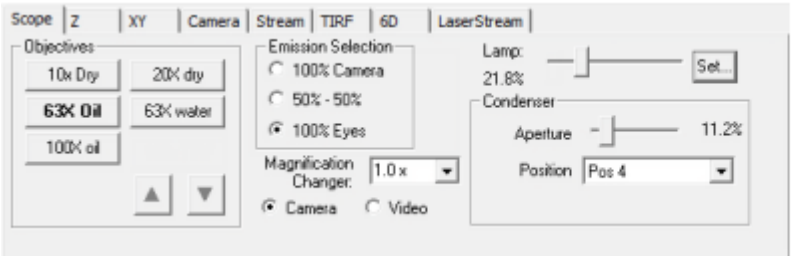
Move the stage in *X*, *Y*, or *Z*.

Adjust movement size.

Test the "Auto Focus".

Neutral density settings.

Scope tab

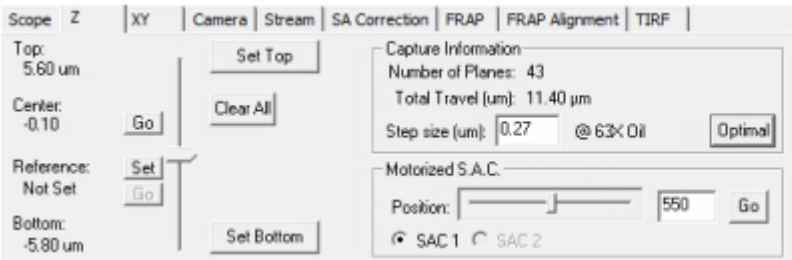


Select objective.

Direct light to eyes or camera.

Adjust transmitted light intensity and condenser.

Z tab

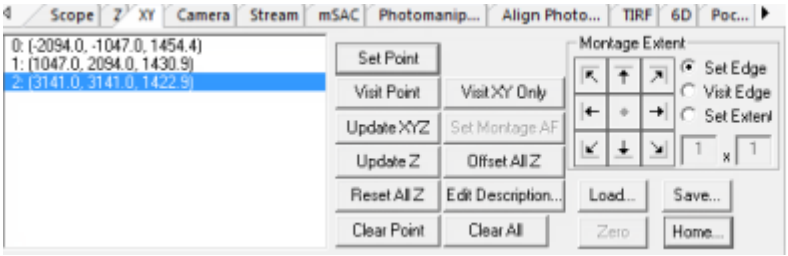


Set top and bottom of *z* range for acquisition.

"Go" to center of range.

Set *z* step size for capture.

XY tab

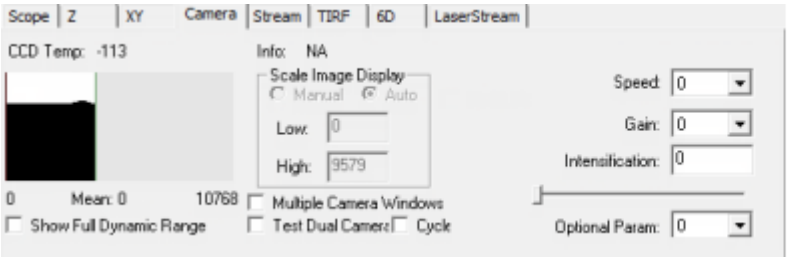


Set *x*,*y*,*z* positions for point visiting experiments.

Set extents for montage imaging.

Edit points.

Camera tab



View intensity information based on sensitivity and exposure time.

Choose manual or auto-scaling display for live window.

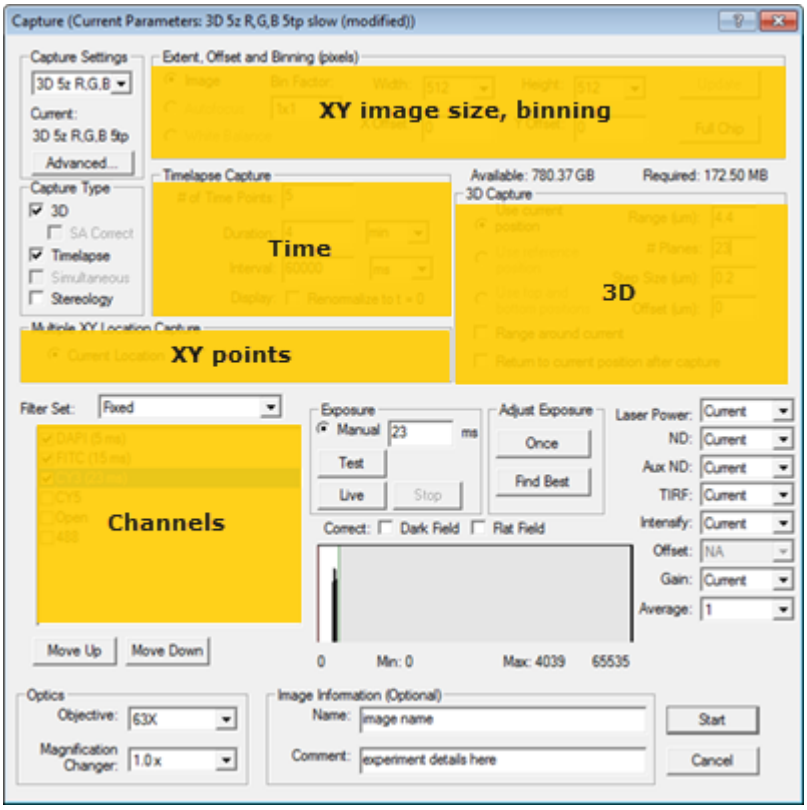
Adjust intensification, speed, and gain for camera readout.

See more on the [Focus Controls Window]

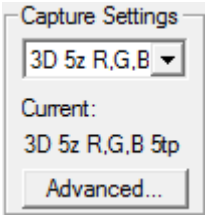
Introduction to Capture Controls

Capture controls are accessed from the ***Capture*** icon in the Home tab, or by pressing **Ctrl + E**.

The Capture Controls dialog provides all necessary tools to set up basic or complex capture sequences. The dialog is broken down into areas to adjust parameters for different dimensions of capture as depicted below.



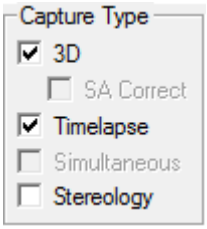
Capture Settings



Save/Load Capture Settings

Access ***Advanced*** options including variable time intervals, photomanipulation, autofocus, periodic imaging, speed optimizations, and triggering.

Capture Type



Select elements to be included in image acquisition then complete relevant fields.

If none of these elements is desired, proceed to Channel Selection and Exposure Time.

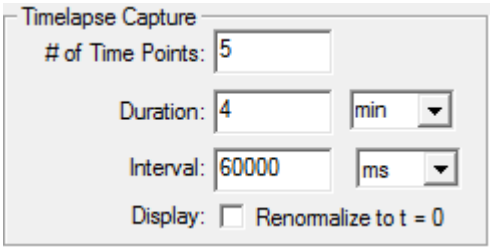
Extent - Offset - Binning

Select a bin factor to increase signal (note this will decrease resolution). Manually select a chip size based on the center of the chip.

OR

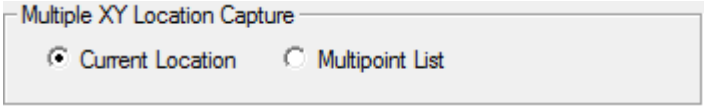
Draw a region to image on the live window using the tool. Click "Update" to apply.

Timelapse Capture



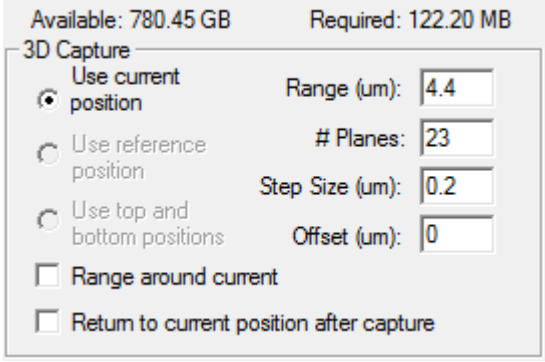
Enter parameters for time series acquisition.

Multiple XY Location Capture



Select a multipoint list if you would like to perform point visiting or capture images for a montage.

3D Capture

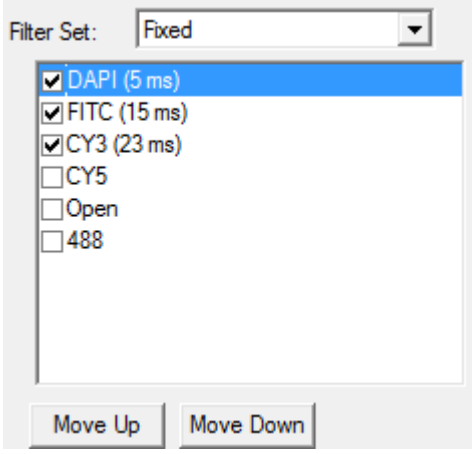


Select elements to be included in image acquisition then complete relevant fields.

If none of these elements is desired, proceed to Channel Selection and Exposure Time.

Be aware of the amount of memory available and required for the capture. It may be necessary to adjust the portion of the chip used, binning, number of time points, or number of Z sections to create a manageable data set.

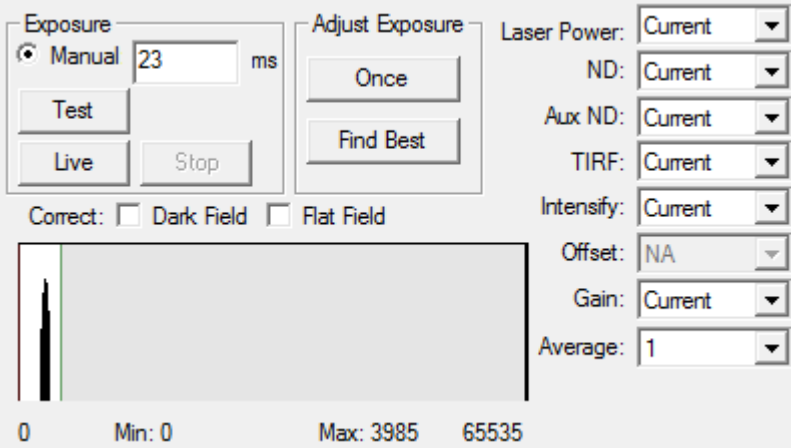
Channels



Check the boxes next to the imaging filters you would like to capture.

Use the buttons below to adjust the order in which the channels are captured.

Exposure



Adjust the exposure time for each channel that will be captured. Use the ***Live*** button should you need to reposition your sample.

Refer to the histogram (see tips below) to determine optimal exposure times.

Try using the buttons in the adjust exposure dialog to allow SlideBook to optimize exposure time for your sample.

Adjust neutral density settings, gain, and intensification for the camera as desired. Use "Current" to use the settings from the focus controls. Different settings can be applied to each channel.

Setting Exposure Time

Setting exposure time appropriately is essential to obtaining the desired imaging results, for live cell experiments, it can be a matter of life and death! When using an EM (electron multiplying) camera, one needs to adjust intensification with exposure time. Here are some basic tips for setting exposure time and intensification appropriately.

IDEAL

ACCEPTABLE FOR LIVE CELL

SATURATED

UNDEREXPOSED

TIPS

For fixed specimens, aim for filling the histogram 2/3 of the way. This gives you room for brighter planes.

Never saturate an image you would like to use for deconvolution or most other quantitative analysis techniques.

For live samples, experiment to determine how much light they can handle. You will often sacrifice intensity for cell viability. It is acceptable to have your signal at twice the background level, depending on the noise in the image.

When imaging live samples with an EM camera, you can increase the signal while keeping the exposure time low by increasing the intensification.

If possible, try to keep the intensification level well below the maximum available.

When using a non-EM, higher resolution camera, another way to increase signal without increasing exposure time is to utilize binning. It is important to note that binning will decrease the lateral resolution of an image.

Example Experiment

This section will step through the process of setting up an example data capture. For brevity, we will walk you through a 4D multipoint capture setup. For less complex experiments, simply exclude the aspects of the experiment that are not relevant to your work.

Getting Started

If you are using an incubation chamber, it should be turned on at least one hour before you begin setting up your experiment. Immersion media for objectives such as distilled water or the microscope manufacturer's immersion oil should be placed in the chamber and allowed to come to temperature as well. It is important that the temperature of the equipment has stabilized to eliminate drift artifacts in the data capture.

Ensure the objective lens is clean and in the lowered (raised for upright microscopes) position. If the objective requires cleaning, use lens tissue or 100% cotton swabs and an approved solvent. Never use general purpose lab or household tissue on optical surfaces. Once the objective is clean, apply the appropriate immersion media*. Ensure there are no bubbles in the media. If there are bubbles during application, remove the fluid using lens tissue and re-apply.

Arrange the specimen on the microscope stage with the coverslip toward the objective lens. Bring the objective toward the sample. For air objectives, stop at about 1mm from the coverslip. For water and oil objectives, stop once the media has contact with the coverslip. Use stage clips, the stage insert, or lab tape to secure the specimen to the stage. Direct light to the specimen and bring the sample into focus looking through the oculars.

* It is important to use the microscope manufacturer's immersion oil to maintain the chromatic properties of high end objectives. For upright microscopes, the immersion media will be applied to the coverslip of the specimen rather than the objective. Alternately, many upright microscopes have "dipping" lenses that can be inserted directly into the specimen's media.

TUTORIAL: Open Focus Controls, Orient Specimen

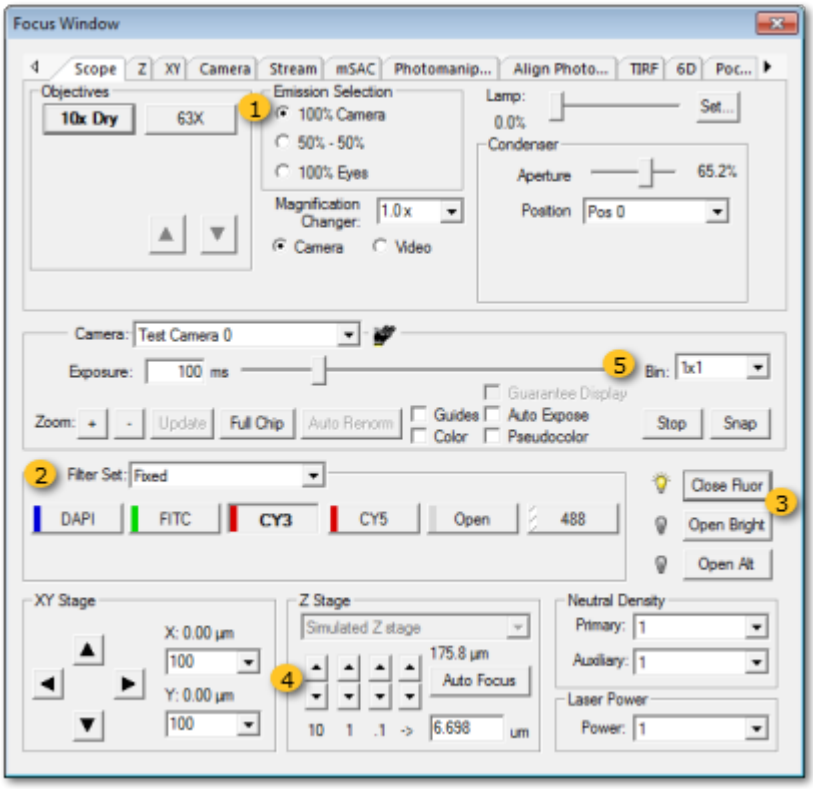
1. In the Scope tab of Focus Controls, set ***Emission Selection*** to 100% Camera.

2. Select filter set and channel you would like to preview.

3. Open the appropriate shutter to direct light to the sample.

4. Adjust the focus using the knobs on the microscope or z stage controls to bring the specimen into focus.

5. If necessary, use binning or change exposure time to alter the appearance of the image on the screen. You will set parameters for final capture later.



TUTORIAL: Set Points for Visiting

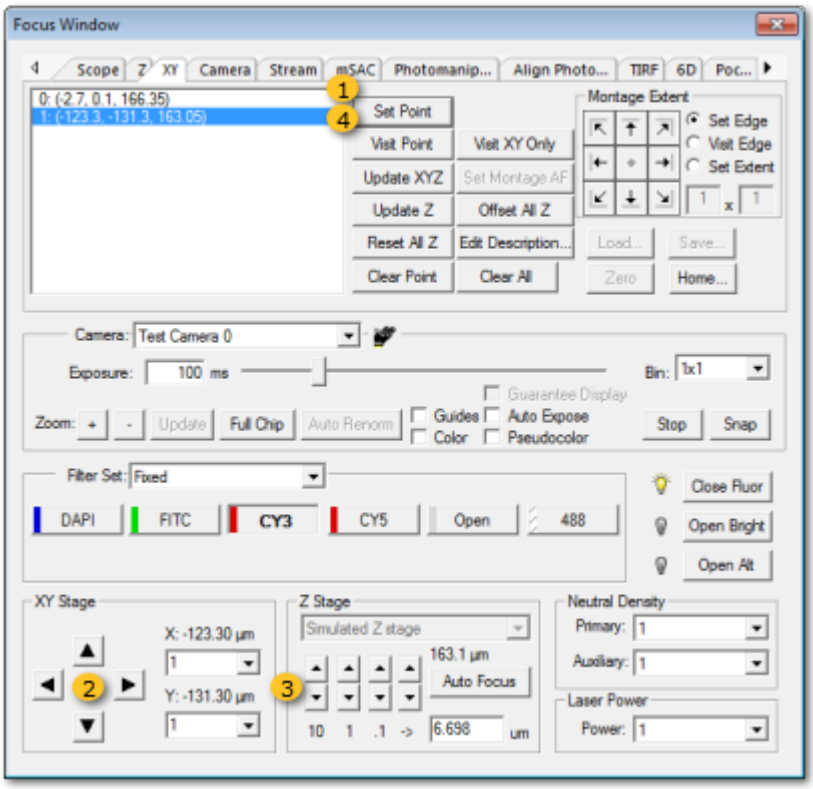
1. Click ***Set Point*** to add the current position to the list for capture.

2. Navigate to the next point using the joystick for the motorized stage or the stage controls.

3. Adjust the z focus to the center of the stack using the focus knob or the z stage controls.

4. Click ***Set Point*** to add the next point to the list.

5. Repeat steps 2–4 until all desired points are set.



TUTORIAL: Set Z Extents

1. Navigate to the top z extent using the z stage controls.

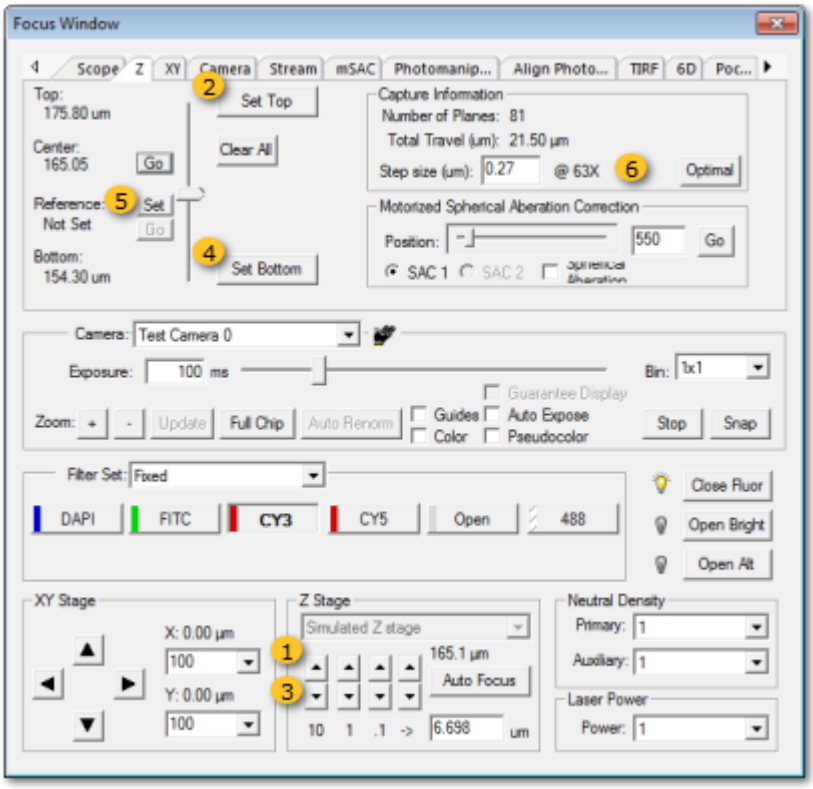
2. Click ***Set Top***.

3. Navigate to the bottom extent using the z stage controls.

4. Click ***Set Bottom***.

5. Click ***Go*** to return to the center of the stack.

6. Set the step size for data collection or click ***Optima***l to use Nyquist sampling of the theoretical axial resolution of the objective.



TUTORIAL: Open Capture Window, Set Exposure Times

1. Open the Capture dialog, check the box next to the channel you would like to capture.

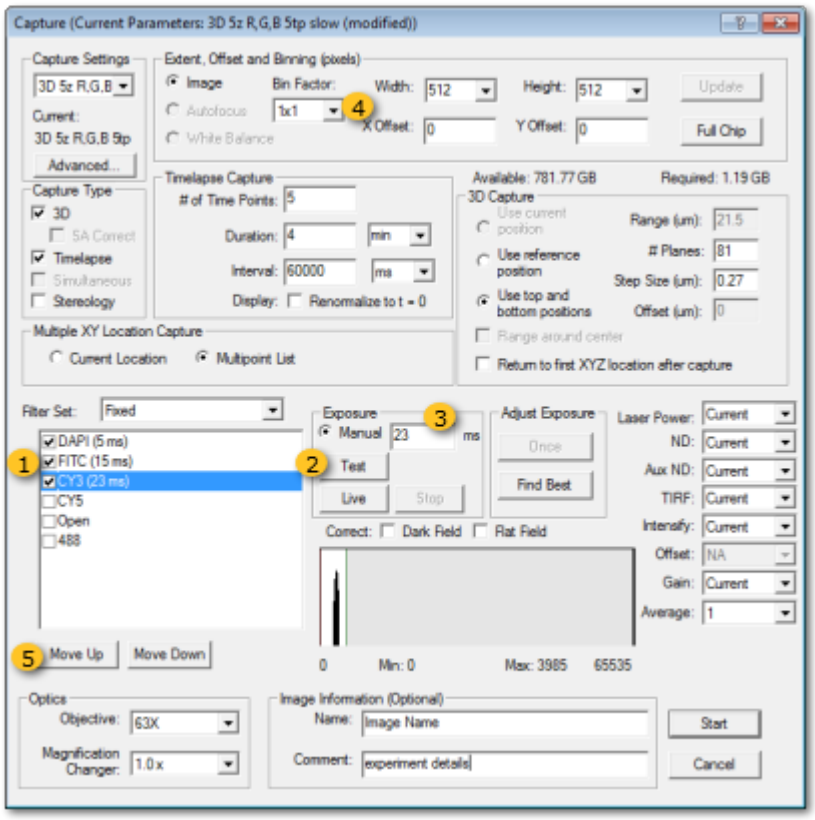
2. Click ***Test***.

3. Adjust ***Exposure*** parameters [see notes here for additional information on setting exposure parameters.]

4. If desired, adjust bin factor to further increase signal, adjust exposure time and intensification accordingly. Repeat 1–3 for additional imaging channels, noting that only one bin factor can be used per experiment.

NOTE: Binning reduces xy resolution.

6. Select channels and use the ***Move*** buttons to change capture order.



TUTORIAL: Set Capture Parameters, Start Acquisition

1. Check the box for ***3D***.

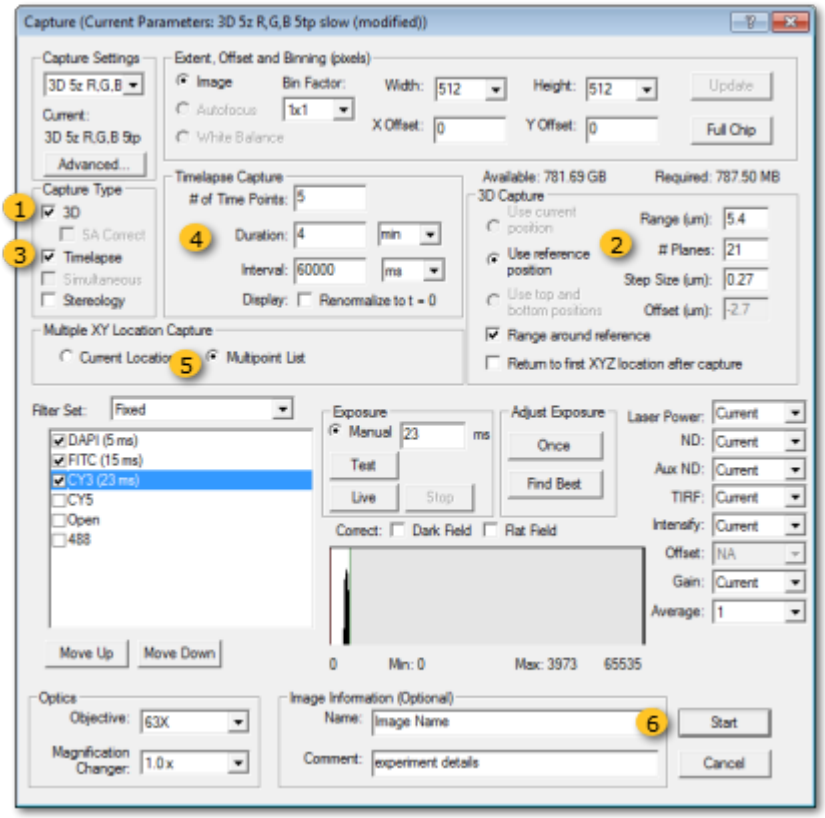
2. Select the type of 3D capture to perform. For multipoint experiments, select ***Use reference position*** and ***Range around reference***. For standard 3D imaging, you will likely select ***Use top and bottom positions***.

3. Check box for ***Timelapse***.

4. Enter ***Timelapse Capture*** details as desired.

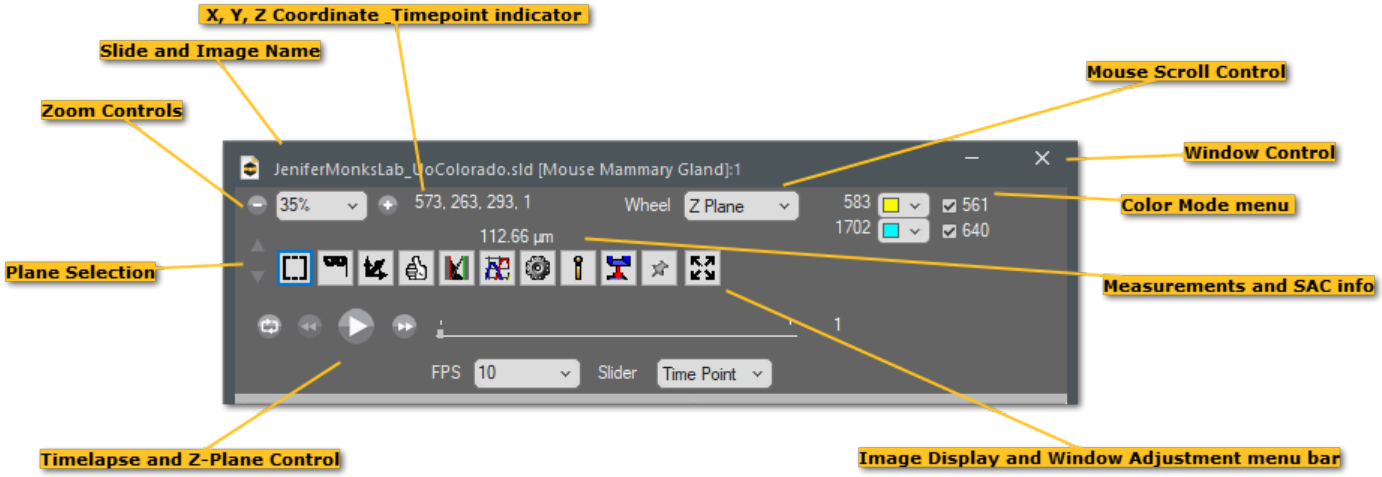
5. Select the radio button for ***Multipoint List*** to direct the capture to the points marked in the XY tab of the Focus Controls.

6. Enter a ***Name*** for the image if desired. Click ***Start***.

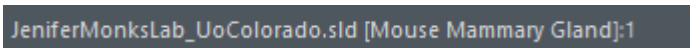


TUTORIAL: Viewing an Image

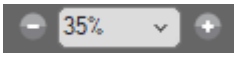
When you open an image by double–clicking on the thumbnail in a slide, a Main View will open. Alternatively, you may open a Main View or a Three View from the View Menu. All views contain many useful tools outlined below.



1 Slide and Image Name



2 Zoom Controls



To determine the desired zoom

3 Mouse Scroll Control

Select if the middle mouse scroll wheel will scroll through either *z*-planes, time or zoom and position if the image is opened in the Multipoint View.
The mouse scroll wheel can also be used with modifiers for each:

- CTRL + mouse wheel → zoom
- Shift + mouse wheel → Z planes
- Shift + CTRL + mouse wheel → Z planes in increments (default 10)
- ALT + mouse wheel → timepoints

4 Plane Selection

Alternative to mouse scroll

5 X, Y, Z Coordinate & Timepoint indicator

6 Color Mode menu

Display depends on the selection in the Home menu > View Mode

7 Timelapse and Z-Plane Control

When an image consists of multiple time points or Z-Planes, additional tools will appear in the ***Main View***.
The ***Playback Controls*** allow you to toggle looping/single playthrough, move back one timepoint or plane, play/pause, or move forward one timepoint or plane. The ***Playback Speed*** controls the speed of the image looping in ***FPS*** or frames per second.
The ***Slider*** displays the current time point or plane both with the small gray box and with a numerical display to the right of the slider bar. A user can move to a desired time point by clicking on the small gray box and dragging it to the desired time point or Z-plane so that you can move forward and backward through time or upwards and downwards in a 3D stack. The ***Slider*** drop down allows selection if the Slider should control timepoints, Z-Planes or Zoom and position if the image is opened in the Multipoint View.

8 Window Control

Minimize (_) or close (***X***)

9 Measurements and SAC info

Measurements when using the ***Ruler*** or ***Angle*** tool (Main View) are displayed here as well as the setting used for motorized Spherical Aberration Correction (SAC) device during image acquisition.

10 Image Display and Window Adjustment menu bar

Contains numerous options to adjust image window size and image display such as Adjust LUT. Please see Main View for detailed explanation.

TUTORIAL: Adjusting Display of an Image

SlideBook offers some basic display tools for adjusting how data is represented on the computer screen.

Renormalization

Renormalization allows you to alter the range of intensities displayed for each channel. To open the Renormalize Image window, click on the ***Adjust LUT*** icon in the ***Home*** tab or ***View*** window.

1. Open the ***Renormalize Image*** window:

A histogram is displayed showing the relative number of pixels on the y axis and intensity values on the x axis for the specified channel. In this image, the selected channel's intensities range from **92** to **24157**.

2. Click on the drop-down ***Channel*** menu and select the channel you would like to adjust.

3. Click on the red and green bars in the histogram window and drag them to the left or right, observing the change in the display of the relevant channel data.

The red and green bars allow you to select the minimum and maximum intensities that correspond to the absence (black) or full saturation of color in the display, respectively. Again, the underlying values in the image are not affected.

4. Now manually enter numbers in the ***Low*** and ***High*** data entry fields. Note that the image does not automatically update as it does when moving the red and green bars.

5. Click ***Apply*** to register the change and update the image.

6. Press ***Reset all to Image Min/Max*** to set the minimum and maximum display values to the minimum and maximum pixel values.

7. Choose the ***Thumbs-Up*** button to re-open the image with the same LUT range values and plane and/or timepoint if applicable next time you decide to view this capture. This will update its thumbnail image but will have no influence on the image data.

8. Choose ***OK*** to exit the Renormalize Image dialog box.

NOTE: When you have several captures open and do not want to have a Renormalize dialog for each capture, click the pin icon. The histogram window will then update to the values of the image in the active (last clicked) viewer window.

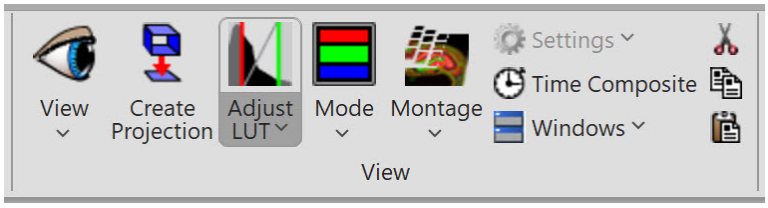
Copy and Paste the LUT Range

You may want to copy the LUT Range (Low, High and Gamma) from one channel of the image to another of the same image or a different capture. For this adjust the range for the first channel (follow steps 2. to 5. above) then select the ***Copy*** icon then select the capture and channel which should have the same values and hit the ***Paste*** icon.

NOTE: If you hold the CTRL key pressed when hitting ***Copy*** the LUT Range values of all channels of this image will be copied by channel order. Then clicking ***Paste*** in the Renormalize window of another capture will paste the LUT range values for all channels in the channel order of the original image - disregarding the channel name.

Adjust the LUT for Multiple Images

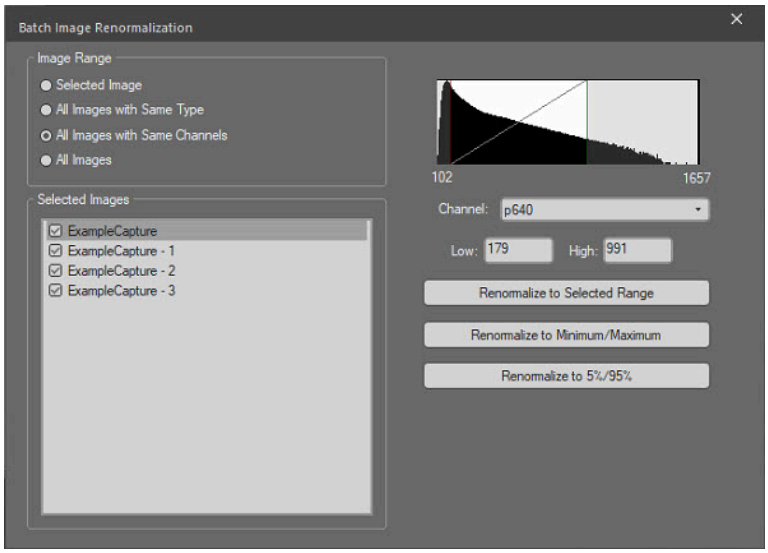
If you want to apply a LUT Range for all images in a slide file. To do so navigate to the drop down of the ***Adjust LUT*** icon in the ***Home*** tab, this will provide you with the following options:



Renormalize all - Once an image is open and renormalized, press the thumbnail icon in the view window then select Renormalize all to apply the current image's renormalization to any matching channel names for all images in the **.sld/ .sldy**. You will need to re-open any images to see the renormalization changes take effect.

Renormalize all to global min/max - Select this option to use the lowest minimum and highest maximum intensity values found in all of the images in the slide file. You will need to re-open any images to see the renormalization changes take effect.

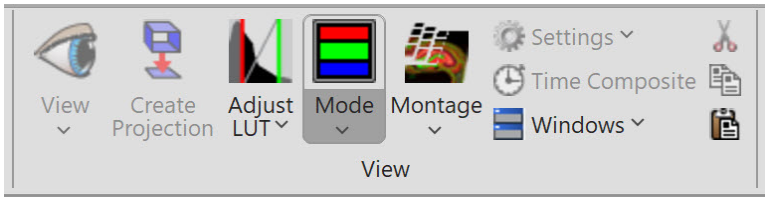
Batch Renormalization - Opens the Batch Renormalization dialog to renormalize multiple images based on selected criteria.



Densitometry - Allows to calibrate, remove or transfer a channel for Densitometry.
Lookup Tables - Allows for the import of customised LUT from e.g. Fiji

Change the Color Scheme

You may also change the basic way channels are displayed in the view window. For example, data can be displayed using a temperature lookup table or in monochrome. Shortcut keys are available for each of the option below or you may find them under the ***Mode*** menu in the ***Home*** tab.



RGB Color - **Ctrl + Shift + R** - Provides three colors (red, green, and blue) for assignment to any of the channels, also enables a background channel to blend a transmitted light image

Monochrome - **Ctrl + Shift + M** - Provides a single channel to display a monochrome image

Inverted Monochrome - **Ctrl + Shift + V** - Provides a single channel to display an inverted monochrome image

Pseudocolor - **Ctrl + Shift + P** - Provides a single channel to display on a hot/cold lookup table, may be gated by a second channel as in ratio data

User-defined Color - **Ctrl + Shift + D** - Provides up to eight channels which can be assigned to a user-defined color selected from a color chart or manually created

LUT X Depth - Provides the display on a hot/cold lookup table depending on sample depth when using the 3D/ 4D Volume Viewer

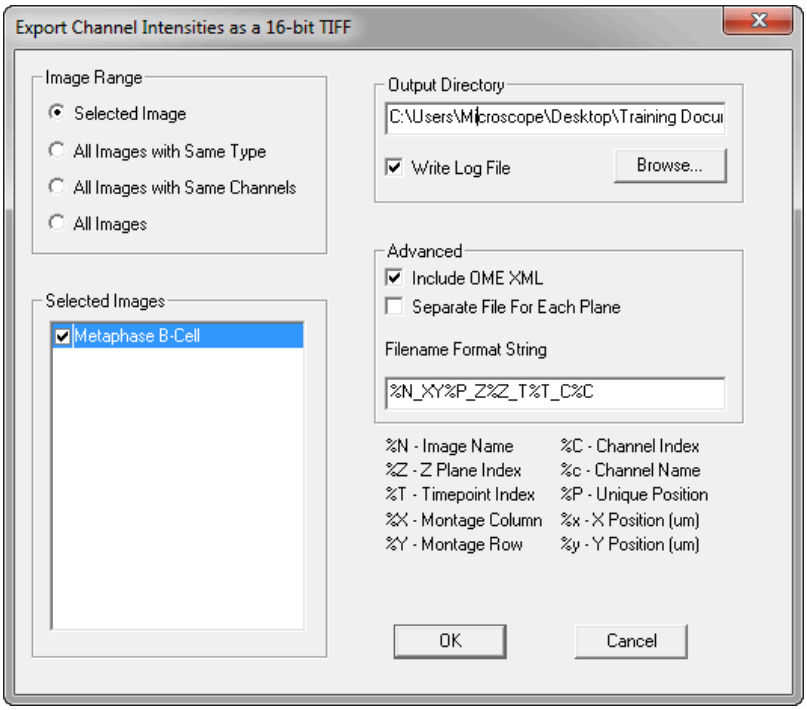
When a transmitted light, greyscale image is collected along with fluorescence, you can combine the channels using ***Blend Background*** (available under Settings in the ***View*** group of the ***Home*** tab. Blend background is compatible with RGB Color and Pseudocolor display types. Degree of blending can be adjusted in the View Settings window.

TUTORIAL: Quantitative Image Export

Sometimes, you will wish to share data with colleagues or back up data in a format that can be read by programs other than SlideBook. In order to do this, you will want to maintain the true intensity values of each pixel of the image. For this reason, there is a 16-bit tiff export option available (see also 16-bit Tiff Export window). This export option is compatible with the OME (Open Microscopy Environment) format.

1. Select the image that you would like to export by clicking on it. You may also start from an open view of the image.

2. In the Home tab, select ***Export > (Quantitative Export) 16-bit TIFF***. A dialog similar to the following will appear.



3. Select the ***Image Range*** as described below. You may also select and deselect images using the checkboxes in the ***Selected Images*** list.

- ***Selected Image*** – Operation will be performed on the selected image only.
- ***All Images with Same Type*** – Operation will be performed on all images in the slide with the same capture type (2D, 3D, 4D, etc.) as the selected image.
- ***All Images with Same Channels*** – Operation will be performed on all images in the slide with the same channels as the selected image.
- ***All Images*** - Operation will be performed on all images in the slide.

4. Select the ***Output Directory*** where the tiffs will be saved by clicking on **Browse** then navigating to the desired location.

5. Check the box for ***Include OME XML*** to include OME metadata in the tiff file (encoded in XML).

6. Check the box for ***Separate File For Each Plane*** if you would like a tiff file for each plane for each channel of the image. If you do not check this box, a multi-plane tiff file will be created for each channel.

7. If desired, format the naming of the tiffs in the ***Filename Format String*** field.

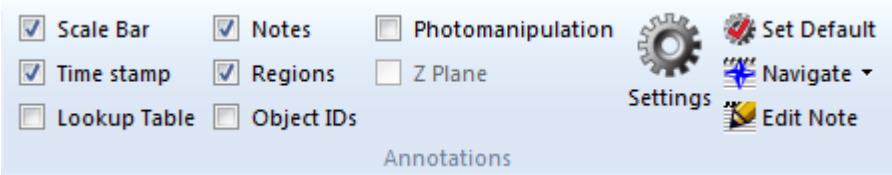
8. Press ***OK***.

TUTORIAL: Displaying and Formatting Annotations

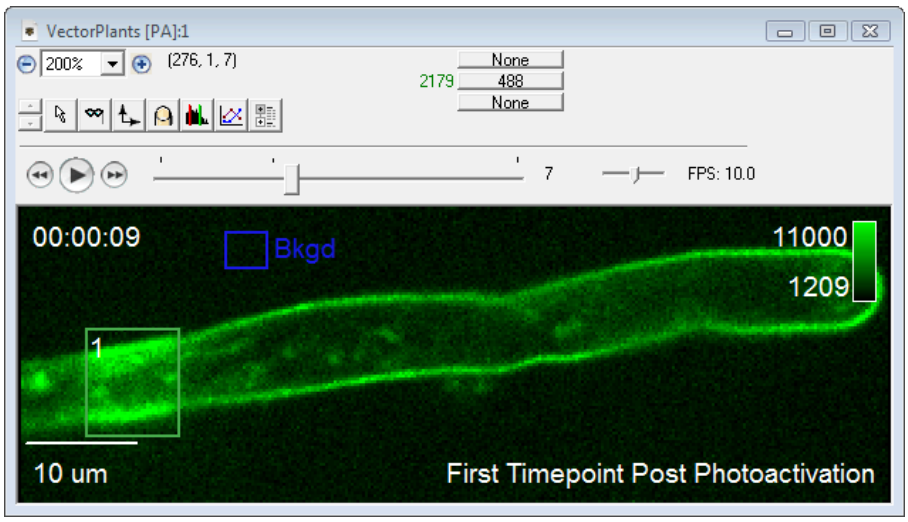
In any of the image views available, SlideBook provides tools to add annotations to displayed images. When annotations are shown in an image, they will be exported with any ***Qualitative*** export of the image. Annotations are not displayed when images are exported using a ***Quantitative*** technique. Additional information on these functions can be found in the Annotations Group.

1. Open a view of the image you would like to annotate.

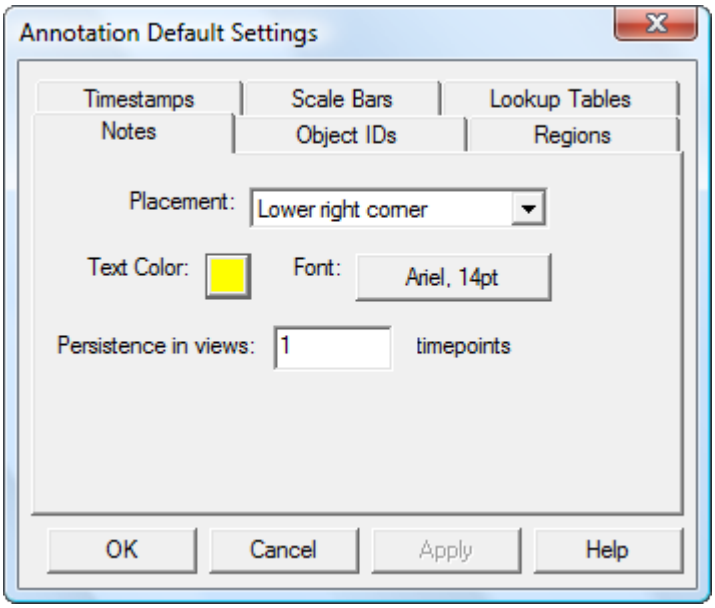
2. Go to the ***Annotations*** group in the ***Home*** tab.



3. Select the annotations you would like to display by checking the boxes. Alternatively, you can use shortcut keys (listed here).



4. Once the annotations are displayed, you may find it desirable to adjust the positioning or appearance of the annotations. To do this, select the ***Settings*** icon in the ***Annotations*** group.



5. This dialog consists of a number of tabs that allow for formatting of each individual annotation. Users can adjust the color, placement, and font used for each annotation. Some annotations have annotation specific settings such as the length setting for scale bars. Adjust all annotations as desired then click ***OK***. The changes will be made in your image window. It may be necessary to click once on the image for the updates to take place.

6. If you are only interested in adjusting annotations for the image you are working with, close the window when finished. Should you choose to apply these annotation settings to every image you open, it is possible to set the default behavior for annotations. To do so, click on ***Set Default Settings*** in the ***Annotations*** group.

> **NOTE:** ***Set Default Settings*** will only set the default annotation settings for the current SlideBook session. If you restart SlideBook, the default annotations will have to be reset.

TUTORIAL: Using Regions to Graph data

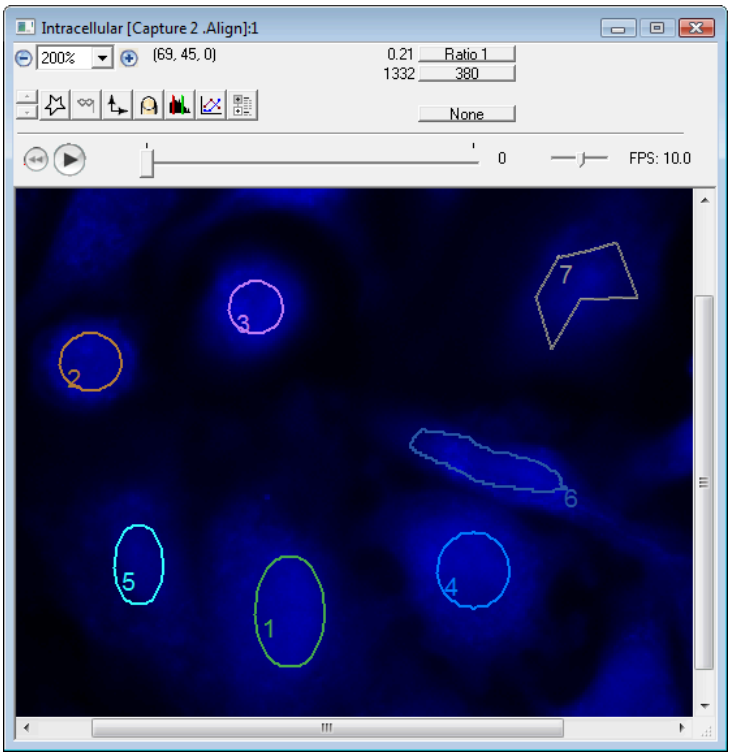
In any open image view, you may draw regions to graph channel intensity information. A drawn region will exist is all *z*-planes and time points of an image. This tutorial reviews drawing and editing regions, setting a background region, and graphing the region data.

1) Open a ***Main View*** of an image.

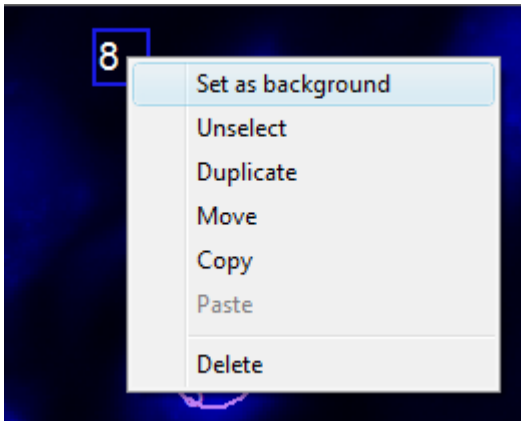
2) Select any of the region drawing tools from the ***Region*** group in the Home tab or from the tool menu in the view window.

NOTE: If regions were drawn and saved with the image in the full SlideBook software, they will be displayed when you select a region drawing tool.

3) Draw regions as desired on the image by clicking and dragging. You may use any combination of the region drawing tools. When using the ⬠ ***Polygon*** tool, double click to close the shape.



4) If desired, draw a region in the background area of the image. Right-click and select ***Set as Background*** from the menu.

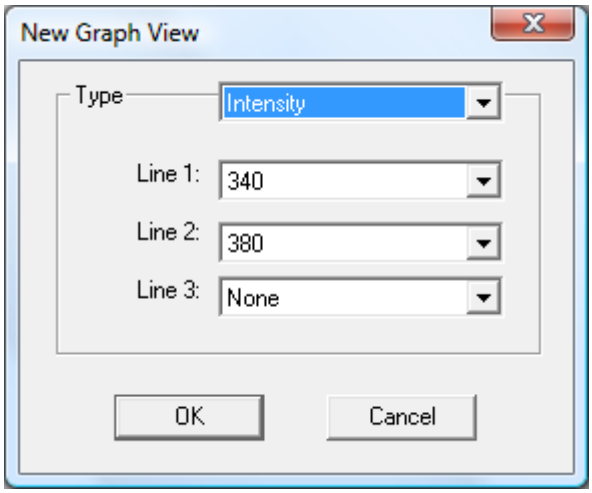


5) To select a region, click within its borders. Once a region is selected, you may drag to to change the location, press **Delete** to remove it, or right-click to copy/paste it. For complete descriptions of all region functions, please review Region Tools.

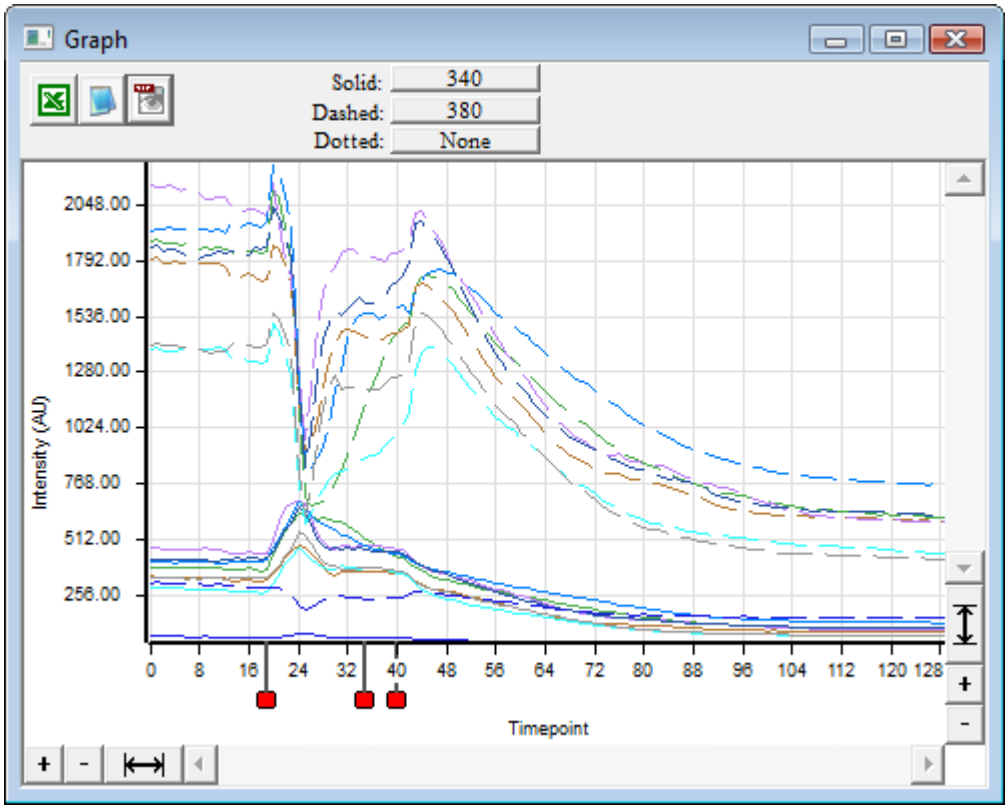
6) Once you are satisfied with the regions, you may graph the data by going to ***Graph*** in the ***Analyze*** tab or by selecting the ***Graph*** icon in the view window.

> **NOTE:** Graph is only available when there are intensity changes over time in 2D Timelapse and 4D data types (not available for 3D or 2D images). For images that do not have a time aspect, you may use Masks for analysis.

7) Select the type of intensity and which channels you would like to graph from the New Graph View window. Click ***OK***.



8) View and explore the ***Graph*** window. For more information on the tools in this window, go to the Graph window page.



9) If desired, export an Excel file, text file, or tiff of the graph data.

SlideBook File Formats

A *slide* contains the individual captures of an imaging session and can be saved as .sld, .sldy or .sldyz.

See also:
Convert and Easy Switch between Slide Formats

SLD

SlideBook's native file type is SLD (***.sld**). A slide file corresponds to a single file on the disk and can be thought of as SlideBook's document type. It can contain many multi-plane, multi-channel, multi-timepoint images in any combination.

- Double-clicking on an image in a slide will open a Main View of the image.

- You may also right-click on an image and perform the following functions: rename, cut, copy, paste, delete. Additionally, you may select ***Properties*** to view information about the image capture. ***Recapture Experiment*** is available for images captured in SlideBook 6 and later. By selecting ***Recapture Experiment***, the Capture dialog will open, populated with the experiment acquisition parameters used in collecting the selected image, thus easily reproducing the acquisition

- In the **SLD**, each image will be represented as a thumbnail with associated image information including comments made during capture or analysis, capture date, capture type, number of channels, physical image dimensions, amount of drive space taken up by image (size), and number of masks. The thumbnail style represents the capture type. Three dimensional images will be represented as a stack of thumbnails. Images taken over time will have a film strip depiction above the thumbnail. The image seen in the thumbnail is a direct representation of the default view of the image
- To adjust how the thumbnail appears in a **SLD**, open the image and adjust the plane, renormalization, lookup table, etc. Once the image appears as desired, click on the thumb icon and ***Save*** the **SLD** to set the default setting for future openings of the slide and images

- A **SLD** must be open in order to run the ***Focus Window*** or set up image capture

- **SLD** can be opened using the OME Bioformats plugin but the the SlideBook6Reader.jar or its components (.class and .dll/ .dylib/ .so) must be individually enabled. In Fiji, this is possible through Help > Update > Manage Update Sites > Enable SlideBook.

SLDY

In 2022 we introduced the **SLDY** (*.sldy) file format, which is an open-source file format designed for very large data sets (>100GB) produced by modern microscope systems.

- The **SLDY** file is a distributed file format that points to a directory where each captured image stored in an independent file (by timepoint) alongside metadata as files that describe the capture

- The distributed nature makes many image manipulation operations, such as removing a channel from an image or removing a selection of time points, nearly instantaneous. Images, masks, and histograms are stored as python-compatible .numpy arrays, and the metadata is stored in the open-source YAML format, enabling users with coding experience to process images directly in Python. Please reach out to support@intelligent-imaging.com for our existing SBread.py.

- All functions available for **SLD** are also available when saving as **SLDY**

- When copying SLDY files into a new slide per CTRL+C and mouse right click > paste there is also the option to Paste via Disk - this avoids going through memory and is faster for larger files. Equivalent to this option some functionalities in SlideBook, like Extract/ Crop, allow the user for faster processing

- A user can set the default slide format to either **SLD** or **SLDY** - Please reach out to support@intelligent-imaging.com if you want to change permanently. Otherwise you can control how a new slide is saved by setting up the menu bar in SlideBook, see Convert and Easy Switch between Slide Formats

- **SLDY** can be opened using the OME Bioformats plugin.

SLDYZ

SlideBook supports lossless compression of the **.sldy** file format with several methods. Those files are saved as **SLDYZ** (***.sldyz**).

- The ZStandard compression algorithm is default. Zstandard is a new lossless algorithm developed at Facebook Labs that works to compress our very large files with a balance of speed and compression ratios that far outperforms many of the standard lossless algorithms. Each .numpy image file is compressed individually, so the structure remains the same, but the overall size of the directory is smaller. Compressed files can be opened natively in SlideBook with no need to decompress them. More information can be found here: https://engineering.fb.com/2016/08/31/core-data/smaller-and-faster-data-compression-with-zstandard/

- For full details of available algorithms please contact support@intelligent-imaging.com for further details

- **SLDYZ** can be opened using the OME Bioformats plugin.

TUTORIAL: Convert and Easy Switch between Slide Formats

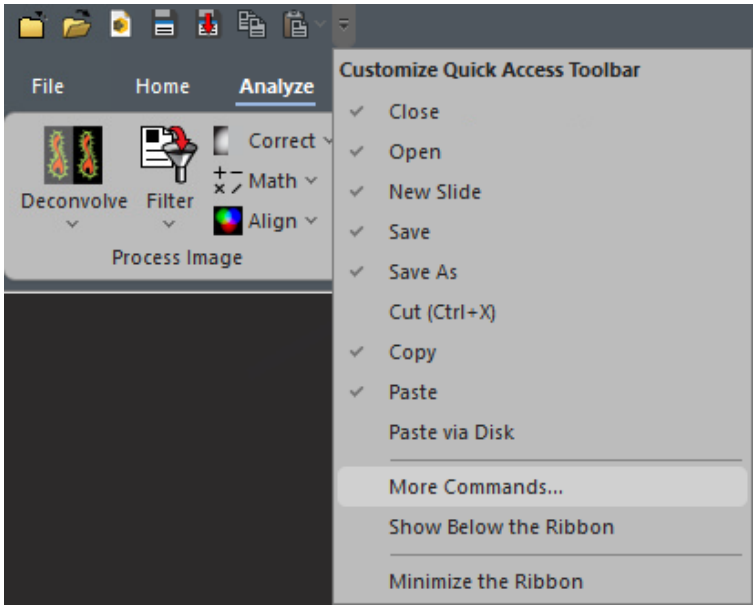
Here are several options which can be used to quickly decide which file format is used and how to convert between the options.

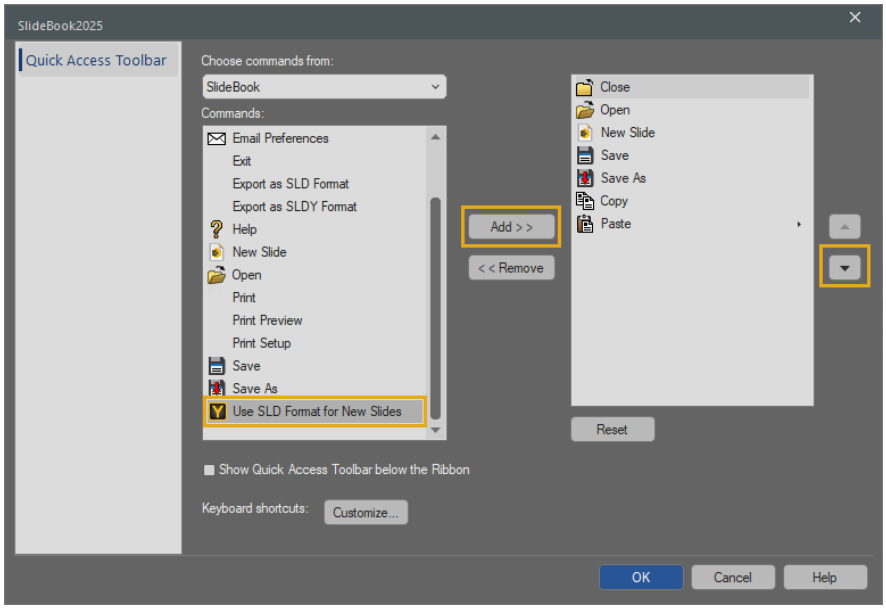
1.) Set Up the Menu Bar

1. Select the arrow in the menu bar to get to the drop down

2. Select ***More Commands...***

3. Highlight "Use SLDY Format for New Slide" and ***Add >>***





4. Sort its position in the menu bar with the up and down arrows > ***OK***

5. The menu bar will now show a clickable icon:
   i. D indicates the slide will be saved as **.sld**
   ii. Y indicates the slide will be saved as **.sldy**

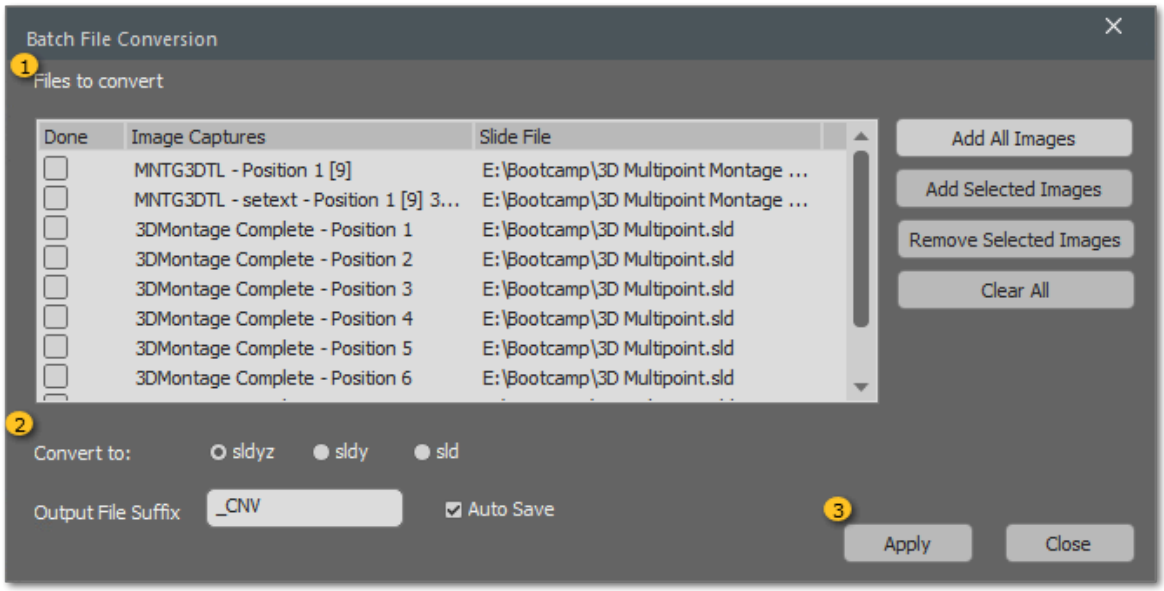
6. Alternatively: With a new unsaved file open, navigate to ***File*** and click ***Use*** **SLD(Y)** ***Format for new Slides***

2.) Convert between Formats

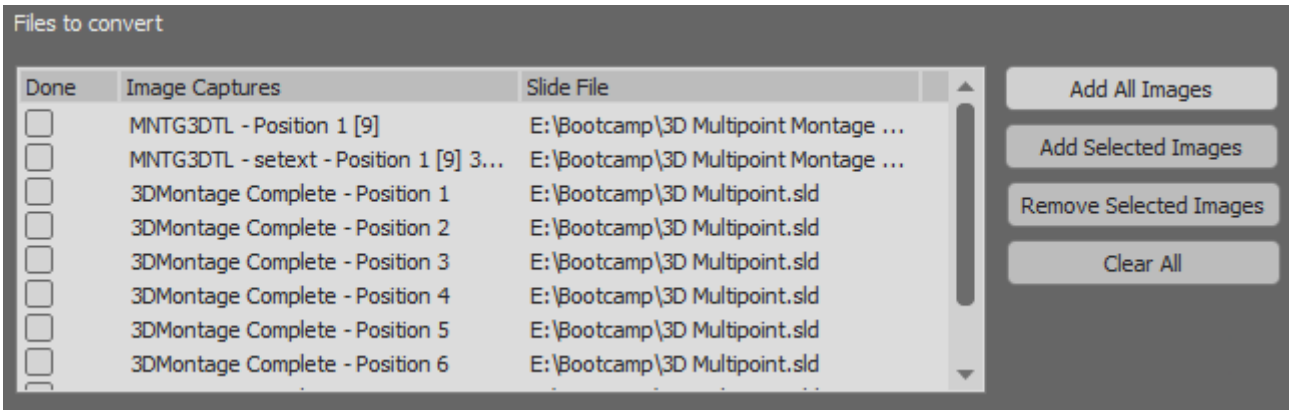
1. To convert to **SLDY**
    a. Open a **.sld** file
    b. Select the images to convert
    c. Navigate to ***File*** > ***Export as*** **SLDY** ***format***
    d. It will create a new slide with the selected images

2. To convert to **SLD**
    a. Open a **.sldy** or **.sldyz** file
    b. Select the images to convert
    c. Navigate to ***File*** > ***Export as*** **SLD** ***format***
    d. It will create a new slide with the selected images

1. To batch convert
    a. Open all **.sld**, **sldy** or **.sldyz** files to convert
    b. Navigate to ***File*** > ***Batch conversion...***

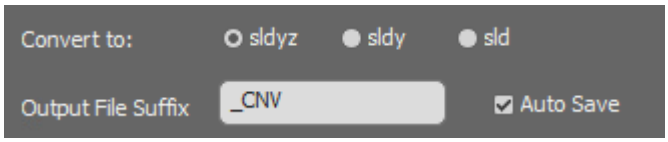


1 File Selection Group



- ***Add All Images / Add Selected Images***: Select all captures from the individual slides and add to the list
- ***Remove Selected Images***: Removes highlighted images from the list
- ***Clear All***: Clears the entire list

2 Conversion Selection Group



Toggle the radio button to convert to either **SLDYZ**, **SLDY** or **SLD.** New slides will be created containing the respective images as selected. The file name will need a suffix, by default this is *OriginalFileName_CNV*
Auto Save makes sure the file is immediately saved after conversion.

3 Apply & Close buttons

Click ***Apply*** to start the process or ***Close*** to leave the window without action.

c. It will create (a) new slide(s) with the selected suffix including selected images respectively.

Contacting Intelligent Imaging Innovations Inc

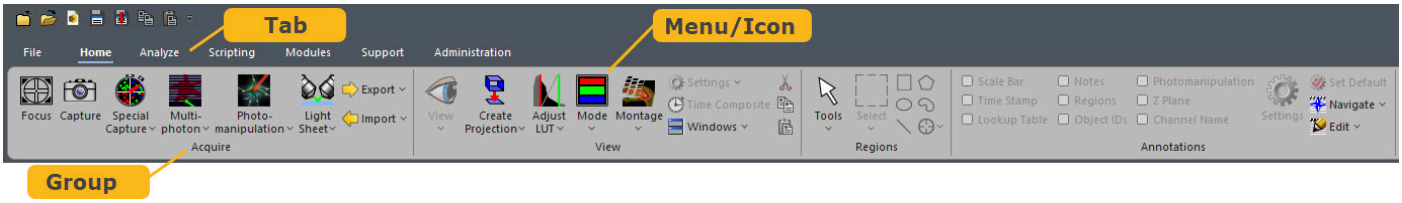
If you have any questions or experience any problems with SlideBook™, please contact our support team:

By Phone: 303.607.9429 ext.2
By Email: support@intelligent-imaging.com
On the web: https://www.intelligent-imaging.com

Help file last edited 8/30/2022

User Interface

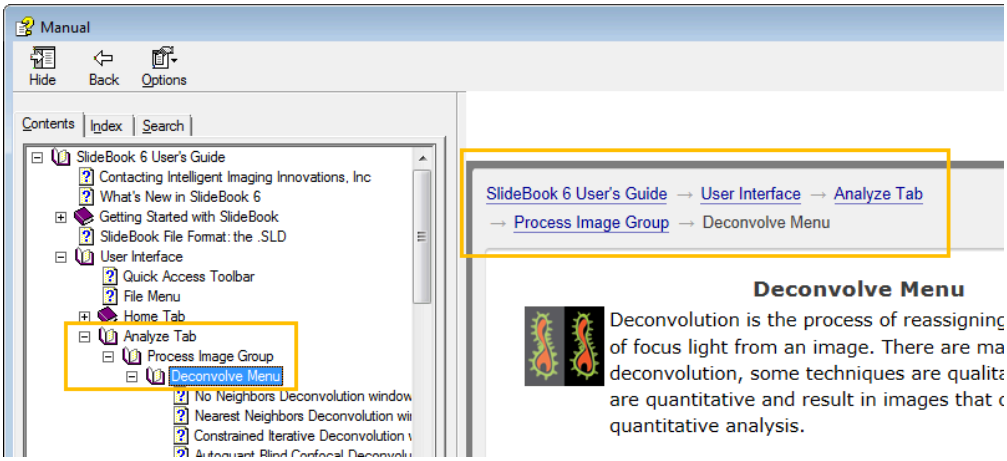
SlideBook is organized using a graphical menu and icon system. There is a customizable ***Quick Access toolbar*** across the top of the application with the rest of the program functionality being found in a tabbed system. The tabs are designed to group functionalities and improve workflow while minimizing the number of clicks required to accomplish tasks.



Terminology used in the manual refers to location by tab and group and often includes an image of the icon. A main portion of this help system is the User Interface section. User interface pages are organized in a structure that mimics the actual user interface:

Tab > Group > Menu/Icon > Function

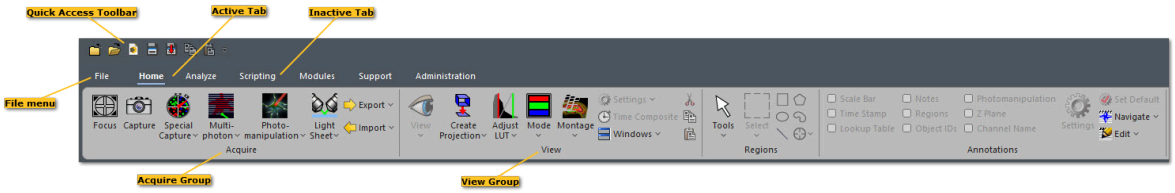
When you are on a specific user interface page of the help system, the location of the page can be found in the organizational tree on the left side of the Help pane, or across the top of the window.



Tabs are the largest organizational feature of SlideBook. Tabs available include:

File - execute normal file functions such as open, close, create and save **.sld** or **.sldy**
Home - control hardware to set up and acquire images, import and export images, view images using basic and advanced techniques, and access region tools and annotations.
Analyze - deconvolve and filter data, perform mask generation and analysis, graph, particle track, smooth curve analysis and analyze using other techniques
Scripting - execute, design, and edit existing scripts
Modules - access purchased analysis modules such as FRET, Ratio, FLIM, and Stereology
Support - easily update software and key, backup configuration, contact 3i technical support
Administration - resource for system administrators that includes setup, maintenance, and configuration tools

SlideBook window



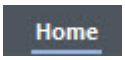
1 Quick Access Toolbar

The Quick Access Toolbar contains frequently used functions and can be customized to suit your needs.

2 File menu

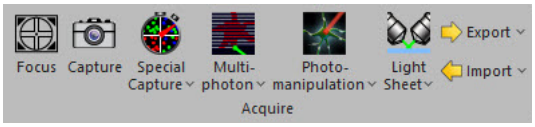
The ***File Menu*** contains common file menu commands such as ***New Slide***, ***Open Slide***, ***Close Slide***, ***Save Slide***, and ***Save Slide As***. You also have access to printing functionality from this menu.

3 Active Tab



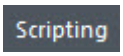
The currently selected tab is highlighted and underlined.

4 Acquire Group



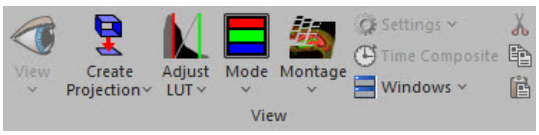
Groups are collections of buttons that share functional similarities. In this example, we show the Acquire Group, which is used to acquire images.

5 Inactive Tab



Inactive tabs are subdued text.

6 View Group

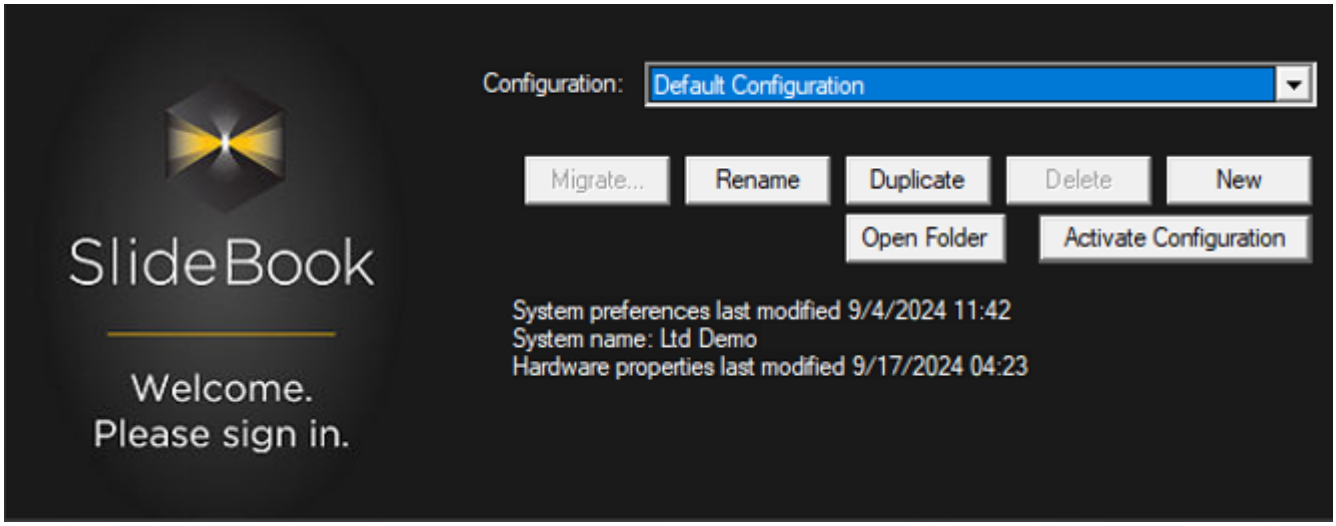


Access to major image display options for viewing, creating projections, changing LUT and display mode, montage as well as window alignment.

Multi-Preference Manager

Depending on individual user requirements SlideBook can be configured with individual hardware properties or preferences. When the SlideBook Multi-Preference Manager is enabled a user can select between default or alternative configurations for their system.

Upon opening SlideBook and the Multi-Preference Manager Interface will open and the drop down will display the last used configuration.



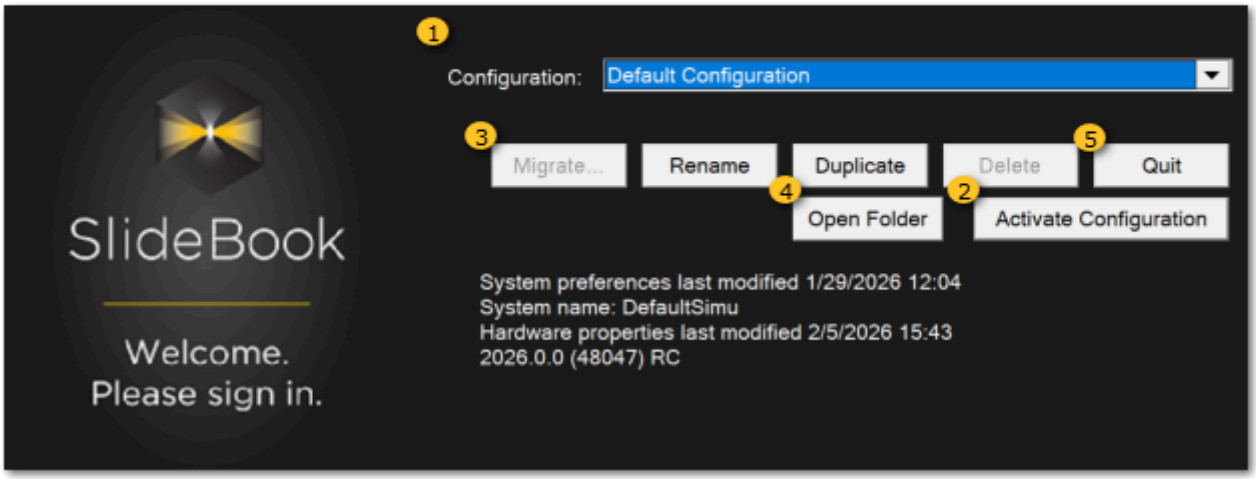
1. Select the desired preference configuration in the drop down
2. Select ***Activate Configuration***
3. SlideBook will initialize and display the selected configuration in the lower right corner as "Prefs: (configuration name)"

NOTE: If you selected the Default Configuration "Prefs: (configuration name)" will not show

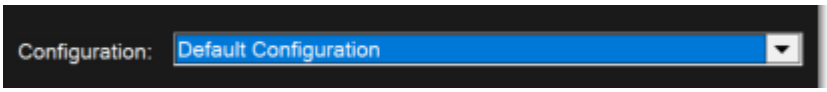
See also:
[The Multi-Preference Manager Window]
[Enabling the Multi-Preference Manager]

The Multi-Preference Manager window

When the SlideBook Multi-Preference Manager is enabled the Multi-Preference Manager interface will open upon starting SlideBook.



1 Current Selected Configuration



The interface will display the last used configuration. Alternative configurations can be selected from the drop down.

2 Activate Configuration

To initialize SlideBook with a selected configuration click the ***Active Configuration*** button. If an alternative (not the default) configuration is selected SlideBook will display "Prefs: (configuration name)" in the lower right corner upon initialization.

3 Manage Configurations

Migrate allows to import configurations preference files from an older version of SlideBook (before 2024.0)
Rename allows to change the name of an alternative configuration
Duplicate allows to duplicate the preference and configuration files of a currently selected configuration in order to create a new alternative configuration. One can then alter this new configuration individually.
Delete allows to delete an alternative configuration if no longer required. It will remove it from the active alternative configurations but not erase is completely. **NOTE:** Deleting a configuration will require entering a password - please contact 3i Support for help.

4 Open Folder button

Opens the directory of stored configurations.

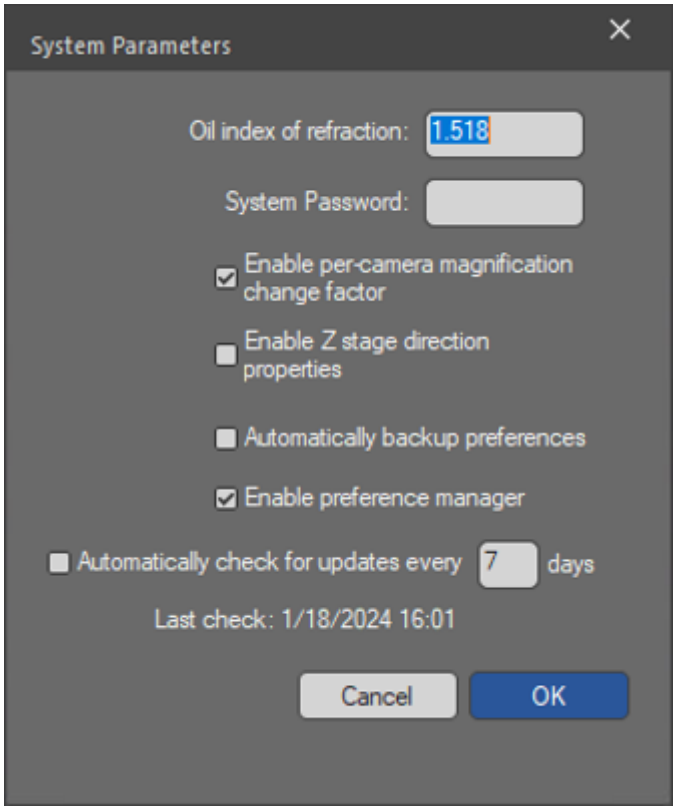
5 Quit button

Exits the program without a selected configuration.

Enabling the Multi-Preference Manager

The Multi-Preference Manager interface will only open upon starting SlideBook if it is enabled as follows.

To enable the Multi-Preference Manager in SlideBook
1. Go to the Administration Tab
2. Select System Parameters
3. Select the tickbox for Enable preference manager
4. Then restart SlideBook.



File Menu

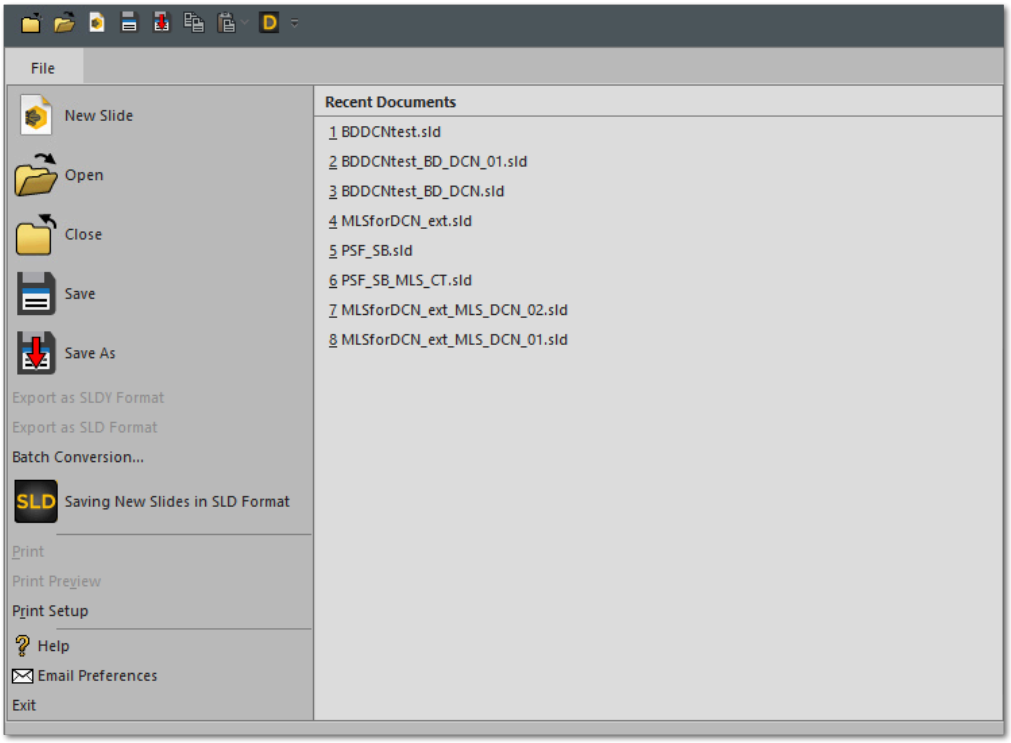
The ***File Menu*** contains common file menu commands such as ***New Slide***, ***Open Slide***, ***Close Slide***, ***Save Slide***, and ***Save Slide As***. You also have access to printing functionality from this menu.

With ***Export as SLD/SLDY*** you can Export **.sld** into **.sldy** or **.sldyz** files or **.sldy** into **.sld** or **.sldyz** if you highlighted the captures in the current slide. ***Batch Conversion...*** is especially useful to convert many slides to lossless compressed (**.sldyz**) files.

When ***Saving New Slides in SLD Format*** is shown, newly created files will be saved in **.sld** format, the the icon '***D***' in the menu bar will indicate this as well.
When ***Saving New Slides in SLY Format*** is shown, newly created files will be saved in **.sldy** format, the the icon '***Y***' in the menu bar will indicate this as well.

There is also a link to the SlideBook F1 Help (***?***) document and to upload your preferences, log or .dmp files per ***Email Preferences***.

At the right of the menu is a list of recently opened slide files for re-opening convenience.



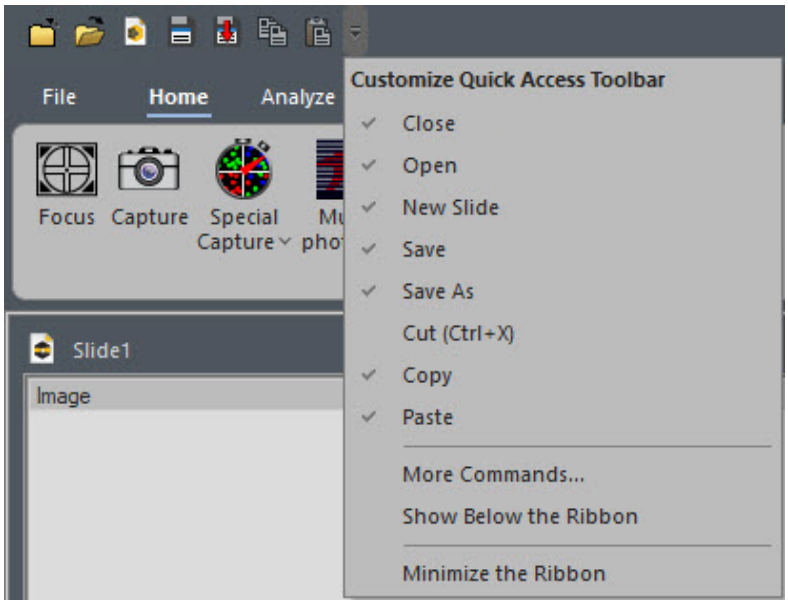
Quick Access Toolbar

The Quick Access Toolbar persists regardless of the active tab. It contains frequently used functions and can be customized in several ways to suit your needs.

Use the dropdown list to customize the toolbar.

Right click on any icon or group shown in the ribbon and select ***Add to Quick Access Toolbar*** to expand functionality.

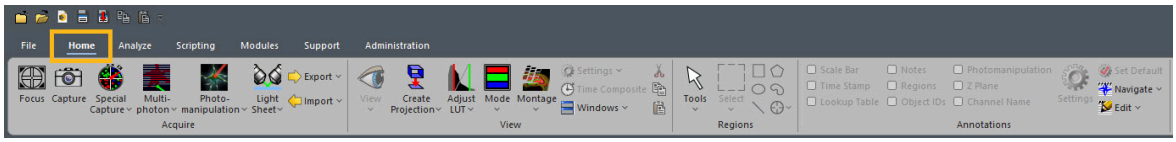
Minimize the Ribbon will hide the ribbon interface when it is not in use. When you click on a tab, the ribbon will re-appear. To end this behavior, uncheck the selection by clicking on it.



Home Tab

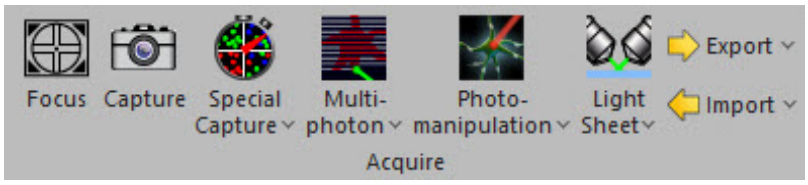
The Home tab contains access to the tools necessary to:

- setup and execute experiments, from basic to very complex designs
- import and export data
- view and adjust the display of images
- create and edit regions
- perform basic measurements
- add and edit annotations



Acquire Group

The ***Acquire*** group of the ***Home*** tab provides access to the ***Focus Controls*** and ***Capture*** window as well as advanced capture setups and complex laser options. It is in this group, that you have the opportunity to import and export data and images in multiple formats.

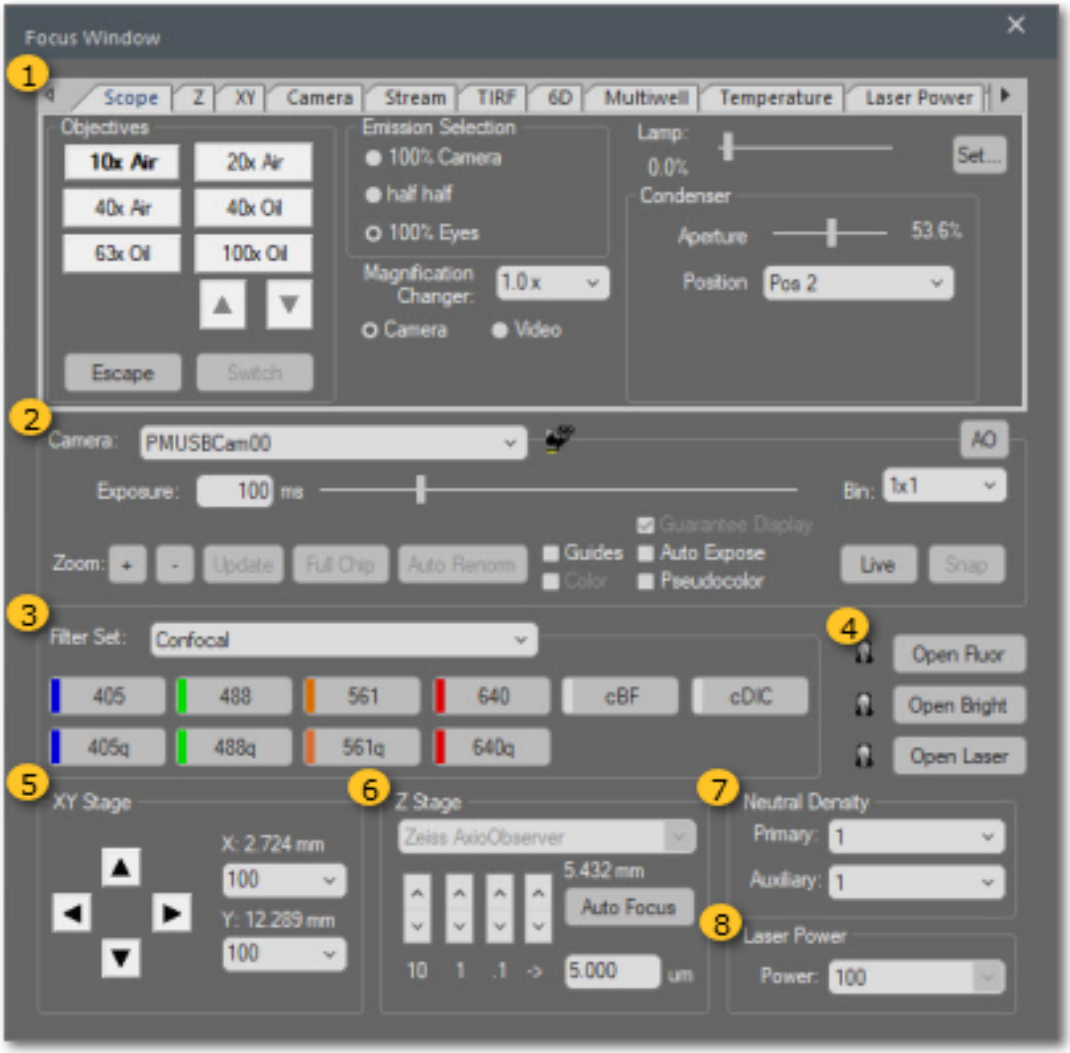


Focus Controls Window

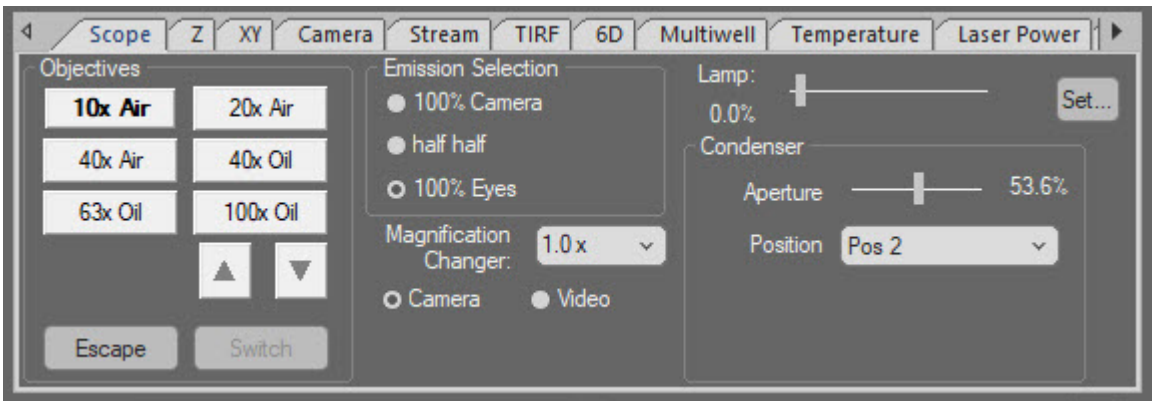
The ***Focus Controls*** are used to set up a variety of parameters before image capture. You may use these controls to help position your specimen, mark the *z* range for capture, calibrate FRAP or a spherical aberration correction (SAC™) device, or set up a point visiting list. The top portion of the window (area 1) is tabbed to allow selection of specific device control. The bottom portion of the window (areas 2-8) remains static to allow control of commonly accessed hardware controls.

See also:

- Scope Tab of Focus Controls
- Z Tab of Focus Controls window
- XY Tab of Focus Controls
- Camera Tab of Focus Controls
- Stream Tab of Focus Controls
- TIRF Tab of Focus Controls
- mSAC Tab of Focus Controls
- 6D Tab of Focus Controls
- Laser Tab of Focus Controls
- LaserStream Tab of Focus Controls
- Using AutoFocus in SlideBook
- Phasor Tab of Focus Controls
- Calibrating Vector in SlideBook



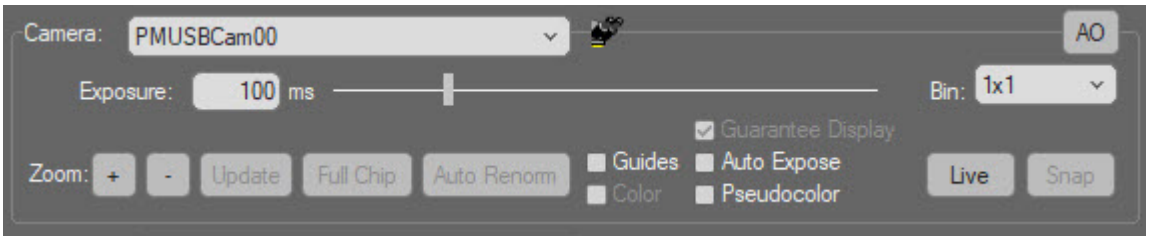
1 Tabbed Controls



The ***Tabbed Controls*** allow control of individual components of the imaging system. Depending on hardware configuration some or all of the following tabs may be present:

Scope, Z, XY, Camera, Stream, Photomanipulation, Photomanipulation Alignment, TIRF, Laser, Multiwell, Focus, 6D

2 Basic Camera Control



Camera: For multi-camera systems, select the camera you would like to use from the dropdown menu.

Exposure: Type an exposure time (ms) in the text field or use the slider bar to control the exposure time in the live ***Camera*** window. There is a limit of **500** ms using the slider bar; this value can be increased when necessary by typing in the edit field. It is important to note that this exposure time is only used in the live ***Camera*** window and exposure times for image capture will be set in the Capture dialog.

Bin: Select a bin factor from the dropdown menu. Binning amplifies signal while decreasing resolution by combining the signal of adjacent pixels before readout. This is useful in the ***Focus Controls*** because it allows shorter exposure times and faster image refresh rates, ultimately making it easier to find a sample and bring it into focus. ***Bin*** factor adjustments are used only for optimizing image display in the live ***Camera*** window and can be set independently in the ***Capture*** dialog.

Zoom: The + - ***zoom*** buttons can be used to zoom in and out on the image in the live ***Camera*** window.

Update - Make a selection using the ***marquis*** tool then click update to zoom to a region of interest in the live ***Camera*** window. Note that this region will not be carried into the ***Capture*** dialog but can be selected at that time for image capture.

Full Chip - Reverts to the full camera chip after zooming or selecting a region and updating in the live ***Capture*** window.

Auto Renorm - When the intensity scaling is set to ***Manual*** in the ***Camera*** tab of ***Focus Controls***, pressing ***Auto Renorm*** will reset the live ***Camera*** window to automatically renormalize the intensity scaling to the minimum and maximum values. Default behavior is for the live image to be automatically renormalized and this button to be inactive.

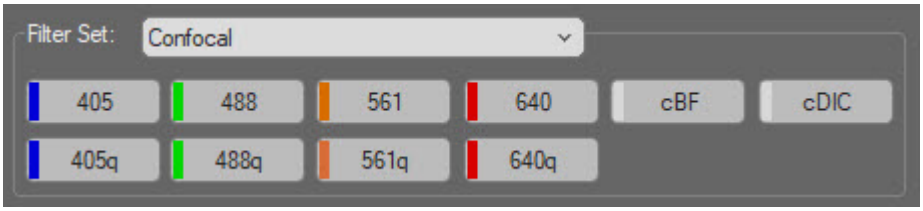
☐ Guides - Checking this box puts yellow guide lines on the live ***Capture*** window. There will be a crosshair in the center with corners marking central regions of 256x256, 512x512, and 1024x1024 pixels.

Color - Checking this box will display a color brightfield image in the live Capture window. Use of this feature requires specific hardware for capturing color images, such as an LCD color filter changer or color camera.

Start Stop - This button toggles to ***Start*** and ***Stop*** real time image viewing in the live ***Camera*** window by controlling the camera readout. In most cases, this will not affect the light being delivered to the sample; instead, the shutter buttons should be used to minimize exposure. In systems with a synchronized LaserStack™, this button will open/close the ***Alternate Source Shutter***.

Snap - Press ***Snap*** to capture a single image in the current channel and save it in the open slide. SlideBook will open the shutter for the associated filter selected and snap an image using the exposure time selected.

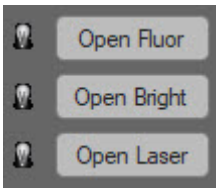
3 Channel Control



Select a ***Filter Set*** from the dropdown menu. Filter sets can be used to organize channels that are used for similar types of imaging such as **Confocal**, **TIRF**, **Widefield**, **Vector**, **Ratio**, or **FRET**. For more on this topic, please review Working with Filter Sets.

Select a channel by pressing the appropriate button. To direct light to the sample, open the associated shutter.

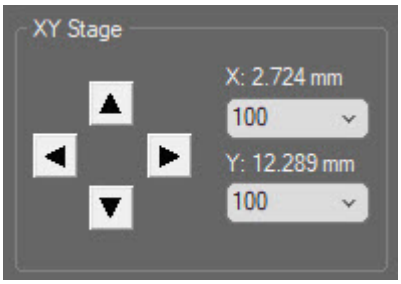
4 Shutter Control



The three shutter buttons correspond to shutters configured in ***Hardware Configuration*** for the ***Fluorescence Lightpath*** (reflected lightpath), ***Transmitted Lightpath***, and ***Advanced Lightpath*** (usually a laser light source) respectively. Shutters are associated with channels in the ***Light Source*** section of the ***Filter Configuration Parameters*** dialog. When a shutter is open, the light icon will turn on .

If you would like your fluorescence shutter to close automatically when you close the Focus window, select ***Edit > Hardware Properties***, then expand the ***Focus Window*** section. Click on ***ShutterUponClose***.

5 XY Stage Control

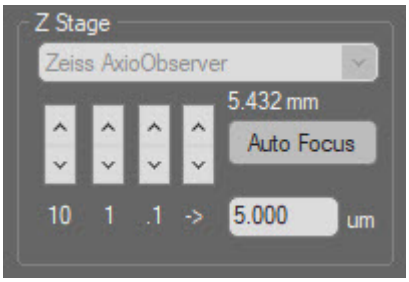


On systems equipped with a motorized *xy* stage, you can use the directional buttons to move the stage in increments specified by the value in the adjacent edit field. This value is always given in microns (or fractions thereof). You can change this increment distance at any time. The defaults are **100**, **10**, **1** and **FOV** (Field of View is an integer value defined by the pixel array of the CCD) which can be selected from the dropdown menu.

The current *x* and *y* positions read from the stage are also displayed in this section.

TIP: If you double-click on an object in the image display, the *xy* stage will move so that the object is centered.

6 Z Stage Control

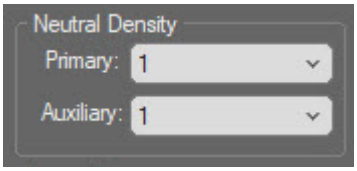


On systems equipped with motorized *z* focusing, you can move the focus up and down using the directional buttons. There are three sets of buttons with predefined stage movement increments (**10**, **1**, and **0.1** microns). In addition, an user-defined stage movement increment value can be entered in the edit field below the last set of buttons on the right. The default value of the edit field will be set to the theoretical *z* resolution for the selected objective.

With few exceptions, the up and down buttons correspond to the direction of mechanical movement. For instance, on an upright microscope with either an integrated or external stepper motor, the direction corresponds to stage movement, so "up" means focusing further past the coverslip and towards the slide since the stage is moving up. On an inverted microscope, "up" corresponds to moving the nosepiece up, and therefore also means focusing further past the coverslip.

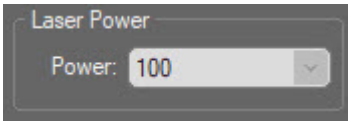
The ***Auto Focus*** button is used to define and test parameters for auto focusing during capture. When you press this button, the Auto Focus window will open.

7 Neutral Density Control



If you have defined ***ND Selection*** and ***Auxiliary ND*** in ***Hardware Configuration***, you may select the position from the ***Neutral Density Primary*** and ***Auxiliary*** dropdown menus. The default value of **100** corresponds to 100% light throughput, a value of **1** corresponds to 1% light throughput.

8 Laser Power Control



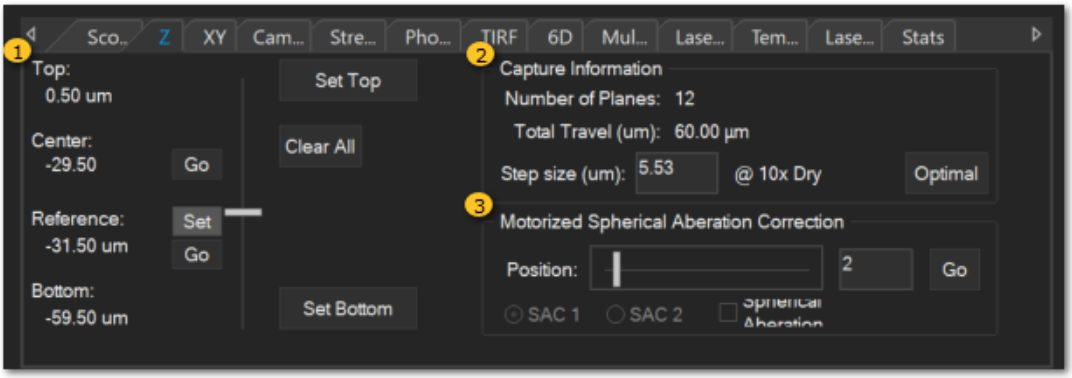
If a laser is being used as the light source, the power can be controlled by this dropdown menu. The default value of **100** corresponds to 100% laser power, a value of **1** corresponds to 1% laser power.

Z Tab of Focus Controls

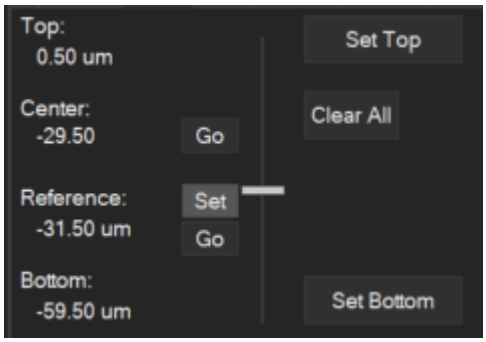
The ***Z Tab*** of Focus Controls window allows you to set up *z* positioning for 3D (or 4D) image acquisition. This dialog also provides information on optimal sampling in *z* and allows control of motorized SAC (Spherical Aberration Correction) devices. This information will be used in the Capture dialog.

See also:

3D Capture group of Capture window



1 Z Range Setup



Navigate to the top of the *z* stack you would like to capture by using the Z Stage Controls in the focus window. Press ***Set Top***.

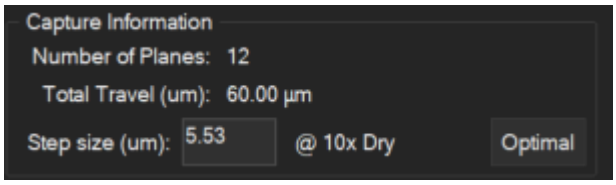
Navigate to the bottom f the *z* stack you would like to capture by using the Z Stage Controls in the focus window. Press ***Set Bottom***.

Press ***Go*** (next to ***Center:***) to go to the center of the defined *z* range.

Reference: Set allows you to ***Set*** a reference *z* position for the point of best focus, or some other position that is of importance for your experiment. Once you set this reference position, it may be used for 3D capture. To visit the reference position, press ***Reference: Go***. This position will be saved until you reset the position or close SlideBook.

Clear All will clear all set *z* positions.

2 Capture Information group

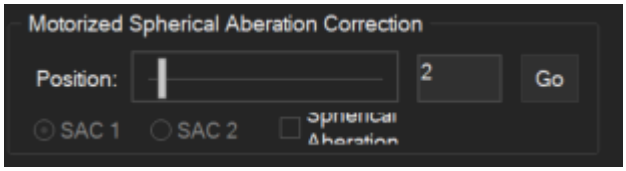


Press ***Optimal*** to calculate the optimal *z* ***Step size*** for image capture (utilizes Nyquist sampling of the theoretical axial resolution of the objective).

Alternatively, type desired *z* ***Step size*** in the text edit field to set this value manually.

Number of Planes and ***Total Travel*** will be automatically calculated from ***Top*** and ***Bottom*** positions and ***Step size***.

3 Motorized S.A.C. group



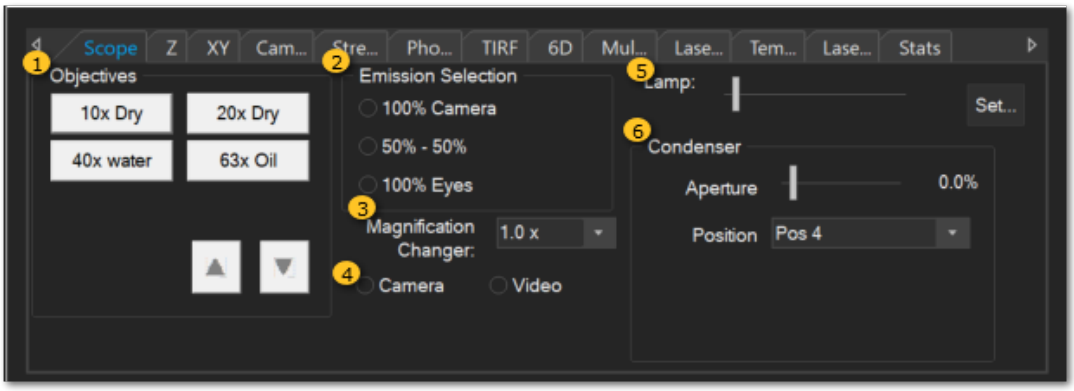
To move the Motorized Spherical Aberration Correction device, move the slider or type a value in the text edit field and press ***Go***. For calibration of the SAC, go to the ***SA Correction*** tab of ***Focus Controls***.

Scope Tab of Focus Controls

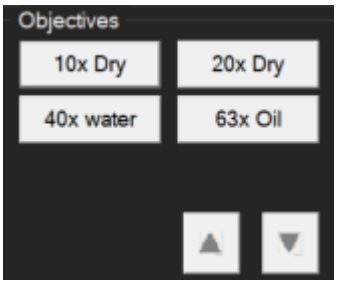
The ***Scope Tab*** of Focus Controls window allows you to control microscope imaging components such as objective, imaging port, optovars, and brightfield imaging techniques. This information will be used in the Capture dialog.

See also:

- Magnification Changer Parameters window
- Configure Objective Lenses window
- Optics Group of Capture Window



1 Objectives group

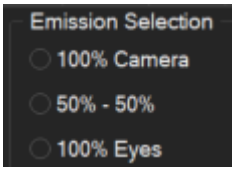


On systems with a motorized objective turret, you can move to a different objective by selecting the corresponding button. (For information on adding objectives to those defined for your system, please refer to the Configure Objective Lenses window).

If you try to move between objectives that use differing immersion media, such as an oil objective to an air objective, SlideBook will bring up a dialog box warning about the pending move.

> **CAUTION:** On most systems with motorized objective turrets, you can safely move between objectives of similar immersion media (e.g., air, oil). However, there are situations where you will definitely not want to move between objectives automatically. For instance, if you are using a water immersion objective on an upright microscope, where the objective is dipped into the chamber, trying to move the objective could break the chamber or damage the motorized turret.

2 Emission Selection group



On systems with a motorized emission light path, these radio buttons let you choose between letting the light go 100% to the camera, 100% to the oculars (eyepieces), or to 50% each (if available). The ***50% - 50%*** option is not desirable for capturing images as the sample is exposed to twice the light necessary for imaging; however, this mode may be useful for teaching purposes.

You may also provide custom labels for the emission selection positions (ocular photo prism positions). To do so, select ***Administration tab > Hardware Properties***, then expand the ***Focus Window*** section. Click on the ***Ocular Photo Prism Pos*** you wish to edit and enter the label in the edit field. Click ***Apply Change***, ***Close***, and restart SlideBook.

3 Magnification Changer

On systems with an encoded magnification changer, this field reports its current position. ***Magnification Changers*** can be configured in the Magnification Changer Parameters window.

4 Camera/Video option

There are two motorized prism controls in the scope: ocular/photo and camera/video. The ocular/photo is a three position prism as described above in the Emission Selection group and camera/video is two position prism. The default labels refer how Zeiss used to label it (film camera, video camera). However, most microscopes have multiple prisms that interact using this interface. You may customize the labels under ***Administration tab > Hardware Properties > Focus Window > Camera Video Prism Pos 1/2 Description***.

5 Lamp Controls

On microscopes with a computer controlled lamp voltage adjustment, the intensity of the brightfield illumination can be controlled using this slider. Moving the slider to the far left corresponds to turning the lamp off, while moving the button to the far right corresponds to turning the lamp to maximum intensity.

6 Condenser group

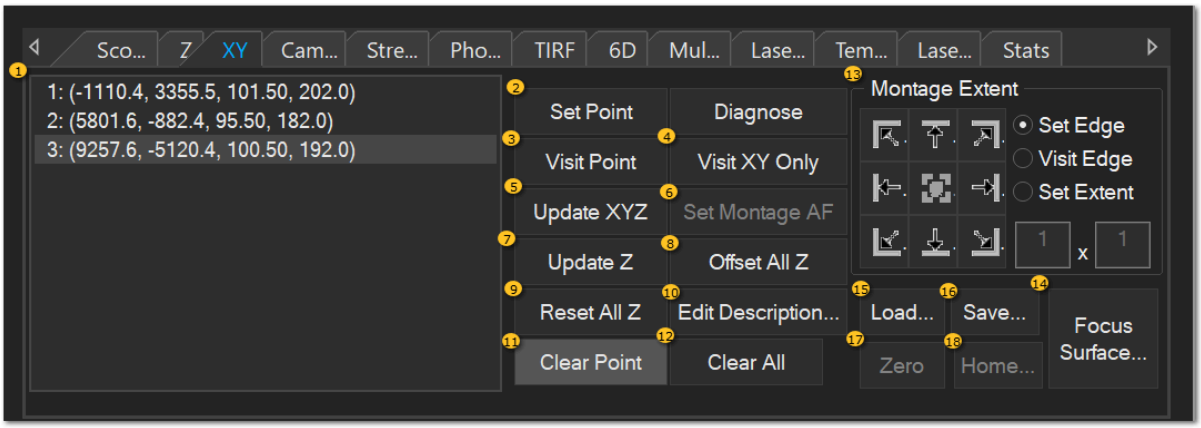
On microscopes with an automated condenser turret, you may select condenser positions from the drop-down menu.

XY Tab of Focus Controls

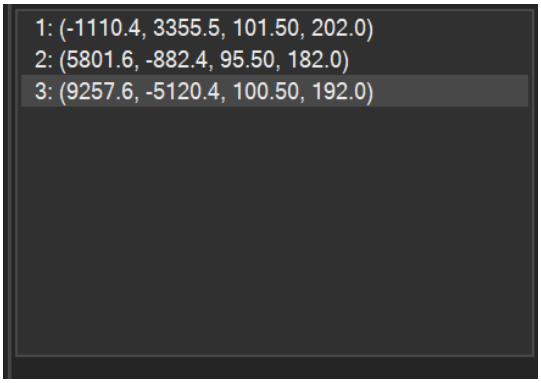
The ***XY Tab of Focus Controls*** allows the user to set up XY locations to visit during an experiment. This maybe part of a point visiting experiment that takes place over time (in 2D or 3D) or part of a montage in which images are acquired then assembled into one large image. Locations (or points) may be saved for future use. It is important to note that before you can save or load a pointlist, you must first ***Home*** the stage.

See also:

[TUTORIAL: Using 6D Scripted Capture](#)
[Capture Window: Multiple XY Location Capture Group](#)
[Multiwell Tab of Focus Controls](#)



1 XYZ Points list

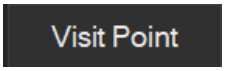


Displays *x*, *y*, and *z* coordinates of points. Selected point is highlighted in blue. A selected point may be visited, updated, or deleted.

2 Set Point button

Add the current stage location coordinates to the point list.

3 Visit Point button

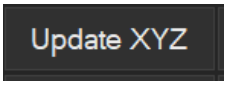


Visit the currently selected point.

4 Visit XY Only button

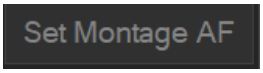
Visit the *xy* location of the selected point and ignores the *z* coordinate.

5 Update XYZ button



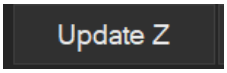
Update the *xyz* coordinates of the selected point with the current *xyz* stage coordinates.

6 Set Montage AF button



Currently a beta feature only available to software keys coded for beta features. When components of a montage have been defined, will convert the full area to a series of *xyz* points rather than just the existing extents in order to utilize hardware-based autofocus.

7 Update Z button

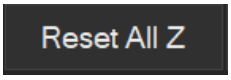


Update the *z* coordinate of the selected point with the current *z* location.

8 Offset All Z button

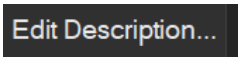
When the z focus position has been changed, will offset all z coordinates by the amount changed. The workflow is to visit a location, adjust the focus in the focus window using the up and down arrow buttons, then click ***Offset All Z***. A message will appear asking if you would like to adjust the focus position of all points by *N* microns, click ***Yes*** to continue or ***No*** to abandon the operation.

9 Reset All Z button



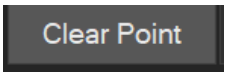
Reset the *z* coordinate of all points with the current *z* position.

10 Edit Description... button



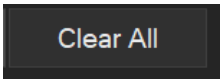
Open a text edit field that allows naming of the location. This will be used in front of - **Position N** in image naming in the **.sld**.

11 Clear Point button



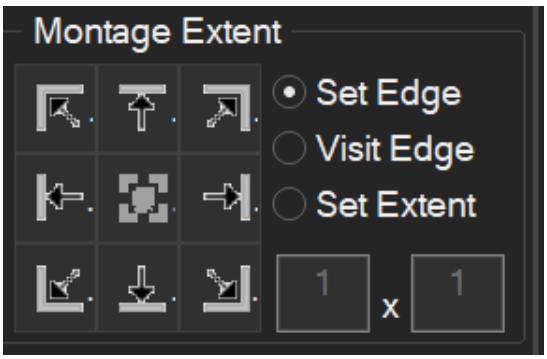
Remove the currently selected point from the list.

12 Clear All button



Delete all points from the list.

13 Montage Extent group



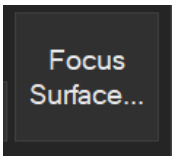
Set a point then use the ***Montage Extent*** buttons to set the point as one of the montage extents. You may use any combination of diagonally opposite corners or corners and edges as long as an extent is defined in each direction.

Use ***Set Edge*** to define a montage by either 2 diagonal corners for a plane that uses the same z in each image or by a 3-point slope interpolation technique. Slope interpolation works best when 3 corners are used (ex. UL, UR, LR). Z positions will be interpolated from a plane that is fit through all 3 points. Before you set up the montage, be sure to select ***Capture > Advanced > Focus > Enable 3 point Z interpolation for montage capture***.

> **NOTE:** This option will be greyed out if you have set any points in the XY tab of Focus Controls. Delete any existing points and try again.

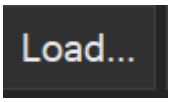
The ***Set Extent*** button enables the boxes where you can define the number of frames in x and y. For example, if you set the extent value to 2 x 2, and then press the (dot) button in the center, you should get a 2 x 2 montage centered at the current location. Similarly, if you press the upper left button you should get a 2 x 2 montage with the current location as the upper left anchor.

14 Focus Surface button



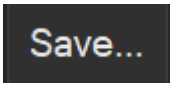
Opens the Focus Surface dialog.

15 Load... button



Load a previously saved point list. Only available after stage has been homed.

16 Save... button

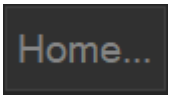


Save the point list. Only available after stage has been homed.

17 Zero button

Zeroes the stage at its current position.

18 Home... button



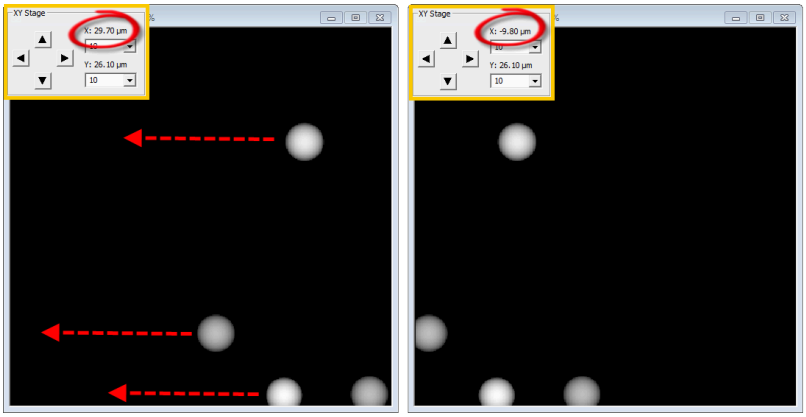
Homes the stage. That is, moves the stage to visit the limits of travel and set the true zero position. Homing the stage enables point lists to be saved and loaded. Before homing the stage, you should ensure the stage is free of all obstructions and clear of objective lenses.

TUTORIAL: Setting the XY Stage-Camera Relationship

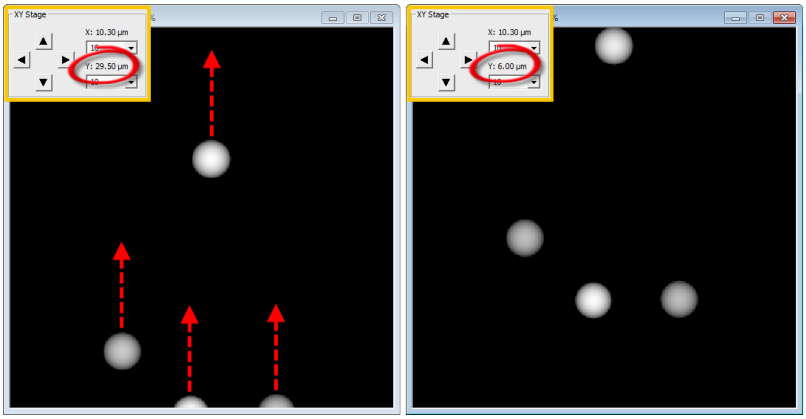
The easiest way to confirm the stage-camera relationship is configured correctly is to double click on an object in the live camera window. If the object is centered in the view, everything is configured correctly. If the object moves in the wrong direction, you may need to adjust ***XY Stage Properties*** as described below. If the object moves too far or too little, you may need to adjust the pixel size for the objective or set a per-camera magnification factor.

1. Bring a sample into view and focus using the Focus Controls.

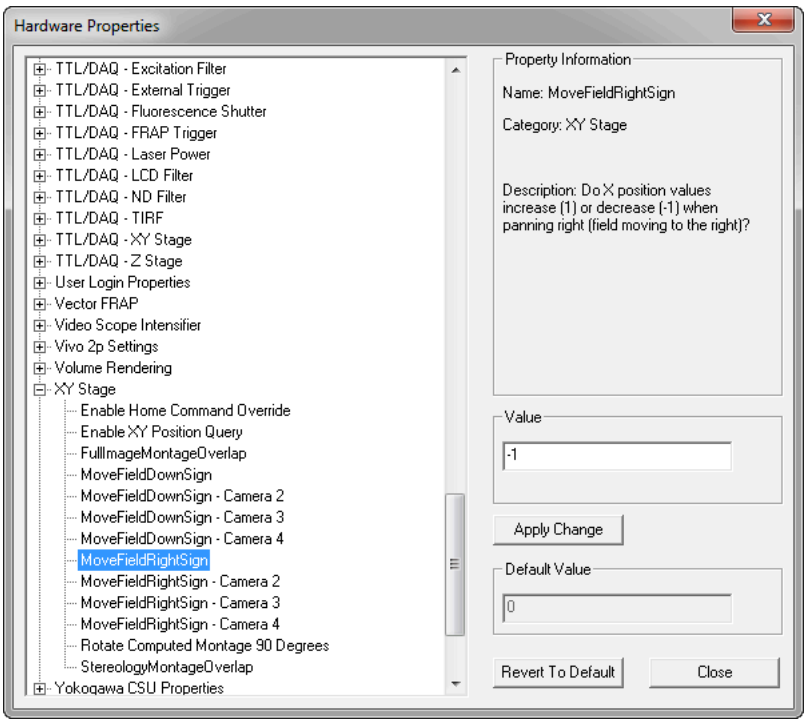
2. Using the joystick, move the stage so that the image on the screen moves to the left (in other words, pan and move the image field to the right). When panning to the right, notice whether the x coordinate position in the ***XY Stage*** section of the ***Focus Controls*** window increases or decreases. In the example below, the x value is decreasing. If the x value decreases, you will use **-1** for the ***MoveFieldRightSign*** hardware property discussed later in this procedure. If the x value increases, you will use **1**.



3. Repeat for the y axis by panning down so that the image appears to move up. Note the change in the y axis coordinate position (increasing or decreasing) when panning. In the example below, the y value decreases. If the y value decreases, you will use **-1** for the ***MoveFieldDownSign*** hardware property discussed later in this procedure. If the y value increases, you will use **1**.



4. Select ***Administration tab > Hardware Properties***. The following ***Edit Hardware Properties*** dialog box will appear. Click on the **+** sign next to **XY Stage** to expand the list.



5. Click on the item in the list titled ***MoveFieldRightSign***. The right side of the dialog box will be updated. Enter the appropriate value (**1** or **-1** based on the change in x position values when panning right) in the edit field and select ***Apply Change***.

6. Click on the item in the list titled ***MoveFieldDownSign***. The right side of the dialog box will be updated. Enter the appropriate value (**1** or **-1** based on the change in y position values when panning down) in the edit field and select ***Apply Change***.

7. If there are multiple cameras on the system, you may repeat for each camera ensuring that the correct camera is selected in ***Focus Controls*** and when setting the ***XY Stage*** properties.

8. Close the dialog box using the ***Close*** button and restart SlideBook when prompted.

9. Test your calibration by double-clicking to center an object in live ***Camera*** window.

TUTORIAL: Collecting a Montage

(Optional) Enable z-Tilt Compensation for the Montage

When collecting a montage, images need to be in a contiguous plane of imaging. For this reason, autofocus is not used during montage acquisition. Instead, you can configure SlideBook to use the z position at 3 corner extents of the montage and calculate the precise z plane for each tile of the image based on this information.

1. Open the ***Capture*** window from the Home tab.

2. Click ***Advanced*** in the upper right of the window.

3. Go to the ***Focus*** tab, check the box to ***Enable 3 point Z-interpolation for montage capture***.
   NOTE: This option will be greyed out if you have set any points in the XY tab of Focus Controls. Delete any existing points and try again.

4. Click ***OK***.

Define the Montage Area

1. Open the ***Focus*** window from the Home tab.

2. Select the objective for making the montage.

3. Scan the specimen to identify the extent of the region to montage.

4. Open the ***XY tab*** in the Focus window.

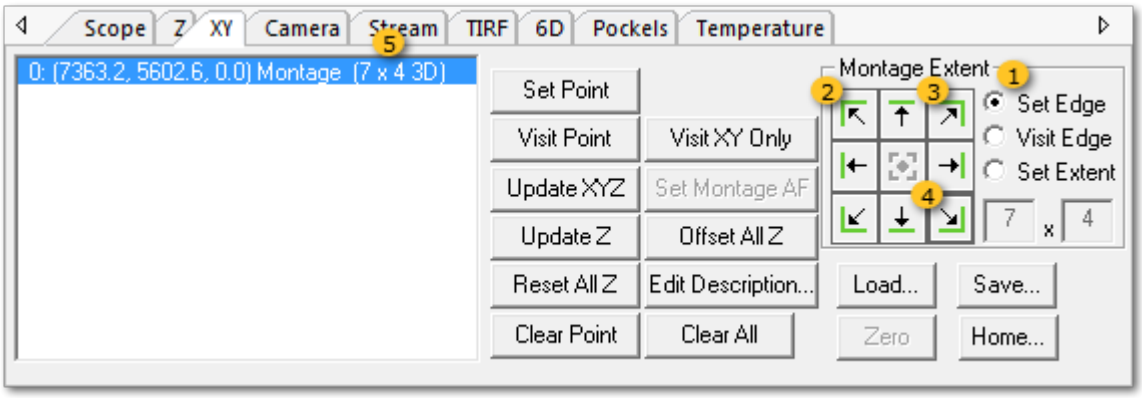
5. Make sure that ***Set Edge*** option is checked in the ***Montage Extent*** group (1 below).

6. Move to the first corner of the area, focus to an identifiable focus position.

7. Click the montage button corresponding to the position of the current field (2 below)

8. Move to each of two other subsequent corners of the area and click on their corresponding buttons (3 & 4 below).

9. The single line for the montage region will display the number for frames for the montage as well as ***3D*** when tilt compensation has been configured and *z* plane was changed in setting the montage extents (5 below)



Tips

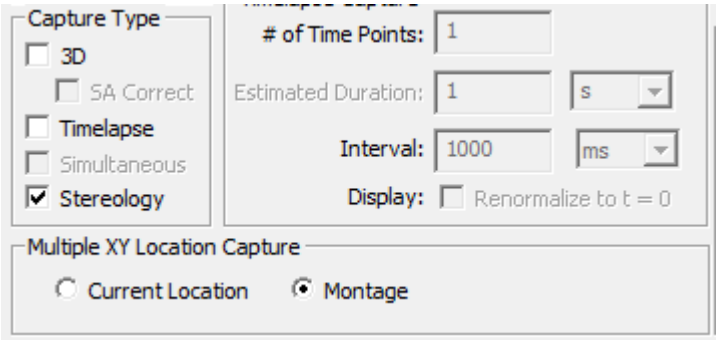
• Each corner must be set to the same focal level in the specimen in order to correctly calculate the degree of tilt for the specimen.
• The corners must be set in sequence, such as UL-UR-LR, or LL-UL-UR, etc.
• If you make a mistake, delete the montage with ***Clear Point*** and start over.
• It may be easier to focus if you enlarge the focus window image by clicking on the ***Zoom***: **+** button, far left, middle of focus. Return to 50% with ***Zoom***: **-**.

Capture Montage

1. Open the ***Capture*** window from the Home tab.

2. Select the channel(s) to be included, set the exposure time for each channel (for more information on this, refer to the Adjust Exposure group).

3. Select ***Montage*** from Multiple XY Location Capture.



4. Name the montage if desired, click on ***Start*** to capture the montage.

5. One image will appear in the slide file, it will be a "timelapse" of image tiles collected for the montage.

Generate the Montage

1. Select the image then click on ***Montage*** in the View group of the Home tab.

2. An subsampled preview montage image will open along with the ***Montage Generation*** window.

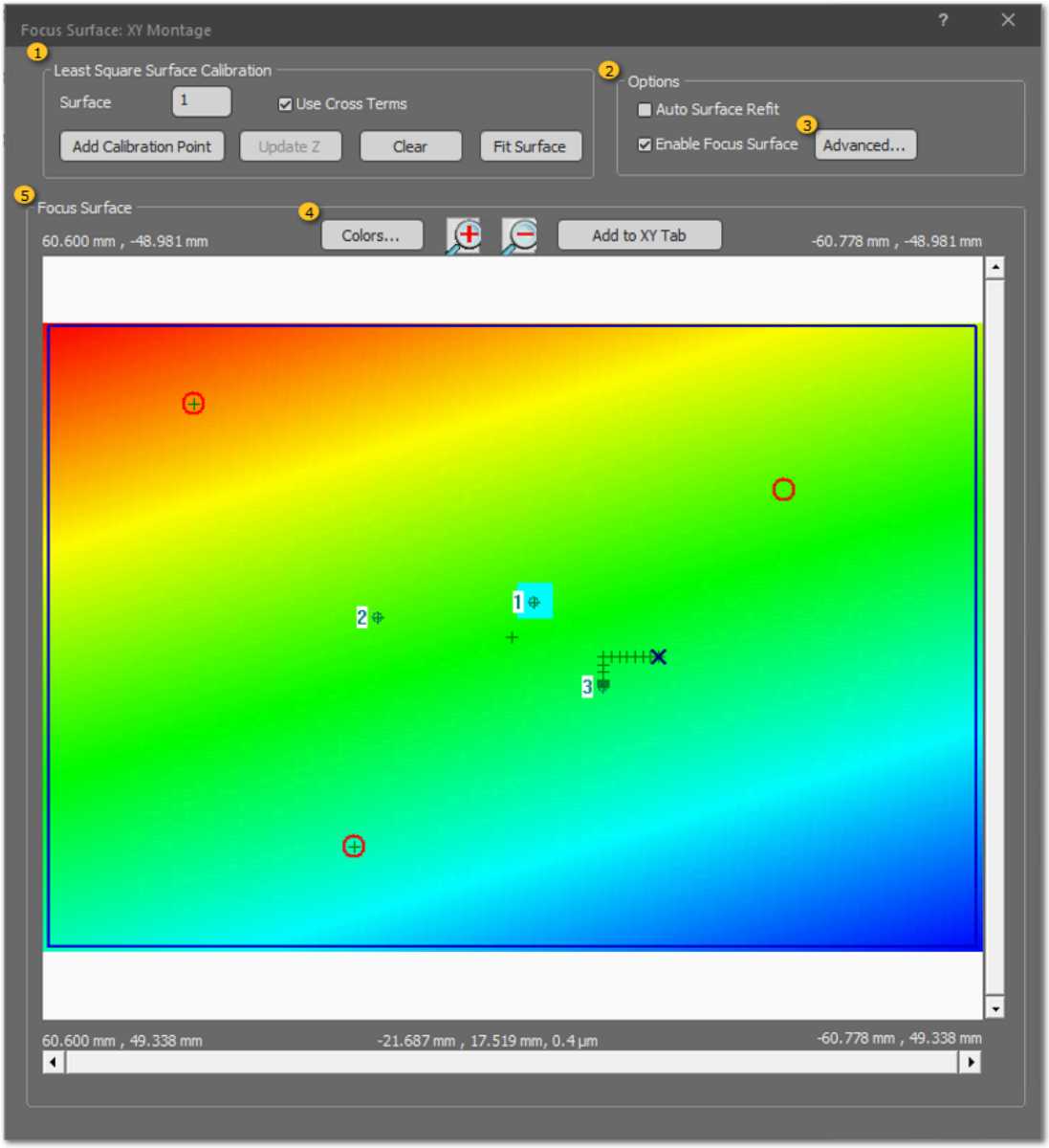
3. Manually adjust image alignment or use automatic functions as described in the Generate Montage window help page.

Focus Surface

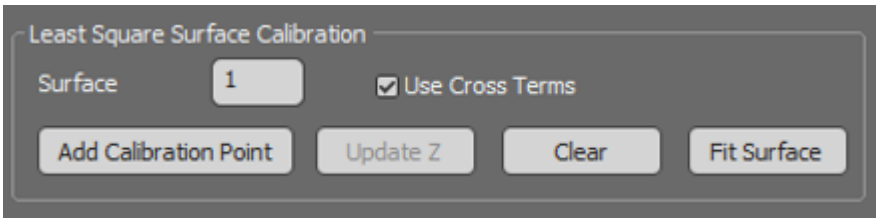
The ***Focus Surface*** dialog allows the user to define XYZ positions in space and then fit a surface to them to correct for non-planar deformities in the sample. It can be used in both XY and Multiwell modes.

See also:

[Capture Window: Multiple XY Location Capture Group]
[Multiwell Tab of Focus Controls]
[TUTORIAL: Setting the Stage Parameter defaults for Focus Surface]



1 Surface Calibration Group



Surface allows you to choose the type of fit that SlideBook will use to compute the surface. Currently only Least Square is supported.

Use Cross Terms will utilize cross terms (or off-diagonal terms) with multiple variables to fit the polynomial. Cross terms also requires more points to be added in the surface diagram.

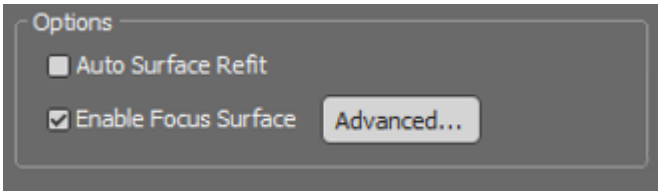
Add Calibration Point Adds the current xyz position to the focus surface fit equation.

Update Z Sets the Z position of a previously added point.

Clear Removes all points and the current fit from the Focus Surface.

Fit Surface Fits a surface to the added points and allows for z position interpolation at intermediate points.

2 Options group

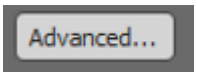


Auto Surface Refit Auto-enables and automatically refreshes the plot as new calibration points are added or points are deleted or edited.

Enable Focus Surface If a surface has been fitted, this checkbox will toggle it on and off.

Advanced... Opens the Advanced Focus Surface Settings window

3 Advanced Surface Focus Settings



Stage group allows the user to enter alternate stage bounds if required. Defaults can be set in the Hardware Properties.

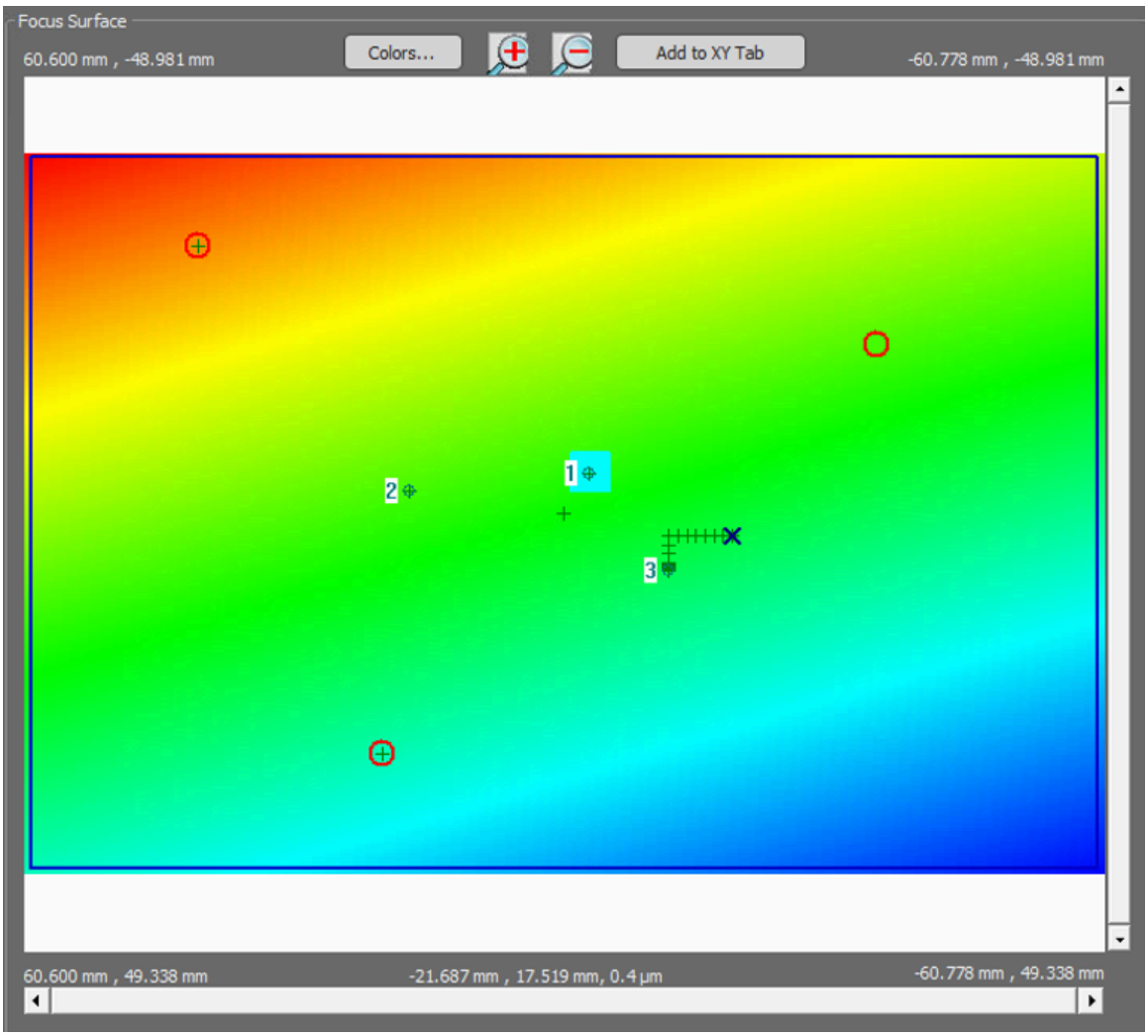
Invert X and ***Invert Y*** Users can toggle these to ensure that the orientation of the Focus Surface image is the same as that of the stage. Default settings are taken from the Hardware Properties and should only need change in rare situations.

Python 3D Surface Plot allows you to use python to visualize the surface. A suitable Miniconda environment must be installed and set in Hardware Properties.

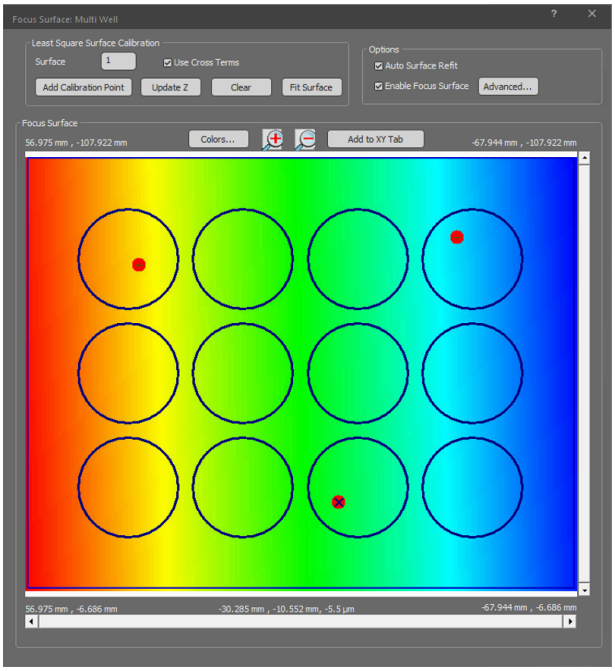
4 Visualization Tools

Colors opens a menu allowing users to customize the appearance of the position, point, and stage trace iconography. The ***magnifying glass (+)*** zooms in, and the ***magnifying glass (-)*** zooms out of the view with regard to the stage bounds.

5 Surface Diagram



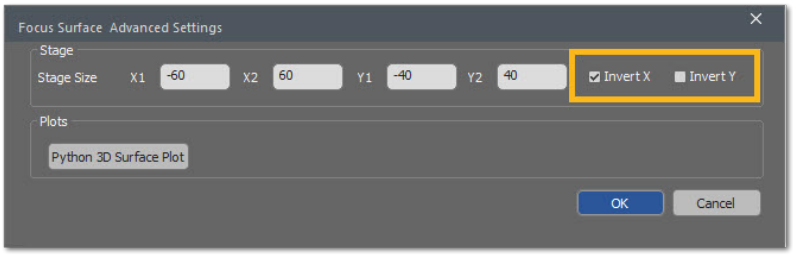
Once a surface is fitted, a colormap will be applied showing the gradients in the image.
The Surface Focus displays current stage position (X), previous stage positions (+) and the path of navigation as well as added points or montages (numbers).
X, Y and calculated Z position are shown in the lower center and updated when hovering the mouse over the Surface Focus area.
Clicking once on a location within the stage bounds (blue frame) will move the stage to that XYZ position.
For Montages the grid of FOVs in regard to the stage bounds is presented (zoom in for visualization). For multiwell applications open the Focus Surface from the Multiwell Tab, when the well plate is calibrated the wells will be shown as well.


Focus Surface: Multi Well
Least Square Surface Calibration
Surface
1
Use Cross Terms
Add Calibration Point
Update Z
Clear
Fit Surface
Options
Auto Surface Refit
Enable Focus Surface
Advanced...
Focus Surface
56.975 mm , -107.922 mm
Colors...
Add to XY Tab
-67.944 mm , -107.922 mm
56.975 mm , -6.686 mm
-30.285 mm , -10.552 mm, -5.5 µm
-67.944 mm , -6.686 mm

TUTORIAL: Setting the Stage Parameters for Focus Surface

There are three main prerequisites for Focus Surface to work as expected.
• The XY Stage-Camera Relationship must be set appropriately for the system and the stage bounds should be updated so that the representation of the sample within the stage is correct.
• The X and Y movement of the stage should be reflected correctly. This can be checked within the Focus Surface window.

1. When opening the Focus Surface the current position will be indicated (X).
2. Use the joystick and move to the left or right and then up and down on your sample then confirm the indicator moves the same direction
3. No action needed if the movements are conform.
4. If one or both directions are indicated inverse navigate to the ***Options group*** > ***Advanced...*** button and invert the axis which is displayed incorrectly.



• Last but not least the stage bounds should be updated so that the representation of the sample within the space of the stage is correct. To alter the current stage bounds navigate to the ***Options group*** > ***Advanced...*** button.
The ***Stage group*** allows the user to enter alternate stage bounds if required.

Alternatively the default stage bounds can be set in the Hardware Properties:

1. Use the microscope touch pad or Escape button in the F***ocus window*** > ***Scope tab*** > ***Objectives group*** to bring the objective to load position (furthest away from the stage) while you move it to it's extreme positions

2. Move the stage so that the objective points to the utmost imageable position in the back left corner of the stage

3. From the XY stage controller display and note down the values for X and for Y

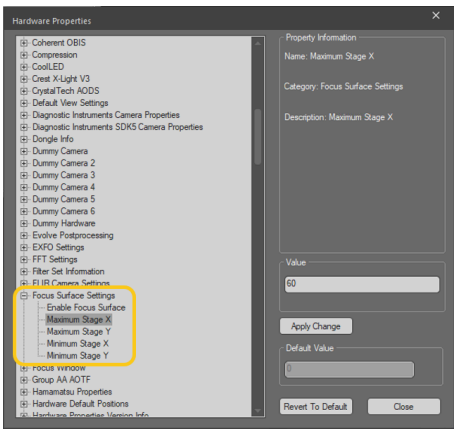
Example: X = 56.9 (going to be "Maximum Stage X") and Y = -20.1 (going to be "Minimum Stage Y")

4. Move the stage so that the objective points to the utmost imageable position in the front right corner of the stage

5. From the XY stage controller display and note down the values for X and for Y

6. Once those positions are recorded return the stage to a central position

7. In SlideBook navigate to the ***Administration Tab*** > ***Hardware Properties*** > ***Focus Surface Settings***



a. Enter the respective <u>positive</u> values for X and Y into '***Maximum Stage X***' then hit ***Apply*** and '***Maximum Stage Y***' then hit ***Apply***

b. Enter the respective <u>negative</u> values for '***Minimum Stage X***' then hit ***Appl***y and '***Minimum Stage Y***' then hit ***Apply***

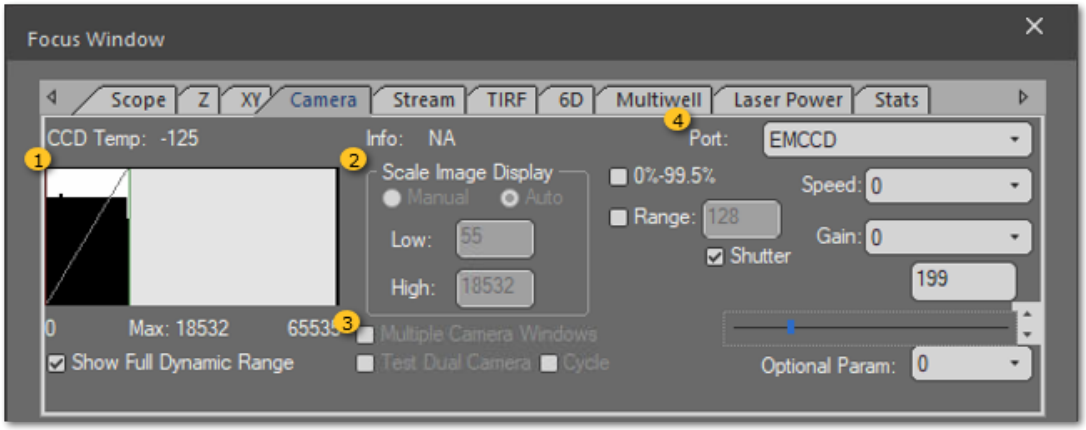
8. Click ***Close***

9. Please restart SlideBook to save those settings for future uses

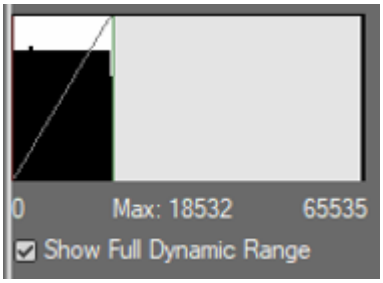
NOTE: Make sure the Maxima stage X and Y positions are positive values and the Minima stage X and Y positions are negative values. Should this not be the case for your system (e.g. Minimum Stage X = 2) set them to the closest positive or negative value of zero (e.g. set Minimum Stage X = -0).

Camera Tab of Focus Controls

The ***Camera Tab*** lets you control various settings on the CCD camera as well as view a histogram of the pixel intensities for the image in the focus window.



1 Histogram area



The histogram shows a plot of pixel intensities values for the focus window image and is continuously updated when the camera is actively acquiring images.

• Pixel intensity values are plotted on the x axis. Numbers below indicate the minimum (e.g. 0) and maximum (e.g. 65535) of the displayed dynamic range as well as the maximum intensity value of the current image (e.g. Max: 18532).

• The numbers of pixels at a given intensity (counts) are plotted on the y axis.

Show Full Dynamic Range checkbox. When this box is checked, the histogram will display minimum and maximum possible intensities based on your camera's readout. If it is unchecked the range will zoom in to the minimum and maximum intensities of the actual image.

2 Scale Image Display group

The histogram can be operated in two modes as shown in the ***Scale Image Display*** region of the window, ***Manual*** and ***Auto***.

Manual mode: drag the red and green bars or enter numbers in the ***Low*** and ***High*** edit fields to set the min and max values that are displayed in the live image.

Auto mode: the live image will automatically calculate the minimum and maximum intensity values of the live image to set the contrast in the image as default.

• Enabling the ***0%-99.5%*** tickbox will adjust the displayed dynamic range from 0% to 99.5% of the available signal

• Enabling the ***Range*** tickbox and entering a specific value will adjust the displayed dynamic range from the data defined minimum to a user defined maximum resulting from minimum + range value

Example: Minimum is 55, Range is set to 128, Displayed dynamic range: 55 - 183

3 Display Options

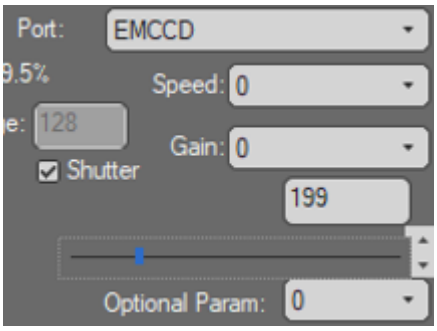
Multiple Camera Windows - This will display each camera in its own unique live window. Changing cameras will open a new window if one does not exist and/or move an existing one up top.

Test Dual Camera (for dual camera configuration) / ***Test Image Splitter*** or ***Test Dual View*** (for dual view configuration) - This will produce an alignment image of the image of one view subtracted from the other.

Cycle - When selected it moves from one camera to the next camera to the next camera, in a circle.

NOTE: The image splitter setting is in ***Hardware Properties*** > ***Simultaneous Capture*** > ***SplitCapture***: ***Yes*** if a dual camera splitter is installed, ***No*** for dual camera.

4 Camera-Specific Options



Port - On cameras that support this adjustment, the CCD read-out port can be adjusted using this dropdown menu.

Speed - On cameras that support this adjustment, the CCD read-out speed can be adjusted using this dropdown menu.

Gain - On cameras that support this adjustment, the gain can be set using this dropdown menu.

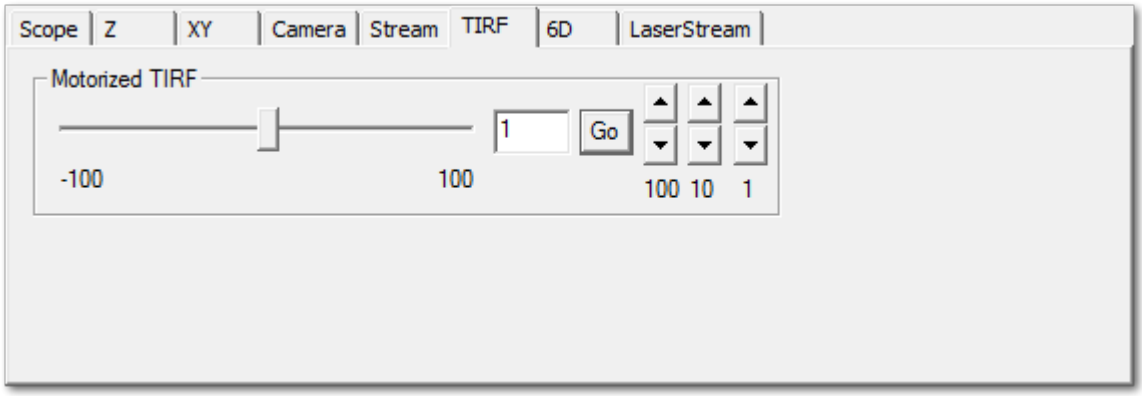
Intensification - On cameras that support this adjustment, the intensification factor can be adjusted using the edit field and slider located below the label.

Optional Parameters - This dropdown menu allows for support of additional features on certain cameras. For instance, on Hamamatsu cameras, the parameters menu allows you to select the camera offset, or you can select the Vertical Speed on certain Andor EMCCD cameras.

Shutter - On cameras that support this adjustment, the internal shutter can be enabled using this tickbox.

TIRF Tab of Focus Controls

When motorized TIRF is installed on a system, you may use the ***TIRF Tab*** of Focus Controls to adjust the TIRF angle. Simply start a live preview of the sample then adjust TIRF using the slider, edit field, or arrow buttons. During experiment setup, you may wish to fine tune TIRF such that you obtain and use a setting for each individual channel. These settings can be applied in the ***TIRF*** dropdown of the Capture Window.

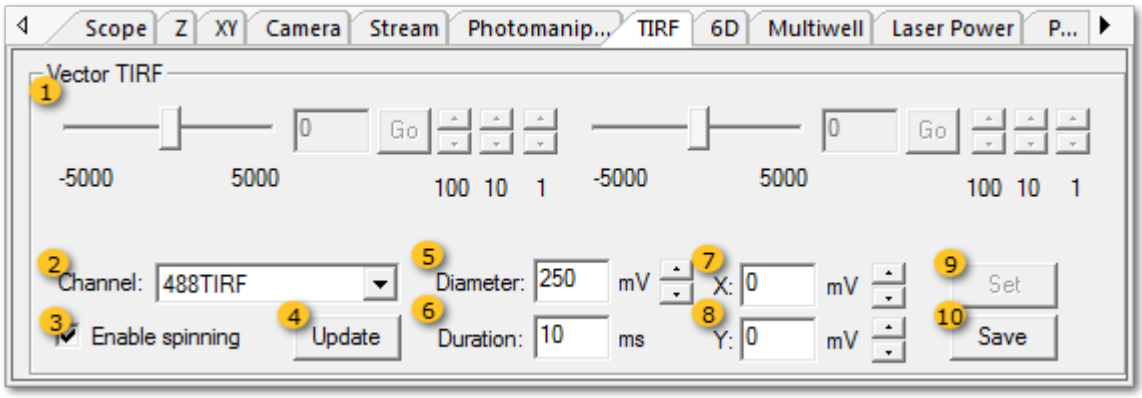


VectorTIRF Tab of Focus Controls

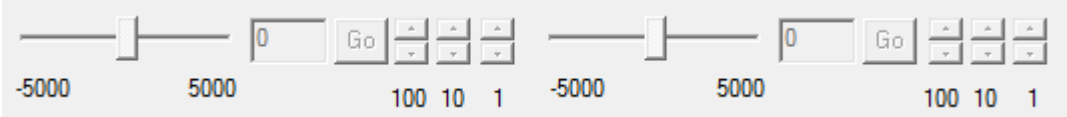
Vector is 3i's patented high-speed laser scan head that utilizes galvanometers to move a laser beam very quickly across a field of view. Vector technology can be used for point scanning photomanipulation or applied toward a VectorTIRF unit. VectorTIRF allows the users to steer a laser to a TIRF angle then use galvanometers to "spin" the point source in a ring of designated diameter in the back focal plane of the objective. The end result is a highly uniform precise TIRF illumination that can be tuned to individual channels and adapted to accomplish various experimental parameters. Simultaneous excitation using a single TIRF angle can also be achieved. For assistance setting up simultaneous laser excitation, please contact 3i support.

See also:

Configuring VectorTIRF Positions



1 XY Center Point Controls group



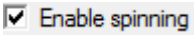
Sets the center location X and Y coordinates for the laser entering the back focal plane of the objective. The location should be the center of the back focal plane of the objective for spinning TIRF. These controls are only enabled when ***Enable spinning*** is NOT checked. Use the sliders or up/down arrows to control the location values. Alternatively, type a value in the text field and click ***Go***. Once values are determined, click ***Set*** to update the X and Y values used for imaging.

NOTE: These sliders are also useful for testing TIRF in non-spinning mode.

2 Channel Selection

Use the Channel selection drop down to select the filter configuration for which you will adjust parameters. See also Configuring VectorTIRF Positions. If you want to test parameters, be sure the matching channel is selected from the ***Filter Set*** selection in the lower half of Focus Controls.

3 Enable spinning check

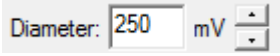


Check this box to move the laser in a circle of set diameter based on the center of the back focal plane of the objective. If this box is not checked, the laser beam will remain stationary at the set X/Y location value, typically the center of the back focal plane of the objective.

4 Update button

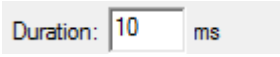
Press Update after changing diameter, duration, X:, or Y: parameters. Pressing update will activate the parameter change(s) for use until SlideBook is restarted. The active parameters will be used for imaging.

5 Diameter field



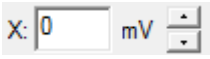
Enter a value (in mV) to be applied to the Vector galvanometers. the larger the value, the larger the TIRF angle will be. This is the distance off center to which the laser beam will be moved. When spinning, the laser beam will be following a circular path based on this diameter.

6 Duration field



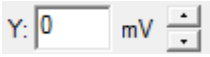
Enter a value in milliseconds for how long it should take to complete full rotation of VectorTIRF. You may want to consider camera exposure time when setting this value. For standard imaging, a duration of 5-10 milliseconds is recommended.

7 X: center field



X center location can be set by applying a voltage to the X galvanometer. For spinning TIRF, this should be the center point of the back focal plane of the objective. For traditional TIRF, this should be the location on the back focal plane that generates TIRF and spinning should be disabled.

8 Y: center field

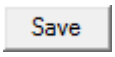


Y center location can be set by applying a voltage to the Y galvanometer. For spinning TIRF, this should be the center point of the back focal plane of the objective. For traditional TIRF, this should be the location on the back focal plane that generates TIRF and spinning should be disabled.

9 Set button

Enabled only when ***Enable spinning*** is unchecked. This button copies the X and Y values set by the sliders in the top half of this tab into the active parameters for the TIRF channel selected.

10 Save button



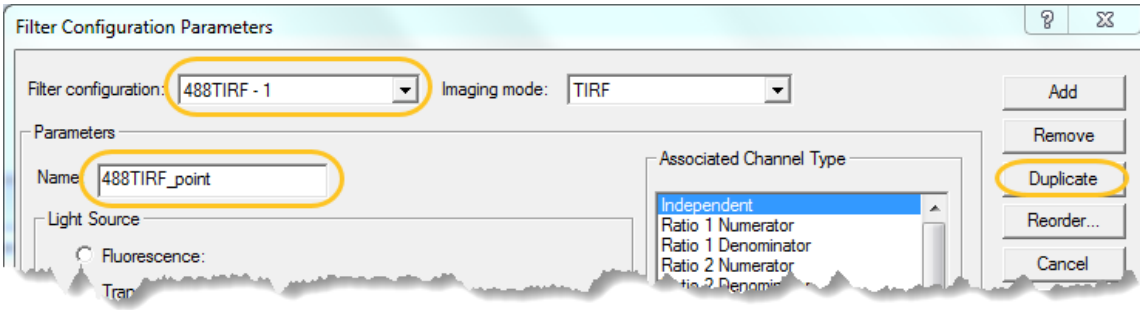
Saves the diameter, duration, X: and Y: parameters to the Hardware Properties file for the position indicated by the selected channel's filter configuration. SlideBook will need to be restarted for these changes to be accessible though they do show up correctly in the hardware properties file. SlideBook will always use the active parameters (parameters last selected when ***Update*** was pressed). When you save new parameters to hardware properties, these are the default values that will be loaded when you initialize SlideBook as a fresh start.

Configuring VectorTIRF Positions

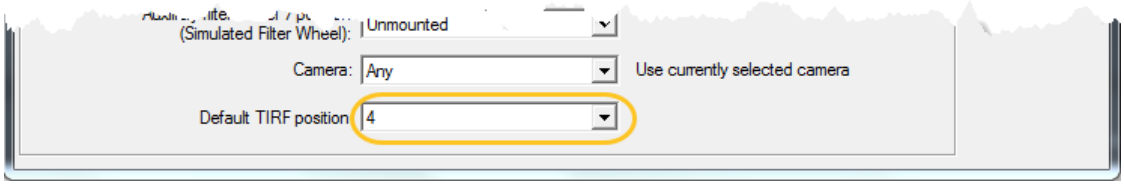
In addition to standard hardware component configurations, there are specific hardware properties that allow users to utilize VectorTIRF for spinning TIRF or traditional single point TIRF. 3i VectorTIRF Position properties allow you to set default values for the parameters found in the VectorTIRF tab of Focus Control. These parameters will control penetration depth, TIRF angle, field uniformity, and other advanced aspects of TIRF.

Administration tab > Filters allows you set up channels for imaging. When a system is installed by 3i staff, they will configure all required filters. Should you have specific research goals that require additional channels, you may wish to duplicate a configured channel then customize advanced parameters.

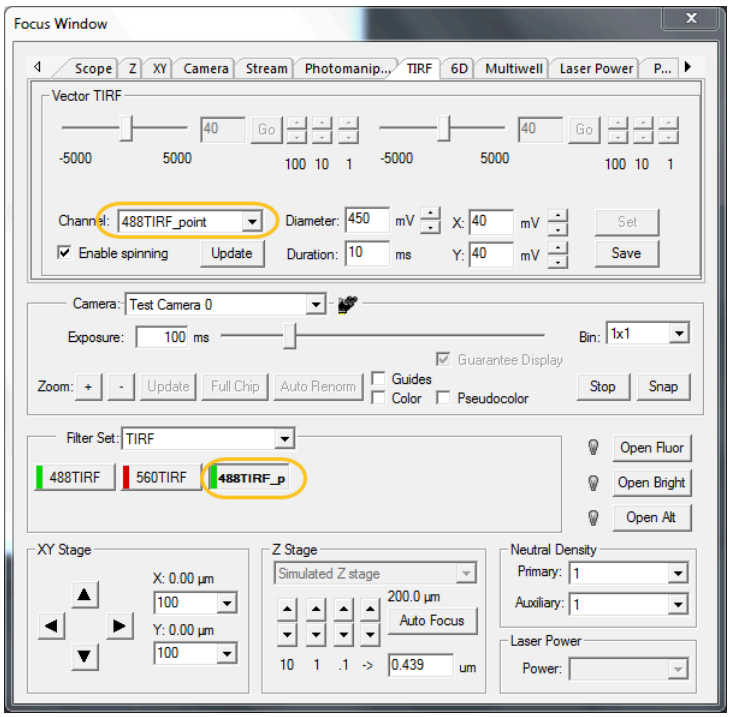
1. Go to ***Administration tab > Filters***. Select the desired TIRF filter from the ***Filter Configuration*** dropdown menu. Edit the name to reflect the changes you will be making. In this example, we will set up a 488 TIRF channel that does not spin and is configured to perform "traditional" objective based TIRF using a collimated laser beam at a single point in the back aperture of the objective.



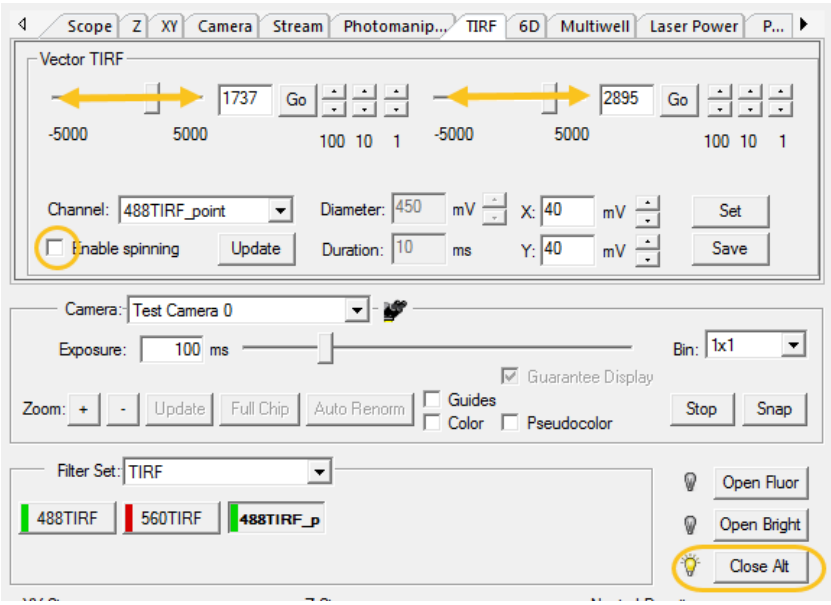
2. Select a value for Default TIRF Position. This value refers to the ***Position*** used in ***Hardware Properties***. You may need to look at other TIRF filter configurations to determine the next available position. Click ***OK*** to save changes and close the dialog. Restart SlideBook as directed.



3. Open Focus Controls and navigate to the TIRF tab. Select the appropriate channel using the channel selection buttons in the lower half of the dialog. Select the matching channel using the channel dropdown in the TIRF tab.



4. Disable spinning by unchecking the box. Use the ***Open Alt*** button to open the shutter. Use the sliders at the top of the dialog to steer the beam into a position that produces the desired TIRF effect.



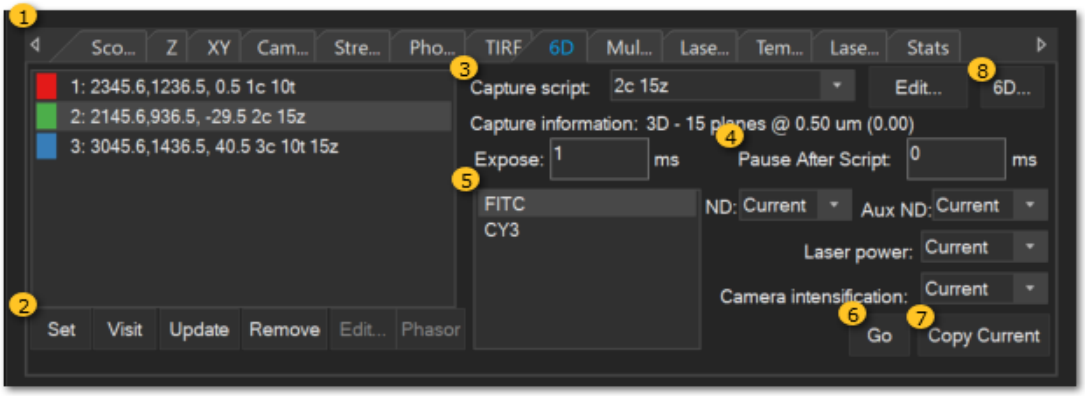
5. Click ***Set*** to copy the X and Y slider values to the parameter fields in the lower portion of the tab. Continue to imaging if desired. Alternatively, click ***Save*** to save the settings to Hardware Properties. Once SlideBook is restarted, these parameters will automatically be linked to the channel.

6D Tab of Focus Controls

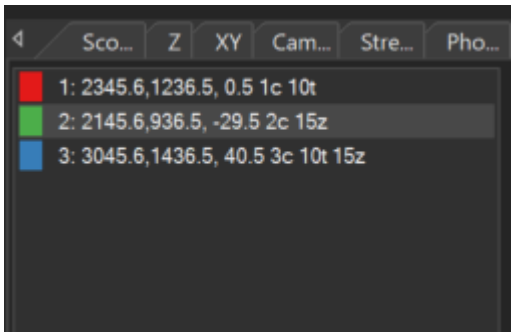
In order to help automate capture, SlideBook includes a heterogeneous 6D capture option. This allows you to visit different physical locations on a sample and run unique capture routines at each location.

See also:

- TUTORIAL: Using 6D Scripted Capture
- 6D MultiCapture window
- Capture Window
- Advanced Capture Preferences window



1 Point list

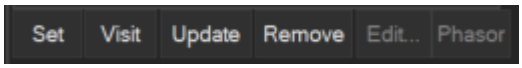


Displays the list of locations, also called points. Points can be ***Set*** in within this interface or within the ***XY tab***. Specifically the XY Tab of Focus Controls is used to load or save point lists and when points are set using the Multiwell tab of Focus Controls.

Points may be selected and edited from this list. To select a point, click on it. A black rectangle is drawn around the selected point, in this example **Point 4** is the selected point. Double-clicking on a point will move to that location. Different color blocks next to each point are used to indicate different capture scripts. Black will appear when no capture script has been assigned to a location.

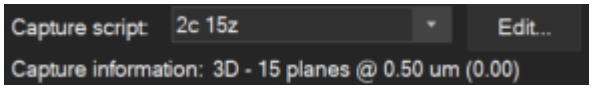
NOTE: You can hold the **Shift** key down and click two points on the list to select a range of points then assign a capture script to multiple points at one time. You can also hold **Ctrl** down to select multiple discontinuous points.

2 Point Command buttons



- ***Set*** - Set the current XYZ position as a point on the list
- ***Visit*** - Move to the currently selected point's XYZ location.
- ***Update*** - Update the currently selected point with the current XYZ location.
- ***Remove*** - Removes the currently selected point from the list.
- ***Edit*** - Not currently active, will enable editing of point name and manual XYZ entering.
- ***Phasor*** - not currently active.

3 Capture Script group

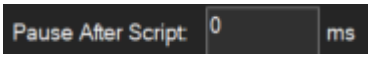


Select a capture script from the dropdown menu. Capture scripts are created by setting parameters in the Capture Window and saving the Capture Settings. Parameters used from the saved script include: extent, bin factor, 3D capture selection and parameters, filters selected and order used, exposure time, associated ND, Aux ND, intensification, and autofocus parameters set in the Focus Tab of Capture Preferences window. Timelapse parameters are used for Sequential 6D Multicapture only. Please click here to see a visual representation of parameters used in heterogeneous 6D capture.

Once a capture script is selected, ***Capture information*** will appear below the dropdown and the channels present will populate the channel list.

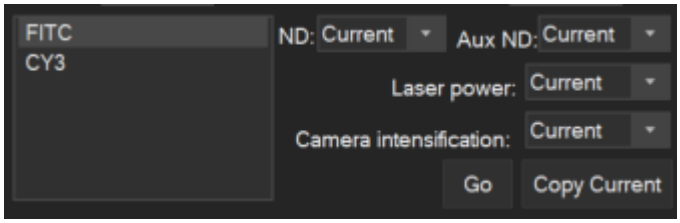
Edit opens the Capture Window with the selected script loaded. You may edit desired parameters such as channels collected, z sectioning, or time lapse intervals then either overwrite or save as a new experiment.

4 Pause After Script



This field allows you to add a pause after the specified script is captured. ***Pause After Script*** is only applied when performing the ***Sequentia***l mode of 6D MultiCapture.

5 Channel settings



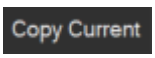
Exposure time, neutral density, auxiliary neutral density, and intensification values are set from the saved capture script but can be edited manually in this area. Click on a channel from the list to adjust exposure time, neutral density, auxiliary neutral density, and intensification.

Double clicking on a channel (or clicking ***Go***) will change the filter settings used in the ***Focus Controls*** window to those selected in the ***6D tab***. Conversely, clicking ***Copy Current*** will pull the values from the Focus Controls into the selected channel.

6 Go button

Changes ***Focus Controls*** window to match the channel settings shown in the ***6D tab***.

7 Copy Current button



Pulls channel setting values for exposure time, neutral density, auxiliary neutral density, and intensification from the ***Focus Controls*** window to use with the current capture script and selected channel. You can do the same thing by double-clicking on a channel in the channel list.

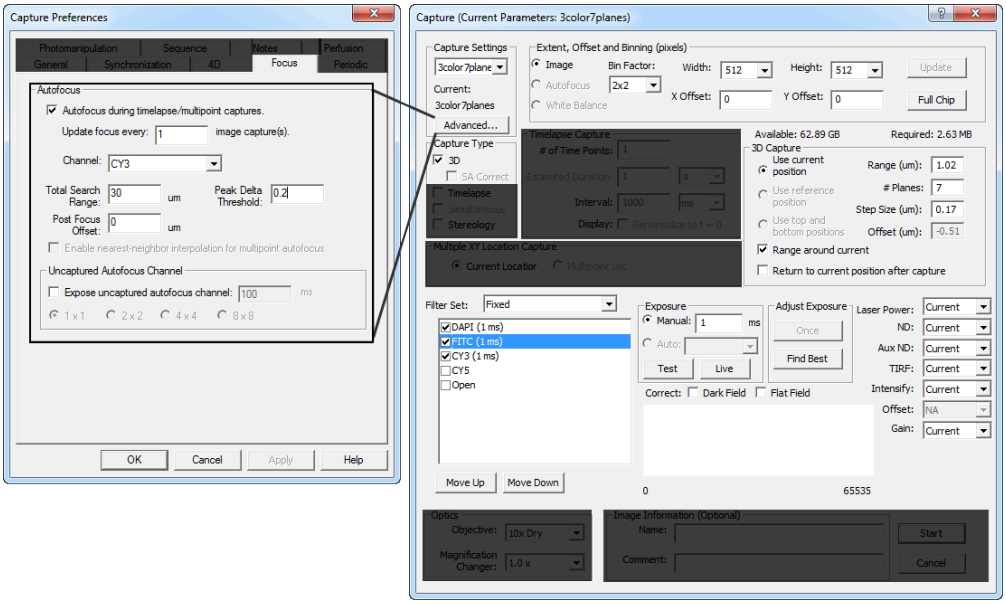
8 6D Button

Opens the 6D MultiCapture window. This is the next step in initiating scripted capture.

Saved Capture Parameters used in 6D Scripted Capture

Capture scripts for 6D MultiCapture are simply saved ***Capture Settings*** created in the Capture Window. The image below is a visual representation of the parameters in saved capture scripts that are used in 6D scripted capture.

NOTE: Timelapse parameters are used for Sequential 6D MultiCapture only (capture an entire experiment at location A, move to location B, cont'd). Interleaved capture uses timelapse parameters defined in the 6D Multicapture dialog.



TUTORIAL: Using 6D Scripted Capture

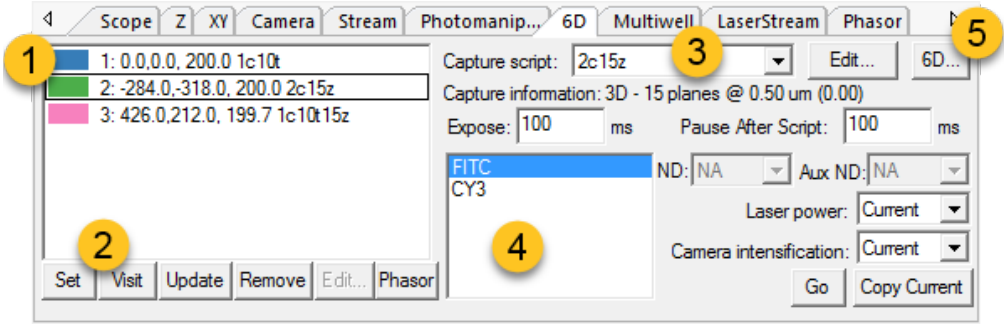
It is often useful to run an automated capture routine that performs different acquisition types at different physical locations. For example, you may wish to capture different channels, different z ranges, or different exposure times at different points then visit those locations over time. This tutorial will review the basic steps required to set up a 6D heterogeneous capture.

A. The first step in setting up 6D scripted capture is to create the capture scripts. This is done using the Capture Window. Simply set up capture parameters as you would in a single location experiment. Parameters used from this interface include CCD chip extent and offset, 3D parameters, channel order, exposure time, and associated filters as well as the autofocus routine set in the advanced options Focus tab. Please visit Saved Capture Parameters used in 6D Scripted Capture to visualize the active parameters.

B. Next, you need to define the locations to visit during the experiment. This can be done several different ways:

- Define the locations within the 6D tab.
- Define the locations or load a saved list in the XY tab.
- Define the locations using the Multiwell tab of Focus Controls and ***Export Points*** back to the XY tab.

C. Now you are ready to associate capture scripts with locations and fine tune acquisition parameters. Go to the ***6D tab*** of the ***Focus Controls*** window.



1. Select a point from the point list by clicking on it once.

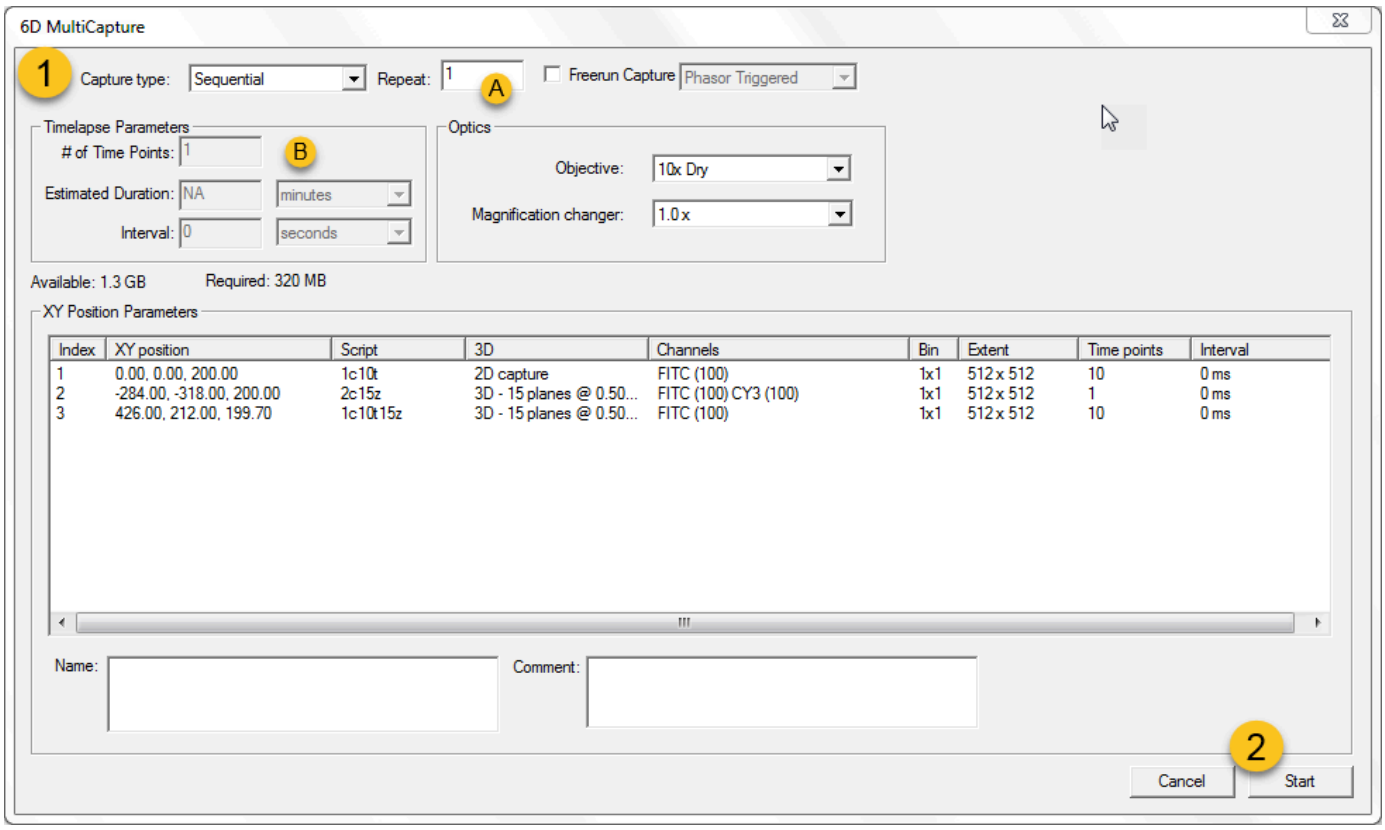
2. Move to that location by clicking on ***Visit*** or double-clicking the point in the list.

3. Select a ***Capture script*** from the dropdown menu.

4. Select a channel by clicking on it. Adjust exposure time, associated neutral density filter, auxiliary neutral density, and intensification as desired. If you need to adjust additional parameters, clicking ***Edit*** will open the Capture Window with the selected script parameters loaded. You may edit parameters as desired then either re-save the experiment or save it under a new name. Click ***Go*** to copy the selected parameters to the live ***Focus Controls***. Conversely, click ***Update*** to populate the capture parameter fields with the settings currently used in the ***Focus Controls***. Repeat for all channels listed.

5. Go to the next location, repeat steps#1-4 until capture scripts and exposure times have been set for all locations. Use the ***6D*** button to directly open the ***6D MultiCapture window*** and proceed to the step D below.

D. After scripts are set for the point list, you are ready to configure the time lapse routine. Click ***6D*** from the 6D tab in the Focus Controls or go to ***Special Capture > 6D MultiCapture*** (in the acquire group of the Home tab). For more on this window, visit the 6D MultiCapture window page.



1. Select the ***Capture type*** from the drop down menu as follows:

   Sequential - Performs a time lapse routine at each location, capturing the assigned script for the entire time lapse duration before moving on to the next location. If Sequential capture is desired, there is an option to add a pause after each location in the 6D tab of Focus Controls.

   A. You may repeat the sequence by entering a value in the ***Repeat*** field.

   Interleaved - Performs a time lapse routine in which the selected capture script is performed at each location in a loop using the time lapse interval between the start of each point visiting loop.

   B. Type a value for the ***# of Time Points*** to acquire and the ***Interval*** between time points. This is the interval between the start of each capture routine. If capture takes longer than the designated interval, it will proceed as quickly as possible. ***Estimated Duration*** will be calculated once the other two parameters are completed.

2. Press ***Start*** to begin acquiring images.

E. During data acquisition, the Capture Controls dialog is open. This window allows you to interact with the microscope and data while images are being acquired. In 6D scripted capture, you may find it particularly useful to make note of interesting events using the Notes tab or to review previously captured images using the ***Show*** button in the Live tab.

Using AutoFocus in SlideBook

AutoFocus is a powerful tool that can help you maintain an imaging plane or z-stack range over time. The instructions below will aid in setting the appropriate focusing parameters for your sample.

1. Ensure the sample is flat in the sample holder and the sample holder is level in the stage. Angles here will dramatically affect the focal plane from one side of the slide to the other.

2. In the ***Focus Controls*** window, select the channel that you will use to autofocus and ensure the focus drive that SlideBook controls is in the center. When using a piezo *z* drive, the piezo *z* should be centered or set to **0.0**. The sample should be hand focused by the microscope focus controls. This is due to the fact that the piezo only has 100 microns travel distance, if the only z-control is the microscope focusing drive, this is less of an issue because there is such a large range of travel. Open the autofocus window by pressing the ***Autofocus*** button in the ***Z Stage*** control area of the Focus Controls window.

3. Run the autofocus algorithm from the ***Focus Controls*** window to ensure the settings are correct. The settings that you need to set are:

a) ***Search Range*** – Depends on sample thickness and fluorescence distribution. More points are better but you will need to find the balance between the needed search distance and photobleaching/phototoxicity or temporal resolution needed for the experiment. You can autofocus on a non-imaged channel, or a bright but less interesting channel to speed this up. Blue shifted filter sets will lead to more photobleaching and phototoxicity than red filter set in general.

b) ***Delta Peak Threshold*** – This is the limit set for the minimum change in the Gaussian curve required to trigger the resetting of the *z* due to the autofocus algorithm. It is required for montages. Imagine a Gaussian curve of z-position vs. sharpness in a 3D stack. The difference in the peak height to the edges is what this parameter is looking at. So in a stage position that has signal, the peak height should be large compared to the base line, but in a stage position without sample (say the edges of a montage), this peak height would be only slightly different than the base line. On the outskirts of the montage you do not want the *z* position to change, but within the sample you may. The delta peak threshold sets the limit at which you accept or ignore the autofocus' suggested sharpest *z* plane. The algorithm is a value between **0.1** and **1**, where the lower the value the more stringent the difference must be to accept the autofocus and the higher the value, the autofocus suggestion is always accepted.

Guidelines:

i) For thin montage sample where signal should be in a similar plane, delta peak threshold should be set to a low value (i.e. **0.1 - 0.4**). Smaller ***Search Range*** okay.

ii) For a thick montage sample where signal could be in any plane, delta peak threshold should be set to a middle range value (i.e. **0.5 - 0.8**). Larger ***Search Range*** needed.

iii) For multipoint/multiwell captures where imaging planes are unrelated, delta peak threshold should be set to a high value (**0.8 - 1.0**).

iv) To always accept the auto-focus suggestion select delta peak threshold of **1.0**.

c) ***Exposure Time*** – Use your judgment here. You want a good dynamic range but the longer time this is set for, the longer it will take to autofocus and the more light you will put on the sample.

4) Press the large ***Autofocus*** button, the stage should move and three values will be placed in the right hand side of the Auto Focus window. These are ***Total Time***, ***Image Time***, and ***Best Z***. You can modify ***Search Range*** and ***Delta Peak Threshold*** to speed up the autofocus, but know there are warnings in doing so. The ***Best Z*** will be either a number meaning the auto focus routine was successful, or one of two error messages:

a) **No Gradient**, means the peak value did not surpass the edges on the Gaussian curve, thus the delta peak threshold criteria was not met for the z-stack. Increase the delta peak threshold.

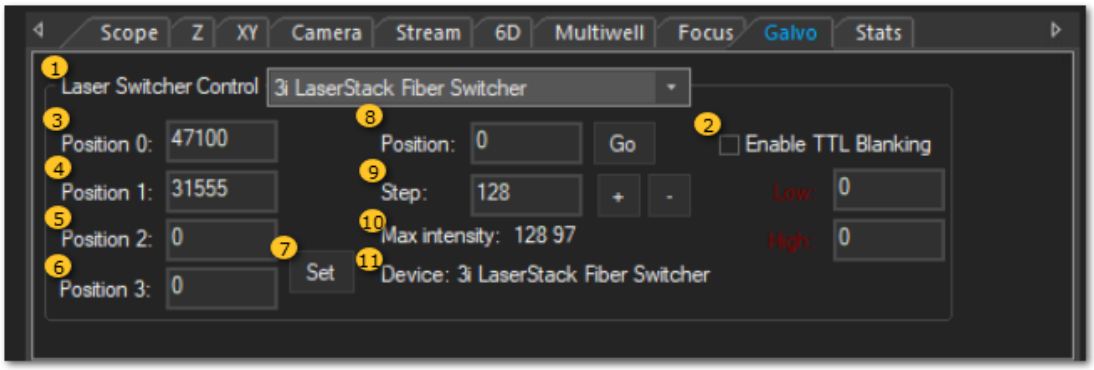
b) **At Edge**, means the peak value reached a limit of the search range and the Gaussian curve was shifted to the far right or far left. Increase the search range. Or the piezo has reached a its limit of movement given your search range is greater than 100 microns or the piezo started at one of its limits, re-center and follow the above steps.

5) Open the ***Capture Window > Advanced > Autofocus tab***. Most of the settings from the ***Focus Controls*** should port over, but adjust these as required. You can save this capture script under upper left hand portion of the capture window from the drop down menu **Default**.

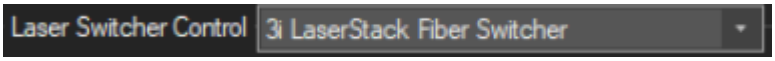
For montage capture the autofocus algorithm must reset z when in a stage location that has interesting sample, but ignored when in a stage position where there is no interesting sample. With a test sample, it is recommend to run the autofocus algorithm to be perfected for a position where sample is interesting, and also perfected for a position where the sample is uninteresting and the algorithm is ignored (i.e. no gradient). While it is easy to get the algorithm to achieve no gradient, it may be a challenge to get the settings to match both requirements. Since every sample is different, it is difficult to give absolute values for the autofocus routine to use. However once the correct balance of search range and thresholding are found, this routine can lead to better focused captures across multiple samples with little user intervention.

Galvo Tab of Focus Controls

When a 3i automated switcher is present on an imaging system, the ***Galvo tab*** will be present in ***Focus Controls***.

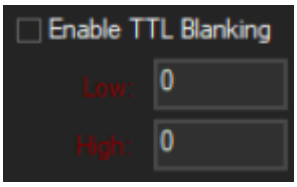


1 Laser switcher control



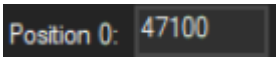
This dropdown menu allows selection of the galvanometer product to be controlled in the ***Galvo Tab*** interface.

2 Enable TTL Blanking



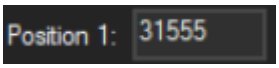
Toggles the active galvanometer between positional control (uncheck, conventional USB) and TTL control (checked). The *Low* and *High* fields allow for entry of positions based on the TTL signal state.

3 Position 0



Upon SlideBook launch, displays the value configured in ***Hardware Properties*** for ***Position 0***. After clicking the ***Set*** button, it displays the Set value (see Set button below) for Position 0.

4 Position 1



Upon SlideBook launch, displays the value configured in ***Hardware Properties*** for ***Position 1***. After clicking the Set button, it displays the Set value (see Set button below) for Position 1.

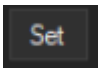
5 Position 2

Upon SlideBook launch, displays the value configured in ***Hardware Properties*** for ***Position 2***. After clicking the Set button, it displays the Set value (see Set button below) for Position 2.

6 Position 3

Upon SlideBook launch, displays the value configured in ***Hardware Properties*** for ***Position 3***. After clicking the Set button, it displays the Set value (see Set button below) for Position 3.

7 Set button



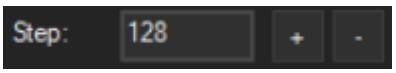
Sets the entered value in the ***Position 0-3*** fields for the duration of the SlideBook session.

NOTE: Does not write to the ***Hardware Properties*** file.

8 Go to Position

Moves the active galvanometer (as selected by the Laser switcher control menu) to the value of the ***Position*** field.

9 Step Size



Enter a value for the galvanometer step size. The +/- buttons move the active galvanometer by the entered value.

10 Max Intensity

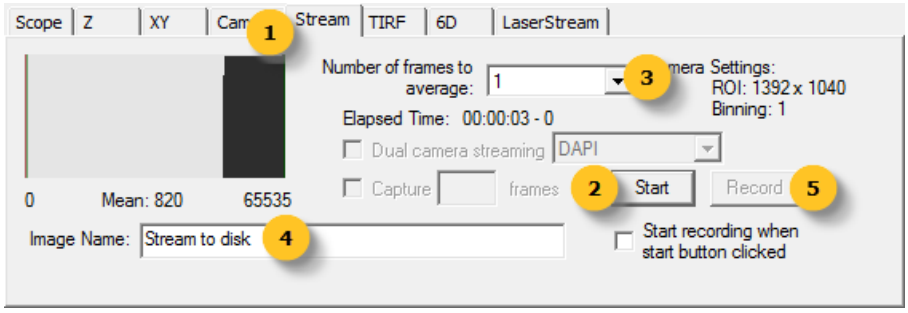
Displays the Minimum and maximum intensity values for the live image.

11 Device

Displays the active galvanometer, should correspond to Laser switcher control selection.

Stream Tab of Focus Controls

The streaming capture function allows you to run your Hamamatsu or Photometrics camera at the fastest possible speed. There are limitations with streaming capture. When a camera is recording in this mode, you may only alter other hardware via the ***Focus Window***. Streaming capture does not work using the ***Capture*** dialog box, so you may not perform automated multi-channel, 3D, 4D, multipoint or montage capture. This capture mode is ideal for rapid 2D timelapse capture (see also Rapid 4D Streaming Capture). To run your camera in streaming capture mode, follow the instructions below.



1. Open the Focus Window and select the ***Stream*** tab (or use the Stream shortcut from Special Capture in the Acquire group of the Home tab).

2. Select the ***Start*** button. A new image capture window will open with a counter for number of images captured.

3. The camera will continuously generate images with the exposure time that was have selected in the ***Focus Window***. If desired, select the ***Number of frames to average*** from the drop-down list.

4. Name the file in the ***Image Name*** field.

5. Press ***Record*** to save the captures to disk. Once you have pressed record, images will be saved to disk and the record button will convert to ***Pause***. To end the recording, press ***Stop***. This will also stop the streaming update. To resume the streaming update, press ***Start***. You will not be able to change camera settings while the camera is streaming. You must press ***Stop*** and make changes to camera settings, then press ***Start*** again.

CAUTION: Recording at this speed will rapidly use computer disk space.

Spool files will be opened automatically once you press ***Stop***. They will also be placed in the open slide with the name designated in Step 4.

Auto Focus window

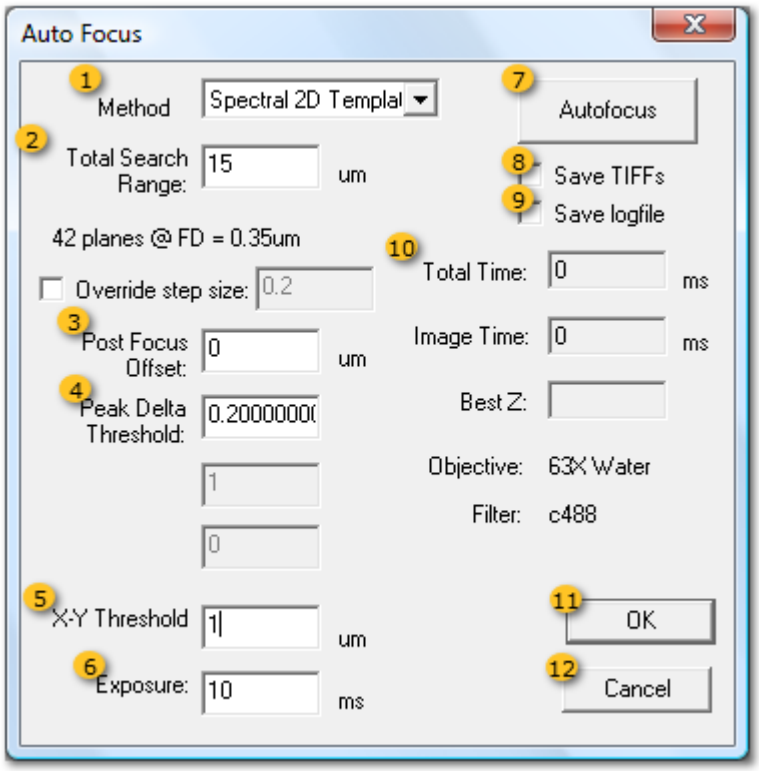
The ***Auto Focus*** window is used to define and test parameters for auto focusing during image capture. Here, you may test different methods and search parameters to determine what works well with your specimen. These values will later be used in the Focus tab of Advanced Capture Preferences.

> **NOTE:** Autofocus is not compatible with montage imaging please refer to TUTORIAL: Collecting a Montage for preferred method.

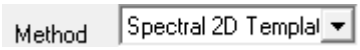
Access ***Auto Focus*** from the Auto Focus button in the Z Stage control area of ***Focus Controls***

See Also:

- Focus tab of Advanced Capture Preferences
- Using AutoFocus in SlideBook
- TUTORIAL: Using Definite Focus for Multipoint Capture
- TUTORIAL: Using ZDC for Autofocus
- TUTORIAL: Using Perfect Focus for Autofocus
- TUTORIAL: Nikon Ti2 Perfect Focus for Autofocus



1 Method



The computational ***Method*** used to evaluate and compare focal planes. Changing the method can affect the success of the auto focus routine. The default method of ***Spectral 2D Template*** will work best in almost all cases. ***Gaussian Derivative*** may prove useful when using a phase contrast channel for auto focus. The other methods are experimental. You should consult 3i Support if you are not having success with the default method.

2 Search Range

Total Search Range: 15 um

42 planes @ FD = 0.35um

☐ Override step size: 0.2

Total Search Range is the total distance in the *z* dimension that will be searched during the auto focus routine starting from half the specified distance below the current *z* position.

SlideBook will automatically compute the number of planes to image based on the total search range and the calculated depth of field (FD) of the microscope objective. This information is displayed below the ***Total Search Range*** edit box. For instance, in the above example in order to span the **15** micron search distance with an objective that has a focal distance of **0.35** microns, a total of **42** planes will be imaged.

To use a different *z* step size, you may check the box to ***Override step size*** and enter a value in microns in the text field.

3 Post Focus Offset

Post Focus Offset: 0 um

The ***Post Focus Offset*** is a positive or negative offset distance that the *z* stage will be moved from the optimal focus position. This feature is useful for always focusing a constant distance above or below the automatically determined focal plane.

4 Peak Delta Threshold

Peak Delta Threshold: 0.20000000

The ***Peak Delta Threshold*** is used to determine when no optimal plane of focus should be computed from a particular image position. When performing a montage capture, for example, some fields might not contain tissue and should not be used to change the current *z* position. This value can range between **0.0** and **1.0**, but should not be decreased below **0.1** in most cases. For captures where you know that each image in the sequence will have sufficient detail to focus, this threshold can be raised. If the value is set to **1.0** then the threshold will be ignored.

5 X-Y Threshold

X-Y Threshold 1 um

This parameter is not currently used.

6 Exposure

Exposure: 10 ms

The ***Exposure*** time used for image capture during the auto focus routine. This edit box will have a default value of the exposure time used in the ***Focus Controls***.

7 Autofocus button

Autofocus

Press the ***Autofocus*** button to initiate a single auto focus scan using the parameters defined in this window. Results will be displayed in the Results area as described below.

8 Save TIFFs

☐ Save TIFFs

Check this box to enable an advanced debugging feature that will generate a series of TIFF files in a numbered directory created in SlideBook program directory.

9 Save logfile

☐ Save logfile

Check this box to enable an advanced debugging feature that creates an autofocus log file in the SlideBook program directory.

10 Results

Total Time: 0 ms

Image Time: 0 ms

Best Z:

After the ***Autofocus*** button is pressed and the routine is finished, SlideBook will report the optimal focal *z* position in the ***Best Z*** information box and automatically move the *z* stage to this location. In addition, timing statistics for the autofocus routine are given in the ***Total Time*** and ***Image Time*** information boxes.

NOTE: If SlideBook is unable to determine the optimal focal plane in the sample it will return one of two messages in the ***Best Z*** information box. The **No Gradient** message indicates that SlideBook was unable to find a focal gradient within the specified search range. In this case, the peak delta threshold used for autofocus computation may need to be increased or the search range may need to be increased. The **At Edge** message indicates that the optimal focal plane appears to lie above or below the specified search range. Since SlideBook will automatically move the *z* stage to the best focal plane within the specified search range, simply running the auto focus routine again (starting from this new *z* position) will often find the optimal focal plane.

11 OK button

OK

Press ***OK*** to save the autofocus parameters for use during ***Capture*** and close the dialog.

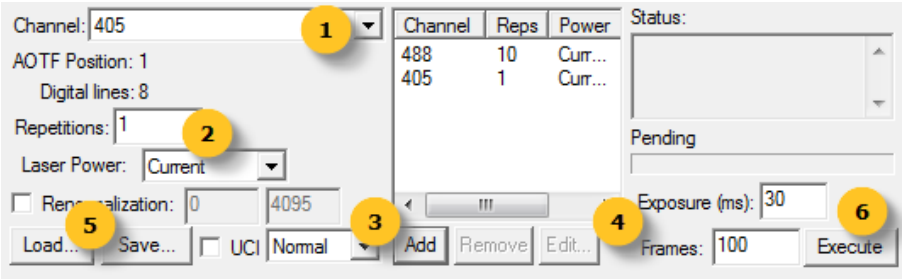
12 Cancel button

Cancel

Press ***Cancel*** to discard changes and close the dialog.

LaserStream Tab of Focus Controls

The ***LaserStream Tab*** of Focus Controls was designed to stream capture using different channels with a repeating pattern (for single channel streaming, you can use the Stream tab). This interface is specifically designed for very fast periodic capture using TTL controlled light sources, particularly the 3i LaserStack or NEOS AOTF. It allows you to set up a channel with the desired number of captures then add up to seven more channels (8 total); the end goal being a looping capture stream of different channels with different number of capture repeats for each channel.



1. Select the filter or ***Channel*** using the dropdown menu. Only 3i LaserStack or NEOS AOTF channels will be available.

2. Enter a value for the desired number of ***Repetitions*** for the selected channel. This will be the number of times the channel is captured in order in the loop of captures. Select a value for ***Laser Power*** using the dropdown menu.

3. Click the ***Add*** button to add the channel to the list of captures that will be looped. If necessary, you may select a channel from the list and use the ***Remove*** button to delete that step of the loop. You may add up to 8 different channels at 8 different repetition intervals. You may not add the same channel twice. Repeat steps 1-3 until all desired channels have been added.

4. Enter a value (in milliseconds) for the ***Exposure*** time. The same exposure time will be used for all channels. Enter a value for the total number of ***Frames***. This will determine how many times the designated sequence will be repeated. For example, if your sequence includes capturing one channel 9 times and a second channel once, 100 frames will repeat the designated sequence 10 times.

5. If desired, ***Save*** the laser stream sequence for future use.

6. Press ***Execute*** to begin the designated stream capture. Images on the screen will be updated approximately three times per second. Actual imaging will proceed as quickly as possible depending on camera frame rate, image size, and exposure times. Execute will become ***Abort*** and allow a safe early termination of the experiment.

> **NOTE:** The capture will be stored differently than normal capture, where each time point includes data from all channels. Due to the potential difference in capture rates, each time point will include a single image from one of the channels and the other channels will be blank.

TUTORIAL: Using Definite Focus for Multipoint Capture

Background: the DF requires a change in refractive index between the coverslip and the sample. Definite Focus projects a grid onto the bottom of the culture vessel, where it is reflected onto a camera chip that is positioned at a slant. If there is a drift, Definite Focus returns the relative position of the grid projection back to the original state and thus counteracts drift.
In SlideBook you can check to see if the DF engaged during a previously taken capture by opening the capture and in the Home tab> Annotations section> Notes checkbox. Checking 'notes' will display 'AF success' or DF: ### in the top right corner if the DF was engaged during that capture. For 3D this will only be shown on the first plane of each timepoint.
The DF will never work with a hard mounted slide and a water/oil objective.

The Focus Tab of Focus Controls provides the tools necessary to interact with hardware-based autofocus systems. Since each system has unique functionality, each user interface is unique. Below, you will find a a tutorial for using the Zeiss Definite Focus system during an xy point visiting experiment setup.

> ***NOTE:*** The specimen must have a glass-water interface for Definite Focus to work optimal.

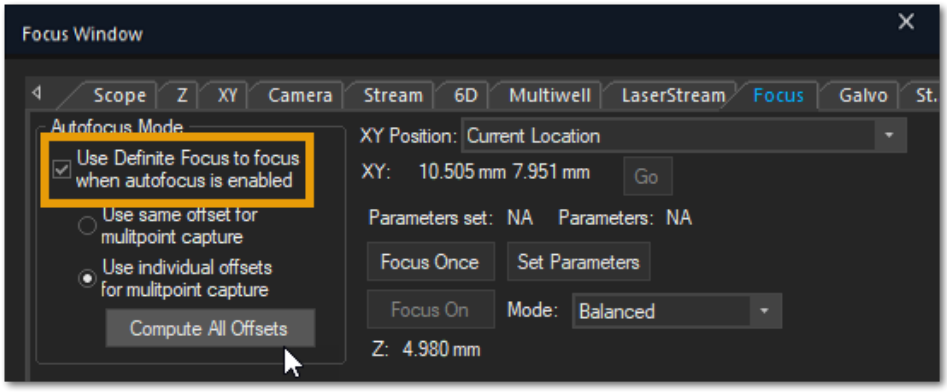
1. Before setting up the XY points or compute points from the multi-well interface navigate to the Focus window > Focus Tab

2. Find the sample using the appropriate channel

   a. ***OPTION 1***: In the ***Focus Tab***, check the box to ***Use Definite Focus to focus when autofocus is enabled***. Select the ***Use same offset for multipoint capture***. This will set the same offset for each point and works the most efficient for multipoint experiments when the sample is equally close to the coverslip or multi-well plate bottom. It is therefore the best choice for best for most applications.
      i. Check with ***Focus Once*** that the sample is in focus, otherwise correct with the microscope Z control
      ii. Select ***Set Parameter*** to set the Definite Focus offset (from the coverslip) required for this sample type.

   b. ***OPTION 2***: In the ***Focus Tab***, check the box to ***Use Definite Focus to focus when autofocus is enabled***. Select the ***Use automatic offset for multipoint capture***. This will set individual offsets for each point and is only required for some applications where the distance of the samples to the coverslip vary dramatically.

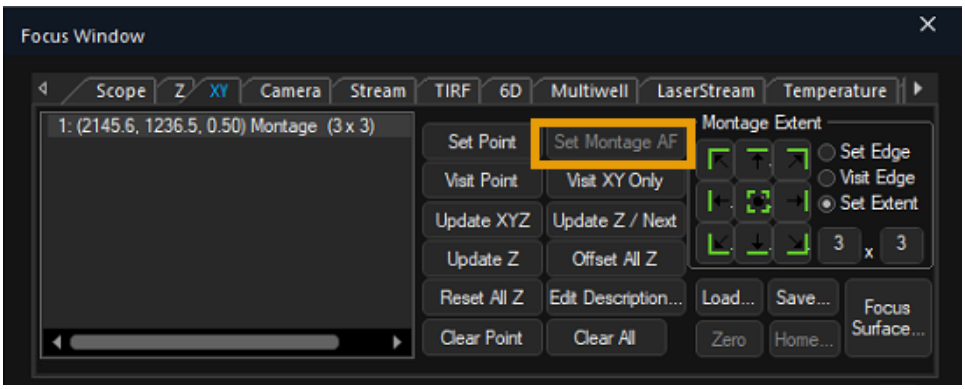
   NOTE: For Definite Focus 2: **No Need** to click ***Compute All Offsets*** yet. Return to this tab after setting the points (3.) and then hit ***Compute All Offsets***



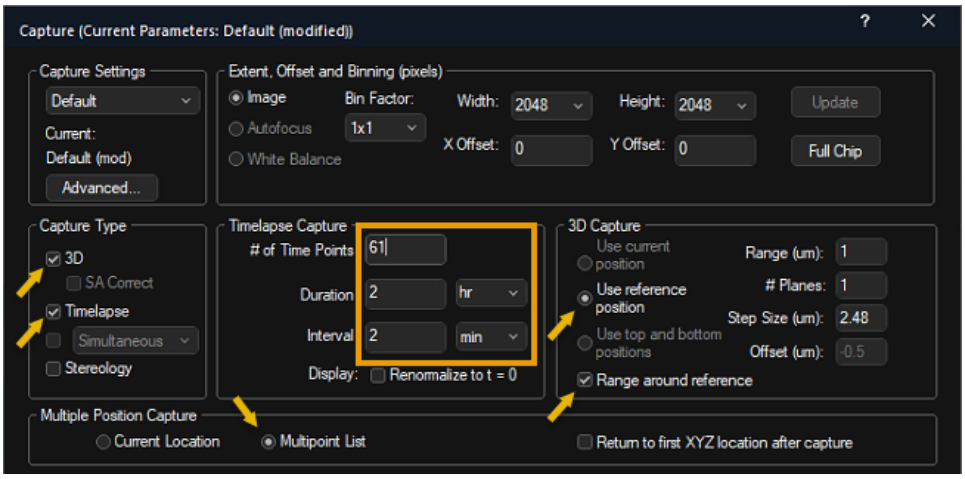
3. After activating Use the XY Tab or the Multiwell Tab to set x,y,z positions for multipoint capture. For this it's useful to also consider to use the Focus Surface.

> **NOTE:** Use the Axio Observer Z (as opposed to a piezo Z device) for setting different Z positions.

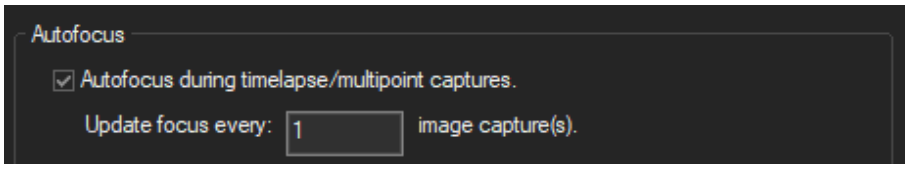
4. If the XY list contains one or several montages enable the autofocus for each montage individually by highlighting the montage and click ***Set Montage AF***.



5. Open the Capture dialog. Set up ***Timelapse Capture*** parameters and select ***Multipoint list***. Set up 3D Capture parameters by selecting ***Use reference position*** and ***Range around reference***.



6. Go to the ***Advanced*** button of the ***Capture*** dialog. Select the ***Focus*** tab. Check the box to enable autofocus and enter in a value for how often you would like to update the focus. You do not have to select a channel or anything else in this dialog, ***Definite Focus*** will automatically be selected as the ***Channel*** when things are configured properly in the ***Focus*** dialog.



7. Click ***OK*** to close the ***Capture Preferences*** dialog. Configure the rest of the Capture settings (such as channels and exposure time) as desired and ***Start*** acquisition.

Troubleshooting Definite Focus

See below for troubleshooting suggestions for Definite Focus. Many of the tips below come from Zeiss and are inherent functionalities of the hardware, not associated with 3i or SlideBook.

- The following order of actions for turning on or off the Defitine Focus must be duly followed under any circumstance:
    1. Turn Definite Focus on or off, shortly press the On/Off pushbutton (Fig. 4-1/1) at the controller front panel.
    2. Turn Axio Observer.Z1 microscope on or off (also refer to microscope operating manual).

- On the TFT, verify that the objective you are planning to use is defined in the AxioObserver by Zeiss part number. This is critical as it sets internal parameters for the Definite Focus.

- Ensure cable ends are firmly seated in their sockets.

- Confirm the z positions for point visiting were set using the Zeiss z focus drive and not a piezo z drive.

- When applicable, ensure the cover glass thickness is correctly set on objectives with a cover glass correction adjustment collar.

- Confirm there is enough immersion media, that it is free of bubbles, and that the objectives are clean.

- Occasionally, Definite Focus may fail when certain conditions are met such as excessively high cell densities, non-planarity of, or scratches in the Petri-dish surface.

- Certain non-aqueous culture mediums may be incompatible with Definite Focus. You need a glass/water interface tofor Definite Focus to work. Dry dishes or glycerol mounted samples will fail.

- Error message on TFT of **out of range**: A selected Z-position is out of working range limits. Its signal cannot be evaluated. Z-drive may have traveled down too far for the current lens focus plane to match any reasonable specimen plane. Definite Focus is not designed to cope with a situation like this. Culture dish was removed during an "On" phase

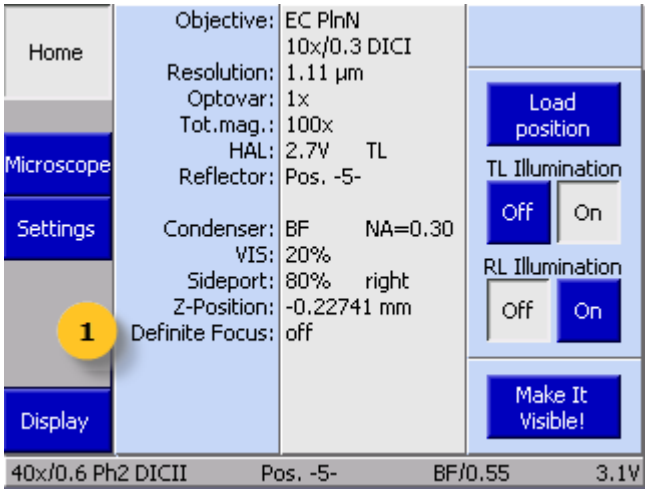
- Error message on TFT of **error**: Culture dish was removed during an "On" phase.

- Error message on TFT of **camera error**: The Sensor cable is not plugged in correctly or the camera chip in the sensor module has a defect, contact 3i technical support.

Definite Focus TFT page

1. Ensure the Definite Focus is powered on/off before the microscope is powered on/off under all circumstances. When the microscope is powered up, you may observe the Definite Focus state on the Home screen of the TFT microscope display as shown below. Definite Focus states are as follows:

Detecting Stand - search for microscope stand is under way
Connected - communication established
Off - Definite Focus ready, inactive



2. Once you get the **Definite Focus: off** confirmation, start SlideBook. Use the Focus Controls to bring the sample into position and focus. The specimen must have a glass-water interface to work.

3. Turn on the Definite Focus using the ***Focus On*** button in the ***Focus*** tab of Focus Controls. Re-adjust the focus using the Axio Observer. This will set the offset of the current position and the Definite Focus will hold focus. Turn off Definite Focus by using the ***Focus Off*** button.

TUTORIAL: Using ZDC for Autofocus

The ZDC2 tab of the Focus Controls window allows you to interact with the Olympus hardware-based autofocus system, ZDC2.

SlideBook + ZDC2

Requirements:

- SlideBook 5.0.0.17 or newer
- Olympus IX81 microscope and ZDC2
- Optional: motorized XY stage

Setup:

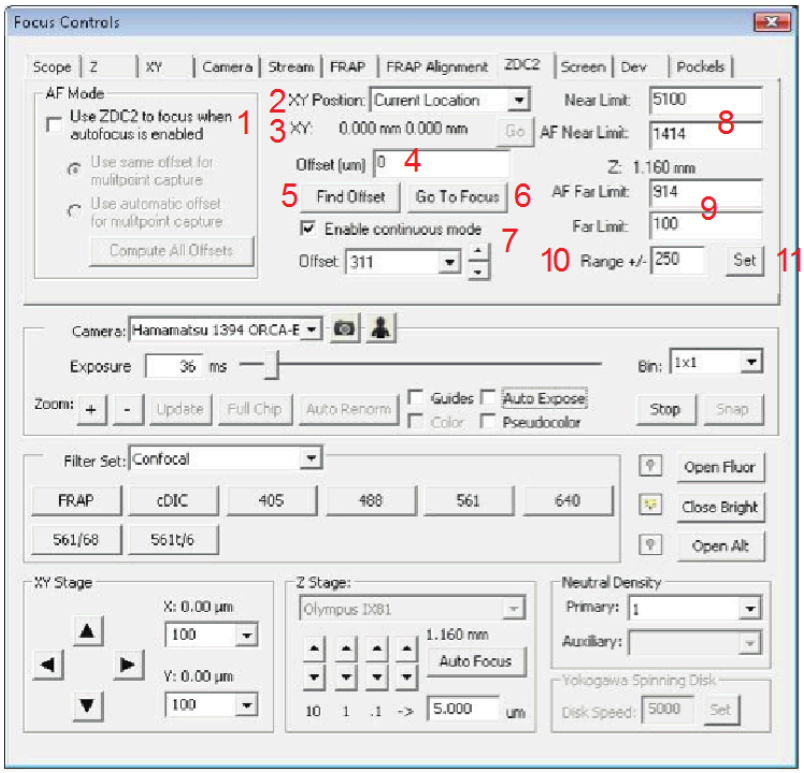
- ***Administration tab > Hardware Properties > Olympus MAL > Enable IX2-ZDC2 > Yes***
- Ensure the file **MalZDC2.ini** is installed in the SlideBook directory
- Ensure objectives have been defined in BX2-BSW software and all objectives are supported by ZDC2 (contact Olympus for compatibility information)

ZDC2 – Hardware Operation

The ZDC2 is a hardware system for autofocus. It has two modes of operation: continuous autofocus and single-shot autofocus. When the system is in continuous autofocus mode the system will keep the specimen in the same Z focus. In continuous mode a focusing lens allows the position to be changed to adjust the plane of focus. In single-shot autofocus mode the system places the focusing lens into an optimal position and then applies an optional offset to return the focus a specific distance from the hardware-based autofocus location.

ZDC2 – Focus Window

If initialized on startup a ZDC2 tab will be added to the focus window:



This tab is used to configure and test the ZDC2 system.

The check box (1) determines if SlideBook will use the ZDC2 for autofocus. By default it is unchecked. To use the ZDC2 for autofocus the user must check this box. If the user would like to use SlideBook's image-based autofocus then they can uncheck this box.

In addition, there are two options for autofocus during capture: same offset and unique offset. If your specimen is the same distance from the coverslip at each XY location (or you do not have a motorized XY stage) then you can use a single offset for each point. However, if you sample is not homogenously spaced off the cover slip you will need to use a "unique offset" per XY location. Once you have setup the entire XYZ point list you will need to click "computer all offsets." This button will visit each XYZ point in the point list and compute the distance between "coverslip autofocus" and "the selected Z position."

The rest of the dialog is used to test and update the settings. To test focus at a particular XYZ location select it from the XY list (2) and click "go." This will move the XYZ stage to the selected location. Clicking "Go To Focus" (6) will execute the single-shot autofocus: it will locate the coverslip and then apply the selected offset (4). Alternatively, if you want to find the offset between the current Z position and the coverslip, click "Find Offset" (5). This is the same as clicking (6) but the offset is calculated based on the starting and ending position. Once the offset is computed it is put into (4) and the Z stage returned to the original position.

The option to engage a "continuous autofocus" is enabled by clicking "Enable continuous mode" (7). This will place the focus lens in a position to maintain the current Z position and engage the autofocus system. Disable continuous focus by clicking "Disable continuous autofocus" (7). When continuous autofocus is engaged the focus position is adjusted by changing the focus lens offset (7). This value ranges from 0 to approximately 3000 and can be changed by clicking up/down or selecting a specific value. Note that the system cannot hold focus in all positions.

In order for the ZDC2 to autofocus the Z position must be within the acceptable range. Parameters 8, 9 and 10 dictate the allowable autofocus range. A larger range will increase the time-to-focus, whereas a smaller range increases the potential that the optimal focus will not be found. To change the parameters edit the values and click "set" (10). Also note that when the current Z position lies outside the acceptable autofocus range it will turn red, indicating an error condition. It will return to black when autofocus will work again.

ZDC2 – SlideBook Operation

To use the ZDC2 during SlideBook capture you must perform the following steps (note that you must have ZDC2 autofocus enabled in the ZDC2 tab in all cases):

2D time lapse capture

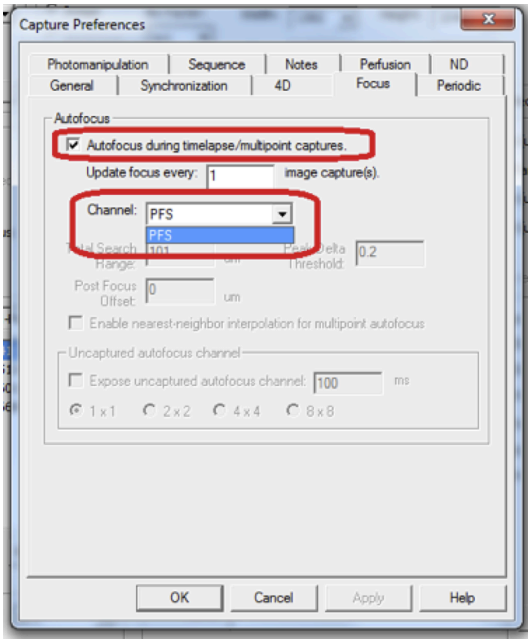
1. Enable the ZDC2 in the ***ZDC2*** tab
2. Compute the desired offset
3. Open the I***mage > Capture*** dialog box. Click on the ***Advanced*** button and go to the ***Focus*** tab. Click the ***Autofocus*** check box and set the frequency of autofocus.
4. Setup the capture and click ***OK***.

At the requested frequency SlideBook will activate the ZDC2 and update the Z position.

Multipoint 2D time lapse capture

1. Enable the ZDC2 using.
2. Move to the first XYZ position and move the sample into focus.
3. In the ***Focus Window > XY*** tab click ***set point***.
4. For each XYZ point repeat steps 2 and 3.
5. In the ***ZDC2*** tab select ***use automatic offset for multipoint captures*** and click ***Compute all offsets***.
6. Open the ***Capture*** dialog box. Click on the ***Advanced*** button and go to the ***Focus*** tab. Click the ***Autofocus*** check box and set the frequency of autofocus. ***ZDC*** will automatically be selected as the ***Channel*** when it is set up properly in the ***Focus*** dialog.
7. Setup the capture and click ***OK***.

SlideBook will move to each XYZ location, activate the ZDC2 system, and then turn it off. The newly updated Z location will be stored after each successful autofocus for future visits to reduce the search time.


Capture Preferences
Photomanipulation
Sequence
Notes
Perfusion
ND
General
Synchronization
4D
Focus
Periodic
Autofocus
Autofocus during timelapse/multipoint captures.
Update focus every:
1
image capture(s).
Channel:
PFS
PFS
Full Search Range:
101
um
Focal Delta Threshold:
0.2
Post Focus Offset:
0
um
Enable nearest-neighbor interpolation for multipoint autofocus
Uncaptured autofocus channel
Expose uncaptured autofocus channel:
100
ms
1 x 1
2 x 2
4 x 4
8 x 8
OK
Cancel
Apply
Help

TUTORIAL: Nikon Ti2 Perfect Focus for Autofocus

Requirements for using Nikon Ti2 Perfect Focus for Autofocus in SlideBook:

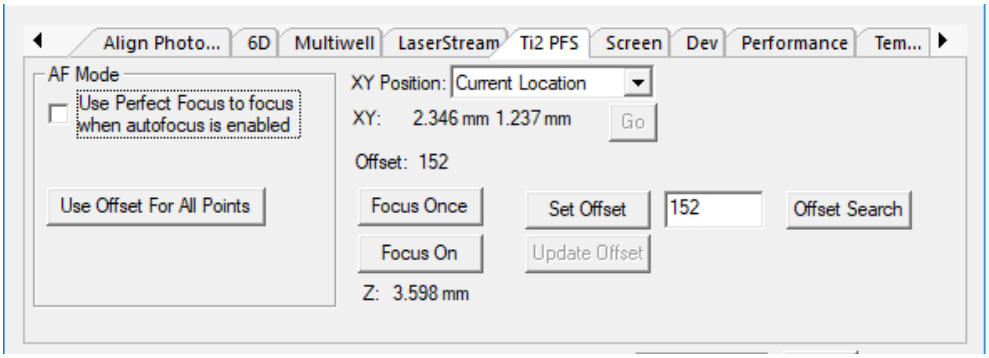
- SlideBook 6.0.14 or newer
- Nikon Ti2 DLL (Ti2_Mic_Driver.dll)
- PFS (Perfect Focus System) compatible objectives - Not all objectives work with Perfect Focus, refer to the Nikon user manual to determine compatibility. Microscope metadata must be configured properly for PFS to work; you can not just swap around objectives without reconfiguring the metadata
- Proper sample - The Nikon manual is an excellent resource on what will and will not focus using PFS

The Nikon Ti2 can operate in two modes:

Normal focus mode, where the Z drive is moved by the Z wheel
PFS mode, where the Z drive is moved by changing the PFS offset

This tutorial will walk you through different use scenarios for using the system in PFS mode. Normal focus mode is controlled by conventional Z methods.

When configured appropriately, the ***Ti2 PFS*** tab will be present in Focus Controls.



PFS works by tracking a reference plane (for example, the coverslip/oil interface for oil immersion lenses). A user defines the desired focal plane and an offset is calculated between the focal plane and reference plane. PFS tracks the reference position over time, maintaining the desired focal plane.

To setup PFS capture you can do one of two things:

(A) Click the PFS button on the stand and use the PFS knob to locate focus. The PFS LED will solid green when the PFS is active.

(B) In SlideBook click the ***Offset Search*** button when the specimen is in focus (assuming the sample is in an acceptable PFS range). This causes the PFS offset to "scan" until the correct offset is located for the current Z position.

To maintain focus in a 2D timelapse capture:

1. Enable the PSF using (A) or (B) above until the reference position is in focus.

2. In the Ti2 PFS tab of Focus controls, check the box to ***Use Perfect Focus to focus when autofocus in enabled.***

3. Open Capture controls. Click ***Advanced*** in the top left corner of the dialog. Select the ***Autofocus*** tab and enable focus using PFS. Check the box to use autofocus and set the frequency of autofocus. Select ***Ti2 PFS*** as the ***Channel***. Click ***OK*** to close Advanced Capture Preferences.

4. Setup the capture parameters as desired and click ***OK*** to run the acquisition.

NOTE: When data is captured the current PFS offset will be used. This means you can use the PFS knob to change focus during capture if needed.

To maintain focus in a 3D timelapse capture:

1. Enable the PSF using (A) or (B) above until the reference position is in focus.

2. In the Ti2 PFS tab of Focus controls, check the box to ***Use Perfect Focus to focus when autofocus in enabled.***

3. Go to the ***Z tab*** of Focus controls window. Click the ***Set*** button for the reference position.

4. Open Capture controls. Click ***Advanced*** in the top left corner of the dialog. Select the ***Autofocus*** tab and enable focus using PFS. Check the box to use autofocus and set the frequency of autofocus. Select ***Ti2 PFS*** as the ***Channel***. Click ***OK*** to close Advanced Capture Preferences.

5. Setup the capture parameters, including 3D capture ***Use Reference Position*** and ***Range around Reference***, and click ***OK*** to run the acquisition.

NOTE: When data is captured the current PFS offset will be used. This means you can use the PFS knob to change focus during capture if needed.

To maintain focus in a multipoint capture:

1. Enable the PSF using (A) or (B) above until the reference position is in focus.

2. Ensure the PFS is activated. The ***Focus On*** button will read ***Focus off*** and the ***PFS LED*** on the stand will be lit.

3. In the Ti2 PFS tab of Focus controls, check the box to ***Use Perfect Focus to focus when autofocus in enabled.***

4. Go to the ***XY tab*** of Focus controls window. Set points by clicking ***Add Point***. Adjust Z using the PFS adjustment knob.

   WARNING: Do not adjust the microscope Z drive of the auxiliary Z drive (if installed). If the PFS is not enabled and you use the PFS adjustment knob, SlideBook will ask if you want to run the ***Offset Search*** command. If the PFS is already enabled, the current PFS offset will be used. To update the offset at a later time, enable PFS on the stand and click Update XYZ of Update Z to use the new offset.

5. Open Capture controls. Click ***Advanced*** in the top left corner of the dialog. Select the ***Autofocus*** tab and enable focus using PFS. Check the box to use autofocus and set the frequency of autofocus. Select ***Ti2 PFS*** as the ***Channel***. Click ***OK*** to close Advanced Capture Preferences.

6. Setup the capture parameters, including ***Multiple XY location capture*** and 3D if desired, and click ***OK*** to run the acquisition.

TUTORIAL: Using Perfect Focus for Autofocus

The Perfect Focus tab of the ***Focus Controls*** window allows you to interact with the Nikon hardware-based autofocus system, Perfect Focus.

SlideBook + Nikon Perfect Focus

Requirements:

- SlideBook 5.0.0.33 or newer
- Nikon Eclipse Ti microscope with motorized Z drive, motorized objective turret and PFS
- Optional: motorized XY stage

Setup:

- ***Administration tab > Hardware Configuration > Objective Turret > Nikon Ti***
- During hardware initialization SlideBook will determine if the PFS is present.

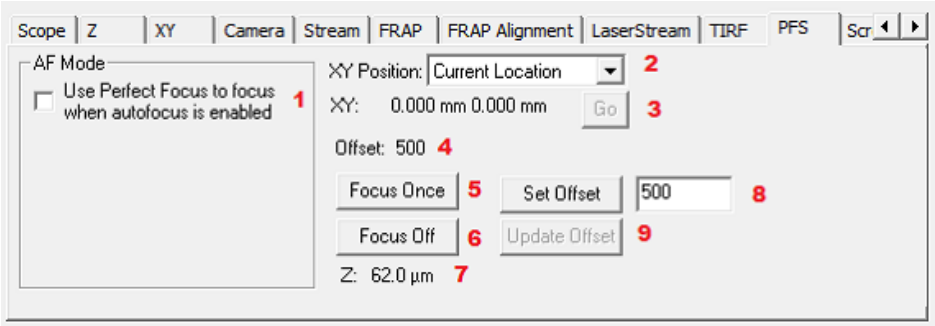
Nikon Perfect Focus – Hardware Operation

The Nikon Perfect Focus System uses the position of the autofocus system to move the objective a specific distance from the slide coverslip. This hardware position is called the offset. When the user moves the PFS knob they are changing this offset position, which is turn will change the Z position of the microscope. Both the PFS offset and the Z position are displayed and updated in this dialog box.

Note that in order for SlideBook to operate the PFS system correctly both the objective and sample must be appropriate and the green "PFS OK" LED on the front of the microscope must be lit. Please refer to the Nikon Eclipse Ti manual for more information.

Nikon Perfect Focus – Focus Window

If detected on startup a PFS tab will be added to the focus window:



This tab is used to configure and test the PFS system.

The check box (1) determines if SlideBook will use the PFS for autofocus. By default it is checked. If the user would like to use SlideBook's image-based autofocus then they can uncheck this box.

While the camera is running the offset (4) and Z position (7) captions are updated. Moving the PFS focus knob will change the offset, and when the PFS is engaged changing the offset will move the Z position.

Clicking on Focus Once (5) will turn the PFS on until focus lock is achieved, and then immediately turn the PFS off. This simulates what happens during capture.

Clicking on Focus Off / Focus On (6) will toggle the state of the PFS. If the user activates or deactivates PFS using the microscope button the caption will change to the appropriate value. For example, when the user activates PFS via the scope or by clicking Focus On the button will change to Focus Off.

The Set Offset (8) button allows the user to set a specific offset value. When the user selects a specific XYZ location from the XYZ Point List (2) the saved offset is displayed in the edit field.

When a specific XYZ Point List (2) location is selected the user can update the PFS offset by clicking the Update Offset (9) button. This will replace the previously saved offset with the current PFS offset.

Nikon Perfect Focus – SlideBook Operation

To use the PFS during SlideBook capture you must perform the following steps (note that you must have PFS autofocus enabled in the PFS tab in all cases):

2D time lapse capture

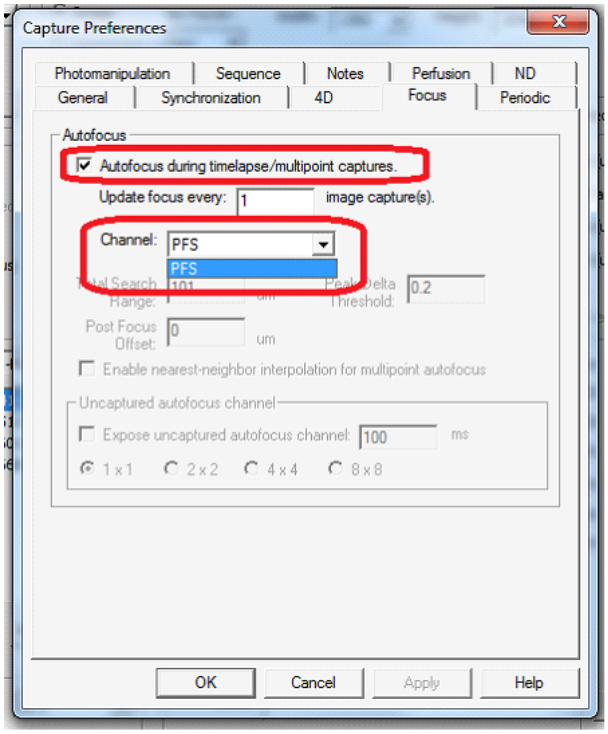
1. Enable the PFS using SlideBook or the microscope body
2. Move the sample into focus using the PFS knob. Optionally disable PFS.
3. Open the ***Image > Capture*** dialog box. Click on the ***Advanced*** button and go to the ***Focus*** tab. Click the ***Autofocus*** check box and set the frequency of autofocus. Select ***PFS*** as the ***Channel***. Click OK to close Advanced Capture Preferences.
4. Setup the capture as desired and click ***OK*** to run the acquisition.

At the requested frequency SlideBook will activate the PFS and update the Z position.

Multipoint 2D time lapse capture

1. Enable the PFS using SlideBook or the microscope body
2. Move to the first XYZ position and move the sample into focus using the PFS knob.
3. In the ***Focus Window > XY*** tab click ***set point***
4. For each XYZ point repeat steps 2 and 3
5. Open the ***Capture*** dialog box. Click on the ***Advanced*** button and go to the ***Focus*** tab. Click the ***Autofocus*** check box and set the frequency of autofocus.
6. Setup the capture and click ***OK***

SlideBook will move to each XYZ location, activate the PFS system, and then turn it off. The newly updated Z location will be stored after each successful autofocus for future visits to reduce the search time.

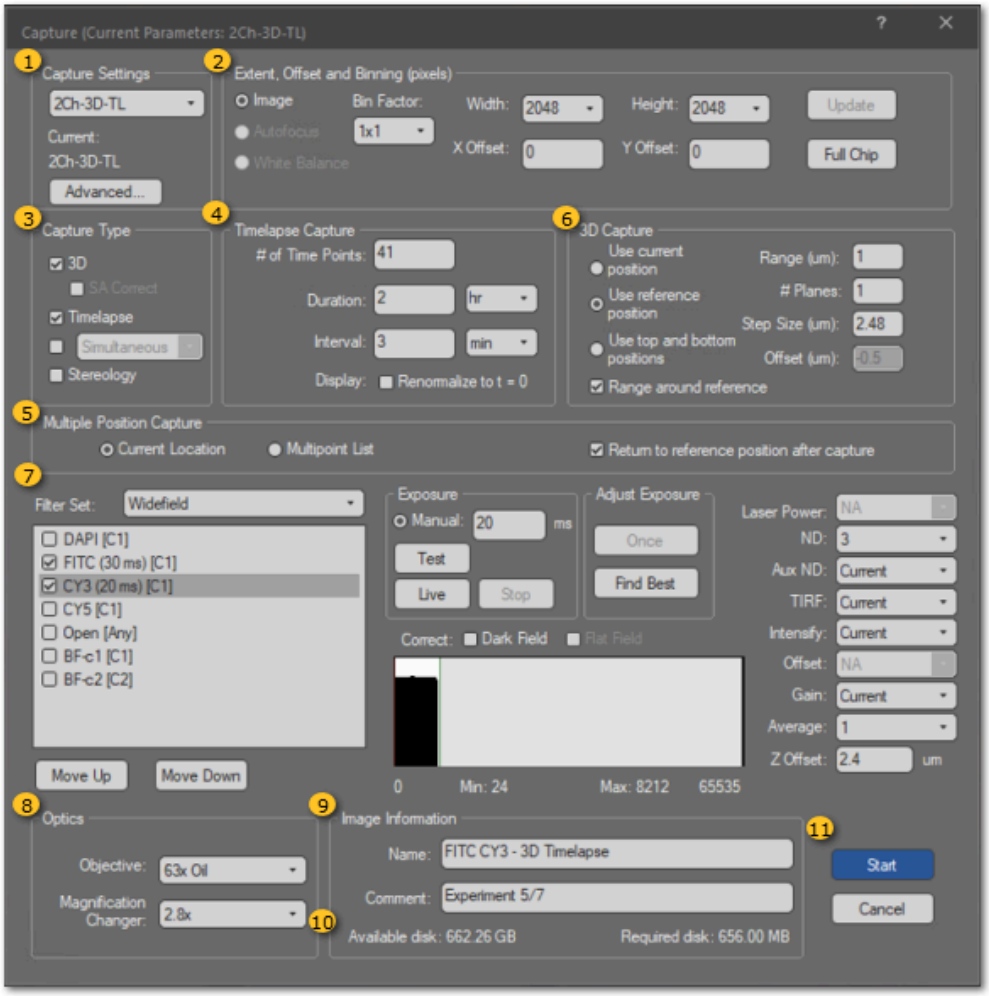


Capture Window

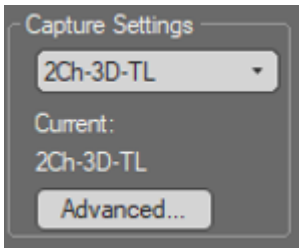
The ***Capture*** window allows you to set up anything from a basic 2D two channel image to a complex 4D experiment with point visiting. Basic controls are outlined below. Advanced controls can be accessed from the ***Advanced*** button.

See also:

- Focus Controls window
- Advanced Capture Preferences window
- Exposure Time Tips
- Setting Capture Parameters for Timelapse Ratio Imaging
- Capture Controls window
- TUTORIAL: Using 6D Scripted Capture
- 6D MultiCapture window



1 Capture Settings group



The dropdown menu allows you to select/load , ***Save***, ***Save as***, ***Rename***, and ***Delete*** experiment capture preferences. Capture settings (**.prefs** file type) can later be found and managed by going to the ***Administration tab > More Options > Open User Preferences***. See also Backing up and Restoring Preferences.

The ***Advanced*** button opens a dialog to set up more complex parameters such as variable time lapse intervals, capturing a mid-volume plane, autofocus, perfusion design, and more. Refer to Advanced Capture Preferences for more information on these options..

2 Extent, Offset and Binning (pixels) group

Select a ***Bin Factor*** from the dropdown menu. Binning amplifies signal while decreasing resolution by combining the signal of adjacent pixels before readout.

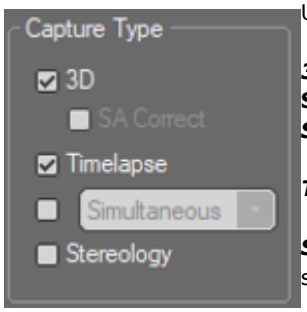
Select the *x*- and *y*-extent of the image from the ***Width*** and ***Height*** dropdown menus. You may also manually enter the desired values in the edit field. If you want to move the region off the center of the CCD, enter offset values for both the *x*- and the *y*-axes in the ***X Offset*** and ***Y Offset*** fields.

Alternatively, you may draw a region for imaging by using the ***Marquis*** tool and clicking ***Update***. To revert to the full imaging area, press ***Full Chip***.

NOTE: You will need to take a ***Test*** capture to update the ***Camera*** window after any of the above changes have taken place.

See the Focus Tab of Capture Preferences window to learn how to select a region for auto focus during capture.

3 Capture Type group



Use the checkboxes to enable different capturing paradigms as follows:

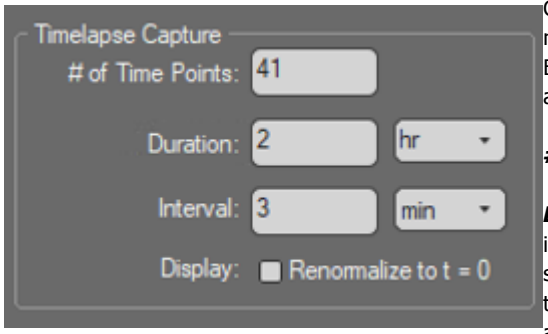
3D - Captures multiple planes in the z axis as designated in 3D Capture
SA Correct - Enables a motorized Spherical Aberration Correction device as configured ***SA Correction*** tab of Focus Controls.

Timelapse - Captures images at designated time intervals as set in Timelapse Capture

Simultaneous - Enables simultaneous dual camera or image splitter operation for systems so equipped

Stereology -Enables capture of stereology images, requires SlideBook Stereology module.

4 Timelapse Capture group



Check the ***Timelapse*** checkbox in the Capture Type section. You now have the option to define a variety of timelapse parameters. Enter the two of the following values and the third value will be automatically calculated.

# of Time Points – Number of timepoints that will be captured.

Duration – Total length of time for the experiment. Units of time, in milliseconds (ms), seconds (s), minutes (m) or hours (h), can be selected from the dropdown menu. Due to the inability to predict time required for hardware movement, this option may not me accurate when the interval for capture is very small.

Interval – Delay between the beginning of one timepoint and the beginning of the next timepoint. The interval unit can be selected from the dropdown menu. If the capture sequence at one timepoint takes longer than the interval, SlideBook will capture the next timepoint immediately following the preceding timepoint.

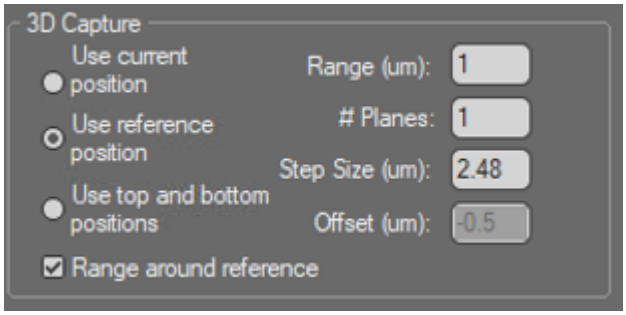
Display: Renormalize to t = 0 – When checked, the minimum and maximum pixel intensity values for the first timepoint in a timelapse capture series will be used to determine the renormalization values of all subsequent timepoints. This option only affects the image display during capture and will not affect the captured image.

5 Multiple XY Location Capture group

Use the radio buttons to select ***Current Location*** or ***Multipoint List***. When Multipoint List is selected, either point visiting or montage parameters will be imported from the ***XY Tab*** of Focus Controls.

For any capture paradigm, check the box for ***Return to Reference/First XYZ location after capture*** if you would like to refocus to the center of the captured *z*-range after image acquisition has finished. If you do not check this box, focus will be at the last plane captured when you resume live sample viewing.

6 3D Capture group



Check the ***3D*** checkbox in the Capture Type section. You now have the option to slect one of three different 3D capture paradigms. Read about each paradigm below carefully to ensure you are selecting the desired behavior.

Use current position is useful when no *z* positions have been set in the Z Tab of ***Focus Controls***. Select the radio button for this option then enter values for two of the three parameters: ***Range***, ***# Planes*** and ***Step Size***. The third parameter will be calculated based on the other two values.

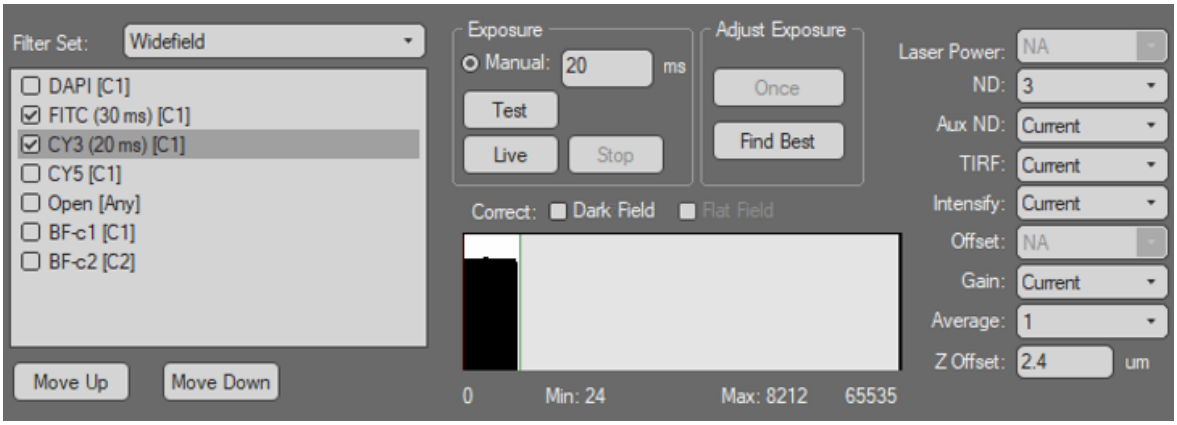
Offset will move down in the *z* direction a specified distance before beginning collection of the *z*-stack. Alternatively, you may check the box for ***Range around center*** to use the current position as the center of the *z*-stack (automatically calculating ***Offset***). If ***Offset*** is left at **0** and the box is not checked for ***Range around center***, image capture will begin at the current position and move up.

Use reference position is used either when a reference position has been set in the Z Tab of ***Focus Controls*** or when an XY point list has been set in the ***XY Tab*** of ***Focus Controls*** and selected in Multiple XY Location Capture.

Select the radio button for this option then enter values for two of the three parameters: ***Range***, ***# Planes*** and ***Step Size***. The third parameter will be calculated based on the other two values. Offset will move down in the *z* direction a specified distance before beginning collection of the *z*-stack. Alternatively, you may check the box for ***Range around center*** to use the reference position as the center of the *z*-stack (automatically calculating ***Offset***). If ***Offset*** is left at **0** and the box is not checked for ***Range around center***, image capture will begin at the reference position and move up.

Use top and bottom positions is useful when top and bottom *z* positions have been set in the Z Tab of ***Focus Controls***. Values from the Focus Controls will populate the ***Range***, ***# Planes***, ***Steps Size***, and ***Offset*** fields. If you choose, you may change the ***# Planes*** and/or ***Step Size*** at this time using the edit fields. ***Range*** and ***Offset*** are not editable as they are based on the ***Top*** and ***Bottom*** positions set in ***Focus Controls***.

7 Adjust Exposure group



Click on the checkbox of the channel that you would like to expose. The channel that you select will be highlighted in blue.

To manually set ***Expose*** time:
Enter an exposure time in the ***Expose*** field and press ***Test***. Pressing the ***Test*** button for a particular filter configuration will give you a sample single-plane image at the specified exposure time for the selected channel.
You may resize the image by dragging and dropping the corners or edges of the live ***Camera*** window. An intensity histogram and the minimum and maximum data values will be displayed in the histogram area to show you where in the dynamic range of the CCD your exposure lies. Continue to evaluate the histogram, adjust ***Expose*** time, and press ***Test*** until an ideal exposure time has been determined. Please refer to Exposure Times Tips for more information on properly setting exposure time and interpreting histograms.

You may find it useful to start a live image preview in the ***Camera*** window. To do so, use the ***Live*** and ***Stop*** buttons in the ***Exposure*** group.

To automatically set ***Expose*** time:
Press the ***Once*** button in the ***Adjust Exposure*** section and notice that the exposure time has been adjusted to take advantage of the CCD camera's full dynamic range. Alternatively, press ***Find Best*** to allow SlideBook to take a series of test images and determine an exposure time to maximize the dynamic range.

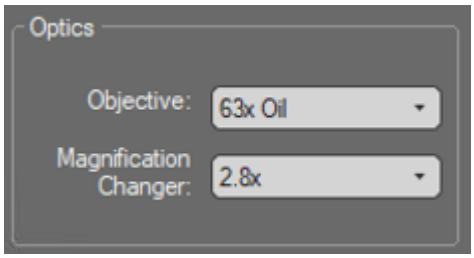
For multi-channel capture:
Simply repeat the process of checking the box/selecting the channel and determining ***Expose*** time for each channel that you wish to expose. You may use the ***Move Up*** and ***Move Down*** buttons with a selected channel to change the order in which channels are captured during data acquisition.

The ***Correct: Dark Field*** check box will cause SlideBook to capture a dark field calibration image for each channel. The dark field image will be acquired with all shutters closed using the exposure times, intensification, gain, and other conditions set in the ***Capture*** window. These images will automatically be subtracted from each captured frame before the image is written to memory. Most cameras available today produce such a low level of dark noise that the fraction of the image noise comprised of dark current is practically negligible.

The ***Correct: Flat Field*** check box will apply flat field correction when flat field setup has been performed (see also Flat Field Calibration).

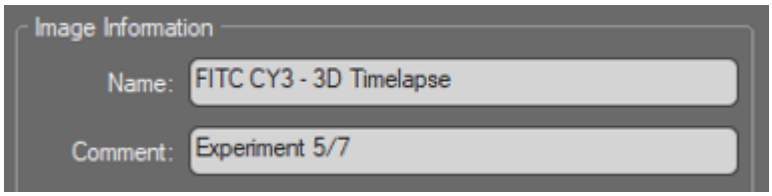
The dropdown menus for ***Laser Power***, ***ND***, ***Aux ND***, ***TIRF***, ***Intensify***, ***Offset***, ***Gain, Average and Z offset*** allow control of specific components of an imaging system that may or may not be present on your system. ***Current*** will utilize the values last used in the ***Focus Controls***.

8 Optics group



Select an ***Objective*** and ***Mag Changer*** from the dropdown menus. For manual microscope systems, you should ensure the value set here match the physical optics that are in place. Motorized microscope systems will automatically use the correct vales. ***Optics*** information is used to assign pixel size for an image.

9 Image Information (Optional) group

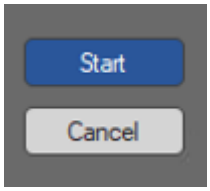


If desired, you may type an image ***Name*** and ***Comment*** in this field. ***Name*** will title the image within the **.sld(y)**. If you perform multiple captures with the same name, the name will be appended with **- 1**, **- 2**, **- 3**, etc. ***Comment***s will be visible from the **.sld(y)** view and editable from ***Image Info***.

10 File Size information

SlideBook will automatically check to see how much memory is ***Available*** and how much will be ***Required*** by the designated experiment. You should check this field before beginning data acquisition. To decrease file size, consider capturing a region rather that the entire field of view.

11 Start/Cancel buttons



Use these buttons to ***Start*** data capture or to ***Cancel*** capture and close the dialog.

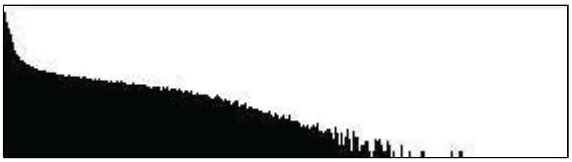
Exposure Time Tips

Setting exposure time appropriately is essential to obtaining the desired imaging results, for live cell experiments, it can be a matter of life and death! When using an EM (electron multiplying) camera, one needs to adjust intensification with exposure time. Here are some basic tips for setting exposure time and intensification appropriately.

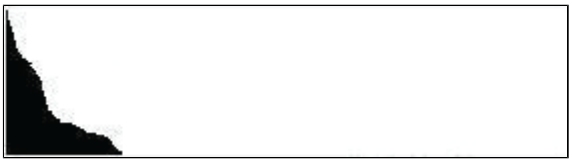
- For fixed specimens, aim for filling the histogram 2/3 of the way. This gives you room for brighter planes.
- Never saturate an image you would like to use for deconvolution or most other quantitative analysis techniques.
- For live samples, experiment to determine how much light they can handle. You will often sacrifice intensity for cell viability. It is acceptable to have your signal at twice the background level, depending on the noise in the image.
- When imaging live samples with an EM camera, you can increase the signal while keeping the exposure time low by increasing the intensification.
- If possible, try to keep the intensification level well below the maximum available.
- When using a non-EM, higher resolution camera, another way to increase signal without increasing exposure time is to utilize binning. It is important to note that binning will decrease the lateral resolution of an image.

Example Histograms

Ideal

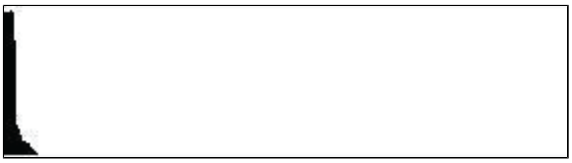


Acceptable for Live Cell Imaging



Saturated (not compatible with quantitative analysis)

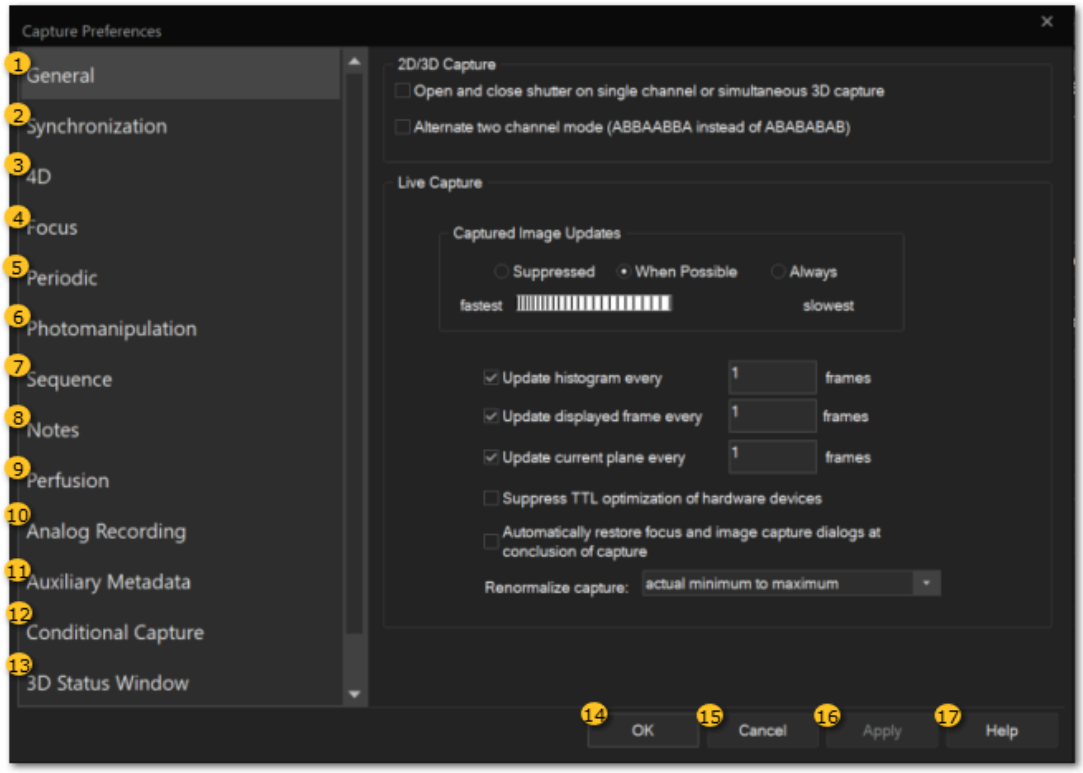
Underexposed



Advanced Capture Preferences window

Advanced ***Capture Preferences*** allow you to easily increase the complexity of experiments by controlling various external and internal hardware during image capture without scripting. Below, you will find an overview of each of the tabs with links to the complete descriptions and application pages.

Access ***Capture Preferences*** from ***Advanced...*** within the Capture window.



1 General tab

General

The General tab contains several options for adjusting shuttering or channel capture order when photobleaching or hardware speed (respectively) are concerns. There are also some options for updating display during capture that may be relevant if your computer is more than three years old.

2 Synchronization tab

Synchronization

Part of the SlideBook TTL Synchronization Module allows TTL pulses to be incorporated into experiments via the Synchronization tab. You can use TTL triggers to start experiments or have experiments send out a TTL pulse or train of pulses at specific times. All activity is logged as ***Notes*** within the image file.

3 4D tab

4D

The 4D tab allows you to capture a single plane in one channel when you are acquiring 3D stacks in another channel or channels. In this tab, you may also set up channel collection order for 3D stacks; that is, you may choose to collect a *z*-stack in before switching channels instead of collecting all channels before switching *z*-plane.

4 Focus tab

Focus

When you would like to use auto focus during time lapse imaging, you may configure auto focus parameters using the Focus tab. This tab is used computer contrast based auto focus algorithms as well as hardware based auto focus

systems including Zeiss Definite Focus, Nikon Perfect Focus, Leica Adaptive Focus Control, and Olympus Z-Drift Compensator.

5 Periodic tab

Periodic

You may choose to capture a single channel at a slower rate than other channels when performing a multi-channel 2D time lapse capture. You may do so by selecting the Periodic tab.

6 Photomanipulation tab

Photomanipulation

The Photomanipulation tab allows you to configure settings for right-click and double-click event initiation as well as advanced event protocols. In this tab you can control everything from laser power to complex triggering of different regions at different time points.

7 Sequence tab

Sequence

You may find it useful to vary the time lapse intervals used during image acquisition. The Sequence tab allows you to configure different stages to capture either automatically in a preset protocol or by manually triggering.

8 Notes tab

Notes

Notes can be configured either prior to or during image capture. When notes are designed prior to capture, they become available buttons in the Notes tab of ***Capture Controls*** during acquisition. To set up notes prior to capturing data, review the Notes tab of Capture Preferences window.

9 Perfusion tab

Perfusion

Complex experiments utilizing TTL driven perfusion devices are effortless in SlideBook. The Perfusion tab provides manual control of up to 24 channels and a scripting dialog for more complex experiments. Manual control of pumps is linked to the ***Notes*** buttons during capture execution. You may also combine manual and automated controls to achieve the desired experimental results.

10 Analog Recording

Analog Recording

Allows for continuous recording of one or more analog inputs during a capture.

11 Auxiliary Metadata

Auxiliary Metadata

Enables recording of temperature outputs from cameras, lasers, and incubation chambers.

12 Conditional Capture

Conditional Capture

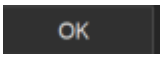
Allows for execution of SlideBook scripts or Matlab functions during or after capture See Conditional Capture for more details.

13 3D Status Window

3D Status Window

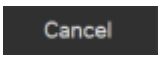
Displays three-view maximum intensity projections at each time point during a 4D acquisition. See 3D Status Window for more details.

14 OK button



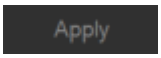
Press ***OK*** to apply changes made in all tabs and close the ***Capture Preferences*** window. You will return to the ***Capture*** dialog to begin image acquisition.

15 Cancel button



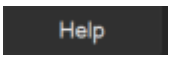
Press ***Cancel*** to discard changes made in all tabs and close the ***Capture Preferences*** window. You will return to the ***Capture*** dialog to begin image acquisition.

16 Apply button



Press ***Apply*** to save the current capture parameter changes and continue working in the ***Capture Preferences*** window.

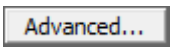
17 Help button



Press ***Help*** to open online help.

Photomanipulation Tab of Capture Preferences

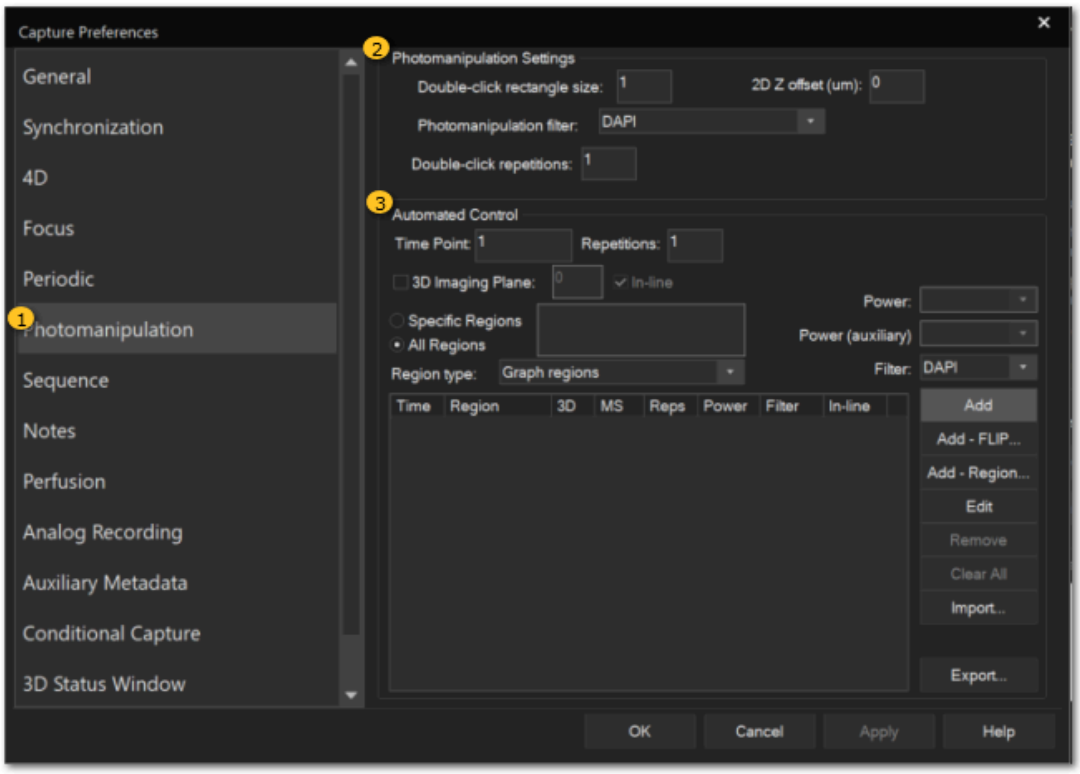
Prior to running an experiment, you may configure various Vector settings to assist in photomanipulation during image capture. The ***Photomanipulation*** tab of ***Capture Preferences*** allows you to configure settings for right-click and double-click event initiation as well as advanced event protocols. Be sure to save your Capture Settings if you would like to use the values set in this tab in future SlideBook imaging session.

To access this window, click 

in the ***Capture*** window.

See also:

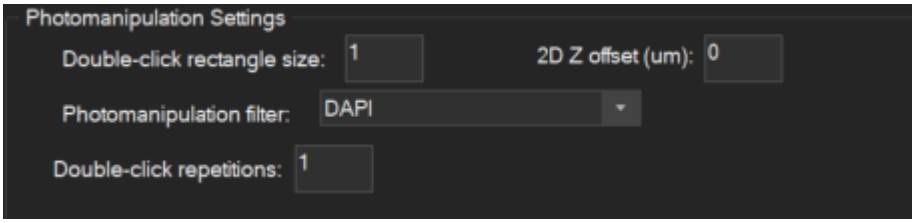
Right-Click to Initiate an Event in the Photomanipulation Module
Double-Click to Initiate an Event in the Photomanipulation Module
Advanced Event Protocols in the Photomanipulation Module



1 Photomanipulation tab

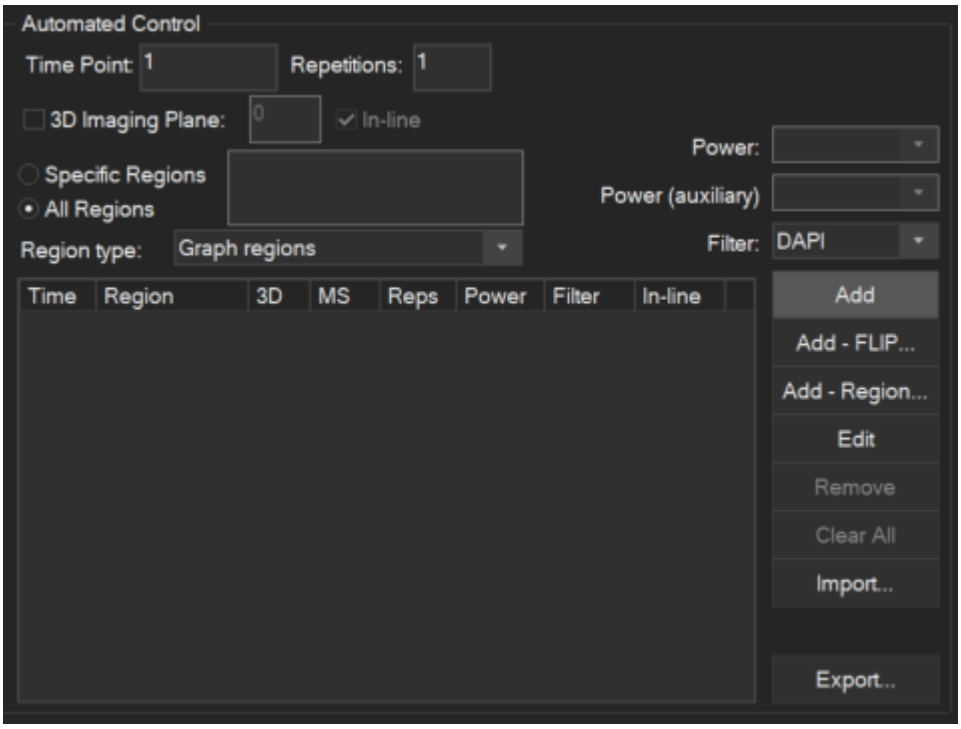
The ***Photomanipulation*** tab contains all photomanipulation settings for experiment design.

2 Photomanipulation Settings group



Photomanipulation Settings are used to configure settings for right-click and double-click event creation and to set the laser used for photomanipulation. Click here to learn more about these settings.

3 Automated Control group



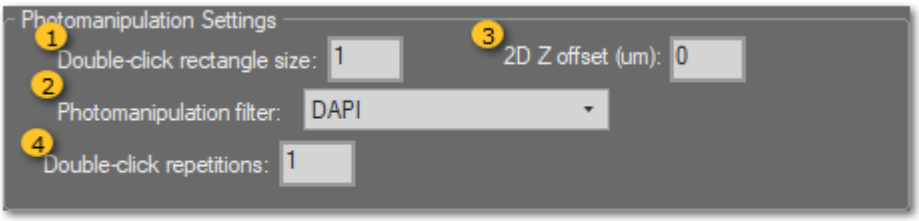
The ***Automated Control*** group contains tools to set up advanced photomanipulation experiments. Click here to learn more about these tools.

Photomanipulation Settings window

This is the upper portion of the ***Photomanipulation*** tab in the ***Capture Preferences*** window. It is used to configure basic photomanipulation settings.

See also:

Right-Click to Initiate an Event in the Photomanipulation Module
Double-Click to Initiate an Event in the Photomanipulation Module
Advanced Event Protocols in the Photomanipulation Module
Photomanipulation tab of Capture Preferences



1 Double-click rectangle size

This is the number of spots that will be hit by the laser is a square pattern when you double-click to initiate an event during image capture. A good starting point to test is the value of the ***Raster block size*** used in the ***FRAP*** tab of ***Focus Controls***.

2 Photomanipulation filter

This is the laser line that will be used for the photomanipulation event(s). Filters that show in this list are those configured with an ***Associated Channel type*** equal to ***Photomanipulation*** in the ***Filter Configuration Parameters***.

3 2D Z offset (um)

The distance in Z that the sample should be moved before a photomanipulation event takes place. This value can be determined using the ***Z Stage*** section of ***Focus Controls***. The value entered can be positive or negative and include a decimal point. Some example values include: **1**, **0.55**, and **-2.1**.

4 Double-click repetitions

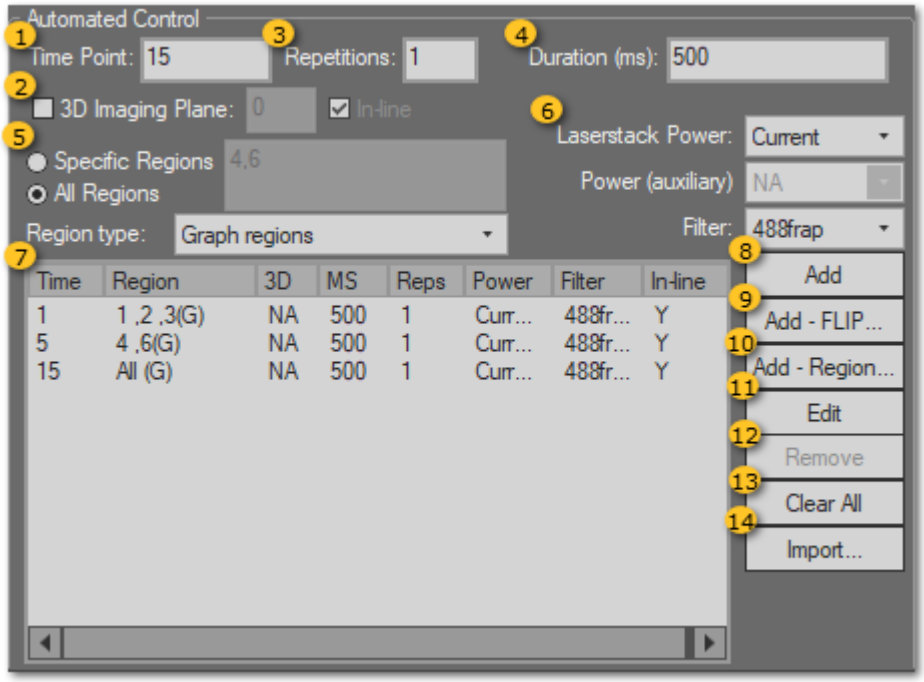
Number of times photomanipulation event will be repeated when a double-click event is initiated.

Automated Control window

This is the lower portion of the ***Photomanipulation*** tab in the ***Capture Preferences*** window. It is used to set up advanced capture protocols utilizing photomanipulation.

See also:

[Advanced Event Protocols](#) in the Photomanipulation Module
[Photomanipulation tab of Capture Preferences](#)



1 Time Point

The time point that will be captured after the event will take place. This is a whole number integer starting with **0** and ending with the value defined under ***# of Time Points*** in the ***Capture*** dialog. For example, if a value of **3** were entered, two time points would be captured then the photomanipulation event would take place then the third time point would be captured.

2 3D Plane

If you are imaging a 3D stack and would like the photomanipulation event to occur on a single plane within the stack, check the box and type the integer value of the Z plane where the event should occur. Not checking this box when imaging a 3D stack will result in the event occurring at the Z plane in the center of the stack.

In-line is always enabled and that means the stimulation occurs during the stack capture. The alternative would be not in-line and would mean you capture a 3D stack, move to plane N, stimulate, return back to top. It is not yet implemented.

3 Repetitions

The number of times the photomanipulation event will be repeated at the time point specified. To repeat an event at another time point or series of time points, click the ***Add- FLIP*** button.

4 Duration

Enter a value for the duration of the specified event.

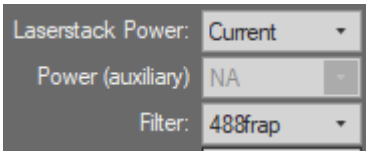
5 Regions

Use the radio buttons as follows:

Specific Regions - Enter the number of the region or regions that will be photomanipulated. Numbers can be formatted using commas and/or dashes such as **1, 4-7, 15**.

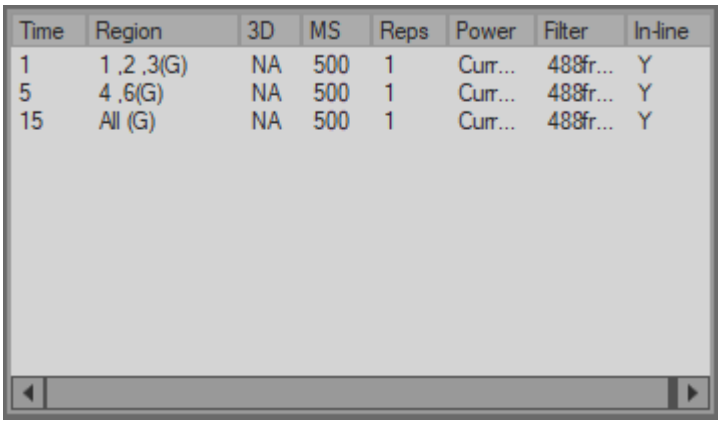
All Regions - All regions drawn in the active ***Camera*** window will be photomanipulated.

6 Power



You may select the laser power level from the dropdown list where ***0*** refers to 0% of the laser power and ***100*** refers to full (100%) laser power. ***Current*** uses the current value configured in the ***FRAP*** tab of ***Focus Controls.***

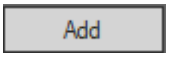
7 Action List



Time	Region	3D	MS	Reps	Power	Filter	In-line
1	1 ,2 ,3(G)	NA	500	1	Curr...	488fr...	Y
5	4 ,6(G)	NA	500	1	Curr...	488fr...	Y
15	All (G)	NA	500	1	Curr...	488fr...	Y

The ***Action List*** field provides a display of all photomanipulation events configured for the current capture sequence. You may remove one or all events from the list using the buttons to the left of the list.

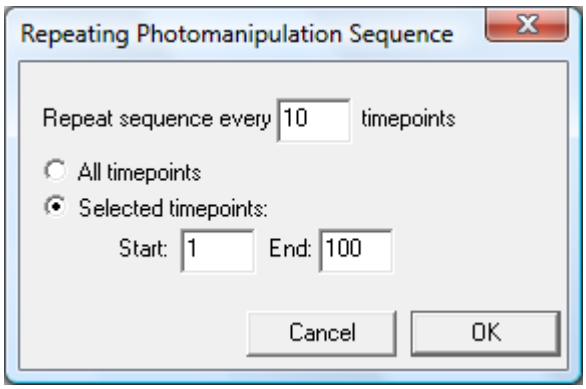
8 Add Button



Once all settings for a photomanipulation event have been configured, click ***Add*** to add the event to the image capture.

9 Add - FLIP button

This opens a dialog that allows you to repeat a photomanipulation event over multiple time points. First, set up a photomanipulation event by setting the time point, 3D plane, repetitions, and regions. Then, click the ***Add- FLIP*** button to open the following dialog:



Repeat sequence every** N **timepoints - Enter an integer value for the number of time points to wait between triggering an event.

All timepoints - Select this radio button to repeat the sequence for the entire experiment.

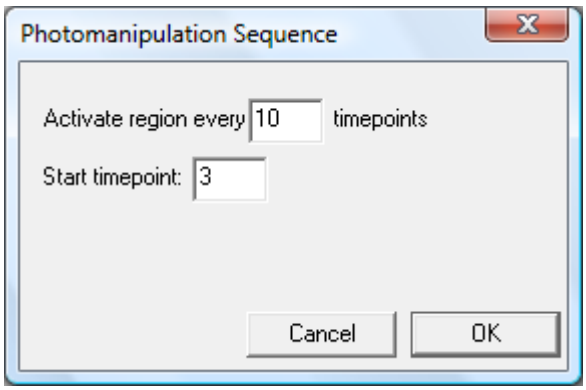
Selected timepoints - Select this radio button to repeat the sequence during a specified range of time points.

Cancel - Closes the dialog and does not add a repeating photomanipulation sequence to the ***Action List***.

OK - Adds a repeating photomanipulation sequence to the ***Action List*** and closes the dialog.

10 Add - Region Button

This opens a dialog that allows you to direct events to a sequence of regions during a sequence of time points. One example of this would be if you wanted to photomanipulate region 1 at time point 3, region 2 at time point 13, and region 3 at time point 23. To do so, you would complete the dialog as follows:



Activate region every** N **timepoints - Defines the interval between triggering photomanipulation events on regions.

Start timepoint - Defines the timepoint before which the first region will be photomanipulated.

Cancel - Closes the dialog and does not add a photomanipulation sequence to the ***Action List***.

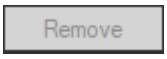
OK - Adds a photomanipulation sequence to the ***Action List*** and closes the dialog.

NOTE: This dialog will create a sequence for all regions that show in the ***Camera*** window at the time ***OK*** is clicked. Regions will be added in numerical order.

11 Edit button

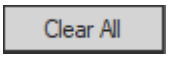
Select an event from the list by clicking on it. Click ***Edit***. This will remove the event from the list and re-populate the event parameters in the top part of the window. Adjust any parameters you would like to change and click ***Add*** to re-add the event to the list.

12 Remove button



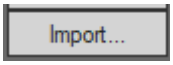
Select an event or event sequence from the ***Action List*** then click ***Remove*** to remove the event from the list.

13 Clear All button



Click ***Clear All*** to remove all events and event sequences from the ***Action List***.

14 Import button



This allows you to import a user generated text file containing photomanipulation information. The import format is a tab-delimited (\t) text file with Windows line endings (\r\n). All fields are required. The field format is:

Start time\Duration\Power 1 (top power menu) (-1 = current / ignore)\Power 2 (lower power menu) (-1 = current / ignore)\Region(s) (comma separated)\Channel name\Description

Example 1:
1000\t50\t100\t-1\t1,5\tPhasor 950\t50 ms ROI 1,5 stim @ t=10000 ms\r\n

Starting at time = 1000 stimulate regions 1 and 5 for 50 milliseconds using channel Phasor 950 at power 50. The comment displayed for this event will be "50 ms ROI 1,5 stim @ t=10000 ms".

Some more complex formats are available for use with Phasor:

1p stimulation

duration@{H,L},duration@{H,L}

Duration in ms or us depending on system timing property (phasor hardware properties/millisecond timing mode)
H = laser on
L = laser off

Example 1: 10@H,5@L
Turn laser on (H) for 10 ms/us, then turn laser off for 5 ms/us

Example 2: 10@H,5@L,10@H
Turn laser on (H) for 10 ms/us, then turn laser off for 5 ms/us, then turn laser on for 10 ms/us

2p stimulation

duration@power, duration@power, duration@power where duration is in ms and power in device units

Example 1: 40@100,60@0,40@200,60@100
40 ms @ 100 power, 60 ms @ off, 40 ms @ 200 power, 60 ms @ off

Example 2:40@100,60@50,40@200,60@50
40 ms @ 100 power, 60 ms @ 50 power, 40 ms @ 200 power, 60 ms @ 50 power

Repeating loops

All of the sequences above can be set to repeat N times using the following format: Nx[sequence]

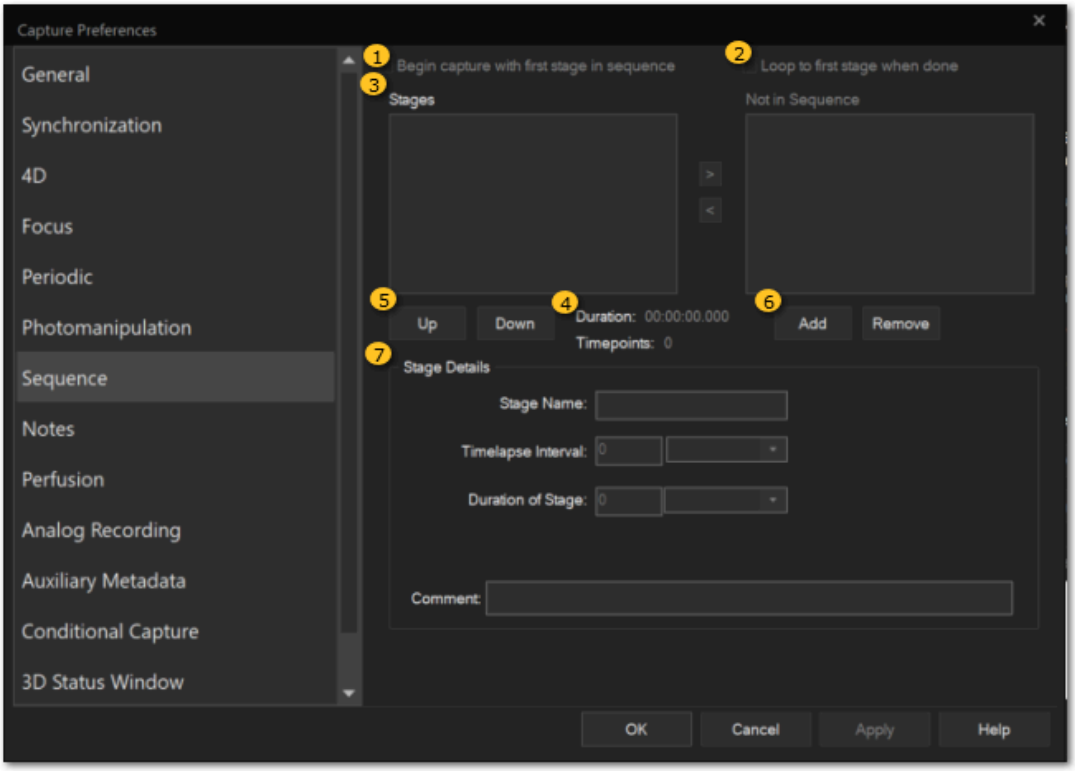
Example 1: 50x[10@H,5@L]
Repeat 10 ms on / 5 ms off 50 times (total duration = 750 ms)

Example 2: 25x[40@100,60@0]
Repeat 40 ms @ 100 power / 60 ms @ off 25 times (total duration = 2500 ms)

Sequence Tab of Capture Preferences window

You may find it useful to vary the time lapse intervals used during image acquisition. The Sequence tab allows you to configure different stages to capture either automatically in a preset protocol or by manually triggering. Be sure to save your Capture Settings if you would like to use the values set in this tab in future SlideBook imaging session.

To access this window, click Advanced... in the ***Capture*** window.



1 Begin capture with first stage in sequence check

Begin capture with first stage in sequence

If ***Begin capture with first stage in sequence*** is checked, capture will start with the first event in the sequence and proceed through the stages until the # of Time Points set in the ***Capture*** dialog is met.

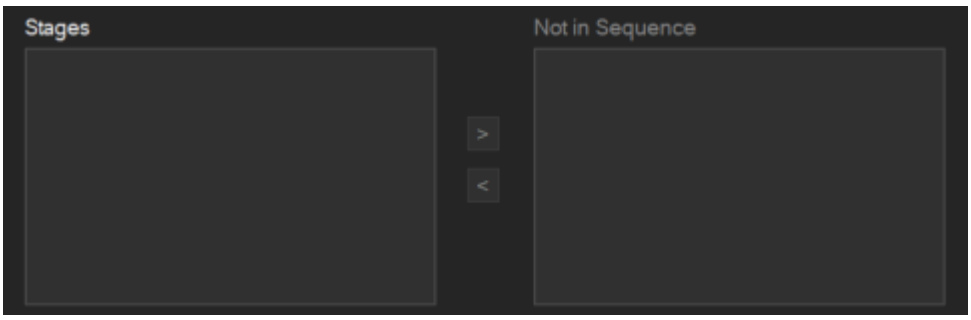
If this box is not checked, SlideBook will begin capture according to time lapse parameters set in the ***Capture*** dialog. You may execute any of the stages manually during capture and SlideBook will automatically progress through the stages until the # of Time Points set in the ***Capture*** dialog is met. Therefore, if you plan on manually executing stages during image acquisition, it is recommended that you set a large value for ***# of Time Points***. When image acquisition is complete, you can end a capture early by pressing the ***Stop*** button, thus ending acquisition and saving the images to the **.sld** before the requested number of time points is complete.

2 Loop to first stage when done check

Loop to first stage when done

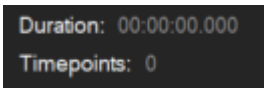
If ***Loop to first stage when done*** is checked, capture will return to the first stage in the list if the # of Time Points set in the ***Capture*** dialog exceeds the number of time points designated in the ***Stages*** area.

3 Stages area



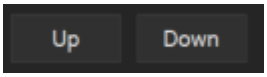
Displays ***Stages*** configured for capture and those ***Not in Sequence***. The ***Stages*** area designates capture sequences that will be performed during image acquisition. As soon as once sequence is complete, capture will move to the next stage in the list. Alternatively, sequences can be placed in the ***Not in Sequence*** area and manually initiated at any time during image acquisition. Stages may be moved between the two lists by selecting the stage (selected stage will be highlighted in blue) and using the **>** and **<** buttons.

4 Experiment Information



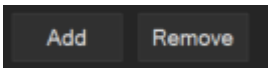
This is a read-only area that calculates experiment ***Duration*** and total number of ***Timepoints*** based on the sequences configured in the ***Stages*** list. This information can be used to help populate the Timelapse Capture parameters for the desired acquisition result.

5 Up/Down buttons



When a stage is selected, the acquisition order of the stages can be changed by using the ***Up*** and ***Down*** buttons.

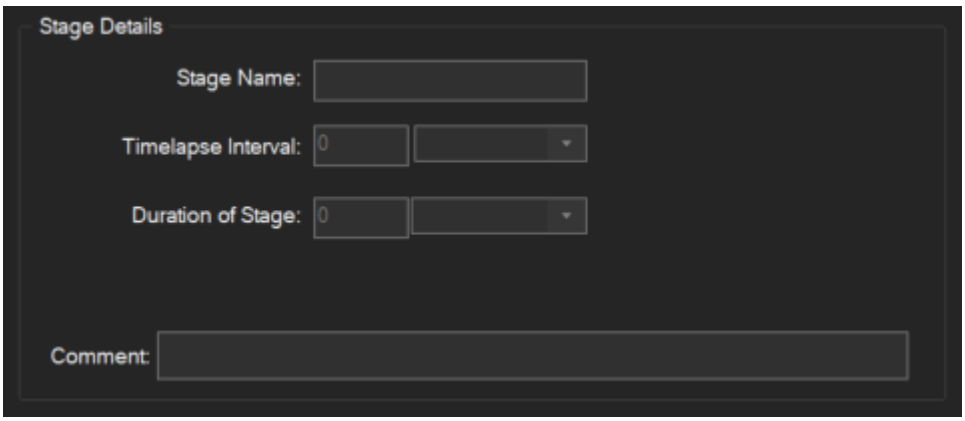
6 Add/Remove buttons



To add a new stage, click ***Add*** then complete the ***Stage Details*** section.

To remove a stage, select it and click ***Remove***.

7 Stage Details group



After pressing ***Add***, you may designate the specifics of a stage in this area. Alternatively, you may edit an existing stage at any time by selecting it (highlighted in blue) and updating the values in this area.

Stage Name: Type the name of the stage here. This value will appear as a ***Note*** in the resulting image when no ***Comment*** is written.

Timelapse Interval: Delay between the beginning of one timepoint and the beginning of the next timepoint. The interval unit can be selected from the dropdown menu. If the capture sequence at one timepoint takes longer than the interval, SlideBook will capture the next timepoint immediately following the preceding timepoint.

Duration of Stage: Enter the a value for how long you would like to acquire images at the requested time interval. Choose either timepoints or units of time from the dropdown menu. If ***continuous*** is selected as the units for ***Duration of Stage***, the stage will end when a different stage is manually initiated.

Comment: Type any additional comments about the sequence here. This information will be displayed as a ***Note*** in the resulting image. If this field is left blank, the ***Stage Name*** will become the note in the image.

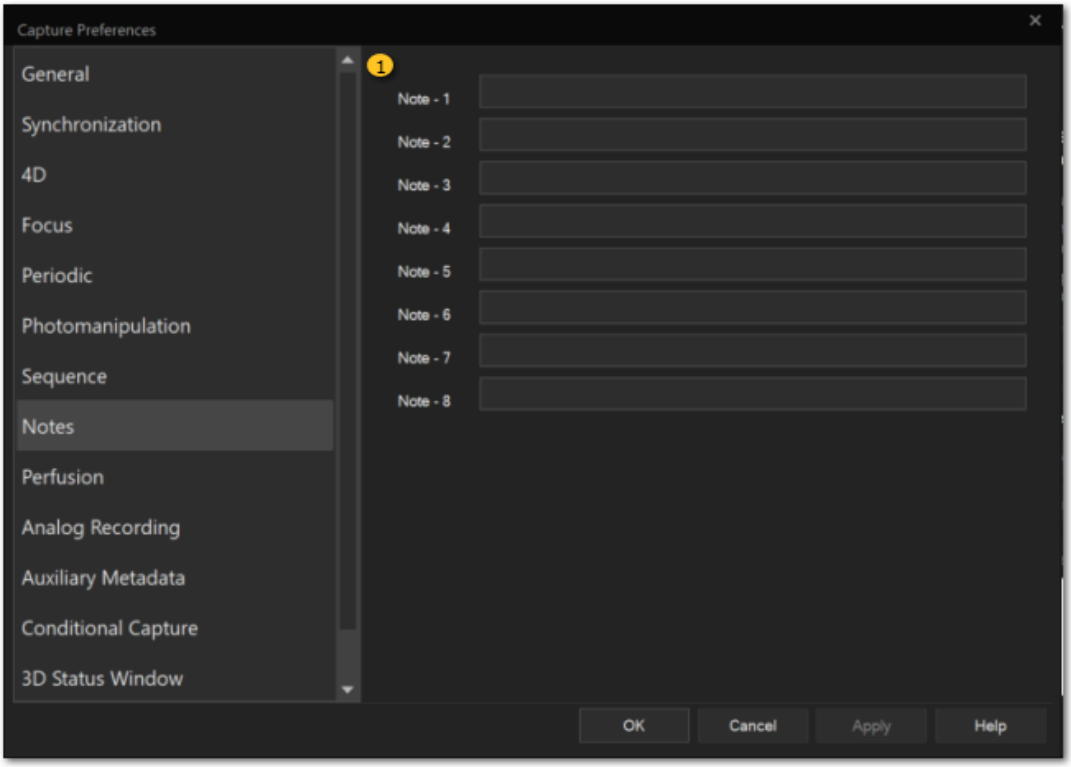
Notes Tab of Capture Preferences window

Notes can be configured either prior to or during image capture. When notes are designed prior to capture, they become available buttons in the ***Notes*** tab of ***Capture Controls*** during acquisition.

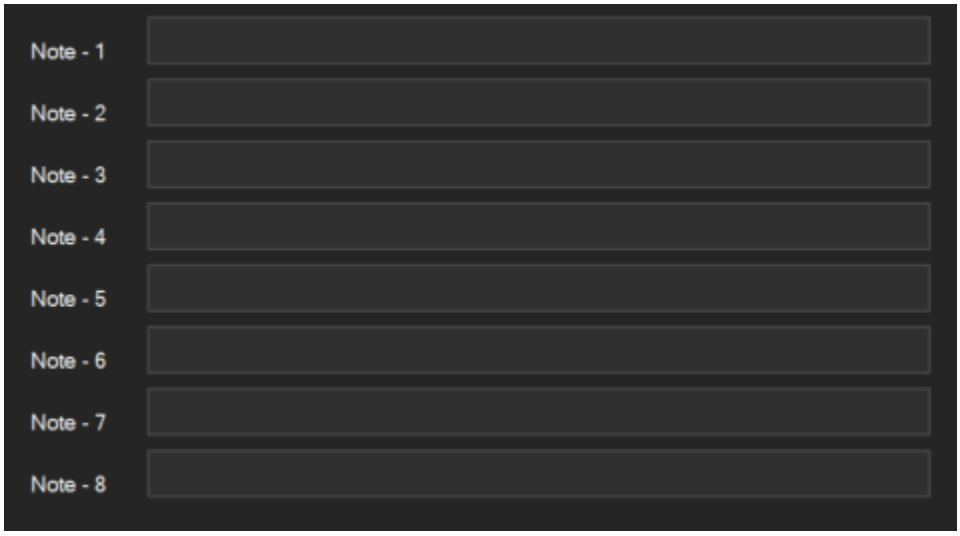
To access this window, click ***Advanced...*** in the ***Capture*** window.

See also:

User Interface, Notes Tab of Capture Controls window
Ratio Module, Setting Capture Parameters for Timelapse Ratio Imaging
Ratio Module, Creating Event Markers (Notes)



1 Note Creation area

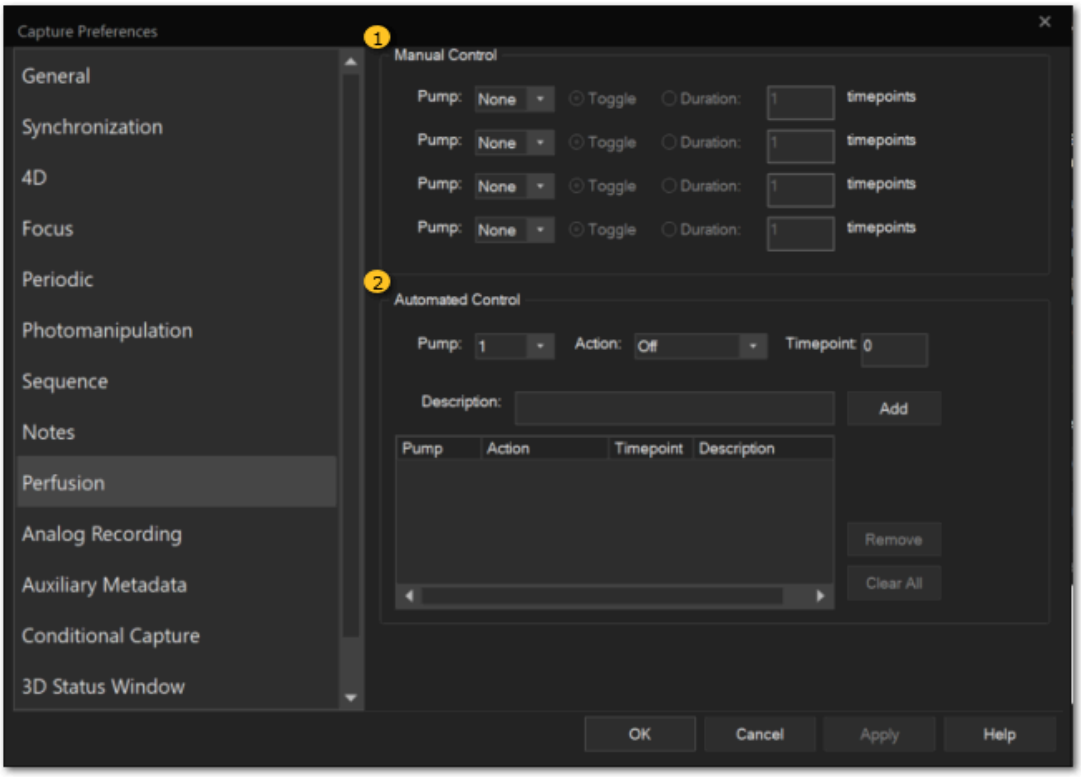


Simply type notes in the provided fields. During image capture, ***Notes*** buttons will be provided. You will simply press the appropriate button to add a note to the image. ***Notes*** can later be displayed as ***Annotations*** or on ***Graphs***. You may also later ***Edit Note*** or explore data using the ***Notes Navigator***.

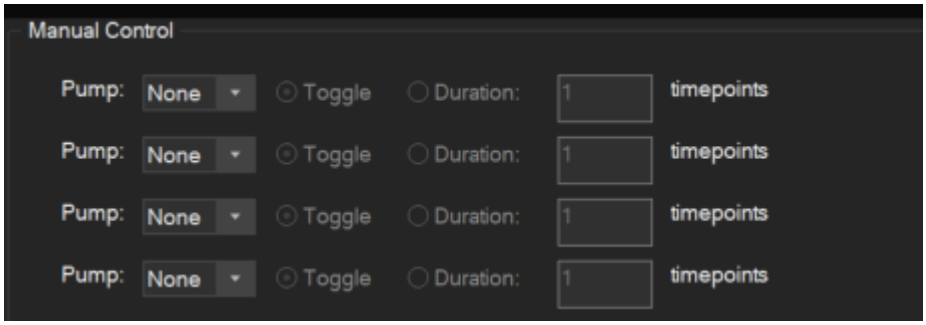
Perfusion Tab of Capture Preferences window

Complex experiments utilizing TTL driven perfusion devices are effortless in SlideBook. The ***Perfusion*** tab provides manual control of up to 24 channels and a scripting dialog for more complex experiments. Manual control of pumps is linked to the ***Notes*** buttons during capture execution. You may also combine manual and automated controls to achieve the desired experimental results.

To access this window, click ***Advanced...*** in the ***Capture*** window.



1 Manual Control group



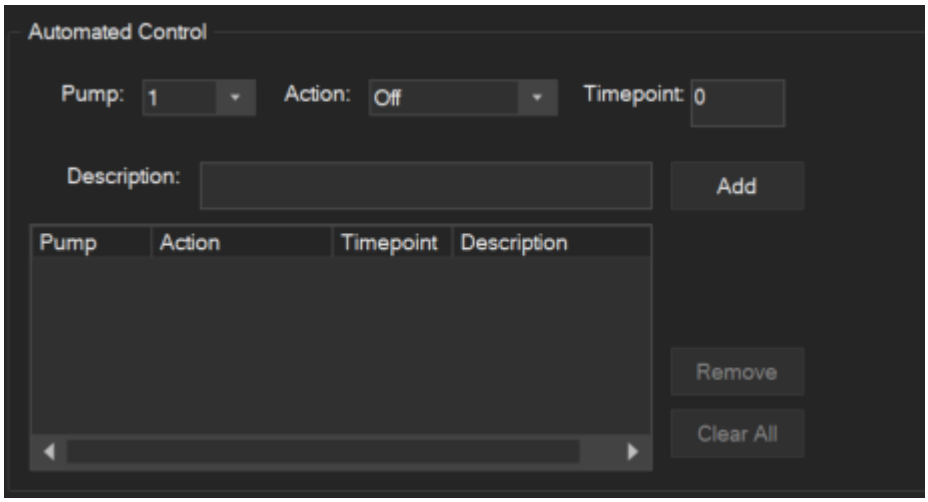
Manual Control allows you to configure actions to be linked to ***Notes*** buttons during image acquisition. Simply select a ***Pump*** from the dropdown menu then choose an action.

Toggle will switch the pump's current status (on/off) when the ***Note*** button is pressed.

Duration will switch turn a pump on for a set number of ***timepoints***.

The four different ***Pumps*** listed correspond to ***Notes 1 - 4*** respectively. It is recommended that you go to the Notes tab after configuring manual perfusion actions to fill in the text for the corresponding notes to label the actions the button will perform.

2 Automated Control group



Automated Control allows you to set up complex control of multiple pumps to be automatically executed during image acquisition.

Select a ***Pump*** from the dropdown menu and and ***Action*** (turn ***On*** or ***Off***). Then select a ***Timepoint*** when you would like the action to occur and write a ***Description*** if desired. Press ***Add*** to add the command to the list.

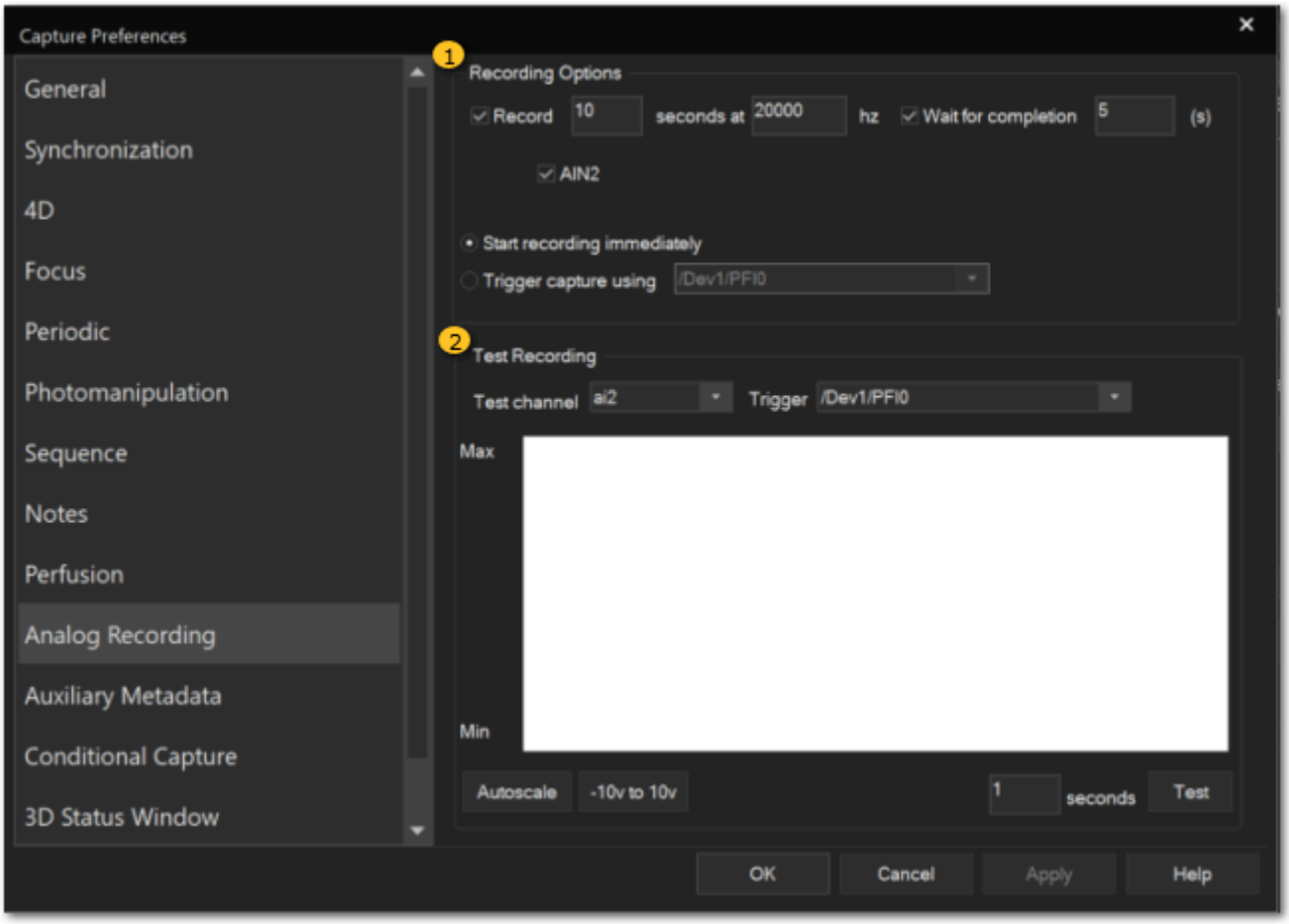
Repeat this process to add all processes to the list of commands. Remember that separate commands must be created to turn a pump on and then off again as there is no duration setting for automated control.

Select a command by clicking on it in the list and press ***Remove*** to delete it from the list. To clear the list and start over, press ***Clear All***.

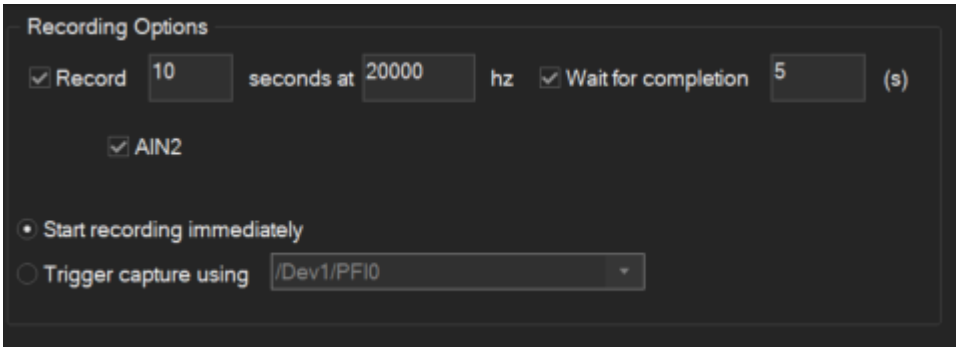
Analog Recording Tab of Capture Preferences window

You may choose to record one or more analog inputs during a capture. You may do so by selecting the ***Analog Recording tab***.

To access this window, click ***Advanced...*** in the ***Capture*** window.

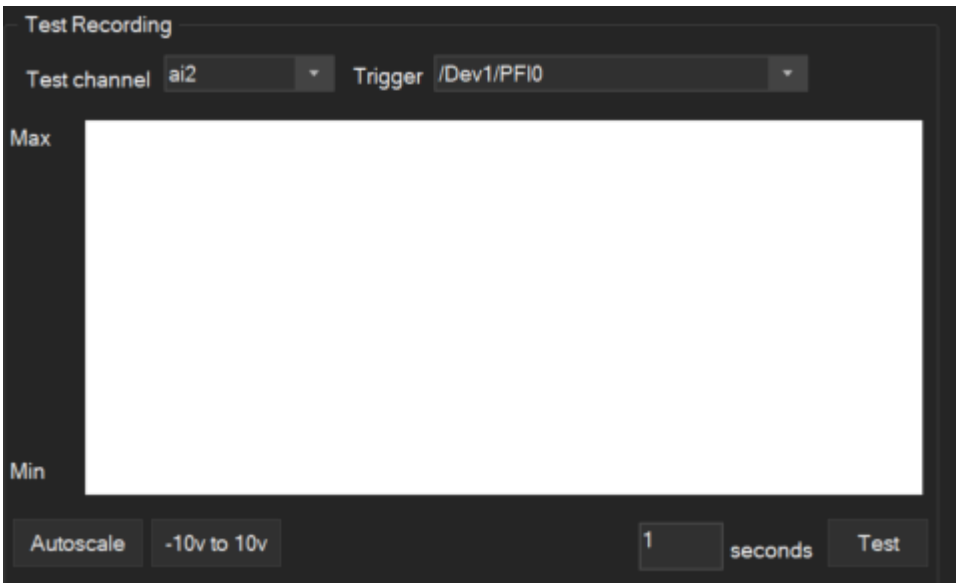


1 Recording Options



Check the box to enable analog recording for the specified duration (in seconds) at the specified frequency. Depending on how many analog in lines are set up in your configuration, one or more will appear below the Record checkbox section. You may either choose to begin the recording immediately, or to have it triggered by a signal from elsewhere in the system.

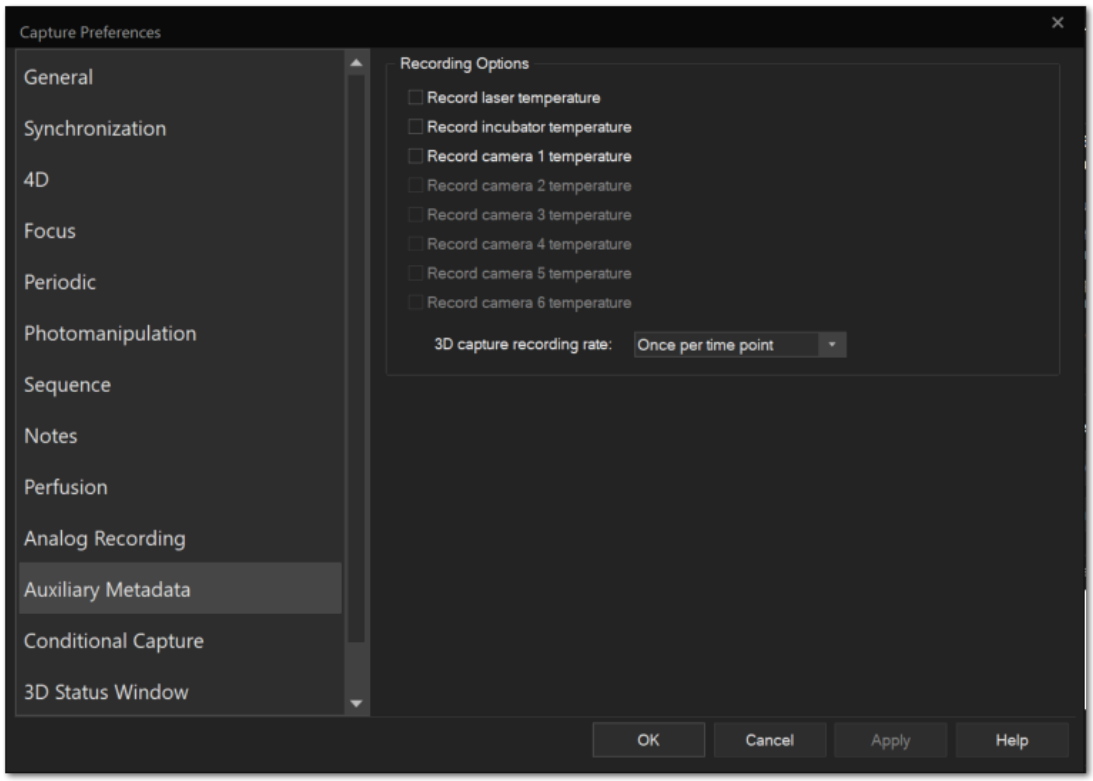
2 Test Recording



Test the recording prior to starting the capture to verify functionality.

Auxiliary Metadata Tab of Capture Preferences window

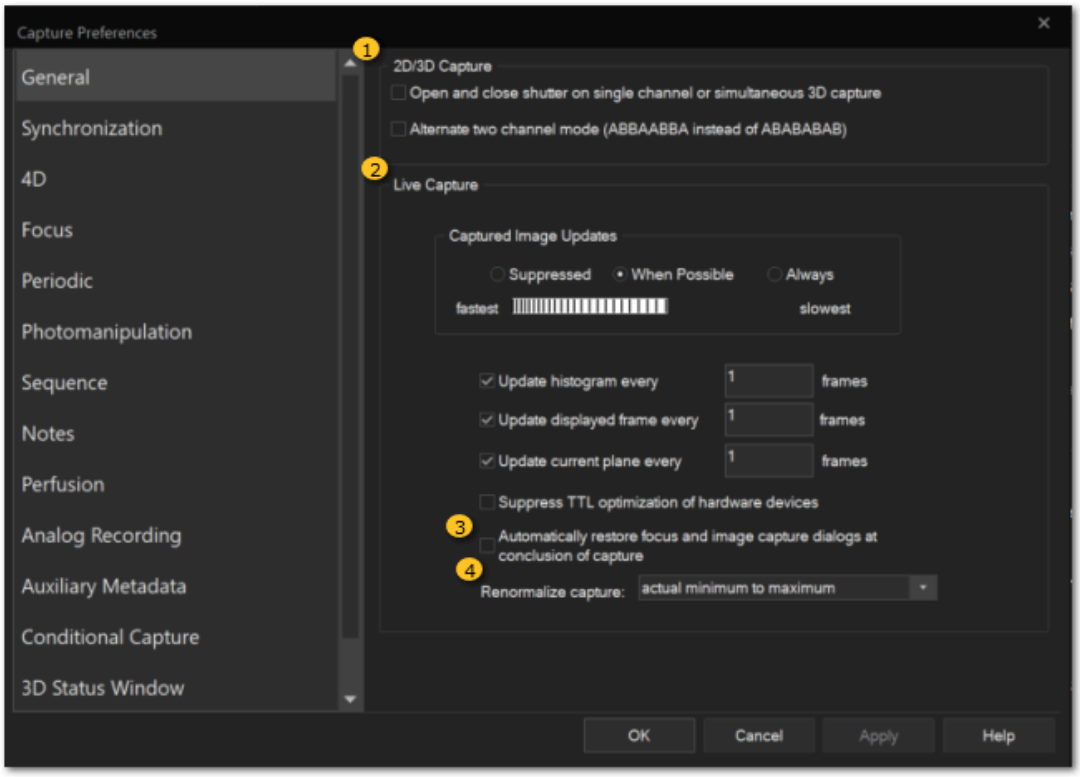
The Auxiliary Metadata tab allows you to record temperature readings from lasers, incubation chambers, and cameras. Simply check the boxes for the recordings you'd like and select a recording frequency from the drop down menu. Post acquisition, this metadata can be displayed, graphed, and exported from any view window by clicking > Auxiliary Data.



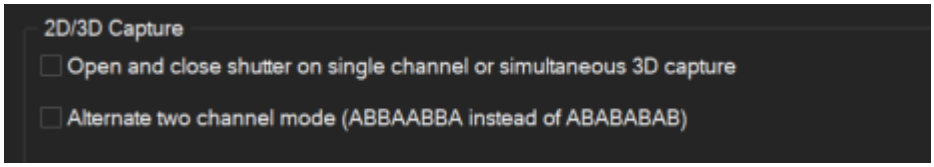
General Tab of Capture Preferences window

The ***General tab*** contains several options for adjusting shuttering or channel capture order when photobleaching or hardware speed (respectively) are concerns. There are also some options for updating display during capture that may be relevant if your computer is more than three years old.

To access this window, click ***Advanced...*** in the ***Capture*** window.



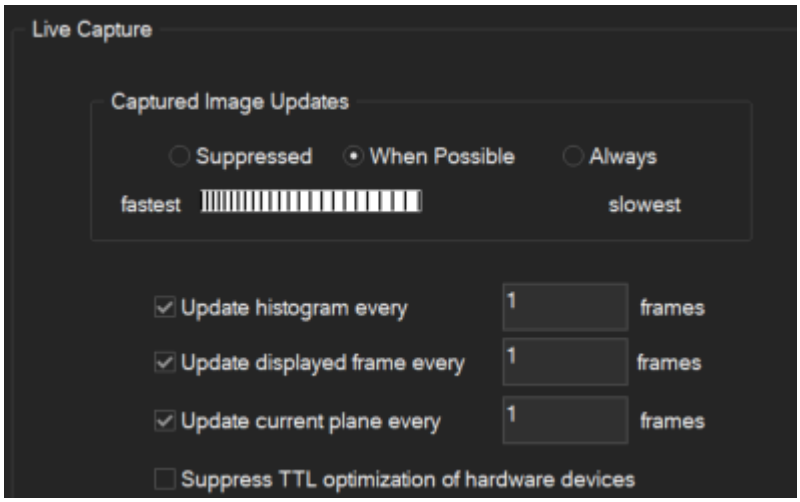
1 2D/3D Capture group



Open and close shutter on single channel or simultaneous 3D capture - If you are performing single channel 3D capture, the default fluorescence shutter action in SlideBook is to leave the shutter open during capture. If you would like the shutter to open and close after every capture, check this box.

Alternate two channel mode (ABBAABBA instead of ABABABAB) - If your multi-channel image capture is slowed down due to change time of a filter wheel or filter turret, you may find it useful to check this box. This will reduce the amount of time spent moving hardware between channels.

2 Live Image Updates

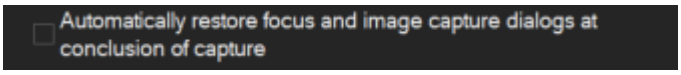


As computers age, their relative efficiency of display during image acquisition may not be optimal. If your computer is more than three years old, you may find it advantagous adjust how captured images and associated data are displayed. Please contact support@intelligent-imaging.com if you would like information/assistance in upgrading the computer.

You may suppress all updates for optimal speed performance. To do so, select the ***Suppressed*** radio button. If ***When Possible*** is selected, updates will be provided when the computer processor allows. Some updates will be dropped in the interest of speed. If ***Always*** is selected, each frame requested will be displayed (at the expense of speed).

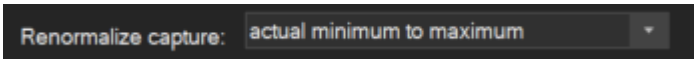
Alternatively, you may choose to suppress updates of individual aspects of the display such as the ***histogram***, ***displayed frame***, or ***current plane***. Use the check boxes to adjust how often these items are updated.

3 Reopen Focus/Capture dialogs



Check this box if you would like the ***Focus Controls*** and ***Capture*** dialogs to automatically reopen after image acquisition is complete.

4 Automatic Renormalization



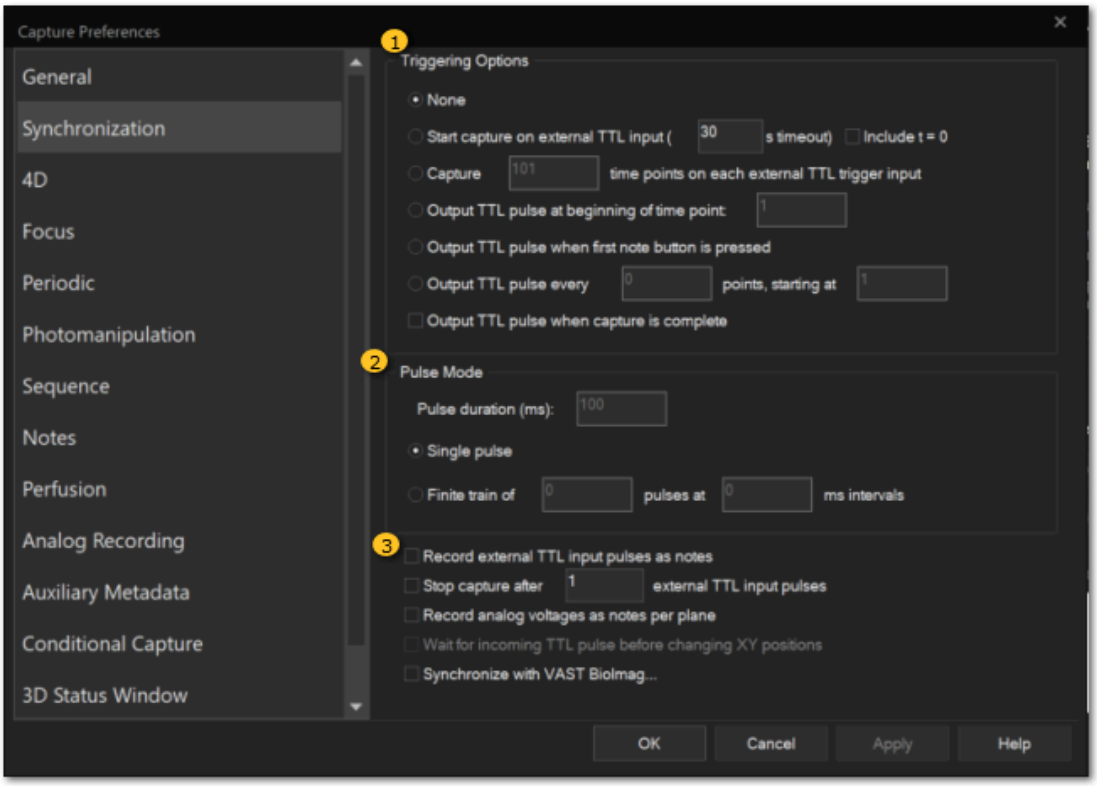
Select a renormalization range for the captured data. This renormalization is not applied until image capture is complete and teh image reopens from the .sld. Options include:

- actual minimum to maximum
- actual minimum to 99.9% maximum
- 0 to camera bit depth
- 0 to 65535

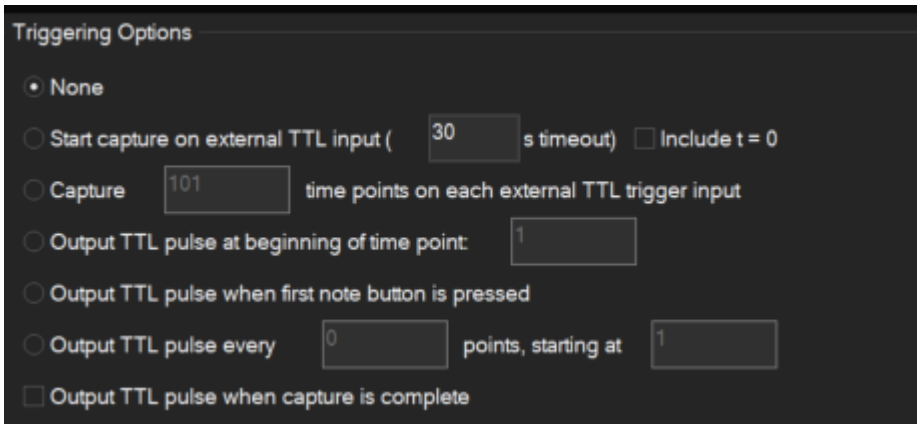
Synchronization Tab of Capture Preferences window

Part of the SlideBook TTL Synchronization Module allows TTL pulses to be incorporated into experiments via the ***Synchronization tab***. You can use TTL triggers to start experiments or have experiments send out a TTL pulse or train of pulses at specific times. All activity is logged as ***Notes*** within the image file.

To access this window, click ***Advanced...*** in the ***Capture*** window.



1 Triggering Options group



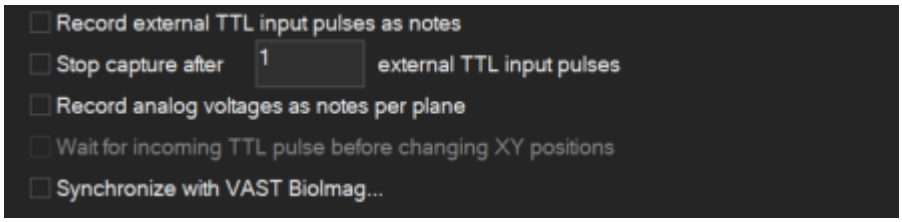
Select a ***Triggering Option*** by clicking on the radio button. You may choose to capture based on TTL trigger input or to output a TTL pulse with a ***Note*** button command or at a specific time point.

When you choose to output a TTL pulse, you may configure the ***Pulse Mode*** below.

2 Pulse Mode group

When an output TTL pulse has been selected as a ***Triggering Option***, you may configure either a ***Single pulse*** or a ***Finite train of pulses*** in this area. For choices to be enabled, one of the three ***Output TTL pulse...*** options must be selected in the group above.

3 General TTL Options



You may check boxes to record either ***external TTL input pulses*** or ***analog voltages as notes*** in the image file. These notes can later be displayed, edited, or further navigated from the ***Annotations*** menu.

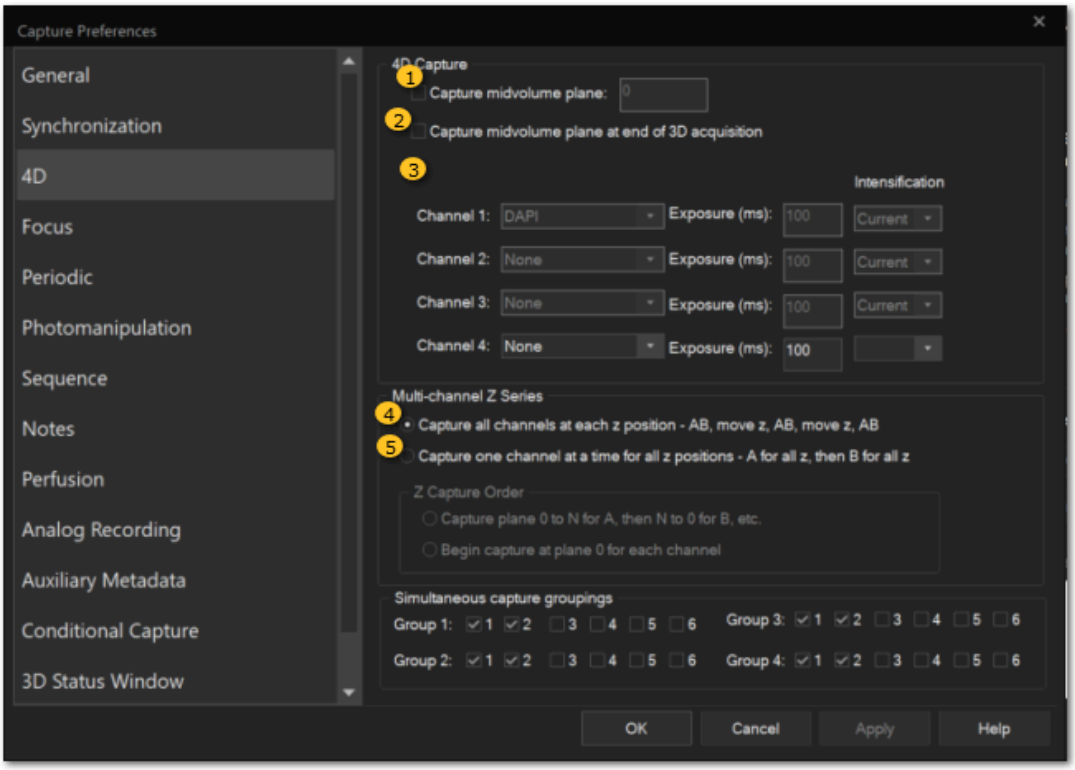
When an XY point visiting experiment is configured, you may check the box to ***Wait for incoming TTL pulse before changing XY positions***.

If you have a VAST BioImager, you can utilize the TTL pulses to synchronize the loading, unloading, and movement of fish from SlideBook.

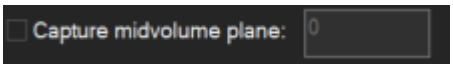
4D Tab of Capture Preferences window

The ***4D tab*** allows you to capture a single plane in one channel when you are acquiring 3D stacks in another channel or channels. In this tab, you may also set up channel collection order for 3D stacks; that is, you may choose to collect a *z*-stack in before switching channels instead of collecting all channels before switching *z*-plane.

To access this window, click ***Advanced...*** in the ***Capture*** window.



1 Capture Midvolume Plane

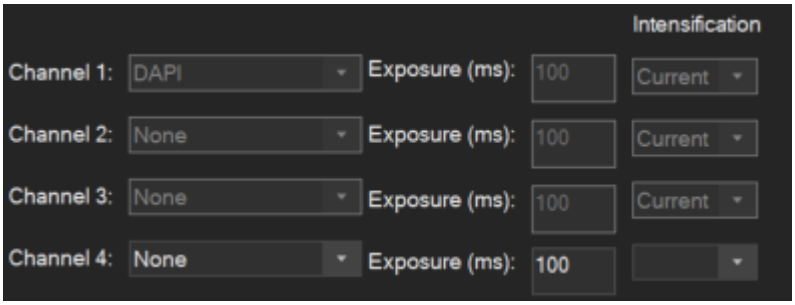


Check the box to capture a single *z*-plane in the *z*-stack. Enter a plane number based on the 3D Capture information in the ***Capture*** window.

2 Capture at End of Acquisition

Check this box to capture the midvolume plane after the *z*-stack has been acquired. If this box is not checked, the midvolume plane will be captured with the rest of the *z*-stack images at the appropriate plane.

3 Channel Selection



You may select up to three channels to capture at a midvolume plane. Select the channel from the dropdown menu and set an ***Exposure*** time in milliseconds. You may test the exposure in the ***Capture*** window.

NOTE: Be sure not to select the channels for midvolume in the ***Capture*** window. If the midvolume plane channel is selected in the ***Capture*** window, the channel will be captured at all *z*-planes.

4 Standard Z Capture Order

Capture all channels at each z position - AB, move z, AB, move z, AB

Select this option for standard *z* acquisition where a plane is collected in all channels, focus moves to the next *z*-plane, a plane is collected in all channels, focus moves to the next *z*-plane, and so on.

5 Capture One Channel at a Time

Capture one channel at a time for all z positions - A for all z, then B for all z

Select this option to capture a *z*-stack in a single channel at a time. This approach may be preferred when the hardware to change channels is slowing down image acquisition.

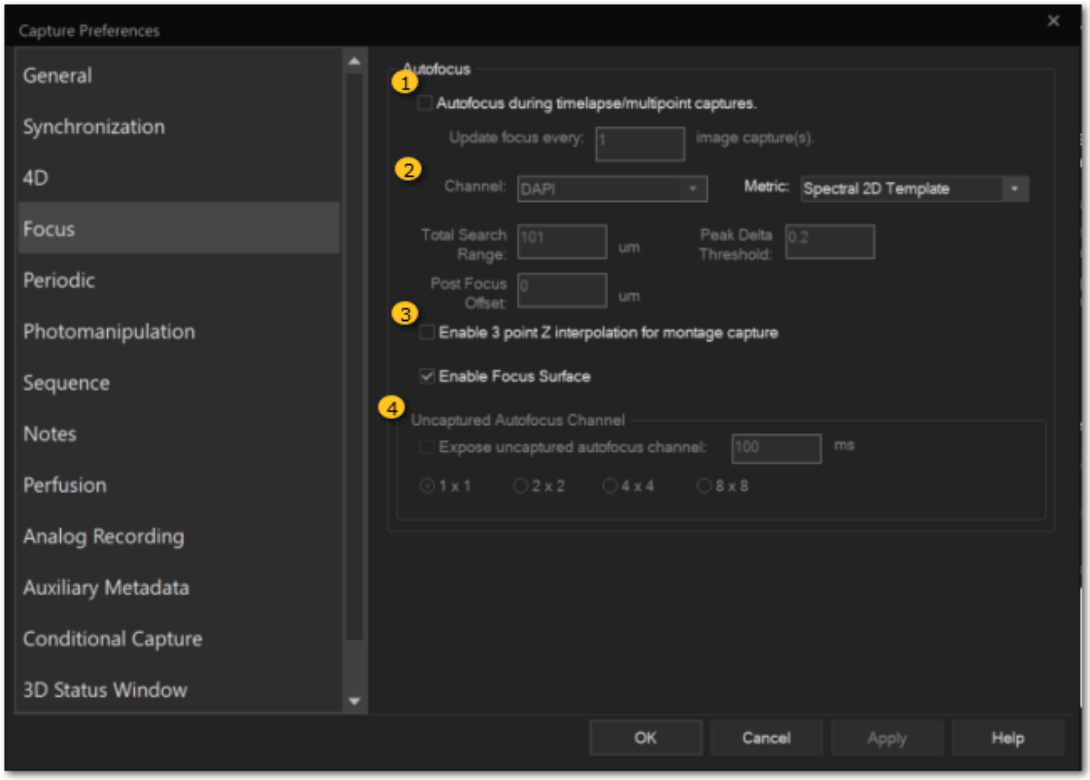
Focus Tab of Capture Preferences window

When you would like to use auto focus during timelapse imaging, you may configure auto focus parameters using the ***Focus tab***. This tab is used computer contrast based auto focus algorithms as well as hardware based auto focus systems such as Zeiss Definite Focus, Nikon Perfect Focus, Leica AFC, or Olympus ZDC.

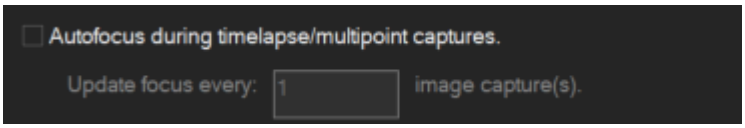
To access this window, click ***Advanced...*** in the ***Capture*** window.

See also:

AutoFocus window of ***Focus Controls***
Using AutoFocus in SlideBook



1 Enable Autofocus



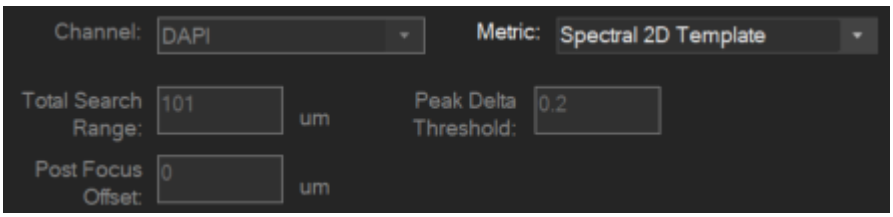
Check the box to enable auto focus during image capture. Enter a value in the edit field to determine how often autofocus should be performed.

If you are using a hardware-based auto focus method such as Zeiss Definite Focus or Olympus ZDC, you do not need to fill out any additional information. These hardware-based devices are configured in the Focus Controls.

Read below to learn how to configure contrast-based auto focus during image acquisition.

NOTE: Software-based Autofocus is not compatible with montage imaging, instead, use Focus Surface or 3 point Z interpolation (described below).

2 For Contrast-Based Algorithms



Select a channel from the dropdown menu to use for contrast-based auto focus. You may use an channel you are not acquiring for autofocus (See Uncaptured Autofocus Channel). Values for the auto focus parameters can be tested and determined in the ***Focus Controls*** by using the Auto Focus window.

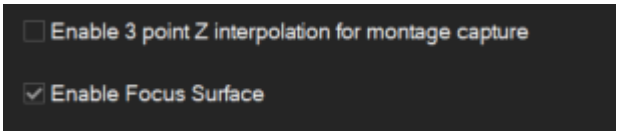
Metric is the computational method used to evaluate and compare focal planes. Changing the method can affect the success of the auto focus routine. The default method of ***Spectral 2D Template*** will work best in almost all cases. ***Gaussian Derivative*** may prove useful when using a phase contrast channel for auto focus. The other methods are experimental. You should consult 3i Support if you are not having success with the default method.

Total Search Range is the distance in microns in the *z* direction that will be searched for best focus.

Post Focus Offset is the distance in microns in the *z* direction that the focus will move after the best focal plane is determined. For example, if you are imaging a cell that is 5 microns thick and the best focal plane is the center of the cell, you would want to set this value to **-2.5** so that focus would move to the bottom of the *z*-stack and you would capture the entire 5 micron distance with the best focal plane in the center.

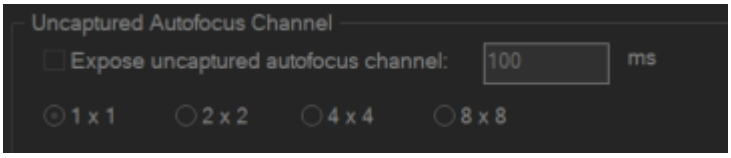
Peak Delta Threshold is used to determine when no optimal plane of focus should be computed from a particular image position. When performing a montage capture, for example, some fields might not contain tissue and should not be used to change the current *z* position. This value can range between **0.0** and **1.0**, but should not be decreased below **0.1** in most cases. For captures where you know that each image in the sequence will have sufficient detail to focus, this threshold can be raised. If the value is set to **1.0** then the threshold will be ignored.

3 For Multipoint Autofocus



Check this box to enable 3 point Z interpolation for montage imaging, allowing the montage to be acquired of a tilted plane calculated by using 3 corner z coordinates. More information can be found in the XY Tab of Focus Controls.

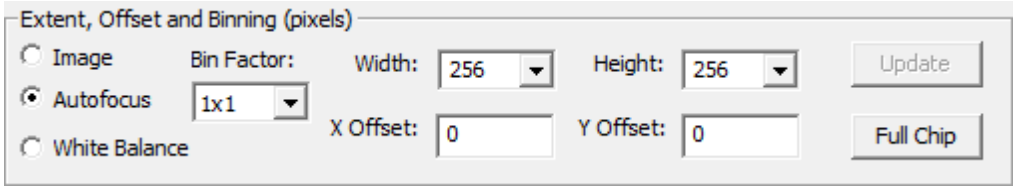
4 Uncaptured Autofocus Channel



If you do not want to capture the auto focus channel, or you want to use a different image extent, bin factor or exposure time for auto focusing, select the ***Expose uncaptured auto focus channel*** checkbox.

Enter the exposure time and select a bin factor for the uncaptured auto focus channel. These parameters will be used to determine image capture for the purposes of auto focusing.

Selecting ***Expose uncaptured auto focus channel*** also provides the ability to specify a region of interest that will be used for auto focusing. This is useful for auto focusing on samples that have features with heterogeneous optimal focal planes. In this case, a full chip image extent can be captured at each timepoint while using only a small subregion to determine optimal focus. Specifying an auto focus region of interest is done in the ***Capture*** dialog box:

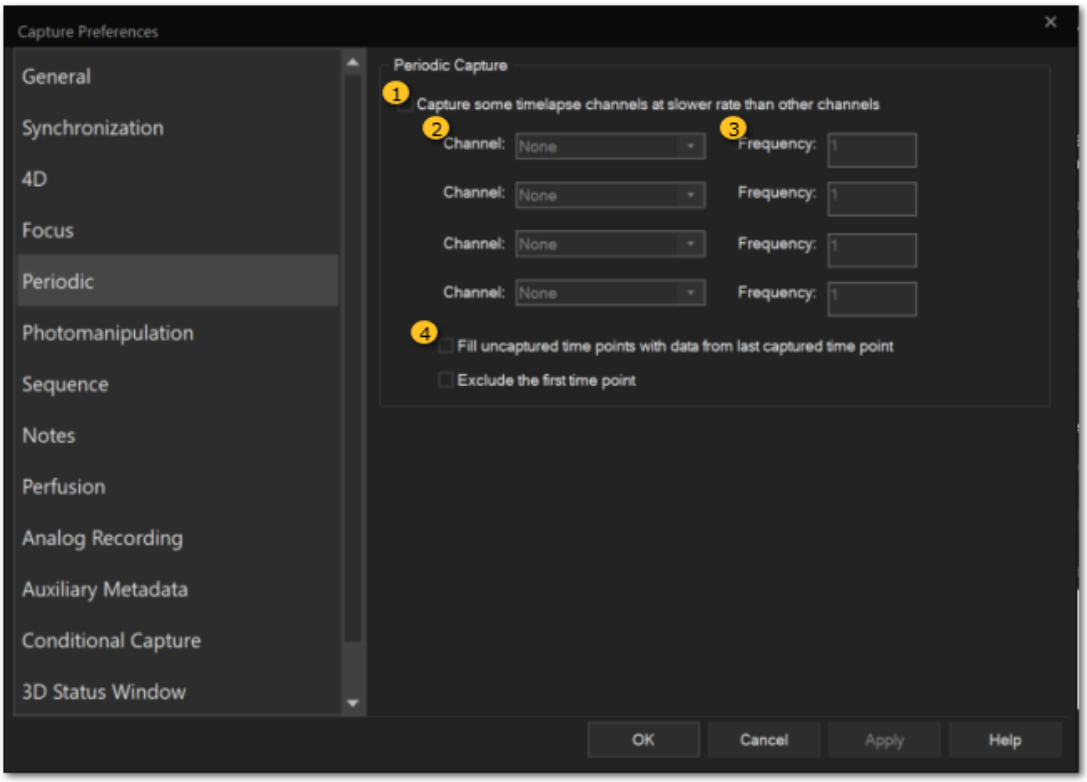


When the ***Expose uncaptured AF channel checkbox*** is selected, the ***Autofocus*** radio button is active in the Extent, Offset, and Binning (pixels) section of the Capture window. This menu is used to alternate between designating the image extent for captured channels and the image extent for auto focus. To define a subregion for auto focus, simply select the ***Autofocus*** radio button and specify a ***Width***, ***Height***, ***X Offset***, and ***Y Offset***. Alternatively, draw a selection in the live ***Camera*** window and press the ***Update*** button.

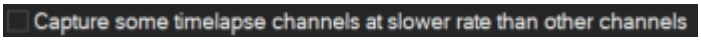
Periodic Tab of Capture Preferences window

You may choose to capture a single channel at a slower rate than other channels when performing a multi-channel 2D time lapse capture. You may do so by selecting the ***Periodic tab***.

To access this window, click ***Advanced...*** in the ***Capture*** window.



1 Enable Periodic Capture



Check the box to enable periodic capture for 2D time lapse image acquisition. This will allow you to capture one channel at a slower rate than the other channel(s). You may choose to do this because you are trying image very fast or because one channel is more phototoxic to the sample.

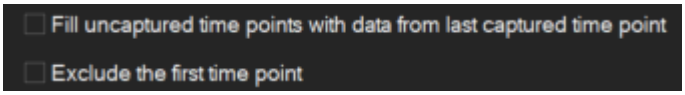
2 Channel Selection

Select the Channel you would like to capture at a periodic rate from the dropdown menu. Be sure to set the Expose time in the ***Capture*** window.

3 Frequency Field

Enter the ***Frequency*** at which you would like to capture that channel. For example, a frequency of 5 indicates that you would like to capture a channel every 5th image/ time point.

4 Image Behavior



If you would like to copy the image of the selected channel to time points where you are not collecting the channel, select the check box to ***Fill uncaptured timepoints with data from last captured timepoint***. Otherwise, the time points where the periodic channel is not captured will not show an image in this channel.

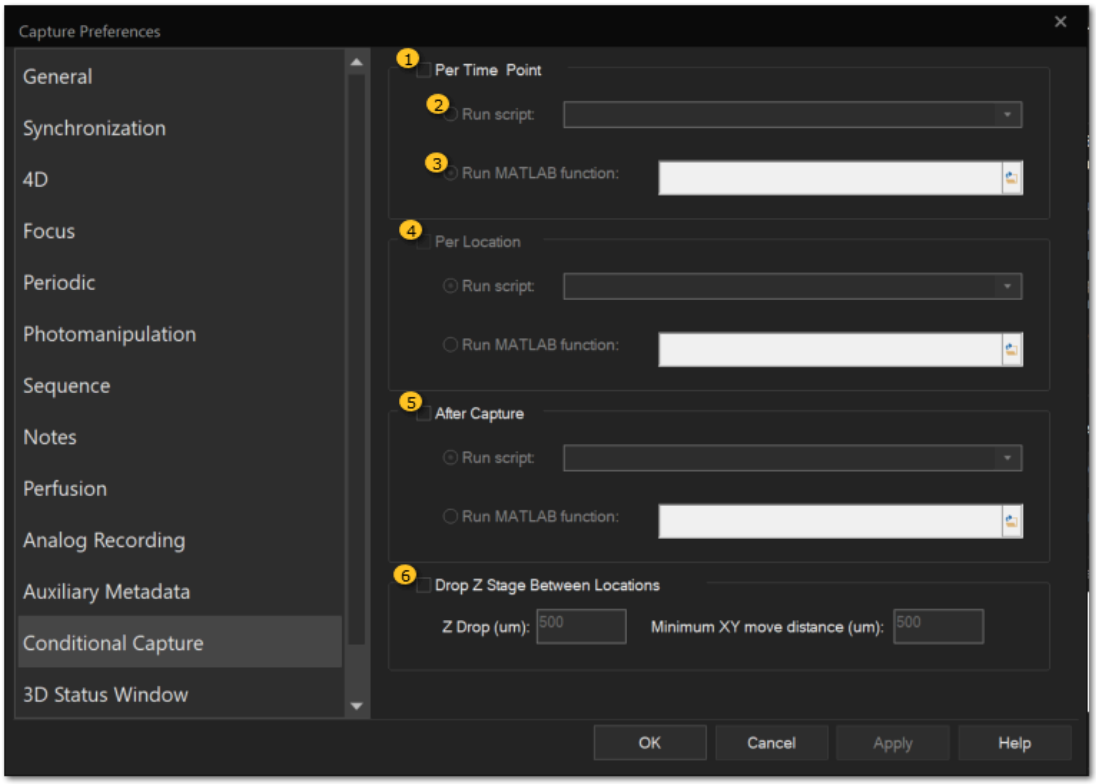
Conditional Capture Tab of Capture Preferences Window

Conditional Capture allows you to run either SlideBook scripts (.sbs files defined in the Scripting Tab) or Matlab functions during and/or after your capture.

To access this window, click ***Advanced...*** in the ***Capture*** window.

See also:

Scripting Tab
Macro Scripting



1 Per Time Point check

Per Time Point

This checkbox will enable the script or function to run at each time point in a timelapse acquisition.

2 Run script: option

Run script:

This radio button will run a SlideBook script (.sbs file) chosen from your saved scripts in the dropdown menu to the right.

3 Run MATLAB function: option

Run MATLAB function:

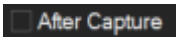
This radio button will run a Matlab function chosen from the file selection dialog to the right.

4 Per Location check

Per Location

Currently under development, this will eventually allow for scripts or functions to be run at each location in a multipoint acquisition.

5 After Capture check



This checkbox enables the chosen script of function to be run at the completion of the capture.

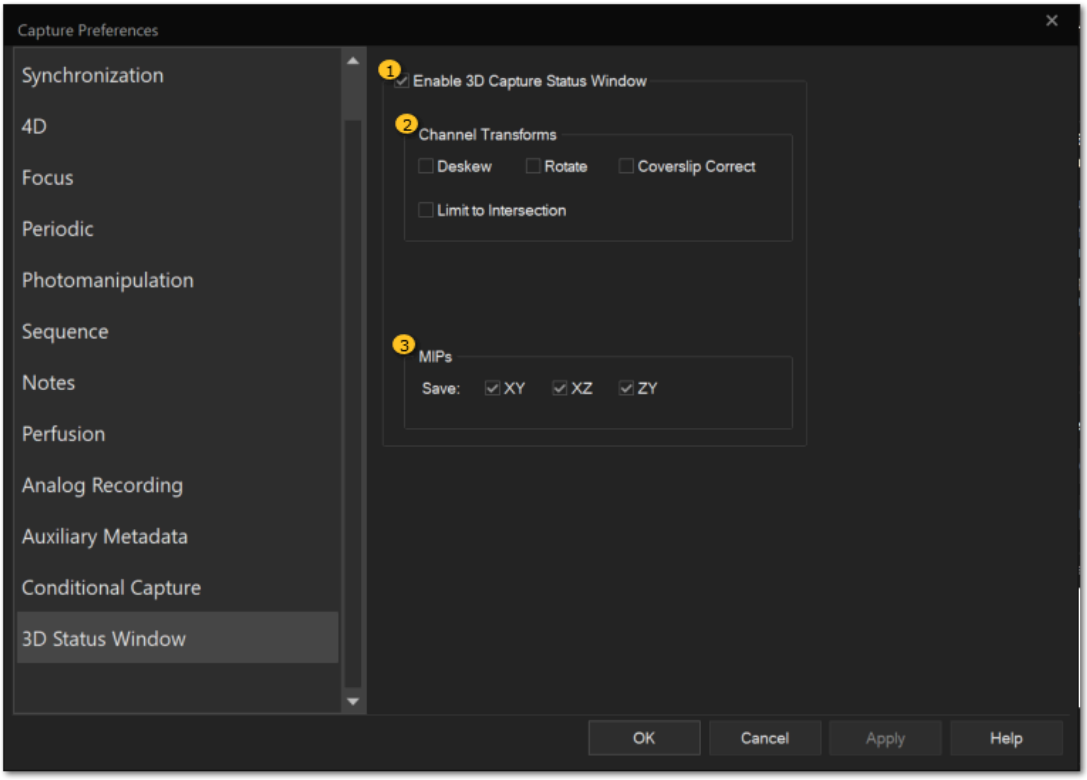
6 Drop Z Stage Between Locations check

Select this checkbox if you would like to drop the Z-stage or objective during a XY move. You can specify how far you would like the stage or objective to drop and the minimum distance between the XY points required for the drop to occur.

3D Capture Status Tab of Capture Preferences Window

The 3D Capture Status window is a three-view that displays at each time point during a 4D capture.

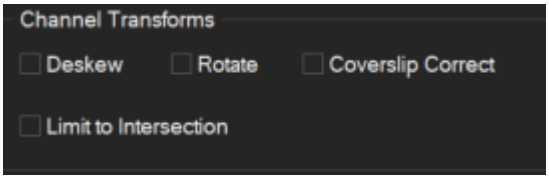
To access this window, click ***Advanced...*** in the ***Capture*** window.



1 Enable 3D Capture Status Window check

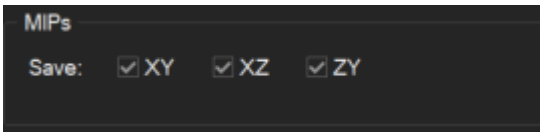
Select this to turn on the 3D capture status viewer.

2 Channel Transforms group



These checkboxes represent channel transforms primarily used in diSPIM imaging. **Deskew** places the data back into a geometrically correct orientation. **Rotate** changes the orientation of the secondary channel to match the first. **Coverslip correct** rotates the entire dataset so that it can be viewed as a normal z-stack. **Limit to intersection** is the volume of both sides of the scan that overlap.

3 MIPs group



Regardless of whether or not these boxes are checked, the three-view will be present. If any of these boxes are checked, then the corresponding max intensity projection will be saved to the slide along with the capture.

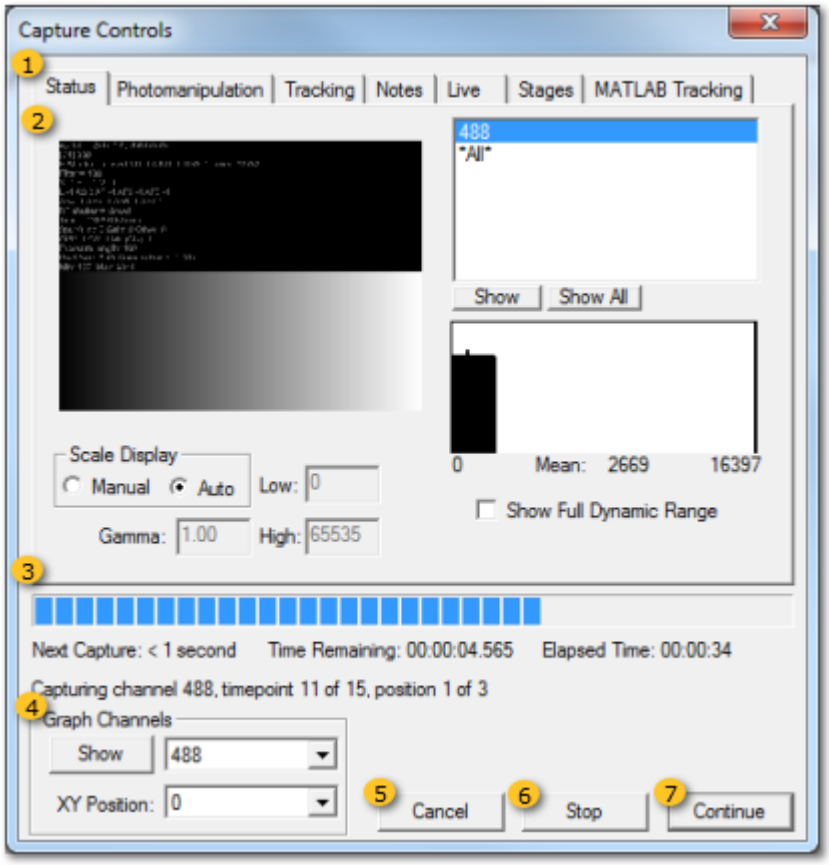
Capture Controls window

The ***Capture Controls*** window opens once image acquisition is initiated in the ***Capture*** window. The top portion of the window changes based on the tab selected while the bottom portion of the window is present at all times. In this window, you can adjust focus, initiate photomanipulation events, mark images with notes, or execute stages.

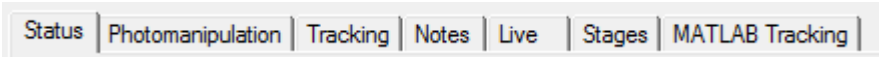
Access ***Capture Controls*** by beginning an image capture in the ***Capture*** window.

See also:

- Graph Display in Ratio Module
- Focusing During Capture in Ratio Module
- Creating Event Markers (Notes) in Ratio Module
- Displaying Region Intensity during Capture in Photomanipulation Module
- Adjusting Vector Settings during Capture in Photomanipulation Module
- Sequence Tab of Capture Preferences window
- TUTORIAL: XY Cell/Region Tracking



1 Function tabs



Status tab - Allows you to adjust the display of captured images during acquisition, see more below.

Photomanipulation tab - During image acquisition, certain photomanipulation parameters can be controlled via the Photomanipulation tab. This tab will be activated if you have a Vector, an Ablate, or another photomanipulation device configured.

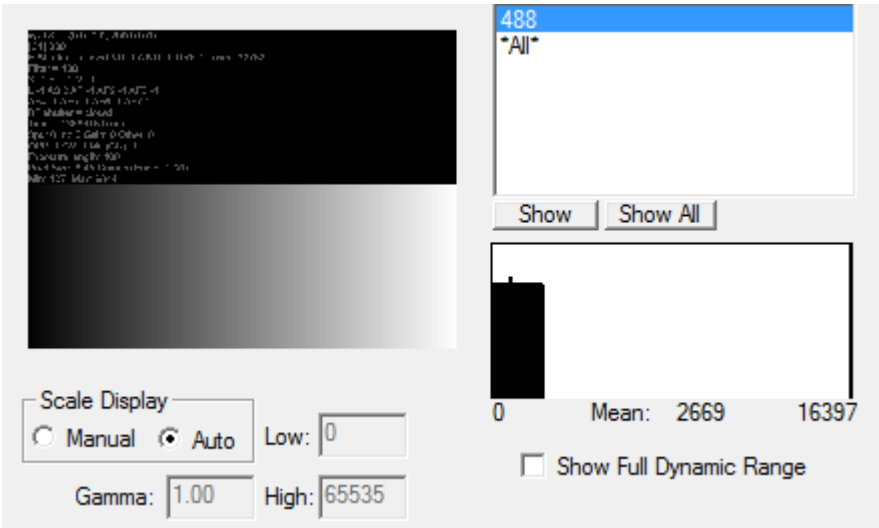
Tracking tab - When imaging a live specimen over time, it may be useful to move the imaging field based on where the object of interest is moving. To assist in this, SlideBook 6 includes real time XY tracking in which a motorized XY stage will move to keep a defined region central in the imaging field. This feature is only available for 2D time lapse acquisitions and can be accessed via the Tracking tab.

Notes tab - Notes set up in the ***Notes*** tab of ***Advanced Capture Preferences*** can be triggered in the Notes tab of ***Capture Controls***. Alternatively, if notes are not previously configured, you may create notes and trigger them using this interface. Notes will show up as an annotation in the data set post-acquisition.

Live tab - The Live tab is used to adjust focus during an experiment and to show previously captured images. When performing a point visiting experiment, you can update any or all *XYZ* points.

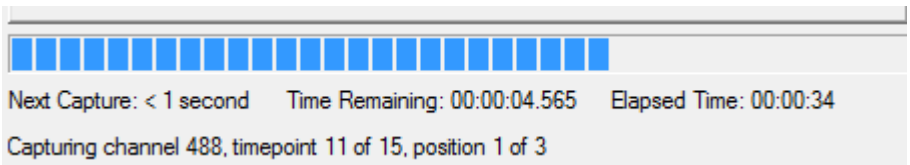
Stages tab - The Stages tab allows you to interact with stages that were configured in the Sequence Tab of ***Advanced Capture Preferences***. You may execute any stage at any time using this dialog regardless of order of events configured before image acquisition began.

2 Status tab



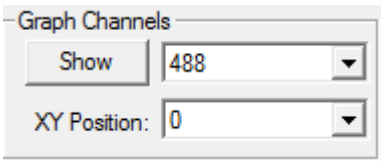
The current captured image is displayed in this tab and you are able to adjust intensity renormalization and well as which channel(s) are displayed. Adjusting renormalization/intensity scaling will only affect the display of the image and not the underlying data.

3 Progress bar



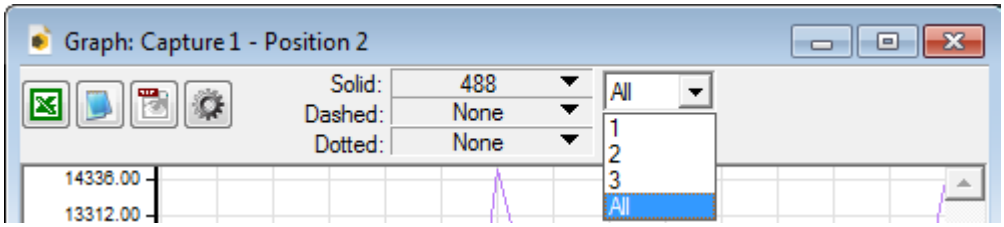
The progress bar displays what portion of the requested image capture is complete. There is also a readout that estimates when the next capture will occur, how much time is remaining in the experiment, and how much time has elapsed. Finally, you are given a status of the current frame that is being captured, including channel, time point, and *z*-plane.

4 Graph Channels group



When regions are drawn on the image, select a channel from the drop down menu and press ***Show*** to graph the mean intensity of each or the regions over time. If a background region is set, it will automatically be subtracted from the calculation. If a ratio is configured, it will be available to graph from the dropdown menu.

When performing an XY Point Visiting capture, use the ***XY Position*** dropdown to select the position you would like to graph. At this time, the same regions will be used to all locations but you can choose to graph one specific region in the graph itself as shown below/



5 Cancel button

Cancel

Cancels capture, discarding images.

6 Stop button

Stop

Stops capture, images captured will be saved.

7 Pause/Continue button

Continue

Pause/Continue toggle button allows you to pause and restart image acquisition. You may wish to pause an experiment to adjust focus, add reagents, etc.

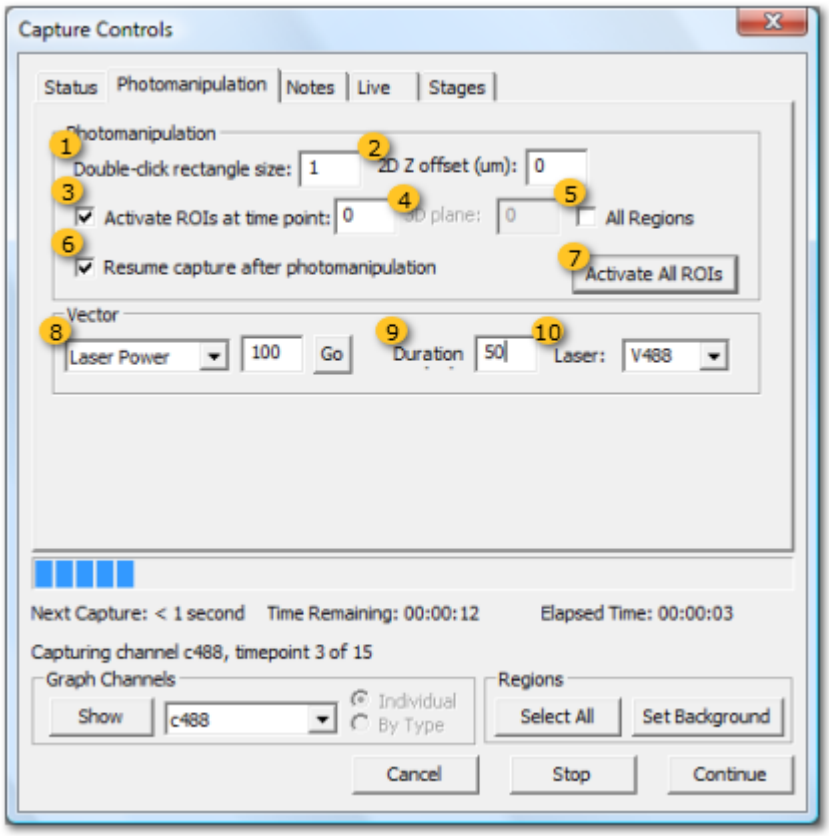
Photomanipulation Tab of Capture Controls

During image acquisition, certain photomanipulation parameters can be controlled via the ***Photomanipulation*** tab of ***Capture Controls***.

Access by selecting the ***Photomanipulation tab*** during image capture.

See also:

[Photomanipulation Tab of Capture Preferences]
[Adjusting Vector Settings during Capture]



1 Double-click rectangle size

Double-click rectangle size: 1

This is the number of spots that will be hit by the laser is a square pattern when you double-click to initiate an event during image capture. A good starting point to test is the value of the ***Raster block size*** used in the ***FRAP*** tab of ***Focus Controls***.

2 2D Z offset (um)

2D Z offset (um): 0

The distance in Z that the sample should be moved before a photomanipulation event takes place. This value can be determined using the ***Z Stage*** section of ***Focus Controls***. The value entered can be positive or negative and include a decimal point. Some example values include: **1**, **0.55**, and **-2.1**.

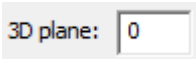
3 Activate regions at time point

☑ Activate ROIs at time point: 0

The time point that will be captured after the event will take place. This is a whole number integer starting with **0** and ending with the value defined under ***# of Time Points*** in the ***Capture*** dialog. For example, if a value of **3** were

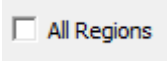
entered, two time points would be captured then the photomanipulation event would take place then the third time point would be captured.

4 3D plane



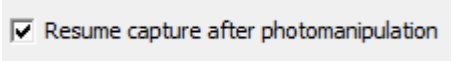
If you are imaging a 3D stack and would like the photomanipulation event to occur on a single plane within the stack, check the box and type the integer value of the Z plane where the event should occur. Not checking this box when imaging a 3D stack will result in the event occurring at the Z plane in the center of the stack.

5 All Regions



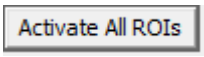
Check this box to photomanipulate ***All Regions*** drawn in the active ***Camera*** window.

6 Resume capture after Photomanipulation



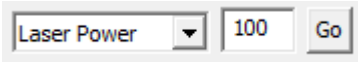
If you have paused image capture, checking this box will automatically signal SlideBook to continue capturing images after the photomanipulation has been completed.

7 Activate All regions button



Clicking this button will immediately pause capture and activate all existing regions in the live window.

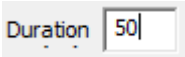
8 Laser Power



You may select the laser power level by entering an integer from **1** - **100** in the text edit field and clicking ***Go***. A value of **0** refers to 0% of the laser power and **100** refers to full (100%) laser power.

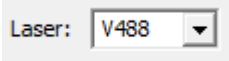
When using Ablate! Be sure to select ***Ablate! ND*** from the Laser Power dropdown menu.

9 Duration



Enter the dwell time in milliseconds for the laser at each point to be photomanipulated.

10 Laser



This is the laser line that will be used for the photomanipulation event(s). Filters that show in this list are those configured with an ***Associated Channel type*** equal to ***Photomanipulation*** in the ***Filter Configuration Parameters***.

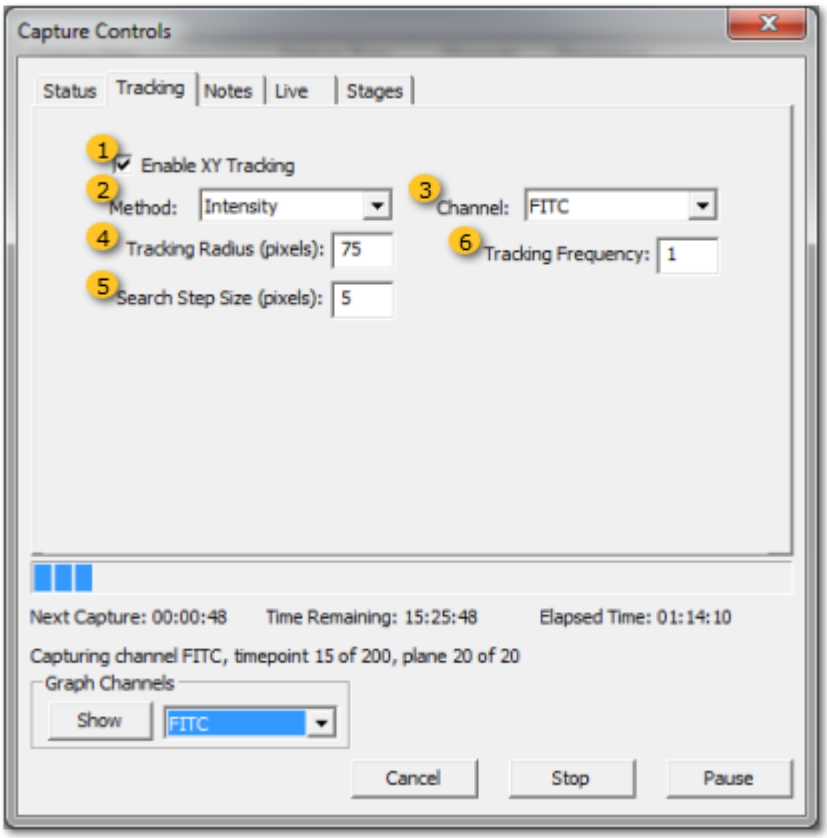
Tracking Tab of Capture Controls window

When imaging a live specimen over time, it may be useful to move the imaging field based on where the object of interest is moving. To assist in this, SlideBook 6 includes real time XY tracking in which a motorized XY stage will move to keep a defined region central in the imaging field. This feature is only available for 2D time lapse acquisitions and can be accessed via the Tracking tab.

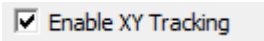
Access by selecting the ***Tracking tab*** during image capture.

See also:

TUTORIAL: XY Cell/Region Tracking
Particle Tracking Menu



1 Enable XY Tracking check



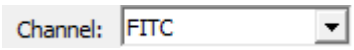
Check the box to enable XY tracking of a region. To define the region to track, draw a region using the Region Tools. Right click on the region and select ***Set XY Tracking Region***.

2 Method

Intensity uses sum image intensity within the region as the metric. It then moves the region in XY and re-calculates. It uses the region shift with the biggest increase in sum intensity. This is useful for finding a bright object surrounded by darkness such as an isolated cell.

Correlation uses a complex mathematical function called cross-correlation to compare two regions. However, in this case the metric is not dependent on intensity but rather dependent on 2D shape.

3 Channel Selection



Channel is used for both methods, only the data in the selected channel is used for the metric.

4 Tracking Radius

Tracking Radius (pixels): 75

Tracking Radius is the maximum distance from the center of the image that will be searched (in pixels).

5 Search Step Size

Search Step Size (pixels): 5

Search Step Size is how far the region will be moved in each direction searching for a match.

6 Tracking Frequency

Tracking Frequency: 1

Tracking Frequency determines how often the region is re-centered according to the method defined. For example, if you enter a frequency of 5, after time point 5 is captured, SlideBook will compare time point 1 with time point 5 to re-center the defined region and move the stage accordingly. This process will then be repeated, comparing time point 10 to 5, 15 to 10, and so on.

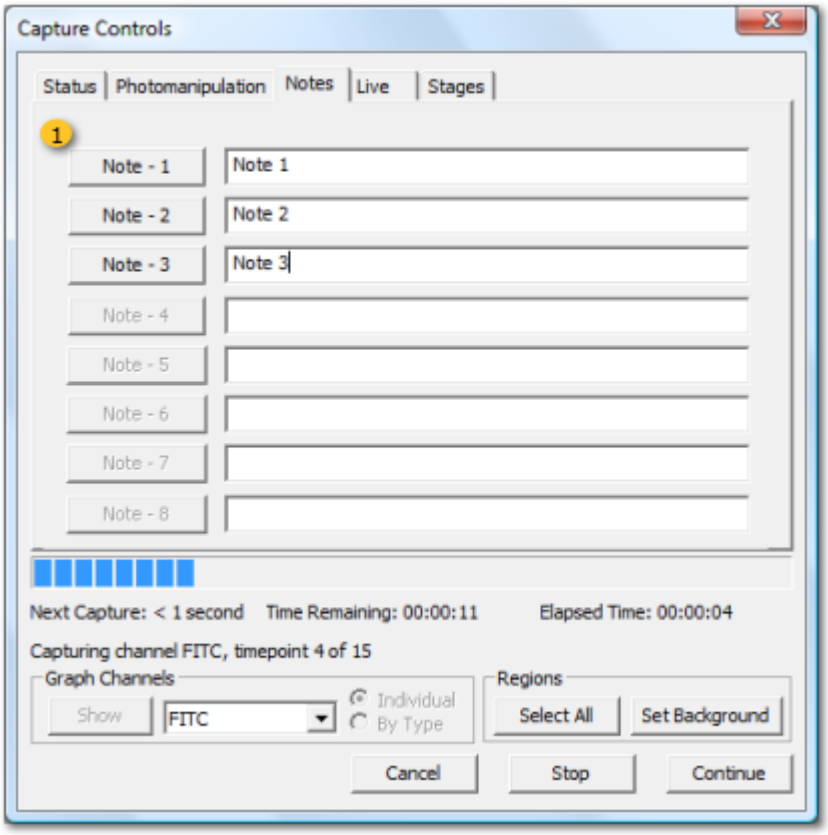
Notes Tab of Capture Controls window

Note set up in the Notes tab of Advanced Capture Preferences can be triggered in the ***Notes tab*** of ***Capture Controls***. Alternatively, if notes are not previously configured, you may create notes and trigger them using this interface. Notes will show up as an annotation in the data set post-acquisition.

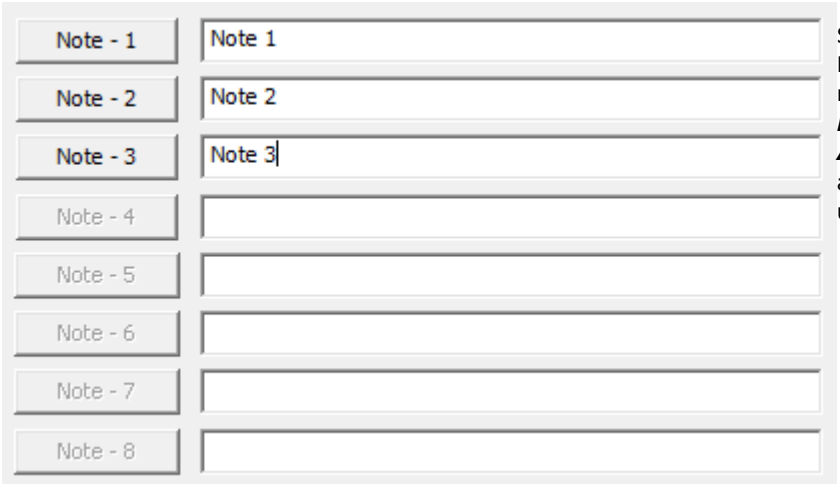
Access by selecting the ***Notes tab*** during image capture.

See also:

[Notes Tab of Advanced Capture Preferences]
Ratio Module, [Setting Capture Parameters for Timelapse Ratio Imaging]
Ratio Module, [Creating Event Markers (Notes)]



1 Notes Panel



Simply type notes in the provided fields. Press the appropriate button to add a note to the image during acquisition. ***Notes*** can later be displayed as ***Annotations*** or on ***Graphs***. You may also later ***Edit Note*** or explore data using the ***Notes Navigator***.

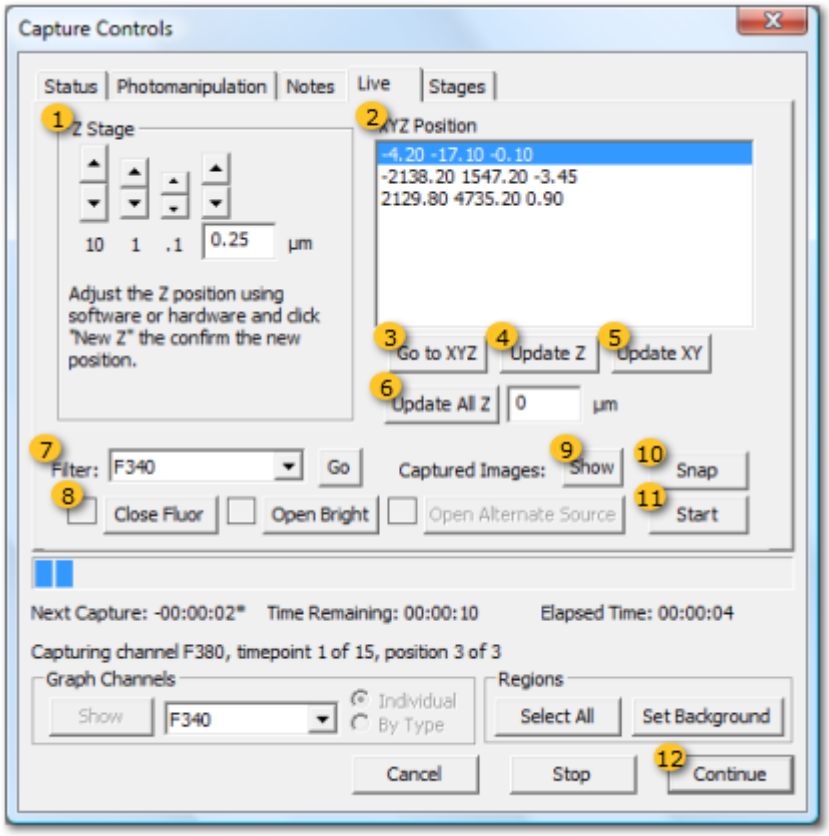
Live Tab of Capture Controls window

The ***Live tab*** is used to adjust focus during an experiment and to show previously captured images. When performing a point visiting experiment, you can update any or all *XYZ* points.

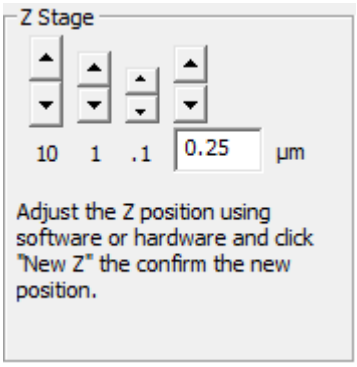
Access by selecting the ***Live tab*** during image capture.

See also:

Ratio Module, [Focusing During Capture]



1 Z Stage group

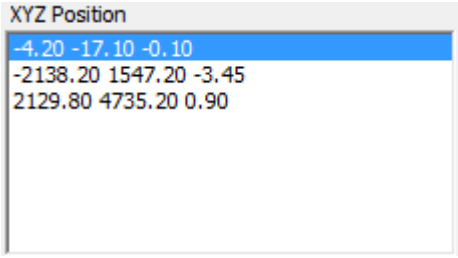


The ***Z Stage*** group allows precise movement of the *z* focus position. You may use the buttons to fine-tune focus during image acquisition. For point visiting experiments, you may update individual *z* positions or all *z* positions.

Simply:

1. ***Pause*** the experiment (12)
2. Select the appropriate ***Filter*** (7)
3. ***Start*** (11) or ***Snap*** (10) or ***Open*** (8) the appropriate shutter
4. Adjust the ***Z Stage*** as desired
5. ***Update Z*** (4) or ***Update All Z*** (6), this step is not necessary when the experiment has only **Current Position**
6. Press ***Stop*** (11) or ***Close*** the appropriate shutter (8)
7. ***Continue*** the experiment (12)

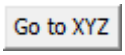
2 XYZ Position list



The ***XYZ Position*** list displays either **Current Position** for single XY point experiments or a list of coordinates for point visiting experiments.

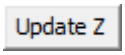
When multiple points are present, you may
1. ***Pause*** the experiment (12)
2. Select a point to ***Go to XYZ*** (3),
3. Select the appropriate ***Filter*** (7)
4. ***Start*** (11) or ***Snap*** (10) or ***Open*** (8) the appropriate shutter
5. Adjust either the ***Z Stage*** (1) or use the motorized stage joystick to adjust the XY position
6. ***Update Z*** (4) or ***Update XYZ*** (5)
7. ***Close*** the appropriate shutter (8)
8. Repeat for additional positions
9. ***Continue*** the experiment (12)

3 Go to XYZ button



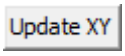
When a point visiting experiment is in progress, you may select a point from the ***XYZ Position*** list and press ***Go to XYZ*** to visit that point.

4 Update Z button



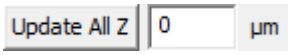
Press ***Update Z*** to update a single *z* position for the selected point on the ***XYZ Position*** list.

5 Update XY button



Press ***Update XY*** to update a single *xy* position for the selected point on the ***XYZ Position*** list.

6 Update All Z button

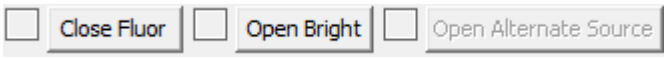


Type an offset in the text field the press ***Update All Z*** to update all *z* positions for all points on the ***XYZ Position*** list.

7 Filter Selection

Select a ***Filter*** from the dropdown menu of channels being acquired. Press ***Go*** to move the hardware to the appropriate filter position.

8 Shutter Controls



Use the ***Open*** and ***Close*** buttons to toggle fluorescence, brightfield, or alternate source shutters; the boxes to the left of the buttons indicate status. The ***Shutter Controls*** are used for adjusting *x*, *y*, or *z* while looking at the specimen through the eyepieces. Alternatively, shutters will be automatically controlled when you use the ***Start*** (11) or ***Snap*** (10) buttons to view the sample via the camera.

9 Show Captured Images button

Show

Press ***Show*** to open the Captured Images window. This window allows you to review previously captured images in the current experiment.

10 Snap button

Snap

Press ***Snap*** to open the shutter for the associated filter selected and snap a single image using the exposure time set in the Capture dialog. Use this instead of ***Start*** if you are concerned about exposing the sample to extra excitation light.

11 Start button

Start

Press ***Start*** to begin a semi-live stream from the camera. You can use this stream to adjust *x*, *y*, or *z* positioning. When you press ***Start***, SlideBook continuously snaps images (opening and closing the appropriate shutter) and displays them in the live ***Camera*** window. Press ***Stop*** to end the semi-live stream.

12 Continue button

Continue

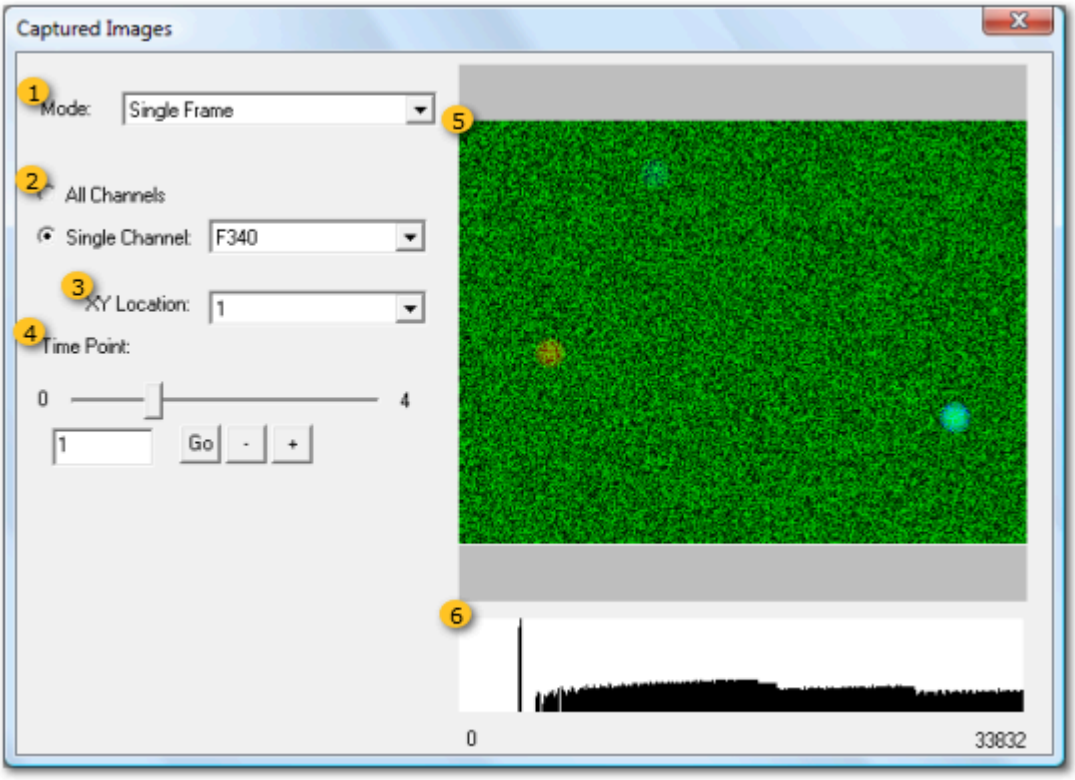
This button toggles between ***Pause*** and ***Continue*** to allow you to pause writing the images to the file when you adjust *x*, *y*, and *z* positioning.

Captured Images window

This window allows you to review previously captured images in the current experiment. You can review 3D images as a maximum intensity projection, select individual or all channels, and review individual time points as well as xy positions.

Access by pressing ***Show*** in the Live Tab of Capture Controls

See also:



1 Mode Selection

Select a ***Mode*** from the dropdown menu as follows:

Single Frame: Displays the individual Z plane, this si the only option for 2D or 2D timelapse captures

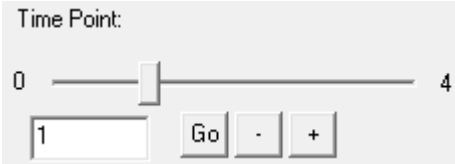
MIP: Displays a maximum intensity projection image for the Z stack at the selected timepoint.

2 Channel Selection

Use the radio buttons to choose to display ***All Channels*** or view a specific ***Single Channel*** that you select from the dropdown menu.

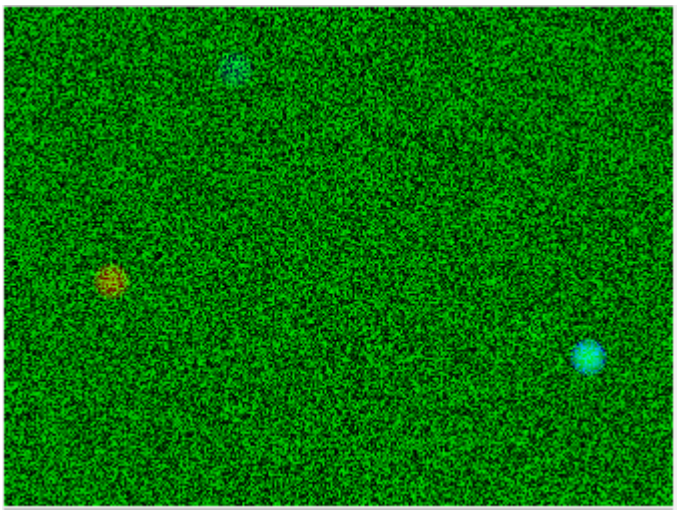
3 XY Location

4 Time Point Selection



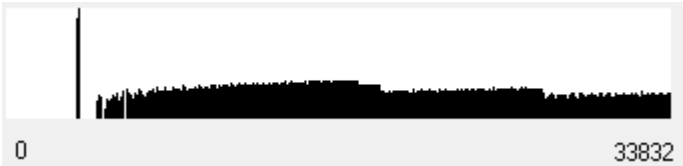
Use the ***Time Point Selection*** area to select a time point to display. You may scroll through time using the slider bar or type a time point in the edit field and press ***Go*** to visit that point. To move a single time point forward or backward, use the **+** and **-** buttons.

5 Image Area



Previously captured images are displayed in the ***Image Area***.

6 Histogram area



When a ***Single Channel*** is selected (2), a histogram of intensity information is displayed.

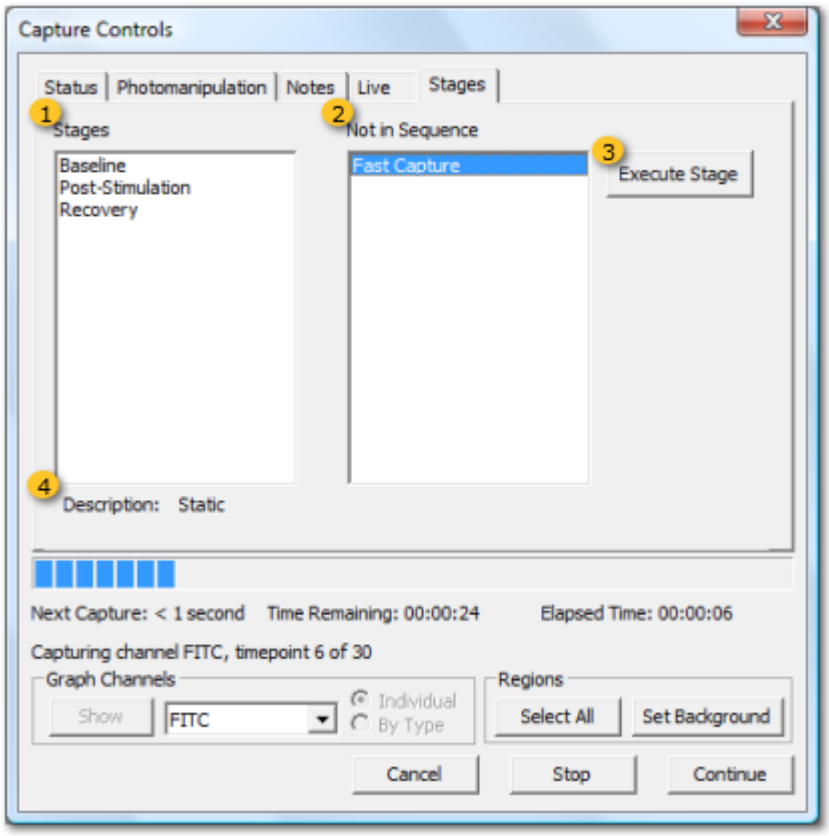
Stages Tab of Capture Controls window

The ***Stages tab*** allows you to interact with stages that were configured in the Sequence Tab of Advanced Capture Preferences. You may execute any stage at any time using this dialog regardless of order of events configured before image acquisition began.

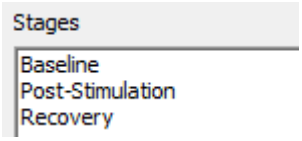
Access by selecting the ***Stages tab*** during image capture.

See also:

Sequence Tab of Advanced Capture Preferences

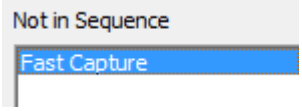


1 Stages list



Lists the stages that were configured in the ***Stages*** list of the Sequence tab of Advanced Capture Preferences. These are stages that will be executed in sequence according to the details configured. At any time you may execute a stage out of order by selecting it from the list (will be highlighted in blue) and clicking ***Execute Stage*** (3).

2 Not in Sequence list



Lists the stages that were configured in the ***Not in Sequence*** list of the Sequence tab of Advanced Capture Preferences. These stages can be manually executed by selecting from the list (will be highlighted in blue) and clicking ***Execute Stage*** (3).

3 Execute Stage button

Execute Stage

When a stage is selected (highlighted in blue) click ***Execute Stage*** to begin capturing according to the stage details. An asterisk (*) will appear next to the stage name when it is in progress.

4 Stage Description

Description: Static

Displays a description of the details and description of the selected stage.

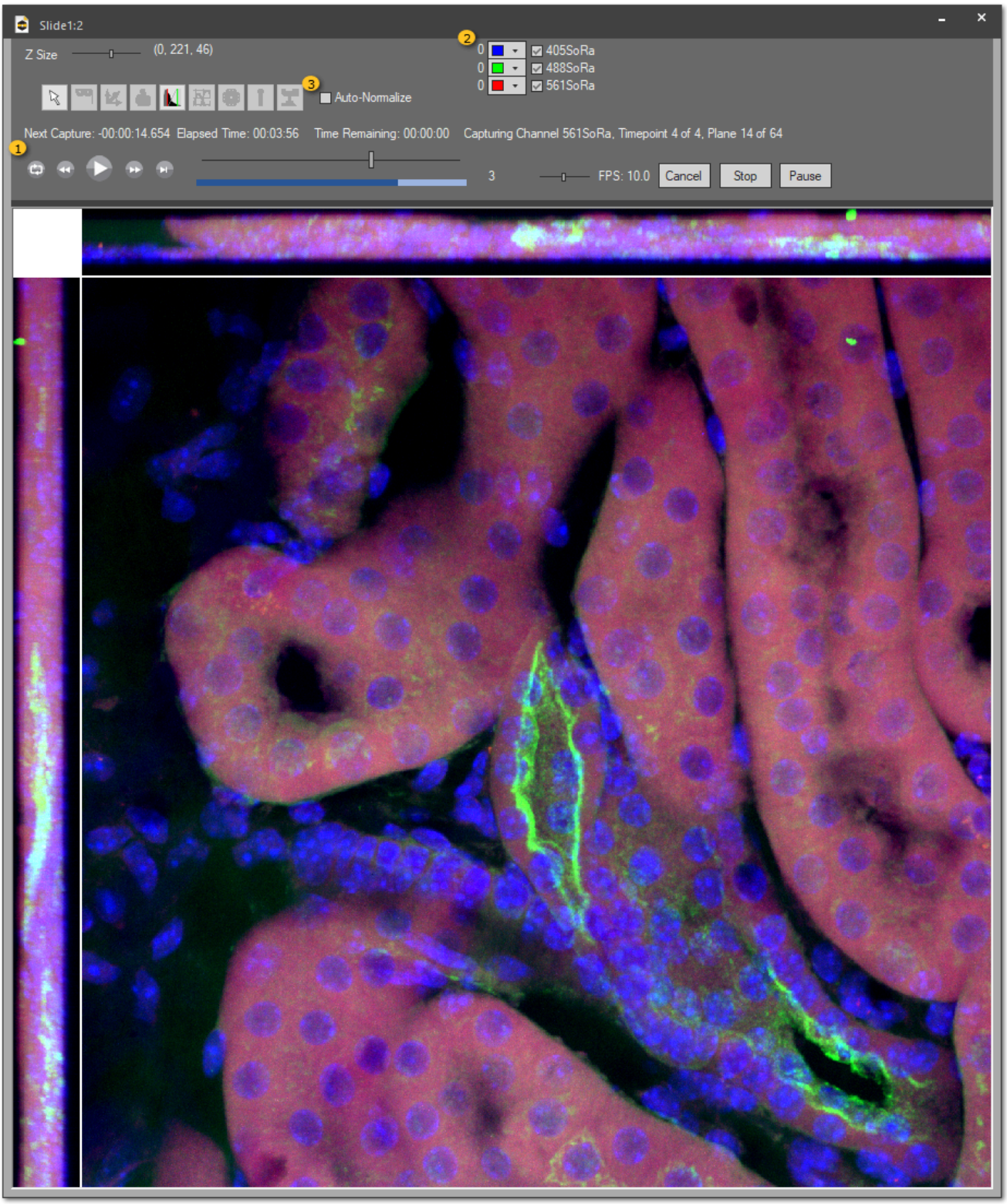
3D Capture Status Window

The ***3D Capture Status Window*** allows you to view and interact with 3D volumes during a 4D acquisition. The window displays a three-view MIP of the desired time point.

Enable by selecting the ***3D Capture Status*** tab in the Advanced Capture Preferences during image capture.

See also:

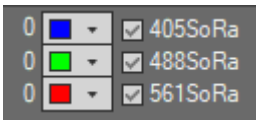
- 3D Capture Status Tab of Advanced Capture Preferences
- Adjust LUT Window
- View Mode



1 Frame Controls

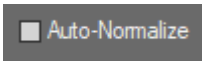
The **Frame Controls** allow you to playback the time points, select one time point to display, change the speed of playback, and pause, stop, or cancel the capture.

2 Channel Selection Controls



The **Channel Selection Controls** allow for changing of channel pseudocolor, and choosing which channels are displayed in the image. The display modes are the same as those available in the View Mode dialog.

3 Auto-Normalize checkbox



Enabled by default, this will auto-normalize the lookup tables. By unchecking this, you can manually manipulate the LUTs via the Adjust LUT window.

TUTORIAL: XY Cell/Region Tracking

When imaging a live specimen over time, it may be useful to move the imaging field based on where the object of interest is moving. To assist in this, SlideBook 6 includes real time XY tracking in which a motorized XY stage will move to keep a defined region central in the imaging field.

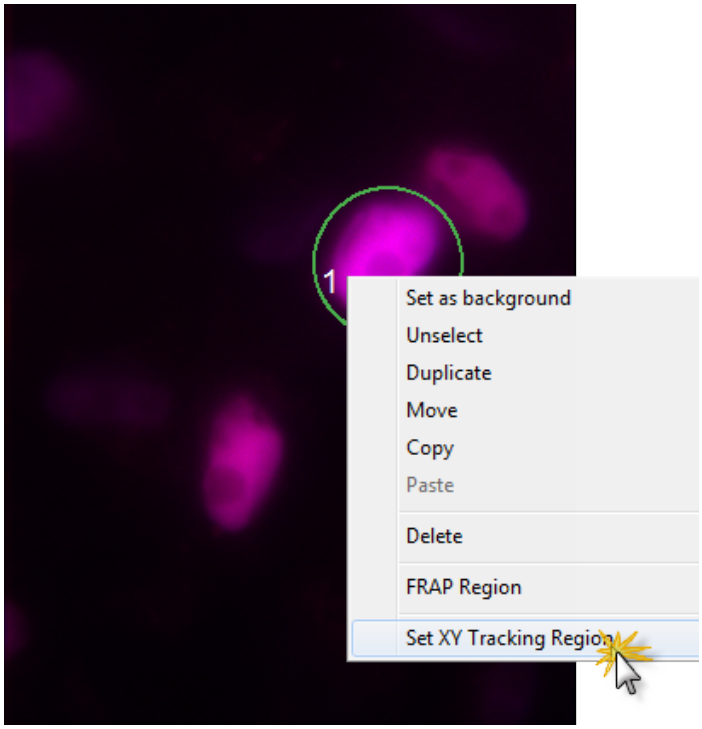
1. Set up a 2D Timelapse Capture as desired using the Capture Window.

NOTE: Tracking will only work on 2D Timelapse Captures.

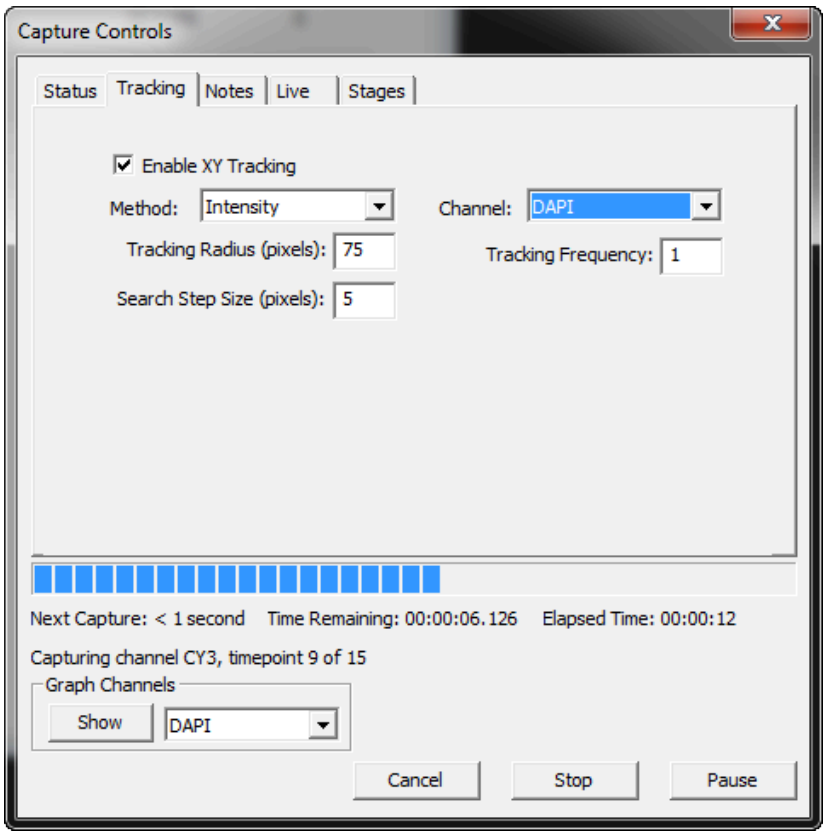
2. Draw a region over the area you would like to keep centralized. For more information on working with region tools, see Region Tools.

3. ***Start*** image acquisition.

4. Right click on the region and ***Set XY Tracking Region***.



4. Go to the ***Tracking*** tab, check the box to ***Enable XY Tracking***.



5. When tracking is enabled, you may select ***Intensity*** or ***Correlation*** as the method. Intensity uses sum intensity of the region and works well for finding a bright area on a dark background whereas Correlation uses a mathematical function (cross-correlation) based on 2-dimensional shape. Either method of tracking will use only the ***Channel*** selected from the drop-down menu.

6. As images are captured, SlideBook will evaluate the image taken ***Tracking Frequency*** number of time points ago. It will compare that image to the current image, specifically the region marked for tracking. The SlideBook will then move the region around in the current image in steps of ***Search Step Size*** pixels until a maximum distance of ***Tracking Radius*** has been reached. The best matching region will then be moved into the center of the field of view before the next image is captured.

Special Capture

At times, it is useful to create more complex capture routines than what is available in the Capture and Advanced Capture windows. There are several options available in the Special Capture menu to help accomplish these goals:

- 2D Stream opens the Stream tab of Focus Controls to capture images in 2D as quickly as possible
- Rapid 4D enables 3D streaming over time
- 6D Multicapture allows you to assign different capture routines to different locations over time
- Multiwell Capture allows you to use standard multiwell format plate specifications to set up a point visiting routine

Rapid 4D Stream Capture window

SlideBook's rapid 4D streaming capture mode permits 4D acquisition, using either one or two cameras, as fast as physically possible. Fully overlapped (streaming) capture is synchronized with a piezoelectric z stage or focusing collar so that the z position is moved each time a frame is captured, without any additional delay.

This mode requires the following components:

- SlideBook™ 5.0 (version 5.0.0.10) or later
- Rapid 4D Streaming Module for SlideBook™ 5.0
- Photometrics camera that produces an EXPOSE OUT line level signal (and corresponding connector)
- TTL Synchronization and LaserStack Control Module for SlideBook™5.0

Performing a rapid 4D streaming experiment involves four steps:

1. Locate the sample using the focus window
2. Set up rapid 4D streaming capture
3. Import resulting spool (.spl) file from disk
4. Convert stream capture into a 4D image

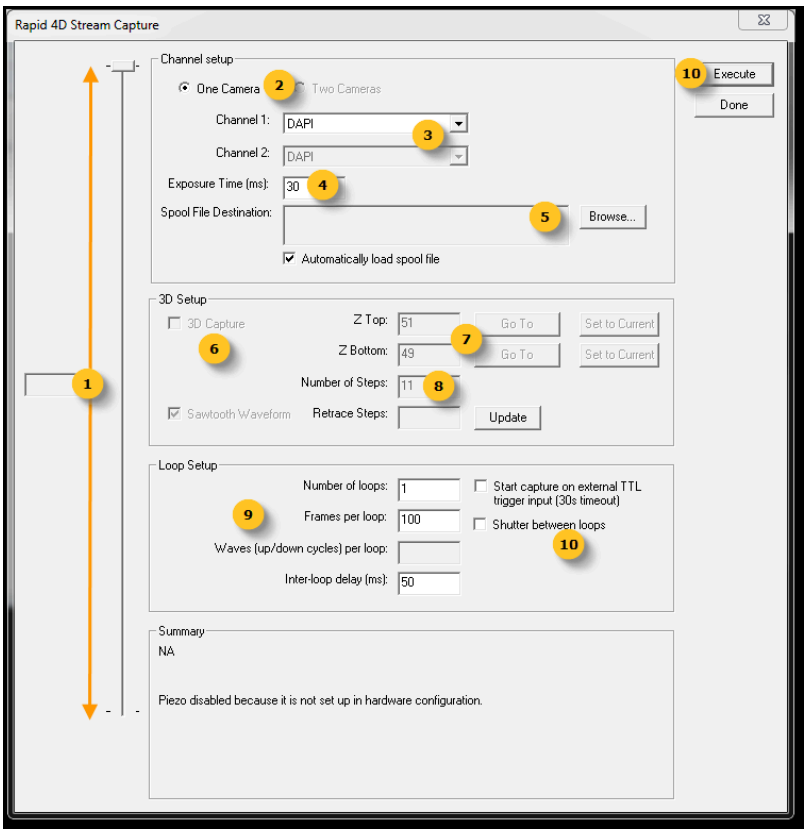
This procedure is more elaborate than setting up normal capture. Depending on camera's frame rate, relative to the magnitude of z motion, it is possible that the actual stage positions either trail their intended positions by some delay or that the stage requires some time to decelerate, switch directions, and accelerate. The interface is designed to give you full visibility (and control) over how the resulting stream capture is interpreted as a 4D image to ensure maximum positional accuracy and linearity.

These effects will be less noticeable during full chip capture of a 512 x 512 camera (such as the Evolve) at 1x1 binning because the frame rate is limited to approximately 30 fps. They become more apparent when using either binning or a small region, or are using a camera capable of much faster frame rates, such as the Cascade 128+.

Setting up rapid 4D streaming capture

1. In order to set up capture, you must first open up the ***Focus Controls*** window and find the sample using the camera(s) you intend to use rapid 4D streaming with. If you want to use binning or only image a subregion of the camera's field, you need to make those selections using the ***Focus Controls*** window.

2. Select ***Special Capture >*** ***Rapid 4D Stream Capture*** from the Acquire group in the Home tab. The ***Streaming 4D Acquisition*** dialog box will open. You can now move the slider on the left side of the dialog to move the piezo up and down. This dialog can be open at the same time as the ***Focus Controls***, so you can select a filter configuration or turn on a light source in the ***Focus Controls*** and use the slider to do live focusing.



2. If you have two cameras, you can choose between ***One Camera*** or ***Two Camera*** capture. Otherwise, ***One Camera*** will be selected automatically.

3. Select the acquisition channel for each camera. When you run the capture, SlideBook will turn on the light source corresponding to ***Channel 1*** and turn it off at the end of capture. The final image will have its channels assigned according to what you select for ***Channel 1*** (and, in the case of two camera capture, ***Channel 2***).

4. Set the exposure time for the capture. Keep in mind that if you select an exposure time that is smaller than what the camera can support in streaming acquisition, it will automatically extend the exposure time to what can be achieved. You can determine whether this adjustment was made by looking at the frame rate achieved in the final image using ***Get Info***.

5. Under ***Spool File Destination*** select a destination folder for resulting spool (.spl) files.

6. Select the ***3D Capture*** checkbox in the ***3D Setup*** group. (There are some modes of operation that do not move a z stage, but these will not be discussed here.)

7. Set the ***Z Top*** and ***Z Bottom*** fields to define the top and bottom positions (in microns) of the full travel in *z*. If you are live focusing on the sample using the slider, you can click on the corresponding ***Set to Current*** buttons to set either of these fields to the current position of the piezo. Conversely, if you want to move the piezo to the value in one of the edit fields, click on the corresponding ***Go To*** buttons.

8. Set the ***Number of Steps*** to the number of steps the piezo will make from the bottom to the top. Remember, the rapid 4D acquisition mode will take one step per camera frame.

> **NOTE:** If you are using the ***Sawtooth Waveform*** mode (which is recommended) the dialog will report how many steps the piezo will take as it makes its way from the top back to the bottom. The corresponding camera frames will be discarded.

9. More details about ***Loop Setup*** will be discussed later. In most situations, you can keep ***Number of loops*** at 1 and choose a ***Frames per loop*** value that gives you the desired number of 3D images. In ***Sawtooth Waveform*** capture, it takes (Number of Steps + Retrace Steps) frames for a single 3D stack. So, if ***Number of Steps*** is 11 and ***Retrace Steps*** is 3, it takes 14 frames per 3D stack (i.e., individual time point in 4D image). If you want a 4D image that has 10 time points, you will need to set ***Frames per loop*** to at least 140.

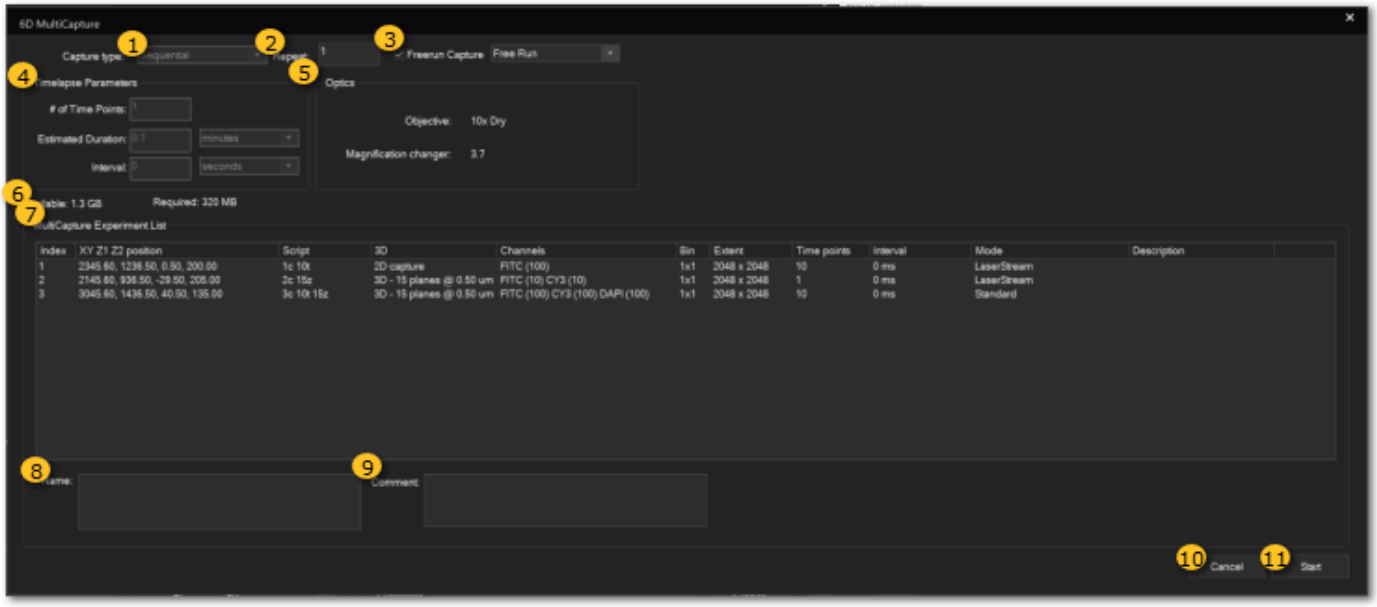
10. Click on ***Execute*** to start the capture. When the acquisition is complete, an alert **Streaming Complete** will appear. Make sure to wait for this before starting another acquisition or closing the dialog box.

6D MultiCapture window

Once capture scripts have been assigned to each location using the 6D Tab of Focus Controls, you can initiate capture using the ***6D MultiCapture*** window. Here, you will set up parameters for the time lapse portion of the experiment and review already configured settings.

See also:

TUTORIAL: Using 6D Scripted Capture
6D Tab of Focus Controls



1 Capture type drop down

Select the ***Capture type*** from the drop down menu as follows:

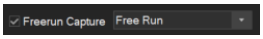
Sequential - Performs a time lapse routine at each location, capturing the assigned script for the entire time lapse duration before moving on to the next location. Uses timelapse parameters in saved capture scripts, ignores ***Timelapse Parameters*** in this dialog.

Interleaved - Performs a time lapse routine in which the selected capture script is performed at each location in a loop using the time lapse interval between the start of each point visiting loop. Uses ***Timelapse Parameters*** in this dialog, ignores timelapse parameters in saved capture scripts.

2 Repeat field

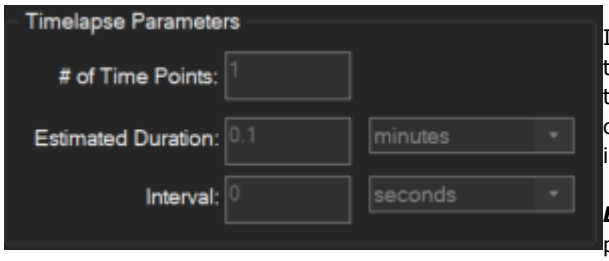
If ***Sequential*** capture type was selected, enter a value here to repeat the sequence multiple times. For this mode of capture, you may find it useful to utilize the ***Pause After Script*** field in the 6D tab of Focus Controls when setting up the point list.

3 Freerun Capture checkbox and menu



Toggles Freerun Capture on and off, and allows for Free Run, Externally Triggered, or Phasor Triggered initiation of the experiment.

4 Timelapse Parameters group



If ***Interleaved*** capture type was selected, type a value for the ***# of Time Points*** to acquire and the ***Interval*** between time points. This is the interval between the start of each capture routine. If capture takes longer than the designated interval, it will proceed as quickly as possible.

Estimated Duration will be completed once the other two parameters are completed.

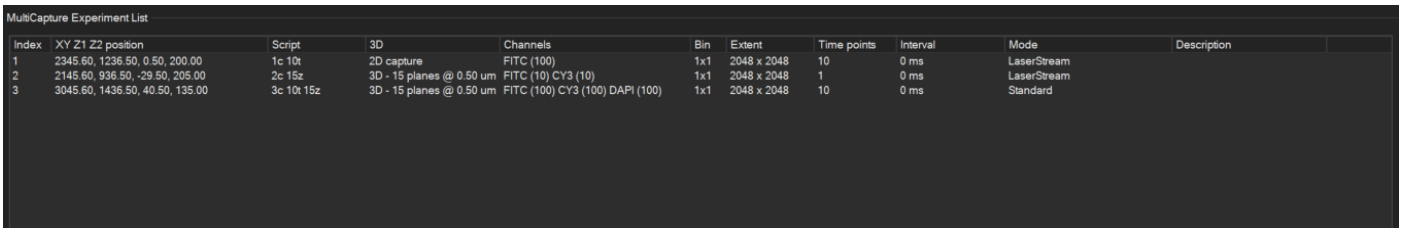
5 Optics group

Displays information on the current objective and magnification changer. The ***Focus Controls*** window can be used to change the optics used in image capture.

6 Space Requirements

The amount of available memory and memory required for the designated image acquisition are displayed here.

7 Location list



Index	XY Z1 Z2 position	Script	3D	Channels	Bin	Extent	Time points	Interval	Mode	Description
1	2345.60, 1236.50, 0.50, 200.00	1c 10t	2D capture	FITC (100)	1x1	2048 x 2048	10	0 ms	LaserStream	
2	2145.60, 936.50, -29.50, 205.00	2c 15z	3D - 15 planes @ 0.50 um	FITC (10) CY3 (10)	1x1	2048 x 2048	1	0 ms	LaserStream	
3	3045.60, 1436.50, 40.50, 135.00	3c 10t 15z	3D - 15 planes @ 0.50 um	FITC (100) CY3 (100) DAPI (100)	1x1	2048 x 2048	10	0 ms	Standard	

The location list displays the XYZ position and their associated capture scripts as designated in the 6D tab of the ***Focus Controls***

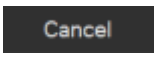
8 Name field

Click on a location from the list and enter an image ***Name*** in this field.

9 Comments field

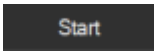
Click on a location from the list and enter any image ***Comments*** in this field.

10 Cancel button



Press ***Cancel*** to discard changes and close this dialog.

11 Start button



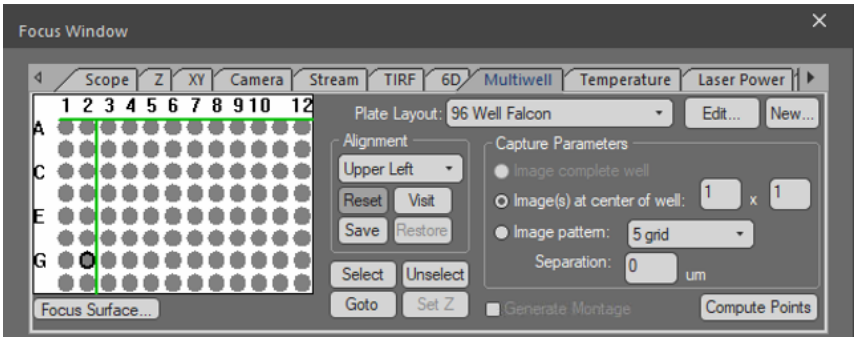
Press ***Start*** to begin the designated 6D scripted image capture.

Multiwell Tab of Focus Controls

The ***Multiwell*** tab of Focus Controls window allows you to set up an automated point visiting experiment based on a multiwell plate configuration.

Before beginning any part of Multiwell setup, it is important to ensure the stage, pixel size, and camera are well calibrated. The easiest way to test this is to double click on an object in the live camera window. If double-clicking causes the object to move to the center of the live window, everything is configured correctly. If the object moves in the wrong direction, you may need to adjust XY Stage Properties. If the object moves too far or too little, you may need to adjust the pixel size for the objective or set a per-camera magnification factor.

To access, open a blank **.sld** then ***Capture Multiwell***



SlideBook comes pre-configured with some common Falcon brand plate formats. If you are using a plate not listed, please follow TUTORIAL: Creating a New Plate Layout.

The first time you use a new plate type (dependant on manufacturer, number of wells, and product type), you will need to confirm the plate design and calibrate the plate layout. This can be done using an empty or used plate and following TUTORIAL: Calibrating a New Plate Type.

For general imaging, plate layouts will be consistent. When you load a new plate (of a previously calibrated type), you will perform a quick fine-tuning based on the placement of the plate on the stage then proceed with identifying wells for imaging. Please follow TUTORIAL: Setting up Imaging with a Calibrated Plate Type.

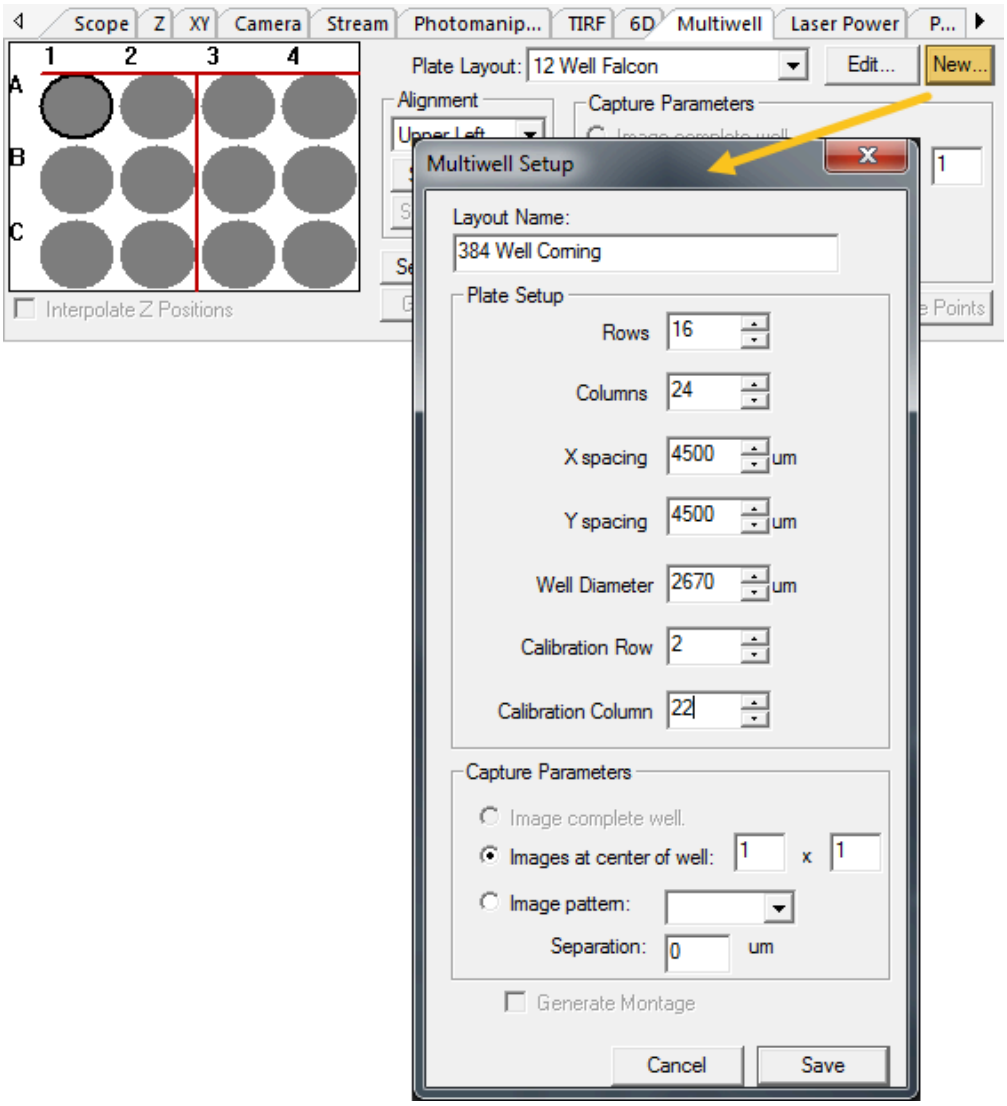
At this point, you can export a point list and set up a standard timelapse experiment, autofocus, or 6D Scripted Capture.

TUTORIAL: Creating a New Plate Layout

SlideBook comes pre-configured with some common Falcon brand plate formats. If you are using a plate not listed, please follow the instructions below.

1. Open a new ***.sld*** file. Open the Focus Controls window and navigate to the ***Multiwell*** tab.

2. Click ***New*** to define a new layout.



- Enter a recognizable name for the new plate type in the ***Layout Name*** field. Use something recognizable for future use. Remember you will be loading this for future use so include any parameters that may help.
- Enter in the number of ***Rows*** and ***Columns*** you count on the plate.
- Use specifications obtained from the manufacturer to enter values for ***X spacing***, ***Y spacing***, and ***Well Diameter***. If values are given in mm, multiply by 1000 to convert to um (i.e. 4.49mm = 4490 um).
- Enter values for ***Calibration Row*** and ***Calibration Column***. These are the locations that will later be used to calibrate placement of the plate. For 96 and 384 well configurations, it is recommended to not use the outermost rows or columns for calibration as the plate design may interfere with imaging at some positions.
- If desired, configure ***Capture Parameters*** for imaging. This can easily be changed later or set up when imaging.
- Click ***Save***.

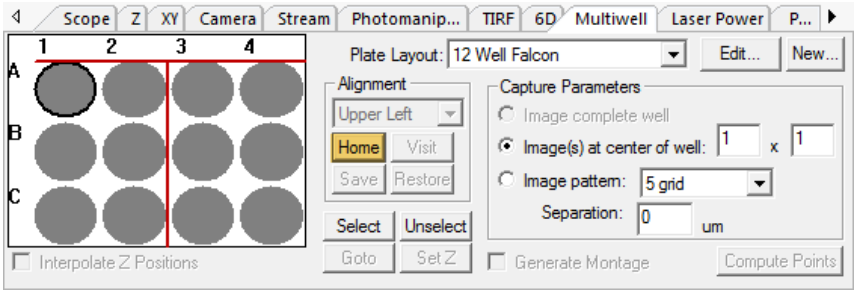
3. Continue on to Calibrating a New Plate Type.

TUTORIAL: Calibrating a New Plate Type

SlideBook comes pre-configured with some common Falcon brand plate formats. If you are using a plate not listed, make sure you have set up the new plate using [TUTORIAL: Creating a New Plate Layout](#).

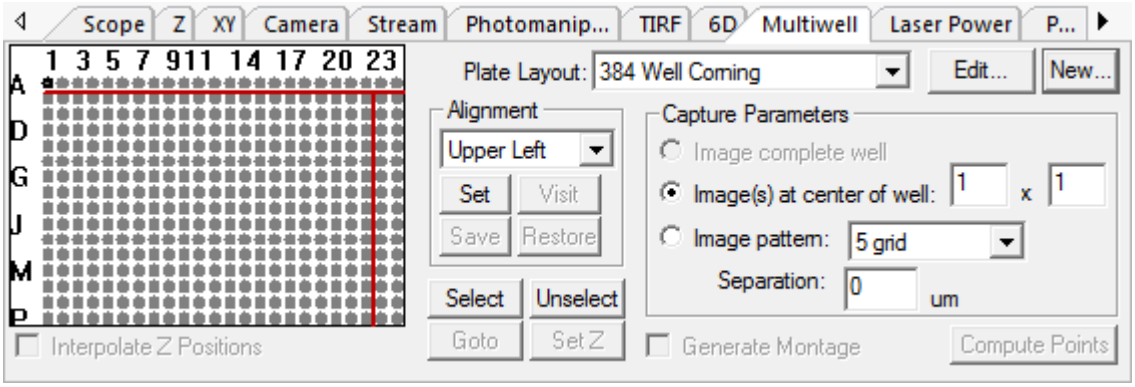
1. Open a new ***.sld*** file. Open the Focus Controls window and navigate to the ***Multiwell*** tab.

2. Click ***Home*** to home the stage. Be sure there is no sample on the stage and it can move freely to all extents.



3. Once the homing process is complete, load the plate onto the microscope stage, an empty or used plate is fine for calibration purposes. Select the desired ***Plate Layout*** from the dropdown menu.

4. Notice the plate diagram is updated for the identified format. There are 2 red lines in the diagram representing the top of the calibration row and the right side of the calibration column. In the Alignment group, ***Upper Lef***t is displayed. This text indicates the calibration position set you are on.



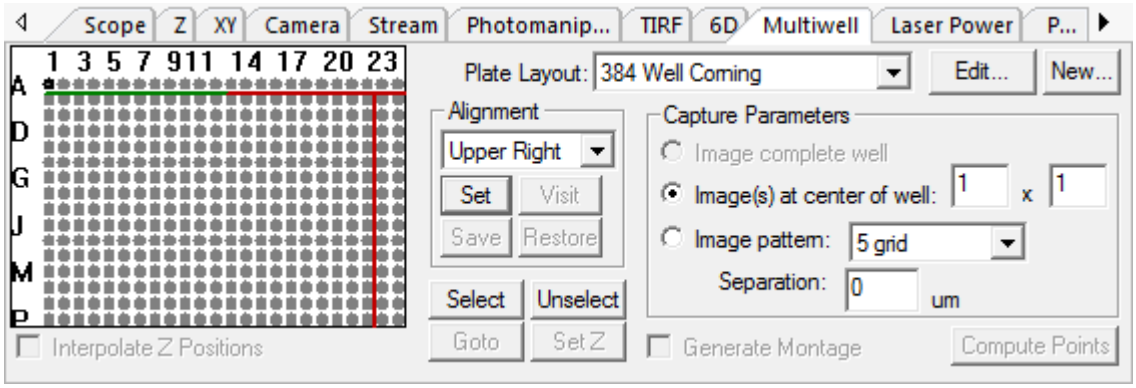
Overview of calibration process:
a. There are 3 calibration positions. Upper Left, Upper Right, and Lower Left.
b. These three locations tell SlideBook how "square" the plate is on the stage and orient the exact position of the plate on the stage for use in imaging.
c. The calibration process includes using the microscope stage buttons/joystick to move to each of the positions then clicking ***Set***.
d. The text in the ***Alignment*** dropdown will display which location the buttons will impact. Once a location is set, the dropdown field will automatically update with the next calibration location.
e. After calibration (and any time during calibration), alignment locations can be revisited or reset as needed. To revisit or adjust calibration locations, select a location from the dropdown list then ***Visit*** to move the stage to the marked location. Adjust the stage position as necessary and click ***Reset***.

5. Using the live image and Focus Controls, navigate to a well on the left side of the alignment row. In the 384 well plate example we are using, wells B-2 through B-7 would be good candidates for ***Upper Left*** locations. Using the crosshairs in the live camera window, center the top of a well. For round wells, this should be center and the top of the well but for square wells, it can be anywhere along the top line. Click ***Set***.

NOTE: To turn on the guide lines in the live window, check the box for ***Guides*** in the center of the [Focus Control](#) window.

Once you click set, several things will happen:

- The left half of the calibration row line will turn green, indicating the Upper Left position was set
- The text in the Alignment dropdown will proceed to ***Upper Right***.

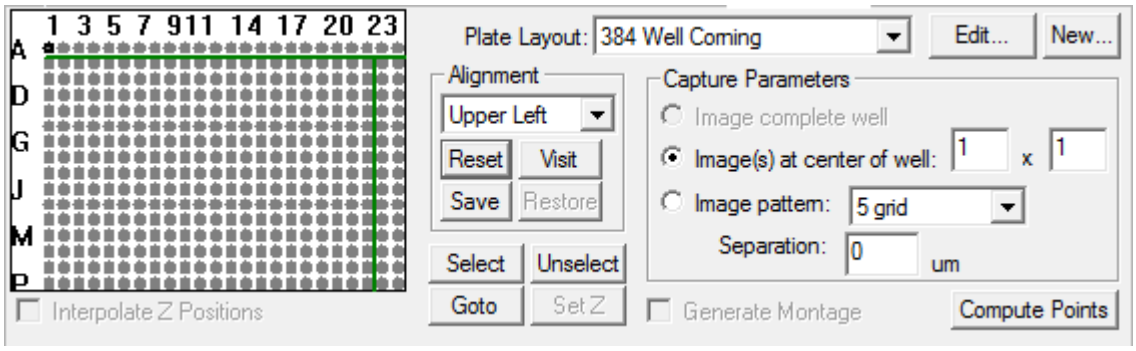


6. Move the stage to the top of a well on the right side of the calibration row. In the 384 well plate example we are using, wells B-14 through B-22 would be good candidates for ***Upper Right*** locations. Using the crosshairs in the live camera window, center the top of a well. For round wells, this should be center and the top of the well but for square wells, it can be anywhere along the top line. Click ***Set***.

Once you click set, several things will happen:
- The right half of the calibration row line will turn green, indicating the Upper Right position was set
- The text in the Alignment dropdown will proceed to ***Lower Right***.

7. Move the stage to the appropriate calibration column as defined in the Plate Layout. In our example, this is column 22. Move down this column toward teh lower half. Remember, the further apart your calibration points, the more accurate the calibration will be. In our example, wells J-22 thorugh O-22 would be good candidates. Locate the right side of the well. For round wells, this should be vertical center and right side of the well but for square wells, it can be anywhere along the right side line. Click ***Set***.

Once you click set, several things will happen:
- The calibration column line will turn green, indicating the Lower Right position was set
- The text in the Alignment dropdown will go back to ***Upper Left***.
- The Set button will become Reset. The Visit and Save buttons will be activated.



8. Now that the plate is calibrated, you can travel freely around the plate using the diagram of the plate. Some functionality available includes:

- Select an alignment position from the dropdown list. Click ***Visit***, the stage will move to the requested position. Adjust the location as necessary and click ***Reset*** to update the calibration point to the current stage location.
- Click on a well in the plate diagram. Click ***Goto***. The stage will move to the center of the selected well.
- Double click on a well in the plate diagram. The stage will move to the center of the selected well.

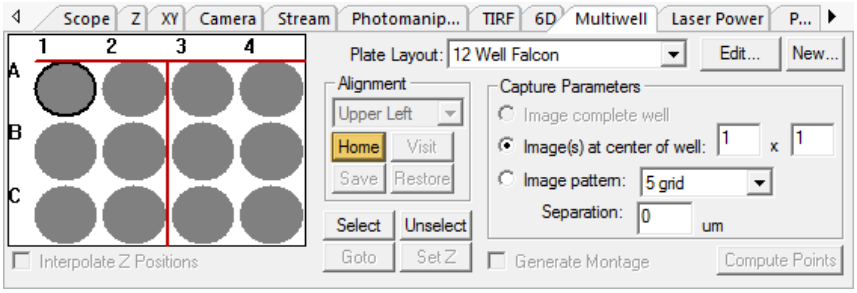
9. Click ***Save*** to save this calibration file to the Plate Layout.

TUTORIAL: Setting up Imaging with a Calibrated Plate Type

Once a plate type has been created and initially calibrated, you will not need to repeat the process. You will simply load the plate, visit and adjust calibration points to account for any placement changes, and set up imaging.

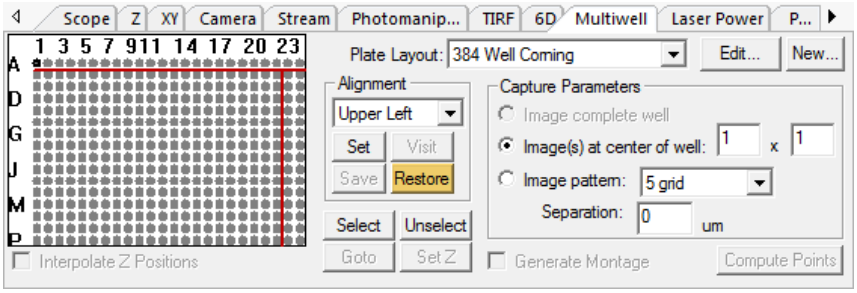
1. Open a new ***.sld*** file. Open the Focus Controls window and navigate to the ***Multiwell*** tab.

2. Click ***Home*** to home the stage. Be sure there is no sample on the stage and it can move freely to all extents.

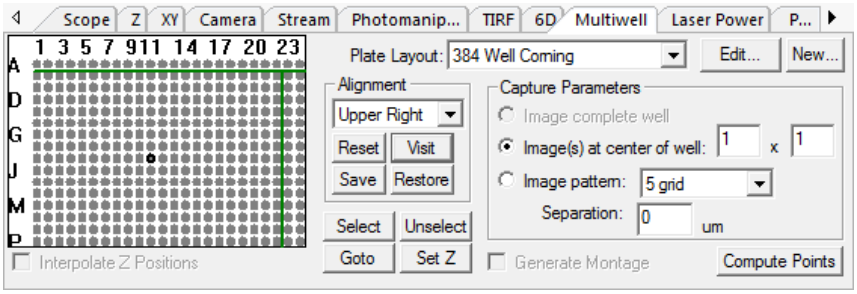


3. Once the homing process is complete, load the plate onto the microscope stage. Select the desired ***Plate Layout*** from the dropdown menu. If the exact plate type is not available in the Plate Type menu, please refer to [TUTORIAL: Creating a New Plate Layout](#).

4. Click ***Restore***. If the Restore button is not available, please refer to [TUTORIAL: Calibrating a New Plate Type](#). After you click restore, a window will open telling you the previous calibration points have been restored and to visit and reset the points before imaging.



5. The red calibration row and column lines will turn green.. Note the alignment position listed is Upper Left. Click ***Visit***. This will move the stage to position the plate in the calibration location used for the upper left point. Open the appropriate shutter to view the plate in the live camera window. Confirm the top of the well in the calibration row centered in the crosshairs (see Guides) of the live view. Adjust the stage as necessary using the joystick or XY Stage buttons. Once the top of the well is centered, click ***Reset***.



6. Notice the alignment position has progressed to Upper Right, click ***Visit***. Repeat the fine tuning described in the previous step for the top of a well on the right side of the calibration row. Click ***Reset***.

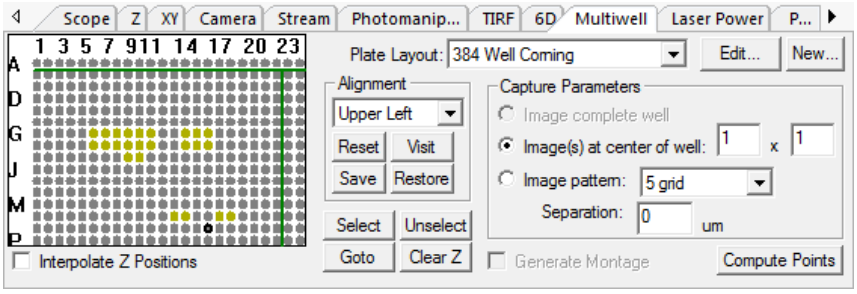
7. Notice the alignment position has progressed to Lower Right, click ***Visit***. Repeat the fine tuning described in step 5 for centering the right side of a well in the lower portion of the calibration column. Click ***Reset***. ***Save*** updated calibration positions if desired.

8. At this point, you can move freely around the plate by either double clicking on wells in the plate diagram or by selecting a well then clicking on ***Goto***. If you notice the sample losing focus when you move around the plate, you will want to calibrate the z plane of the plate. To do so, you will need to set a z position in 3 locations.

a. Visit a well, adjust the focus using the Z Stage buttons. Click ***Set Z***.
b. Visit a second well, adjust the focus using the Z Stage buttons. Click ***Set Z***.
c. Visit the third well, adjust the focus using the Z Stage buttons. Click ***Set Z***.
d. The ***Interpolate Z Positions*** checkbox will become available to check. Check the box to have SlideBook find a plane through the three z calibration points. Z values will be applied to all locations in the plate using these interpolated values.

NOTE: The further apart the z calibration points are, the more accurate the resulting interpolation. This z plane interpolation will not help if the problem is due to an uneven imaging plane such as what can occur when substrate is used in inconsistent thicknesses well-to-well. Using Set Z for individual locations without using Interpolate Z Positions will not export the z coordinates of individual wells. This must be done from the XY tab of Focus Controls.

9. With the plate fully calibrated, the next step is to select locations for imaging. The well select tools work like painting. Click on a well you would like to image. Click ***Select***. The well will turn a light green color. Click on this well and drag (or paint) the green onto adjacent wells you would like to image. Once more than one well is selected, you can click on any green well and drag to select more wells for imaging. ***Unselect*** works in the same manner returning wells to the grey, unselected state.



10. Once the desired wells are highlighted, configure Capture Parameters to take one or more images in each well. To change the default capture parameters for the plate type, click ***Edit*** and refer to Creating a New Plate Layout.

11. Click ***Compute Points***. This will export all requested imaging locations as a list to the XY tab of Focus Controls where you can refine z positions for individual wells if needed or proceed to Capture. At this point, you may also find the following help pages useful:

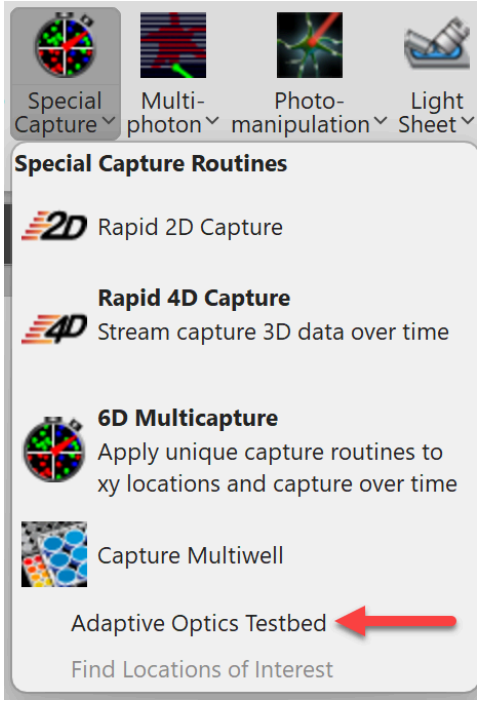
TUTORIAL: Using 6D Scripted Capture
TUTORIAL: Using Definite Focus for Multipoint Capture
TUTORIAL: Using ZDC for Autofocus
TUTORIAL: Nikon Ti2 Perfect Focus for Autofocus

NOTE: Only click ***Compute Points*** once. The list is generated in the XY tab every time you click this button so clicking more than once will result in duplicating the point list. If this happens, proceed to the XY tab, click ***Clear Points***, then return to the Multiwell tab and click Compute Points.

Adaptive Optics Testbed

SlideBook has placed the Adaptive Optics functionality in an UI called the Adaptive Optics Testbed.

This UI can be found by selecting a Slide then selecting Special Capture and Adaptive Optics Testbed.



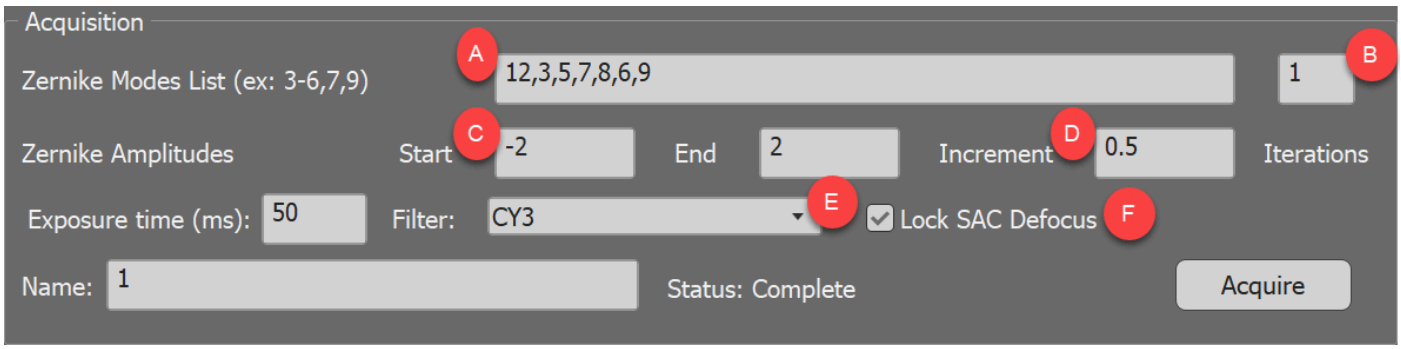
Current Adaptive Optics settings can be saved using the drop down. C-Shaper can be swapped between Bypass and the Deformable Mirror using the radio buttons.

The SAC-DM calibration must be performed when using the Adaptive Optics to correct for spherical aberration. A in all cases should be close to 0, while B varies depending on the index of refraction of the sample.

A correct SAC-DM calibration should result in minimal focal change while adjusting the spherical correction.
Compute performs this calibration automatically.
The quality of the fit can be visualized using Plot.

Acquisition Settings

To perform an adaptive optics correction a series of images spanning a range of Zernike Correction Amplitudes must be taken and analyzed. The image acquisition settings are set using the UI found below.

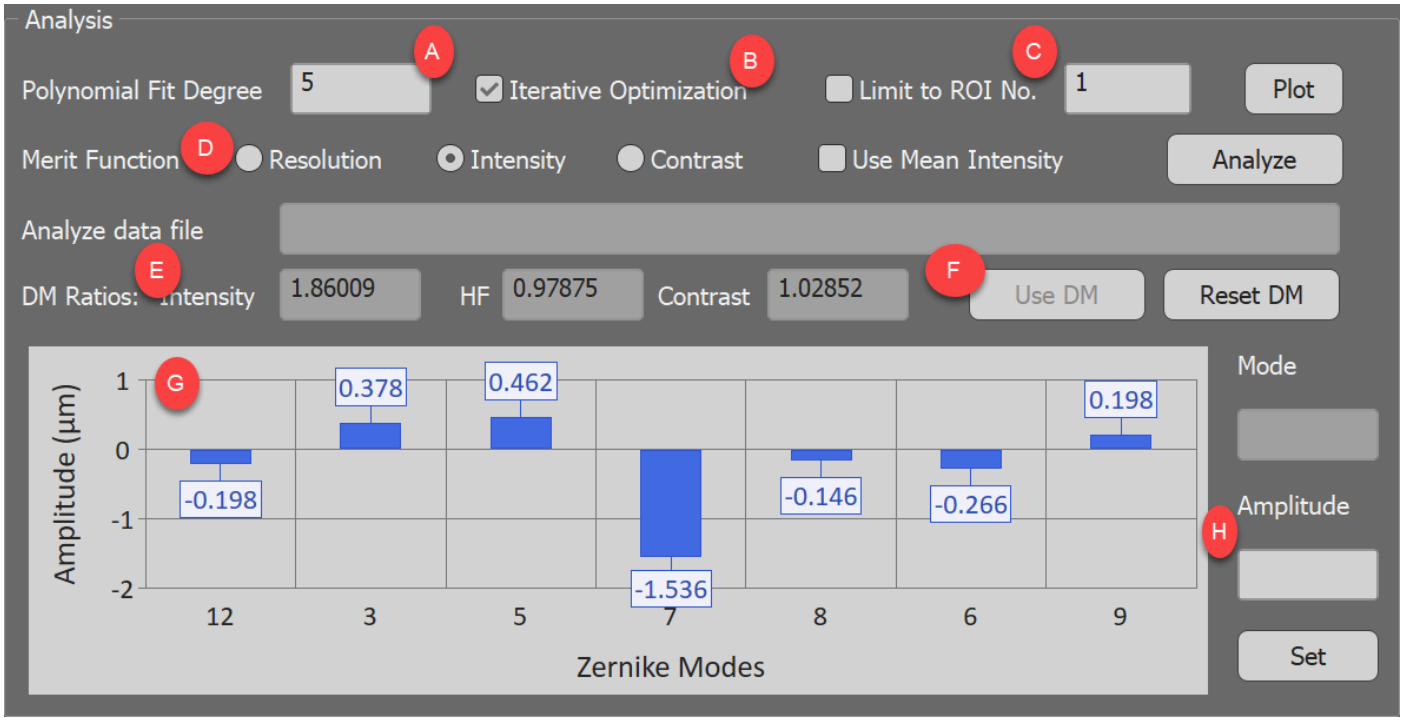


A. Numbers entered in this box determine which Zernike Modes will be optimized.
B. Sets the number of iterations to be performed during the acquisition process.
C. Sets the amplitude range for each Zernike Mode optimization.
D. Sets the increment for each Zernike Mode optimization. A smaller increment will give more accurate results, but require more images.
E. Sets the Exposure time and Filter to be used for the image acquisition.
F. When checked utilizes the SAC-DM calibration to prevent focal change when performing a spherical correction. This should always be checked when performing a spherical correction (Zernike Mode 12).

Acquire will take the image series once the settings are chosen.

Analysis Settings

After an image series is taken it must be analyzed to determine the optimal Zernike Amplitudes for each Zernike Mode. The Analysis settings are set using the UI found below.



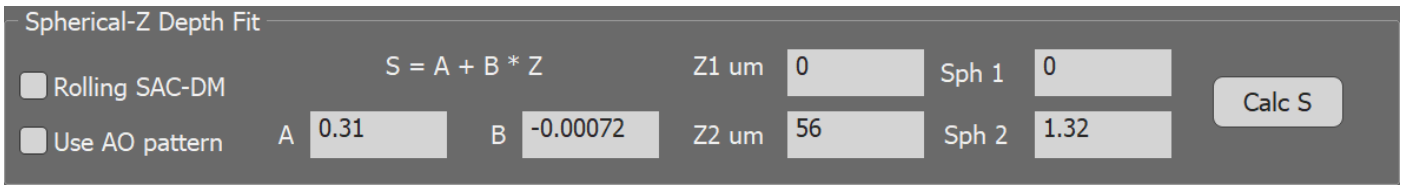
A. Sets the number of degrees used for the polynomial fit.
B. If checked performs an iterative optimization for the fit.
C. Limits the optimization to a Region of Interest in the image.
D. Chooses the merit function to be used for optimization.
E. Shows the improvement in the merit function before and after adaptive optics correction.
F. Use DM turns the adaptive optics correction On, while reset DM sets it to a flat mirror.
G. Graphical illustration of the Zernike Amplitudes applied to each mode.
H. Allows a user to manually enter an amplitude and apply it to the DM.

GO AO

This button will sequentially run the acquisition and analysis to perform an adaptive optics correction for your image.

Rolling Spherical Correction

The UI found below is used to set up a Rolling Spherical Correction. To perform this you will need to know the correct spherical correction at two different Z positions.



The user inputs the spherical correction at two different Z-positions (Sph1 and Sph2 at Z positions Z1 and Z2).
Calc S calculates the rolling spherical correction to use for the 3D Stack and needs to be clicked prior to performing the 3D stack.
Checking the Rolling SAC-DM box will use the rolling spherical correction when performing a 3D stack.

Multiphoton

The ***Multiphoton*** menu gives you direct access to multiphoton scanning acquisition functions. These include the following:

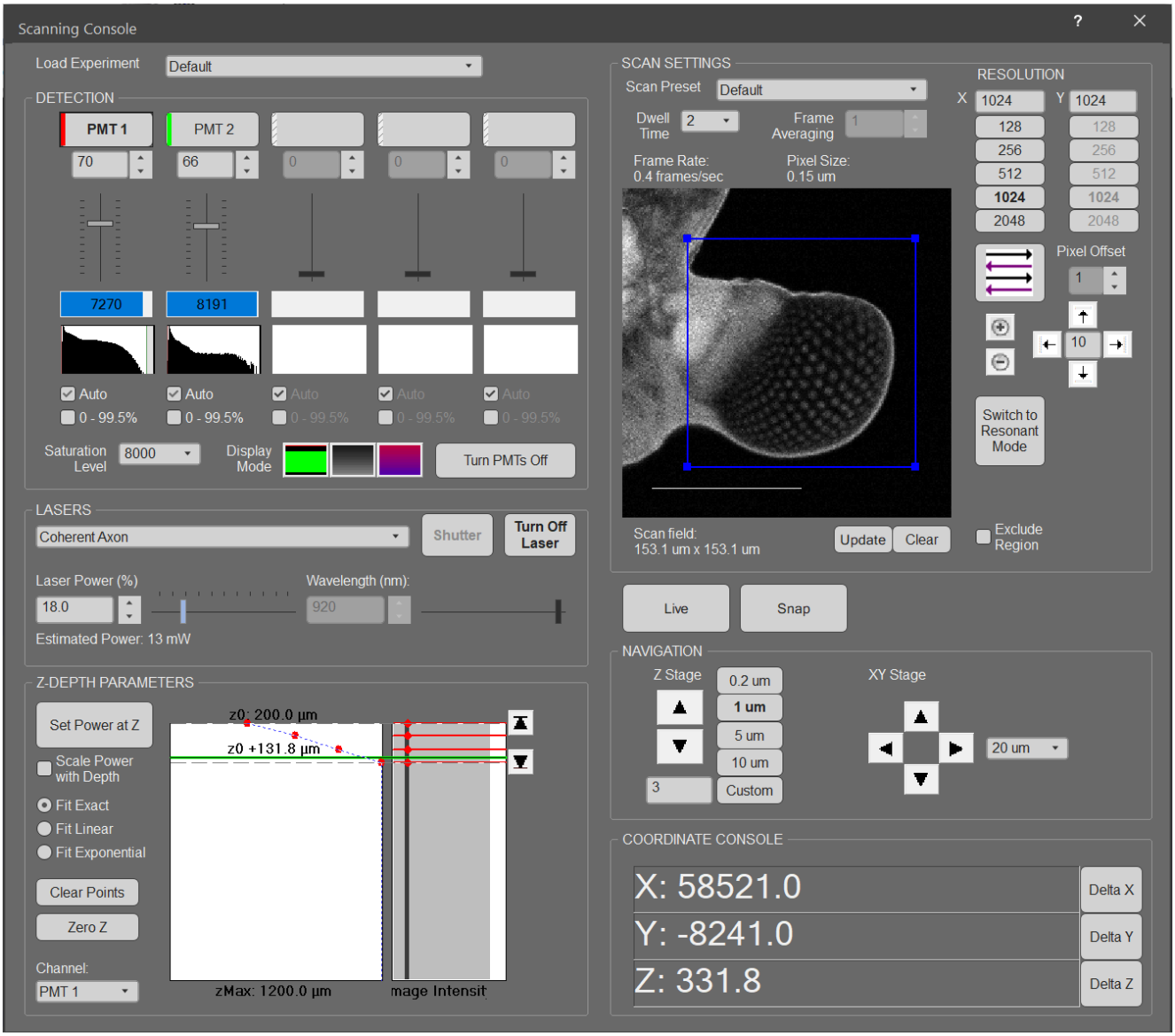
Scanning - Point scanning multiphoton imaging

Line Scanning - Image lines using lasers

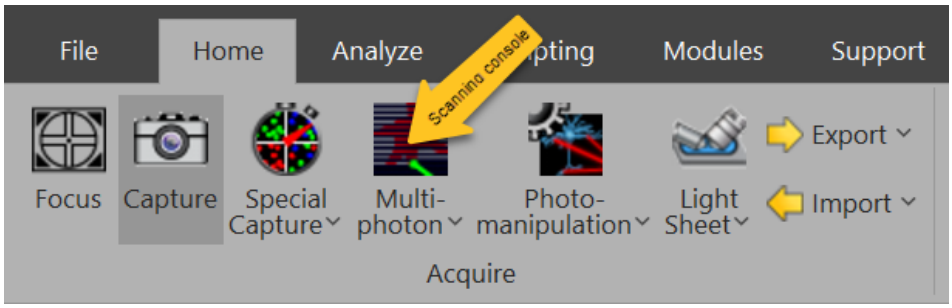
See also Multiphoton Scanning Module

2P Scanning Console

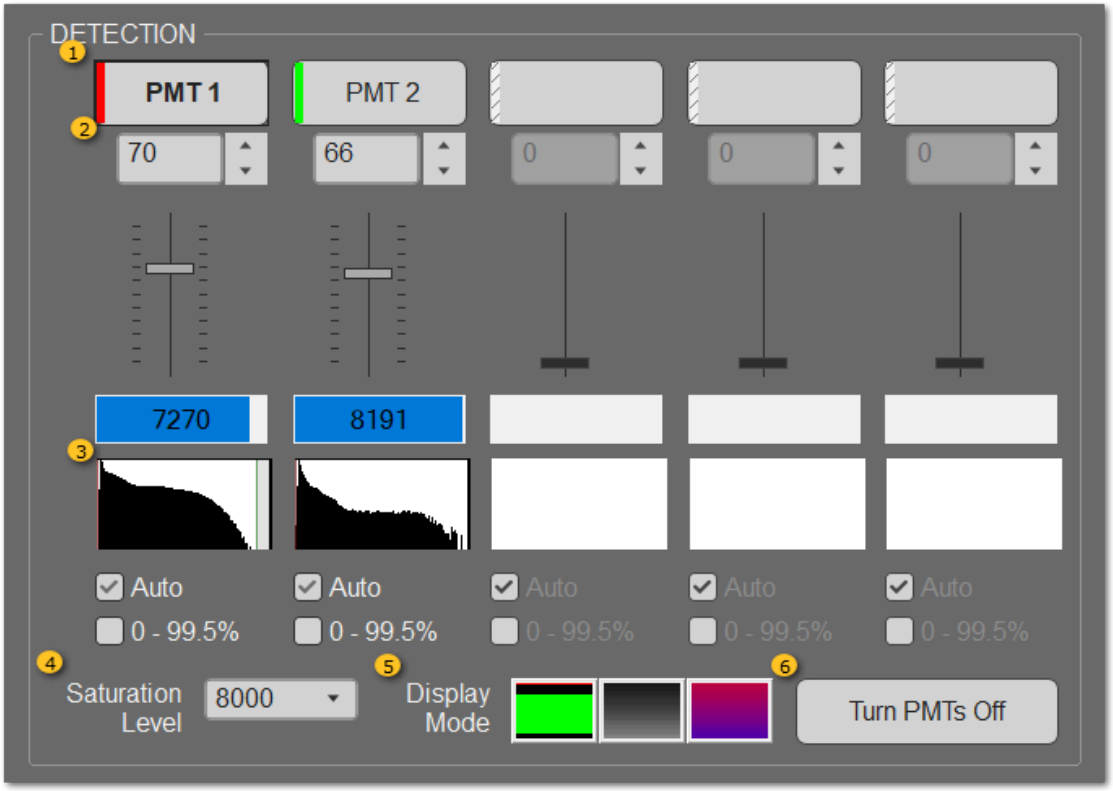
SlideBook has placed all of the common adjustments to the scanning parameters, including PMT gain and laser intensity, in the ***2P Scanning Console*** box. Much like the focus window, this dialog will remain open while you are locating where and what you want to acquire, and while you are dialing in the best parameters for acquisition.



To access the 2P Scanning Console, simply click the **Multiphoton** icon in the **Home** tab in SlideBook.



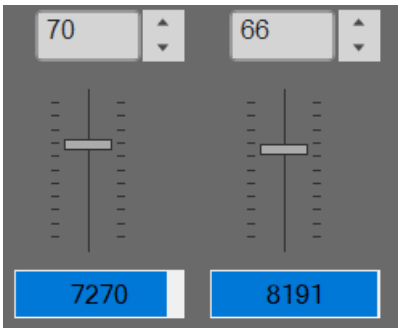
Detection Group



1

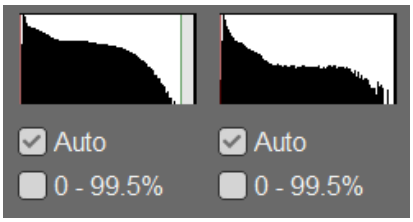
Clicking on the main ***PMT buttons*** (which denote both the PMT's assigned number and associated color) determines which PMT is displayed in **(A)** the live focus window and **(B)** the small full-field scan window located in the ***Scan Settings*** dialog box. To generate a multi-PMT, overlaid image in the live focus window, first click on one PMT, then hold down the ***Ctrl*** button on your keyboard and click on the other PMT you'd like to add to the overlay. If PMT1 (red) and PMT2 (green) are now both selected, you will get a green/red pseudocolored image that will display colocalized pixels in yellow.

2



Each PMT has independent ***Gain Control*** located below the PMT buttons. The gain can be modulated by (1) manually entering a gain value and pressing enter, (2) clicking the up/down toggle buttons, or (3) moving the gain slider up or down. In a typical two PMT system, there is a separate set of controls for the green PMT and the red PMT. Directly below the gain slider is a button to reset a PMT if it has reached an overload condition (indicated by the signal display becoming solid red).

3



The dynamic histogram, located below the PMT reset button, displays the pixel values across the scanned image. ***Auto*** scales the histogram to the min/max pixel values and ***0-99.5% check box*** removes the top 0.5% of pixel values when autoscaling the histogram.
When you uncheck the ***Auto*** checkbox, you can grab the slider on each side of the histogram to adjust the visualization of the live image in the live window and small window in the Scan Setting dialog box. When a capture is initiated, the adjusted histogram settings as seen in the live window will be carried over to the capture window. Rechecking the ***Auto*** checkbox will return the histogram settings to auto-scaling.

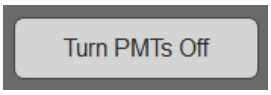
4

The ***PMT Saturation Level*** drop down menu or manually entering a saturation level value allows you to select which grayscale value will display red saturation pixels in the live camera window. The saturation level also adjusts the top level of the histogram. This feature gives the user flexibility in finding an optimal dynamic range based on your current imaging conditions.

5

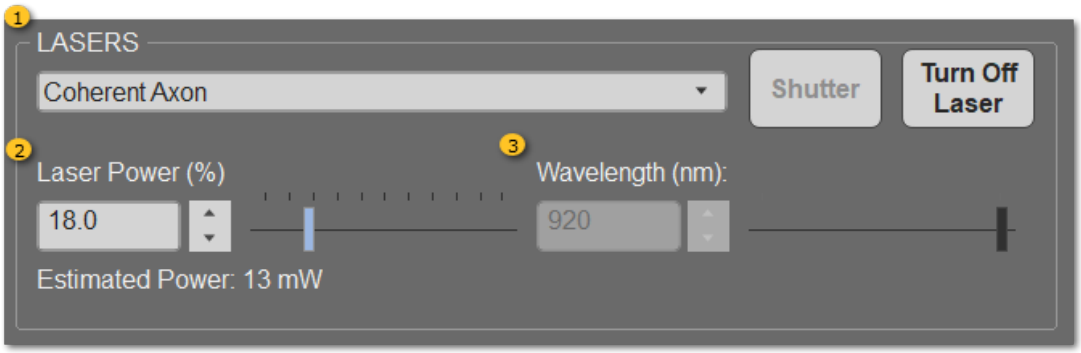
The ***Display Mode*** LUT presets will pseudocolor the live image only a single channel when a single PMT is selected.

6



All PMTs Off button allows you to turn PMTs off by eliminating all voltage being applied to any PMTs. To turn the PMTs back on, click the button when it says "Turn PMTs On." You can tell when PMTs are off if the PMT and gain slider are grayed out and no histogram is displayed.

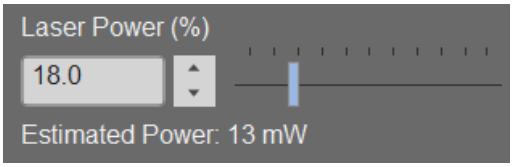
Lasers Dialog



1

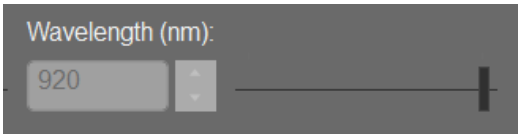
To control a specific laser (on/off, wavelength, power %), first select the laser you would like to control in the drop down menu. To open or close the shutter, click the Open Shutter or Close Shutter button, respectively. To turn the laser ON or OFF, click the "Turn On Laser" or "Turn Off Laser," respectively. These buttons will always display what action will be taken when the button is clicked.

2



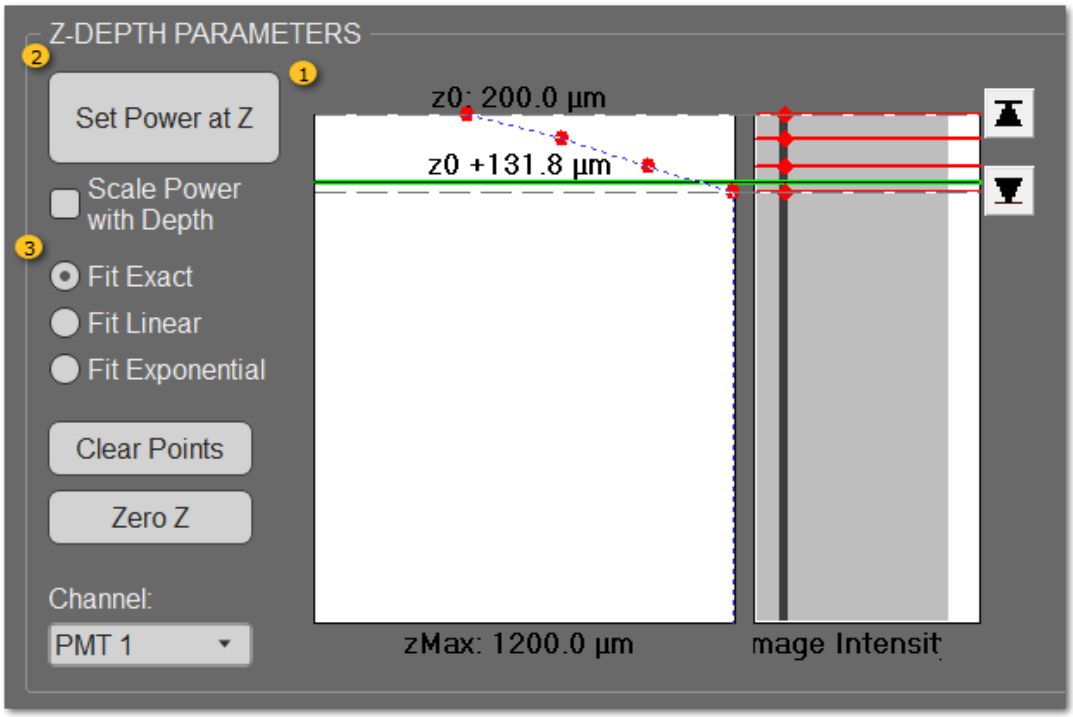
In some instances, ***Laser Power %*** will internally modulate the power of a laser if has internal TPC (total power control). If the laser uses a Pockels cell for modulating the power, then SlideBook can modulate the laser intensity into the system by sending a signal to the Pockels cells, shown in % of total range. When a power map has been generated with a power meter for a specific wavelength, an ***Estimated Power*** in mW will be displayed below the power controls.

3

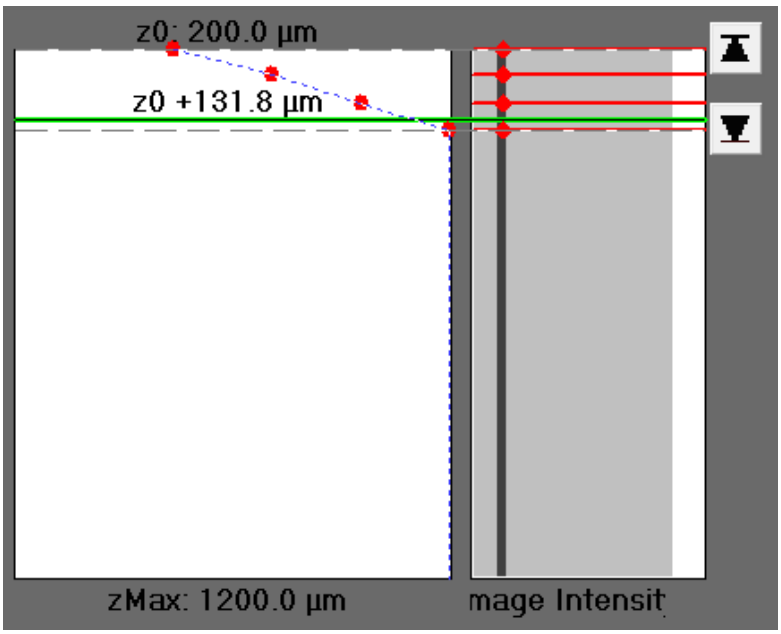


SlideBook can control ***Wavelength*** if the laser is tunable; the wavelength can be changed by (1) manually entering a wavelength value and pressing enter, or (2) using the slider to decrease (slide left) or increase (slide right) the output wavelength. This wavelength set in SlideBook can be confirmed by looking at the front LED display on the laser control box. For a fixed wavelength laser, the ability to change the wavelength will be grayed out.

Z Depth Parameters

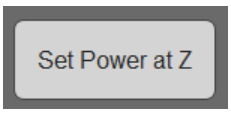


1



The horizontal colored line (red when out of specified depth range, green when within specified depth range as seen above) functions as a slider that can be dragged to change the objective's position and thus the focal plane in the Z axis. By default, the maximum amount of stage movement allowed in one step is 0.5 mm. To set ***Top and Bottom*** extents for captures involving 3D volumes, click the Top and Bottom buttons on the right side of the Z-depth dialog.

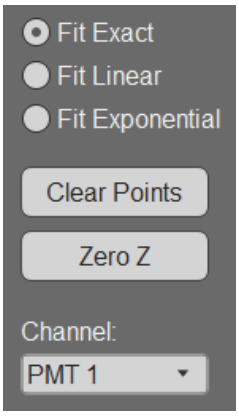
2



Clicking ***Set Power at Z*** assigns a specific laser power to a specific plane in Z, and can be clicked at multiple locations throughout the Z axis.

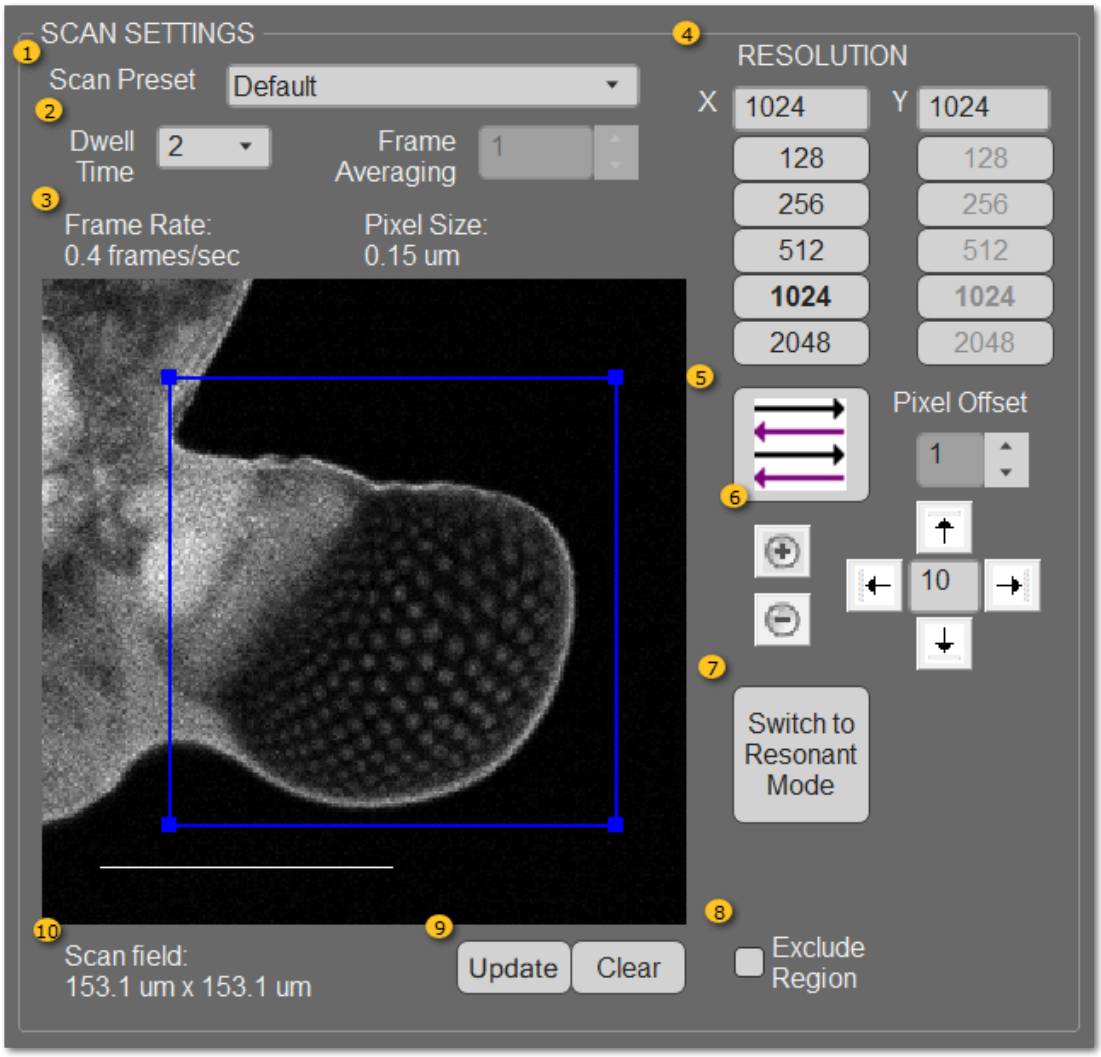
If both a fit function is selected and the checkbox next to ***Scale Power with Depth*** is checked, then the power will scale with Z depth in both (1) live scanning in Z using the focus control knobs or Z stage buttons and (2) during a 3D capture.

3



The ***Clear Points*** buttons will clear all previously logged power/Z planes. The ***Zero Z*** button changes where the top of the power depth map is set, and sets it to the current Z location of the Z stage. The PMT selected in the Channel dropdown menu will display the image intensity of that PMT on the horizontal axis in order to best match intensities at different Z-depths by adjusting the laser power for each Z location.

Scan Settings Dialog



1

Saving a ***Scan Preset*** using this dropdown menu allows the user to save specified scan settings such as resolution, dwell time, pixel offset, scan field size and location, and scan mode. If settings are changed or modified, you can select save in the dropdown menu to overwrite the selected preset, or click Save As to save as a new preset.

2

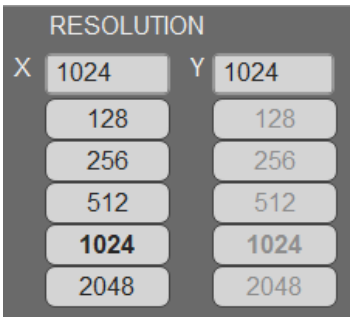
The ***Dwell time*** can be adjusted by entering a value in the field or with th***e*** drop down menu. This allows the user to specify the dwell time of a single pixel in microseconds. Additionally, a dwell time value can be typed in and applied by hitting enter. Default = 2 us.

Frame Averaging is not yet implemented in this version of SlideBook. To average frames, utilize the Frames feature in the Focus or Capture window.

3

Frame Rate displays a live value calculated from on the resolution and dwell time. The displayed ***Pixel Size*** is calculated by dividing the extent in microns but the resolution.

4



Setting the Resolution:
Changing the resolution will maintain a square pixel size and adjust the field aspect ratio accordingly. If you want to change the overall resolution of the scan (how many pixels the scan is divided into), then you should manually change the ***Resolution X*** or ***Y*** field then hit ***Enter*** for the new dimensions to take effect or select one of the preset resolution buttons.

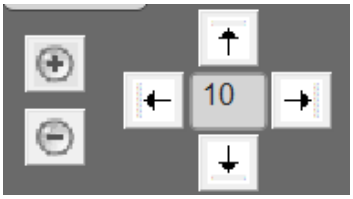
In resonant scanning mode, only allows for a square field to be selected by the Zoom + button, or a selected region that is longer in X than it is in Y (to take advantage of the direction of the resonant scanner).

5

Unidirectional vs. Bidirectional scanning can be changed by clicking on the multi-arrow button (arrows will change to display unidirectional or bidirectional of the current scanning paradigm). Unidirectional scanning only available in Dual-Galvo scanning.

Pixel Offset: The up/down toggle button will change the interlace offset in both Dual Galvo or Resonant Scanning by one integer. Once the ideal interlace offset value is found, this offset will be applied to all images captures in regular capture or streaming.

6

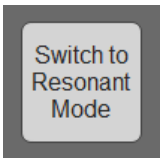


Clicking the + button will generate a blue box centered inside of the full field (see above); this blue box is the updated area being scanned and displayed in the Focus window. Clicking the + button multiple times will continue zooming into the center of the full field and get smaller and smaller. Clicking ***Clear*** underneath the scan field window will reset the blue box to full field scanning (see point 10 below). Clicking the - button will zoom out once for each time it is clicked.

To move the blue box's scan position, without changing its extent or resolution, use the up, down, left, and right arrows. See that the blue box in the image above is shifted to the right within the full FOV. The buttons will move the scan field in those directions within the full field. The default step size of this movement is 20 microns, but you can choose a different step size by manually entering a value and clicking enter.

Users can also select their own ROI within the scan field. Use the mouse cursor draw a square or rectangular ROI encompassing the desired region to image. This region will now be displayed in the live focus window.

7



This buttons directly controls which scan mode (Dual Galvo Scanning to Resonant Scanning) is currently being utilized. This button will always display what action will be taken when the button is clicked.

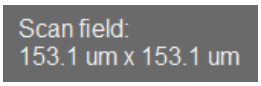
8

When the ***Exclude Region*** checkbox is checked, a square ROI drawn in the focus window will allow for the laser to be blanked at that location and therefore no excitation throughput will be observed at those pixels.

9

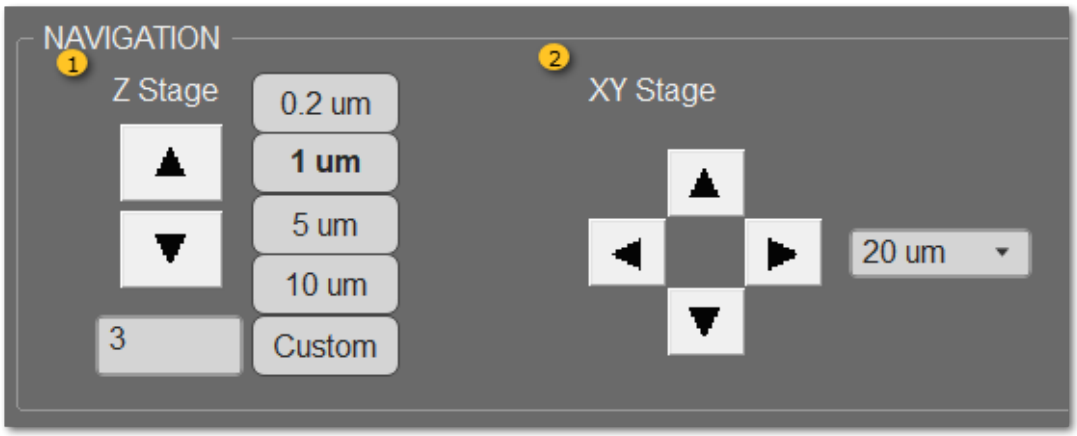
This displays the the size of the current scan field, either when scanning a full frame or a selected ROI within the full field. The ***Update*** button will scan a single full-field size frame to update the scan-field inset window; this allows for the user to update their scan area without exposing their sample to sustained 2P scanning. Clicking the ***Clear*** button will reset the subset of the full field specified with blue box back to to full field scanning.

10

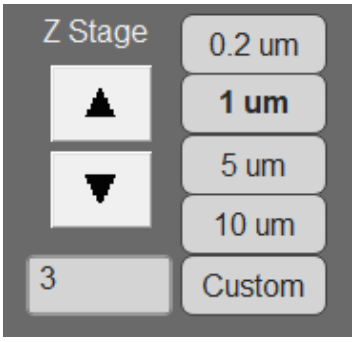


This displays the the size of the current scan field, either when scanning a full frame or a selected ROI within the full field.

Navigation

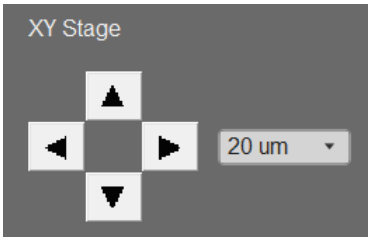


1



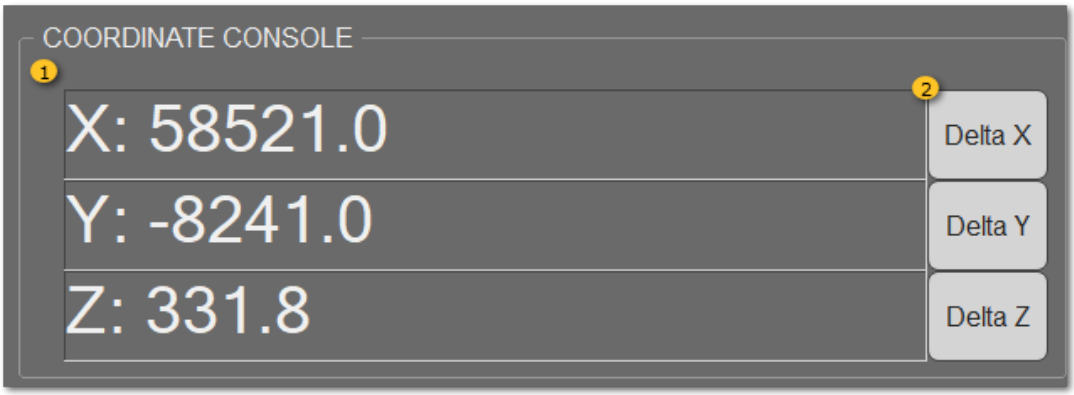
Z Stage navigation allows for remote control of the Z Position of the objective. Select either pre-determined distances (0.2, 1, 5, 10 um) or a custom distance that can be typed in. Use the up/down toggle buttons to move the selected distance.

2

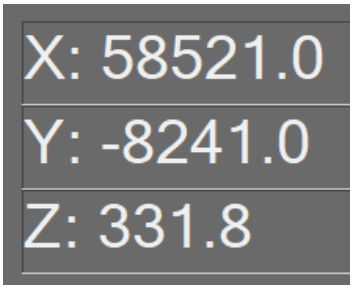


XY Stage navigation allows for remote control over XY position of the stage. Select either pre-determined distances from the drop-down menu or type in a custom distance. Use the up/down/left/right toggle buttons to move the selected distance.

Coordinate Console



1



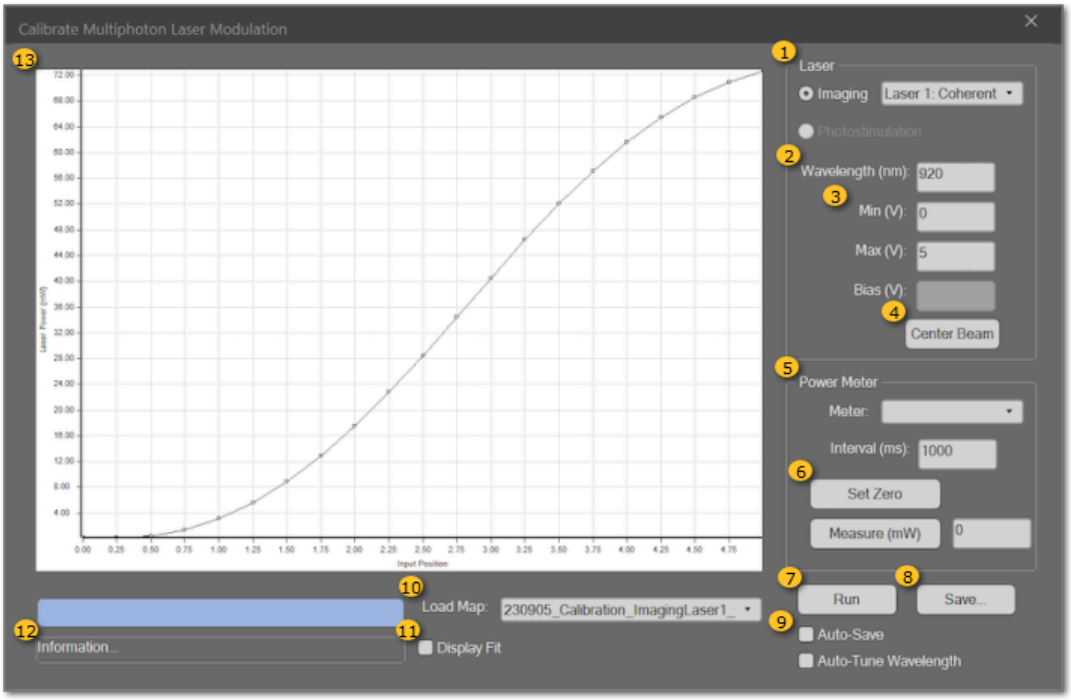
X, Y, Z *coordinates* of the stage are displayed as either an absolute position (X above) or relative position (Rel Y and Rel Z above).

2

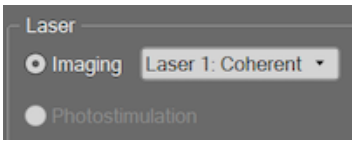
To toggle back and forth between absolute and relative position, click ***Delta X, Y, or Z***. If relative position is displayed, it will show 'Rel' in front of the coordinates.

Multiphoton Laser Calibration

Access the Calibration Dialog through the ***Modules*** tab of SlideBook. Click on the ***Multiphoton*** Icon and navigate to ***Calibrate Multiphoton Laser Calibration***.



1

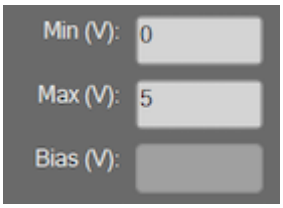


The ***Imaging Laser*** drop down is populated from imaging lasers specified in Advanced Laser Configuration (Modules > Multiphoton > Advanced Laser Configuration) and are identified by name and position in the list. Changing this laser updates the power maps associated with the laser itself, including a power map for all wavelengths captured and saved. This is reflected by changing the Power Map List (10). The laser list drop down only applies to imaging lasers, but a single photostimulation laser line can be selected by using the option labeled "Photostimulation"

2

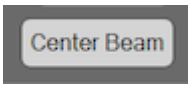
The ***Wavelength*** field is populated automatically by the current setting on the laser. Adjusting this field also automatically adjusts the wavelength of the power meter. If this is set to a different wavelength than the laser and the laser has tunable wavelength, this dialog will *not* attempt to change the wavelength of the laser itself.

3



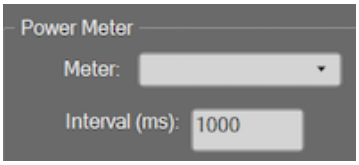
This section controls the minimum and maximum values from which the laser will be tuned between when running the calibration protocol. The units of the input position are in the units recognized by the hardware. For example, AOM/ Pockels' cell this will be in Volts as seen above, while Thorlabs/NewFocus rotation stages, it will be degrees. For built-in control, such as through a serial interface, the units will be percentage of input power. The units displayed in the parentheses will update based on the units used by the power control object of the laser. If a photostimulation laser is selected, these units will always be in centiVolts (cV).

4



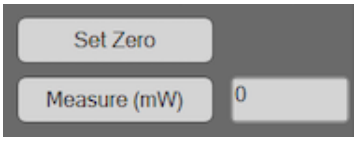
The ***Center Beam*** button moves the XY galvo mirrors to their center position. Centering the galvos are required when taking a power measurement with a power meter located ***after*** the galvo scanners. When a power meter is elsewhere in the path (eg. just after a Pockels cells), the user does not need to center the galvos.

5



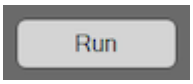
The ***Power Meter*** section allows the user to select which power meter (as set in Hardware Configuration) in the drop down menu that will be used during laser calibration. Changing the ***Interval*** will change the time interval between collected data points.

6



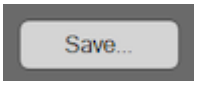
The ***Set Zero*** button will attempt to reset the minimum level of the power meter. Make sure the power meter is in position and the laser is shuttered when clicking "Set Zero". The ***Measure*** button will take a single measurement from the power meter and display it, for diagnostic purposes. The value field shows the measured power and will update during the calibration run.

7



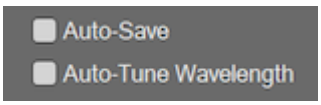
The ***Run*** button starts a calibration, and when a calibration is running, all other controls will be disabled. The ***Run*** button label changes to ***Stop*** until the calibration process ends. Clicking ***Stop*** will abandon the calibration and return the other buttons to the previous state.

8



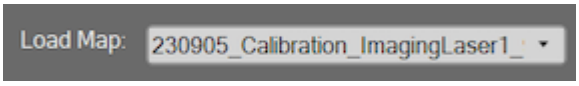
Click the ***Save*** button to save the calibration displayed on the plot to an external file. If the file already exists in the Power Calibrations folder with the same name, then a incrementing suffix is added to the default file name.

9



If the ***Auto-Save*** checkbox is enabled, each newly acquired power map will be automatically saved using the default file name schema. If ***Auto-tune Wavelength*** checkbox is selected when ***Run*** is clicked, then a compatible laser system that can change wavelength will run through a range of wavelengths, acquiring and saving a map for each one. At the conclusion, the user will be prompted if they want to replace all of their current power maps with the newly acquired ones.

10



The ***Load Map*** drop down is repopulated whenever the currently selected laser is changed. The drop down shows all available power maps loaded for that laser, with a maximum of one per unique wavelength. The name refers to the file in which the power map was stored. If a new map has been acquired, it will show at the end of the list with the prefix "(New)" added to the default file name.

If there is a "New" power map available and selected, the Use as Current Map button will be available. Clicking this button will prompt the user for confirmation, and afterwards will attempt to save the current map using the default file naming schema and then overwrite the existing hardware properties for this laser at the selected wavelength. If successful, the hardware properties are saved with a backup and the power maps for the laser are reloaded, with the "New" power map as the current map, replacing the previous map if it already existed. The original power map file will not be affected.

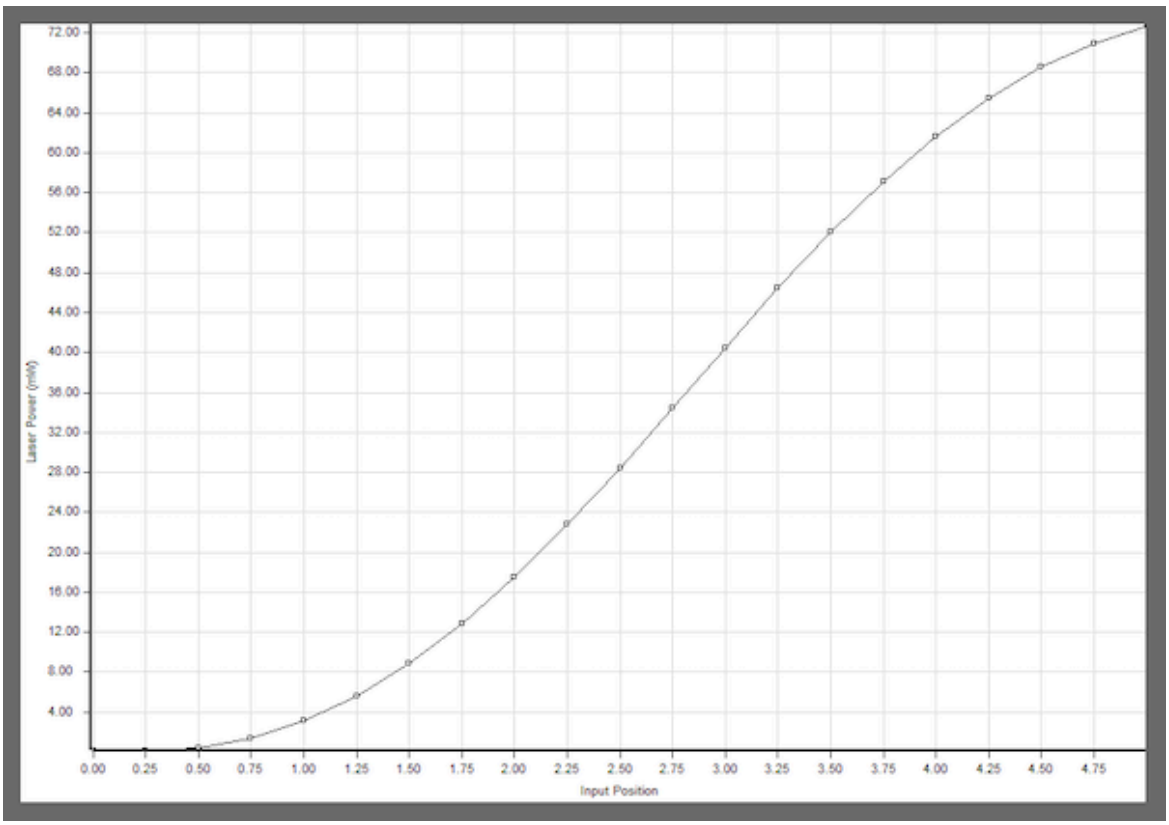
11

The ***Display Fit*** check box enables the displays the fit as a red line on the graph view.

12

The information text box can display warnings, errors, and the current state to the user. This is particularly useful when auto-tuning wavelengths as it will show how far along it is in that process and which wavelength it is working on.

13



The graph view re-scales to the vertical axis. Points are also shown for the individual measurement data. It is redrawn properly when the display fit option is enabled or disabled, or if a different power map is selected from the list.

Photomanipulation

The Laser Options menu gives you direct access to acquisition related laser functions including imaging and photomanipulation techniques, many of which require 3i hardware modules. These include:

Phasor - Opens the Phasor tab of the Focus Window for setting up Phasor streaming Point Scanning

Phasor Settings - Calibrate and test Phasor

Vector - Configure vector photostimulation parameters to use during capture

Laser Settings - Access advanced laser control settings

See also Photomanipulation Module

Phasor Tab of Focus Controls

The ***Phasor Tab*** of Focus controls is designed to create fast streaming captures using a spinning disk confocal or 2 photon system while stimulating using Phasor. Stimulation can only be coupled with imaging in cases where the imaging channel is defined as Alternate Source and the digital line is set high during acquisition. No hardware is moved when imaging through this dialog so it inherently requires an AOTF/LaserStack. For other types of imaging with photostimulation, see Photomanipulation Tab of Capture Preferences. The advantage of using this dialog over standard the capture dialog is that this is designed to use clock time for precision timing instead of the conventional timepoint timing approach. The stimulation events are precisely executed and lend themselves to coordination with electrophysiology setups while imaging proceeds independently at the best rate available.

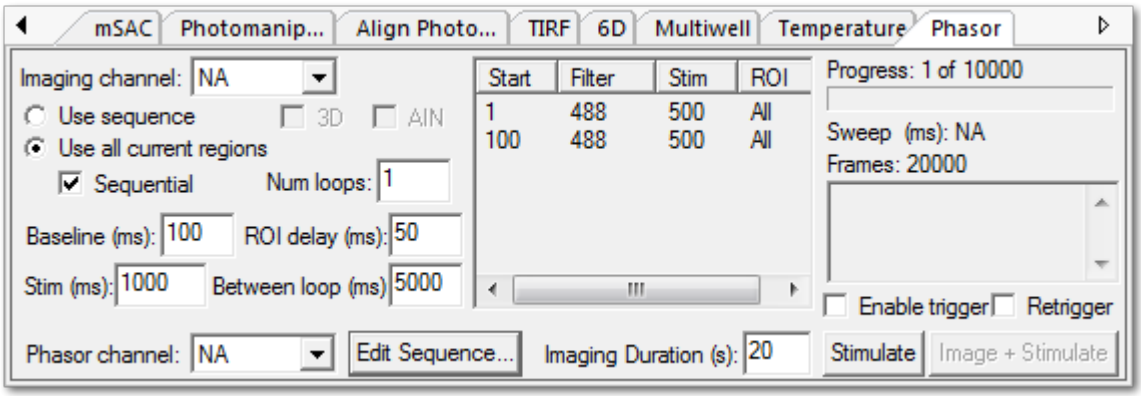
There are two ways to set up a stimulation using this tab:

> BASIC - Uses the options on the left side of the tab to stimulate all regions at once or all regions in sequence with the option to establish a baseline and loop the process.
>
> ADVANCED - Uses the ***Edit Sequence*** dialog to create more complex stimulation routines in which you define the exact region or regions to stilmulate at specific timepoints with the option of looping the routine.

Whether you set up basic or advanced stimulation, there are four different ways to execute the action:

1. Stimulate without a trigger
2. Stimulate with a trigger
3. Image + Stimulate without a trigger
4. Image + Stimulate with a trigger

Each of these executions requires a specific wiring configuration. Please contact support for assistance in configuring the system for your requirements.

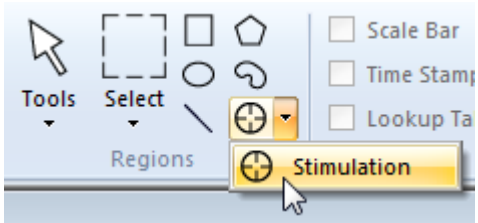


TUTORIAL: Basic Phasor Streaming

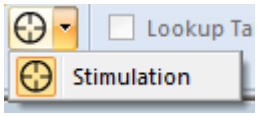
From the Phasor tab of Focus controls, you can set up basic phasor streaming experiments to stimulate all regions either at once or in sequence.

1. Bring the specimen into focus using visualization channels in the Focus Controls.

2. In the Region group of the Home tab, click the drop down button next to the ***Spot*** region tool. Select ***Stimulation***. This will convert the standard Region Tools into Stimulation Region tools.

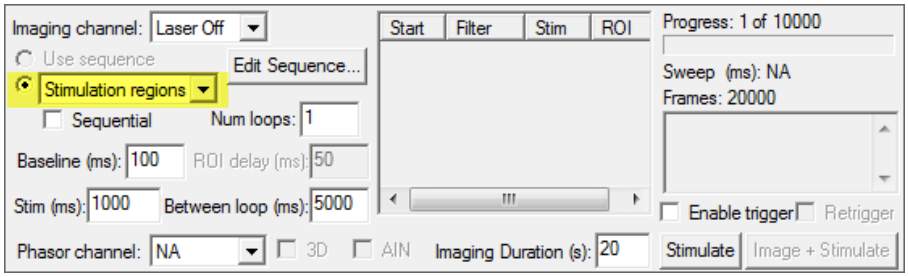


3. Once selected, the spot icon in the drop down will be outlined and highlighted in orange/yellow as displayed below.



4. Select the desired region drawing tool to draw ***Stimulation Regions*** in the Live window. Stimulation regions are numbered with an "S" then number as in S1, S2, S3, etc.

5. Select the ***Phasor*** tab in Focus Controls. Select ***Stimulation Regions*** from the region type dropdown menu highlighted below.



6. The default behavior is to use Phasor to stimulate all the regions at the same time. Alternatively, you have the option to stimulate the regions in sequence with a small delay between stimulations. To stimulate the regions in sequence, check the box for ***Sequential*** and enter a value in the ***ROI delay (ms)*** field. ROI delay is the amount of time between sequential region stimulations. The minimum value is 50ms. If you want to create a more complex stimulation routine, review TUTORIAL: Advanced Phasor Streaming.

7. Enter a value for ***Baseline (ms)***. This is the amount of time to wait before the initial stimulation.

8. Enter a value for ***Stim (ms)***. This is the amount of time the region(s) will be stimulated.

9. If you would like to repeat the stimulation sequence as a loop, enter a value in ***Between loop (ms)*** to define how long between the start of the first sequence and the start of the repeat sequence. Next, enter a value for ***Num loops*** to define how many times the sequence will be repeated. The amount of time between loop should be at least ***Baseline + Stimulation time*** when stimulating all regions at once or ***Baseline + #regions(Stimulation time + ROI delay)*** when stimulating regions sequentially.

10. Select the ***Phasor channel*** from the drop down to identify the Phasor channel that will be used for stimulation.

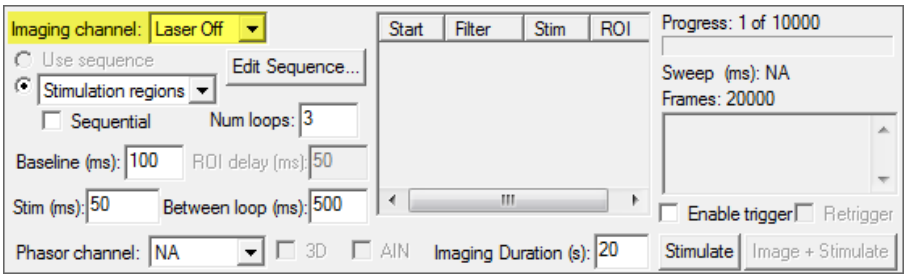
11. ***Enable Trigger*** is used if you would like to begin the process when an external trigger is received. One would check Enable Trigger then click ***Stimulate***. The stimulation routine would not begin until an external TTL pulse is

received. This can help to synchronize other external stimulation or recording devices. Checking ***Retrigger*** will reset equipment so that it is ready for another round of stimulation after the routine completes.

12. Decide if you will ***Stimulate*** only or image while stimulating. If you will only be stimulating, you can click ***Stimulate*** at this time to start the process.

13. If you will ***Image + Stimulate***, you will first need to define ***Imaging Channel***. Imaging Channel should be selected based on imaging system type as follows:

- Spinning Disk Confocal systems - select the confocal channel from the Imaging Channel drop down. The channel name will be the same as the button you would select to display the channel in the Focus Controls.
- Multiphoton system - leave the value at ***Laser Off*** and the system will use the current PMT imaging mode as selected in the Scanning dialog.



11. Set ***Imaging Duration***. This value is independent of the stimulation setup. That is, if your stimulation routine takes 15 seconds and the imaging duration is set to 10 seconds, the stimulation routine will run to completion but imaging will stop 10 seconds in. Therefore, it is important to be aware of the approximate amount of time the stimulation routine will run to ensure the imaging duration is long enough. See #9 above and multiply by the number of loops.

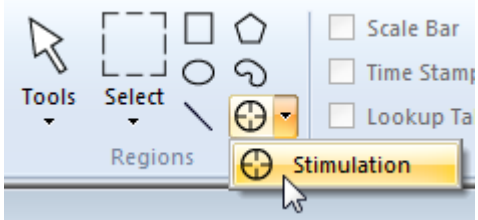
12. Click ***Image + Stimulate*** to begin the process.

TUTORIAL: Advanced Phasor Streaming

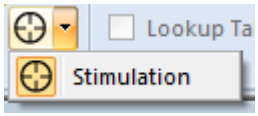
From the Phasor tab of Focus controls, you can set up advanced Phasor streaming experiments to to create more complex stimulation routines in which you define the exact region or regions to stimulate at specific time points with the option of looping the routine.

1. Bring the specimen into focus using visualization channels in the Focus Controls.

2. In the Region group of the Home tab, click the drop down button next to the ***Spot*** region tool. Select ***Stimulation***. This will convert the standard Region Tools into Stimulation Region tools.

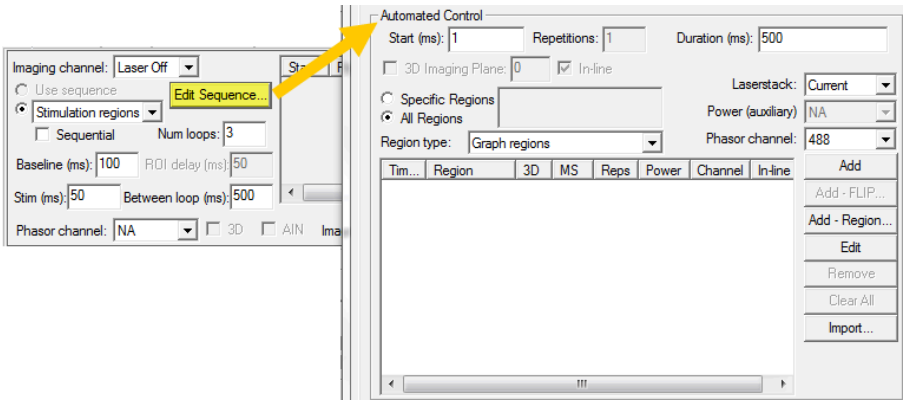


3. Once selected, the spot icon in the drop down will be outlined and highlighted in orange/yellow as displayed below.



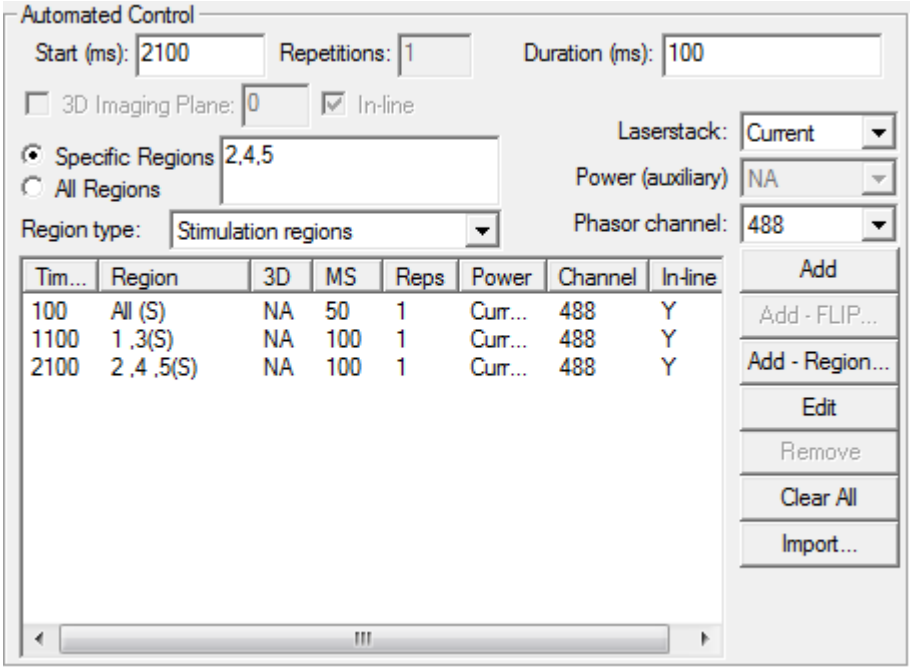
4. Select the desired region drawing tool to draw ***Stimulation Regions*** in the Live window. Stimulation regions are numbered with an "S" then number as in S1, S2, S3, etc.

5. Select the ***Phasor*** tab in Focus Controls. Click ***Edit Sequence*** to bring up the Photomanipulation Settings dialog. You will be using the lower half of this dialog as shown below.



6. Begin creating events by entering the ***Start time*** (in ms) when the first event will occur. This value is elapsed time that will have passed in milliseconds before the event will occur. The first start time is essentially the baseline for the experiment. Subsequent start times will be total elapsed time in the experiment before the event occurs.

7. Select ***All Regions*** to stimulate all existing regions for this event. Alternatively, select ***Specific Regions*** to define which region(s) should be stimulated (e.g. 1,2,6-8). Be sure to select ***Stimulation regions*** from the Region type drop down menu.



8. Set the ***Duration (ms)*** of the stimulation event.

NOTE: It is possible to create complex stimulation sequences by entering a string of values in the duration field. This will apply to whichever region(s) are identified in step #7. The formatting string is **repeatx[duration@power,duration@power...]**. Some examples follow:

- Example 1: **40@100,60@0,40@200,60@0**
  40 ms @ 100 power, 60 ms @ off, 40 ms @ 200 power, 60 ms @ off (total duration = 200ms)

- Example 2: **40@100,60@50,40@200,60@50**
  40 ms @ 100 power, 60 ms @ 50 power, 40 ms @ 200 power, 60 ms @ 50 power (total duration = 200ms)

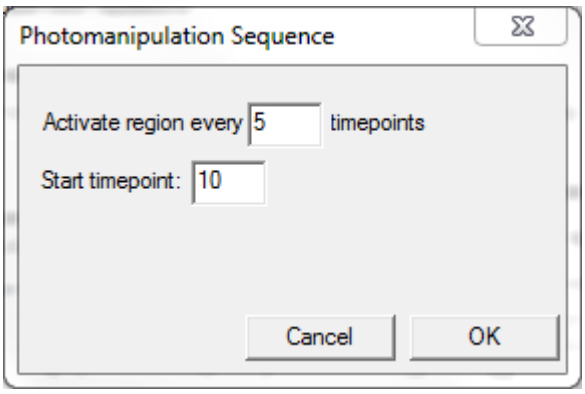
- Example 3: **50x[10@75,5@0]**
  10 ms @ 75 power, 5 ms @off repeated 50 times (total duration = 150 ms)

- Example 4: **25x[40@100,60@0]**
  40 ms @ 100 power, 60 ms @ off repeated 25 times (total duration = 2500 ms)

9. Select the ***Phasor channel*** to use for stimulation from the drop down menu. Click ***Add*** to save the event.

NOTE: You can also add automated sequential region stimulation to the event list by clicking ***Add-Region...*** to open the Photomanipulation Sequence dialog. The dialog will activate each of the existing regions in sequence beginning at a designated time and repeating with the next region at a designated interval until all regions are stimulated. The settings for Duration and Phasor channel as described earlier will be applied to these events.

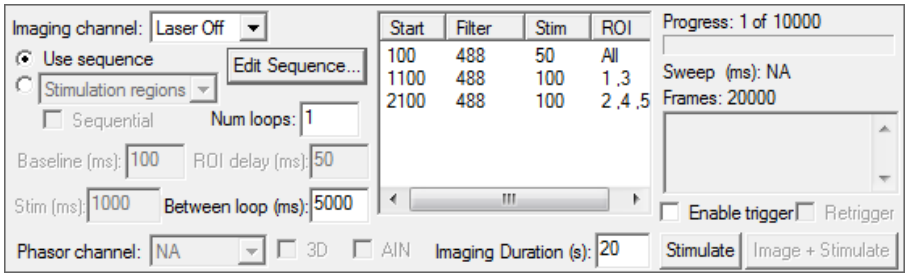
Important: Currently the dialog reads "timepoint" but should read time (ms). Fill out the fields as "Activate region every x milliseconds starting at y milliseconds elapsed time.



10. Create additional events as desired by repeating steps 6-9. If you need to edit an event, select it from the list and click ***Edit***. Alter as desired then click ***Add*** to add it back to the list. To delete an event, select it from the list and click ***Remove***.

11. When all events are entered, click ***OK***. This will close the ***Edit Sequence*** dialog and take you back to the Phasor tab of Focus controls.

12. Notice that the sequence field now contains the event list and the ***Use Sequence*** radio button is selected. In the screenshot below, all regions will be stimulated for 50ms after waiting 100ms, 1 second later regions 1 and 3 will be stimulated for 100ms, 2 seconds after the baseline regions 2, 4, and 5 will be stimulated for 100ms.

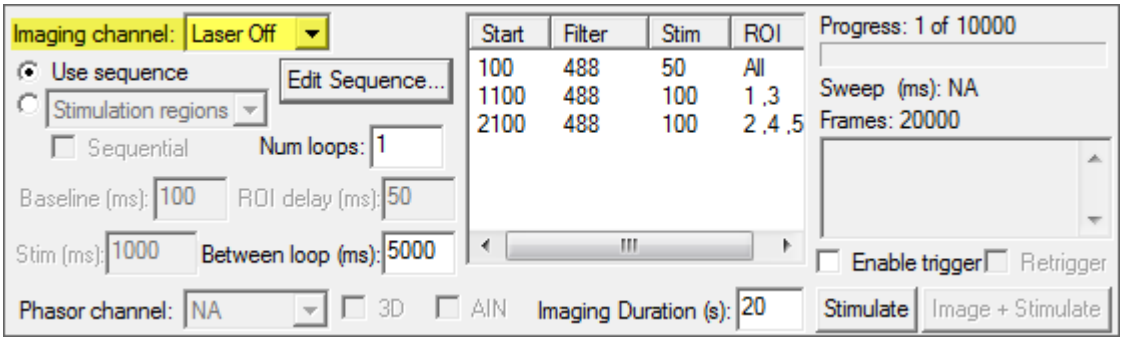


13. ***Enable Trigger*** is used if you would like to begin the process when an external trigger is received. One would check Enable Trigger then click ***Stimulate***. The stimulation routine would not begin until an external TTL pulse is received. This can help to synchronize other external stimulation or recording devices. Checking ***Retrigger*** will reset equipment so that it is ready for another round of stimulation after the routine completes.

14. Decide if you will ***Stimulate*** only or image while stimulating. If you will only be stimulating, you can click ***Stimulate*** at this time to start the process.

15. If you will ***Image + Stimulate***, you will first need to define ***Imaging Channel***. Imaging Channel should be selected based on imaging system type as follows:

- Spinning Disk Confocal systems - select the confocal channel from the Imaging Channel drop down. The channel name will be the same as the button you would select to display the channel in the Focus Controls.
- Multiphoton system - leave the value at ***Laser Off*** and the system will use the current PMT imaging mode as selected in the Scanning dialog.



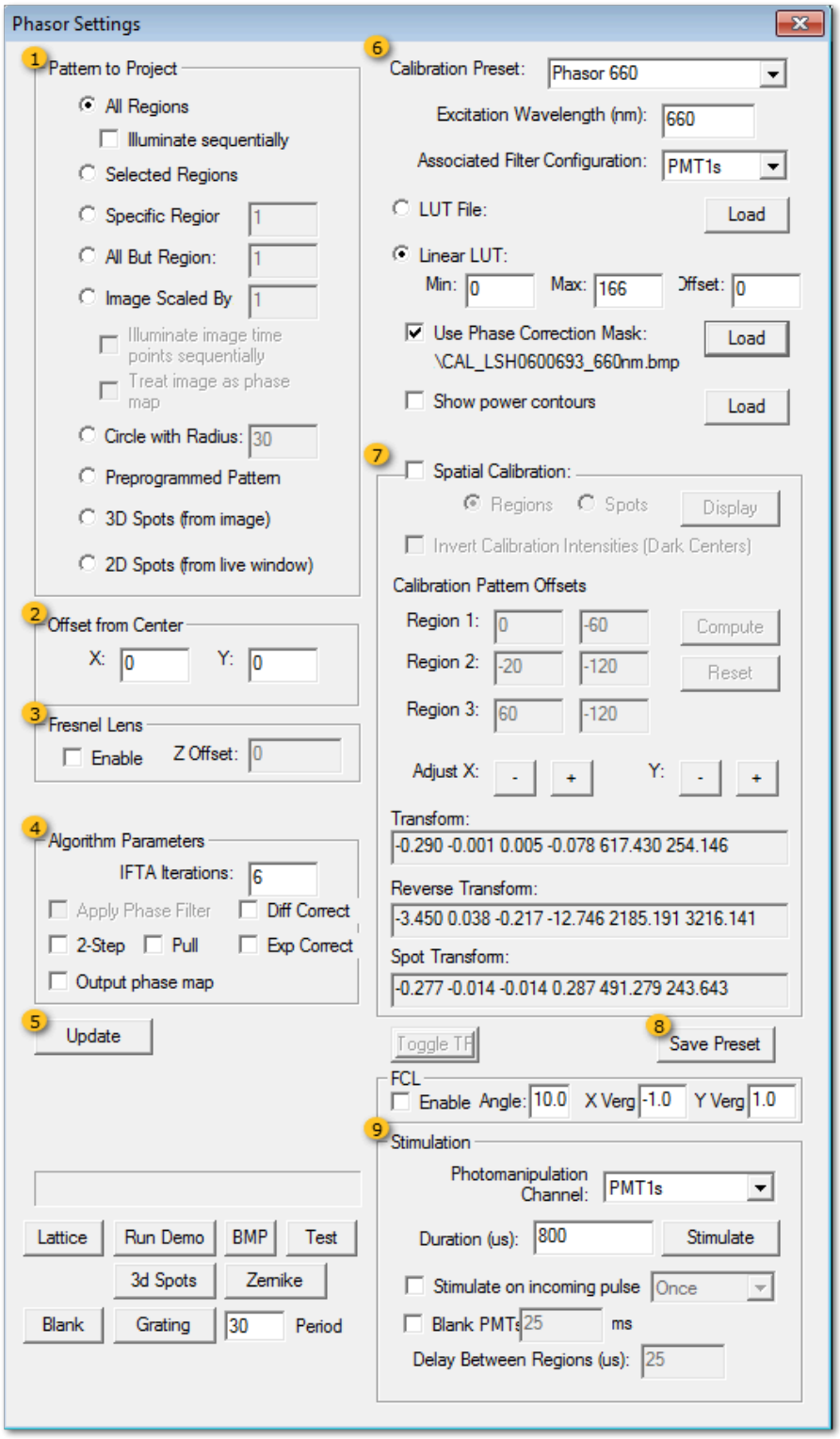
16. Set ***Imaging Duration***. This value is independent of the stimulation setup. That is, if your stimulation routine takes 15 seconds and the imaging duration is set to 10 seconds, the stimulation routine will run to completion but imaging will stop 10 seconds in. Therefore, it is important to be aware of the approximate amount of time the stimulation routine will run to ensure the imaging duration is long enough.

17. Click ***Image + Stimulate*** to begin the process.

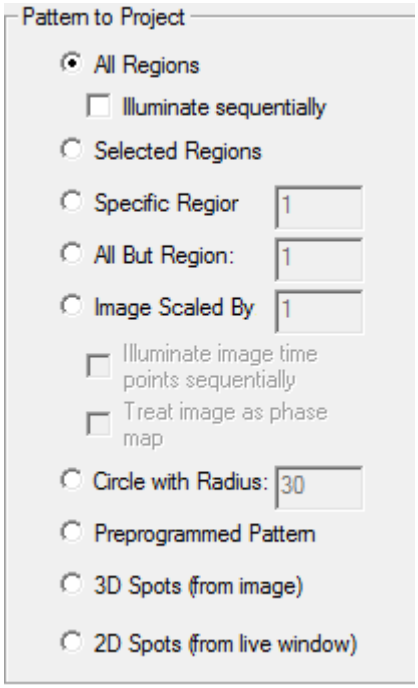
Phasor Settings window

The Phasor Settings window is a general utility window that allows for testing, calibration, and advanced Phasor manipulation.

Access from the Home tab, Acquire group, ***Photomanipulation*** menu > ***Phasor Settings***.



1 Pattern to Project group



Select the pattern Phasor will generate as follows:

All Regions - All regions drawn in the Live camera window will be illuminated simultaneously. Check the box to ***Illuminate sequentially*** to illuminate each of the regions in number order.

Selected Regions - Illuminate only selected regions (depicted by a white region number). To select multiple regions, hold the **Ctrl key** and click on regions.

Specific Region - Type a region number in the box to select the region to illuminate.

All But Region - Type a region number in the box to exclude a single region but illuminate all other regions simultaneously.

Image Scaled By - This is an unused development feature

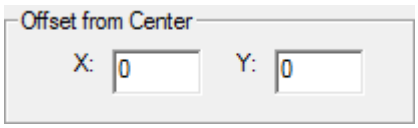
Circle with Radius - Type a value (in pixels) to illuminate a centered circle with given radius. To offset the circle from center, use the ***Offset from Center*** edit fields described below.

Preprogrammed Pattern - Under development - uses a mask in a z-stack to create 3D holograms

3D Spots (from image) - Acquire a 3D image and draw spot regions in desired locations. Open this image and select this option to illuminate spots in 3D.

2D Spots from live window) - Use the ***Spot*** region tool to draw spots to illuminate in the live window.

2 Offset from Center group

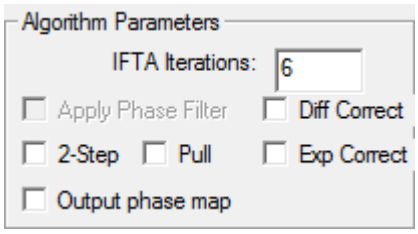


When ***Circle with Radius*** is selected as the pattern to illuminate, use these fields to enter pixel values in X and Y to offset the circle from the center of the field.

3 Fresnel Lens group

Check the box to generate a virtual fresnel lens to apply to the SLM, thus displacing the hologram from the focal plane of the objective. Enter a value (in microns) for the desired ***Z Offset***.

4 Algorithm Parameters



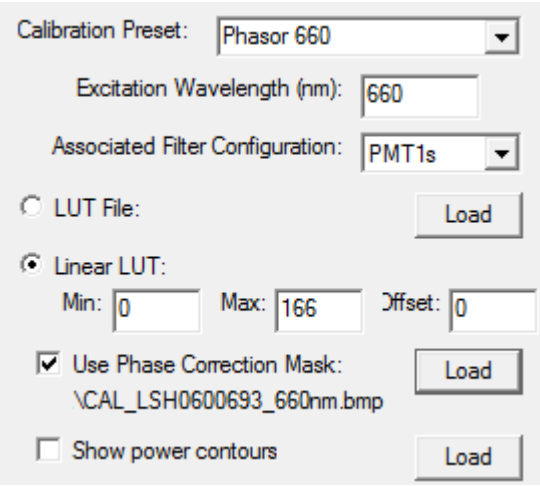
IFTA iterations is the number of iterations the software is performing before converging to a good resolution hologram. 6 iterations should be enough.

Check the box for ***Output phase map*** to create an image in SlideBook that is the phase profile.

5 Update button

Pressing ***Update*** will illuminate the requested pattern and update the live image.

6 Calibration Preset selection



A ***Calibration Preset*** is tied to objective, wavelength, and imaging device. Select a preset from the dropdown menu. You can edit the preset name by typing in the field.

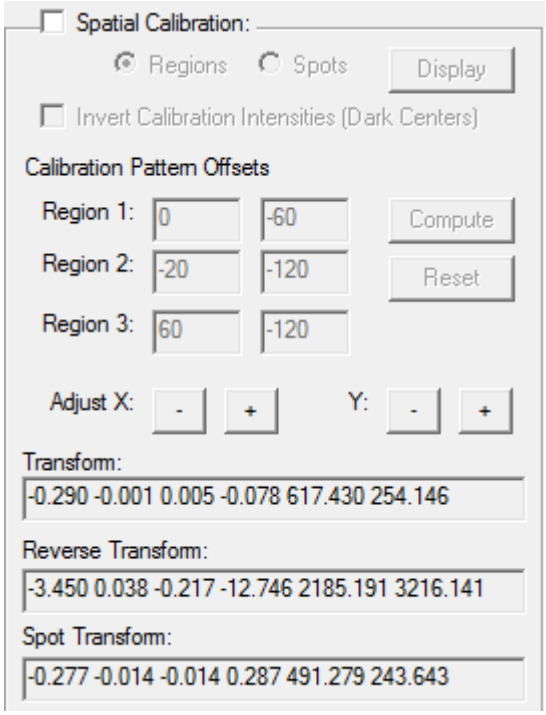
Verify or edit the ***Emission Wavelength (nm)*** and ***Associated Filter Configuration*** as needed.

> ***NOTE:*** Associated Filter Configurations available are any channels that have been configured as ***Photomanipulation Channel Type*** in Filter Configuration Parameters.

Presets are pre-configured at installation and contain all important information regarding LUTs. ***Linear LUT*** should be selected and the values populated automatically. Use ***Phase Correction Mask*** should be checked and a loaded file should be indicated.

If you need assistance changing or creating a new preset, please contact support@intelligent-imaging.com for assistance.

7 Spatial Calibration group



To project the three circles, check ***Spatial Calibration*** and choose the ***Regions*** radio button. Click ***Display*** and the three circles should appear in the Live Camera window once you turn on the appropriate laser illumination.

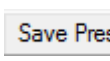
Using the ***Ellipse*** region tool, create regions of interest in the Live Camera window. The orientation of the circles may vary depending on the way the SLM projects to the camera, but by default it should be a "bent L" where circle 1 is at the end of a straight leg (with respect to the camera), circle 2 is at the inflection, and circle 3 is at the end of the bent leg.

Click on ***Compute*** to compute the transformation matrix (and reverse transformation) between the SLM coordinate system and the camera coordinate system.

Should Calibration Pattern Offset default values need to be changed, do so from the Administration tab, H***ardware Properties > Phasor Settings >Calibration Pattern Coordinates***.

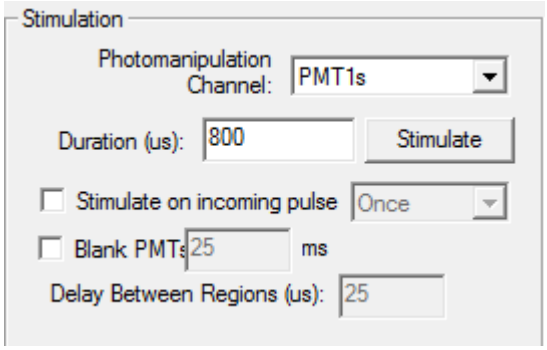
Refer to TUTORIAL: Phasor Direct Calibration for more information.

8 Save Preset button



Save ***Spatial Calibration*** to a preset selected in #6 above.

9 Stimulation group



Primarily, Phasor is run from the Phasor tab of Focus Controls or through Photomanipulation Configuration as part of an experiment design. SHoudl you need to run a quick test without imaging, this group provides tools to set up the test stimulation as follows:

Photomanipulation Channel - Choose the configuration to use for Phasor illumination. Available channels listed will be any channels configured as Photomanipulation Channel Type in Filter Configuration Parameters.

Duration (us) - The length of time the selected regions will be stimulated. Illuminated area was selected in the Pattern to Project group in the top left of this dialog.

Stimulate on incoming pulse - Check this box if you would like to trigger the stimulation from an external TTL pulse. Frequency options include: Once Multiple, or Free Run.

Blank PMTs - Check this box to designate a time to blank the PMTs. Blanking is very useful when 2P imaging is used to image the effects of photostimulation. When photostimulation is occurring and the 1P or 2P laser is on, the PMTs should be blanked to avoid saturating them.

Delay Between Regions (us) - This value is used when you check the box to Illuminate Sequentially in the Pattern to Project group.

TUTORIAL: Phasor Calibration

Overview

For the most accurate photomanipulation possible, it is important to calibrate Phasor regularly. There are several variations for spatial calibration. The best way to calibrate Phasor is dependant on the hardware associated with the system and the application you will be using.

Variations for calibration:

A) Is the imaging technique you will use for the photomanipulation experiment using a camera or Multiphoton imaging. If you will use a camera for imaging the samples, you will use direct calibrations (illuminating locations). If you will be using Multiphoton to image the sample, you will use indirect calibration (bleaching locations) as outlined in Step 5 and Step 6 of the following instructions.

B) Will the photomanipulation target have some lateral extent or be constrained to finite spots? This will determine the type of calibration region you will use in Step 4 of the following instructions.

C) Will you be able to manipulate the calibration sample (either exciting or bleaching) using the desired wavelength? If not, you will need to follow Step 9 in the following instructions for interpolating a calibration from a different wavelength.

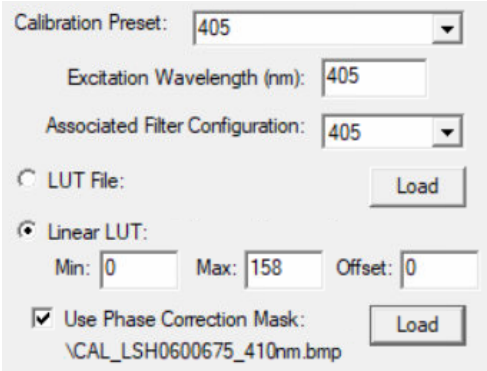
NOTE: It should be noted that spatial calibrations should be done for each wavelength/channel for each objective. While an associated channel is saved with the preset, objective information is not saved. Therefore, if you use more than one objective, you should include the objective information in the Calibration Preset name.

Instructions for Calibration

1. In the Home tab, Acquire group, open ***Photomanipulation*** menu > ***Phasor Settings***.

2. Spatial calibration of Phasor maps the SLM coordinates to camera or scanning coordinates based on system configuration. A ***Calibration Preset*** is tied to objective, wavelength, and imaging device. Select the ***Calibration Preset*** from the dropdown menu in the top right corner of the Phasor Settings dialog. Verify the ***Excitation Wavelength (nm)*** and ***Associated Filter Configuration***.

> ***NOTE:*** When your system is installed, presets will be generated for all options. You may use these or generate your own. If you select, for example, Preset 7, from the dropdown list, you can then highlight the name and type to rename the preset. Be sure to assign the excitation wavelength and associated filter configuration. Associated Filter Configurations available are any channels that have been configured as ***Photomanipulation Channel Type*** in Filter Configuration Parameters. Each preset will have values assigned for Linear LUT and a file assigned for the Phase Correction Mask. If you are working with a preset that does not have these parameters, please contact 3i Support for assistance.



3. You will find Spatial Calibration midway down the dialog on the right side. Check the box for ***Spatial Calibration***.

4. Identify the types of locations you will use for spatial calibrations:

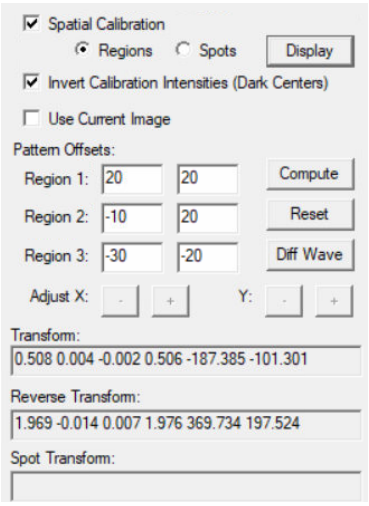
Variations:

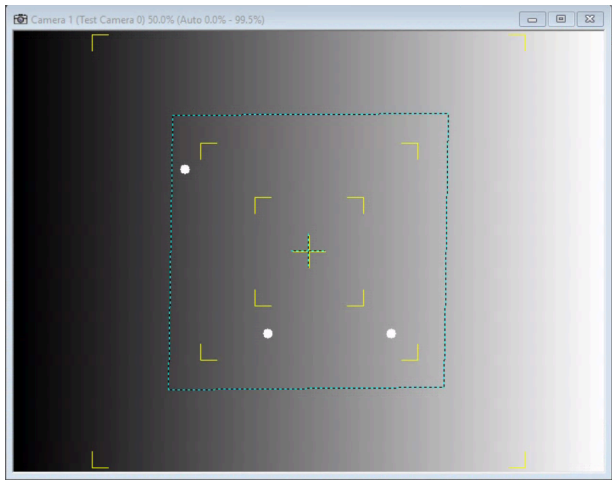
- Choose ***Regions*** if the ultimate photomanipulation target has some lateral extent.

- Choose ***Spots*** if you will be photomanipulating the smallest, most finite spot available.

5. Is the imaging technique you will use for the photomanipulation experiment using a camera or Multiphoton imaging. If you will use a camera for imaging the samples, you will use direct calibrations (illuminating locations). If you will be using Multiphoton to image the sample, you will use indirect calibration (bleaching locations). If you will be bleaching the locations instead of illuminating them, check the box for ***Invert Calibration Intensities (Dark Centers)***.

6. To project the three calibration locations, click ***Display***. Turn on the photomanipulation laser (in the Focus Window), three illuminated locations should appear in the Live Camera. In the case of Multiphoton imaging, keep the photomanipulation laser on for a short amount of time (~1 second) then switch back to the imaging channel/PMT. Three bleached location should appear in the Live Camera Window.

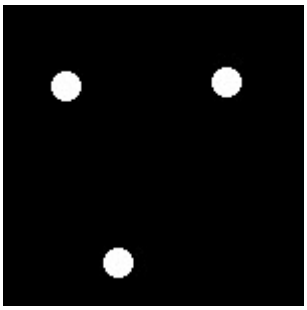




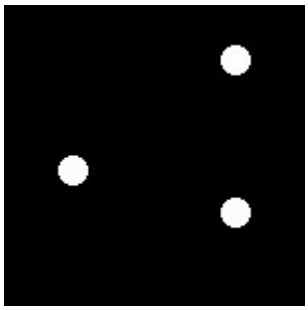
7. SLM coordinates, not image coordinates - The next step is to identify and draw regions over the illuminated or bleached circles. The orientation of the circles may vary depending on the way the SLM projects to the camera, but by default it should be a "bent L" where circle 1 is at the end of a straight leg (with respect to the camera), circle 2 is at the inflection, and circle 3 is at the end of the bent leg. This makes it possible to identify the ordering of the circles regardless of any rotation or reflection between the SLM's coordinates and the camera's coordinates. Misidentification of the region order will result in an incorrect calibration. To further aid in the identification of regions, please review the steps below. Two examples are given to aid in these instructions.

1. After clicking display, note the appearance of 3 spots.

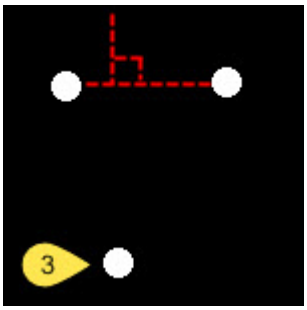
Example 1

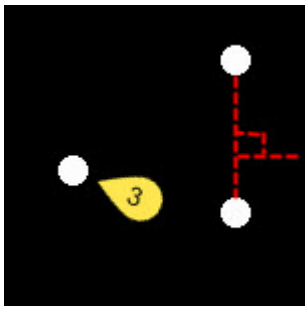


Example 2

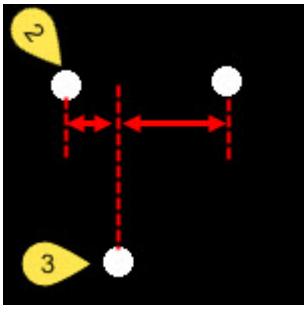


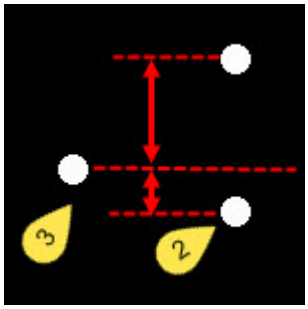
2. Two of the spots (regions 1 and 2) will lie in a line that is either horizontal or vertical. Identify these spots, you still won't know which is which at this point. The other spot will be Region #3.



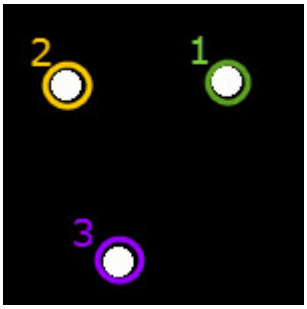


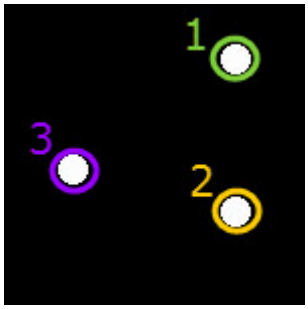
3. Next, determine which spot is closer to spot 3, this will be Region #2.





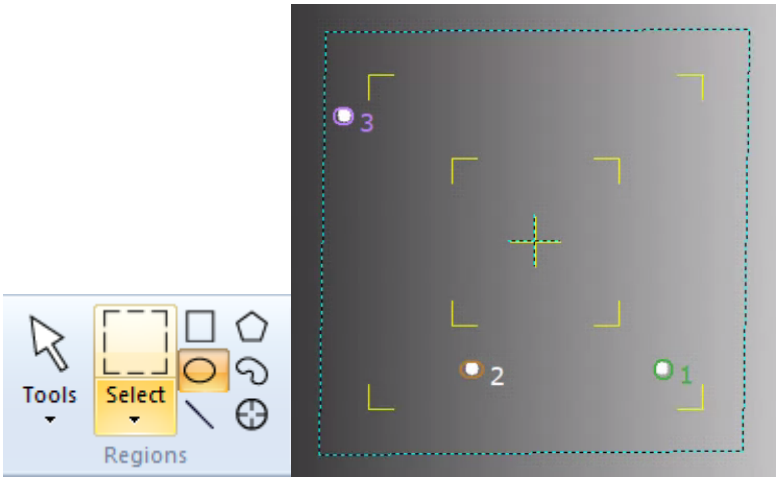
4. The remaining spot will be Region #1. Draw your ellipses slightly larger than the spots (see the following step on drawing regions).





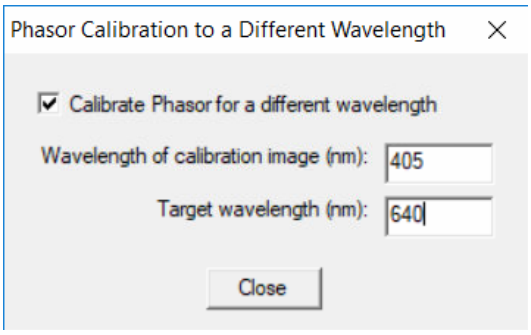
8. Using the ***Ellipse*** region tool, create regions of interest in the Live Camera window so that the numbers of the region correspond to the circle numbers described above. The calibration algorithm uses weighted center of intensity value inside the region so be sure to draw regions at least as large as the spots displayed or bleached.

NOTE: If you selected ***Spots*** in step 4, you will use the ***Point*** region tool to mark the locations.

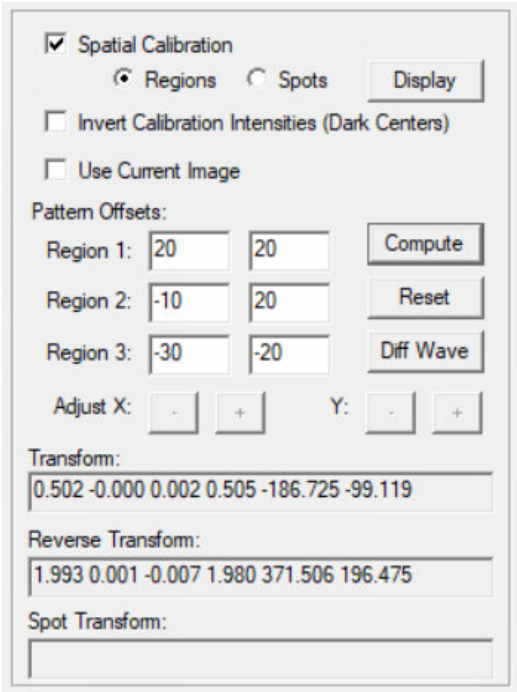


9. For some applications, the wavelength of light you will use for Phasor photomanipulations (i.e. 1P red wavelengths over 600nm) will not easily bleach spots, an essential part of the calibration routine. If this is the case, you can perform calibration

using a different wavelength then extrapolate the spatial calibration to the wavelength you will be using. Click ***Diff Wave*** in the Spatial Calibration group. Check the box to ***Calibrate Phasor for a different wavelength***. Enter the wavelength for the channel you will use for spatial calibration in the ***Wavelength of calibration image field***; this will match the excitation wavelength entered in Step 2. Enter the wavelength you will be using for Phasor photomanipulation (the wavelength you want to interpolate to) in the ***Target wavelength*** field. Click ***Close***.

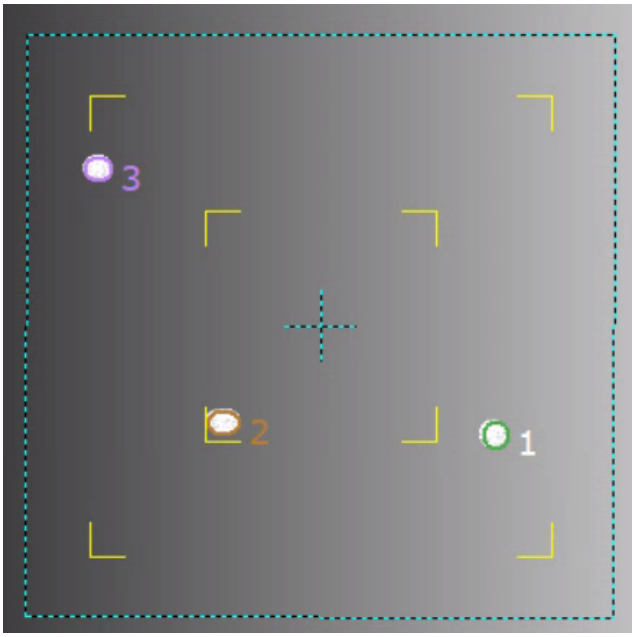


10. Click on ***Compute***. This will compute the transformation matrix (and reverse transformation) between the SLM coordinate system and the camera coordinate system.



11. Confirm the calibration by deselecting the check box for ***Spatial Calibration***. Click and drag each region to move it slightly off the calibration location. selecting the ***All Regions*** radio button (in the upper left of the dialog in Patterns to Project group). Click ***Update*** (found in the lower left quadrant of the dialog). The new pattern should exactly match the new locations of the regions that you created.

> ***NOTE:*** When you have Spatial Calibration checked, Update will be grayed out. To exit the spatial calibration mode and use any of the normal pattern options, uncheck Spatial Calibration.



12. Evaluate the regions you tested. If the illuminated regions are off slightly, repeat steps 8-10.

LightSheet

The LightSheet menu offers acquisition and processing options for lightsheet-based microscopy systems including Lattice LightSheet and Marianas LightSheet. Additional information on using these systems can be found in their system-specific manual sections: Lattice LightSheet Module and Marianas LightSheet Module.

For an introduction on acquisition methodology, please watch this brief movie Understanding Lattice LightSheet Image Acquisition and Deskewing.

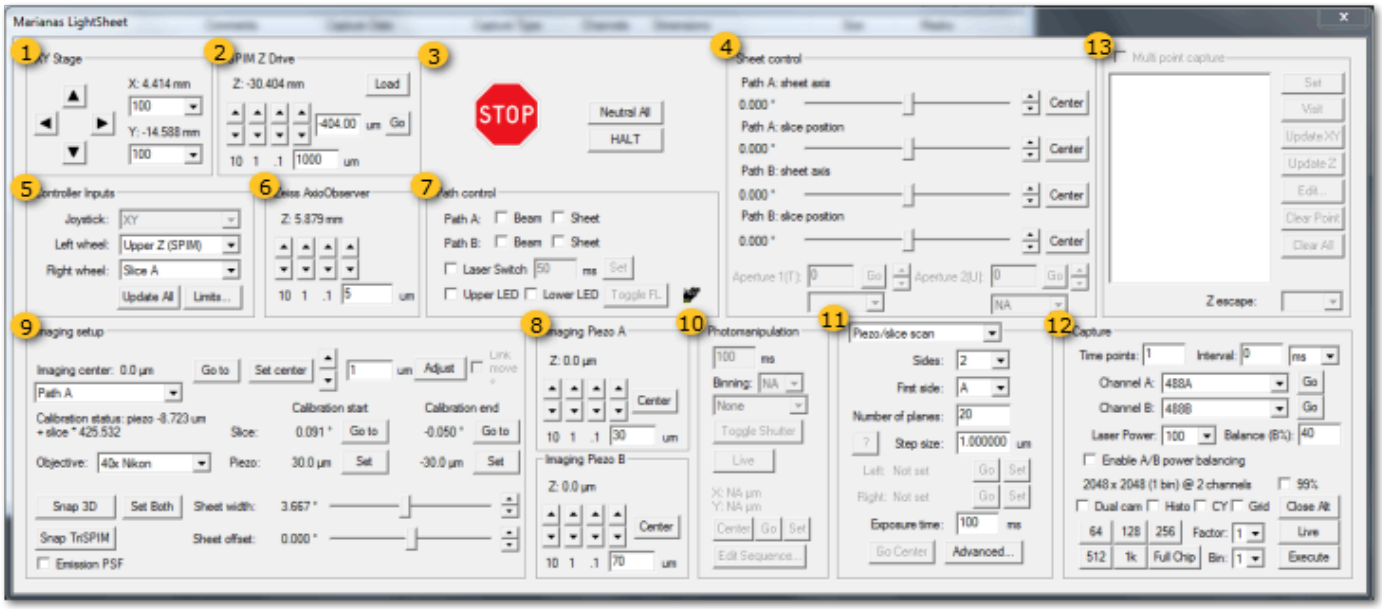
Marianas LightSheet Window

The ***Marianas LightSheet Window*** is used to align your MLS system prior to experiments, set up all SPIM capture parameters and initiate SPIM image capture. You may use these controls to change settings of the microscope, stage and SPIM devices in order to find and position your specimen, mark the *z* range for capture, calibrate scanner to piezo movement, set up FRAP parameters and initiate capture.

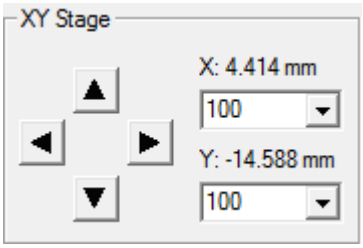
To open the ***Marianas LightSheet*** controls within SlideBook, go to the Home tab, Acquire group > ***LightSheet*** > ***Marianas LightSheet.***

See also:

[Marianas LightSheet Hardware](#)
[Marianas LightSheet Alignment and Calibration](#)
[Marianas LightSheet Operation](#)

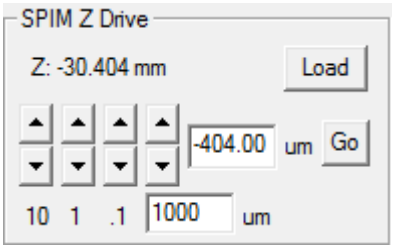


1 XY Stage group



- ***Arrows*** Move the XY stage stepwise in X or Y
- Increment of steps (µm) can be selected by typing values or select in the drop down
- When running in live mode the current ***X*** and ***Y*** positions are updated

2 SPIM Z Drive group

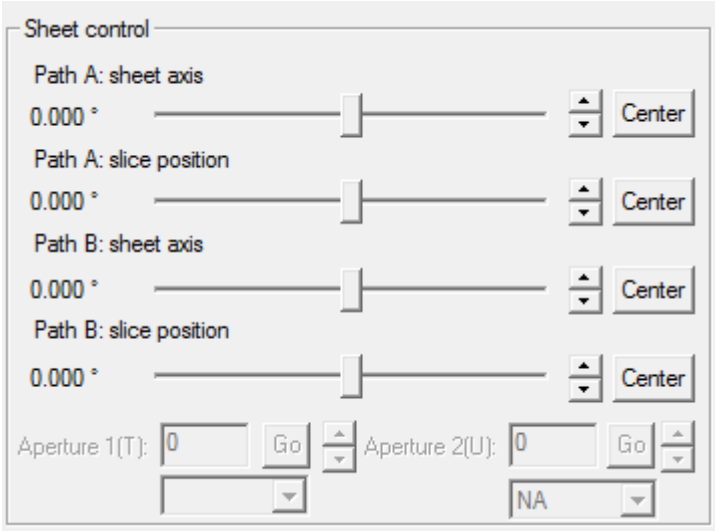


• ***Arrows*** Lower (arrow down) and withdraw (arrow up) the SPIM drive towards or from the sample for 10 µm, 1 µm, 0.1 µm or a given value (e.g. 1000-50000 µm) respectively
• ***Load*** brings the SPIM Z drive to its most upper position to enable sample loading. This button will change to Return when a sample height was engaged before
• ***Return*** will bring the SPIM Z drive to its previously visited lower position at the coverslip
• ***Go*** brings the SPIM Z drive to the value specified in the field left to the button - ***NOTE:*** the value is in µm

3 Stop and Zero group

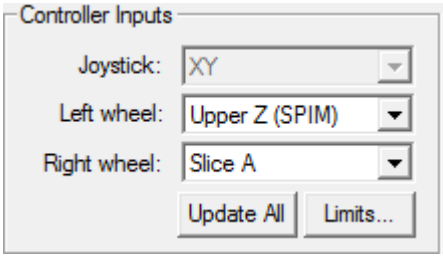
• ***Neutral All*** brings the scanner position in all directions as well as the piezos to their center position (initial alignment point for most range).
• ***STOP*** and ***HALT*** instantly stops all SPIM Z drive movement, stage movement, scanner and piezo movement.

4 Sheet control group



• Adjust the ***Sheet axis*** or ***Slice position*** by sliders and arrows. ***NOTE:*** Changing the slice position by using the joy stick wheel is recommended for easier use.
• ***Center*** will bring each sheet axis or slice position to centre individually.
• When scanners with motorized irises are installed one can control the ***Aperture*** for each scanner (***T*** for scanner A, ***U*** for scanner B) either by typing a specific value and click ***Go*** or selecting a percentage (how far open) in the drop down menu.

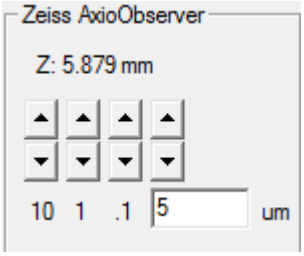
5 Controller Inputs group



• ***Joystick*** controls the XY movement of the stage (no alternative selection available)
• Select which device (scanners, piezos, SPIM Z Drive) is controlled by the ***Left wheel*** of the Joystick controller box
• Select which device (scanners, piezos, SPIM Z Drive) is controlled by the ***Right wheel*** of the Joystick controller box
• ***Update All*** will update the selection (not required when in live mode)

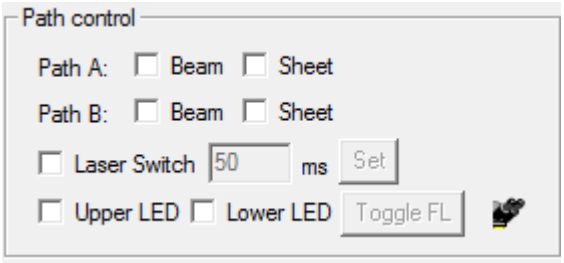
• ***Limits...*** will open an additional interface allowing to change further settings, such as Stage limits, Grid spacing, Window size, Scan Frequency & other Scan settings as well as it contains a command line to communicate to the ASI Tiger Controller

6 Lower Z Stage group



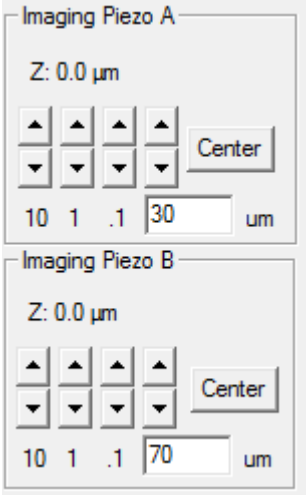
• ***Z*** indicates the current position of the lower / sub-stage objective, microscope nose piece
• ***Arrows*** Lower (arrow down) and raise (arrow up) the lower objective for 10 µm, 1 µm, 0.1 µm or a given value (e.g. 5 µm) respectively

7 Path control group



• ***Path A:*** tick-boxes to enable Beam or Sheet (dithered beam) for scanner of side A
• ***Path B:*** tick-boxes to enable Beam or Sheet (dithered beam) for scanner of side B
• The ***Laser Switch*** switches the lasers faster than the cameras, enter a value in ***ms*** and click ***Set*** to change the laser switch frequence
• ***Upper LED*** to toggle the LED on the diSPIM frame
• ***Lower LED*** to toggle the LED below the stage (RAMM frame systems only)
• ***Toggle FL*** to switch on the Epi-Fuorescence
• switches the microscope light path to the Oculars

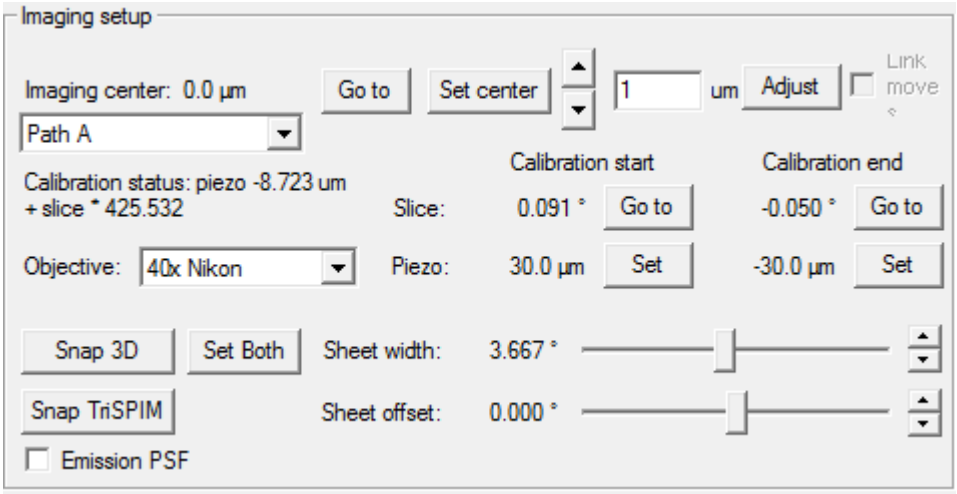
8 Imaging Piezo A/B group



• ***Z*** indicates the current piezo position

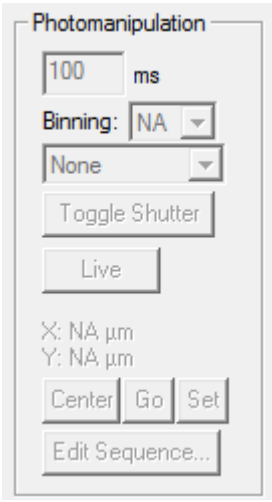
• ***Arrows*** Retract (arrow down) and expand (arrow up) either piezo for 10 µm, 1 µm, 0.1 µm or a given value (e.g. 70 µm) respectively
• ***Center*** brings the piezo to zero position (0 µm) - ***NOTE:*** holding the piezo in position is active, piezos will go to sleep/ relax after 5min without activity

9 Imaging setup group



• Select (***Set center***) a piezo position for and visit (***Go to***) the set ***Imaging center*** (default: zero position of piezo A and B)
• Indicates selected ***Path***
• ***Calibration Status***: indicates whether a calibration is saved for the path and shows the calibration curve
• Indicates selected ***Objective*** - ***NOTE:*** Requires manual change after changing objectives
• Indicates the ***Slice*** position for the respective ***Piezo*** position at ***Calibration start*** and ***Calibration end***
• ***Arrows*** (top of the group) move both piezo and slice position stepwise according to the calibration curve, the piezo step distance (um) can be altered by typing a value
• ***Adjust*** (calibration check) positions the slice Position of the scanner of the selected path (drop down) to the selected piezo position of this path according to the calibration curve (e.g. after clicking ***Neutral All***, ***Adjust*** brings the slice position from zero to it's calibrated position for piezo center position)
• ***Sheet width*** adjusts the width of the sheet over the field of view (slider or arrows) - ***NOTE:*** holding Ctrl while clicking the arrows will move it with 10x increment
• ***Sheet offset*** adjusts how centered the sheet is scanned over the field of view (slider or arrows) - ***NOTE:*** holding Ctrl while clicking the arrows will move it with 10x increment

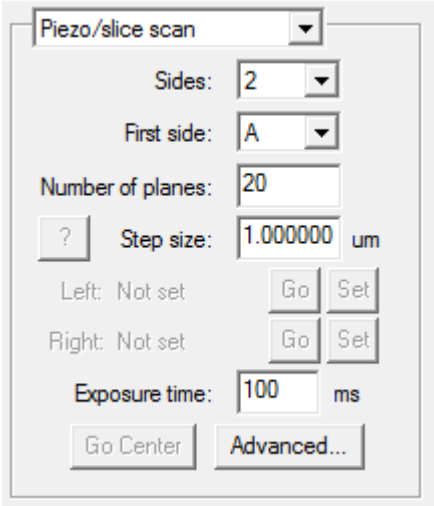
10 Photomanipulation group



• Select parameters (***Exposure time*** in ms, ***Binning***, ***Channel***) for imaging with the finder Camera
• ***Toggle Shutter*** opens or closes the selected Epi-port excitation

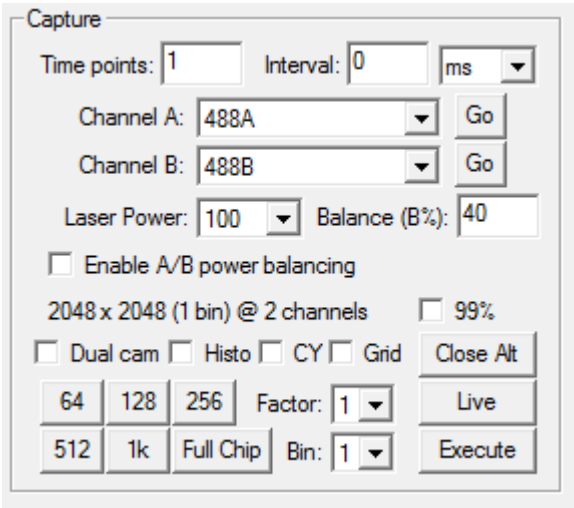
- ***Live/ Stop*** enables and disables the Live mode of the camera (separate view window)
- ***X*** & ***Y*** indicate the set stage coordinates for the photomanipulation
- ***Center*** will move the stage to the center between the start and end coordinates of a stage scan
- ***Go*** moves the stage to the set photomanipulation coordinates
- ***Set*** will set the photomanipulation coordinates to the current stage possition
- ***Edit Sequence...*** select and set photomanipulation channel and parameters before capture

11 Scan Configuration group



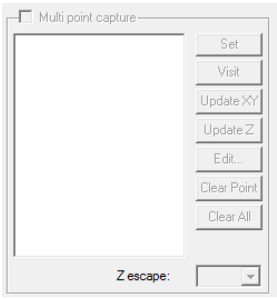
- Select the SPIM capture mode - ***Piezo/ slice scan*** or ***Stage scan*** - in the drop down
- ***Sides*** select how many views are going to be captured ('1' for a single view SPIM capture, '2' for a dual view SPIM capture)
- ***First side*** allows to choose between sides for a single view SPIM capture
- ***Number of planes*** type the required number of Z-planes for each stack for Piezo/slice scans, for Stage scans it will indicate the number of planes as calculated from Step size and Scan distance
- ***Step size*** enter a value for the inter-plane spacing (real Z, <u>not</u> stage step for stage scan)
- ***Left*** stage coordinates for stage scan start, ***Go*** will move the stage to the saved position, ***Set*** will set the stage scan start coordinates to the current stage position
- ***Right*** stage coordinates for stage scan end, ***Go*** will move the stage to the saved position, ***Set*** will set the stage scan end coordinates to the current stage position
- ***Go Center*** will move the stage to the center between the start and end coordinates of a stage scan
- ***?*** will present calculated scan parameters as the Total scan distance, Stage velocity, Scan duration, Slice (frame) timing and number of Total Images (computed)
- ***Advanced...*** will open the MLS Advanced Acquisition Parameters interface allowing to enable further channels, set their parameters and choose from different scan options

12 Capture group



• For timelapse captures enter the number of ***Time points*** and the desired ***Interval***, select the interval unit (e.g. ms) from options in the drop down
• ***Channel A:*** drop down to select the laser line for side A, ***Go*** enables the laser
• ***Channel B:*** drop down to select the laser line for side B, ***Go*** enables the laser
• Drop down selection of Laser power (in %)
• Option to ***Enable A/B power balancing*** to achieve similar intensities for each side
• ***Balance (B%)*** enter a value (% of power side A) to regulate the laser powers of each side separately when power balancing is enabled
• Indicates which ***chip size***, ***binning*** and how many ***channels*** are currently activated
• ***99%*** tick box to restrict the camera intensity range to the 99% of the brightest signal
• ***Close Alt/ Open Alt*** enable and disable lasers
• ***Live*** enables live mode of cameras and opens a separate viewer
• Tick boxes for operation:
    • ***Dual cam*** enables switching between side A and B for simultaneous view in live mode
    • ***Histo*** shows the minimum and maximum intensities for each side in the camera live view window
    • ***CY*** enables the cyan and yellow look up
    • ***Grid*** plots a grid over the live view window to assist alignment
• Select a chip size
    • ***64*** - 64 x 64 pixel (px)
    • ***128*** - 128 x 128 px
    • ***256*** - 256 x 256 px
    • ***512*** - 512 x 512 px
    • ***1k*** - 1024 x 1024 px
    • ***Full Chip*** - 2048 x 2048 px
• ***Factor*** crops image size in Y only, select options in drop down
• ***Bin*** enables binning, select options from drop down

13 Multi Point Capture group



Multi point capture for MLS is currently under development.

Export

Images and movies can be exported from the native **.sld** format to other file formats from the ***Export*** menu. ***Quantitative Export*** will export true pixel values. ***Qualitative Export*** will export the image as it appears on the screen, including any renormalization and annotations. ***Movie Export*** can be used to render qualitative movies of images.

The following options will directly open a ***Save As*** window. All other options will open a dialog as linked below.

RGB TIFF (24-bit) - Exports a TIFF of the currently selected image at the current plane and time point exactly as it appears.

TIFF Series - Exports a series of TIFFs dependant on capture type as follows:

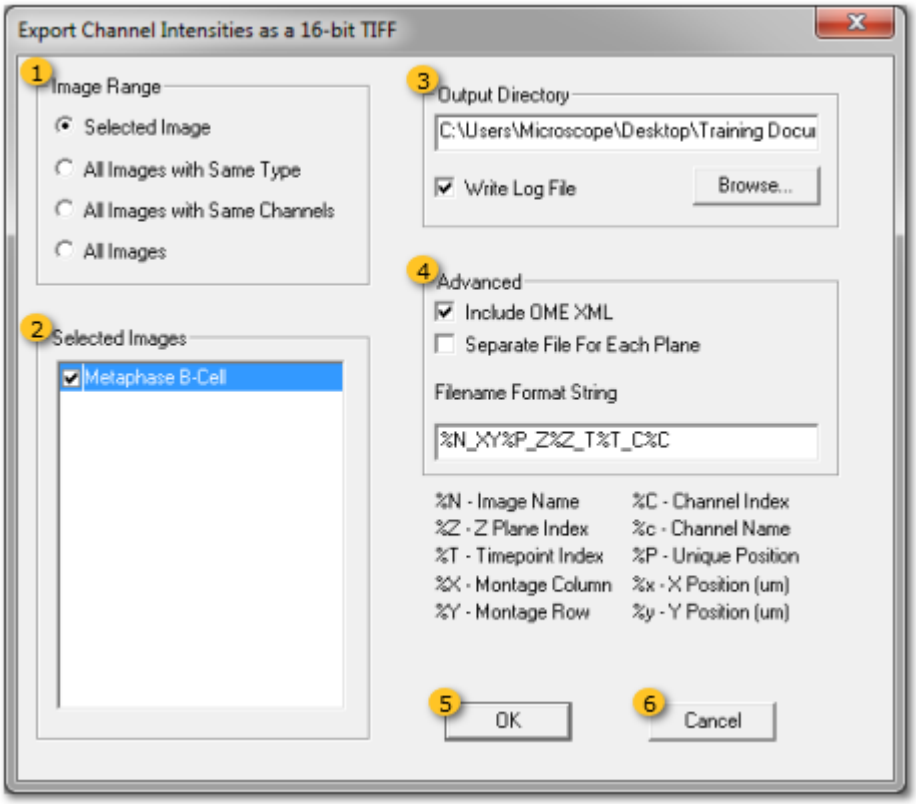
3D Capture - Exports a TIFF for each *z*-plane
2D Timelapse - Exports a TIFF for each time point
4D Capture - Exports the a TIFF of the current *z*-plane at all time points

Default Views of all Images as TIFFS - Exports the default view of all images on the slide as a TIFF. The default view of an image is set by applying the renormalization and saving the slide or by using the ***Thumbnail button*** to set a default view.

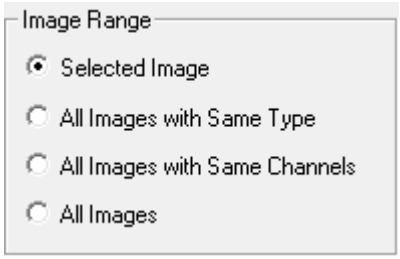
Export all channels as 8-bit TIFFS - Exports a single 8-bit (grey scale) TIFF for each channel of the currently displayed plane.

16-bit Tiff Export window

When you wish to export **.sld** data quantitatively for collaboration or archiving purposes, you will need to export 16-bit tiffs. This mode of export results in tiffs with data ranging 0 - 65535. It is important to note that data from 12-bit or 14-bit cameras will need to be scaled appropriately or the images will appear black. Because this is a quantitative export, renormalization and annotations will not be included in the resultant data.



1 Image Range group



Select the ***Image Range*** as described below.

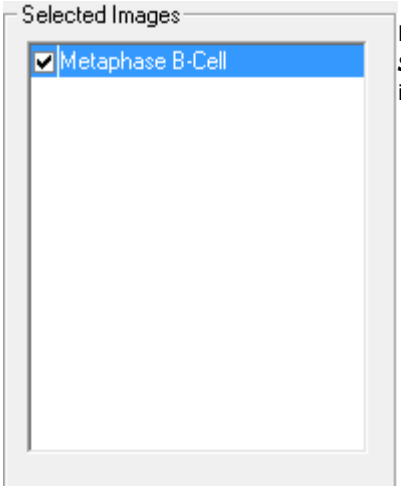
Selected Image – Operation will be performed on the selected image only.

All Images with Same Type – Operation will be performed on all images in the slide with the same capture type (2D, 3D, 4D, etc.) as the selected image.

All Images with Same Channels – Operation will be performed on all images in the slide with the same channels as the selected image.

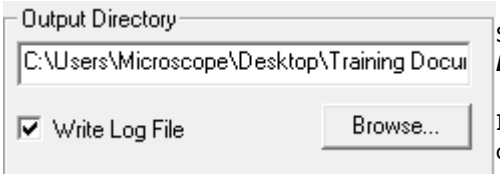
All Images - Operation will be performed on all images in the slide.

2 Selected Images group



Depending on the image range selected, one or more images will populate the ***Selected Images*** list. You may click to check or un-check boxes to select images for batch export.

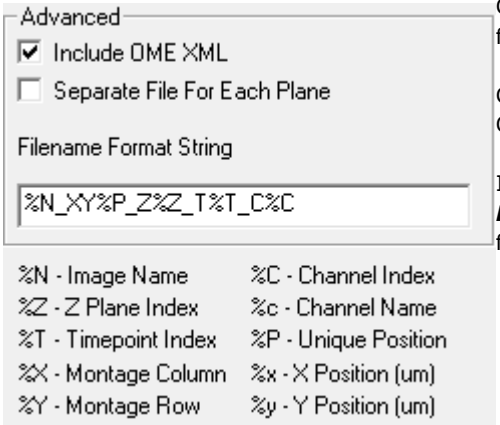
3 Output Directory group



Select the ***Output Directory*** where the tiffs will be saved by clicking on ***Browse*** then navigating to the desired location.

If the ***Write Log File*** box is checked, a **.txt** file will be written that contains information about the image(s) being exported.

4 Advanced group

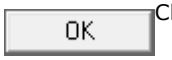


Check the ***Include OME XML*** box to include OME metadata in the tiff file (encoded in XML)

Check the box if you would like a ***Separate File For Each Plane***. Otherwise, a multi-plane tiff will be written for each channel.

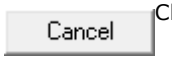
If desired, you may customize the file naming schema by editing the ***Filename Format String*** using the guide provided below the edit field.

5 OK button



Click ***OK*** to export tiffs as per the settings in the dialog.

6 Cancel button



Click ***Cancel*** to discard changes and close the window.

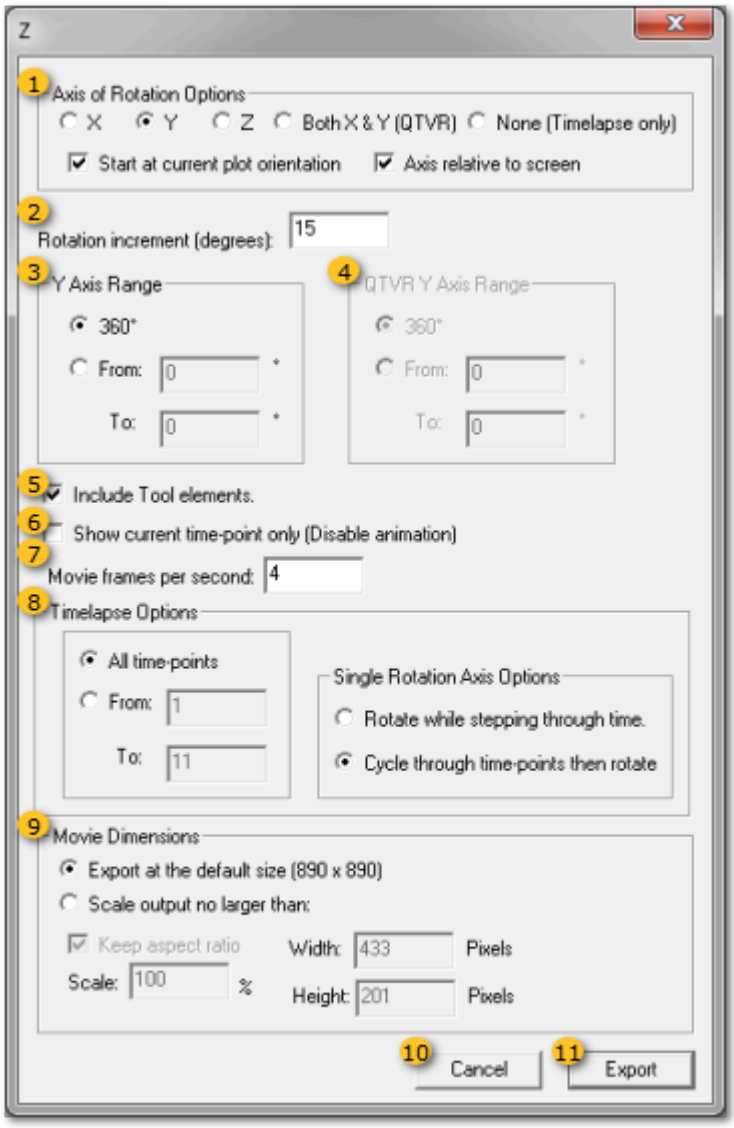
Export Movie window

Movies can be exported from volume views, surface rendering, or 2D Timelapse Intensity Plot with a variety of rendering options.

Access with a Volume View, Surface View, or 2D Timelapse Intensity Plot selected.

See also:

- Create Series Movie
- 3D/ 4D Volume View
- 3D Surface View
- 2D Timelapse Intensity Plot window



1 Axis of Rotation Options group

Axis of Rotation Options – you may generate **.mp4** movies where your volume or surface rotates around a single axis (x, y, or z) or QTVR movies where you may rotate your volume as desired using QuickTime. QTVR is only avaialable in 32 bit operating systems.

Start at current plot orientation – check this box if you want to start your movie with the view that is currently displayed. If left unchecked, the movie will begin with a view with surfaces at right angles to the selected axis.

Axis relative to screen – check this box if you would like the image to rotate around an axis relative to the screen. If left unchecked, the image will rotate around the image axis.

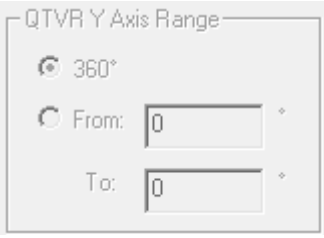
2 Rotation Increment

Rotation Increment - enter the desired rotation increment in the edit field. The rotation range that you selected (below) is computed in the increments you provide. Choosing a smaller increment increases the smoothness of the rotation and adds to the depth cue provided by moving the image. Projection is an expensive computation, so choosing a larger increment will make the projection process faster. To generate a single head-on projection of the entire image, choose 360.

3 Y Axis Range group

Y Axis Range – select 360 to rotate the data completely. You may select a smaller range if desired.

4 QTVR Axis Range



QTVR Axis Range – when QTVR export is chosen, select 360 to rotate the data completely. You may select a smaller range if desired.

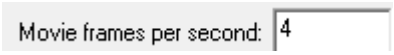
5 Tool Elements option

Check the box to include tool elements from the rendering such as the lighting element, lookup tables, and grid size.

6 Current Time-point option

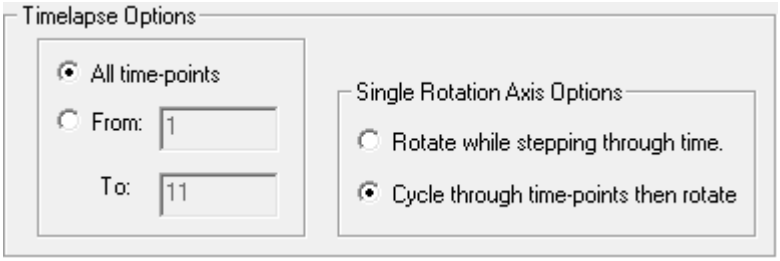
Available for 4D images only, check the box to render a single time point to conserve time and memory.

7 Frames per Second



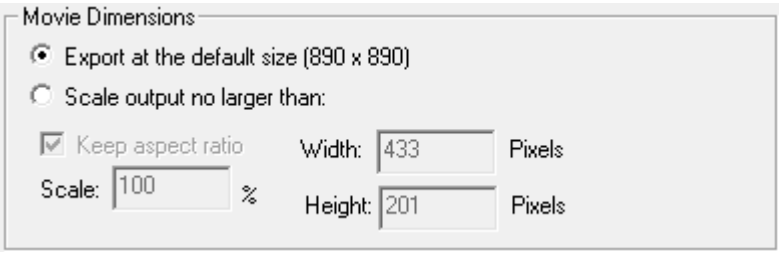
Movie frames per second – adjust the speed of your movie

8 Timelapse Options group



Timelapse Options – select the time points you would like to render and how you would like your time lapse movie to proceed (either step through time as the object is rotating, or complete a rotation before proceeding to the next time point).

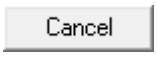
9 Movie Dimensions group



Movie Dimensions – allows you to change the dimensions of the movie

Keep Aspect Ratio – keeps the current height/width ratio
Scale – change the size of the movie with respect to the original image dimensions
Width/Height – manually enter the dimension you wish to export

10 Cancel button



Discard changes and close the window

11 Export button

Begin export, a standard ***Save As*** dialog will open.

Create Series Movie

You may export an MPEG-4 (**.mp4**) movie file to view animated data outside of SlideBook (alternatively, playback controls are available in most view windows). The exported movie will use the current renormalization settings and include any annotations that are displayed.

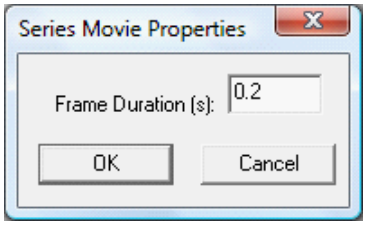
See also:

Main View: Time Controls
Annotations Settings

1. Open a Main View, Three View, Tile View, or Channel View of the image and adjust the look of the data as desired. This may include altering renormalization parameters, cropping the image, or adjusting the zoom factor.

2. Add any desired annotations from the ***Annotations*** group or by using the shortcut keys. Adjust the look and location of the annotations by using ***Annotation Settings***.

3. Select ***Export > Create Series Movie***. The ***Series Movies Properties*** dialog box will appear.



4. Enter a value in the ***Frame Duration*** field. This is the amount of time that a single time point will be displayed during the movie playback. Use smaller values for faster playback. For example, a value of **0.2** will play back at a rate of 5 frames per second.

5. Click ***OK***.

6. A standard ***Save As*** dialog box will open. Name the file and select a ***Save as type***. Options are:
   Windows Media File (*.wmv) - higher bitrate, uses the "high" profile for encoding
   H.264 File (*.mp4) - uses the "Main" profile for encoding

7. Click ***OK***.

Import

By importing files, SlideBook is able to display and analyze data captured in other programs.

Import options are as follows:

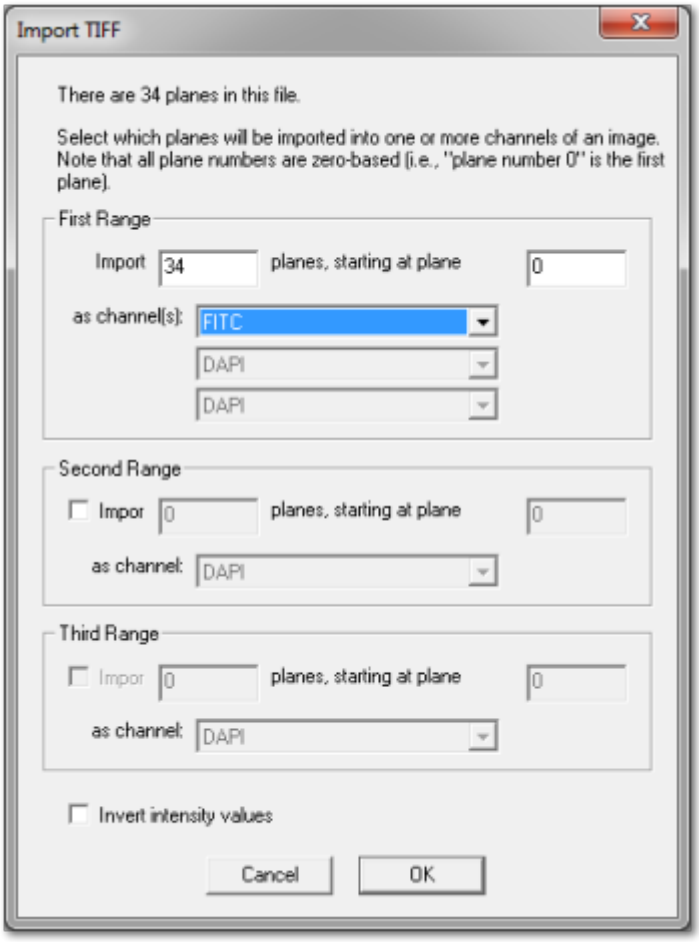
- TIFF
- TIFF Sequence
- SlideBook Spool
- LSM 5
- FV1000
- DV/MRC
- Raw
- PIC
- ZVI
- STK
- UltraVIEW Raw

Import TIFF window

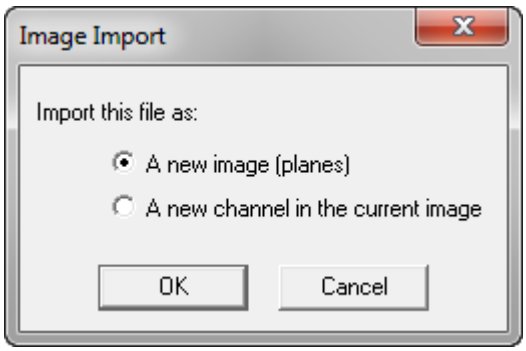
When you select ***Import > TIFF*** for a blank slide the following dialog will open. The first line in the window will tell you how many planes are present in the TIFF, these could be time points or z planes. In some cases multiple channels will be present in a single tiff either as consecutive ranges of planes or as individual channels.

> When channels are presented individually, select the number of planes to import, enter a start plane, and select the appropriate channel. Repeat the import process following the instructions on the bottom of this page.
>
> When channels are presented sequentially in a single TIFF, select the range for each channel and the appropriate channel for each range.
>
> When multiple channels are present in each plane, as in the case of 24 bit RGB TIFFS, select a range of planes and a channel assignment for the red, green, and blue colors. Be aware that 24bit RGB tiffs are likely qualitative only and should not be used for quantitative analysis or deconvolution.

After the TIFF is imported, the Get Info/ Image Info window will open should you need to assign an objective, adjust interplane spacing, or other modify how the data is presented.



If images are present in the **.sld**, the following dialog will appear:

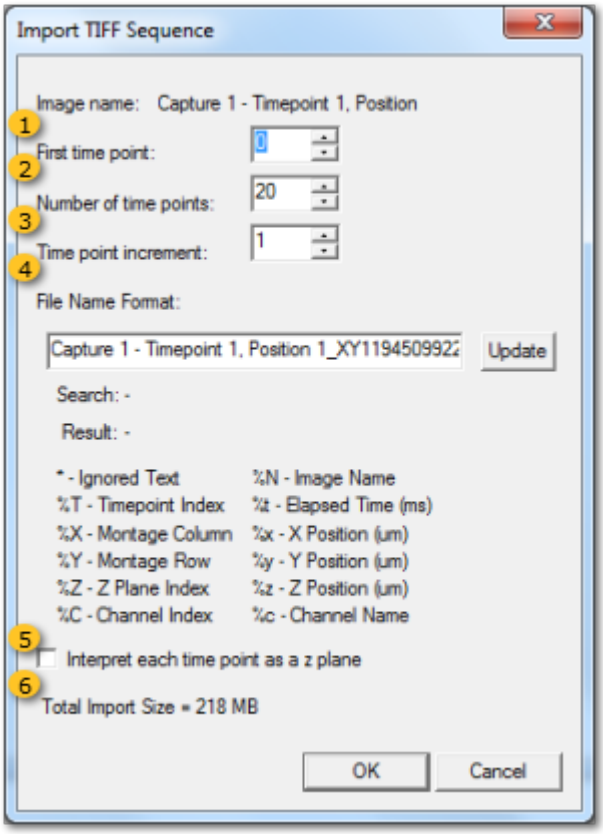


Select ***A new image (planes)*** to create a new, separate image in the **.sld**.

Select ***A new channel in the current image*** to add a channel to an existing (selected) image in the **.sld**.

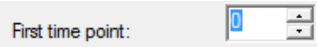
Import TIFF Sequence

When single planes (either z or time) are saved as a sequence of TIFFs, SlideBook can automatically import them into a single multi-plane file by selecting ***Import > TIFF Sequence***. A file browser dialog will open allowing you to navigate to and select the first image in the TIFF sequence. Once a TIFF is selected, the following dialog will open:



In many cases, the input fields will be automatically populated based on the tiff sequence itself. Confirm or edit the values as desired and click ***OK***.

1 First time point

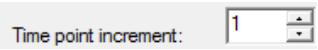


Enter the starting time point (or z plane) for the import. To skip the first three time points of a series, you would enter a value of 3.

2 Number of time points

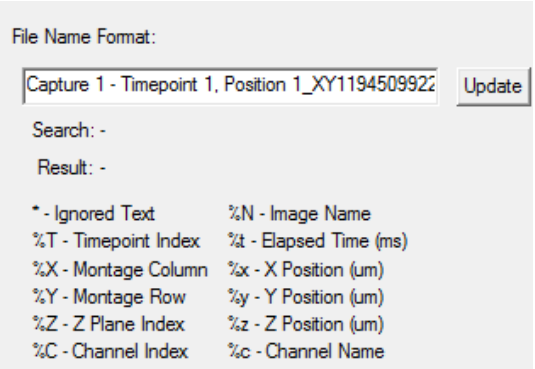
Enter the total number of time points (or z planes) to import.

3 Time point increment



Enter the time point (or z plane) increment.

4 File Name Format



File name format refers to how the tiffs are named and aids in SlideBook image organization. Format tokens available are listed in the dialog.

For example, if your files are of the format:

1_ch0_stack0000_488nm_0000000msec_0016054308msecAbs.tif
1_ch0_stack0001_488nm_0002500msec_0016056808msecAbs.tif

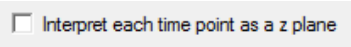
The default File Name Format is

1_ch%C_stack0000_488nm_0000000msec_0016054308msecAbs.tif

Which you can change to

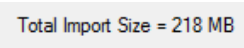
1_ch%C_stack%T_488nm_*.tif

5 Interpret time points as z



Check the box if you are importing z planes instead of time points.

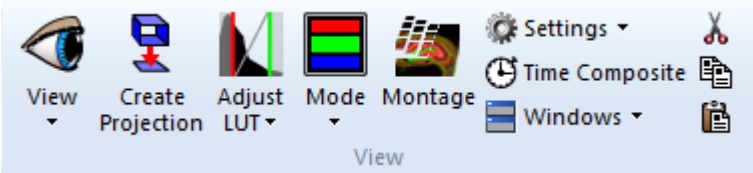
6 Import Size



Displays the size of the requested import

View group

The View group of the Home tab allows you to access the many rendering types available in SlideBook as well as adjust renormalization and color assigned to each channel. It is here that you can combine tiles into a montage, blend background to merge a transmitted light channel with fluorescent channels, create a time composite channel to show paths, and other view related settings.



View menu

SlideBook includes a plethora of viewing options to explore your data. Data views (including Main, Three, 3D Tile, Timelapse Tile, and MultiD Channel views) can be used for quantitative analysis as well as qualitative figure generation and export. Display views (including Volume Views, Surface View, and 2D Timelapse Intensity Plot) are used for visualization and rendering only. Masks and regions may be displayed in some display views but cannot be edited or analyzed.

		2D Capture	3D Capture	2D Timelapse	4D Capture
DATA VIEWS	**Main**	X	X	X	X
	Three		X	X	X
	3D Tile		X		
	Timelapse Tile			X	X
	MultiD Channel	X	X	X	X
DISPLAY VIEWS	**3D Volume**		X		X
	4D Volume				X
	3D Surface		X		X
	2D Timelapse Intensity			X	

When viewing images, the middle mouse scroll wheel can aid in image navigation. In 2D time lapse and 4D data types, the middle scroll wheel will move through time points. In a 3D stack the middle scroll wheel will move through *z* planes. In a 4D series holding down the control key plus the mouse scroll wheel will move through *z* planes.

Main View

The ***Main View*** is the default viewing window for all images within SlideBook and opens any time you double-click on an image in a **.sld**. In this view, you can explore anything from a basic 2D, single channel snapshot to a complex 4D multichannel data set. The top portion of the image frame contains a variety of tools that are available in all data views.

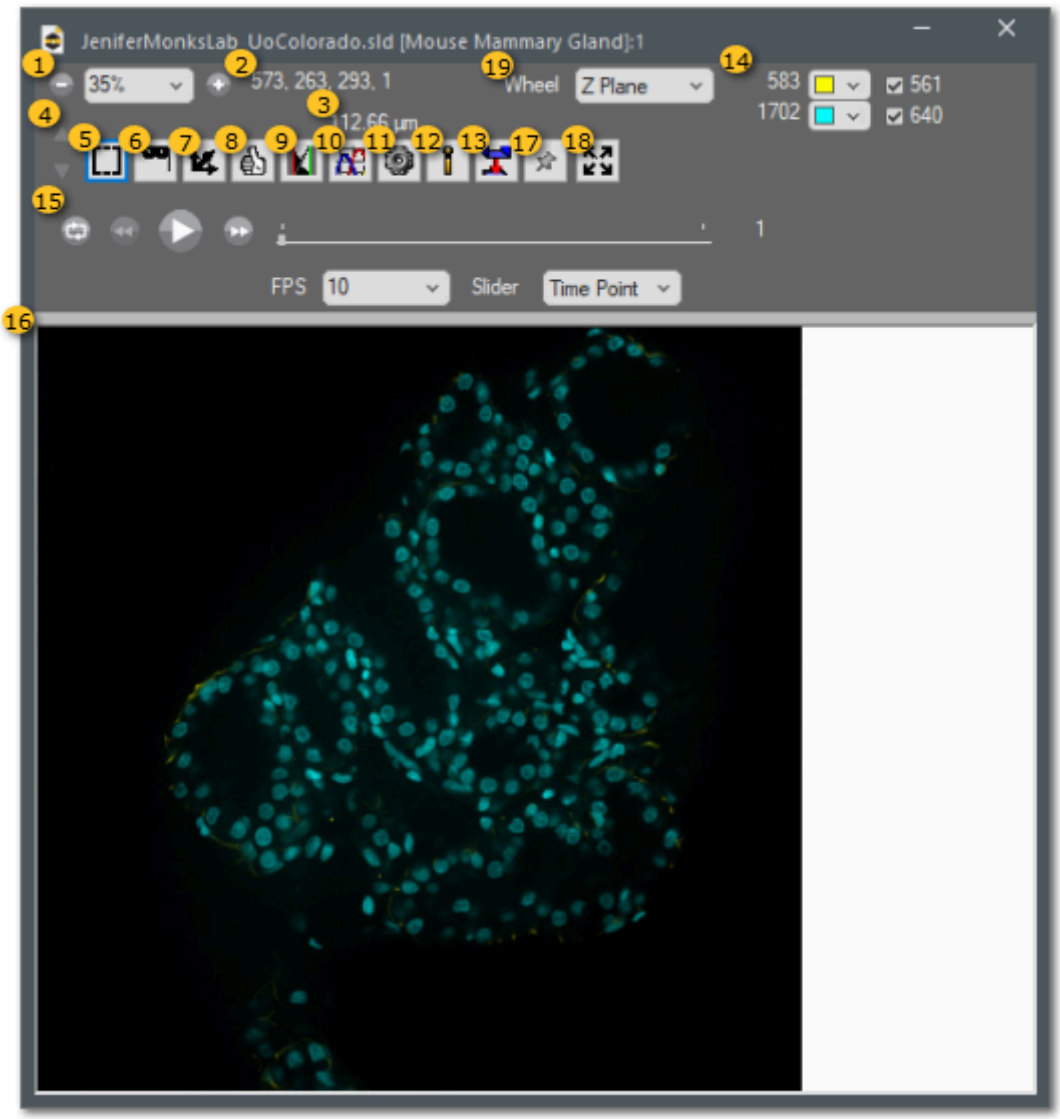
When viewing images, the middle mouse scroll wheel can aid in image navigation. In 2D time lapse and 4D data types, the middle scroll wheel will move through time points. In a 3D stack the middle scroll wheel will move through *z* planes. In a 4D series holding down the control key plus the mouse scroll wheel will move through *z* planes.

See also:

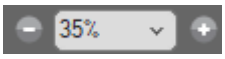
- Graph window
- View Settings window
- Renormalize Image window
- Annotation Settings window

Further viewer options:

- Three View
- 3D Tile View
- Timelapse Tile View
- Montage Tile View
- Multidimensional Channel View
- Multipoint View
- 3D/4D Volume View
- 3D Surface View
- 2D Timelapse Intensity Plot



1 Zoom Control



Indicates current image display magnification. Type or select a magnification factor for display from the drop down menu or use the + and - buttons to move up/down the list.

2 Coordinate Info

Displays the current cursor location in the image. Coordinates are *x*, *y*, *z*, *t* (time). Clicking on the text of the coordinates will change the values to actual stage locations.

3 Measurement & SAC Info

Displays measurements computed using the ***Ruler*** and ***Angle*** tools (6) and settings used for motorized Spherical Aberration Correction device during image acquisition if applicaple.

4 Z Plane Control

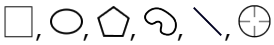
Use the up/down buttons to scroll up and down through z. Alternatively, you may click on the image and use the middle mouse scroll wheel to navigate through *z* for 3D images; for 4D images, hold **CTRL** and scroll to navigate through *z* planes.

5 Tool Menu

Selecting the ***Tool Menu*** will open a dropdown menu of commonly used tools that may include all or most of the tools listed below:

Marquis Tool - Allows you to select an area of the image. One use of this tool is in selecting an area to crop from the image.

Pointer Tool - This tool is used to select regions and for certain functionality in the ***Focus Controls***.

 ***Region Tools*** - These tools are used to draw regions for analysis. They are also found in the main SlideBook toolbar.

Hand Tool - The hand is used to grab and drag an image when the displayed portion of an image is in a window smaller than the extent of the displayed axes.

Zoom Tool - This tool can be used to zoom in on the image, re-centering at the location where you click. Default will display a **+** in the center of the magnifying glass to indicate zooming in. If you hold the **SHIFT** key, the **+** will turn to a **-** and you can zoom out.

Crosshair Tool - This tool is used to retrieve coordinate or intensity information for a single pixel. Press **CTRL** + click on a region in a 2D timelapse image to display an intensity over time graph of that pixel.

Ruler Tool - The ruler is used to draw a line on the image for distance or velocity measurements. Time lapse images will give distances only for the first frame of the time series, subsequent time points will give velocity measurements. By holding down the **CTRL** key, you can draw a multi-segment line. When measurements are made, a value (in um or um/sec) is displayed in ***Measurement Info*** (3). You can retrieve intensity across this line by going to the ***Analyze tab > Evaluate group >*** Line Intensity. To export multiple line measurements, you should use the region line tool then go to the ***Analyze tab > Evaluate group > Extract Measurements > Export Line Lengths***.

Angle Tool - You may select this tool then click three times to draw two connected lines. The angle between the two lines is measured and displayed in ***Measurement Info*** (3). To export the angle measurement information, first go to ***Analyze tab > Evaluate group > Extract Measurements > Export Angle Measurements to Excel***. This will open an Excel file. Every time you draw an angle, the corresponding data will be written to the file.

Mask Tools - These tools are used to draw, erase, and fill when you create a mask.

6 Mask Menu

This menu allows you to create a new mask by segmentation or drawing. You may also choose to display any of the existing masks or **None**.

7 Axis button

You may select the dimension for scrolling planes. For example, the default value is ***Z***. That means that you view *XY* of the image and may scroll through *Z* planes. To look at the stack from one of the sides, you may select ***X*** or ***Y***.

8 Thumb-up button

Pressing the ***Thumb-up*** button resets the thumbnail view in the **.sld/.sldy** and the way the image opens as default. Parameters saved with this button include renormalization and plane.

9 Renormalize button

Opens the Renormalize Image window for use in adjusting the intensity display of the image.

10 Graph button

When regions are drawn, opens the Graph window to display intensity or ratio data over time.

11 Settings Menu

Provides access to the following windows or options:

View Settings - Used to adjust several factors that affect display in the View window. These parameters include array information for tile views, background blending, looping speed for image playback, and background color for Tile and Three Views.

Annotation Settings - Used to adjust the display of annotations in the view.

Blend Background - Blends a background channel (such as **DIC**) into the display using parameters set in View Settings.

Invert Background - Inverts the intensity values of the background channel.

Extract Subseries to New Image - Available when the image includes multiple time points, allows you to extract a subseries of time to a new image. It is important to note that image cropping is not available with this function.

Loop Planes - Continuously plays through the z planes in a loop.

Go to Plane - Opens a dialog to advance the data set to a typed z plane.

Go to timepoint - Opens a dialog to advance the data set to a typed time point.

12 Info Menu

Provides access to the following windows:

Get Info - Provides information about the data including imaging parameters, notes, and processing parameters.

Auxiliary Data - Displays advanced metadata and capture information such as real-time analog recording, temperature recording, and optical parameter metadata about the hardware. You can configure these recordings in the Auxiliary Metadata Tab of Capture Preferences window.

13 Projection Menu

The Projection Menu allows you to quickly view a projection image of 3D data without having to generate a new image as done with the Create Projection Image dialog.

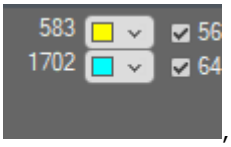
Maximum Z Projection - Uses the maximum intensity found in each channel in the column of pixels

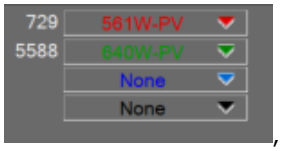
Sum Z Projection - Uses the sum intensity found in each channel in the column of pixels

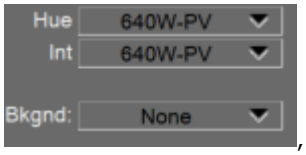
Average Z Projection - Uses the average (mean) intensity found in each channel in the column of pixels

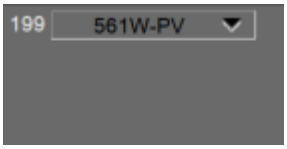
No Projection - Turns off projection view

14 Channel Menus



, 

, 

, 

Channel menus are shown above for the following display types: ***User-defined***, ***RGB, Pseudocolor***, and ***Monochrome/Inverted Monochrome***. Behavior is as follows:

User-Defined - Allows you to select a color from the full palette to assign to each channel. Blending is done automatically.

RGB - Four buttons represent red, green, blue, and background display. You may click on any of the buttons and select the desired channel or **None** from the dropdown menu. To blend a background channel, select it from the fourth button and then go to ***View > Blend Background***.

Pseudocolor - In the pseudocolor view mode, there are two channel selections. The first channel selects the hue of the display and should be set to the ratio channel. The second channel selects what determines the intensity, or brightness, of the displayed colors. If it is set to the numerator or denominator channel, the pseudocolored display will be scaled by the selected fluorescence intensity. An area with high pixel intensity for the ratio channel and low pixel intensity on denominator channel will appear as dim red. An area with low pixel intensity for the ratio channel and high pixel intensity on the denominator channel will appear as bright blue. This helps distinguish areas with real data from background. This is often desirable as computed ratio values for regions of the image that are just slightly above background can vary greatly.

Monochrome/Inverted Monochrome - This mode provides a single button where you can assign a channel to the black and white display.

15 Time, Plane & Zoom Controls

The ***Playback Controls*** allow you to loop, rewind, play/pause, or fast forward the time series. When the play button is used, the image will continually loop until the pause button is selected or the loop button is toggled.

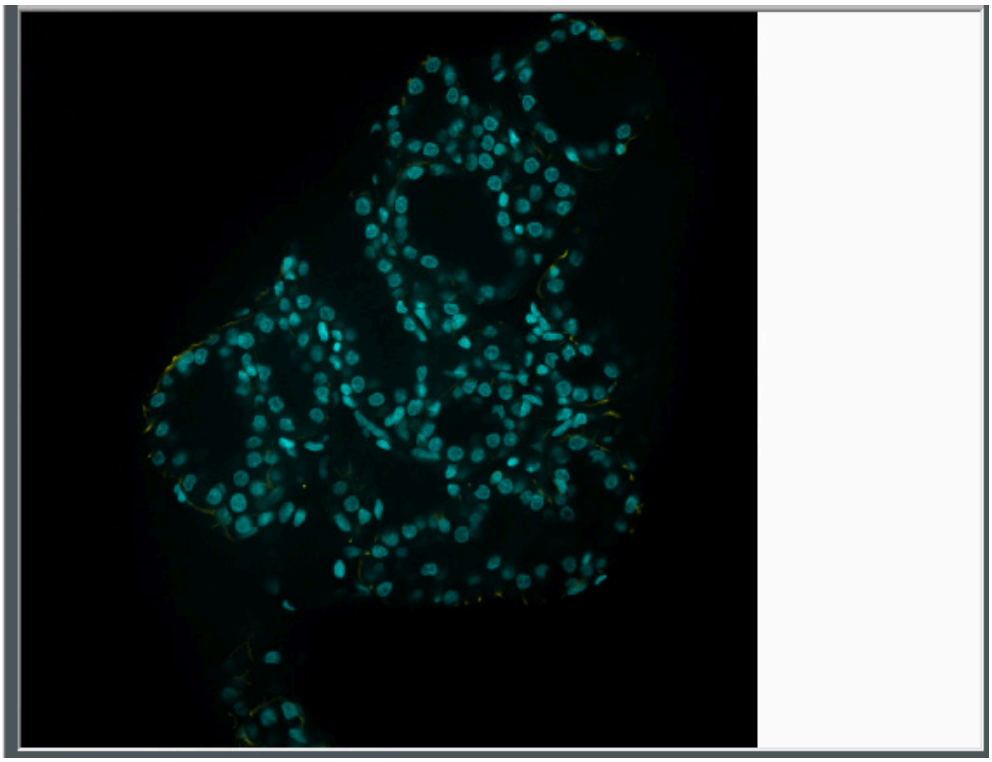
The ***Slider*** automatically shows for 4D images but can also be enabled for 3D only images. It displays the current time point, Z Plane or Zoom - depending on the drop down selection selection below - both with a numerical display to the right of the slider bar. In the example above, the user is at time point 7, about half of the way through the entire time series. A user can move to a desired time point by clicking on the small gray box and dragging it to the desired time point. When the time slider control is selected, it will be surrounded by a dotted line box. Once selected, you can move forward and backward through time by using the middle scroll button on the mouse.

Notice that tic marks indicate the beginning and end of the time series as well as any events or notes created during image capture. If you do not have tics representing events or notes in your time slider, go to Administration tab ***> Hardware Properties > Default View Settings > Show Tics for Notes***.

The ***FPS*** drop down allows to select the speed of the image looping in frames per second.

The ***Slider*** drop down lets you select if going through timepoints, Z planes or Zoom when moving the slider.

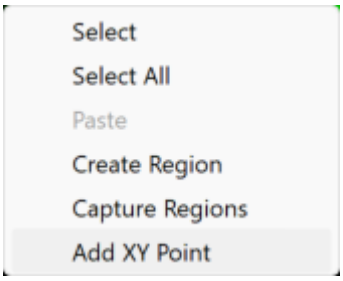
16 Image area



The image will be displayed in the available client area. If the image is smaller than the viewing area, background will be the color assigned in the View Settings window. If the image is larger than the available client area, you can move around using the navigation bars on the border of the image or the ***Hand*** tool from the ***Tool Menu*** (6).

Right Click Options:
When clicking the right mouse button further tools become available

• Select: Selects the specific region underneath the cursor
• Select All: Selects all Regions in the image
• Copy/ Paste: Copies the selected Regions or pastes previously copied Regions from the clipboard
• Create Region: Creates a Region
• Capture Region: Places the coordinates of defined Regions in the Focus window > XY Tab
• Add XY Point: Adds a new point into the Focus window > XY list with the location of the cursor at right click in the center



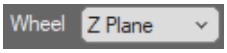
17 Pin Button

Pin the window size of the image to the current size.

18 Expand or Shrink Menu

Expand the image size over the entire screen or return to previous size.

19 Wheel Drop Down



Select if changing Z Plane, zoom or timepoints when scrolling the mouse wheel. If not selected the following keys can also allow for the scrolling:

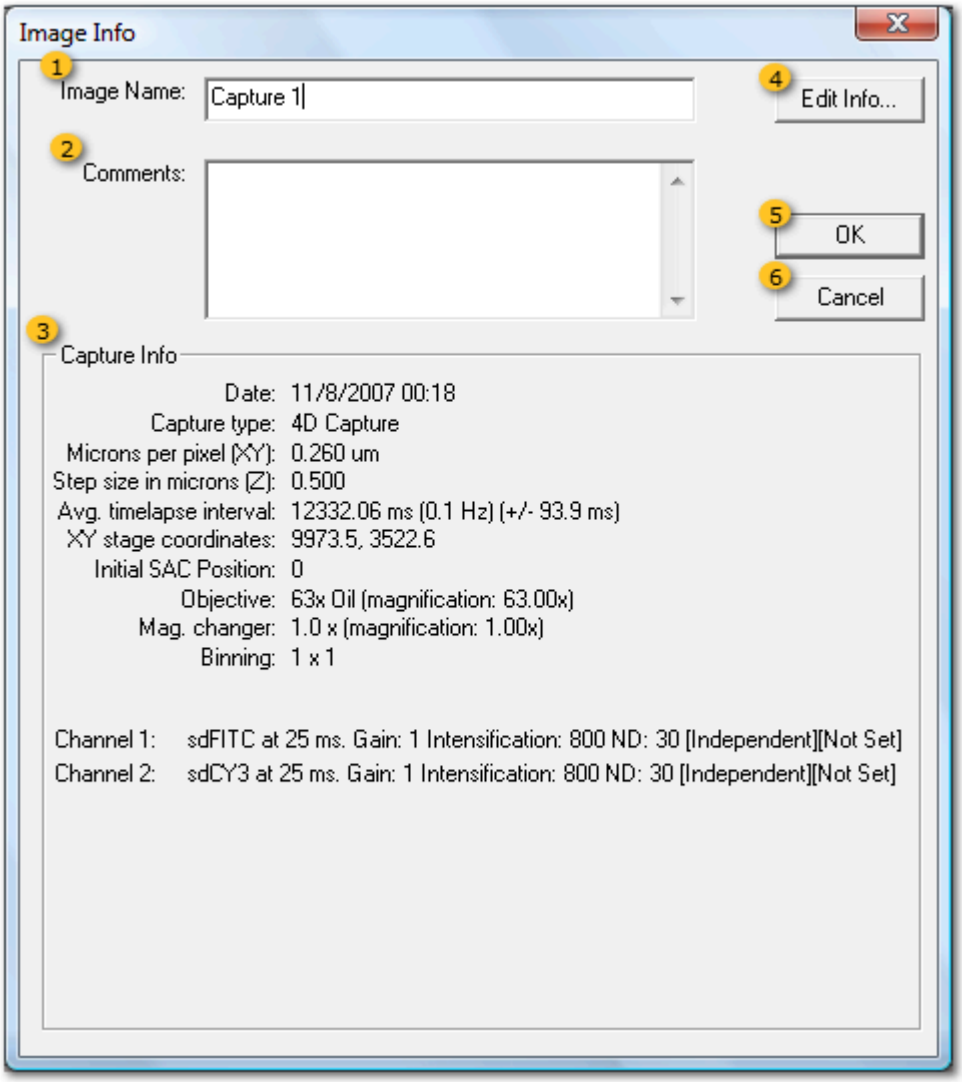
- CTRL + mouse wheel → zoom
- Shift + mouse wheel → Z planes
- Shift + CTRL + mouse wheel → Z planes in increments (default 10)
- ALT + mouse wheel → timepoints

Get Info/ Image Info window

Any image on a **.sld** has a name and information associated with it. You may rename, view, and edit image information in this window. Additional details can be found and edited by pressing ***Edit Info***. This may be particularly important to define voxel size and channels on imported images.

Access by selecting an image form a **.sld** then pressing ***Ctrl + I*** or **> *Get Info*** in the view window.

See also:



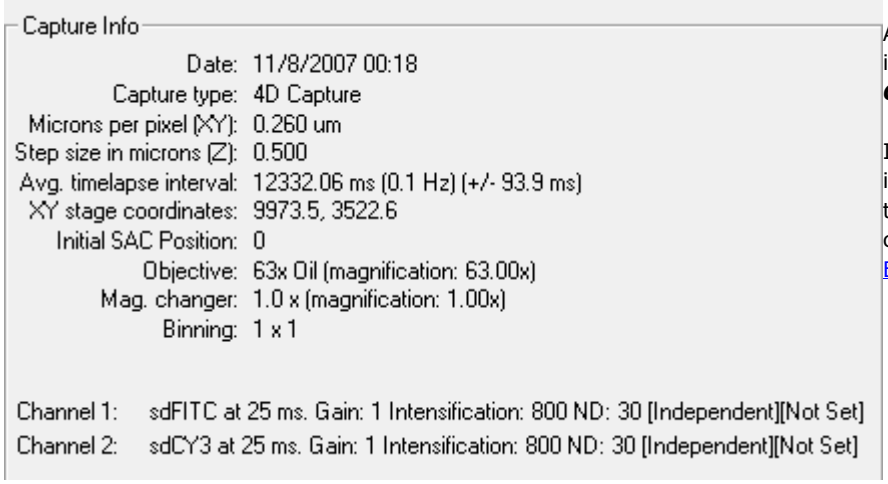
1 Image Name

View and rename your image here by typing in the ***Image Name*** field. Images can also be named upon their creation in the Capture window.

2 Comments field

The ***Comments*** field is a place where you type any comments or notes you would like to make about the image. This may include experimental conditions, sample information, or notes about the image capture. When images have been processed (for example, deconvolved), information about the processing will populate this field. Comments can also be added before image capture in the Capture window.

3 Capture Info



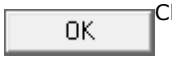
All available information regarding image capture will be displayed in ***Capture Info***.

If the image is imported data, all information may not be displayed in this area. You may edit or add capture information by clicking on Edit Info.

4 Edit Info button

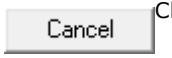
Clicking ***Edit Info*** will open the Edit Info window. Where you can add or edit information about the image capture such as magnification, channels, or pixel size.

5 OK button



Click ***OK*** to save the changes and close the window.

6 Cancel button



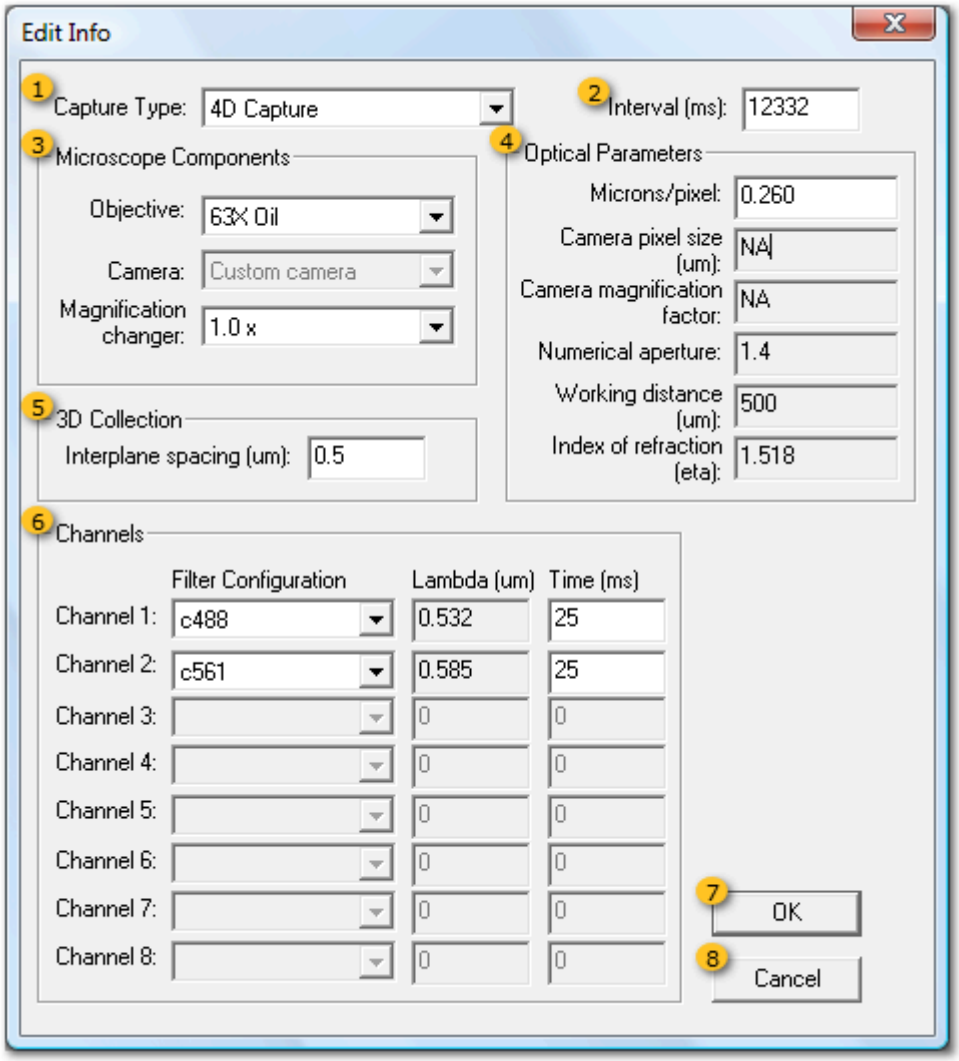
Click ***Cancel*** to discard changes and close the window.

Edit Info window

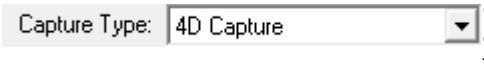
You may edit image information such as microscope components and optical parameters. This may be particularly important for imported images.

Access by selecting an image form a **.sld** then pressing ***Ctrl + I*** or **> *Get Info* > *Edit Info***in the view window.

See also:



1 Capture Type

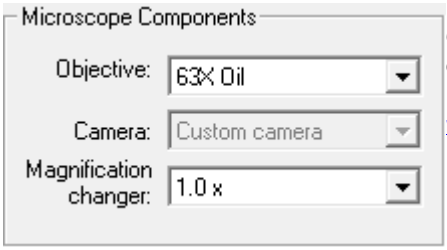


Capture Type describes the type of data acquisition used for the image collection. Options include Unknown, 2D Capture, 2D Montage, 2D Timelapse, 2D Multipoint, 3D Capture, 4D Capture, and 2D Stereology Montage. Capture type will designate how the data is displayed within SlideBook and what views and image processing are available.

2 Interval field

The ***Interval*** field tells the time interval (in milliseconds) between the beginning of one time point and the beginning of the next time point.

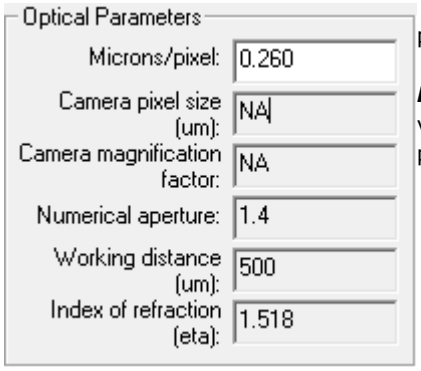
3 Microscope Components group



The ***Microscope Components*** group allows you to designate the ***Objective***, ***Camera*** (on multi-camera systems) and ***Magnification changer*** used when capturing the data. You may need to edit these parameters if you have a manual microscope and did not designate the correct optics in the Capture window. If you have imported data and the required optics are not present, you may configure optics under ***Administration tab > Objectives***.

NOTE: If you have multiple images on a slide that need the objective information changed, you may change one image using the Edit Info dialog, click ***OK*** and ***Save***, then go to ***Administration tab > More Options > Copy Optics Information***. This will copy the optics information from the selected image to all images in a slide.

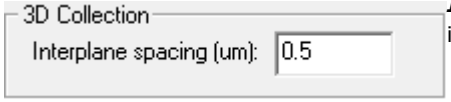
4 Optical Parameters group



The ***Optical Parameters*** group uses microscope component information to populate various values.

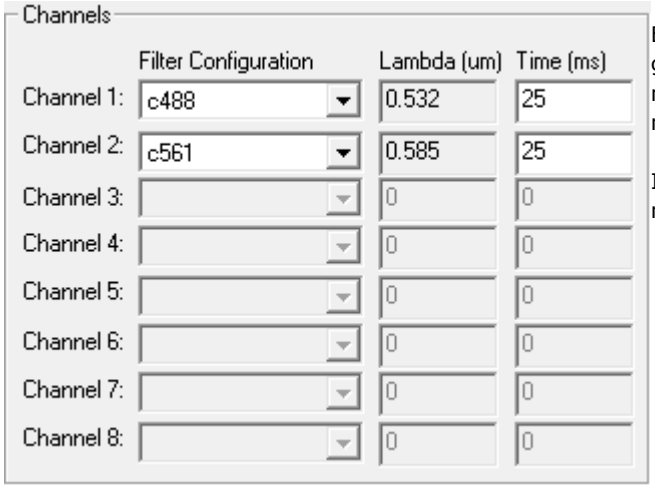
Microns/pixel will be automatically calculated or you may manually enter this value. It is essential to have the correct value in this field if you will be performing any calculations or image processing.

5 3D Collection group



Interplane spacing is the distance between z-planes (in microns) when 3D images are acquired.

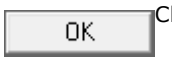
6 Channels group



Each channel of the image is listed in the ***Channels*** group. You may select a channel from the drop down menu and the emission wavelength will be displayed. You may also review/edit the exposure time for each channel.

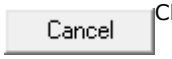
If the required channel is not present, you may create a new channel under ***Administration tab > Filters***.

7 OK button



Click ***OK*** to apply changes and close the dialog.

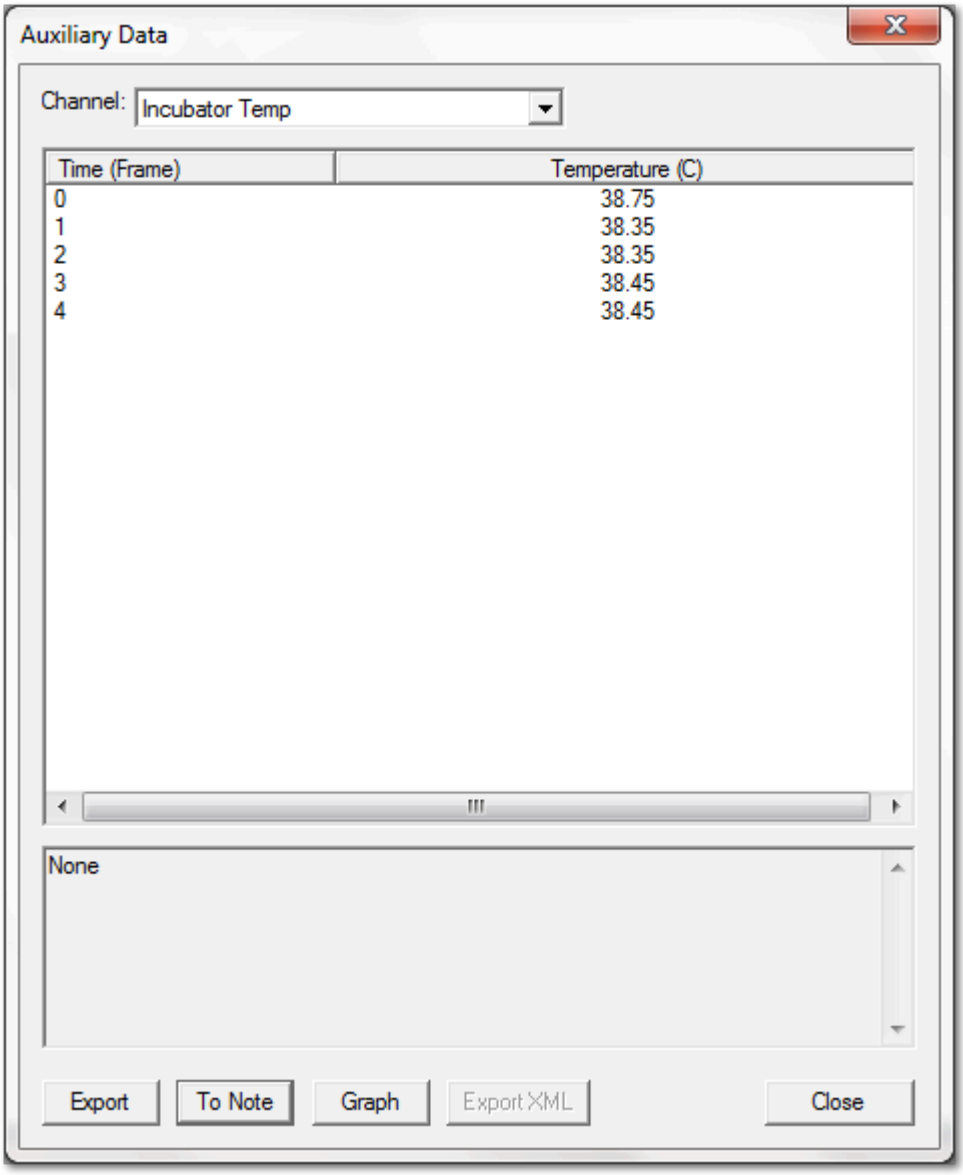
8 Cancel button



Click ***Cancel*** to discard changes and close the dialog.

Auxiliary Data Window

The Auxiliary Data window displays external recordings and experimental setup information. External recordings such as camera temperature, laser temperature, and incubation chamber temperature can be configured during acquisition in Auxiliary Metadata Tab of Capture Preferences window. Any of this information can then be exported or graphed. Selecting ***To Note*** allows you to display the information as a ***Note*** annotation in the image view.



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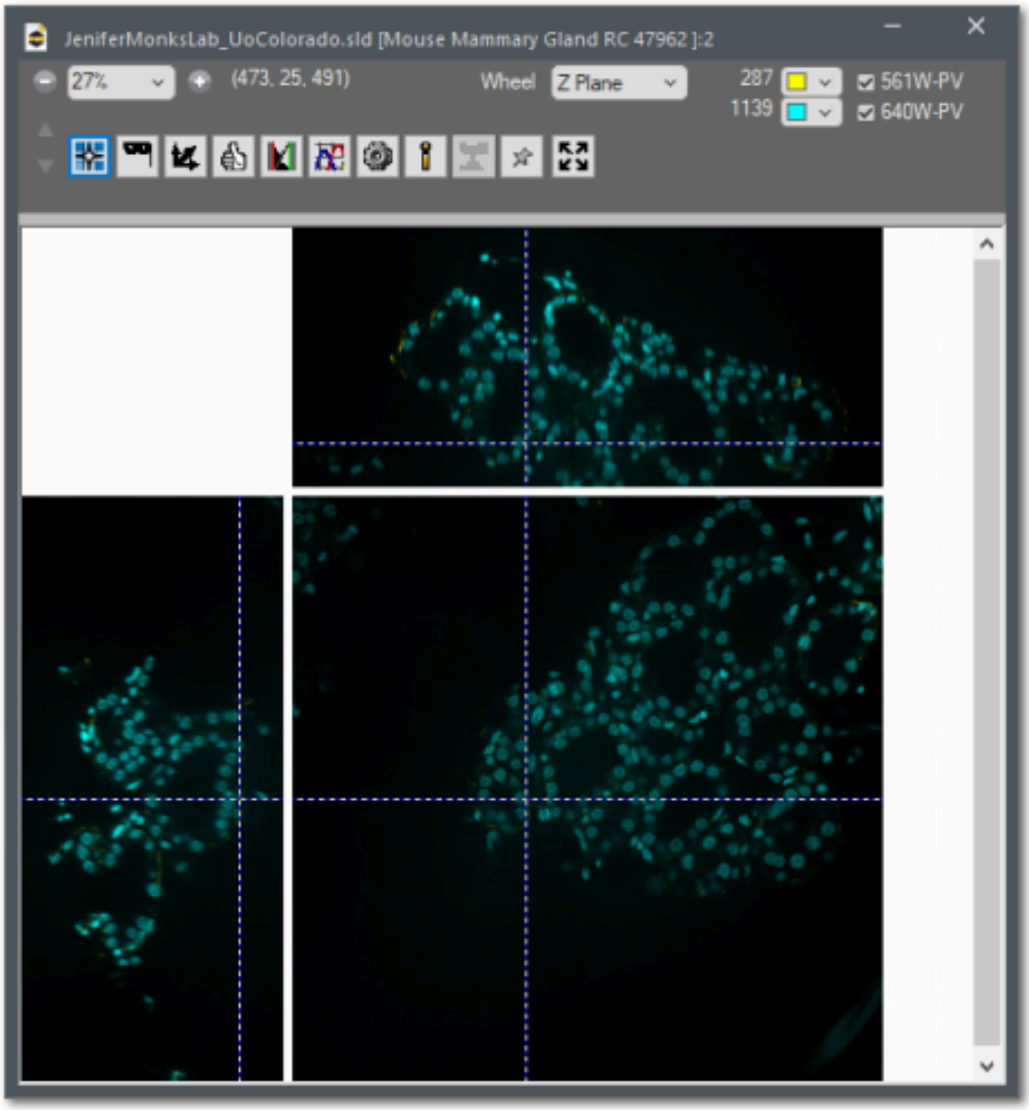
Three View

The ***Three View*** is used to display orthogonal views of a z-stack. This can be particularly useful in examining the interior of a 3-dimensional image. The top portion of the view window contains the standard tools from the Main View. Features unique to this view are listed below.

See also:

- Main View
- 3D Tile View
- Timelapse Tile View
- Montage Tile View
- Multidimensional Channel View
- Multipoint View
- View Settings



• Use the ***Pointer tool*** to select a point in the XY, XZ, or YZ area. The other areas will update to focus around the selected voxel. Alternatively, you may click and drag through a selected area to create a flythrough in other dimensions.

• When you zoom in on the image, all panels will zoom. You may use the ***Hand tool*** from the Tool Menu to adjust image orientation within the panels when zoomed in.

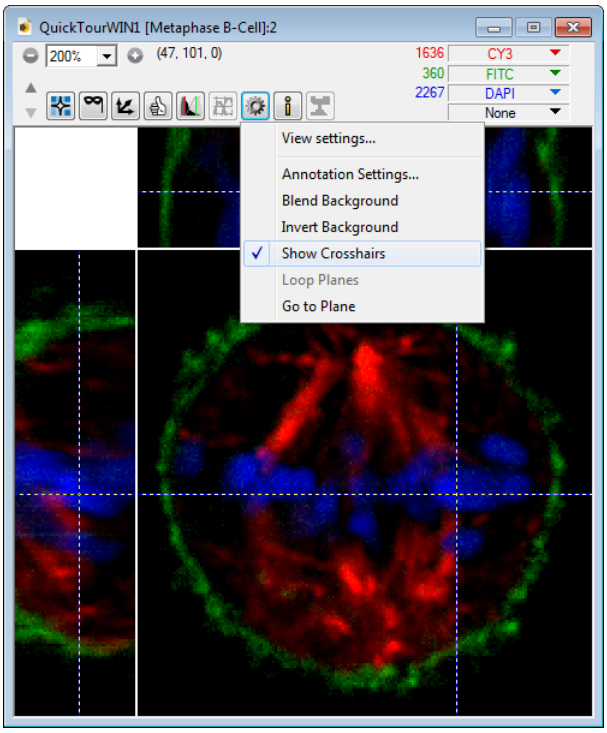
• To highlight an area of interest, you may find it useful to first select the area of interest in a view using the ***Marquee Tool***, then press **Ctrl +B** to select all planes. Once a selection has been made, open a ***Three View***. Only the selected area will be rendered.

• Annotations can be added and their display adjusted from the Annotations Menu.

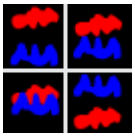
• The background color (gridlines between areas) can be changed from View Settings.

• A TIFF of the image as it appears with annotations and tiles can be exported from the Qualitative Export options. It is important to note that the entire view will be exported, not just the portion you are viewing.

• Click on ***View settings >*** and remove the tick from ***Show crosshairs*** to disable the marker lines that indicate the XYZ coordinates displayed



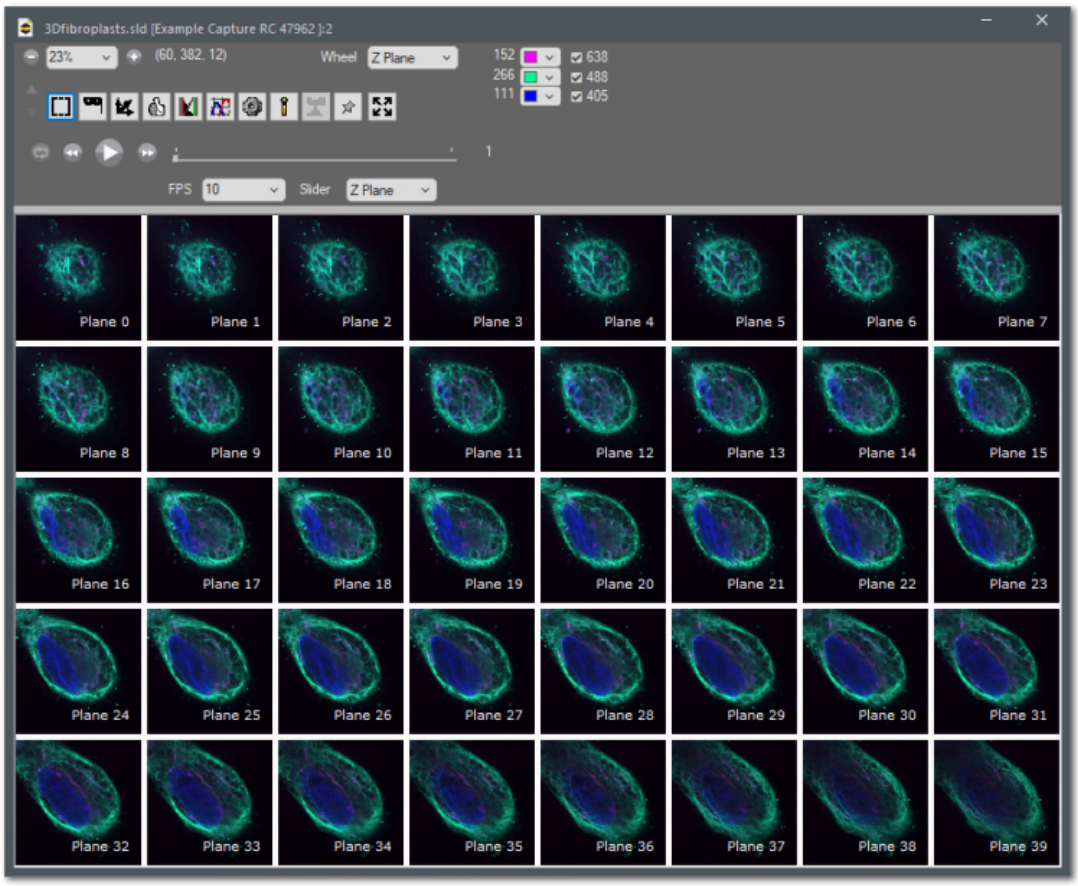
3D Tile View



The ***3D Tile View*** is used to display *z*-planes as an array of images. This can be particularly useful in preparing your data for print. The top portion of the view window contains the standard tools from the Main View. Features unique to this view are listed below.

See also:

- Main View
- Three View
- Timelapse Tile View
- Montage Tile View
- Multidimensional Channel View
- Multipoint View
- View Settings



• When you zoom in on the image, all panels will zoom. You may use the ***Hand tool*** from the Tool Menu to adjust image orientation within the panels when zoomed in.

• To highlight an area of interest, you may find it useful to first select the area of interest in a view using the ***Marquee Tool***, then press **Ctrl +B** to select all planes. Once a selection has been made, open a ***3D Tile View***. Only the selected area will be rendered.

• Annotations can be added and their display adjusted from the Annotations Menu.

• The background color (gridlines) and number of tiles displayed horizontally can be changed from View Settings.

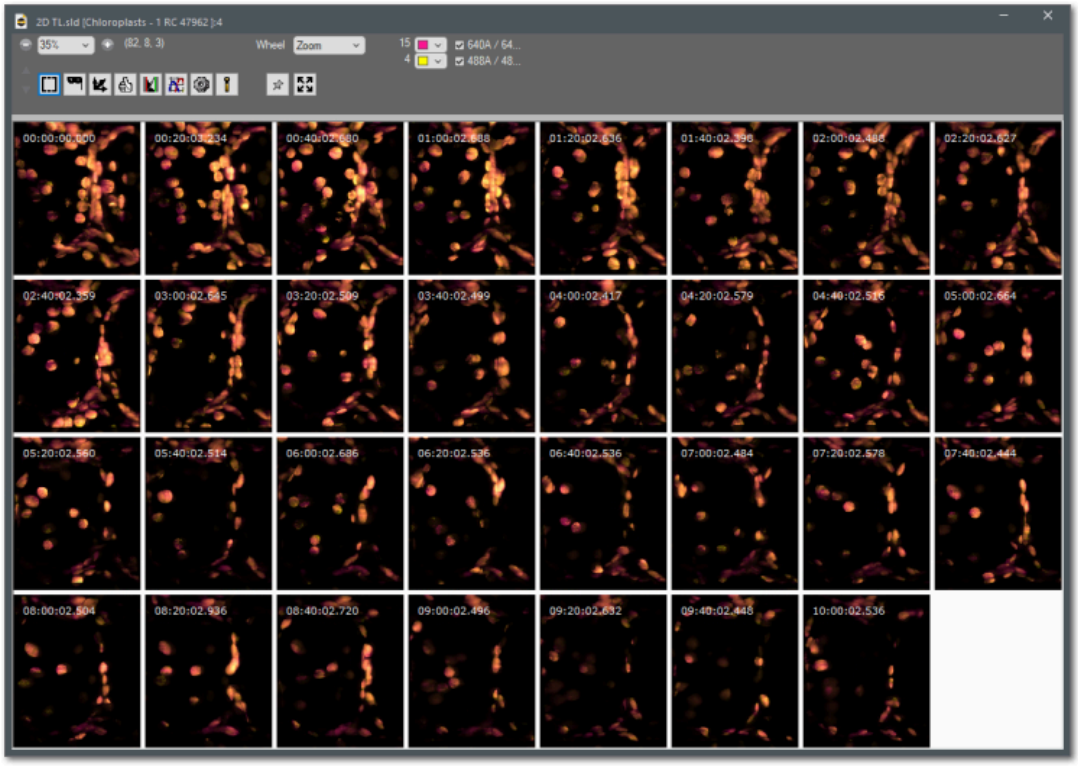
• A TIFF of the image as it appears with annotations and tiles can be exported from the Qualitative Export options. It is important to note that the entire montage of *z*-planes will be exported, not just the portion you are viewing.

Timelapse Tile View

The ***Timelapse Tile View*** is used to display time points as an array of images. This can be particularly useful in preparing your data for print. The top portion of the view window contains the standard tools from the Main View. Features unique to this view are listed below.

See also:

- Main View
- Three View
- 3D Tile View
- Montage Tile View
- Multidimensional Channel View
- Multipoint View
- 2D Timelapse Intensity Plot window
- View Settings



• When you zoom in on the image, all panels will zoom. You may use the ***Hand tool*** from the Tool Menu to adjust image orientation within the panels when zoomed in.

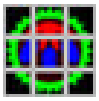
• To highlight an area of interest, you may find it useful to first select the area of interest in a view using the ***Marquee Tool***, then press **Ctrl +B** to select all planes. Once a selection has been made, open a ***Timelapse Tile View***. Only the selected area will be rendered.

• Annotations can be added and their display adjusted from the Annotations Menu.

• The background color (gridlines) and number of tiles displayed horizontally can be changed from View Settings.

• A TIFF of the image as it appears with annotations and tiles can be exported from from the Qualitative Export options. It is important to note that the entire montage of time points will be exported, not just the portion you are viewing.

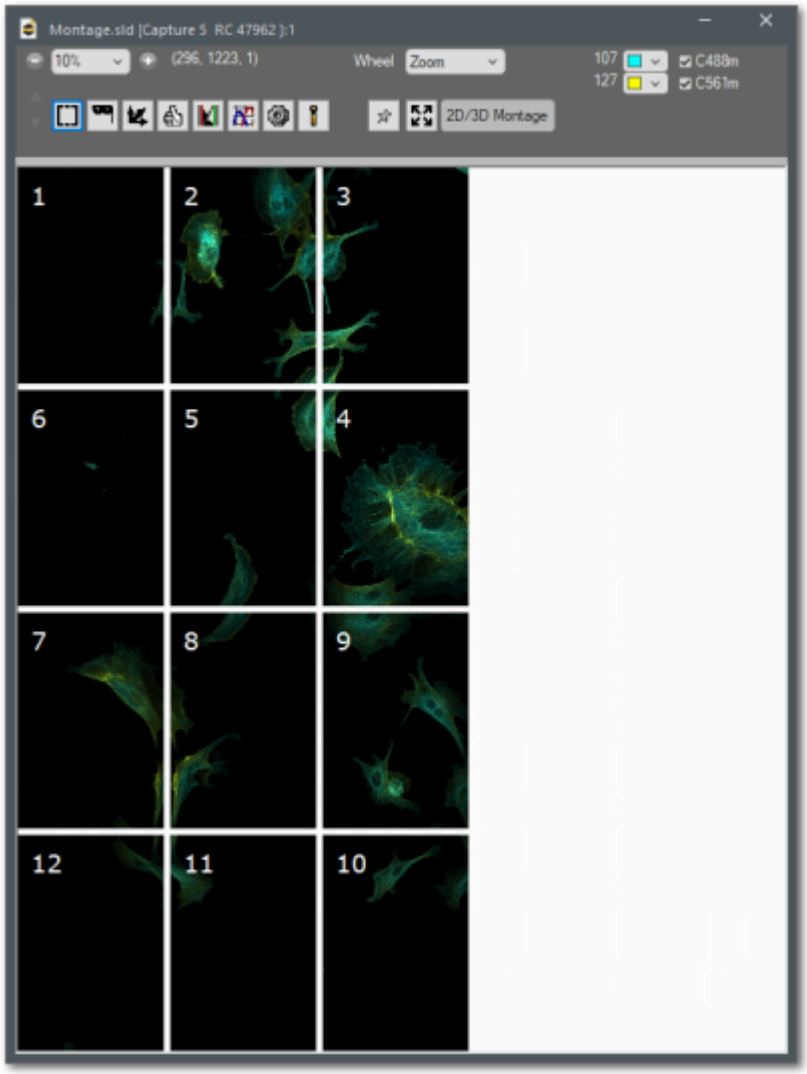
Montage Tile View



The ***Montage Tile View*** is used to display time points as an array of images. This can be particularly useful in preparing your data for print. The top portion of the view window contains the standard tools from the Main View. Features unique to this view are listed below.

See also:

- Main View
- Three View
- 3D Tile View
- Timelapse Tile View
- Multidimensional Channel View
- Multipoint View
- 2D Timelapse Intensity Plot window
- View Settings



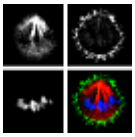
• Annotations can be added and their display adjusted from the Annotations Menu.

• The background color (gridlines) and number of tiles displayed horizontally can be changed from View Settings.

• A TIFF of the image as it appears with annotations and tiles can be exported from from the Qualitative Export options. It is important to note that the entire montage of time points will be exported, not just the portion you are viewing.

- Option to open the 2D/3D Montage console directly to view the data for overview while adjusting the stitching algorithm.

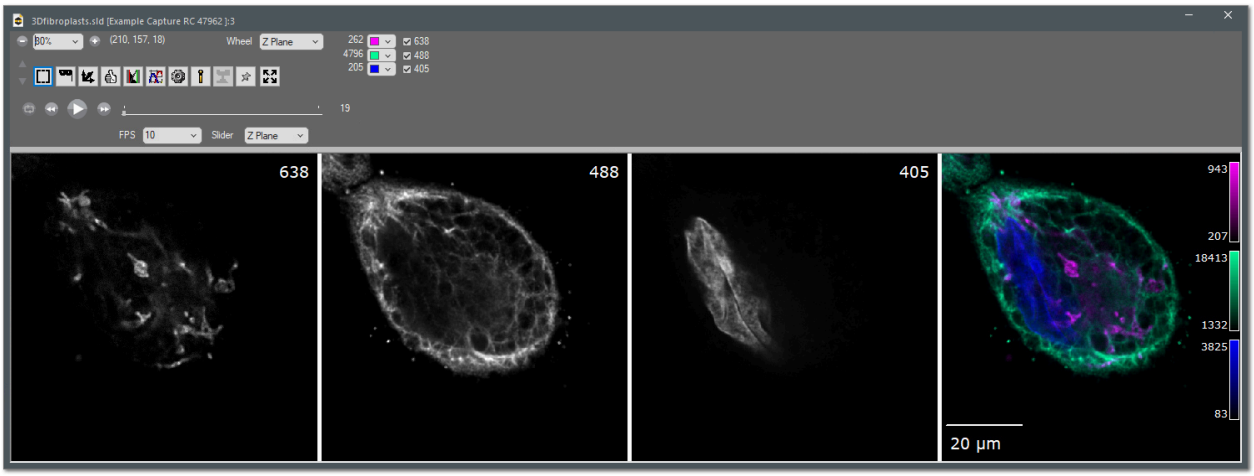
Multidimensional Channel View



The ***Multidimensional Channel View*** provides an excellent rendering to examine individual channels and prepare data for print. This view showcases each channel as a monochrome image as well as a pseudocolored composite image. There are also Multidimensional Channel View settings available that allow you to display individual channels using the default lookup table, tile channels vertically, or display multiple timepoints.

See also:

- Main View
- Three View
- 3D Tile View
- Timelapse Tile View
- Montage Tile View
- Multipoint View
- Multidimensional Channel View Settings
- View Settings



• When you zoom in on the image, all panels will zoom.

• To highlight an area of interest, you may find it useful to first select the area of interest in a view using the ***Marquee Tool***, then press **Ctrl +B** to select all planes. Once a selection has been made, open a ***Multidimensional Channel View***. Only the selected area will be rendered.

• Annotations can be added and their display adjusted from the Annotations Menu.

• The background color (gridlines) and number of tiles displayed horizontally can be changed from View Settings.

• A TIFF of the image as it appears with annotations and tiles can be exported from the Qualitative Export options.

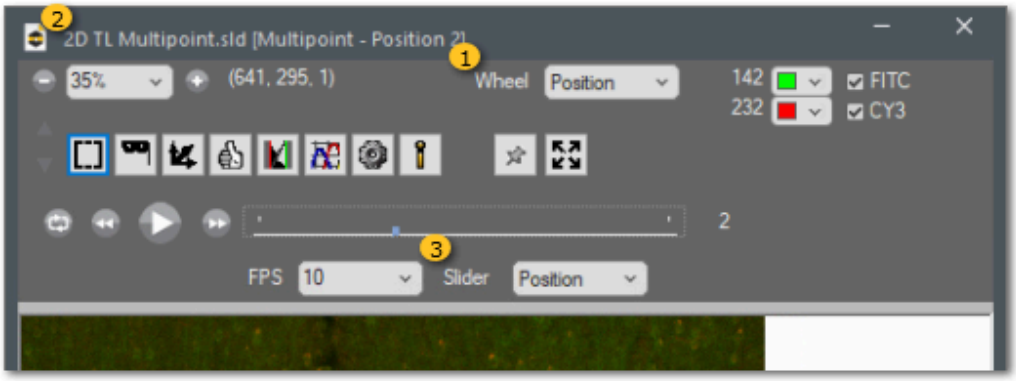
MultiPoint View

The MultiPoint View allows you to view multi-position captures in one convenient interface without having to open multiple windows for each position.

When viewing images, the middle mouse scroll wheel can aid in image navigation. In 2D time lapse and 4D data types, the middle scroll wheel will move through time points. In a 3D stack the middle scroll wheel will move through *z* planes. In a 4D series holding down the control key plus the mouse scroll wheel will move through *z* planes.

See also:

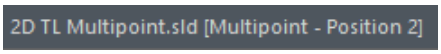
- Main View
- Graph window
- View Settings window
- Renormalize Image window
- Annotation Settings window



1 Mouse Scroll Wheel Controls

The mouse scroll wheel can be set to easily scroll through positions.

2 Position Indicator

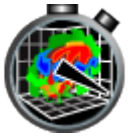


The image name indicates which position is displayed.

3 Slider Control

The Slide can be set to control positions, timepoints, Z-Planes and Zoom individually from the mouse scroll.

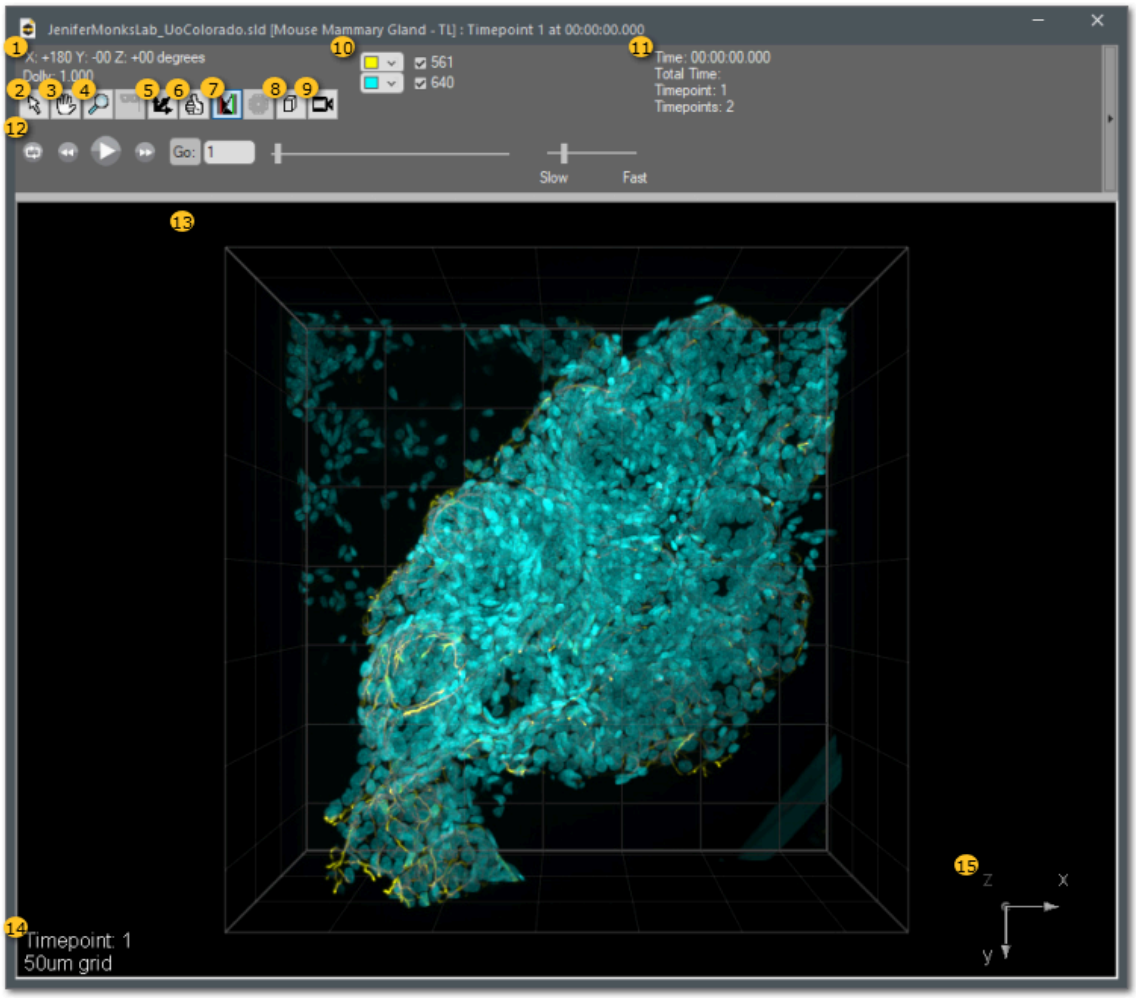
3D/ 4D Volume View



Depending on the image type the volume viewer opens either a 3D or 4D Volume View. This rendering allows you to see 3 dimensional data and 4 dimensional rendered over time. Movies can be exported with various combinations of rotation and time playback from ***Export > Movie***.

See also:

- Volume View Settings window
- Main View
- View Mode
- Export Movie
- Bounding Box
- Movie Maker
- Three View
- 3D Tile View
- Open a selected volume of an image in the 3D/ 4D Volume Viewer



1 Axis Readout

X: +180 Y: -00 Z: +00 degrees
Dolly: 1.000

Displays a readout of the angle of the view in ***X***, ***Y***, and ***Z***. ***Dolly*** refers to the magnification factor of the display.

2 Rotate button

Select this button to turn the cursor into a rotate tool.

Click and drag in the display area to adjust the angle of the image.

A "click and flick" motion will set the image spinning. To "click and flick", click on the image and drag the mouse in the direction you would like the image to rotate. Release the mouse button while it is still in the display area.

To stop the rotation, simply click on the image again.

It is possible to move through time while the image is rotating.

3 Translate button

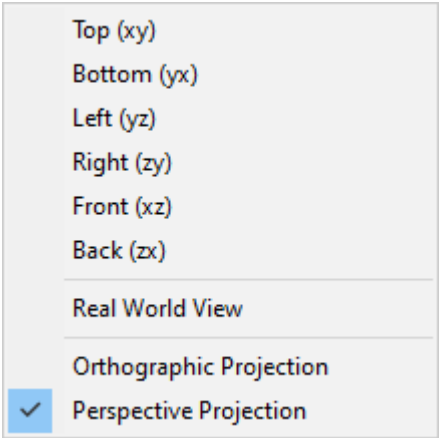
Select this button to change the cursor to a translation hand tool. Once selected, you may click and drag on the image to change the center axis position around which the image rotates.

4 Zoom button

Select this button to turn the cursor into a zoom tool. Using this tool, you may hold down the left mouse button and move the mouse away from you to zoom out (decrease magnification) or move the mouse towards you to zoom in (increase magnification).

5 Axis button

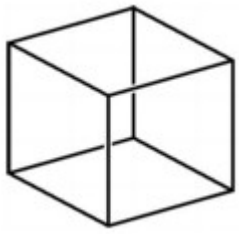
Opens the ***Axis Menu***:



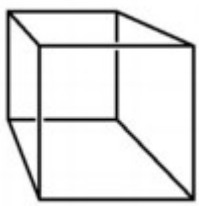
Top, ***Bottom***, ***Left***, ***Right***, ***Front***, and ***Back*** refer to specific angles for display of the data where ***z*** is the depth referring to intensity.

Real World View changes the indicators to represent the the sample's rotation in real world coordinates (not image parameter dependent).

Orthographic Projection is a 2D projection of the 3D data set where the third dimension lines are drawn parallel, that is the object remains the same size as it is further from the viewer.



Perspective Projection is a 2D projection of the 3D data set where the third dimension lines are drawn to a vanishing point, that is the object gets smaller as it is further from the viewer.



6 Thumb-up button

Pressing the ***Thumb-up*** button resets the thumbnail view in the **.sld/.sldy** and the way the image opens as default. Parameters saved with this button include renormalization and plane.

7 Renormalize button

Opens the Volume View Settings window. In this window, you can adjust numerous display parameters for the rendering such as intensity scaling and background color.

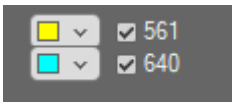
8 Bounding Box button

Opens the Bounding Box menu, which allows for orthogonal slicing of a 3D or 4D volume. This is particularly useful to highlight a certain area in a volume or define a box in 3D for data cropping.

9 Movie Maker Button

Opens the Movie Maker window, which allows for creation of custom movies from a 3D or 4D volume by establishing keyframes and rendering the interstitial frames automatically.

10 Channel Info



Volume View uses the RGB lookup table. You may assign a channel or **None** to each color: red, green, and blue. Refer to View Mode for all options.

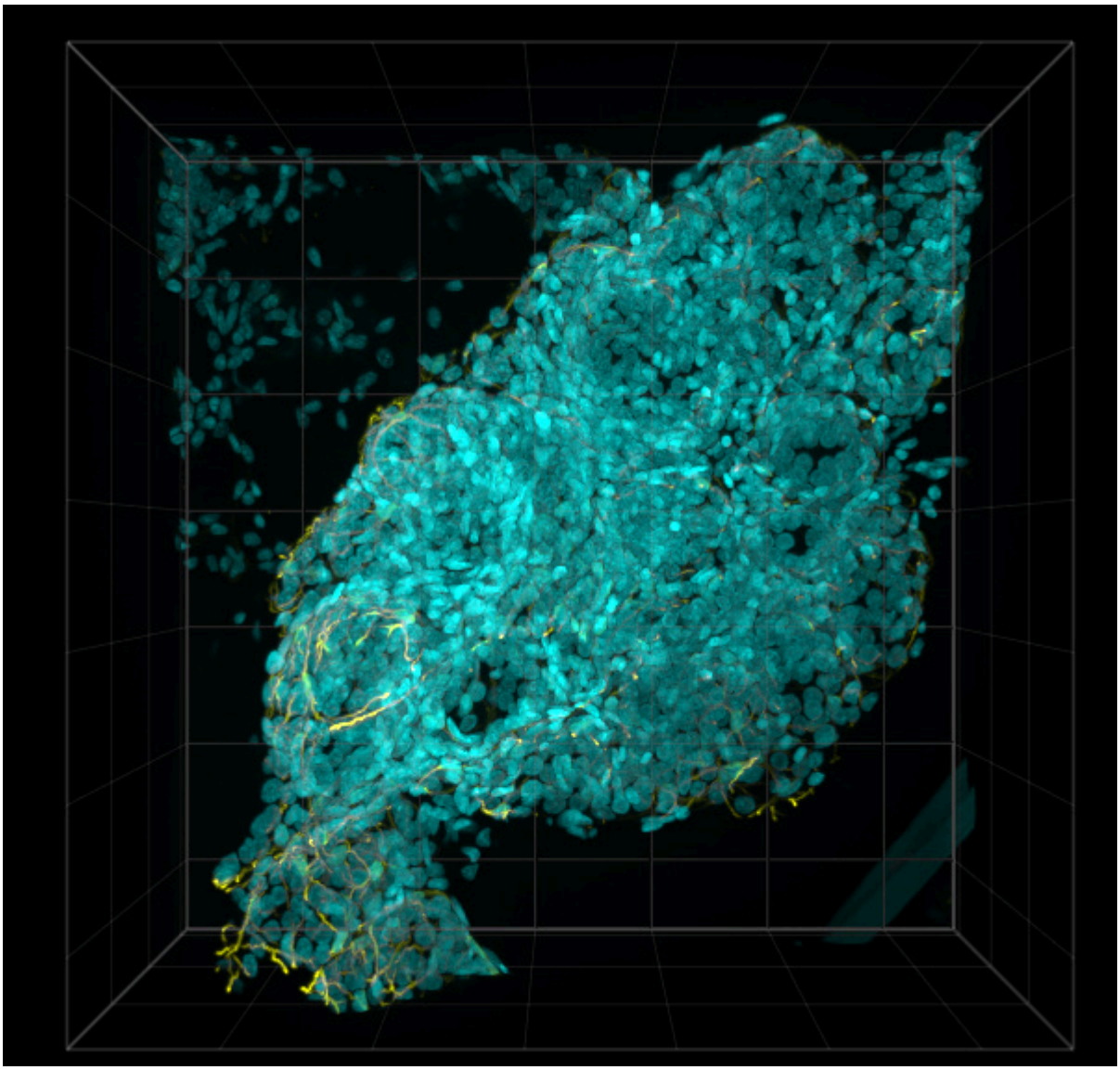
11 Time Readout

Displays a readout of time information for the data set.

12 Time Controls

Control navigation through time.

13 Rendering Area

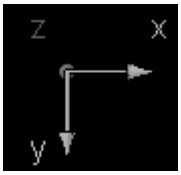


Interactive rendering area.

14 Status

Displays the current elapsed time, timepoint number, and size of gridlines on the rendering. Grid display can be adjusted using Volume View Settings window.

15 Axis Display



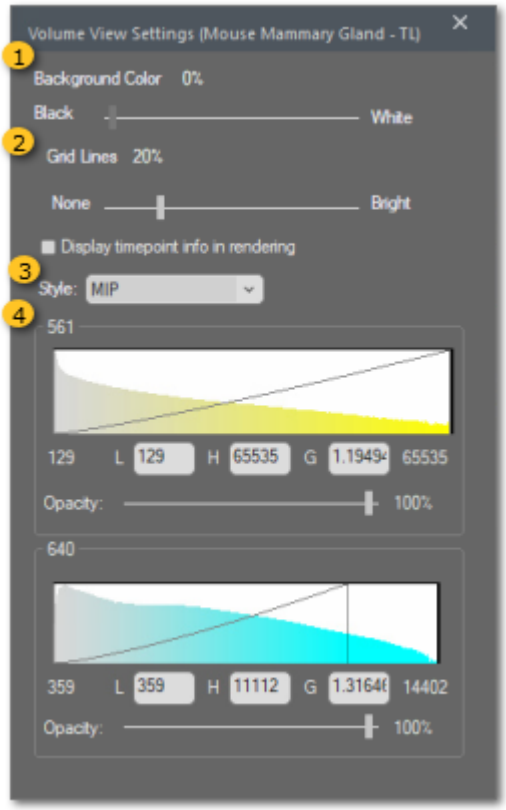
Displays the current angle being rendered. You may adjust the rendered angle by clicking and dragging on this display or the rendered image.

Volume View Settings window

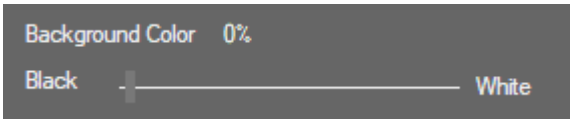
The appearance of channels and rendering approaches in ***3D/4D Volume View*** can be adjusted using the Volume View Settings dialog.

See Also:

[3D/ 4D Volume View](#)
[Renormalize Image window](#)

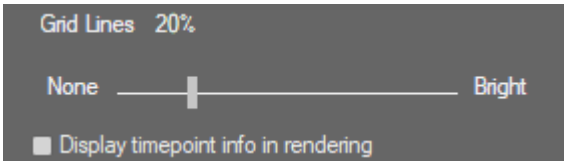


1 Background Color Control



Adjusts the background color of the rendering area from black to white.

2 Grid Lines Control



Adjusts the appearance of the grid lines in the rendering area from not being displayed to being displayed in a bright contrasting color. For example, when using ***Black*** background color, ***Bright*** gridlines will be rendered in white. When using ***White*** background color, ***Bright*** gridlines will be rendered in black.

3 Display Style menu

Select a ***Style*** from the drop down menu as follows:

Dynamic Lighting – allows you to illuminate your object as you wish by allowing control of the lighting angle. Useful for determining surface characteristics.

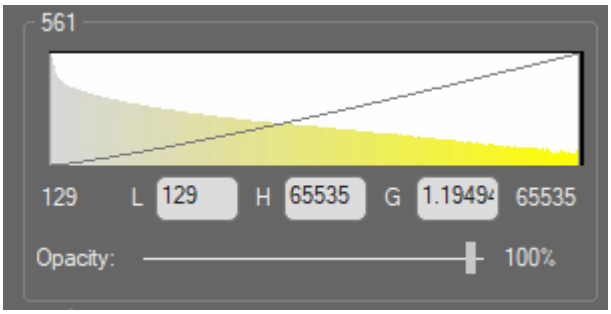
Fixed Lighting – uses a fixed lighting pattern for shading, approximates surface characteristics.

MIP – Displays the pixel of maximum intensity along the axis perpendicular to the viewing plane. This type of rendering does not require an advanced graphics card; however, performance of certain functions will be improved with one.

X Ray – Displays the summed intensity along the axis perpendicular to the viewing plane.

You may change the default rendering ***Style*** by going to ***Administration tab > Hardware Properties > Volume Rendering***.

4 Renormalization Control



Each channel has its own renormalization histogram. The histogram is a graphical representation of intensity values of the pixels of an image. The x-axis represents intensity value in gray levels; the y-axis represents number of pixels. By adjusting ***Low***, ***High***, and ***Gamma*** values, you can alter the way the data is displayed without altering the underlying data.

The left vertical line designates a ***Low*** value for display. You may click and drag on this line to adjust the low intensity value. Pixels with intensity values at or below the low level will be displayed as black. Alternatively, you may type a value in the ***Low*** (left) edit field.

The right vertical line designates a ***High*** value for display. You may click and drag on this line to adjust the high intensity value. Pixels with intensity values at or above the high level will be displayed as fully saturated. Alternatively, you may type a value in the ***High*** (right) edit field.

The black diagonal line between the low and high value lines controls ***Gamma*** scaling. By clicking and dragging on this line, you are adjusting gamma or scaling the displayed pixels nonlinearly to emphasize very dim (line arches up, value < **1.0**) or very bright features (line curves down, value > **1.0**). If you click and drag the line outside the bounds of the histogram, gamma will return to a value of **1.0**.

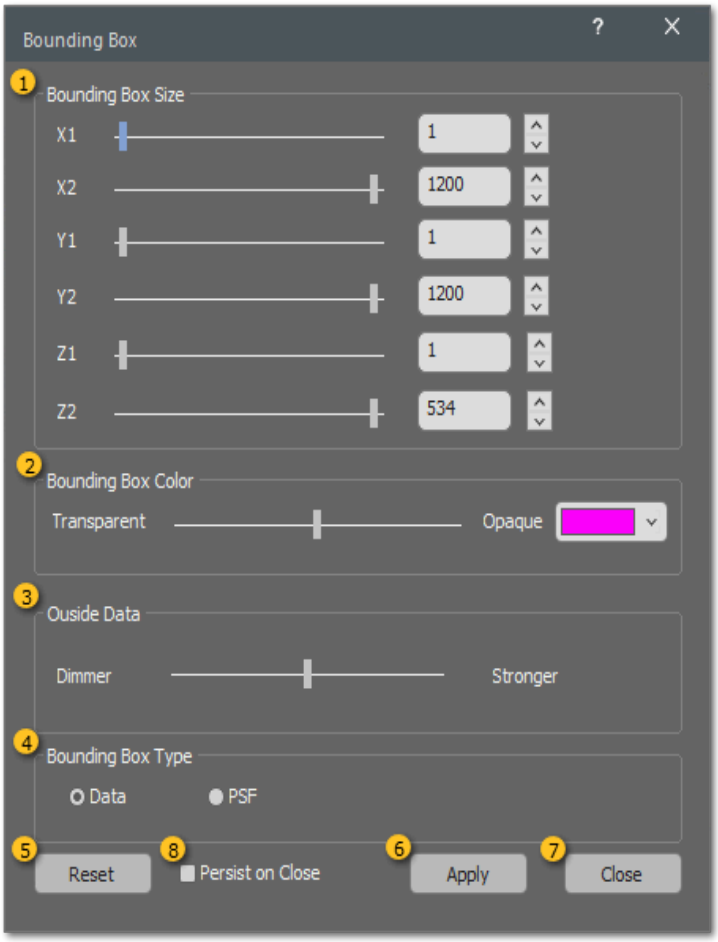
The ***Opacity*** slider allows you to control the transparency of the channel.

Bounding Box

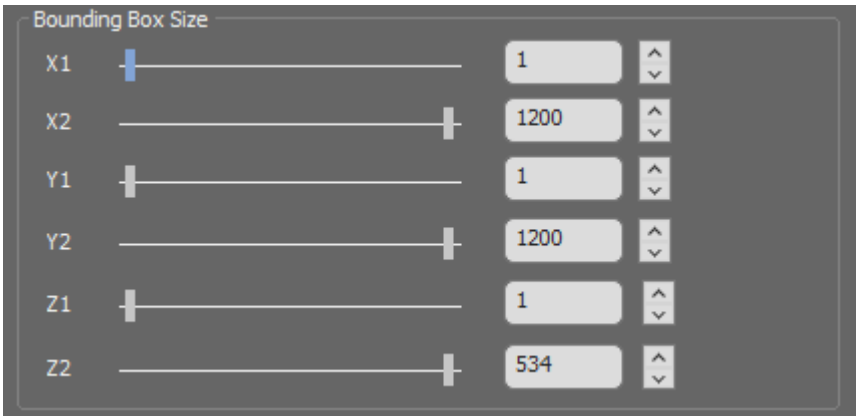
The ***Bounding Box*** allows for orthogonal slicing of a 3D or 4D volume. This is particularly useful to highlight a certain area in a volume.

See also:

[3D/ 4D Volume View]



1 Bounding Box Size group

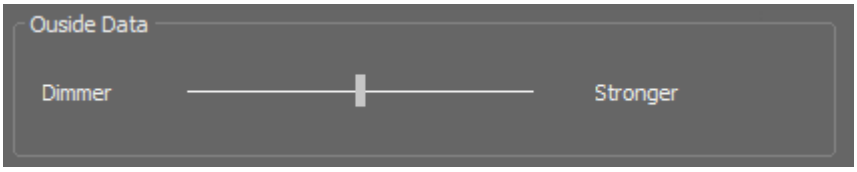


Each of the six slider bars corresponds to one of the sides of the volume cube. You can also type in a value or use the arrow buttons to increment the position of the bounding box in smaller steps.

2 Bounding Box Display group

Changes the transparency of the wireframe of the bounding box. The drop down menu at the right allows you to change to color of the wireframe.

3 Outside Data Display group

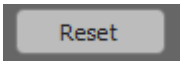


Changes the brightness of the data outside of the bounding box. Dimmer will reduce the brightness of the data, and stronger will increase the brightness.

4 Bounding Box Type

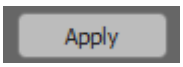
The Data Bounding Box is the volume that will be used for downstream applications, like Extract/Crop or Joint Deconvolution and Channel Transforms. The PSF bounding box will extract the volume to be used as a PSF. It is possible to have two separate bounding boxes in the same image.

5 Reset button



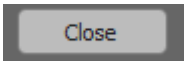
Resets all bounding box controls.

6 Apply button



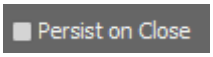
Applies the bounding box settings to the volume and saves with the capture when you save the slide.

7 Close Button



Closes the window.

8 Persist on Close



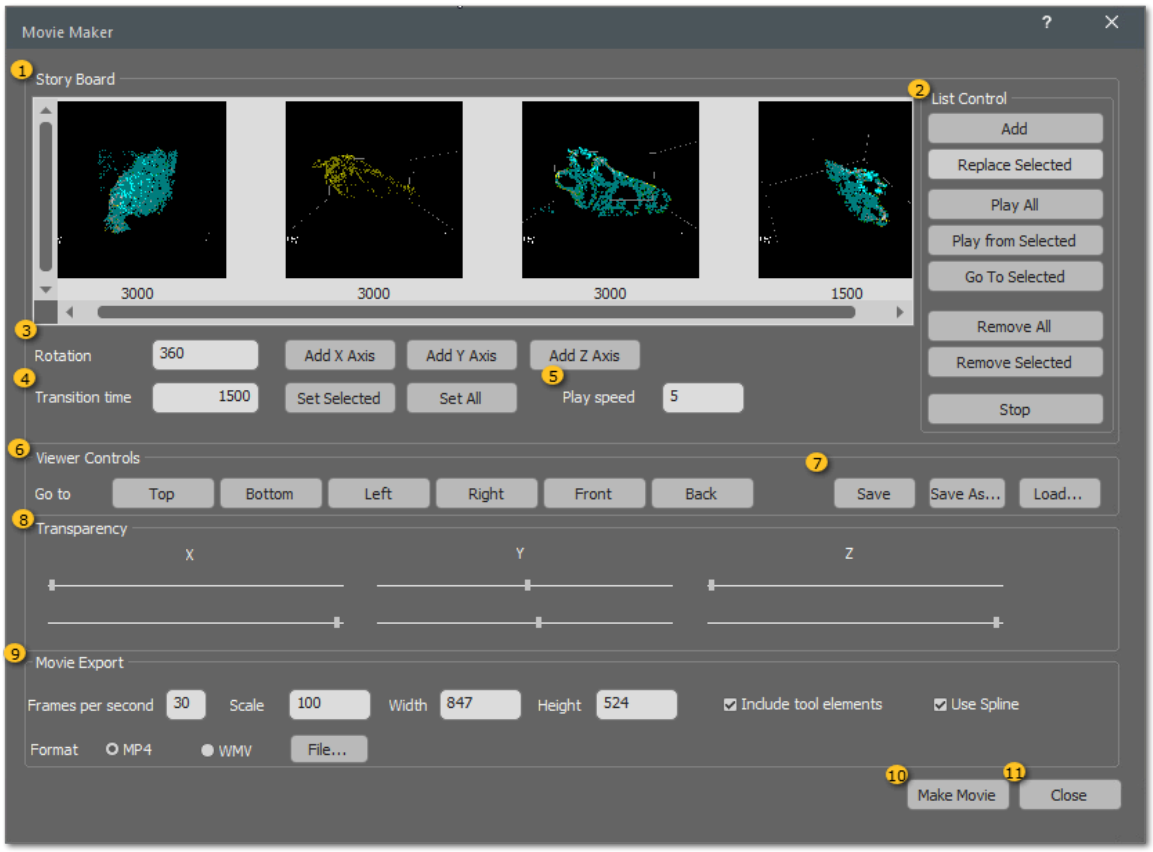
Will keep the Outside Data display as adjusted but remove the bounding box frame on closing the window. This allows to view image slices in the 3D Volume rendering.

Movie Maker

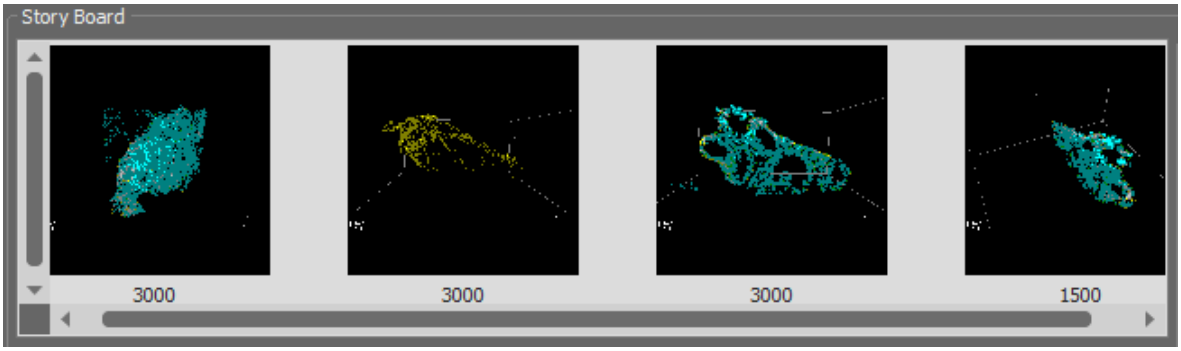
The ***StoryBoard*** allows for creation of custom movies from a 3D or 4D volume by establishing keyframes and rendering the interstitial frames automatically.

See also:

[3D/ 4D Volume View]



1 Story Board



Displays keyframes added to the storyboard. Keyframes are displayed with their position and transition time.

2 List Control

- ***Add:*** Adds a new keyframe to the storyboard
- ***Replace Selected:***
- ***Play All:*** Plays a movie through all keyframes from the beginning
- ***Play from Selected:*** Plays a movie from the currently selected keyframe
- ***Go to Selected:*** Moves the volume view to the position and orientation of the selected keyframe

- ***Remove All:*** Removes all keyframes from the storyboard
- ***Remove Selected:*** Removes the selected keyframe from the storyboard

- ***Stop:*** Stops the movie from playing

3 Rotation Controls Group

Adds a rotation over the desired number of degrees about whichever axis is clicked to add. For example, a 270 degree rotation about the Z axis would be added by typing 270 into the Rotation controls text box and then clicking Add Z Axis.

4 Transition Controls Group

Sets the transition time and play speed between either selected keyframes or all keyframes. Time units are in milliseconds.

5 Play Speed

Controls the speed of playback. A speed of 1 is 1x playback, 2 is 2x, etc.

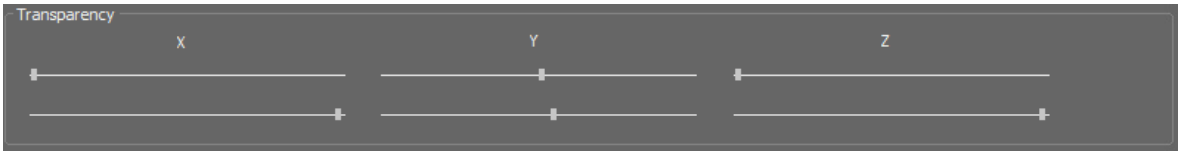
6 Viewer Controls Group

Snaps the orientation and position of the volume view to the corresponding position.

7 Save/Load Group

Save and load the storyboard for later use.

8 Transparency Group

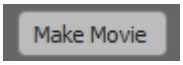


Allows the adjustment of transparent planes in X, Y and Z to display image slices in 3D.

9 Movie Export Group

Specifies playback frames per second (a greater number of fps results in a smoother playback between keyframes), and the scale, height, and width can be adjusted. Tool elements (volume view wireframe and gridlines, axes) can be turned on and off with the checkbox, as well. The export supports either MP4 or WMV format, and the File... button specifies the location and filename of the output movie. Use Spline allows for smooth frame interpolation, giving a much smoother animation.

10 Make Movie button



Executes the rendering and export to the file designated in the Movie Export Group.

11 Close button

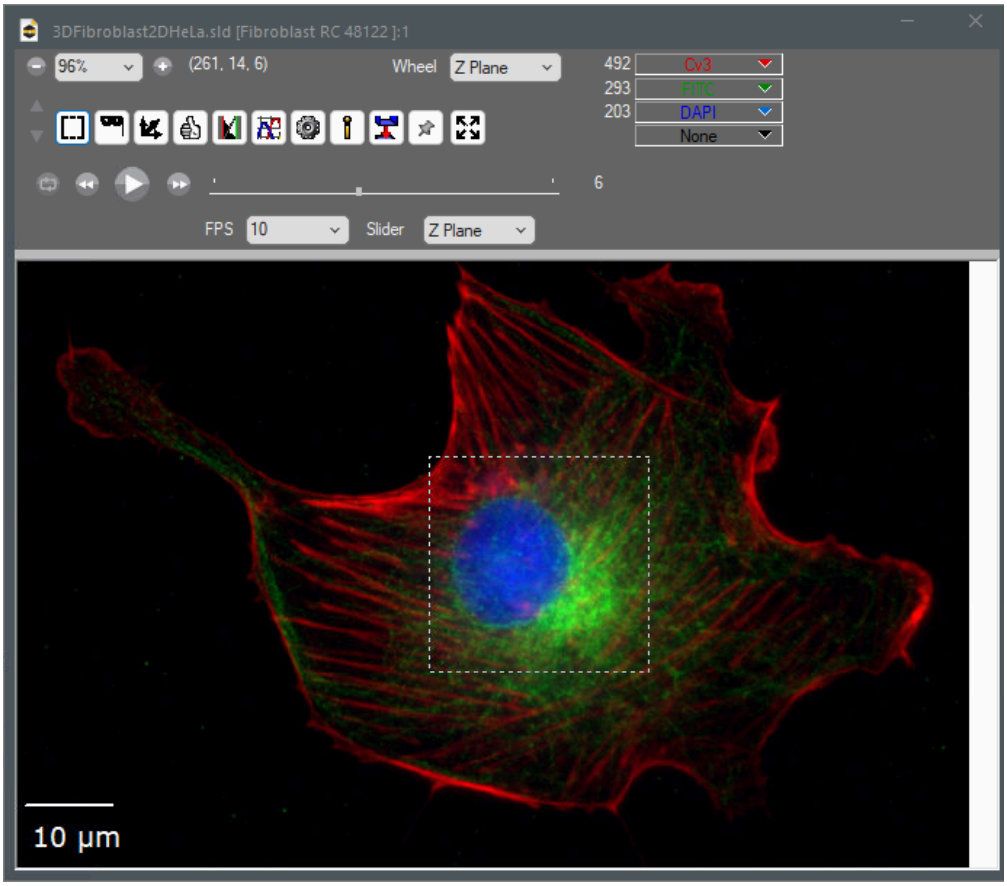
Closes the window without action.

TUTORIAL: Open a smaller volume of an image in the 3D/ 4D Volume Viewer

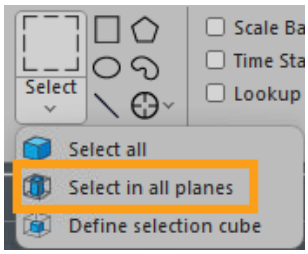
This tool is designed to view a smaller portion of your image in the highest resolution in the 3D/4D Volume Viewer.

1. Open the capture of interest in Main view by double-click on the capture in the **.sld/.sldy** file

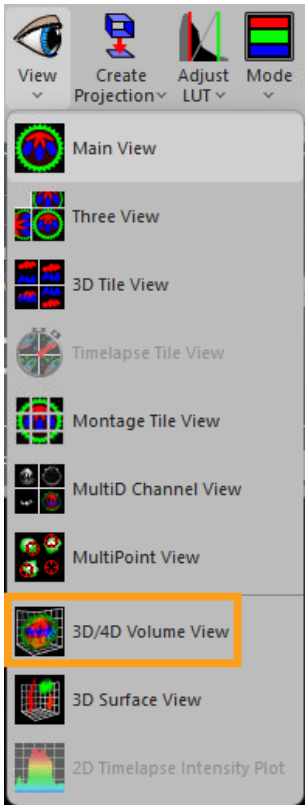
2. Use the ***Marquis Tool*** and select the area of interest



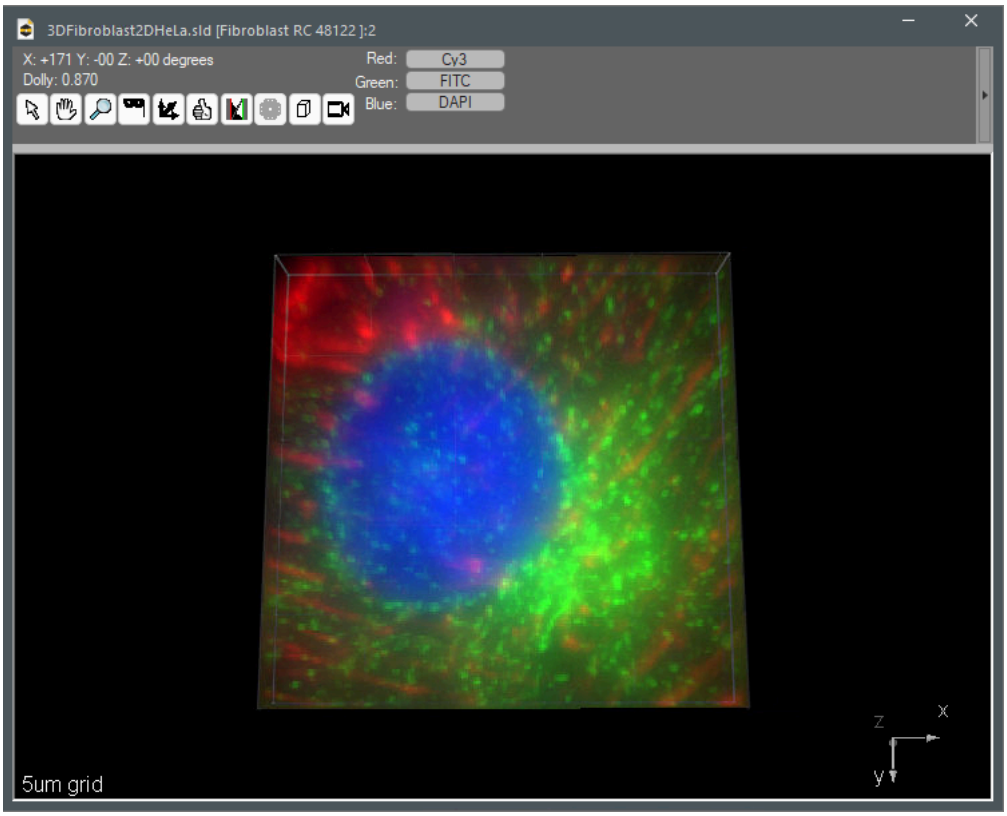
3. Navigate to the main menu bar > ***Regions*** group > ***Select*** drop-down > Select in all Planes



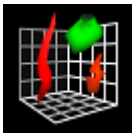
4. With the capture still highlighted navigate to the main menu bar > ***View*** group > ***View*** drop-down > 3D/ 4D Volume View



5. The 3D/ 4D Volume View will show the selected volume only



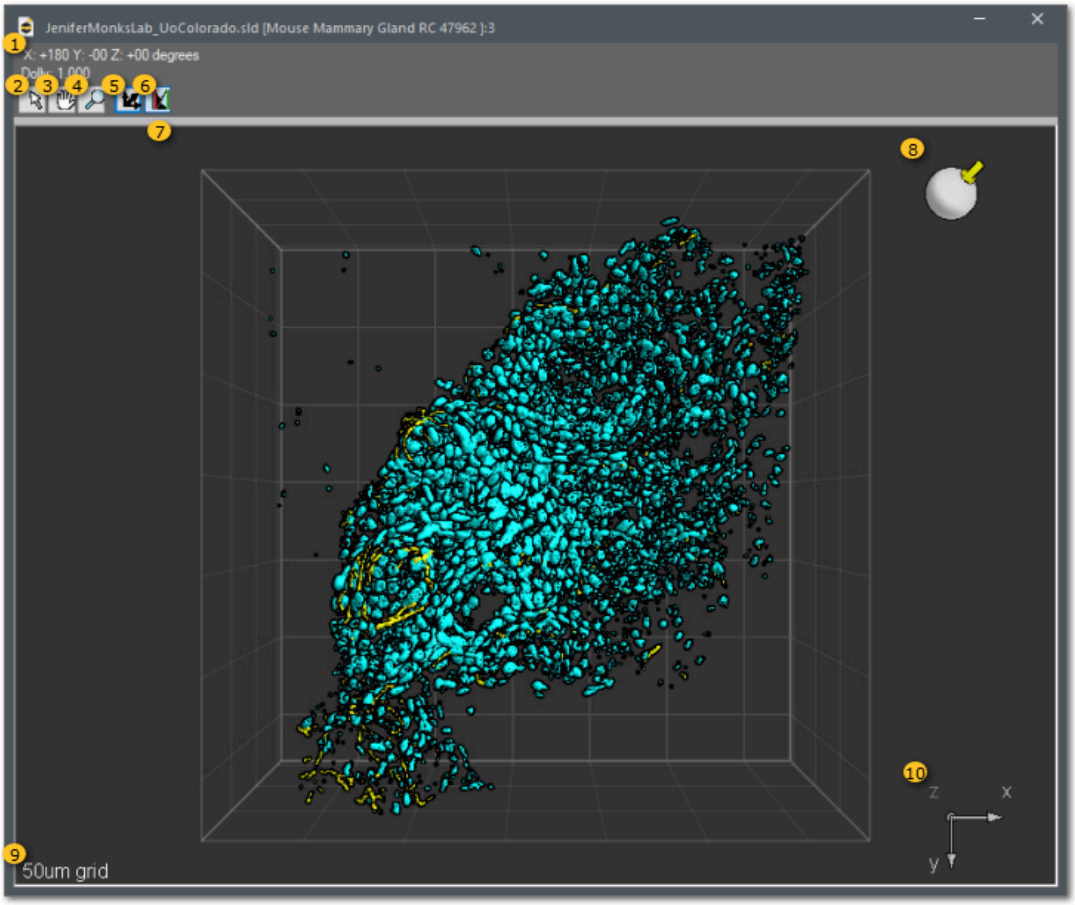
3D Surface View



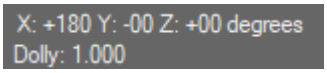
The ***3D Surface View*** is a useful approach for modeling three dimensional data. By adjusting outlines, scaling, and transparency, you can create renderings that aid in advanced visual analysis. In surface rendering, pixels are either displayed or not based on a threshold value. Due to this nature, it is possible to render mask objects in a surface view.

See also:

- 3D Surface View Settings window
- Main View
- Export Movie
- 3D/ 4D Volume View
- Three View
- 3D Tile View



1 Axis Readout



Displays a readout of the angle of the view in ***X***, ***Y***, and ***Z***. ***Dolly*** refers to the magnification factor of the display.

2 Rotate button

Select this button to turn the cursor into a rotate tool.

Click and drag in the display area to adjust the angle of the image.

A "click and flick" motion will set the image spinning. To "click and flick", click on the image and drag the mouse in the direction you would like the image to rotate. Release the mouse button while it is still in the display area.

To stop the rotation, simply click on the image again.

3 Translate button

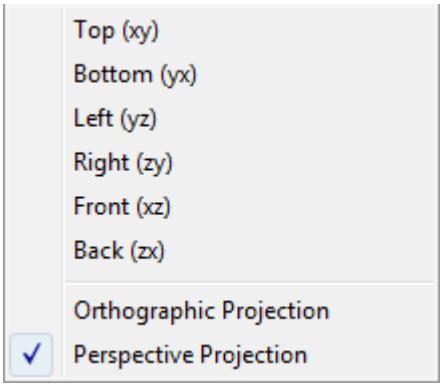
Select this button to change the cursor to a translation hand tool. Once selected, you may click and drag on the image to change the center axis position around which the image rotates.

4 Zoom button

Select this button to turn the cursor into a zoom tool. Using this tool, you may hold down the left mouse button and move the mouse away from you to zoom out (decrease magnification) or move the mouse towards you to zoom in (increase magnification).

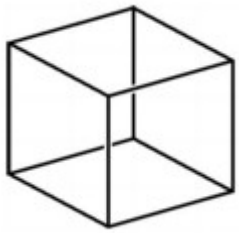
5 Axis button

Opens the ***Axis Menu***:

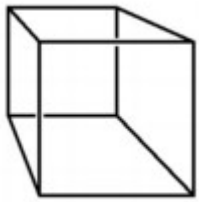


Top, ***Bottom***, ***Left***, ***Right***, ***Front***, and ***Back*** refer to specific angles for display of the data where ***z*** is the depth referring to intensity.

Orthographic Projection is a 2D projection of the 3D data set where the third dimension lines are drawn parallel, that is the object remains the same size as it is further from the viewer.



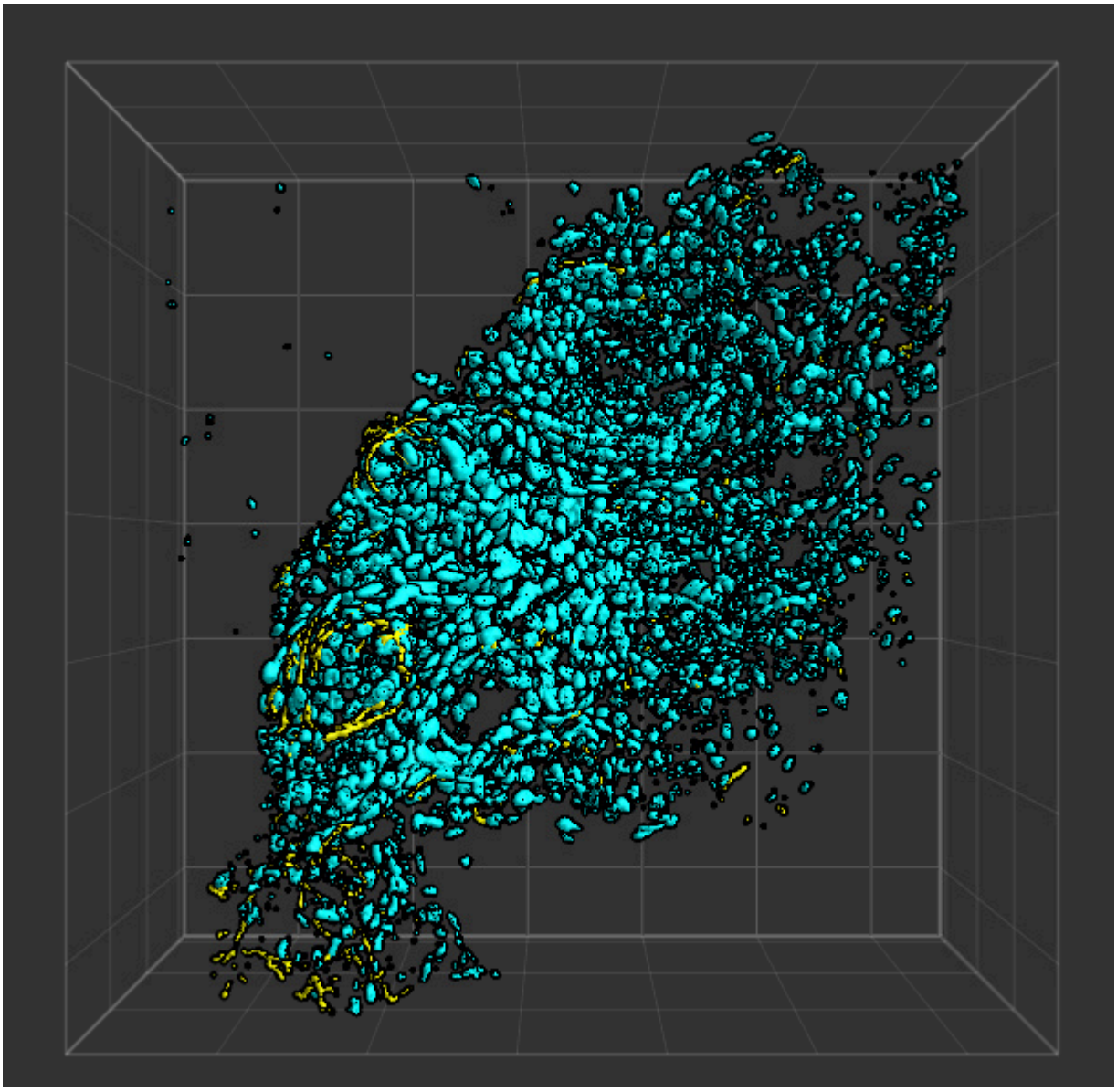
Perspective Projection is a 2D projection of the 3D data set where the third dimension lines are drawn to a vanishing point, that is the object gets smaller as it is further from the viewer.



6 Renormalize button

Opens the 3D Surface View Settings window. In this window, you can adjust numerous display parameters for the rendering such as intensity scaling and background color.

7 Rendering Area



Interactive rendering area.

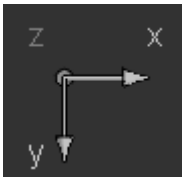
8 Lighting Control

Click and drag the ***Lighting Control*** to adjust the angle of lighting and shadows effects.

9 Grid Size

Displays the size of one square of the grid. Grid display can be adjusted using 3D Surface View Settings window.

10 Axis Display



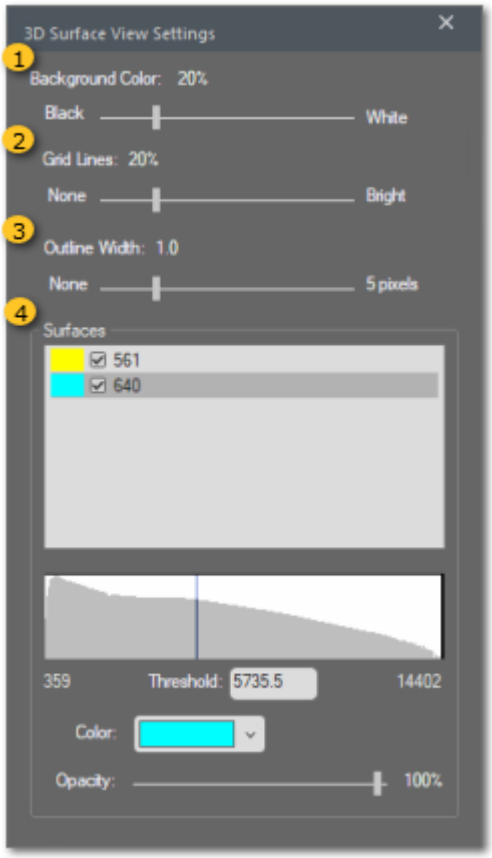
Displays the current angle being rendered. You may adjust the rendered angle by clicking and dragging on this display.

3D Surface View Settings window

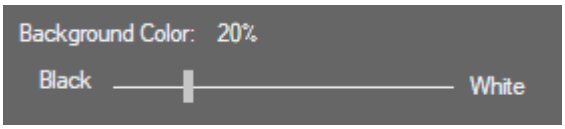
The appearance of channels and rendering approaches in ***3D Surface View*** can be adjusted using the 3D Surface View Settings dialog.

See Also:

[3D/ 4D Volume View](#)
[Renormalize Image window](#)



1 Background Color Control



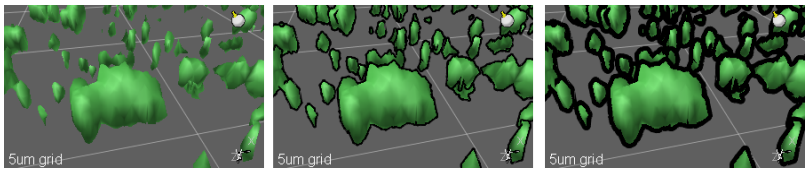
Adjusts the background color of the rendering area from black to white.

2 Grid Lines Control

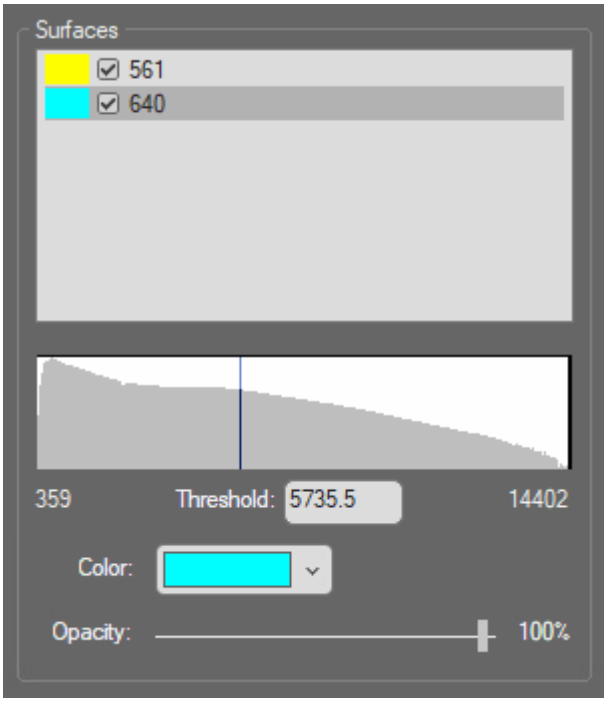
Adjusts the appearance of the grid lines in the rendering area from not being displayed to being displayed in a bright contrasting color. For example, when using ***Black*** background color, ***Bright*** gridlines will be rendered in white. When using ***White*** background color, ***Bright*** gridlines will be rendered in black.

3 Outline Width

Select a ***Outline Width*** by using the slider to select between ***None*** and ***5 pixels***. This is the width of the outline of objects as illustrated below. From left to right examples of **None**, **2.0 pixels**, and **5 pixels**.



4 Renormalization Control



Renormalization control in surface rendering is slightly different from previously discussed renormalization approaches. In surface rendering, each pixel is either displayed or not based on a threshold value.

In the ***Surfaces*** field, check the box for each channel or mask you would like to render. Select a channel or mask for adjustment by clicking on it in the list. In the example above, the **561Pos3** channel is selected.

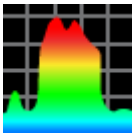
Click and drag the line in the histogram to adjust the ***Threshold*** value for the selected channel. Alternatively, you may type a value in the edit field. It may take a second or two for the rendering to update after threshold adjustment.

To adjust the rendered color of the channel of mask, simply click on the ***Color*** button and use the standard Microsoft Windows Color dialog.

The ***Opacity*** slider allows you to control the transparency of the channel. Because each pixel is either displayed or not, you may find it necessary to adjust opacity in order to display internal objects.

Repeat adjustments as desired for each channel and mask.

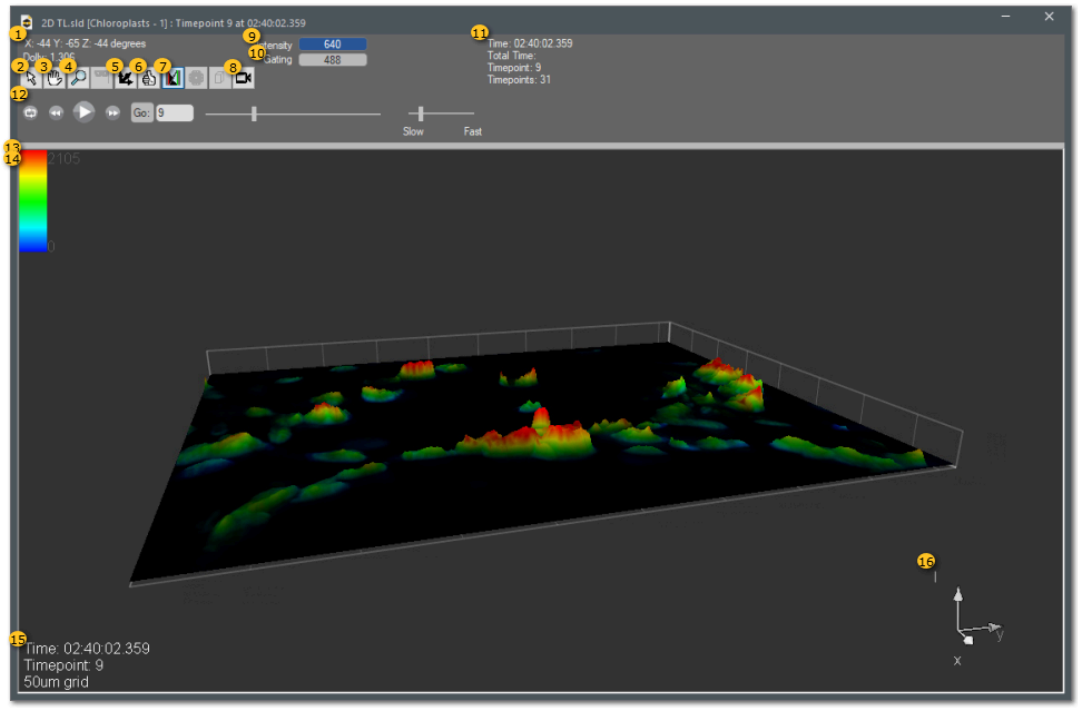
2D Timelapse Intensity Plot window



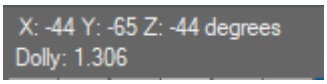
The ***2D Timelapse Intensity Plot*** is a powerful way to illustrate intensity changes over time. This tool can be particularly useful when visually analyzing photomanipulation or ratio data.

See also:

[Timelapse Tile View]
[The 2D Timelapse Intensity Plot]
[Exporting from the 2D Timelapse Intensity Plot]



1 Axis Readout



Displays a readout of the angle of the view in ***X***, ***Y***, and ***Z***. ***Dolly*** refers to the magnification factor of the display.

2 Rotate button

Select this button to turn the cursor into a rotate tool.

Click and drag in the display area to adjust the angle of the image.

A "click and flick" motion will set the image spinning. To "click and flick", click on the image and drag the mouse in the direction you would like the image to rotate. Release the mouse button while it is still in the display area.

To stop the rotation, simply click on the image again.

It is possible to move through time while the image is rotating.

3 Translate button

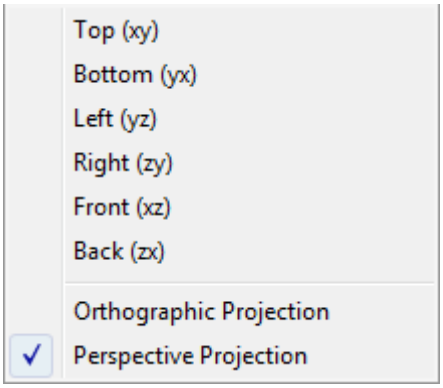
Select this button to change the cursor to a translation hand tool. Once selected, you may click and drag on the image to change the center axis position around which the image rotates.

4 Zoom button

Select this button to turn the cursor into a zoom tool. Using this tool, you may hold down the left mouse button and move the mouse away from you to zoom out (decrease magnification) or move the mouse towards you to zoom in (increase magnification).

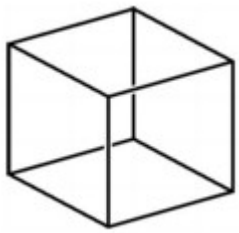
5 Axis button

Opens the ***Axis Menu***:

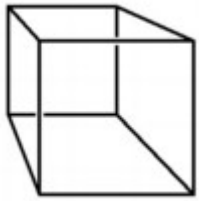


Top, ***Bottom***, ***Left***, ***Right***, ***Front***, and ***Back*** refer to specific angles for display of the data where ***z*** is the depth referring to intensity.

Orthographic Projection is a 2D projection of the 3D data set where the third dimension lines are drawn parallel, that is the object remains the same size as it is further from the viewer.



Perspective Projection is a 2D projection of the 3D data set where the third dimension lines are drawn to a vanishing point, that is the object gets smaller as it is further from the viewer.



6 Thumb-up button

Pressing the ***Thumb-up*** button resets the thumbnail view in the **.sld/.sldy** and the way the image opens as default. Parameters saved with this button include renormalization and plane.

7 Renormalize button

Opens the ***2D Timelapse Intensity Plot Settings*** dialog. In this window, you can adjust numerous display parameters for the rendering such as intensity scaling and background color. Read more about adjusting settings here. Pressing the ***Thumb-up*** button resets the thumbnail view in the **.sld/.sldy** and the way the image opens as default. Parameters saved with this button include renormalization and plane.

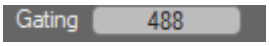
8 Movie Maker button

Opens the Movie Maker window, which allows for creation of custom movies from a 3D or 4D volume by establishing keyframes and rendering the interstitial frames automatically.

9 Intensity Selection

Click the button to select a channel to show in the rendering. Only one channel may be rendered at once.

10 Gating Selection



Click the button to select a channel to gate the intensity channel by. This option is used primarily when visualizing ratio data. For more information on how gating works with ratio data, refer to Viewing Ratio Data in SlideBook. While this tutorial covers a main view, information on how gating impacts the display is discussed in steps 5-7.

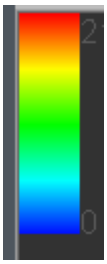
11 Time Readout

Displays a readout of time information for the data set.

12 Time Controls

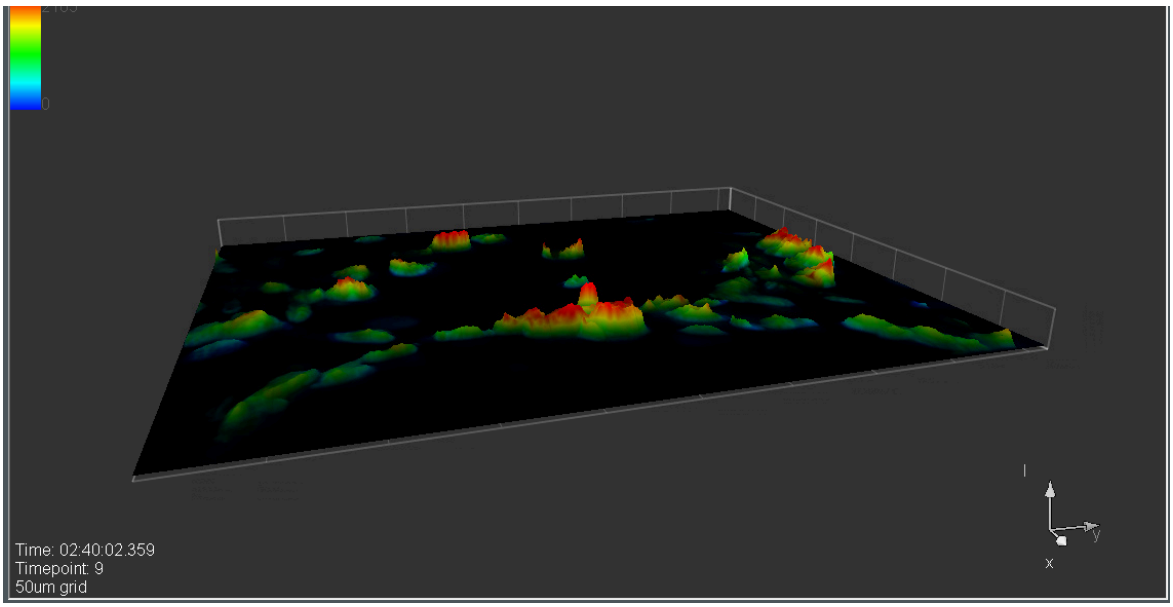
Control navigation through time.

13 Lookup Table



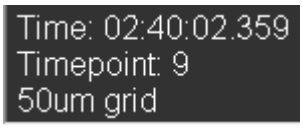
Displays the color map for image intensity information, can be adjusted using the 2D Timelapse Intensity Plot Settings.

14 Rendering Area



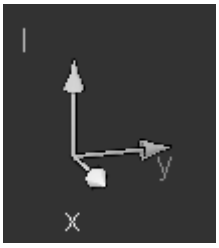
Rendering of 2D time lapse intensity data.

15 Status Display



Displays the current time point number and size of gridlines on the rendering. Timepoint display can be turned off using the 2D Timelapse Intensity Plot Settings.

16 Axis Display



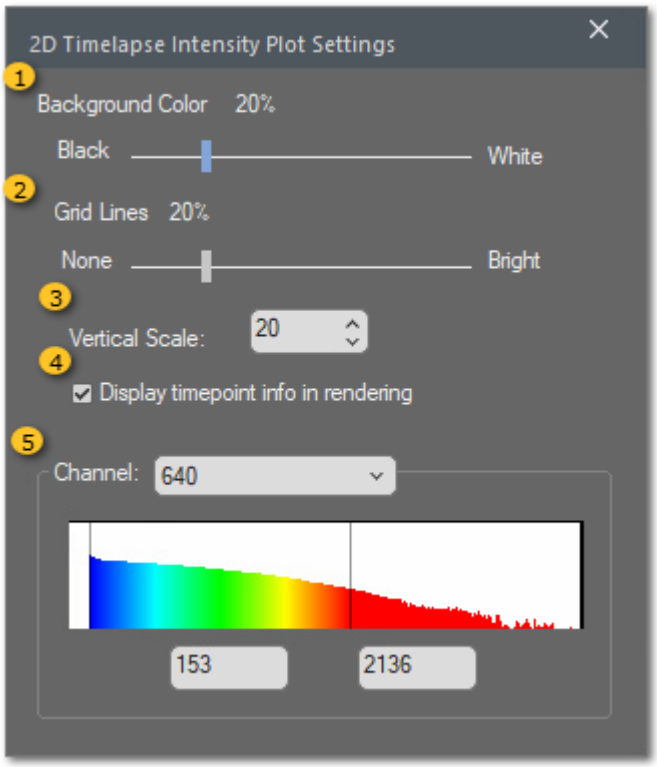
Displays the XY coordinates and intensity axis of the current angle being rendered. You may adjust the rendered angle by clicking and dragging on this display.

2D Timelapse Intensity Plot Settings window

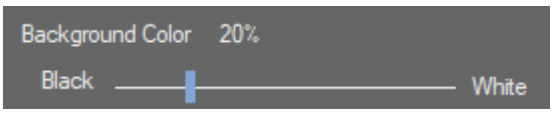
The appearance of channels and rendering approaches in ***2D Timelapse Intensity Plot*** can be adjusted using the 2D Timelapse Intensity Plot Settings dialog.

See Also:

[2D Timelapse Intensity Plot window]



1 Background Color Control



Adjusts the background color of the rendering area from black to white.

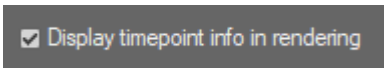
2 Grid Lines Control

Adjusts the appearance of the grid lines in the rendering area from not being displayed to being displayed in a bright contrasting color. For example, when using ***Black*** background color, ***Bright*** gridlines will be rendered in white. When using ***White*** background color, ***Bright*** gridlines will be rendered in black.

3 Vertical Scale

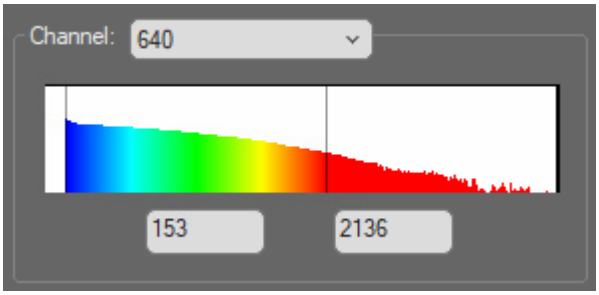
Adjusts the depth for intensity display. A value of **0** will give a flat rendering.

4 Display timepoint info in rendering check



Check the box to display the current time point number in the rendering as shown in Status Display.

5 Renormalization Control

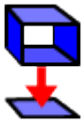


Use the drop-down menu to select the channel for renormalization.

Use the two black bars to adjust renormalization for the selected channel. The x-axis of the graph represents intensity value; the y-axis represents number of pixels.

Pixels with values to the left of the range or below the low value will be displayed in blue. Pixels with values to the right of the range or above the high value will be displayed in red.

Create Projection Image window

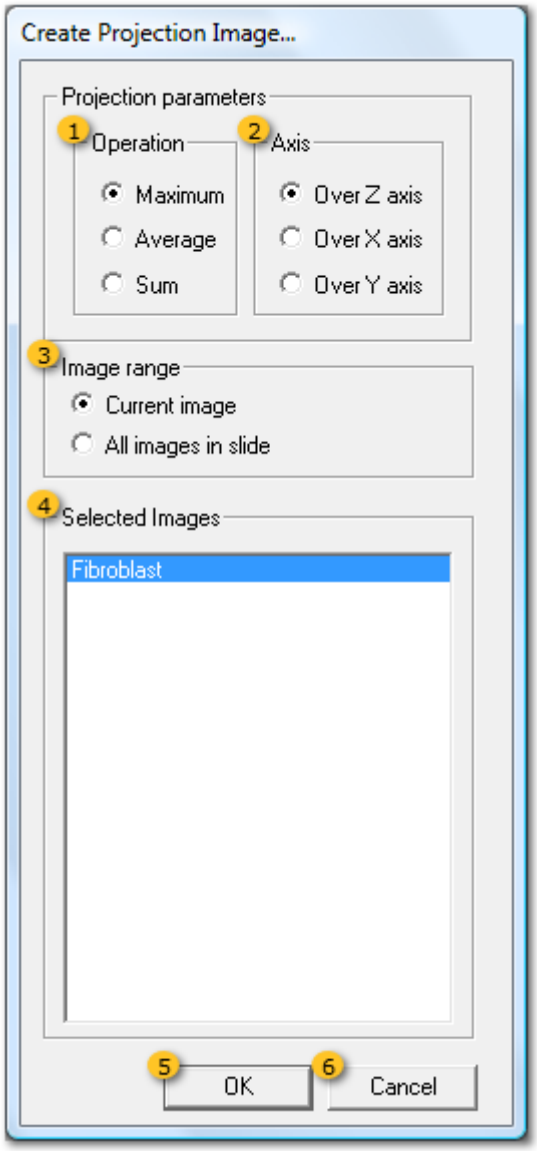


Often it is necessary to display 3D data in a 2D format. One popular way to do so is to create a projection image of the data set. The most popular type of projection image is a qualitative maximum intensity projection. This method projects a ray through a stack of vowels to determine the maximum intensity pixel then uses that pixel to create a 2D image. This is performed for each channel at each pixel location. Alternatively, average or sum projections can be created in this matter.

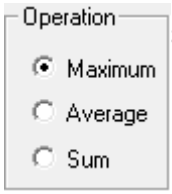
NOTE: Selecting this operation from the main ribbon will generate a new image in the **.sld**. Should you wish to simply view your data as a projection over z, you can toggle the projection icon in a ***Main View***.

See also:

- Three View
- 3D Tile View
- 3D/ 4D Volume View



1 Operation group



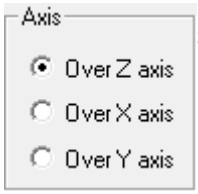
Select the desired ***Operation*** to create the projection image as follows:

Maximum - Uses the maximum intensity found in each channel in the column of pixels in the axis designated

Average - Uses the average (mean) intensity found in each channel in the column of pixels in the axis designated

Sum - Uses the sum intensity found in each channel in the column of pixels in the axis designated, resulting image can be used quantitatively. It is important to note that the sum value is limited to 16 bit space (maximum intensity 65535). If the true summed value exceeds this amount, all values will be rescaled accordingly on a per channel basis. Scale factors can be found in the ***Comments*** section of Image Info. Simply multiply the reported 16 bit value by the scale factor to retrieve the true summed value.

2 Axis group

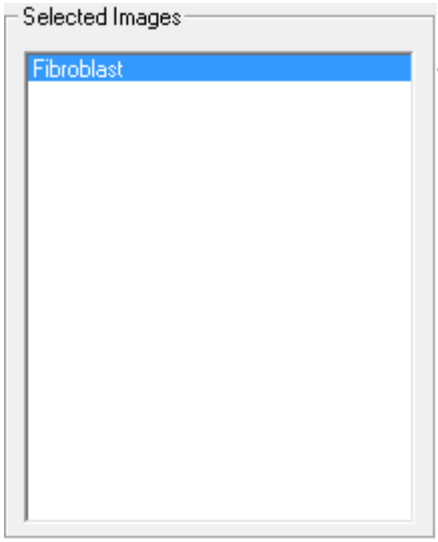


Select the axis over which you would like to create a projection image.

3 Image range group

Choose to create projection images for just the selected image or all images in the slide.

4 Selected Images group

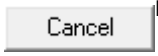


Displays a list of images that corresponds to the ***Image Range*** selected above.

5 OK button

Press ***OK*** to create projection image(s) as directed. New images will be written to the slide with the type of projection noted in the ***Comment*** section of ***Image Info***.

6 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

Adjust LUT window

Image renormalization allows you to adjust how the intensity values of an image are displayed. Adjusting renormalization does not affect the data values, only the display. Display settings can be saved for when the file is opened in the future and will be applied when exporting qualitative TIFFS under the ***View*** menu.

Clicking on the icon in the ***Home*** tab of SlideBook will open the Renormalize dialog as shown below. Alternatively, you may click on ***Adjust LUT*** to access a menu with the following options:

Renormalize all - Once an image is open and renormalized, press the thumbnail icon in the view window then select Renormalize all to apply the current image's renormalization to any matching channel names for all images in the select Renormalize all to apply the current image's renormalization to any matching channel names for all images in the **.sld/ .sldy**. You will need to re-open any images to see the renormalization changes take effect.

Renormalize all to global min/max - Select this option to use the lowest minimum and highest maximum intensity values found in all of the images in the slide file. You will need to re-open any images to see the renormalization changes take effect.

Batch Renormalization - Opens the Batch Renormalization dialog to renormalize multiple images based on selected criteria.

Densitometry functions allow you to generate calibration curves that can be used to convert intensity measurements into known concentrations. The most common biological application of this tool is to rescale images according to a set of standards that correspond to optical density, although other uses exist. To use this, you must capture an image with multiple regions of known concentrations. A calibration curve can then be generated and subsequently applied to sample data.

Lookup Tables - Allows for the import of customised LUT from e.g. Fiji

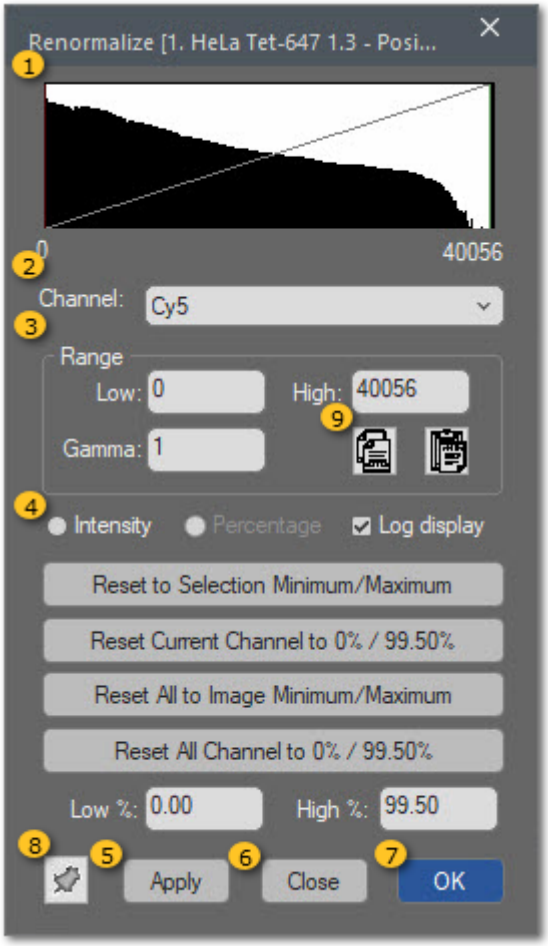
By default, image renormalization is displays the histogram for the pixels in the entire image. If you prefer, you can select an area using the marquee tool . When you open the ***Renormalize*** dialog, only the pixels in the selected area will be shown in the histogram. The renormalization will be applied to the entire image.

This function can also be accessed from the ***Renormalize*** icon in a view window.

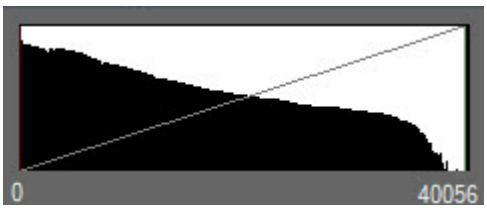
To save the renormalization settings for subsequent viewings of the data set, press the thumbs-up icon in the view window.

See also:

TUTORIAL: Adjusting Dislay of an Image
Ratio Module, TUTORIAL: Viewing Ratio Data in SlideBook



1 Histogram area



The histogram is a graphical representation of intensity values of the pixels of an image. The x-axis represents intensity value in gray levels; the y-axis represents number of pixels. By adjusting ***Low***, ***High***, and ***Gamma*** values, you can alter the way the data is displayed without altering the underlying data.

The red line designates a ***Low*** value for display. You may click and drag on this line to adjust the low intensity value. Pixels with intensity values at or below the low level will be displayed as black. Alternatively, you may type a value in the ***Low*** edit field.

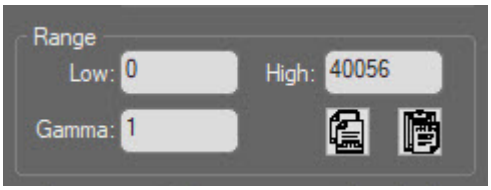
The green line designates a ***High*** value for display. You may click and drag on this line to adjust the high intensity value. Pixels with intensity values at or above the high level will be displayed as fully saturated. Alternatively, you may type a value in the ***High*** edit field.

The black diagonal line controls ***Gamma*** scaling. By clicking and dragging on this line, you are adjusting gamma or scaling the displayed pixels nonlinearly to emphasize very dim (line arches up, value < 1.0) or very bright features (line curves down, value > 1.0). If you click and drag the line outside the bounds of the histogram, gamma will return to a value of 1.0. Alternatively, you may type a value in the ***Gamma*** edit field.

2 Channel dropdown

Select a channel to renormalize from the dropdown menu. All channels will be present in the dropdown menu regardless of whether they are displayed in the image; be sure you are adjusting renormalization on a channel that is displayed in the image.

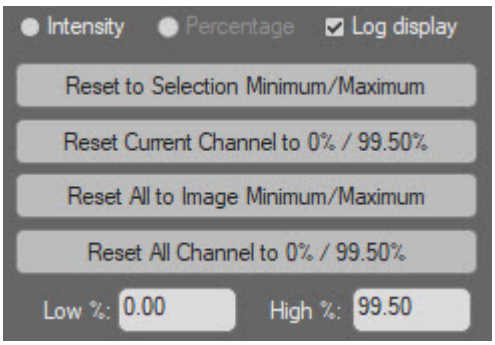
3 Range group



The ***Range*** group allows to you type in values for ***Low***, ***High***, and ***Gamma*** in order to adjust the way the data is displayed. When you type values in these fields, you must click ***Apply*** (5) to update the scaling on the image.

Alternatively, you may click and drag on the lines in the histogram as described above; this will immediately update in the image.

4 Method option



The ***Method*** option allows you to use image intensity properties to adjust the display rather than manually setting ***Low***, ***High***, and ***Gamma*** values.

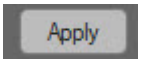
The ***Intensity*** radio button designated that you will be using intensity values for renormalization as opposed to percentages of pixels. ***Percentage*** is no longer an active renormalization approach.
Toggling ***Log display*** will change the histogram display between linear and logarithmic.

Reset to Selection Min/Max will use the minimum and maximum intensity for the channel selected to set the low and high values with a gamma of **1.0**.

Reset to 5%/95% Percentile is no longer an active renormalization approach.

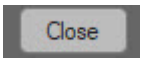
Reset all to Image Min/Max will use the lowest minimum intensity value of all channels present in the image and the highest maximum value of all channels present in the image to set the low and high values of all channels identically with a gamma of **1.0**.

5 Apply button



Press ***Apply*** to apply ***Low***, ***High***, and ***Gamma*** values set in the ***Range*** group. If you adjust renormalization by clicking and dragging lines in the histogram or by using the ***Method*** options, you will not need to press ***Apply***.

6 Close button



Choose ***Close*** to exit the dialog. If you would like subsequent views of the image to contain the same renormalization parameters, click on the thumbs-up button and re-save the **.sld/ sldy**.

7 OK button

Choose ***OK*** to exit the dialog. If you would like subsequent views of the image to contain the same renormalization parameters, click on the thumbs-up button and re-save the **.sld/ sldy**.

8 Pin button

Choose to ***Pin*** down a specific Renormalization window to use this window to display the respective histogram of all open images. The pinned Renormalization window will display the values of the image in the active (last clicked) viewer window.

9 Copy & Paste buttons

Choose ***Copy*** to copy the LUT Range (Low, High and Gamma) from one channel of the image, then select another channel of the same image or a different capture and ***Paste*** to enter the values into this channel's LUT Range.

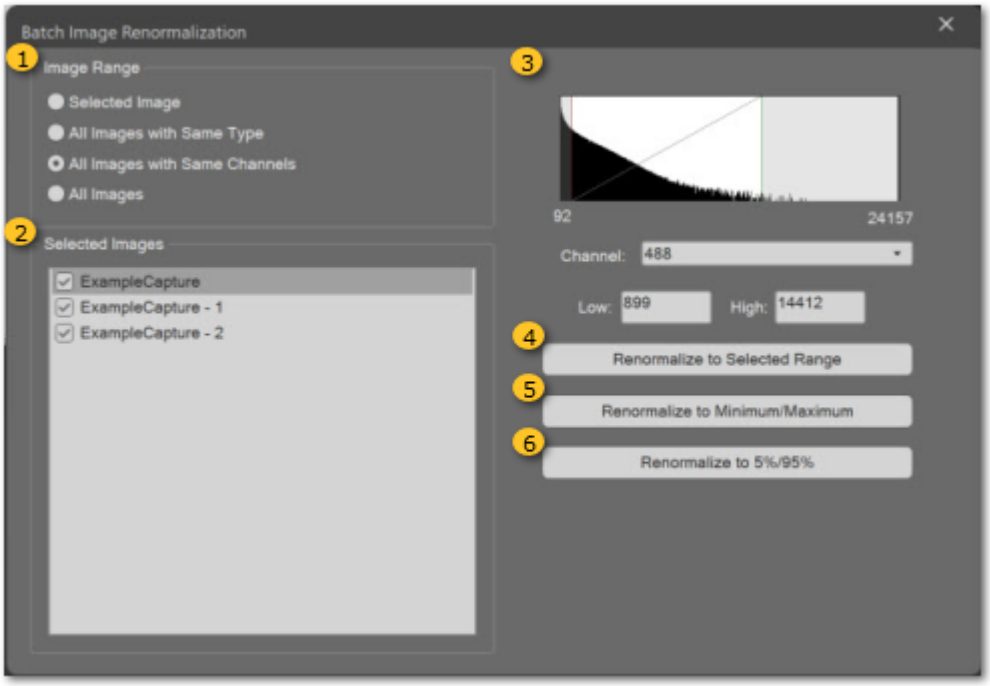
NOTE: If you hold the CTRL key pressed when hitting Copy the LUT Range values of all channels of this image will be copied by channel order. Then clicking Paste in the Renormalization window of another capture will paste the LUT range values for all channels in the channel order of the original image - disregarding the channel name.

Batch Image Renormalization window

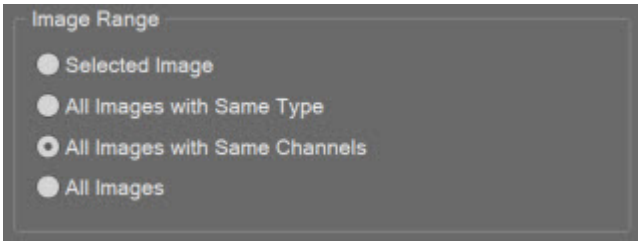
Batch Image Renormalization allows you to adjust brightness and contrast of multiple images within a **.sld** at one time.

See also:

[Renormalize Image window]



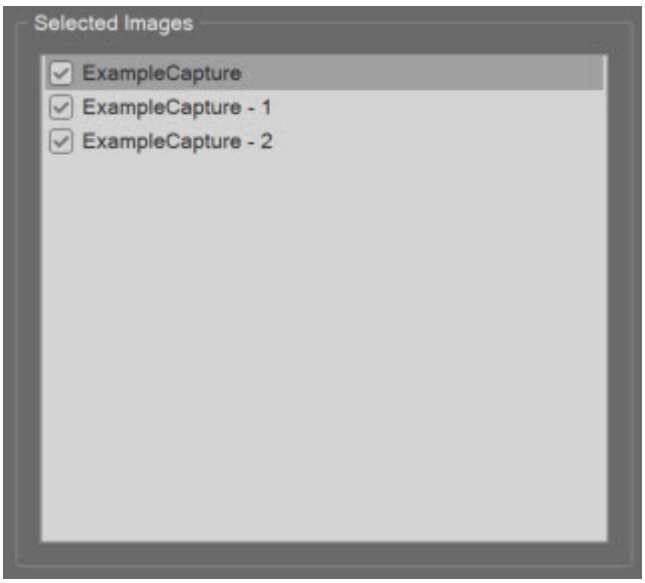
1 Image Range group



Select image range as follows:

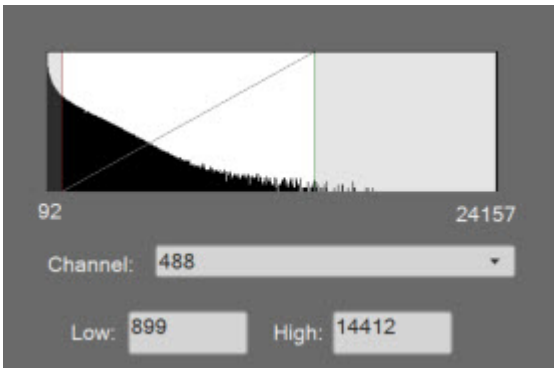
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Selected Images group



Check or uncheck boxes to select which images will be renormalized.

3 Histogram area



The histogram is a graphical representation of intensity values of the pixels of an image. The x-axis represents intensity value in gray levels; the y-axis represents number of pixels. By adjusting ***Low***, ***High***, and ***Gamma*** values, you can alter the way the data is displayed without altering the underlying data.

The red line designates a ***Low*** value for display. You may click and drag on this line to adjust the low intensity value. Pixels with intensity values at or below the low level will be displayed as black. Alternatively, you may type a value in the ***Low*** edit field.

The green line designates a ***High*** value for display. You may click and drag on this line to adjust the high intensity value. Pixels with intensity values at or above the high level will be displayed as fully saturated. Alternatively, you may type a value in the ***High*** edit field.

The black diagonal line controls ***Gamma*** scaling. By clicking and dragging on this line, you are adjusting gamma or scaling the displayed pixels nonlinearly to emphasize very dim (line arches up, value < 1.0) or very bright features (line curves down, value > 1.0). If you click and drag the line outside the bounds of the histogram, gamma will return to a value of 1.0. Alternatively, you may type a value in the ***Gamma*** edit field.

Select a ***Channel*** to renormalize from the dropdown menu. All channels will be present in the dropdown menu regardless of whether they are displayed in the image; be sure you are adjusting renormalization on a channel that is displayed in the image.

4 Renormalize to Selected Range button

Pressing ***Renormalize to Selected Range*** button will apply the selected renormalization to the selected images.

5 Renormalize to Min/Max button

Renormalize to Min/Max will use the lowest minimum intensity value of all channels present in the selected images and the highest maximum value of all channels present in the selected images to set the low and high values of all channels identically with a gamma of **1.0**.

6 Renormalize to 5%/95% button

Renormalize to 5%/95% will use set the lower threshold at 5% of the pixels and the highest threshold value at 95% of all of the pixels. That is, the lowest 5% of pixels will be displayed as black and the highest 5% of pixels will appear saturated with a gamma of **1.0**.

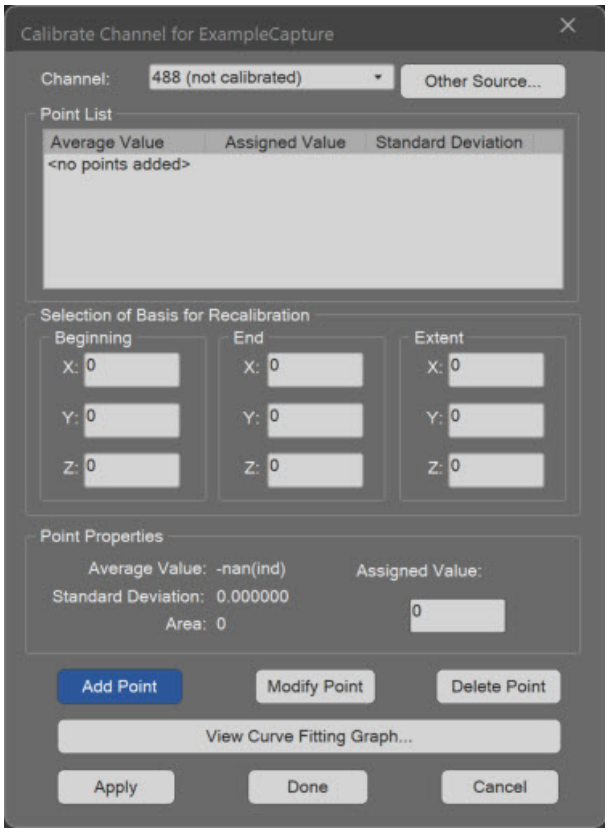
Densitometry

Denistometry functions allow you to generate calibration curves that can be used to convert intensity measurements into known concentrations. The most common biological application of this tool is to rescale images according to a set of standards that correspond to optical density, although other uses exist. To use this, you must capture an image with multiple regions of known concentrations. A calibration curve can then be generated and subsequently applied to sample data.

Generating a Calibration Curve

To generate a calibration curve:
1. Open a Main View of an image containing regions of data to be used for calibration.

2. Select ***Densitometry > Calibrate Channel*** from the Adjust LUT menu in the View group of the Home tab. The Calibration dialog box will appear:

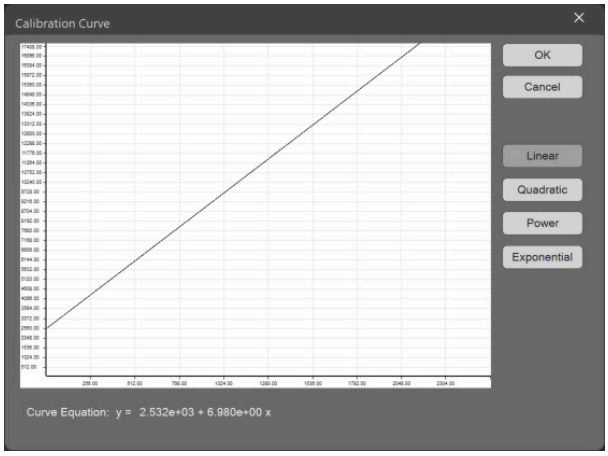


3. Use the marquee tool to highlight an area of known concentration. The ROI dimensions will appear in the X, Y, and Z edit fields.

4. Enter the concentration in the ***Assigned Value*** edit field.

5. Select ***Add Point***. The region and corresponding data will appear in the ***Point List***. Repeat steps 3-5 for additional concentrations/areas.

6. If you are not satisfied with a point in your list, select the point from the ***Points List*** by clicking on it, make the desired changes and then select ***Modify Point***. You may choose to redraw the region or alter the assigned value. If you wish to remove the point completely, simply select ***Delete Point***. Once you are satisfied with your list of points, select ***View Curve Fitting Graph***. The ***Calibration Curve*** dialog box will appear.



7. Select one of the curve fitting options on the right hand side of the dialog box. A graph will appear with the associated equation of fit.

8. If you are satisfied with the curve fit, select ***OK***. If not, select other fit options as necessary. After selecting ***OK***, you will return to the ***Calibration*** dialog box.

9. Select ***Apply*** to calibrate the image based on the calibration curve. You may scroll around in the image to see the results of the calibration. If you are not satisfied, you may repeat steps 3-8 as necessary.

10. Click ***Done*** to save the calibration information with the image.

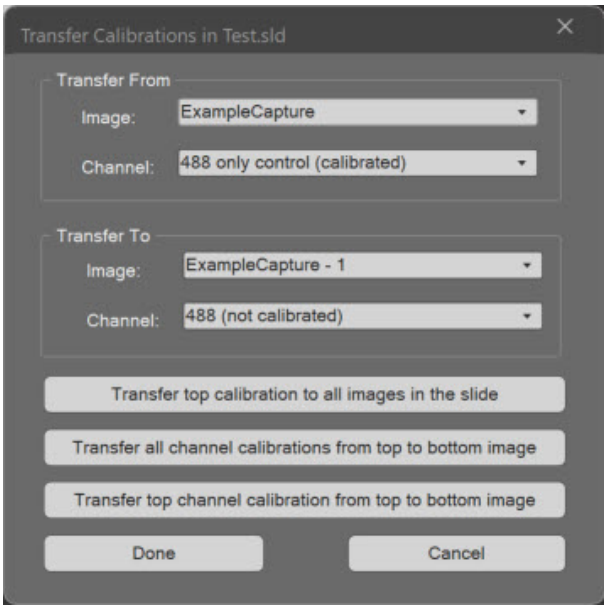
11. To obtain density statistics, simply generate statistics using a mask that you created manually (please see Mask Generation).

Transferring Calibration Data

You may calibrate images using calibration data generated from another image using SlideBook, but you should do so with caution. Image intensities may vary from image to image due to fluctuations in the light source (especially for non-xenon light sources), imperfections in the slide or the coverslip, or any other number of changes that introduce significant differences in imaging conditions. It is preferable to use internal calibration data.

To transfer calibration data (only available within a slide):
1. Open a Main View of the data that you wish to calibrate.

2. Select ***Densitometry > Transfer Calibration*** from the Adjust LUT menu in the View group of the Home tab. The following dialog box will appear.



This dialog box gives you several calibration options.

Transfer the calibration chosen in the drop-down menus at the top of the dialog box to all images in the slide by clicking the ***Transfer top calibration to all images in the slide***

Transfer multiple channel calibrations from one image to another by selecting ***Transfer all channel calibrations from top to bottom image***

Transfer a calibration from a single channel to another image, single channel by using the drop-down menus and click ***Transfer top channel calibration from top to bottom image***

3. Select the appropriate transfer procedure and click ***Done***. The calibration curve data will automatically be transferred. The data in the newly calibrated image can be viewed by selecting ***Densitometry > Calibrate Channel***.

Removing Calibration Data

To remove calibration data:

1. Open a Main View of the image.

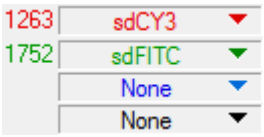
2. Select ***Densitometry > Remove Channel***. The calibration data will be removed.

View Mode

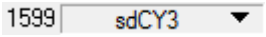
SlideBook provides a variety of lookup tables for use in any of the view windows. Display options include the following:

Icon	Lookup Table	Hot Key	Description
	RGB Color	**Ctrl + Shift + R**	Provides three colors (red, green, and blue) for assignment to any of the channels, also enables a background channel to blend a transmitted light image
	Monochrome	**Ctrl + Shift + M**	Provides a single channel to display a monochrome image
	Inverted Monochrome	**Ctrl + Shift + V**	Provides a single channel to display an inverted monochrome image
	Pseudocolor	**Ctrl + Shift + P**	Provides a single channel to display on a hot/cold lookup table, may be gated by a second channel as in ratio data
	User-defined Color	**Ctrl + Shift + D**	Provides up to eight channels which can be assigned to a user-defined color selected from a color chart or manually created
	LUT X Depth		Provides the display on a hot/cold lookup table depending on sample depth when using the 3D/ 4D Volume Viewer

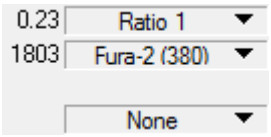
Channel menus are shown below for the different display types.



RGB - Four buttons represent red, green, blue, and background display. You may click on any of the buttons and select the desired channel or **None** from the dropdown menu. To blend a background channel, select it from the fourth button and then go to ***View Settings > Blend Background***.



Monochrome/Inverted Monochrome - This mode provides a single button where you can assign a channel to the black and white display.



Pseudocolor - In the pseudocolor view mode, there are two channel selections. The first channel selects the hue of the display and should be set to the ratio channel. The second channel selects what determines the intensity, or brightness, of the displayed colors. If it is set to the numerator or denominator channel, the pseudocolored display will be scaled by the selected fluorescence intensity. An area with high pixel intensity for the ratio channel and low pixel intensity on denominator channel will appear as dim red. An area with low pixel intensity for the ratio channel and high pixel intensity on the denominator channel will appear as bright blue. This helps distinguish areas with real data from background. This is often desirable as computed ratio values for regions of the image that are just slightly above background can vary greatly.

User-Defined - Allows you to select a color from the full palette to assign to each channel. Blending is done automatically.

Generate 2D/3D Montage

After setting up and acquiring a set of montage tiles (see also TUTORIAL: Collecting a Montage), you will want to stitch the tiles from a 2D, 3D, or 4D Montage together into a montage image. To do so, you can use the ***2D/3D Montage*** window. If you have several files to stitch please use the ***2D/3D Batch Montage*** Window. SlideBook still has the ***Generate Montage (Legacy)*** window enabled as well which will be familiar to all users of SlieBook6. The montage option of your choice can be accessed when highlighting a capture with 2D Montage image type selected.

The 2D/3D Montage provides you several options to shift and merge a montage capture and output the stitched file.

2D/3D Montage

1. Select an image in a file and select ***Montage > 2D/3D Montage***. The 2D/3D Montage window will open.

2. The ***Stage Orientation*** should automatically be populated using the file properties.

3. Select an ***Alignment*** Method and desired parameters (***Maximum Expected Shift, Channel Selection for Alignment, Z Plane Range for Alignment***) if applicable. Or select a subset of the overall image with custom-entered shift values using ***Test Mode***.

4. Select the ***Output*** format and file.

5. Click ***Merge*** or ***Calculate Shifts and Merge***, available depending on the alignment algorithm you selected.

2D/3D Batch Montage

1. Select one or several images in a file select ***Montage > 2D/3D Batch Montage***. The Batch 3D Montage window will open with the selected files in the list.

2. You can add further montage captures to the list by highlighting the image(s) in the file and click ***Add Capture***.

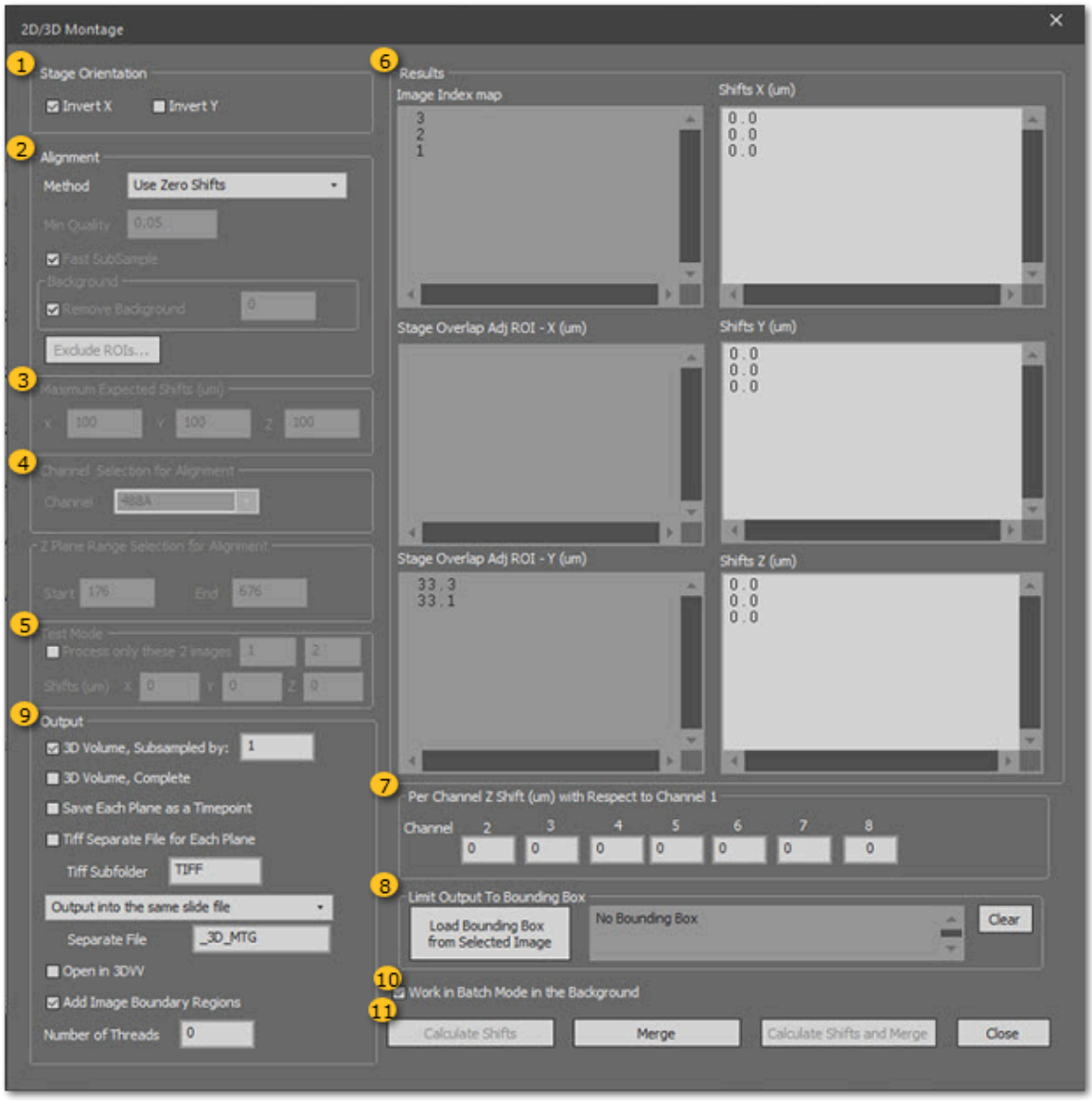
3. You can remove montage captures form the list by highlighting the image in the ***Batch 3D Montage*** list and click ***Remove Selected***.

4. Click ***Start*** to continue and the 2D/3D Montage window will open.

2D/3D Montage window

After setting up and acquiring a set of montage tiles (see also TUTORIAL: Collecting a Montage), you will want to stitch the tiles together into a montage image. To do so, you will use the ***2D/3D Montage*** window, accessed when you have a 2D, 3D, or 4D Montage image type selected.

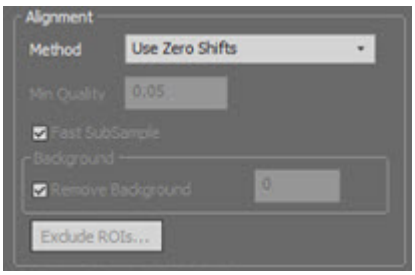
The ***2D/3D Montage*** window provides options for generating a montage with multiple alignment methods and multiple potential outputs. Users can also choose the number of threads dedicated to processing the montaging, and have it work in batch mode in the background.



1 Stage Orientation Group

The ***Stage Orientation*** group allows for changing the orientation of the stage, so that acquired images are placed in the correct order for stitching. This is also a hardware property that can be permanently set. The ***2D/3D Montage*** window will parse this information from the file when opening and set the correct values by default.

2 Alignment Group

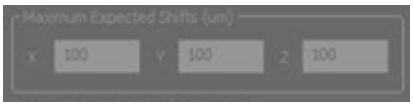


The ***Alignment*** group contains selections for the Alignment Method: Zero-shifts (no shifts are calculated, only the image positions are used), Subsampled Spatial Normalized Cross Correlation (utilized max-intensity projections to calculate shifts and blend the images), and 3D Correlation (used the entire volume to calculate shifts and blend the image). Any of the methods can be used for 2D, 3D, or 4D montages, however 2D montages will not have any additional volumetric data used in the calculation.

Remove Background allows for reduction of the background intensity based on the value entered in the adjacent field.

Exclude ROIs allows for exclusion of specified regions of interest in the shift calculations.

3 Maximum Expected Shifts



Maximum Expected Shifts allows for entry of the maximum values that the images should be shifted.

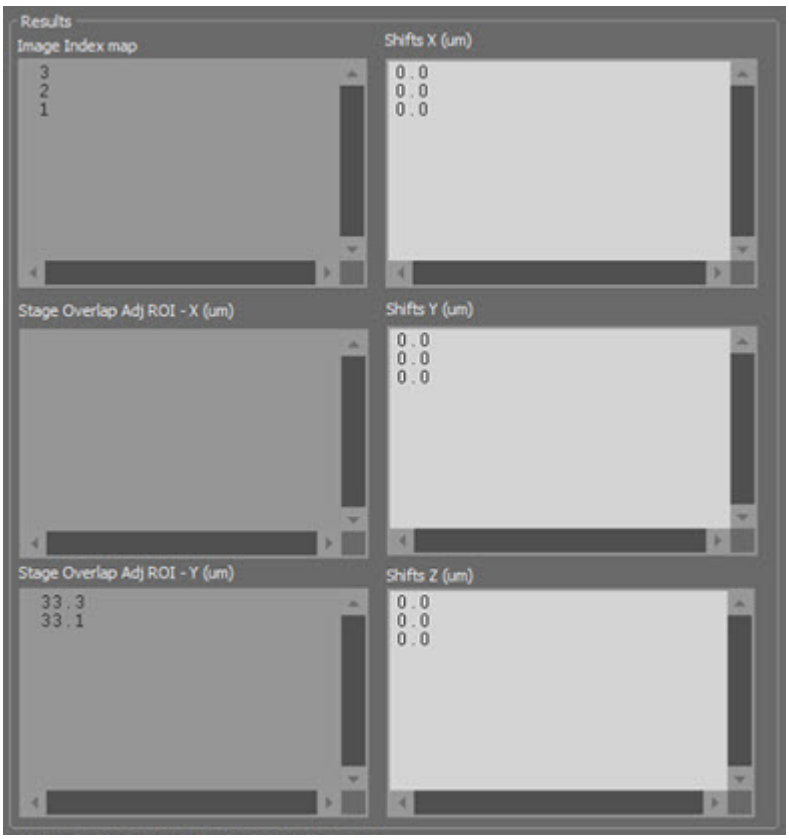
4 Channel and Z Plane Range Selection for Alignment

Choose which channel and a single Z plane or range to use for the alignment.

5 Test Mode

Using ***Test Mode***, users can choose to process on a subset of the overall image with custom-entered shift values. This is especially useful on very large datasets.

6 Results



The ***Results*** section displays the results of the calulated shifts, as well as the relative positions of the images in the image index.

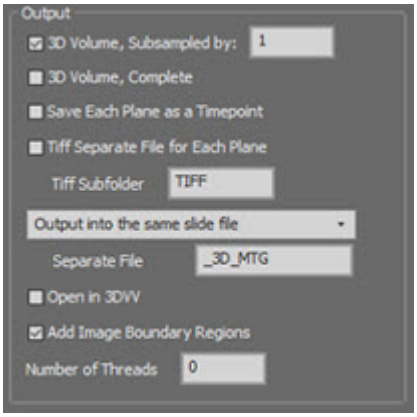
7 Per Channel Z Shift

This enables users to enter z-shift values for channels relative to channel 1.

8 Limit Output to Bounding Box

If a bounding box has been selected, then ***Limit Output to Bounding Box*** will restrict the alignment and stitching to the extent of the bounding box.

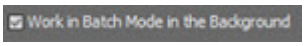
9 Output Group



The ***Output*** group contains controls to choose the type of output from the montage process.

• ***3D Volume, Subsampled*** outputs a total montage (2D, 3D, or 4D) that has been subsampled by the factor entered in the adjacent field.
• ***3D Volume, Complete*** outputs the complete montage with no subsampling.
• ***Save Each Plane as a Timepoint*** exports a multiplane montage as a series of 2D planes into a separate SlideBook file.
• ***Tiff Separate Separate File for Each Plane*** exports a multiplane montage as a series of 2D tiff files to the folder defined in the filed below.
• ***Open in 3DVV*** will open the constructed montage in the 3D/ 4D Volume Viewer.
• ***Add Image Boundary Regions*** adds ROIs to the completed montage displaying the boundaries of the constituent images.
• ***Separate File*** with Suffix exports the montage to a new slide file with the image name as the filename and the suffix appended.
• ***Number of Threads*** determined the number of processing threads dedicated to the alignment and stitching process. Default is 0, which is all threads.

10 Work in Batch Mode in the Background



This enables the montage to work in the background when 2D/3D Batch Montage is selected.

11 Calculate Shifts, Merge, and Close

These buttons allow for calculating shifts and merging the montage independently, or calculating and merging in one process. The Close button closes the 2D/3D Montage window, and this button changes to "Go" in the 2D/3D Batch Montage window.

2D/3D Batch Montage window

After setting up and acquiring a set of montage tiles (see also TUTORIAL: Collecting a Montage), you will want to stitch the tiles together into a montage image. To do so, you will use the ***2D/3D Montage*** window, accessed when you have a 2D, 3D, or 4D Montage image type selected.

The ***2D/3D Batch Montage*** window provides options for generating a montage with multiple alignment methods and multiple potential outputs. Users can also choose the number of threads dedicated to processing the montaging, and have it work in batch mode in the background.

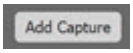
1 Image Captures

The capture names of selected images are listed in the ***Image Captures*** .

2 Slide

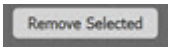
The name of the original Slide file of a listed image are listed in the *Slide* .

3 Add Capture



Add Capture will add one or several highlighted captures from a slide into the ***Batch 3D Montage*** list.

4 Remove Selected



Remove Selected will erase a highlighted image from the ***Batch 3D Montage*** list.

5 Start /Close

Start continues the batch process and opens the 2D/3D Montage window to select montage options. ***Close*** will close the Batch 3D Montage window and erase the list.

6 Done

A tick in the tickbox in the ***Done*** will indicate which capture has already been processed during the batch process.

7 Status

Status will indicate the progress of the batch process with Not Started, Processing (which image) and Finished.

Generate Montage (Legacy) window

After setting up and acquiring a set of montage tiles (see also TUTORIAL: Collecting a Montage), you will want to stitch the tiles together into a montage image. To do so, you can use the ***Generate Montage (Legacy)*** window, accessed when you have a 2D Montage image type selected.

This dialog provides you with a list of the individual images that make up the montage and the ability to move each image individually or automatically. When you open the ***Generate Montage*** window, a subsampled image will also be generated to see a rough estimate of the final montage image.

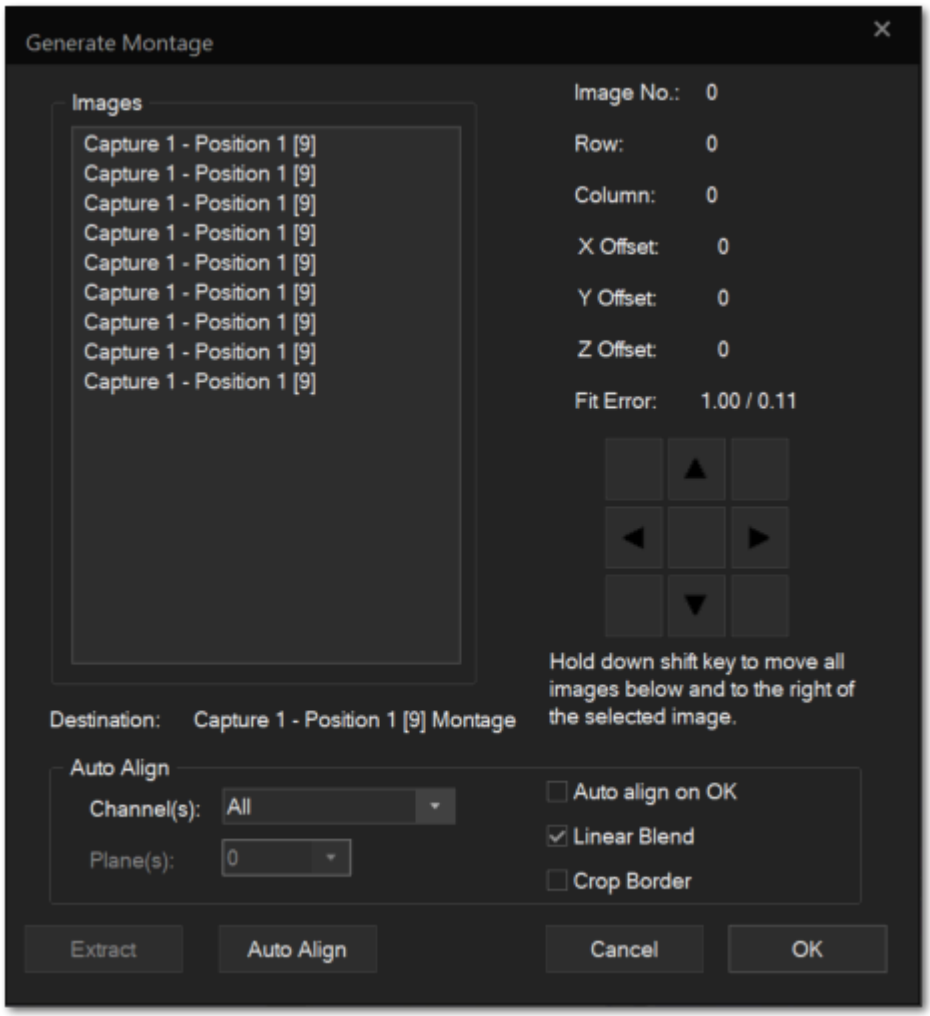
Manual Adjustment

1. Select an image from the ***Images*** list.

2. Use the arrows to shift the image location in the displayed montage. Hold the **Shift** button while clicking the arrows to shift the selected image as well as all images below and to the right of the selected image. Repeat steps 1 and 2 until the subsampled preview is aligned properly.

3. Uncheck the box for ***Auto Align on OK***.

4. Click ***OK***. A new image will be written to the slide with the assembled montage.



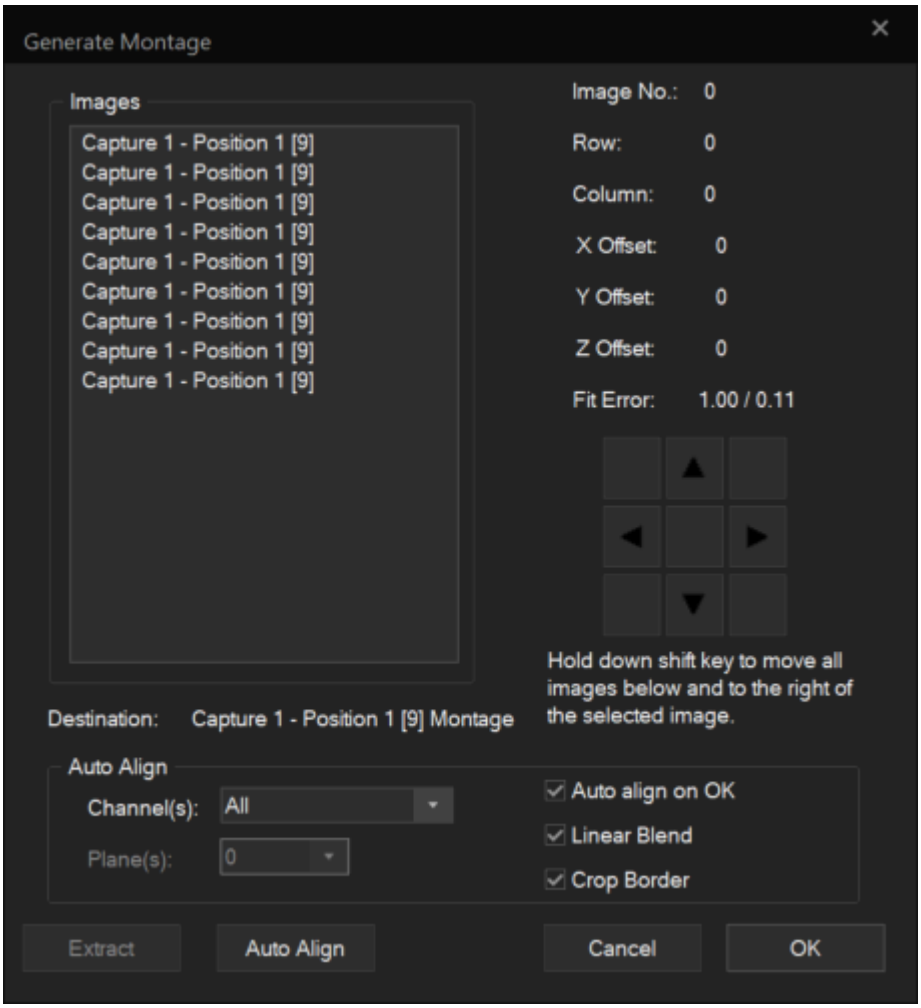
Automatic Alignment

1. Select an individual channel to use for alignment or ***All***.

2. If there are multiple z planes, select the best plane to use for the alignment from the ***Auto Align Plane*** drop down menu.

3. Click ***Auto Align*** to preview the automatic alignment.

4. Press ***OK*** to generate the final alignment and write a new image to the slide.



View Settings Menu

Many aspects of the display can be adjusted from the Settings menu in the View group.

View Settings opens the View Settings dialog that includes array information for tile views, background blending, looping speed for image playback, and background color for Tile and Three Views.

MultiDimensional Channel View Settings opens the MultiDimensional Channel View Settings dialog to adjust how this view is displayed and exported. You may find this particularly useful in preparing data for print materials.

Blend background is executed upon selection. Applicable for RGB Color and Pseudocolor display types, will blend a transmitted light image (such as DIC) into the colors displayed. Degree of blending can be adjusted in the View Settings window.

Invert Background is executed upon selection. Inverts the monochrome lookup table for the background channel.

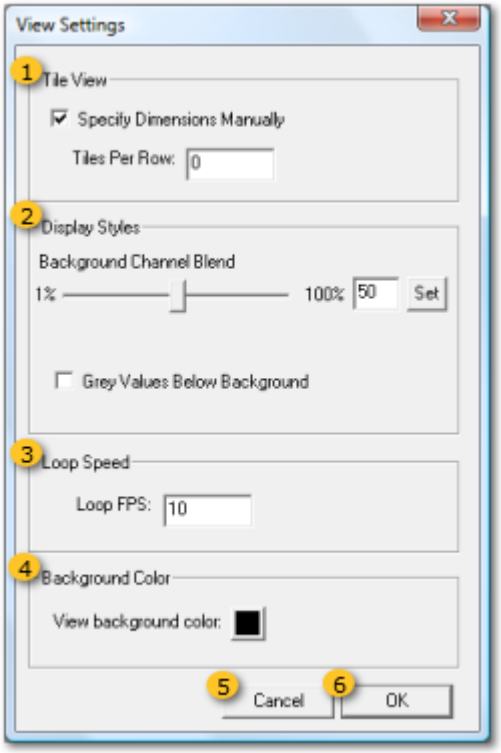
Set best planes from Autofocus (4D) is executed upon selection. It will set the ideal focus position for each time point in a 4D data set using an autofocus algorithm. This plane is then used for Create Series Movie.

View Settings window

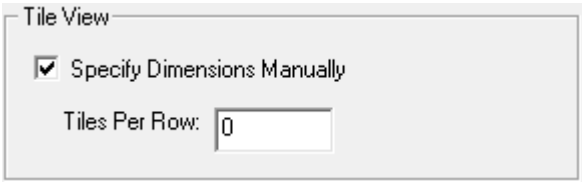
View Settings are used to adjust several parameters for specific views. These parameters include array information for tile views, background blending, looping speed for image playback, and background color for Tile and Three Views.

See also:

[The Timelapse Tile View]



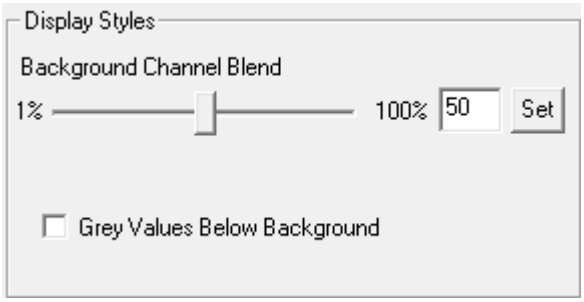
1 Tile View Group



Specify Dimensions Manually - Check this box to manually enter a the number of tiles displayed per row when viewing a ***Timelapse Tile View***.

Tiles Per Row - Enter the number of tiles you would like to see displayed per row of the ***Timelapse Tile View***.

2 Display Styles Group



Drag the slider to select the percentage of blending that will be performed on a background channel such as **DIC**. Alternatively, type a value in the text field and click ***Set***. This is the percentage of the greyscale background that is overlayed with the fluorescence signal.

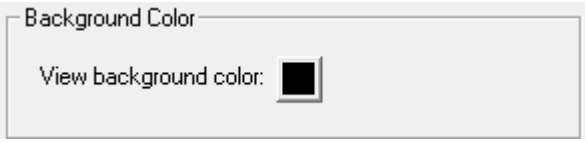
Check this box if you would not like to display grey values that fall below a designated background value.

To change the stored default Blend Background value, go to ***Hardware Properties*** in the Administration tab. Find the section ***Default View Settings*** and expand it by pressing the "**+**". Select ***Default Blend Background Percentage***, enter a ***Value*** in the edit field and click ***Apply***. You will need to restart SlideBook for the changes to take effect.

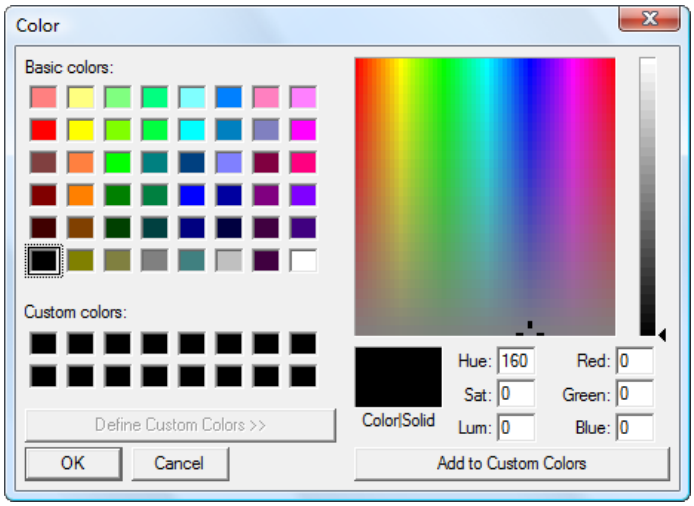
3 Loop Speed

Enter an integer to determine how quickly (in ***FPS***, frames per second) an image will play when in is looped. To loop an image, go to ***View > Loop timepoints***, ***View > Loop planes***, or press the play button in the image window .

4 Background Color



The background color is the color that appears in the blank space behind an image. This color is particularly prominent when viewing a specialty view type such as a ***Three View*** or ***Tile View***. When you click on the color button, the following dialog will open:

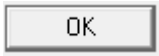


The Background Color default can be changed by noting the Red, Green, and Blue values from this dialog then going to ***Hardware Properties*** in the Administration tab. Find the section ***Default View Settings*** and expand it by pressing the "**+**". Set each of the three default background color fields, clicking ***Apply*** after each one. Restart SlideBook for the changes to take effect.

5 Cancel button

Discards ***View Settings*** changes and closes the dialog.

6 OK button



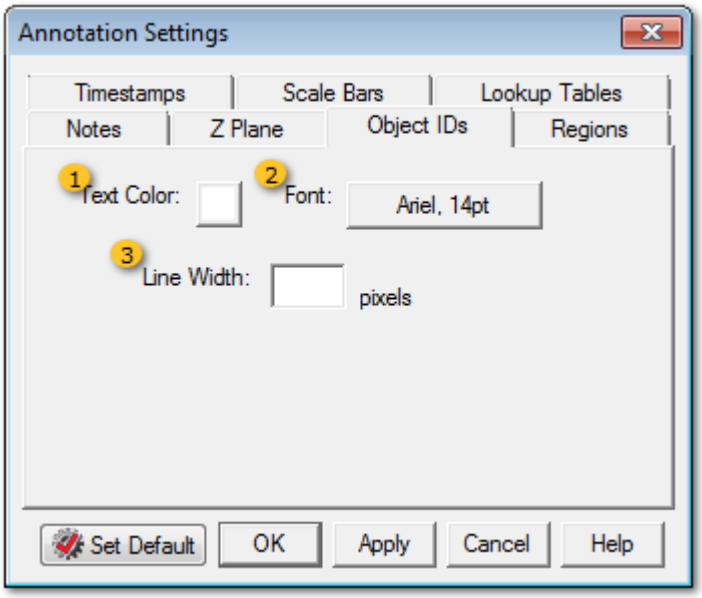
Applies ***View Settings*** changes and closes the dialog.

Annotation Settings Object IDs tab

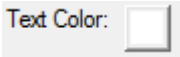
The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

TUTORIAL: Displaying and Formatting Annotations
Annotations Menu

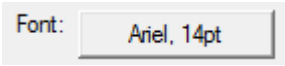


1 Text Color Selection



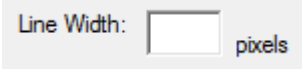
Select the color of the text of the annotation by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

2 Font Selection



Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

3 Line Width



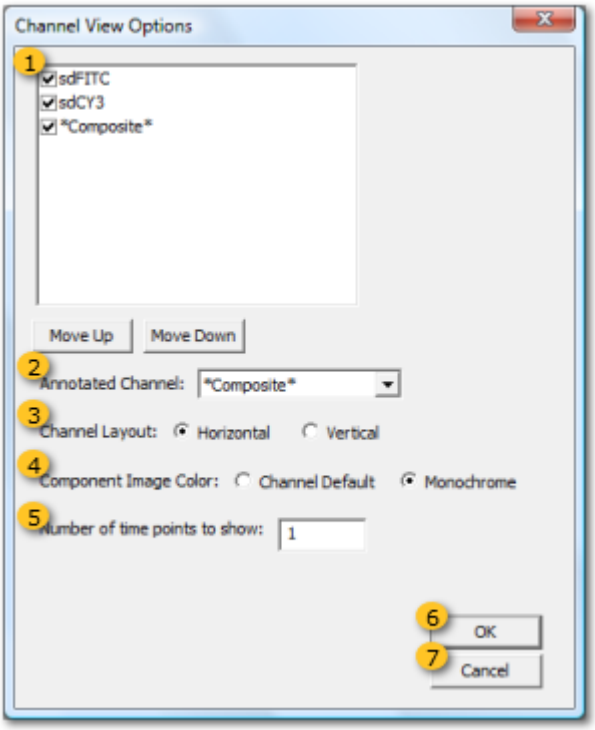
Masks are normally displayed as a blue field without an outline. When pseudocolor display is selected, the mask is depicted as an area bound by a white outline. You may change the thickness of this outline by adjusting the ***Line Width***.

Multidimensional Channel View Setttings window

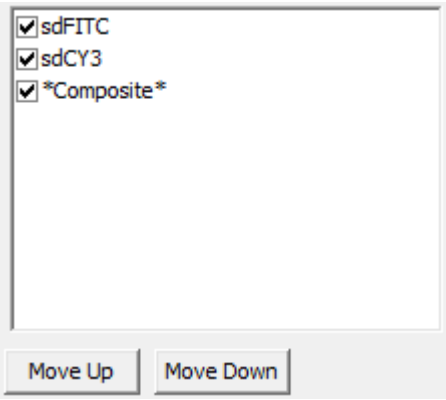
Multidimensional Channel View Settings allow you to adjust how this view is displayed and exported. You may find this particularly useful in preparing data for print materials.

See also:

[Multidimensional Channel View]
[View Settings window]
[Annotations Settings]



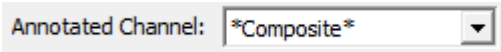
1 Channel Selection and Controls



Click on each box to toggle the channels on/off in the image view. Check marks indicate displayed channels. ***Composite*** will consist of all present channels regardless of the display status in this view. T

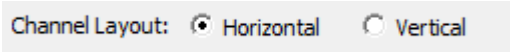
Click on a channel to select it, selected channels will be highlighted in blue. Use the ***Move Up*** and ***Move Down*** buttons to adjust the order the channels are displayed.

2 Annotated Channel Selection



When annotations are displayed, you may select the channel tile where they will appear. One exception for this is scale bars. Scales bars (when displayed) will appear in each tile.

3 Channel Layout option



Select if you would prefer to tile the channels horizontally or vertically.

4 Component Image Color option

Component Image Color: ○ Channel Default ◉ Monochrome

Select how you would like the individual channels to be displayed. ***Channel Default*** uses the default color display set up in Filter Configuration. ***Monochrome*** will display individual channels in greyscale.

5 # of Time Points field

Number of time points to show: 1

If a time series is present, you may choose to display multiple time points by entering a value in this field.

6 OK button

OK

Press ***OK*** to apply changes and close the dialog.

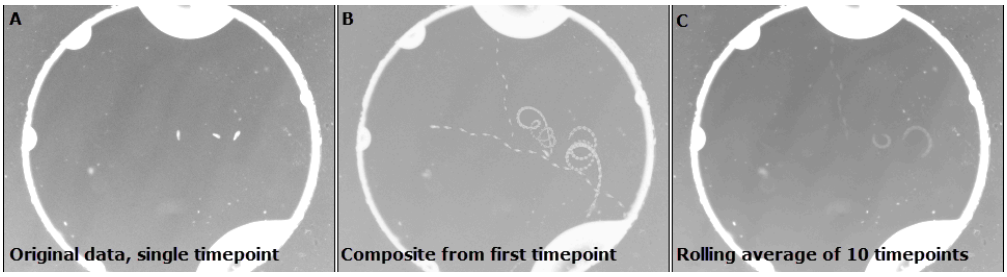
7 Cancel button

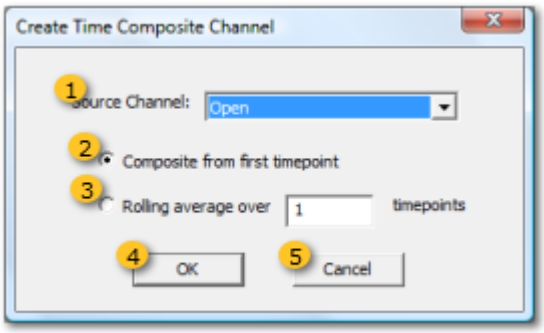
Cancel

Press ***Cancel*** to discard changes and close the dialog.

Create Time Composite Channel window

When analyzing time lapse images, you may find it useful to display a composite of multiple timepoints. For this purpose, you may create a time composite channel. The image below shows a single time point of an image with a time composite channel of all time points or a time point range.





1 Drop down

Source Channel: Open

Select the source channel from the drop down menu. An additive projection image will be created for each time point. This image will be created as a new channel in the image with **comp** appended to the channel name.

2 Composite from first timepoint option

Composite from first timepoint

The composite channel will be built with all time points starting at 1. Thus, if the image has 100 time points, the 50th image would be a projection of time points 1-50 and the last image would be a projection of time points 1-100.

3 Rolling average over option

Rolling average over 1 timepoints

The composite channel will be built with with a user-defined number of previous time points. Thus, if ***Rolling average over 10 time points*** was selected and the image has 100 time points, the 50th image would be a projection of time points 40-50 and the last image would be a projection of time points 90-100.

4 OK button

OK

Press ***OK*** to create the designated channel and close the dialog.

5 Cancel button

Cancel

Press ***Cancel*** to discard the changes and close the dialog.

Navigation Tools

Many of the navigation tools available in view windows are available as hot keys or menu items. A list of these commands follows:

Previous plane - **Ctrl+[** - - Move to the previous *z* plane

Next plane - **Ctrl+]** - - Proceed to the next *z* plane

Go to plane - **Ctrl+G** - Open a dialog to type a specific z plane to view

Loop planes - - Animate the view window to continuously loop through the *z* planes

Previous timepoint - **Ctrl+Shift+[** - - Move to the previous time point

Next timepoint - **Ctrl+Shift+]** - - Proceed to the next time point

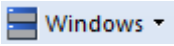
Go to timepoint - **Ctrl+Shift+G** - Open a dialog to type a specific time point to view

Loop timepoints - - Animate the view window to continuously loop through time

NOTE: When viewing images, the middle mouse scroll wheel can aid in image navigation. In 2D time lapse and 4D data types, the middle scroll wheel will move through time points. In a 3D stack the middle scroll wheel will move through *z* planes. In a 4D series holding down the control key plus the mouse scroll wheel will move through *z* planes.

View Properties

In addition to the previously discussed viewing parameters, there options available for the how windows are positioned on the screen and the default way that images are zoomed in the view windows.



The ***Windows*** menu provides the following options:

Cascade - Moves all open view windows to a cascading position starting in the upper left corner of the workspace and layering down with images overlapping previous views.

Tile Horizontal - Resizes views in the height aspect to create non-overlapping tiles.

Tile Vertical - Resizes views in the width aspect to create non-overlapping tiles.

Arrange Icons -

Close All Views - Closes all open view windows, slide files will remain open.

Windows Manager - Opens a list of all open slides with the ability to ***Save***, ***Activate***, ***Close***, or ***Minimize*** the selected slide in the list.

Default View Settings are also accessed in the ***Administration tab*** under ***Hardware Properties***.

Alternative Image Cascading - Gives an option to use a newer cascading approach that opens view windows more towards the center of the workspace.

Automatically Zoom New Views - If set to ***Yes***, SlideBook will automatically zoom images as large as possible so that the entire image will fit on the screen without scroll bars. If set to ***No,*** SlideBook will use the ***Manual View Zoom Target Size*** described below.

Bring Matching Image View to Top - If this is set to ***Yes***, when you try to open an image that is already open in a Main View, SlideBook will bring the open view to the front of the windows instead of opening the image again. This will only work for Main Views. Hold down the **Ctrl** key to override this behavior.

Default Background Color and Blending - more information on this is found in View Settings Display Styles and Background Color.

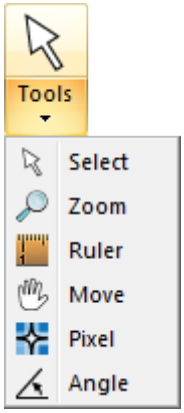
Manual View Zoom Target Size - This is used when ***Automatically Zoom New Views*** is set to ***No***. Use a value of 1 to display images at the true resolution such that 1 pixel from the camera = 1 pixel on the monitor. A 1024x512 image will be displayed at 1024x512.

Show Tics for Notes - If set to ***Yes***, displays tic marks on the time slider in view windows to mark notes or events during capture.

Regions Group

In the Regions group of the Home tab, you can draw regions to track, photomanipulate, or analyze. You also have access to standard view tools such as zoom, angle and ruler measurements, and pixel analysis. With the marquee selections tool, you can select a portion of the image to view, analyze, or crop.

Tools Menu



Pointer (Select) Tool - This tool is used to select regions and for certain functionality in the ***Focus Controls***. Holding **Shift** and clicking on multiple regions will allow you to select more than one region. Clicking and dragging on a selected region(s) will move the selected region(s). If the region number is displayed in white, this indicates that the region is selected.

Zoom Tool - This tool can be used to zoom in on the image, re-centering at the location where you click. Default will display a **+** in the center of the magnifying glass to indicate zooming in. If you hold the **SHIFT** key, the **+** will turn to a **-** and you can zoom out.

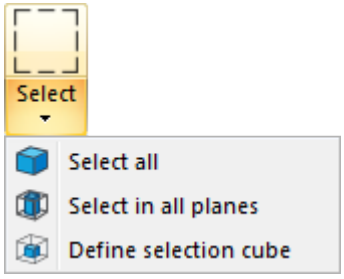
Ruler Tool - The ruler is used to draw a line on the image for distance or velocity measurements. By holding down the **CTRL** key, you can draw a multi-segment line. When measurements are made, a value (in um) is displayed in ***Measurement Info*** (3). You can retrieve intensity across this line by going to the ***Analyze tab > Evaluate group >*** Line Intensity. To export multiple line measurements, you should use the region line tool then go to the ***Analyze tab > Evaluate group > Extract Measurements > Export Line Lengths***.

Hand Tool - The hand is used to grab and drag an image when the displayed portion of an image is in a window smaller than the extent of the displayed axes.

Pixel Tool - This tool is used to retrieve coordinate or intensity information for a single pixel. Press **CTRL** + click on a region in a 2D timelapse image to display an intensity over time graph of that pixel.

Angle Tool - You may select this tool then click three times to draw two connected lines. The angle between the two lines is measured and displayed in ***Measurement Info*** (3). To export the angle measurement information, first go to ***Analyze tab > Evaluate group > Extract Measurements > Export Angle Measurements to Excel***. This will open an Excel file. Every time you draw an angle, the corresponding data will be written to the file.

Select Menu



Marquis Tool - Allows you to select an area of the image, useful in the ***Live*** camera window to select an area for imaging or in the ***Main*** view to select an area to crop.

Select all - Selects the entire image in *x*, *y*, *z*, and time.

Select in all planes - uses the marquis selection defined and extends the selection to all planes of the image, useful for cropping an image in *x/y*.

Define selection cube - opens the Define Selection Cube window where you can type in the exact dimensions/coordinates of the selection area.

Regions

□, ⬭, ⬠, ⬬, ╲, ⊕ ***Region Tools*** - These tools (rectangle, ellipse, polygon, freehand, line, and point) are used to draw regions for analysis. More information on using regions can be found in the Region Tools help page.

Region Tools

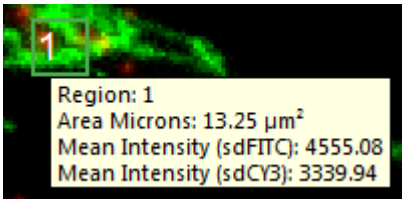
Regions are a key analysis tool provided in SlideBook that can be drawn before image capture, while images are be acquired, and/or post capture. Regions persist over all time points and *z* planes and are used primarily to monitor intensity changes over time (either in real-time or post-acquisition). Mean background intensity can be subtracted from analysis by assigning a region as background. The region drawing tools are found in the ***Regions group*** of the Home tab. Should you want access in other tabs, this group or individual items can be added to the Quick Access Toolbar, by right-clicking and choosing ***Add to Quick Access Toolbar***. Region selection and usage tools are found by right-clicking on a region in the image.

Region drawing tools include:

Marquee tool - useful in the ***Live*** camera window to select an area for imaging or in the ***Main*** view to select an area to crop. You may change the color used to draw the Marquee in the Administration Tab > Hardware Properties > Default View Settings > Default Marquee Color Red/Green/Blue.

NOTE: you will need the RGB values for the color you select when using Hardware Properties.

Region Selection tool - use to select a region. Holding **Shift** and clicking on multiple regions will allow you to select more than one region. Clicking and dragging on a selected region(s) will move the selected region(s). If the region number is displayed in white, this indicates that the region is selected. With a single region selected, you may hold the cursor over the region to obtain basic region statistics as shown below.



Rectangle tool - click and drag to draw a rectangle; alternatively, click once on the image to open a dialog to define the region dimensions in pixels or microns or to define a rotation angle for the region

Ellipse tool - click and drag to draw an ellipse; alternatively, click once on the image to open a dialog to define the region dimensions in pixels or microns or to define a rotation angle for the region

Polygon tool - click to draw a series of straight lines, double-click to close the shape

Free Hand tool - click and drag to draw freehand, release mouse button to close the shape

Line tool - click and drag to draw a line; hold **Ctrl** and click for a multi-segment line, double click to complete multi-segment line

Point tool - click to draw a point

Region selection tools (right-click on a region in the image to access)

Set as background – uses the selected region for background subtraction during graphing. The method used is to take the mean intensity value of the background region and subtract it from each individual pixel in the region of interest. Pixels in the region of interest with values >0 will then be used to calculate statistics for the region of interest.

Unselect – de-selects the selected region(s)

Duplicate – creates a copy of the selected region(s) that can be dragged to a new location

Move – allows the region(s) to be moved

Copy – copies the region(s) to allow for pasting, specifically when copying to a different image

Paste – pastes the copied region(s) in the same location on a different image

Delete – removes the selected region(s)

NOTE: You can use the defined background region for background subtraction in Mask Statistics by following instructions in Configuring Background Subtraction with Mask Statistics.

When regions are drawn on timelapse images, you may graph them using the ***Graph*** icon in the Evaluate group of the Process tab or by the ***Graph*** icon in view windows. See also:

New Graph View window
Graph window

Define Selection Cube window

The ***Define Selection Cube*** window can be used independently or in conjunction with the ***Marquee tool***. This dialog allows you to specify *x*, *y*, and *z* dimensions for selecting an area to render or crop.

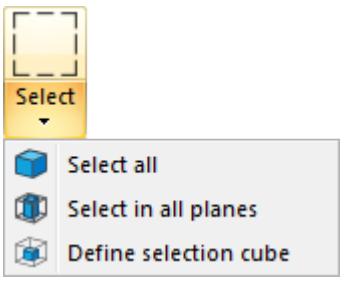
See also:

- Crop Image from Selection Cube
- Bounding Box
- 3D/4D Volume View
- 2D Timelapse Intensity Plot
- 3D Surface View

1) Open an image in the Main View.

2) If desired, select the ***Marquee tool*** and click and drag to draw an *xy* area on the image.

3) Go to the Select menu > ***Define Selection Cube***.



4) Review and edit the ***X*** and ***Y*** values for ***Beginning***, ***End*** and ***Extent***. You may choose to leave the values that were selected by the marquee tool or manually enter new values or use the X and Y ***Shift*** buttons at the bottom of the window to shift the entire selection but maintain the dimensions. You may wish to use the extent values to specify the resultant image dimensions.

5) Review and edit the ***Z*** value for ***Beginning***, ***End*** and ***Extent***. You may find it useful to eliminate out of focus *z*-planes or *z*-planes containing objects that may obscure those of interest.

6) Alternatively you can apply the values defined by the Bounding Box in 3D/4D Volume View.

6) Press ***Apply*** to apply the selection to the image and ***OK*** to close the dialog.

7) Proceed with rendering (3D/4D Volume View, 3D Surface View, or 2D Timelapse Intensity Plot), cropping or extracting from the image.

Graph Regions vs. Stimulation Regions

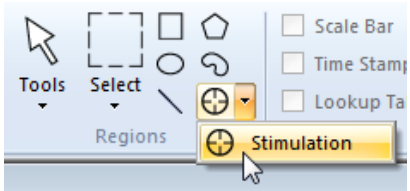
When performing photomanipulation experiments, you may find it useful to utilize ***Stimulation Regions*** instead of the traditional ***Graphing Regions***. The main advantage of Stimulation regions over Graph regions is that they can not be moved or altered post-acquisition so the experiment data integrity can not be compromised. In addition to this, stimulation regions can be drawn outside of the imaging field in *xy* and/or *z*. A full chart comparing the two region types can be found below.

	Stimulation Regions	**Graph Regions**
Used to create photostimulation events	**YES**	**YES**
Used for right-click initiated photostimulation events	**NO**	**YES**
Used in advanced capture photostimulation sequence	**YES**	**YES**
Can exist outside *xy* extents of the imaging field (for 2P only)	**YES**	**NO**
Can exist outside of *z* range of imaging	**YES**	**NO**
Compatible with Vector	**YES***	**YES**
Compatible with Phasor	**YES**	**YES**
Has *z* coordinate component	**YES**	**YES**
Can be graphed during acquisition	**NO**	**YES**
Can be graphed post-acquisition	**NO**	**YES**
Can be moved or edited post acquisition	**NO**	**YES**

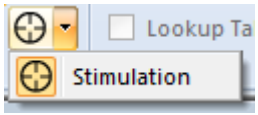
* Vector Stimulation regions do not have z component

How to Use Stimulation Regions

1. In the Region group of the Home tab, click the drop down button next to the ***Spot*** region tool. Select ***Stimulation***. This will convert the standard Graph Region tools into Stimulation Region tools.

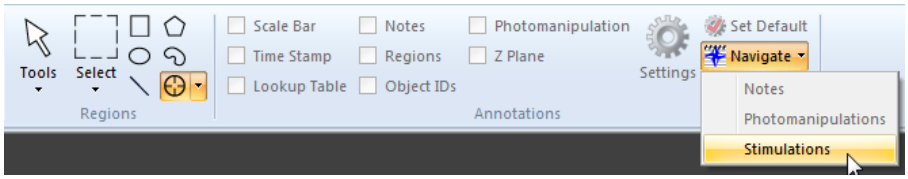


2. Once selected, the spot icon in the drop down will be outlined and highlighted in orange/yellow as displayed below.



3. Select the desired region drawing tool to draw ***Stimulation Regions*** in the Live window. Stimulation regions are numbered with an "S" then number as in S1, S2, S3, etc.

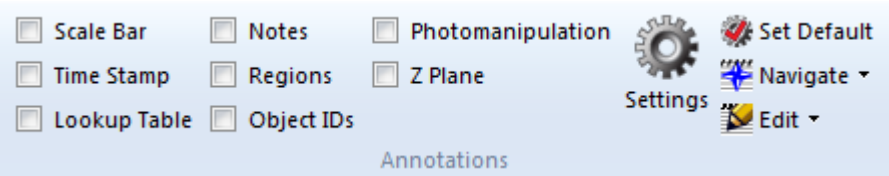
4. Once Stimulation regions have been drawn in the live window, you can open the ***Stimulation Navigator*** from the Navigation menu in the Annotations group to display the regions with their coordinate information.



5. To visit the *xy* or *z* location of a Stimulation region, simply double-click on the region in the list. Alternatively, click once to select the region in the list then click the ***Go To*** button.

Annotations Group

The ***Annotations Group*** controls the display and settings for various image annotations. Annotations can easily be added to the active view window by checking the boxes in the Annotations group of the Home tab.



Alternatively, shortcut keys are available:

Scale Bar	Ctrl + Shift +S
Time Stamp	Ctrl + Shift + T
Lookup Table	Ctrl + Shift + L
Notes	Ctrl + Shift + N
Regions	Ctrl + Shift + G
Object IDs	Ctrl + Shift + O

Color, size, font, and location of the annotations can be changed from the ***Settings*** dialog.

Clicking ***Set Default*** will apply the settings to all annotations used during the current session; restarting SlideBook will reset the settings.

The ***Navigate*** menu provides access to Notes Navigator, Photomanipulation Navigator, and Stimulations Naviagtor.

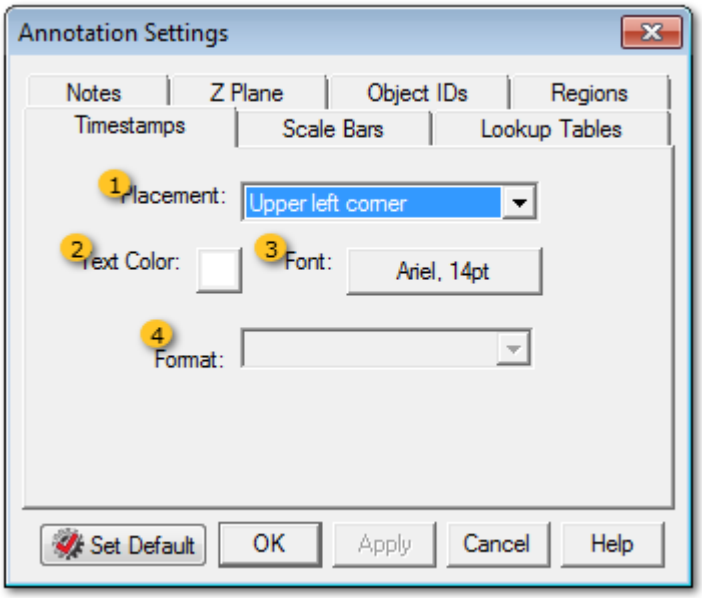
The ***Edit*** menu provides access to Edit Notes and Edit Timestamps (a tool that lets you set the "**0**" timepoint).

Annotation Settings Timestamps tab

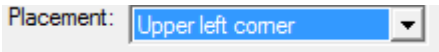
The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

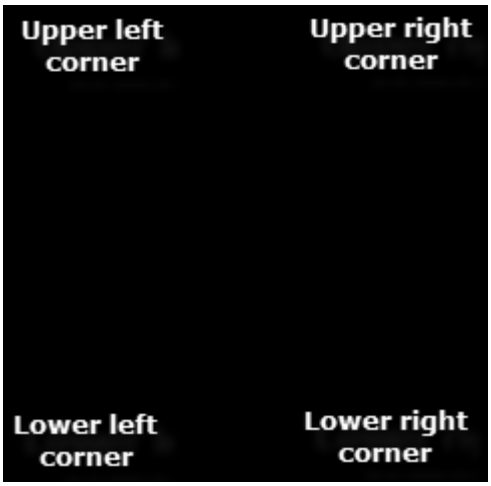
TUTORIAL: Displaying and Formatting Annotations
Annotations Menu



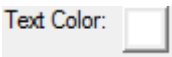
1 Placement Selection



Select the placement of the annotation as follows:

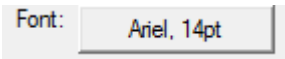


2 Text Color Selection



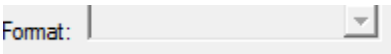
Select the color of the text of the annotation by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

3 Font Selection



Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

4 Format Selection



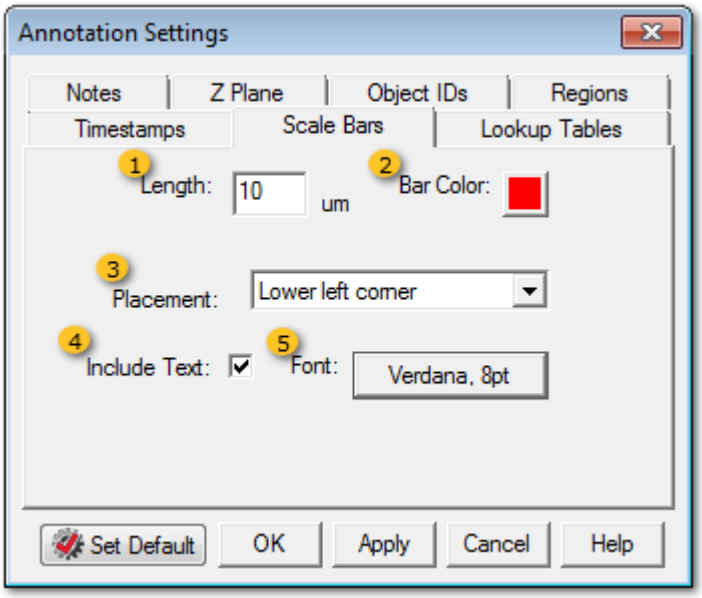
Select the desired format from the dropdown menu.

Annotation Settings Scale Bars tab

The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

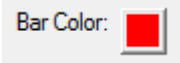
TUTORIAL: Displaying and Formatting Annotations
Annotations Menu



1 Length

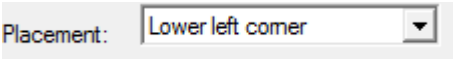
Enter the desired length (in microns) in the text edit field.

2 Bar Color Selection

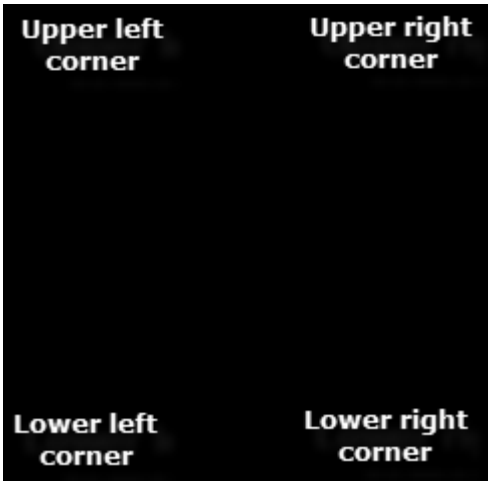


Select the color of the scale bar by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

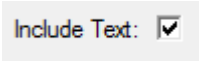
3 Placement Selection



Select the placement of the annotation as follows:

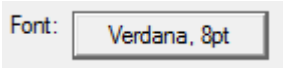


4 Include Text Check Box



Check the box to include the size of the scale bar.

5 Font Selection



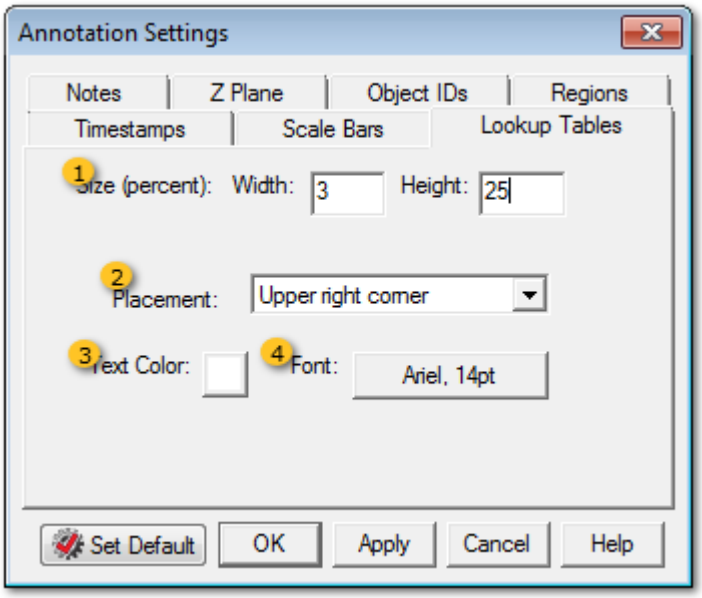
Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

Annotation Settings Lookup Tables tab

The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

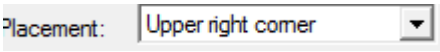
TUTORIAL: Displaying and Formatting Annotations
Annotations Menu



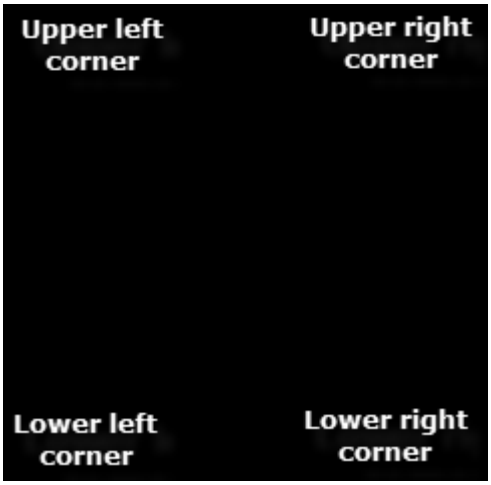
1 Size Selection

Enter values for the dimensions of each of the lookup tables. A lookup table will be provided for each of the channels present in the image.

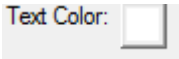
2 Placement Selection



Select the placement of the annotation as follows:

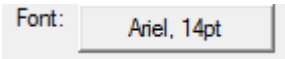


3 Text Color Selection



Select the color of the text of the annotation by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

4 Font Selection



Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

Annotation Settings Notes tab

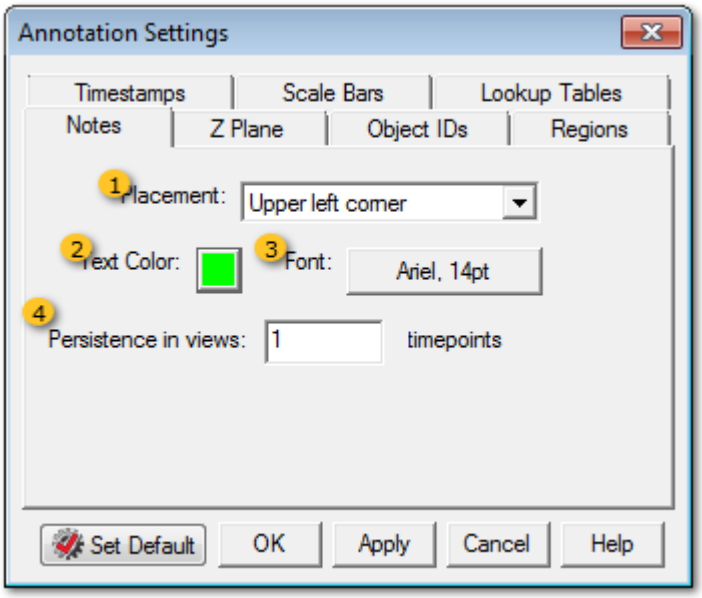
The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

In view windows, you can display tic marks on the Time Slider by going to ***Edit > Hardware Properties > Default View Settings > Show Tics for Notes***.

> Notice that tic marks indicate the beginning and end of the time series as well as any events or notes created during image capture. If you do not have tics representing events or notes in your time slider, go to ***Edit > Hardware Properties > Default View Settings > Show Tics for Notes***.

See also:

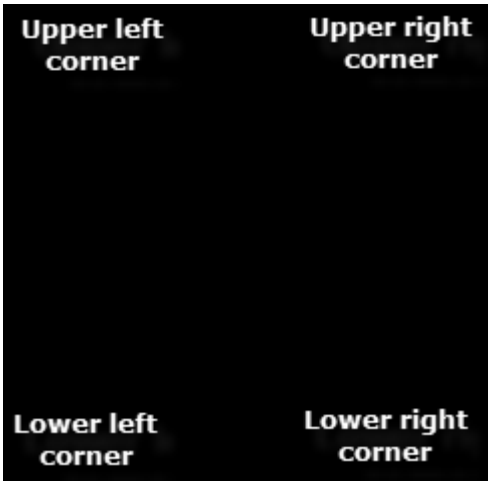
TUTORIAL: Displaying and Formatting Annotations
Annotations Menu



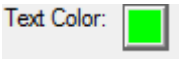
1 Placement Selection

Placement: Upper left corner

Select the placement of the annotation as follows:

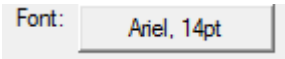


2 Text Color Selection



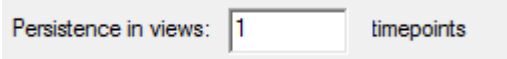
Select the color of the text of the annotation by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

3 Font Selection



Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

4 Persistence Selection



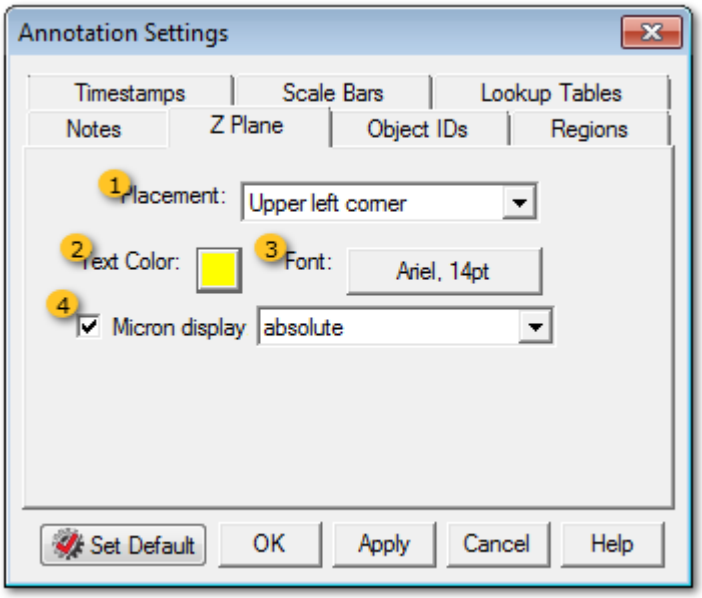
When playing back a time lapse movie, you may find it useful to display notes for longer than a single time point. To do so, enter a value for the number of time points to persist in the view.

Annotation Settings Z Plane tab

The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

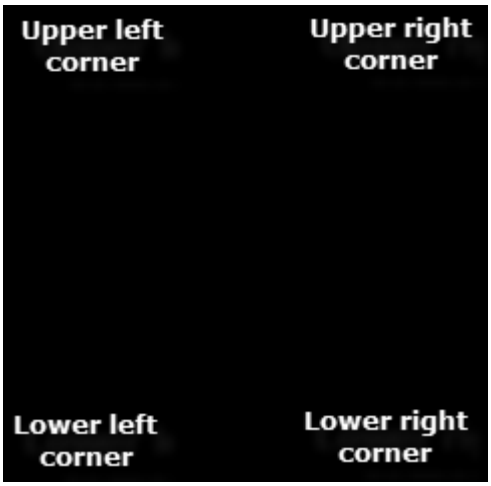
TUTORIAL: Displaying and Formatting Annotations
Annotations Menu



1 Placement Selection

Placement: Upper left corner

Select the placement of the annotation as follows:

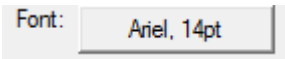


2 Text Color Selection

Text Color:

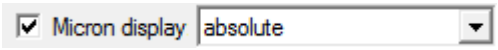
Select the color of the text of the annotation by clicking on the square color button. The ***Color*** dialog will open. Select the desired color from this utility.

3 Font Selection



Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

4 Format Selection



If the box is not checked, the actual plane will be displayed such as "**Plane 7**"

To use ***Micron display***, check the box and select:

absolute - to show the actual micron position of Z stage

relative reference - to show stage position relative to the reference position (or start position if there is no reference position). For example, if you select "range around current position" and capture a 20 um volume the starting position will be -10 um (it moved -10 um down when you clicked start) and the final plane will be 10 um (the final position was 10 um above the starting position)

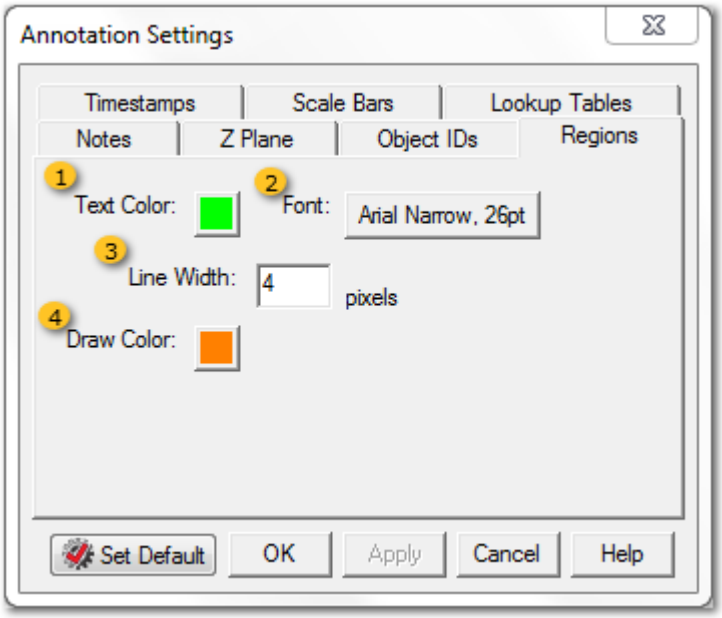
relative plane 0 - displays the distance moved from plane zero For example, if you did a 3D capture with 1 um step size plane 0 would be 0 um, plane 1 would be 1 um, showing the total distance moved from the start of the experiment

Annotation Settings Regions tab

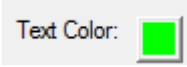
The ***Annotation Settings*** dialog allows you to alter the way annotations are displayed in the view window. You may change the color, font, location, and format of each type of annotation by selecting the desired annotation tab and setting the relevant parameters. To use these settings for an entire SlideBook session, first adjust the annotation settings and click ***Apply*** then ***Set Default***. The annotations will be displayed the way you configured them until SlideBook is closed and restarted.

See also:

TUTORIAL: Displaying and Formatting Annotations
Annotations Menu

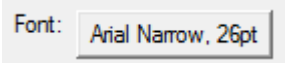


1 Text Color Selection



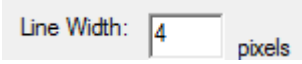
For regions, this color is the color of the selected region(s) and can not be modified.

2 Font Selection



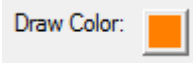
Select the desired font and size by pressing the button to open the ***Font*** selection dialog.

3 Line Width



You may change the thickness of the drawn region(s) by adjusting the ***Line Width***.

4 Draw Color



Draw Color controls the color of the marquee line you see when you are drawing a region. This does not control the color of the region once it is drawn. ***Apply*** and ***Set Default*** will set this color for the current session. To have the setting persisit for future sessions go to Administration Tab > Hardware Properties > Default View Settings > Default Marquee Color Red/Green/Blue.

NOTE: you will need the RGB values for the color you select when using Hardware Properties.

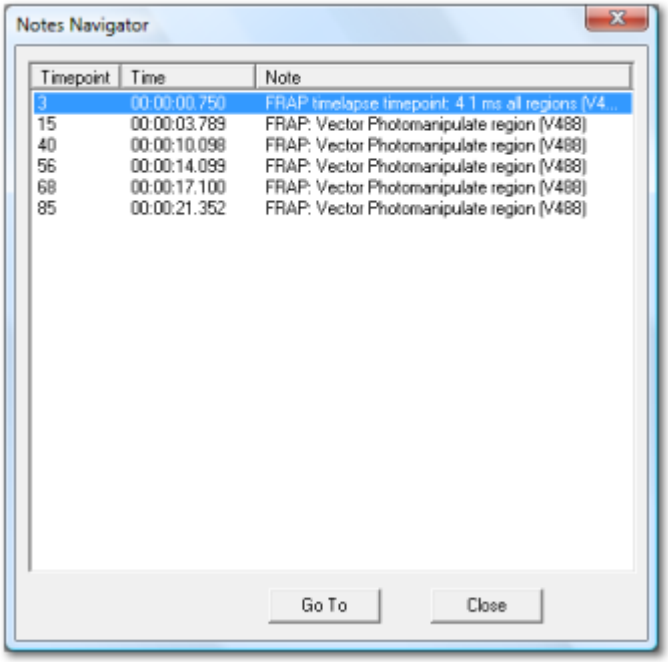
Notes Navigator window

When you have a large data set or many notes in a data set, the ***Notes Navigator*** will allow you to view the notes as a list and easily move to a specific note in the data set.

Access by ***Navigate > Notes***

See also:

- Annotation Settings Notes tab
- Edit Note window
- Notes Tab of Capture Preferences window
- Notes Tab of Capture Controls window
- Ratio Manual: Creating Event Markers (Notes)
- Photomanipulation Manual: Designing Experiments Using Vector



To navigate to a desired event either:

Select an event by clicking on it in the list. The selected time point will be highlighted in blue as shown above. Click ***Go To*** to proceed to the selected event.

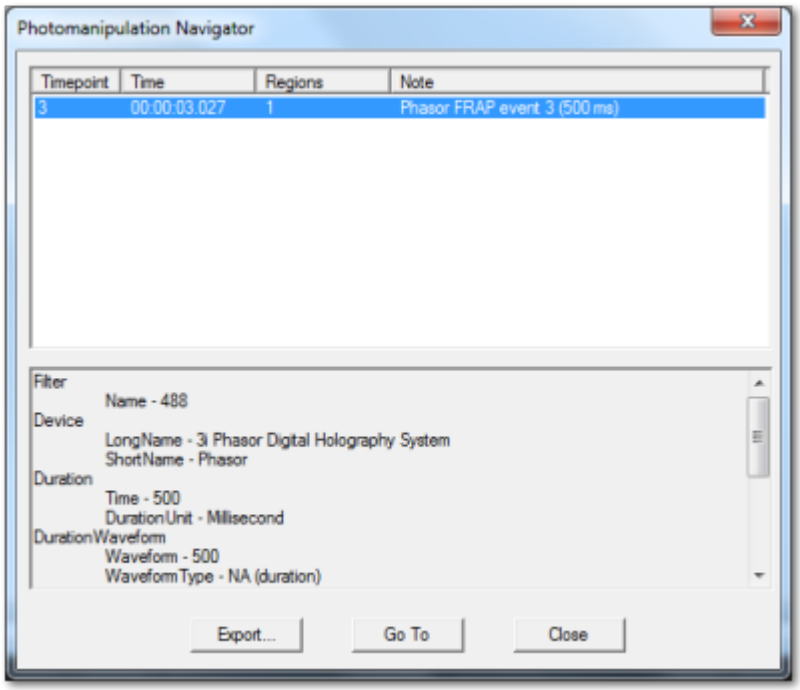
Alternatively, double-click on an event to advance the data set to the desired event.

When finished, click ***Close*** to close the dialog.

Photomanipulation Navigator window

When you have a large data set or many photomanipulation events in a data set, the ***Photomanipulation Navigator*** will allow you to view the events as a list and easily move to a specific time point in the data set. It is also important to note that photomanipulation metadata is not exported with tiffs so the only way to export this information out of SlideBook is through this interface.

Access by ***Navigate > Photomanipulation***



To navigate to a desired event either:

> Select an event by clicking on it in the list. The selected time point will be highlighted in blue as shown above. Click ***Go To*** to proceed to the selected event.
>
> Alternatively, double-click on an event to advance the data set to the desired event.

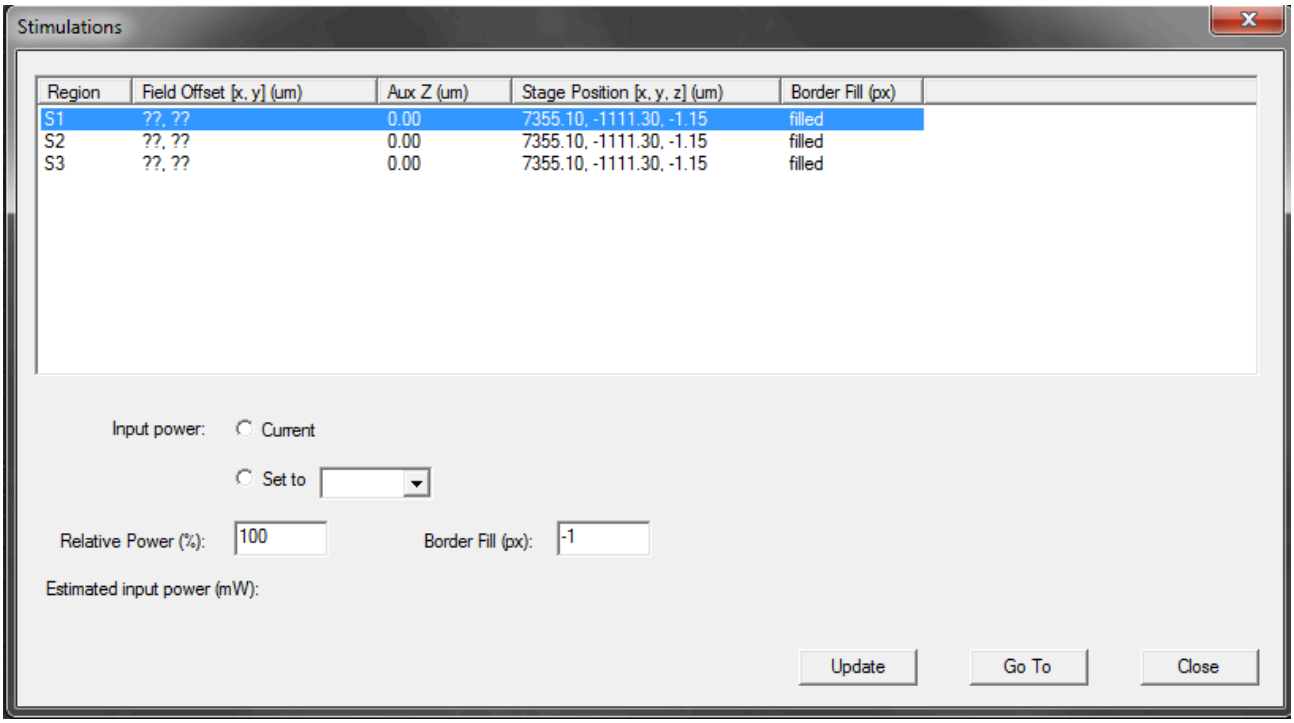
Click ***Export*** to export a text file of the photomanipulation event details.

When finished, click ***Close*** to close the dialog.

Stimulations Navigator window

Complex photomanipulations may require specific setting be applied to individual regions.

Access by ***Navigate > Stimulations***



Click on a stimulation region to select it from the list.

Adjust parameters as desired:

Input power: Select ***Current*** to use the value set in the Focus Controls window. Alternatively, select ***Set to*** and choose a value from the drop down menu.

Relative Power (%):

Border Fill (px): When an ellipse region is used as a stimulation region, a value of **-1** or **1** will fill the ellipse. A value of ***2*** or greater will use a ring of the designated pixel width for ring FRAP.

Estimated Input power (mW):

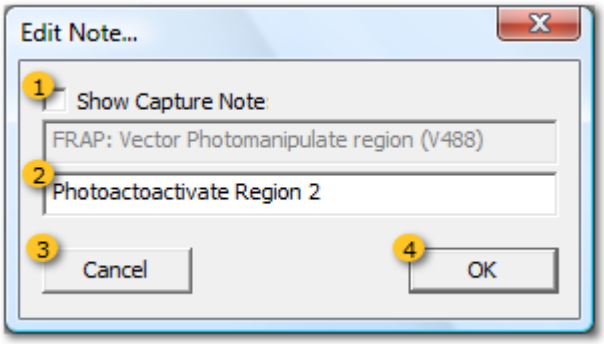
Edit Note window

Post acquisition, you may find it useful to edit the text of notes created during capture. This can be done easily using the ***Edit Note*** dialog.

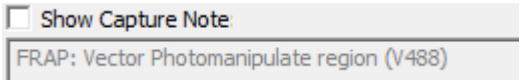
Access by ***Edit > Notes***

See also:

- Annotation Settings Notes tab
- Notes Navigator window
- Notes Tab of Capture Preferences window
- Notes Tab of Capture Controls window
- Ratio Manual: Creating Event Markers (Notes)
- Photomanipulation Manual: Designing Experiments Using Vector

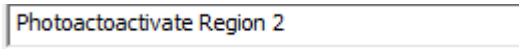


1 Original Capture Note options



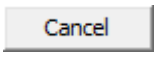
Check the box to display the original note text that is shown in the text field below. Should you choose to display the original note , the new note text will be displayed below the original.

2 New Text field



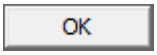
Type the new note text in the second edit field.

3 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

4 OK button



Press ***OK*** to apply the changes and close the dialog.

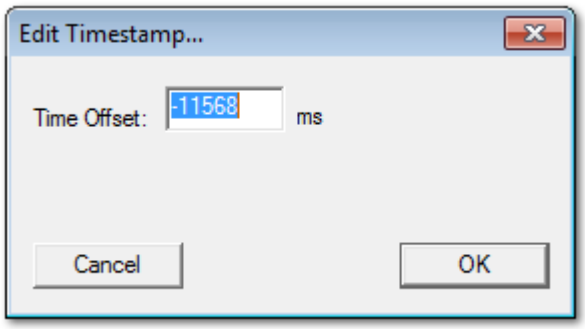
Edit Timestamp window

Post acquisition, you may find it useful to reset the "**0**" timepoint. This can be done easily using the ***Edit Timestamp*** dialog.

Access by ***Edit > Timestamps***

See also:

[Annotation Settings Timestamps tab]



1. Open a timelapse image.

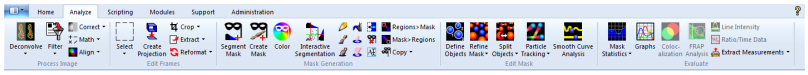
2. Advanced the image to the time point you wish to make "**0**".

3. Open the ***Edit Timestamp*** dialog. The ***Time Offset*** field will automatically be populated with the value that will set the current timepoint to "**0**".

4. Click ***OK***.

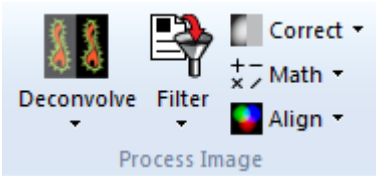
Analyze Tab

The Analyze tab contains many of the tools formerly found in the Image and Mask menus. The workflow moves from processing and cropping the data to generating masks that select voxels for analysis, through mask manipulation and refinement, ending with extracting statistical data.



Process Image Group

The Process Image group contains a variety of tools to help prepare your data for analysis. From quantitative calibrations and deconvolution to filtering approaches that aid in masking to mathematical operations on channels or images, this group has many processing techniques available.



Deconvolve Menu

Deconvolution is the process of reassigning or subtracting out of focus light from an image. There are many different types of deconvolution, some techniques are qualitative while others are quantitative and result in images that can be used for quantitative analysis.

Some deconvolution processes are very fast while other are much more computationally expensive. For the more complex deconvolution approaches, using an newer high speed processor (computer) will speed up the results. Types of deconvolution offered in SlideBook include:

No Neighbors Deconvolution - No Neighbors Deconvolution is the only algorithm included in the base package of SlideBook. All other algorithms are included in either the Deconvolution Module or Autoquant Blind Deconvolution Module contact 3i to learn more about purchasing these modules.

Nearest Neighbors Deconvolution - This algorithm is more computationally intensive and accurate than the No Neighbors algorithm. An image must have at least three planes in order to be deconvolved with the Nearest Neighbors algorithm.

Constrained Iterative Deconvolution - The constrained iterative approach is the most computationally expensive form of deconvolution and, if performed properly, can result in the best final image. All statistical analysis of intensities should be performed on images that have been deconvolved with the constrained iterative algorithm. Constrained iterative deconvolution can be very time consuming as well as memory intensive. Some users find no neighbors or nearest neighbors desirable for every day deblurring; however, there are certain cases where only the constrained iterative approach yields a reasonable result.

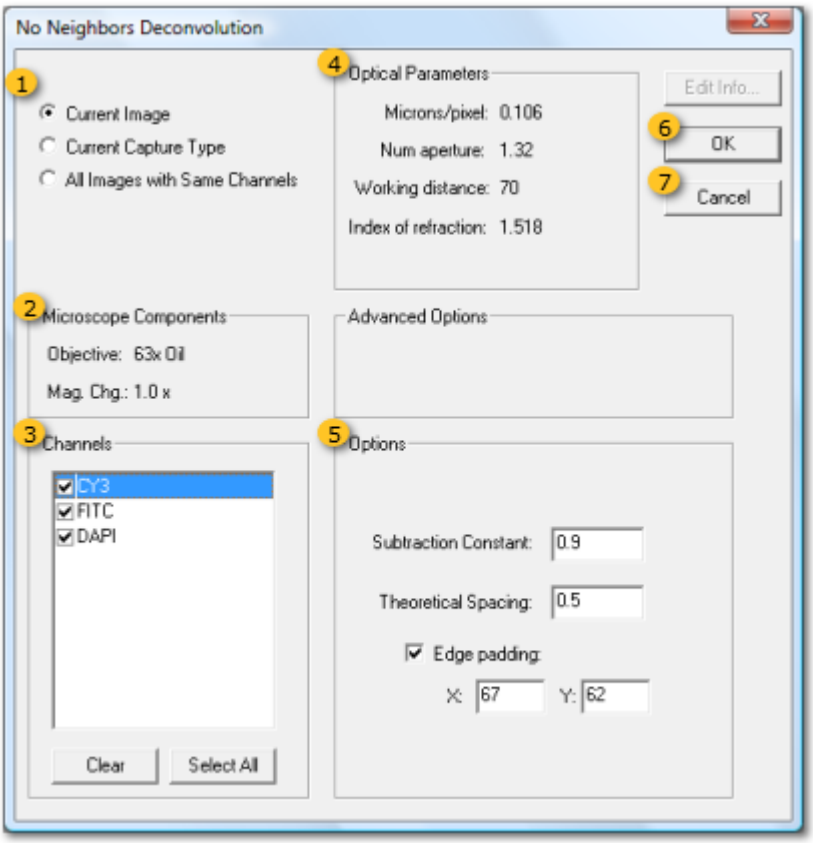
Autoquant Blind Deconvolution (for either Confocal or Widefield images) - Blind deconvolution is a class of constrained iterative in which not only the resultant image but also the PSF is computed by algorithm. Blind Deconvolution can be used with Widefield Fluorescence, Point Scanning Confocal, and Spinning Disk Confocal.

No Neighbors Deconvolution window

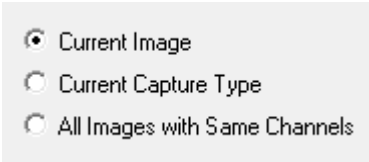
No neighbors is the least computationally intensive method of deconvolution offered in SlideBook and the only method that can be used on 2D images. While no neighbors deconvolution can be applied to three dimensional data sets, it does not use information across multiple planes to calculate and remove out of focus information. Rather, it functions by creating a theoretical blurred image from the original two dimensional image. This blurred image is then subtracted from the original image, leaving objects that are more likely to be in focus.

See also:

Deconvolution Module



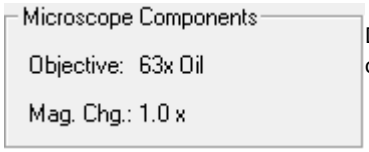
1 Image Range



Select image range as follows:

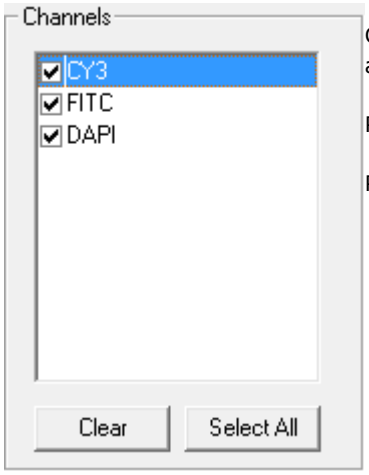
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels group

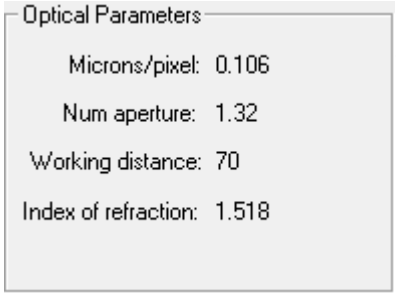


Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

Press ***Clear*** to uncheck all boxes.

Press ***Select All*** to check all boxes.

4 Optical Parameters group



Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing.

5 Options group

These options allow you to adjust values used in deconvolution calculations as follows:

Subtraction Constant - The subtraction constant is the multiplier of the extent to subtract the blurred image from the raw data. It is a value that will weight how much impact the blurred image has on the end result. Increasing the subtraction constant will increase the amount of deblurring or the amount of signal subtracted.

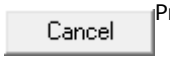
Theoretical Spacing - Theoretical spacing tells SlideBook how much to blur the original data before subtracting that blurred image to create the no neighbors deconvolved image. Increasing this value will result in increasing the out of focus information to subtract, decreasing this value will result in less subtraction. If the theoretical distance is set to **0**, the unaltered image is subtracted (modulated by the subtraction constant). If the spacing is large, then you approach the constant value of the image average with no features. If you notice that real features are being lost when no neighbors deconvolution is performed, decrease this value.

Edge Padding - Checking this box allows you to add pixels to the *x* and *y* dimension using mirror padding. This is particularly useful when fluorescence is present at the edges of the image. If you notice ringing around objects near the edge of the image, you should increase the amount of edge padding used.

6 OK button

Press ***OK*** to start no neighbors deconvolution and close the dialog. An image with **.NoN** extension will be written to the slide.

7 Cancel button



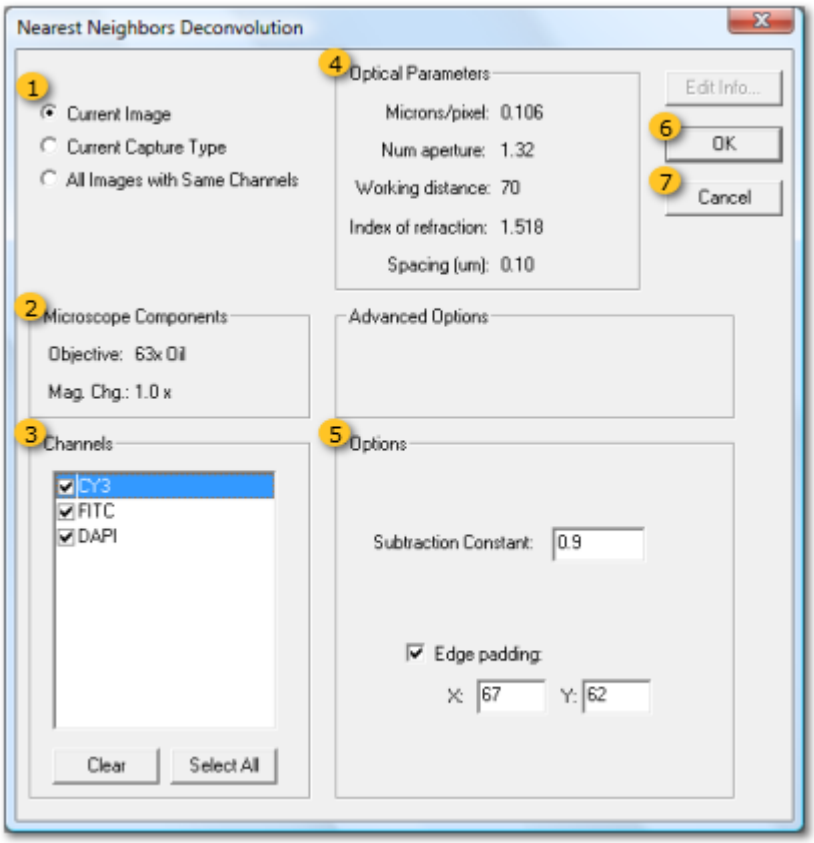
Press ***Cancel*** to close the dialog without initiating deconvolution.

Nearest Neighbors Deconvolution window

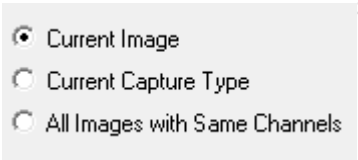
Nearest neighbors deconvolution is a deblurring technique that uses a simplifying assumption that most of the out of focus blur in an image is due to the planes directly neighboring the plane of interest (i.e. directly above and below). To perform nearest neighbors deconvolution, one must start with a data set that is at least three planes deep. The algorithm computes how much blur the neighboring planes contribute to the captured image and subtracts the blur out. Since the algorithm is purely subtractive, it tends to delete very low intensity features. However, it is considerably less sensitive than constrained iterative deconvolution to low intensity image defects such as dust in the optical path. The largest single factor that affects the result of nearest neighbor deconvolution is the distance between neighboring planes. A smaller distance between neighboring planes will cause the algorithm to subtract more from the plane of interest.

See also:

Deconvolution Module



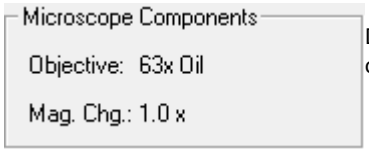
1 Image Range



Select image range as follows:

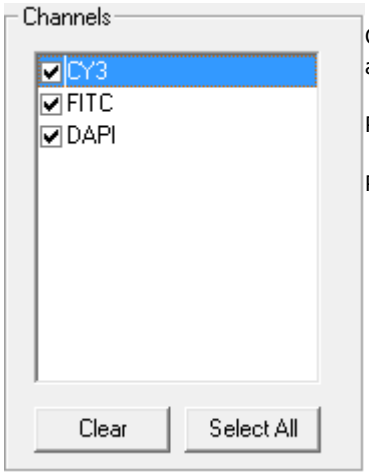
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels group

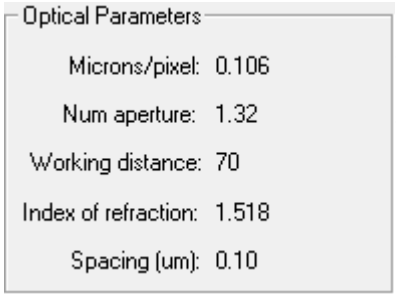


Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

Press ***Clear*** to uncheck all boxes.

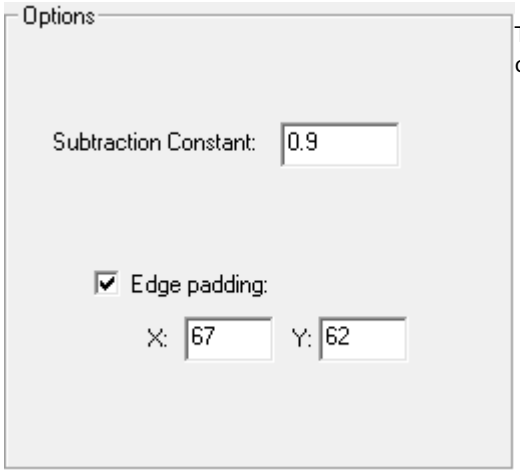
Press ***Select All*** to check all boxes.

4 Optical Parameters group



Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing. If optical parameters are incorrect, go to ***Get Info*** to adjust as necessary.

5 Options group



The options allow you to adjust values used in deconvolution calculations as follows:

Subtraction Constant - Increasing the subtraction constant will increase the amount of deblurring. The subtraction parameter adjusts the amount of light subtracted.

Edge Padding - Checking this box allows you to add pixels to the *x* and *y* dimension using mirror padding. This is particularly useful when fluorescence is present at the edges of the image. If you notice ringing around objects near the edge of the image, you should increase the amount of edge padding used.

6 OK button

Press ***OK*** to start nearest neighbors deconvolution and close the dialog. An image with **.NearN** extension will be written to the slide.

7 Cancel button

Press ***Cancel*** to close the dialog without initiating deconvolution.

Constrained Iterative Deconvolution window

The constrained iterative approach is the most computationally expensive form of deconvolution and, if performed properly, can result in the best final image. All statistical analysis of intensities should be performed on images that have been deconvolved with the constrained iterative algorithm. Essentially, the algorithm makes sequential guesses about the true distribution of fluorescence in an unblurred three dimensional data set. It then compares its computationally blurred guess with the original data and modifies its next guess appropriately. Sequential guesses should converge on a unique solution. The user must determine how many iterations or guesses the algorithm makes.

Because constrained iterative deconvolution considers all of the data to perform its guesses, the approach is very sensitive to the condition of the original data. If performed improperly, the constrained iterative approach will result in a highly artifactual data set. To ensure good results with this approach the following conditions should be met:

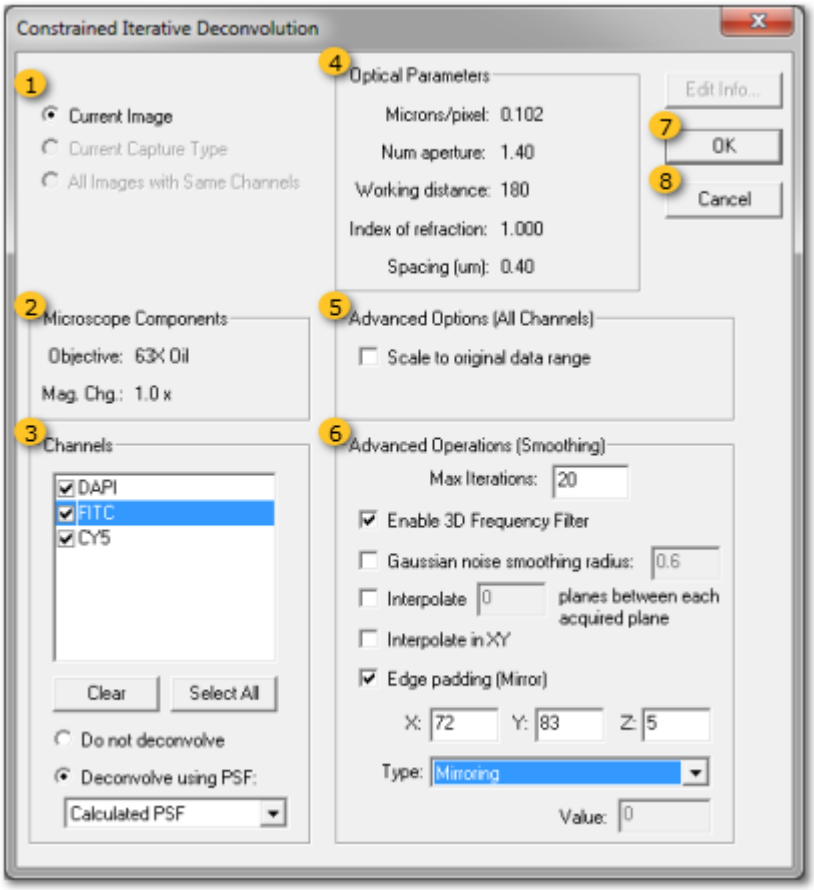
- The original data set should span the sources of fluorescence in the *z* dimension. If there are significant sources of fluorescence that reside above or below the original data set, the algorithm will not be able to properly assign their source within its next guess.

- The data should be flat field corrected. If there are any constant artifacts within each plane of the original data (e.g. dust specks), the algorithm must guess 3D structures which will account for them. This usually results in fictitious tube structures in the final image set.

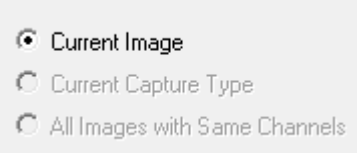
- The data, as always, should reside in the linear range of the camera (not saturated) and should be well above the noise floor of the camera. Very low intensity data sets have a poor signal to noise ratio. This will result in a "scratchy looking" final guess.

See also:

Deconvolution Module



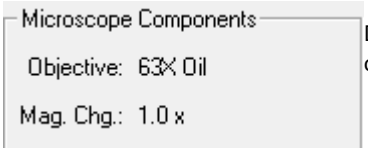
1 Image Range



Select image range as follows:

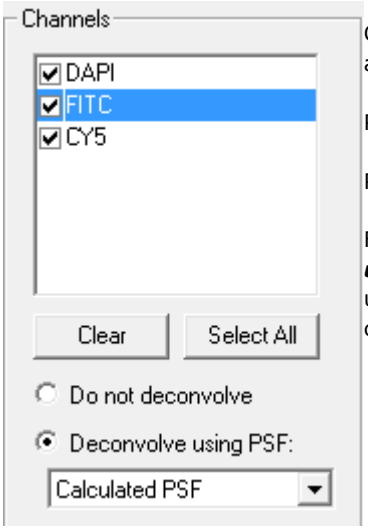
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels group



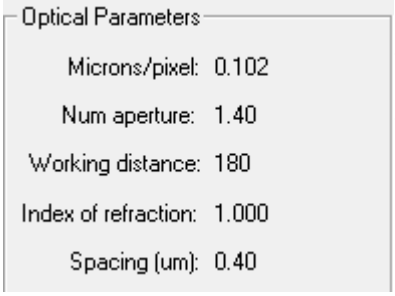
Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

Press ***Clear*** to uncheck all boxes.

Press ***Select All*** to check all boxes.

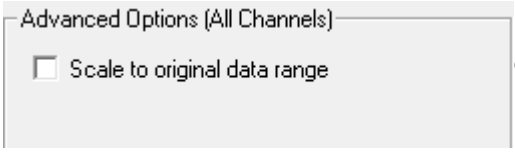
For each individual channel, you may select ***Do not deconvolve*** or ***Deconvolve using PSF***. When you choose to deconvolve a channel, you have the option of using a Calculated PSF or measured PSF that matches the channel and optical components of the image. See also PSF Guide.

4 Optical Parameters group



Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing. If optical parameters are incorrect, go to ***Get Info*** to adjust as necessary.

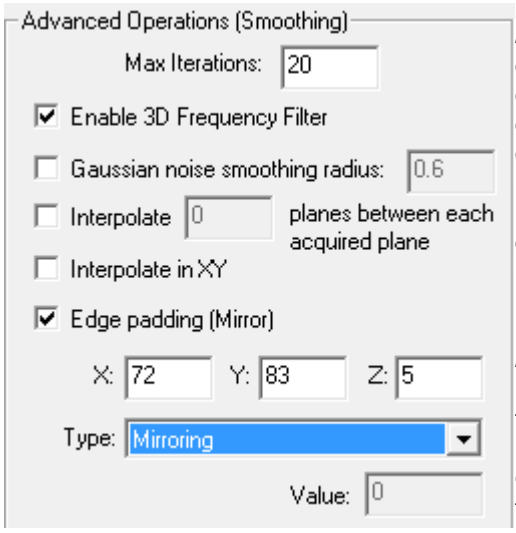
5 Advanced Options (All Channels) group



Because constrained iterative deconvolution reassigns out of focus light rather than subtracting it, the ending maximum intensity value can be much higher, ultimately changing the bit depth of the image. For example, an image acquired using a 12-bit camera with intensity range of 0-4095 may fill 16-bits of intensity space or 0-65535.

To rescale the image back to the original intensity range, check the box to ***Scale to original data range***.

6 Advanced Operations (Smoothing) group



Max Iterations - The default is 20, but the deconvolution will stop earlier if the algorithm reaches the optimal solution. The deconvolution algorithm operates by generating a guess, blurring the guess with the point spread function, and then comparing the blurred guess with original image. If the comparison determines that the image is not improving, the algorithm is stopped. Increasing this number can generate a better set of guesses: but often, the comparison condition will stop the algorithm before completing the iterations. Therefore, you should only increase this parameter if the previous deconvolution completed all of the specified iterations.

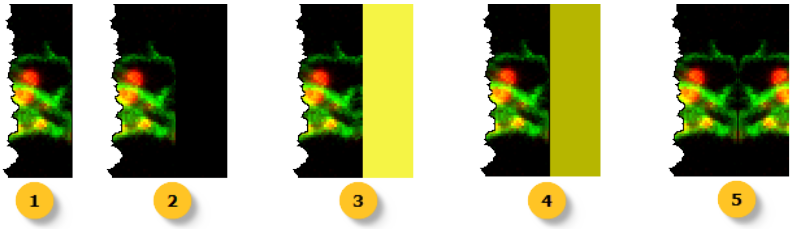
Enable 3D Frequency Filter - Applies a low pass filter in 3D to remove excess single pixel (or smaller than optically possible) features from building up in the image.

Gaussian noise smoothing radius - Turning this function on helps to minimizes artifacts due to noise and hot pixels. Turn this on or increase the value if the deconvolved image looks too grainy.

Interpolate planes between each acquired plane - This is useful when the data is too sparse in *z* to properly perform deconvolution. Interpolation in z is particularly useful when features start to disappear in deconvolution due to under-sampled data.

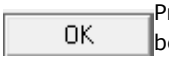
Interpolate in XY - This option is useful if excessive binning was used for image capture or the data is too sparse in *xy*. This is rarely necessary.

Edge Padding - Checking this box allows you to add pixels to the *x* and *y* dimension using a variety of padding types described below. This is particularly useful when fluorescence is present at the edges of the image. If you notice ringing around objects near the edge of the image, you should increase the amount or type of edge padding used. Types of padding are illustrated and described below



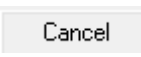
(1) None - Uses the image in its original form
(2) Zero - Uses a zero intensity value for all pixels of each channel in the edge padding area
(3) Constant - Uses a user-defined intensity value for all pixels of each channel in the edge padding area, based on the ***Value*** edit field below
(4) Most Common Pixel - Uses the most common pixel intensity (median value) in each channel (across the entire image? defined edge area?) for every pixel in the edge padding area
(5) Mirroring - Uses a mirror of the pixel intensities in each channel

7 OK button



Press ***OK*** to start constrained iterative deconvolution and close the dialog. An image with .CI extension will be written to the slide.

8 Cancel button



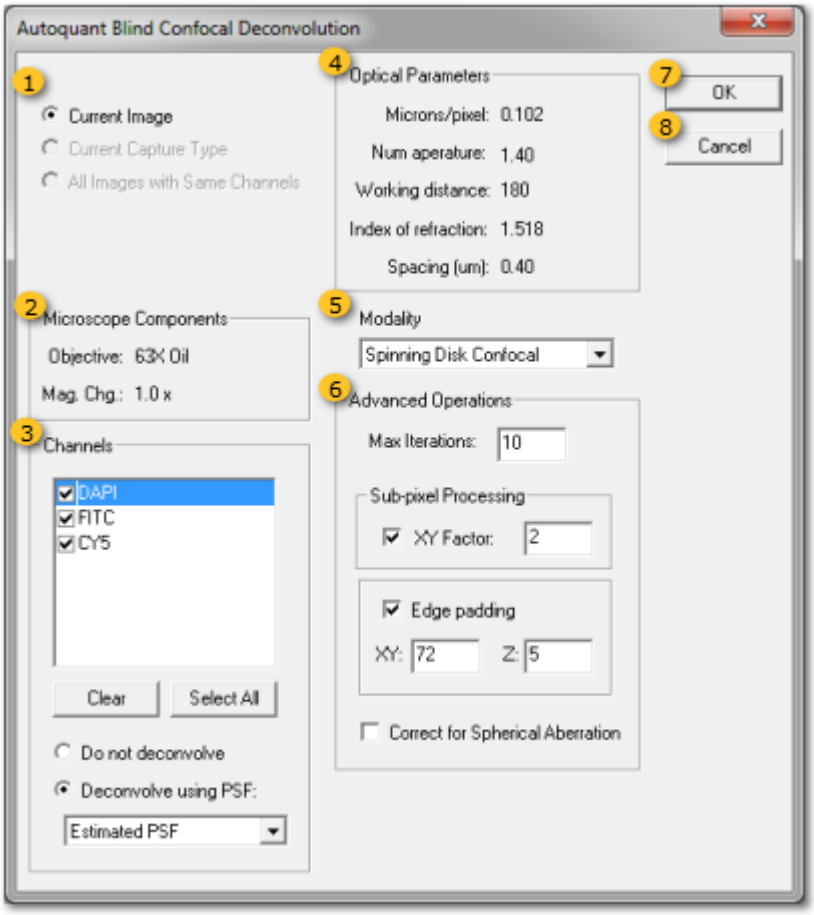
Press ***Cancel*** to abandon deconvolution and close the dialog.

Autoquant Blind Confocal Deconvolution window

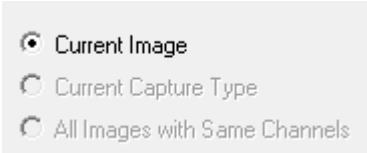
3D Blind Deconvolution is an iterative and constrained algorithm. It is iterative in the sense that it repeats the same computational operations many times while converging to the enhanced image solution. It is constrained in the sense that it only accepts deconvolved images that have the correct mathematical properties of nonnegativities, (that is, it does not allow the tracer concentration to have negative values) and smoothness (suppresses snowy-like noise due to low-light levels). 3D Blind Deconvolution is a method of deconvolution that adapts itself to the real PSF of the microscope system (which can be significantly different from the theoretical PSF and from the previously measured PSFs) due to specimen and instrument variations. The Autoquant Blind Deconvolution system is able to adapt to PSF changes within a specimen itself. Thus, the deconvolved results are superior to those methods which utilize theoretical or previously measured PSFs.

See also:

Deconvolution Module



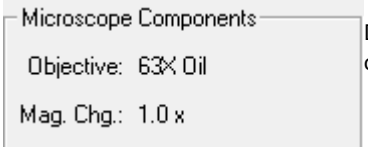
1 Image Range



Select image range as follows:

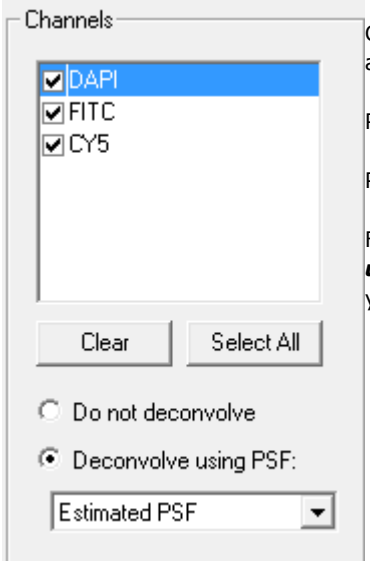
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels list



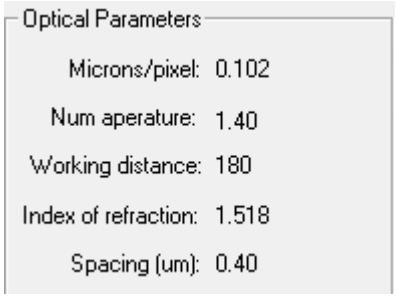
Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

Press ***Clear*** to uncheck all boxes.

Press ***Select All*** to check all boxes.

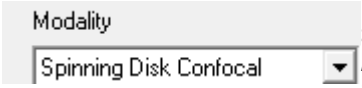
For each individual channel, you may select ***Do not deconvolve*** or ***Deconvolve using PSF***. When you choose to deconvolve a channel using blind deconvolution, you will use an Estimated PSF.

4 Optical Parameters group



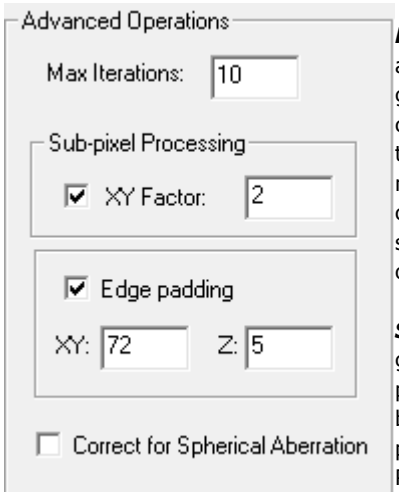
Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing. If optical parameters are incorrect, go to ***Get Info*** to adjust as necessary.

5 Modality Drop down



Select the confocal modality used to capture the image. Options include: ***Spinning Disk Confocal***, ***Two Photon***, and ***Point Scanning Confocal***. This parameter is important in estimating the amount of out of focus light present in the raw image.

6 Advanced Operations group



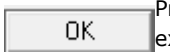
Max Iterations - The default is 10, but the deconvolution will stop earlier if the algorithm reaches the optimal solution. The deconvolution algorithm operates by generating a guess, blurring the guess with the point spread function, and then comparing the blurred guess with original image. If the comparison determines that the image is not improving, the algorithm is stopped. Increasing this number can generate a better set of guesses: but often, the comparison condition will stop the algorithm before completing the iterations. Therefore, you should only increase this parameter if the previous deconvolution completed all of the specified iterations.

Sub-pixel Processing - This option will perform deconvolution on a sub-pixel grid to achieve super-resolved results. It will also, however, result in longer processing times. If the feature has been checked, the ***XY Factor*** feature will become enabled. This feature allows you to select the number by which the pixels will be divided. The options are **1** (which is for when the Sub-pixel Processing feature is not activated), **2** and **3**, with three being the highest resolution, and longest processing time.

Edge Padding - allows you to add pixels to the *x*, *y*, and *z* dimension using mirror padding. This is particularly useful when fluorescence is present at the edges of the image.

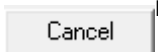
Correct for Spherical Aberration -

7 OK button



Press ***OK*** to start Autoquant blind confocal deconvolution and close the dialog. An image with **.Blind** extension will be written to the slide.

8 Cancel button



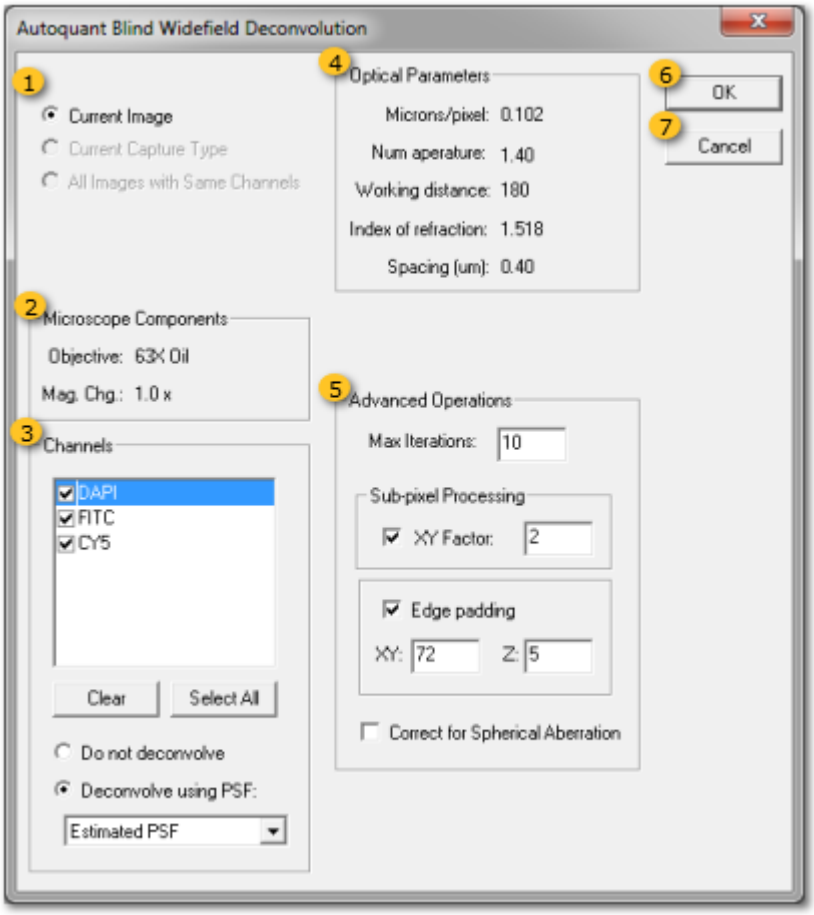
Press ***Cancel*** to abandon deconvolution and close the dialog.

Autoquant Blind Widefield Deconvolution window

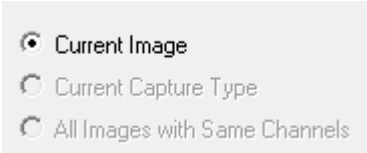
3D Blind Deconvolution is an iterative and constrained algorithm. It is iterative in the sense that it repeats the same computational operations many times while converging to the enhanced image solution. It is constrained in the sense that it only accepts deconvolved images that have the correct mathematical properties of nonnegativities, (that is, it does not allow the tracer concentration to have negative values) and smoothness (suppresses snowy-like noise due to low-light levels). 3D Blind Deconvolution is a method of deconvolution that adapts itself to the real PSF of the microscope system (which can be significantly different from the theoretical PSF and from the previously measured PSFs) due to specimen and instrument variations. The Autoquant Blind Deconvolution system is able to adapt to PSF changes within a specimen itself. Thus, the deconvolved results are superior to those methods which utilize theoretical or previously measured PSFs.

See also:

Deconvolution Module



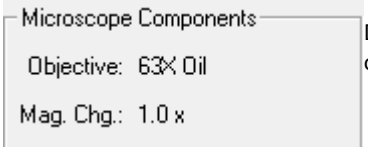
1 Image Range



Select image range as follows:

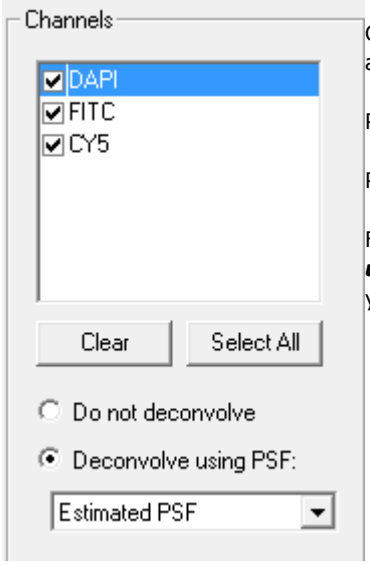
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide.

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels list



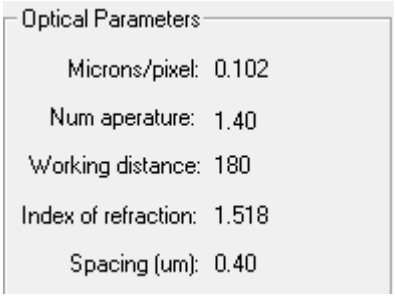
Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

Press ***Clear*** to uncheck all boxes.

Press ***Select All*** to check all boxes.

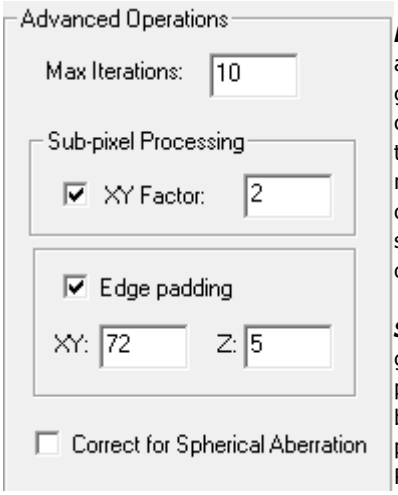
For each individual channel, you may select ***Do not deconvolve*** or ***Deconvolve using PSF***. When you choose to deconvolve a channel using blind deconvolution, you will use an Estimated PSF.

4 Optical Parameters group



Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing. If optical parameters are incorrect, go to ***Get Info*** to adjust as necessary.

5 Advanced Operations group



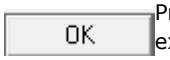
Max Iterations - The default is 10, but the deconvolution will stop earlier if the algorithm reaches the optimal solution. The deconvolution algorithm operates by generating a guess, blurring the guess with the point spread function, and then comparing the blurred guess with original image. If the comparison determines that the image is not improving, the algorithm is stopped. Increasing this number can generate a better set of guesses: but often, the comparison condition will stop the algorithm before completing the iterations. Therefore, you should only increase this parameter if the previous deconvolution completed all of the specified iterations.

Sub-pixel Processing - This option will perform deconvolution on a sub-pixel grid to achieve super-resolved results. It will also, however, result in longer processing times. If the feature has been checked, the ***XY Factor*** feature will become enabled. This feature allows you to select the number by which the pixels will be divided. The options are 1 (which is for when the Sub-pixel Processing feature is not activated), 2 and 3, with three being the highest resolution, and longest processing time.

Edge Padding - allows you to add pixels to the *x*, *y*, and *z* dimension using mirror padding. This is particularly useful when fluorescence is present at the edges of the image.

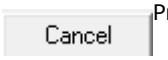
Correct for Spherical Aberration -

6 OK button



Press ***OK*** to start Autoquant blind confocal deconvolution and close the dialog. An image with **.Blind** extension will be written to the slide.

7 Cancel button

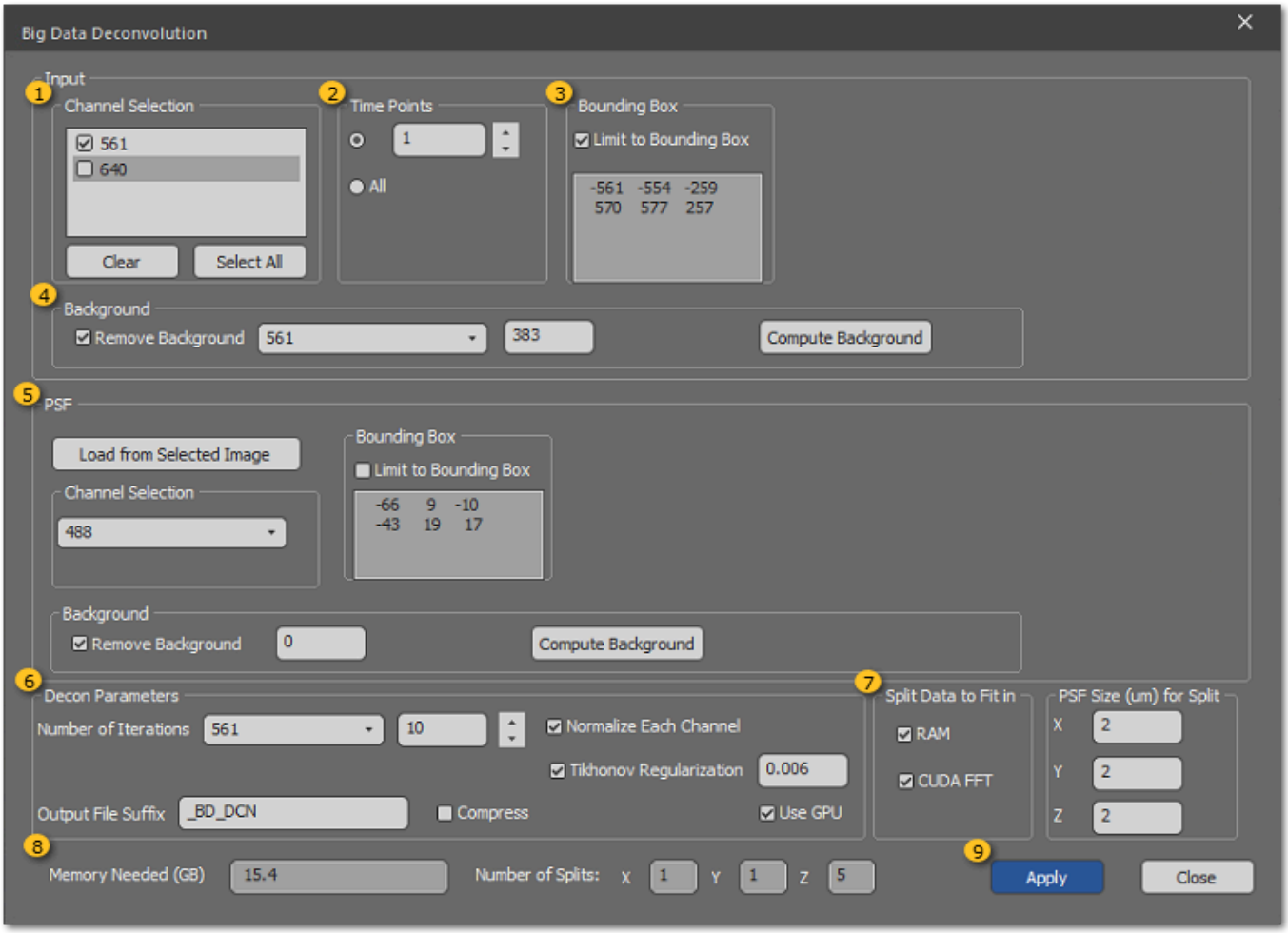


Press ***Cancel*** to abandon deconvolution and close the dialog.

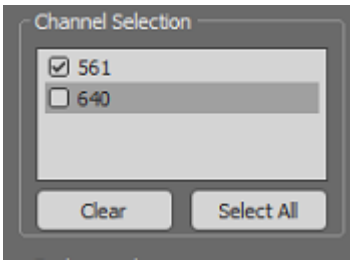
Big Data Deconvolution

The Big Data Deconvolution provides a 3D deconvolution for fluorescent microscopy images as a module SlideBook. The algorithm is Luci-Richardson based similar to conventional algorithm of the Fiji Multiview Reconstruction plugin and requires point spread functions (PSFs) for light-sheet, widefield, confocal, or two-photon microscopes and processing is GPU accelerated. Alternatively, very large images can be split into smaller sub-volumes and processed separately if the GPU(s) do not have sufficient memory to process the whole image.

You will find the Big Data Deconvolution when highlighting your capture of interest and select ***Analyze > Deconvolution > Big Data Deconvolution***. The Dialogue will open to enable the user to set the deconvolution parameters.



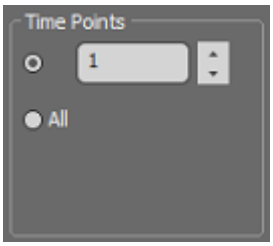
1 Channel Selection



Select the channel(s) you want to deconvolve. It is possible to deconvolve all channels in the image with a PSF of the shortest wavelength.

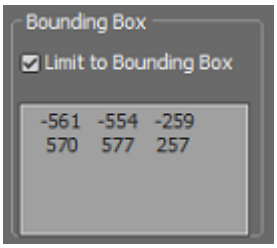
NOTE: To only copy a a channel into the deconvolved image select it and run only one iteration.

2 Time Points



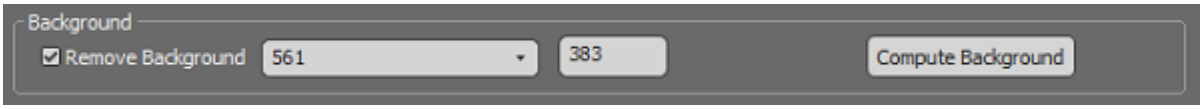
Time Points: Select/type in a single time point or tick ***All*** if you want to deconvolve all time points of a time lapse.

3 Bounding Box



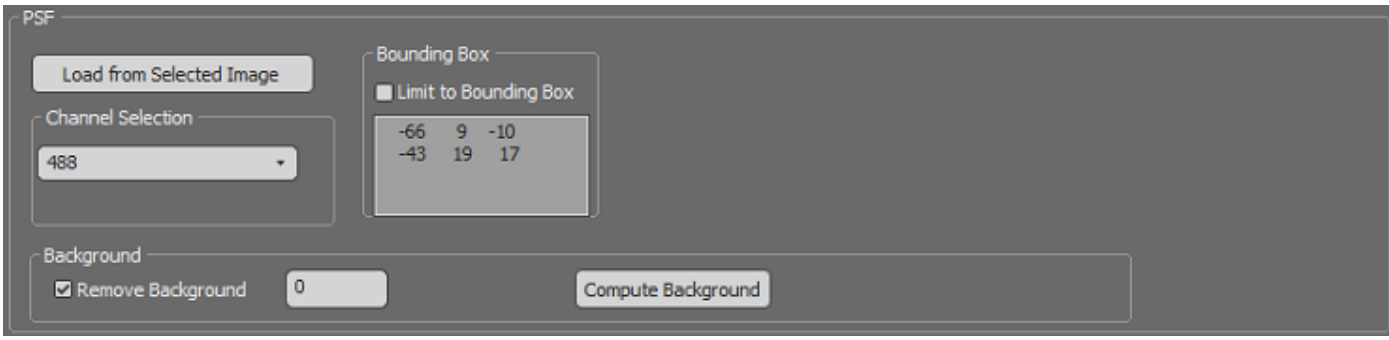
Bounding Box will show you the defined values for this capture if a bounding box was defined e.g. through the 3D/ 4D Volume View > Bounding Box Icon
Limit to Bounding Box: Enable this tick box if the deconvolved file should only include the data within the selected bounding box

4 Background



Enable this box and enter a value or choose ***Compute Background*** to subtract the background noise before deconvolution. For some captures this can greatly improve the deconvolution result like edge padding.

5 PSF Group



You can use beads in the captured file, a bead image captured with the same magnification or a generated PSF file as long as the parameters match the data to deconvolve.
When using from a bead from the same file as PSF define the PSF Bounding Box using the tool in 3D/ 4D Volume View > Boundi . Tick PSF in the bounding box dialogue and select ***Limit to Bounding Box*** here, in this case.

• Open a **.sld** file with a beads capture, with assigned registration and PSF bounding box or use the same file as you want to deco an applied PSF bounding box around a beads pare within.
• Highlight the file with the PSF bounding box and hit ***Load from Selected Image***.
• You can choose a channel in ***Channel Selection***. It is best practice to use a PSF from beads imaged at the same wavelength fo channel in your capture to deconvolve. For deconvolving several channels in one go select the shortest wavelength present in th your PSF
• ***Bounding Box*** will show you the defined values for the PSF capture.

• ***Background:*** Tick the check box and enter a value or choose ***Compute Background*** to subtract the background noise of the deconvolution. This is recommended especially when splitting the data to fir in RAM or CUDA FFT (see below).

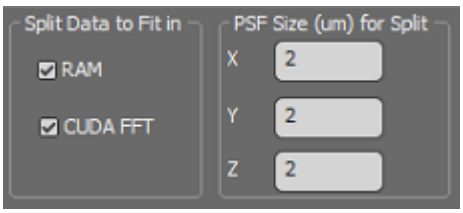
6 Deconvolution Parameters

Deconvolution Parameters:
• Check the box to ***GPU*** to use CUDA accelerated processing.
• Select the number of ***Iterations*** per channel. The default of 10 usually gives a good result. Increasing to 15 or 20 may provide further improvements. For a quick preview or just copy a channel into the deconvolved file, choose 1 or 2 iterations.

NOTE: This deconvolution is using an Luci-Richardson algorithm without threshold or correlation - this means that the optimal number of iterations needs to be adjusted per capture and/or capture conditions. On timelapses we recommend to establish the value with the first timepoint prior to deconvolving the entire time series.

• ***Normalize Each Channel*** will normalize the intensity per channel over all time points if applicable.
• ***Tikhonov Regularization*** is recommended to be applied at the default value.
• ***Compress***: Will automatically create a **.sldyz** file with lossless compression
• ***Output File Suffix***: When a suffix is entered the output **.sld** or **.sldy** will carry the original file name ending with this suffix. When removing the suffix the output file will be created in the same file as

7 Split to Fit



Split Data to Fit in RAM or CUDA FFT: Enable these tick boxes when the ***Memory Needed (GB)*** indicated at the bottom of the window is larger than the RAM of the PC.
• Check the box(es) for ***RAM*** only or RAM and ***CUDA FFT*** to use the CUDA for deconvolution.
• Enter a ***PSF size*** for the coordinates you want to split the image. Recommended values are between 1um and 5um. The algorithm will inform you if the selected values are not appropriate.
• ***NOTE:*** Setting the PSF thickness correctly is required to properly re-montage the slabs after deconvolution. For captures with the 40x objectives PSF sizes of 0.1-0.5 um, for captures with the 10x objectives values about 5 um are recommended

8 Process Information

Displays the ***Memory Needed (GB)*** and the ***Number of Splits*** when splitting the data to fit into RAM and CUDA FFT is enabled.

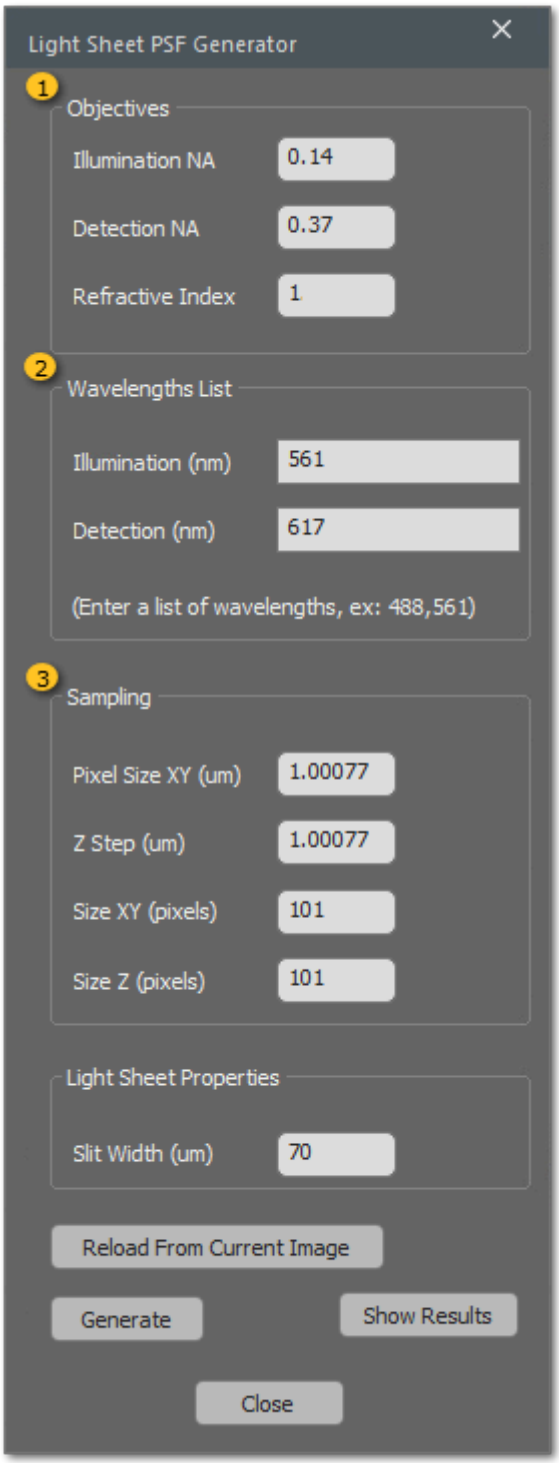
9 Action Buttons

Apply: Starts the deconvolution process
Close: Closes the Big Data Deconvolution deconvolution interface without processing

LightSheet PSF Generator

The Lightsheet PSF Generator creates specific PSFs for the deconvolution of CTLS captures. It is particularly well-suited for Axially Swept Light-sheet (AxL) imaging, where the excitation PSF is uniform across the field of view. The tool creates an individual 3D image within a slide to then be used with the Big Data Deconvolution.

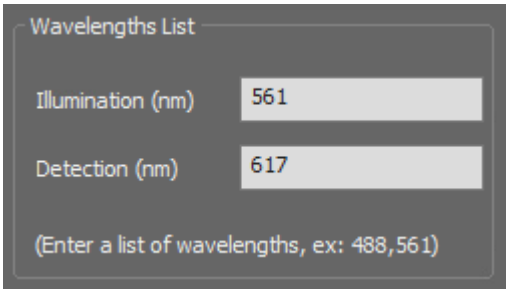
You will find the generator under ***Analyze > Deconvolution*** ***> Light Sheet PSF Generator***. When a capture is highlighted before opening the Lightsheet PSF generator the window will automatically be populated with the required details from the metadata. Alternatively values can be entered if required.



1 Objectives Group

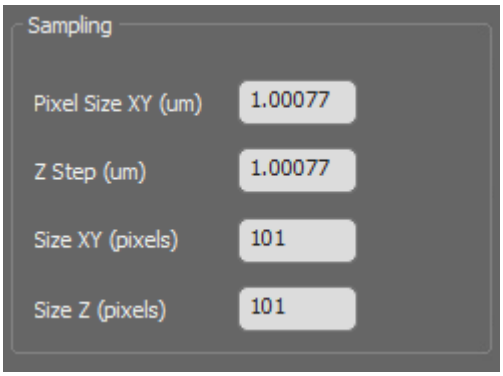
Displays the required objective and imaging parameters.

2 Channel Wavelength Group



Displays the channels available in the capture. If the capture contains several channels it will show a list for each ***Illumination*** and ***Detection*** wavelength in nm. The generator will create a PSF for each wavelength. You can also enter a list of wavelengths if required.

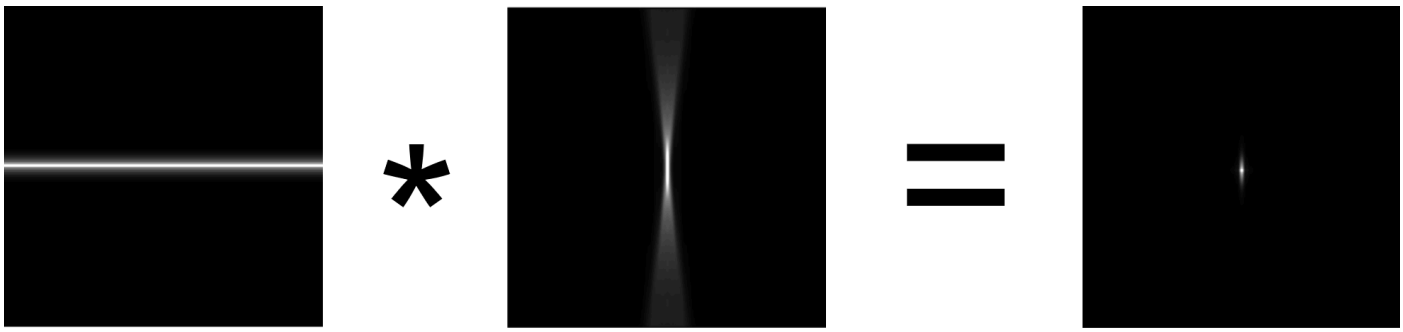
3 Sampling Group



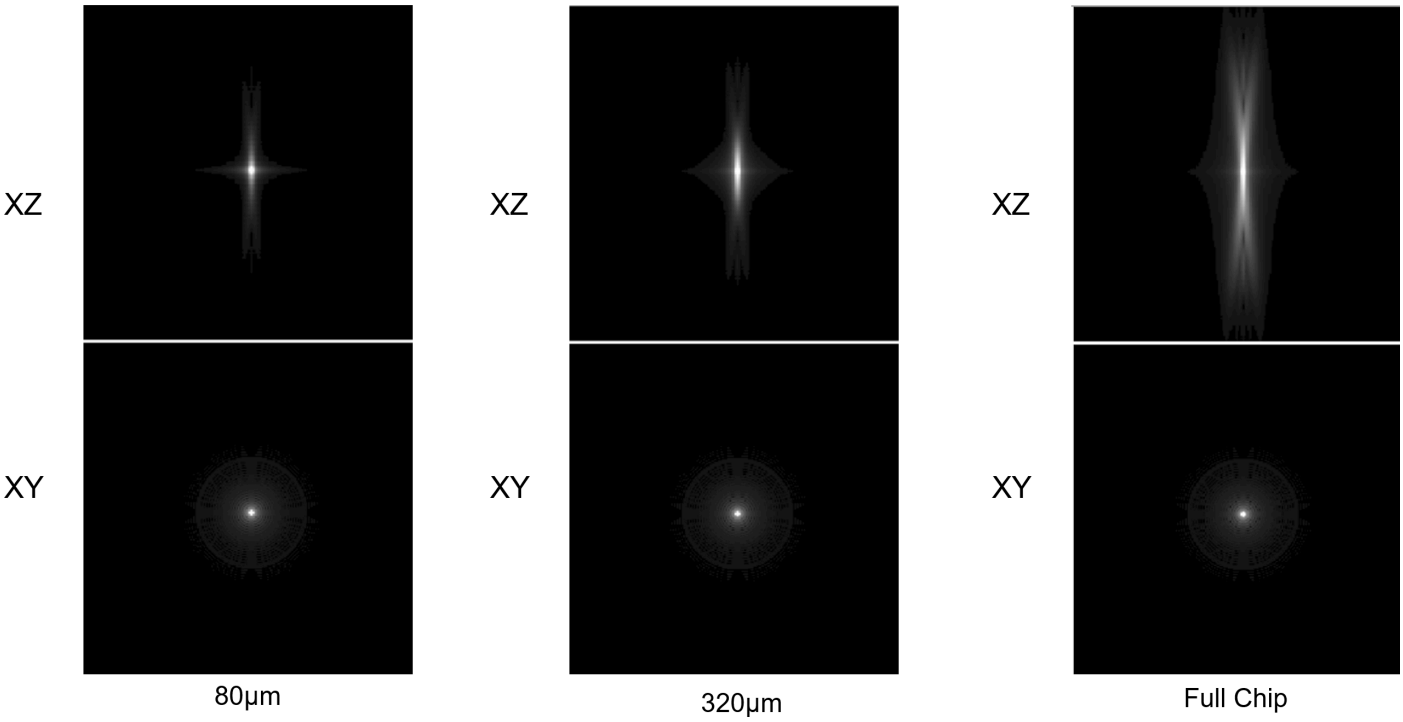
Displays the X,Y and Z sampling rate.

The generator calculates the overall PSF by multiplying the excitation PSF with the detection PSF. This approach is especially accurate for AxL, where the excitation is consistent across the imaging field:

- Excitation PSF: Represents the shape and intensity distribution of the light sheet.
- Detection PSF: Describes the optical properties of the detection optics.
- Combined PSF: Multiplying the two produces the effective 3D PSF.



NOTE: The Slit Width parameter controls how much of the excitation beam is used to generate the excitation PSF (see also Chapter Confocal Slit)
Smaller slit widths create sharper excitation PSFs, improving the optical section. Larger slit widths increase the illuminated volume but reduce axial confinement.

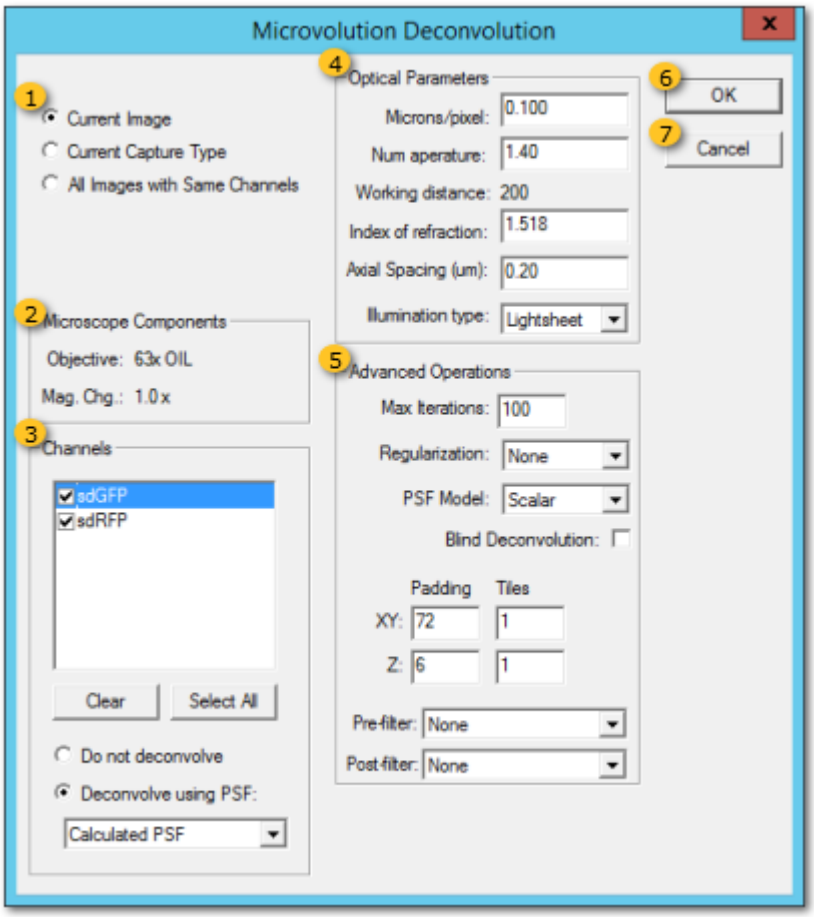


Microvolution Deconvolution window

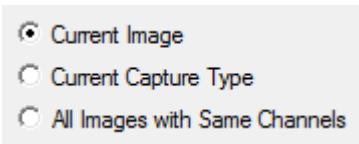
Microvolution software provides rapid 2D and 3D deconvolution for fluorescent microscopy images as a plugin for SlideBook. The software also mathematically generates point spread functions (PSFs) for widefield, confocal, and two-photon microscopes. An option can be purchased to enable blind deconvolution. This software runs exclusively on NVIDIA graphics cards, see minimal requirements here. An option to utilize multiple graphics cards in parallel can be purchased for certain configurations. Alternatively, very large images can be split into smaller sub-volumes and processed separately if the GPU(s) do not have sufficient memory to process the whole image.

See also:

Deconvolution Module



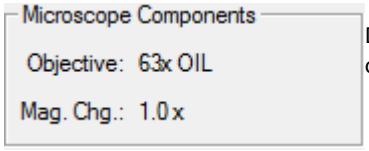
1 Image Range



Select image range as follows:

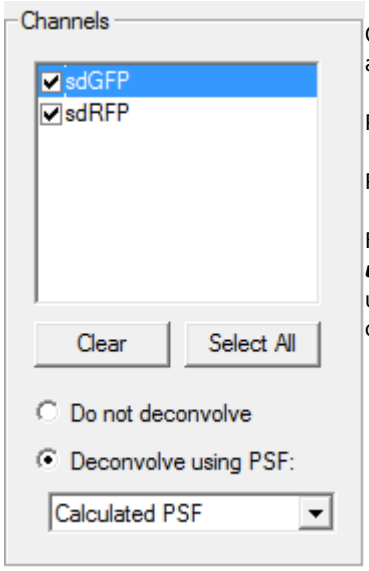
Current Image - Deconvolves only the currently selected image
Current Capture Type - Deconvolves all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Deconvolves all images captured using the same channels as the selected image in the current slide

2 Microscope Components group



Displays microscope optical components. It is essential that this information is correct for accurate image processing.

3 Channels group



Check the boxes next to the channels you would like to deconvolve. Channels that are not checked will be transferred unprocessed in the new image.

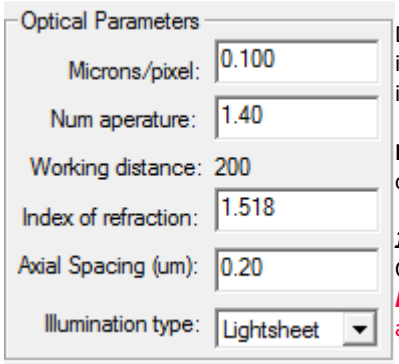
Press ***Clear*** to uncheck all boxes.

Press ***Select All*** to check all boxes.

For each individual channel, you may select ***Do not deconvolve*** or ***Deconvolve using PSF***. When you choose to deconvolve a channel, you have the option of using a Calculated PSF or measured PSF that matches the channel and optical components of the image. See also PSF Guide.

NOTE: When you add a PSF for use with Microvolution, you must select ***Section of entire image*** and NOT radially averaged for the second step of the PSF Guide. If the PSF is radially averaged, deconvolution will fail with an error that Microvolution does not accept radially averaged PSfs.

4 Optical Parameters group

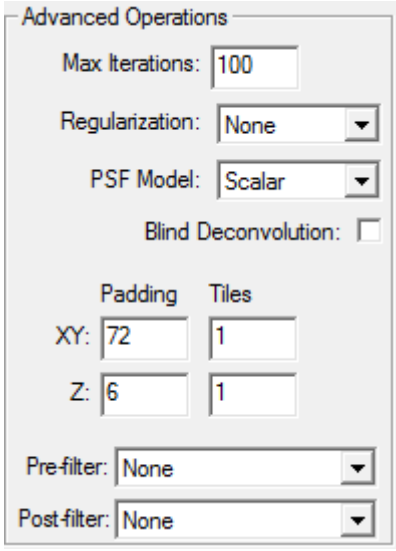


Displays optical parameters from the image capture. It is essential that this information is correct for accurate image processing. If optical parameters are incorrect, type in the edit fields to correct as necessary.

NOTE: You may wish to permanently change objective configuration if these optical parameters are incorrect.

Illumination type - Options include: Point Scanning Confocal, Spinning Disk Confocal, Two Photon, Widefield, Brightfield, Lightsheet, and Unknown. If using ***Lightsheet***, an additional parameter for the effective light sheet illumination NA appears WHERE??

5 Advanced Options (All Channels) group



Max Iterations - Number of deconvolution iterations to run per channel. The required number of iterations needed is dependent upon the quality of the image and the desired amount of deblurring. For many images, 10 iterations is a good starting point. If the chosen image is a hyperstack (multiple channels), then selectors for the number of iterations for
each channel appear. If the selected image has multiple time points, 7 then the specified iterations are run for each timepoint. To skip a channel (e.g. transmitted light), set this parameter to 0.

Regularization - Regularization to suppress amplification of noise and ringing artifacts. Current options:

- No regularization
- Total Variation (TV)
- Entropy

Entropy regularization is a more advanced algorithm than total variation, and is thus recommended over TV. In either case, selecting the ***Automatic regularization*** checkbox that appears after choosing Entropy or TV is highly recommended.

PSF Model - Two mathematical approximations of microscope diffraction are provided: ***Scalar*** and ***Vectorial***. The vectorial model is more accurate than the scalar model, particularly with high NA objectives, and is thus recommended

for most circumstances. In addition to the objective's NA and refractive index, the vectorial model requires knowledge of the sample's refractive index. The scalar model does not include the sample RI as a parameter, and thus cannot account for any refractive index mismatches.

Blind Deconvolution - Enable blind deconvolution. If blind deconvolution option is not licensed, then this panel is not displayed.

Padding - Reduce edge artifacts by adding a padded boundary of the specified number of pixels to the edges in the x, y, and/or z directions. Z padding in particular can give the biggest visual impact (as artifacts in this dimension are visible across the whole slice). This padding is added to both sides of the image. For many images, at most 10 pixels of padding are usually sufficient. Note that increasing the padding, particularly if the dimension is already a power of two, may cause the image to deconvolve slower than otherwise.

Tiles - Break the input image into smaller sub-volumes for images that are too large to deconvolve directly with the available devices. Tiling artifacts (i.e. lines of different intensity where the sub-volumes meet) are rarely seen, but can be at least reduced by increasing the padding in the same dimension.

Pre-filter -Filtering applied to image before deconvolution. Current options:

- No filtering
- Gaussian filter
- Gaussian filter, PSF
- Median filter

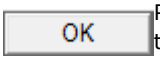
To suppress high frequency noise and compensate for any associated loss of high frequency signal, the third option (Gaussian filter image + PSF) is recommended for high noise images.

Post-filter - Filtering applied to image after deconvolution. Current options:

- No filtering
- Gaussian filter
- Median filter
- Sharpening filter

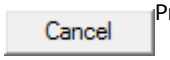
For most users, none of these options are recommended.

6 OK button



Press ***OK*** to start deconvolution and close the dialog. An image with **.Microvolution** extension will be written to the slide.

7 Cancel button



Press ***Cancel*** to abandon deconvolution and close the dialog.

Microvolution Minimum Requirements

Microvolution can be purchased as a SlideBook add-on through sales@intelligent-imaging.com.

Minimum requirements include:

- 64-bit operating system
  - Windows 7 or greater
  - OS X 10.11 or greater
  - Linux with GCC 4.6 support (e.g. Ubuntu 12.04, Fedora 16, RHEL/CentOS 7, openSUSE 12.1 or greater)
- NVIDIA graphics card with CUDA capabilities 2.0 or greater
  - https://developer.nvidia.com/cuda-gpus
  - Multi-GPU option requires multiple graphics cards
- Up-to-date NVIDIA drivers (minimum version 369)
- Minimum RAM depends on size of image, but 4+ GB recommended

PSF Guide

Measuring Point Spread Functions (PSFs)

SlideBook stores point spread functions (PSFs) in a data file. The PSF data file is accessed during constrained iterative deconvolution that specifies the use of measured PSFs. PSFs are computed using 3D images of fluorescent beads. In order to measure a PSF, the following steps are carried out:

1. Prepare a slide containing fluorescent beads (0.2 µm)
2. Capture a 3D image stack of an isolated bead. We suggest 7µm above and below the bead (14 µm total) at 0.1µm spacing.
3. Perform measurements on that stack using the PSF guide.

This sequence should then be repeated for each combination of objective, optovar, and fluorescence channel. You may also store more than one PSF for each optical configuration. This is useful for storing PSFs captured under different sample media conditions, different users, or different brightfield optic settings, for example.

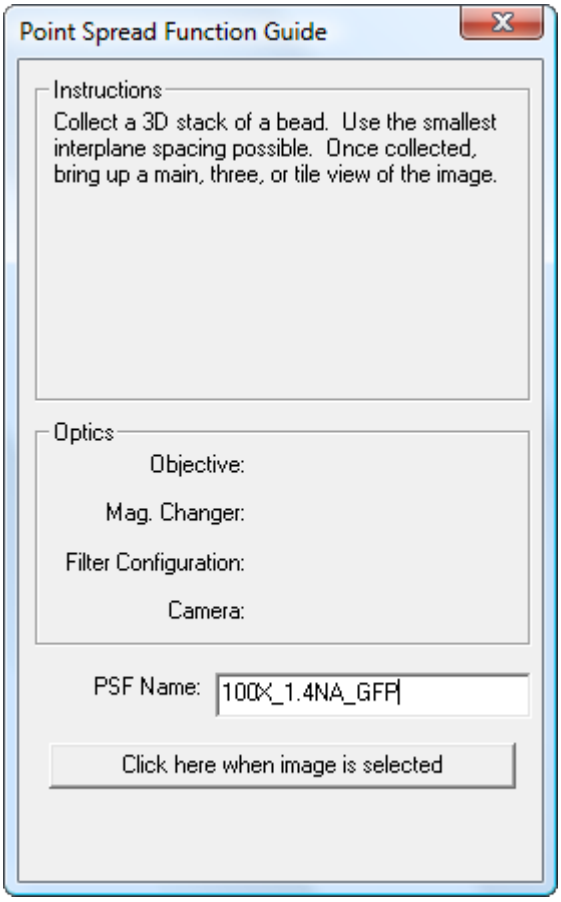
Capturing a 3D Stack of Fluorescent Beads Using SlideBook

The 3D bead image may either be captured using SlideBook or imported from another program. To perform 3D capture, a total depth of 14 µm taken at 0.1 µm intervals is sufficient for capturing a 0.2 µm bead. In order to reduce the amount of memory required for capture, you may wish to capture a single bead. Be sure to allow ample room around the single bead (at least fifty pixels on any side) so that all of the light from the bead is captured. Additionally, the image used to generate a PSF should be flat field corrected (see Flat Field Guide). To import an image, please see Home tab, Acquire group > Import Menu. Make sure that the image information (see Image Information) is correct before proceeding.

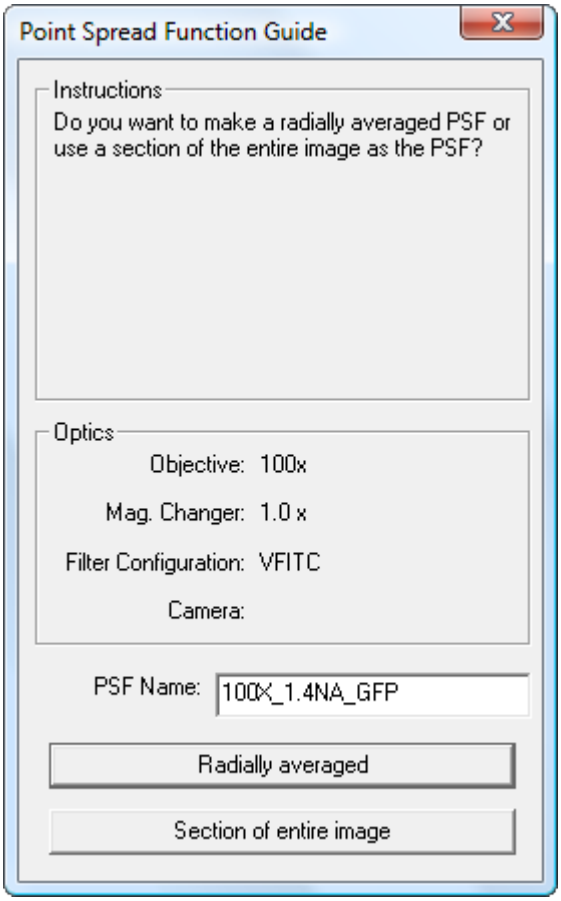
Generating a PSF using the PSF Guide

Once the 3D image stack has been collected, a PSF entry can be created. To do so:

1. Select Analyze tab, Process group, ***Deconvolve > PSF Guide***. The following dialog box will appear.



2. As directed by the guide, open a main, three, or tile view of the image stack and then click on the button in the dialog box. Enter a unique name for your PSF (this is the name you will select from the drop-down menu in the Constrained Iterative Deconvolution dialog). The guide will advance to the next step.

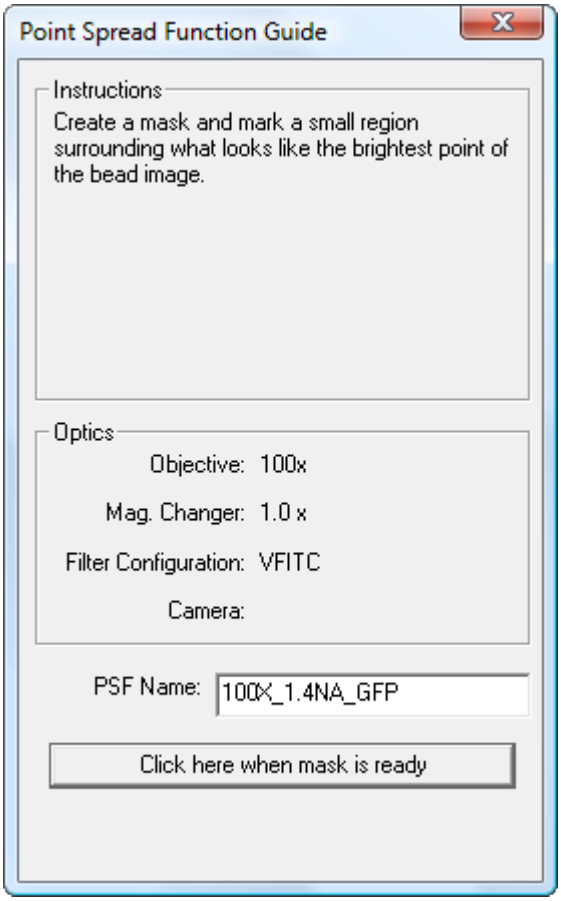


3. Select either ***Radially averaged*** or ***Section of entire image***.

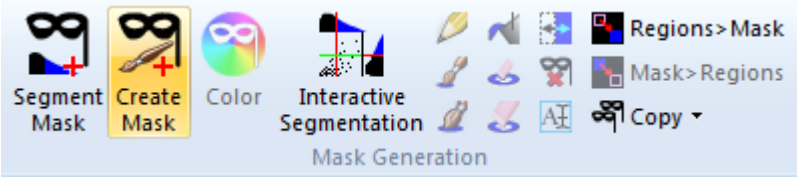
Radially averaged – uses one half of a PSF that is radially averaged. This is good for noisy PSF data.

Section of entire image – uses a 101x101x101 section of the image. This is best for images with good signal and accounts for more aberrations than a radially averaged PSF. This is the method to use if you will be using the PSF for Microvolution deconvolution.

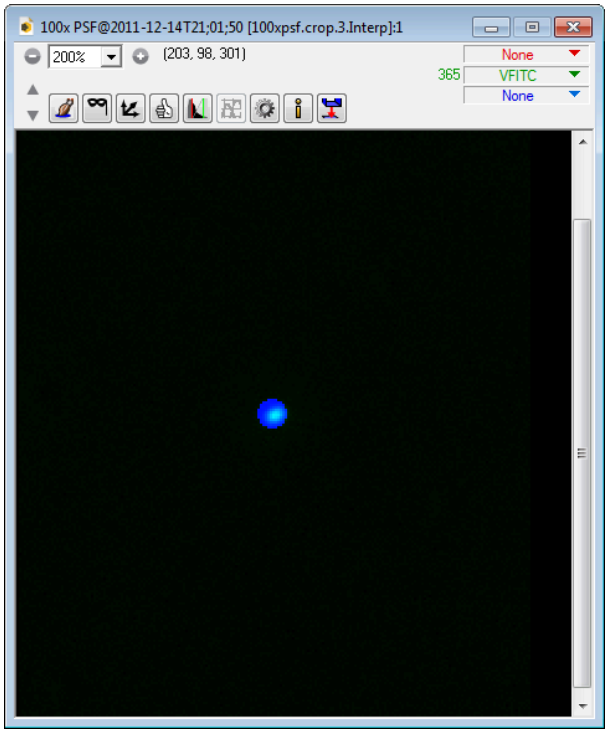
When you have made your selection, the following dialog box will appear.



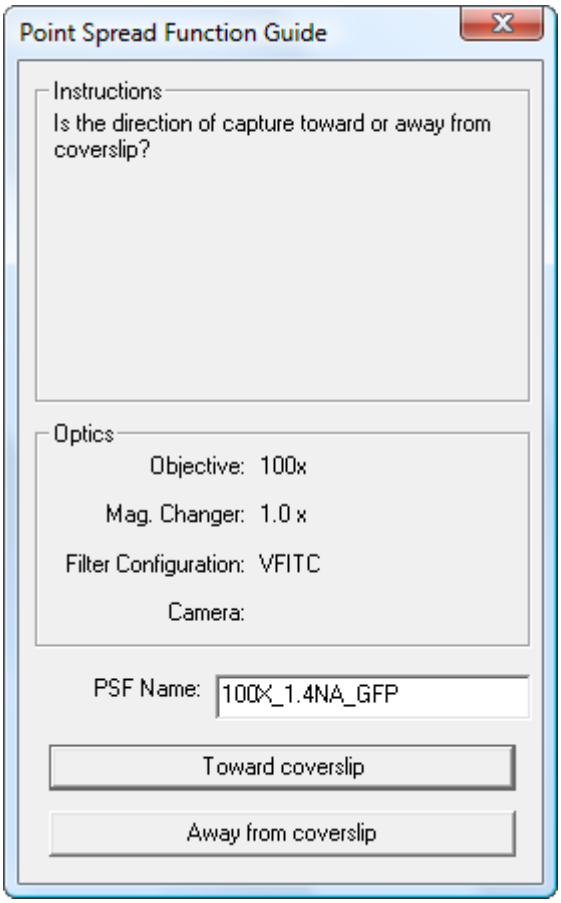
4. Create a new mask by selecting ***Mask Generation group > Create Mask***



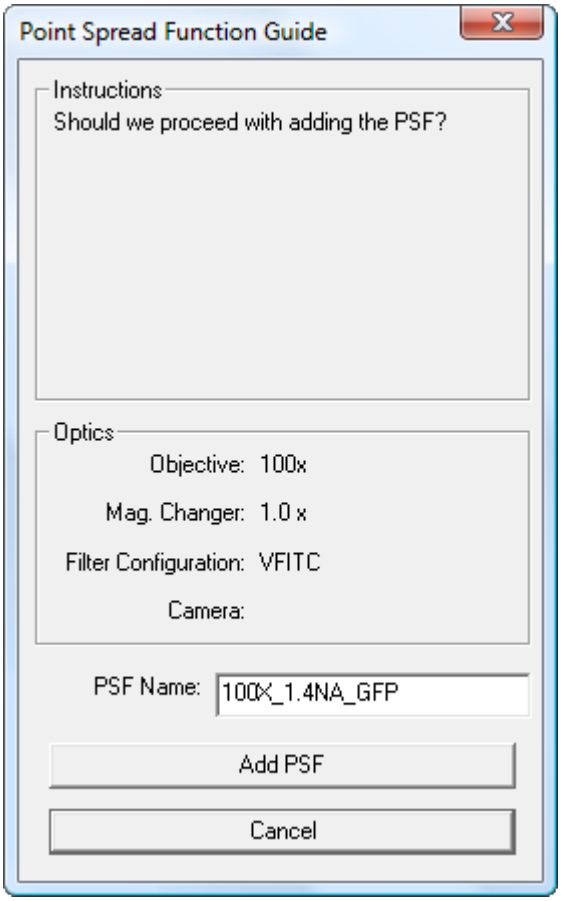
5. Select the large pencil mask drawing tool and mark the center of the bead at what is approximately the brightest region.



6. Advance to the next dialog box by clicking the button ***Click here when mask is ready*** at the bottom of the Point Spread Function Guide dialog box, and then choose the direction of objective movement.

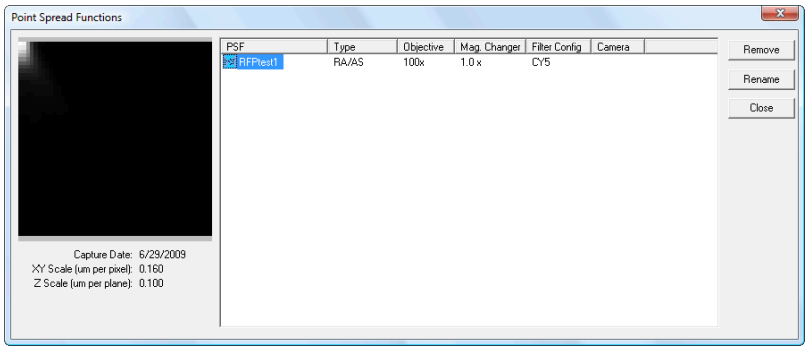


7. In the final dialog box, check that the desired objective, optovar, and fluorescence channel are selected, and then choose ***Add PSF***. The measured PSF will be added to the PSF database.


Point Spread Function Guide
Instructions
Should we proceed with adding the PSF?
Optics
Objective: 100x
Mag. Changer: 1.0 x
Filter Configuration: VFITC
Camera:
PSF Name:
100X_1.4NA_GFP
Add PSF
Cancel

Manage PSFs

The PSFs that are stored in SlideBook can be viewed by selecting ***Manage PSFs*** from the Deconvolution menu. A table of objectives, optovars and channels will be displayed. To view the PSF for a particular combination, click on the PSF icon for the desired optical combination, then choose ***Display***. To remove a PSF from the list, select the PSF then click ***Remove***. When finished managing PSFs, click ***Close*** to close the window.



Filter Menu

Image filtering techniques are used to alter the pixel data of an image. Filters may be used to remove artifacts, better detect objects, eliminate noise and more. SlideBook offers a variety of filtering options, including a user-defined kernel and semi-defined Gaussian, Gaussian derivative, hot pixel, median, and mean filters. These filters perform local, rather than global, calculations on a pixel by pixel basis. In other words, each pixel is altered based on the intensities of the neighboring data.

Filters offered include:

Convolution - A general purpose filtering tool with a user-entered kernal.

Gaussian - Uses a gaussian kernal of a given radius to remove noise.

Gaussian Derivative - Uses a gaussian kernal of a given radius to find edges.

Mean - Replaces the current pixel with an average of the pixels within a given radius.

Median - Replaces the current pixel with a median pixel value within a given radius.

Hot Pixel - Replaces the current pixel with the mean value if the pixel is some factor greater than the mean of its neighbors.

Frequency (Low/High Pass) - Removes low/high frequencies from an image with an option to allow the edges of the image to be Blackman tapered.

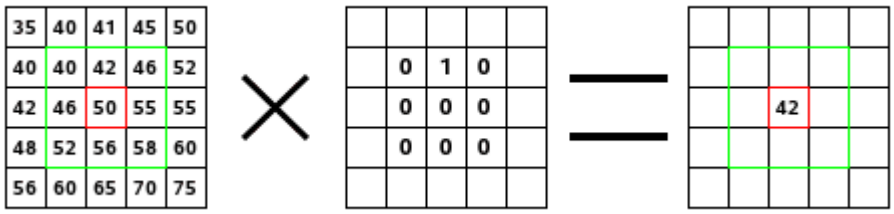
Laplacian - A convolution that determines the first derivative of the channel.

3D Edge Detection - Uses a Brejl kernal with an odd radius to find edges.

Unsharp Mask - Corrects pixels that vary tremendously from the gaussian blur of the image.

Convolution Filter window

The convolution filter is a general purpose filtering tool with a user-entered kernal. The filter studies successively every pixel of the image. For each of them, which we will call the "initial pixel", it multiplies the value of this pixel and values of the 8 surrounding pixels by the kernel corresponding value. Then it adds the results, and the initial pixel is set to this final result value. So, for a 3x3 matrix, we can consider the following:



On the left is the image matrix: each pixel is marked with its value. The initial pixel has a red border. The kernel action area has a green border. In the middle is the kernel and, on the right is the convolution result.

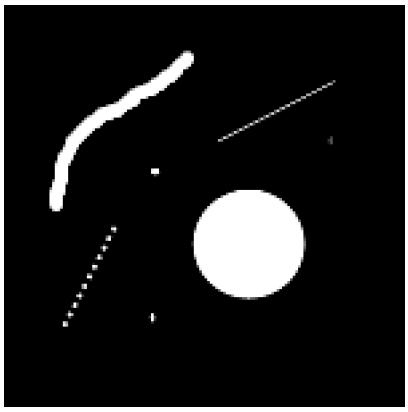
Here is what happened: the filter read successively, from left to right and from top to bottom, all the pixels of the kernel action area. It multiplied the value of each of them by the kernel corresponding value and added results. The initial pixel has become 42: ***(40*0)+(42*1)+(46*0) + (46*0)+(50*0)+(55*0) + (52*0)+(56*0)+(58*0) = 42***. As a graphical result, the initial pixel moved a pixel downwards.

The resultant pixel value can be further manipulated as follows:

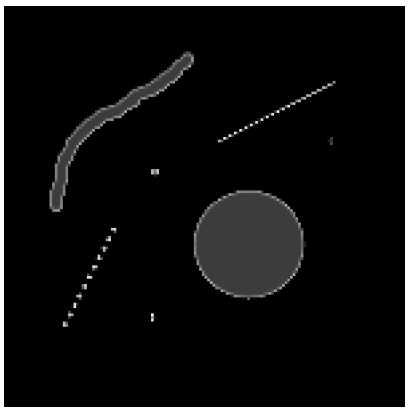
> ***Coefficient*** - the resultant pixel value will be multiplied by the coefficient value. For example, if you would like to average the pixel values of a 3x3 matrix, you would use **1/9** or **0.11111** as the coefficient. Coefficients can be used to rescale the resulting data/image.
> ***Offset*** - the offset value will be added to the resultant pixel value. You may enter a negative value to subtract. Offsets can be used to rescale the resulting data/image.

Some common kernals are given below with examples, filtering techniques can be particularly useful for better defining a mask:

Original:



Sharpen:



0	0	0	0	0
0	0	-1	0	0
0	-1	5	-1	0
0	0	-1	0	0
0	0	0	0	0

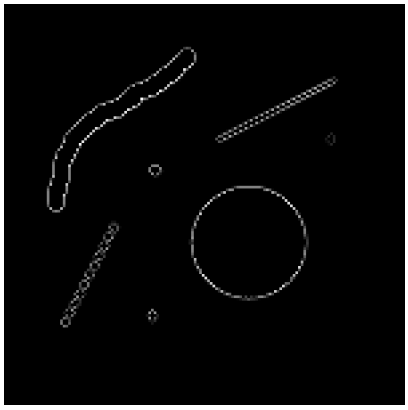
Blur:

0	0	0	0	0
0	1	1	1	0
0	1	1	1	0
0	1	1	1	0
0	0	0	0	0

Edge Enhance:

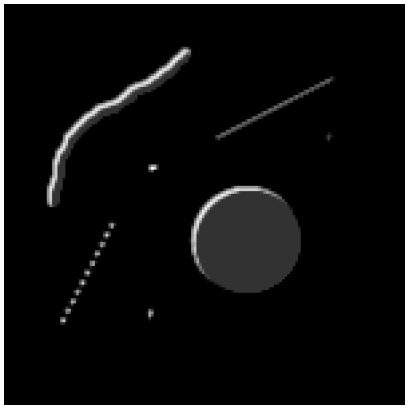
	0	0	0	
	-1	1	0	
	0	0	0	

Edge Detect:



	0	1	0	
	1	-4	1	
	0	1	0	

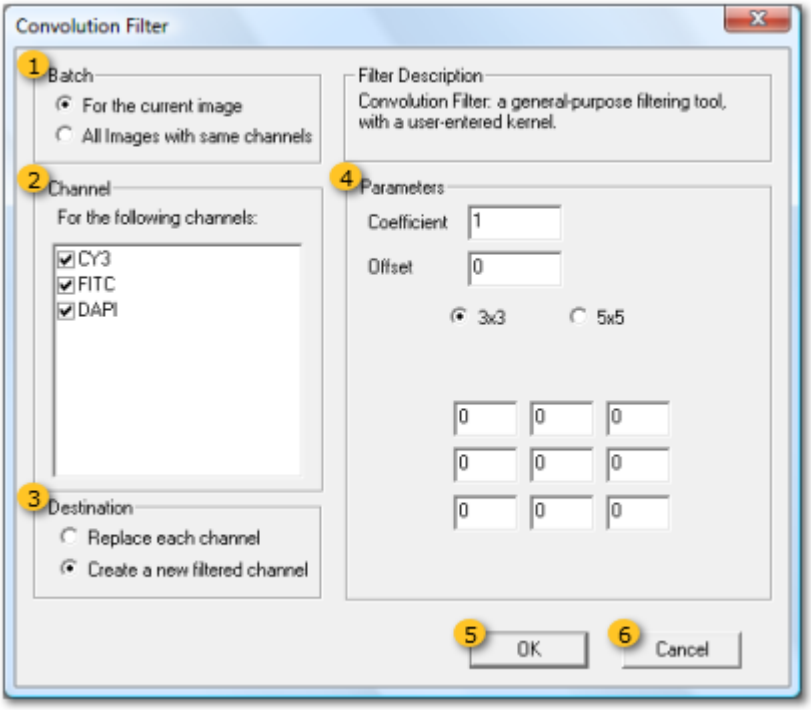
Emboss:



	-2	-1	0	
	-1	1	1	
	0	1	2	

Access by ***Image > Filter > Convolution***

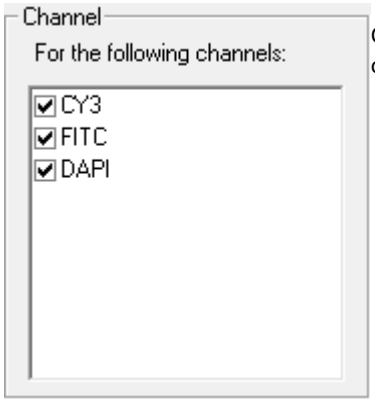
Channel name appended by **conv**.



1 Batch group

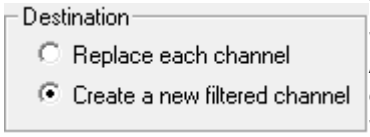
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



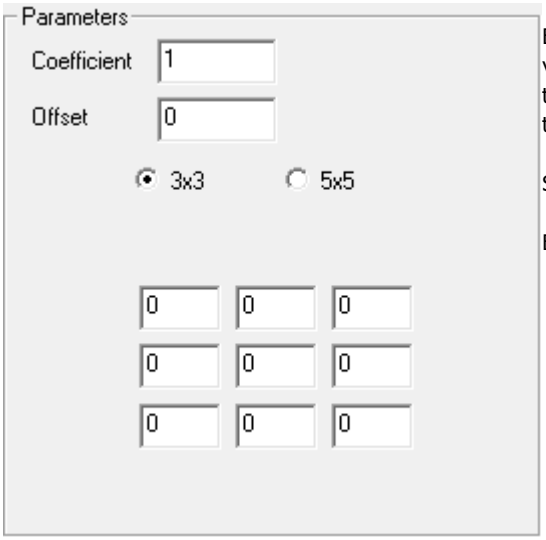
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **conv** added to the channel name.

4 Parameters group



Enter the desired ***Coefficient*** and ***Offset*** in the edit fields. The output value of the kernel will be multiplied by the coefficient and added to the offset. These parameters can be used to scale the convolved data to a desired range and to account for negative kernel output values.

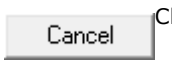
Select either a ***3x3*** or a ***5x5*** kernel size.

Enter the desired matrix elements in the array of edit fields.

5 OK button

Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button



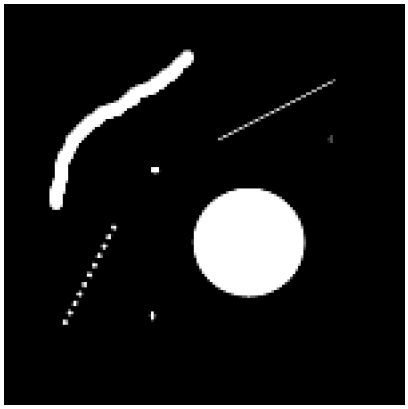
Click ***Cancel*** to abandon the filter and close the dialog.

Gaussian Filter window

A gaussian blur filter uses a gaussian kernal of a given radius to remove noise. It can be quite useful when images are noisy or grainy and you are having a hard time creating a mask by the threshold/segment technique.

Examples are shown below, notice that the "dotted" structure becomes a solid line as the sigma value increases:

Original:

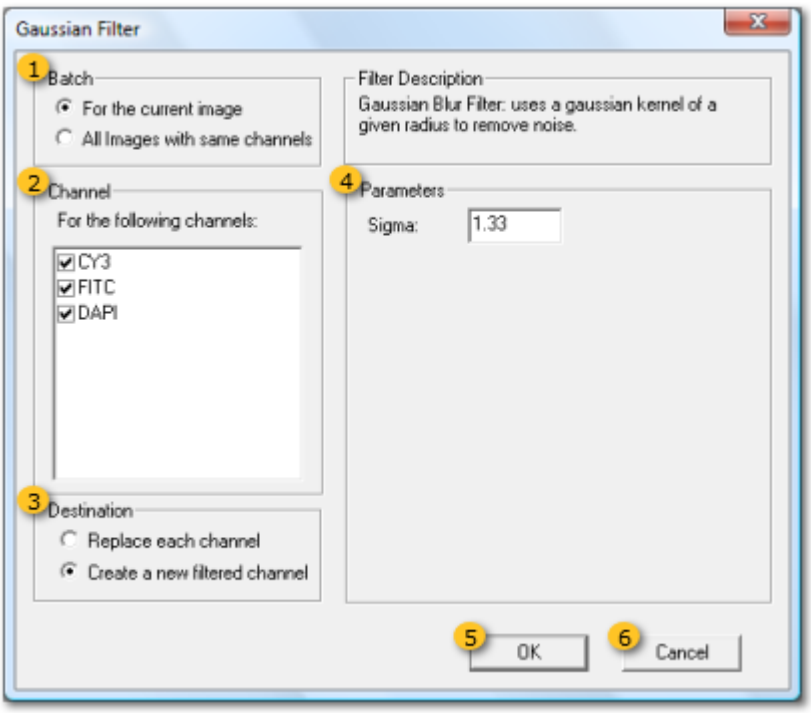


Gaussian filter where Sigma = 1.33

Gaussian Filter where Sigma = 2

Access by ***Image > Filter > Gaussian***

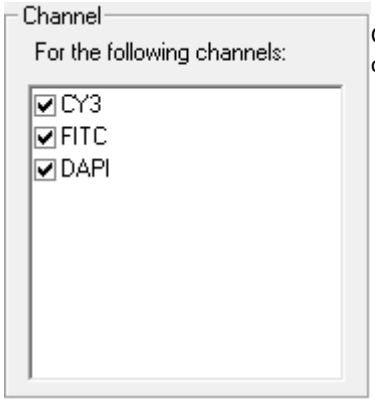
Channel name appended by **gb.**



1 Batch group

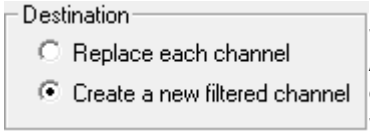
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



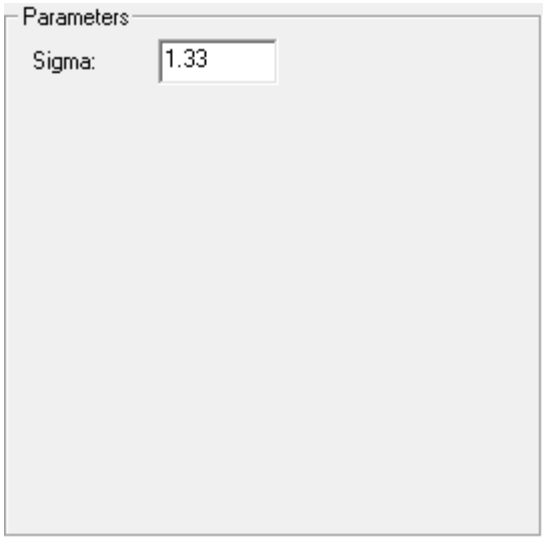
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the original raw data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **gb** added to the channel name.

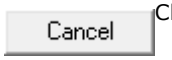
4 Parameters group



5 **OK button**

Click ***OK*** to run the filter as specified and close the dialog.

6 **Cancel button**



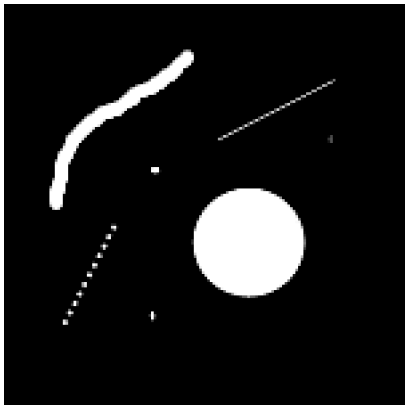
Click ***Cancel*** to abandon the filter and close the dialog.

Gaussian Derivative Filter window

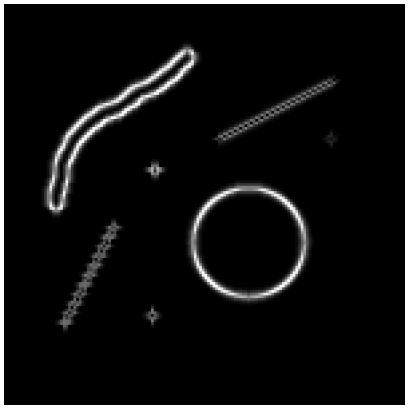
The gaussian derivative filter uses a gaussian kernal of a given radius to find edges.

Examples follow:

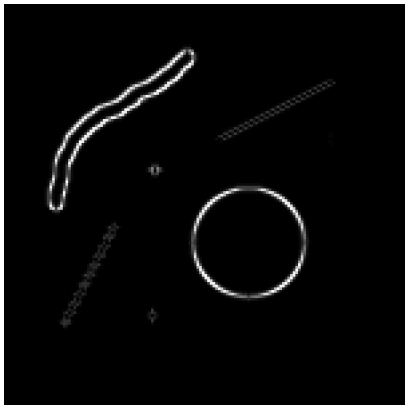
Original data:



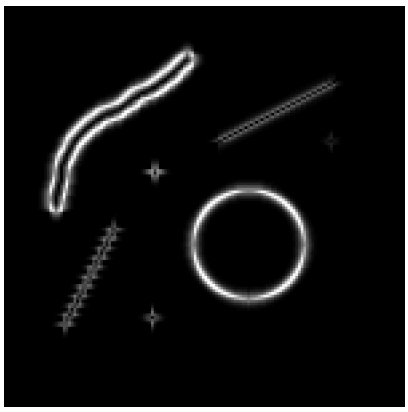
Gaussian Derivative - Radius 2, Scale Shift 0:



Gaussian Derivative - Radius 2, Scale Shift 1:

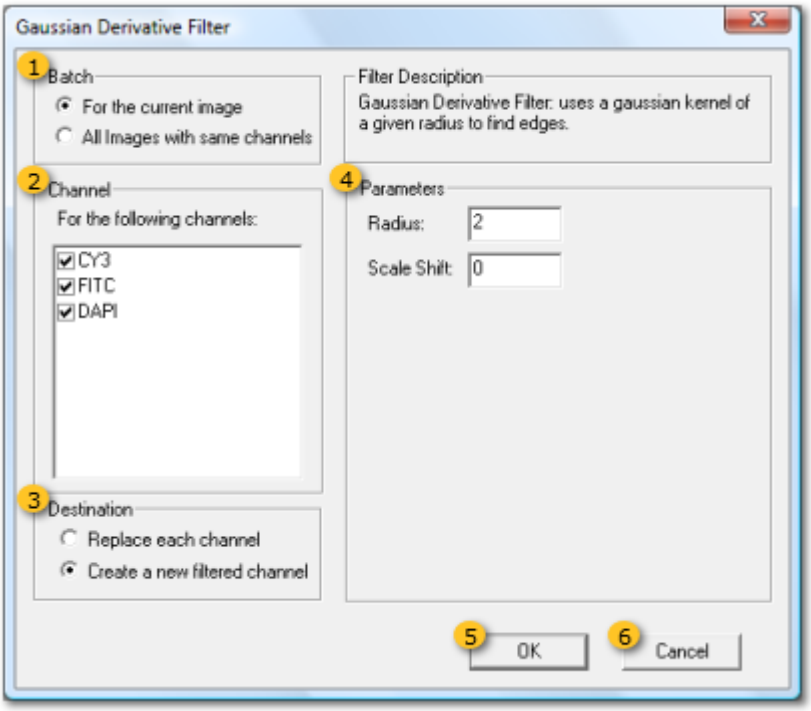


Gaussian Derivative - Radius 5, Scale Shift 0:



Access by ***Image > Filter > Gaussian derivative***

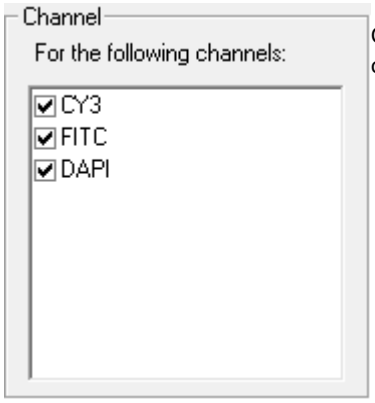
Channel name appended by **gd**.



1 Batch group

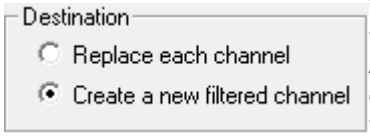
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



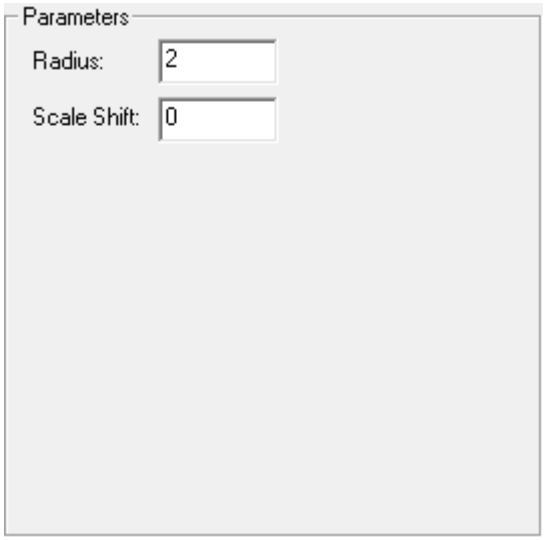
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group

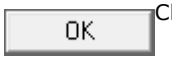


You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **gd** added to the channel name.

4 Parameters group



5 OK button



Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button

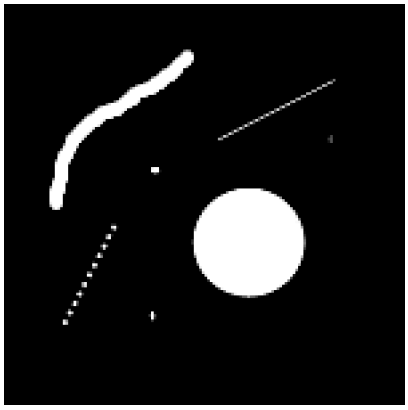
Click ***Cancel*** to abandon the filter and close the dialog.

Mean Filter window

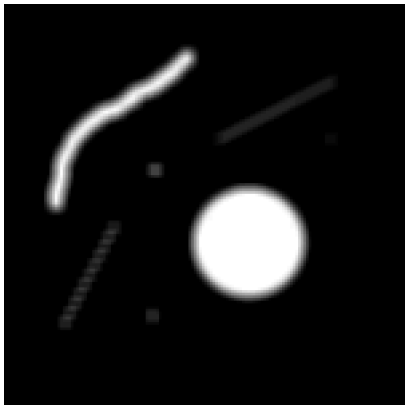
The mean filter replaces the current pixel with an average of the pixels within a given radius.

Examples follow:

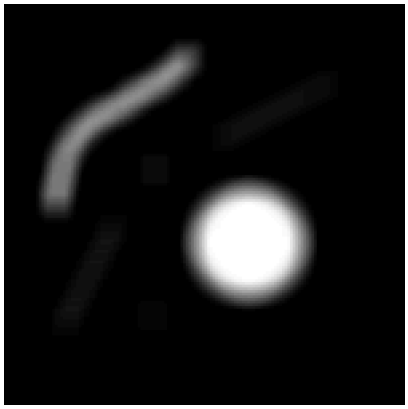
Original data:



Mean Filter - Radius = 2:

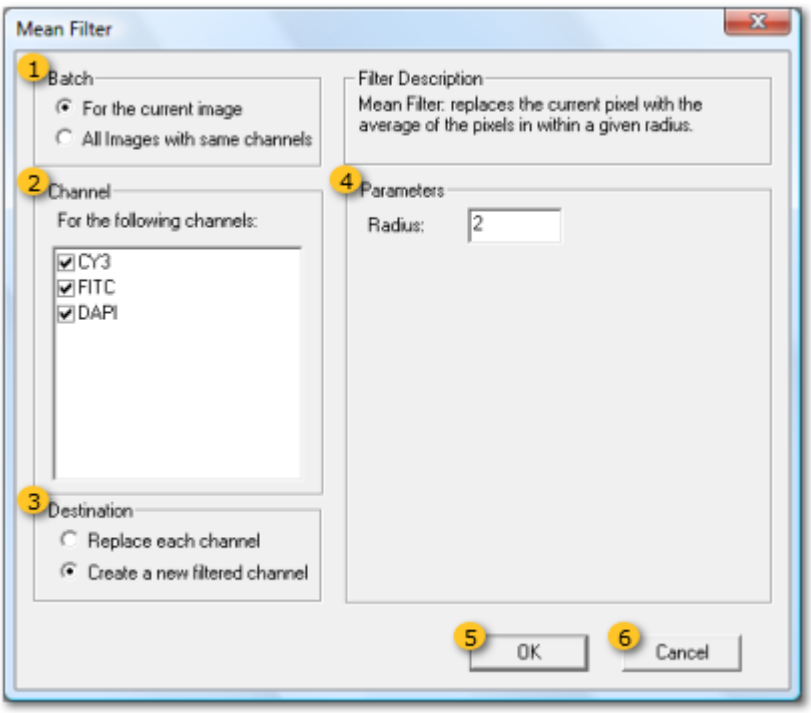


Mean Filter - Radius = 5:



Access by ***Image > Filter > Mean***

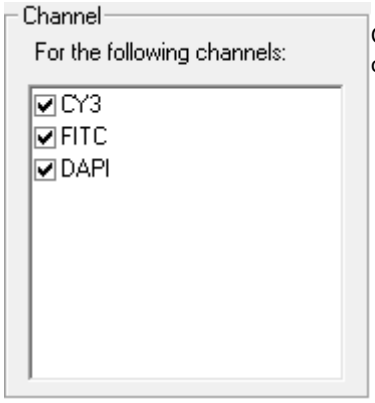
Channel name appended by **mean**.



1 Batch group

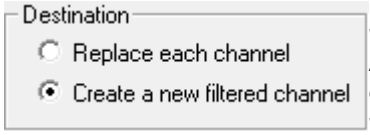
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



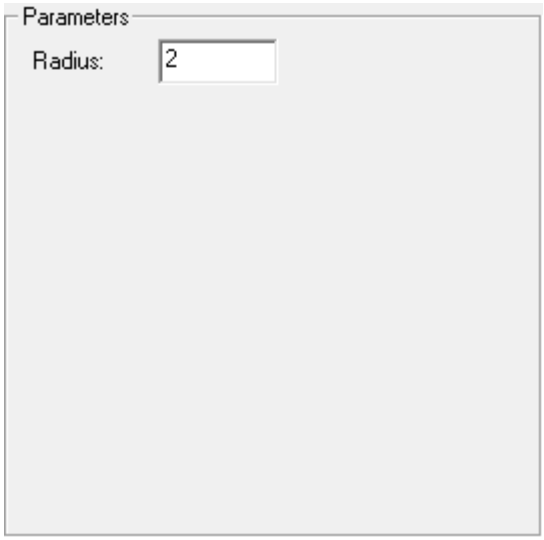
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **mean** added to the channel name.

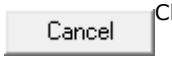
4 Parameters group



5 **OK button**

Click ***OK*** to run the filter as specified and close the dialog.

6 **Cancel button**



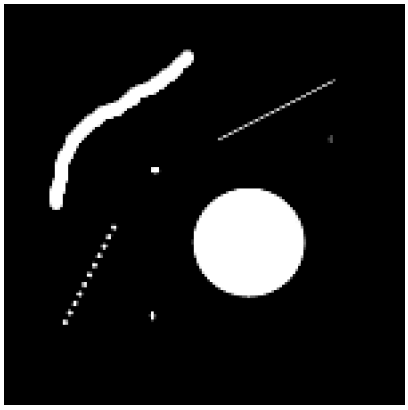
Click ***Cancel*** to abandon the filter and close the dialog.

Median Filter window

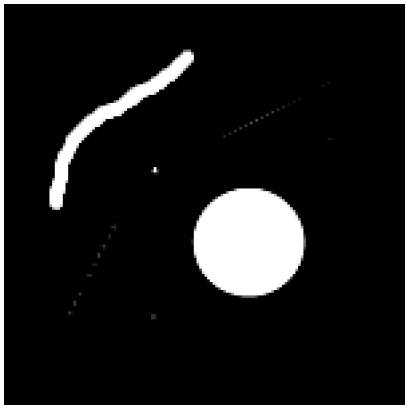
The median filter replaces the current pixel with the median pixel value within a given radius.

Examples follow, notice that small features disappear when the radius is increased:

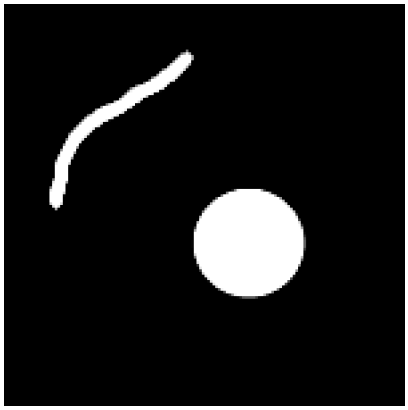
Original data:



Median Filter - Radius = 1:

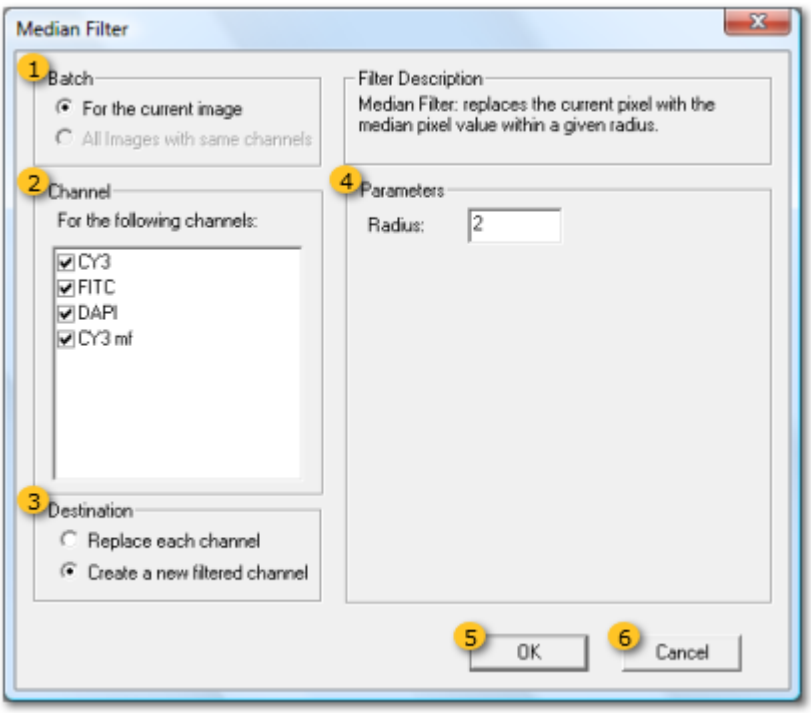


Median Filter - Radius = 2:

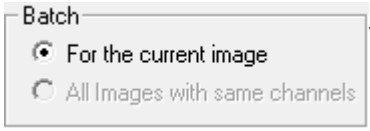


Access by ***Image > Filter > Median***

Channel name appended by **mf**.

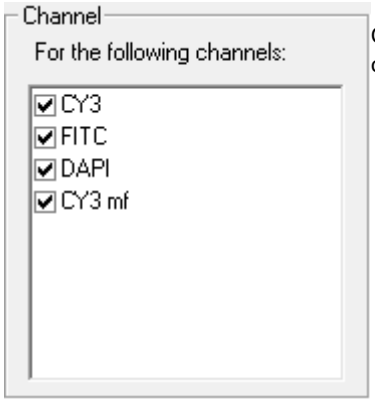


1 Batch group



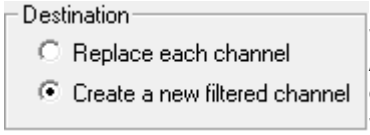
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



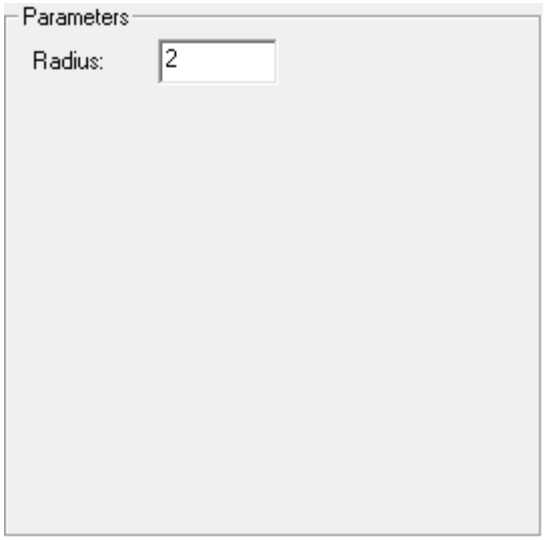
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **mf** added to the channel name.

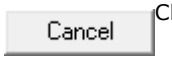
4 Parameters group



5 **OK button**

Click ***OK*** to run the filter as specified and close the dialog.

6 **Cancel button**



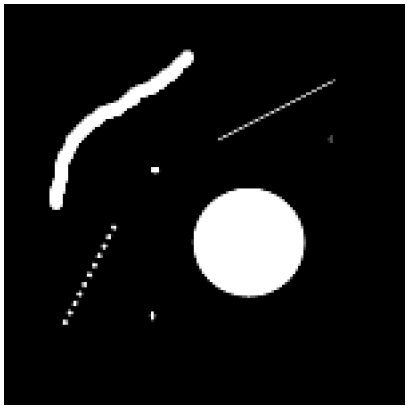
Click ***Cancel*** to abandon the filter and close the dialog.

Hot Pixel Filter window

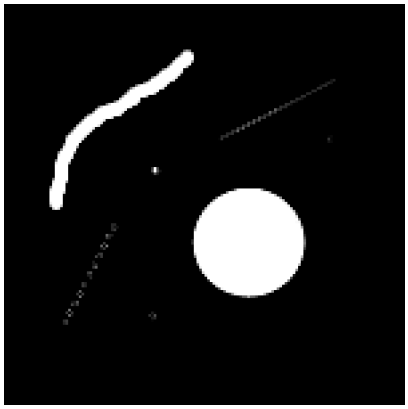
The hot pixel filter replaces the current pixel with the mean value if the pixel is some factor greater than the mean of its neighbors.

Examples follow, note that larger factor values are more likely to only detect and correct actual hot pixels on the sensor while lower factor values may distort small features:

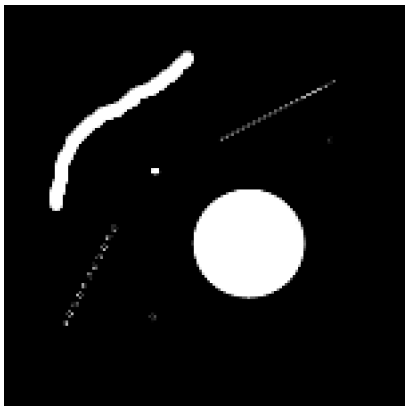
Original data:



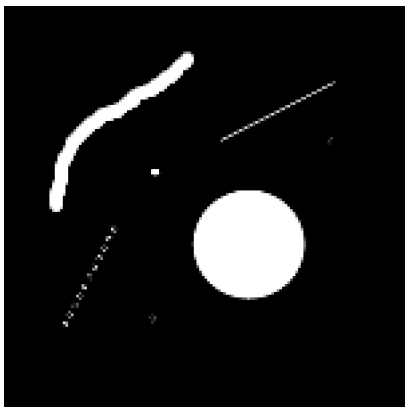
Hot Pixel - Factor = 2:



Hot Pixel - Factor = 4:

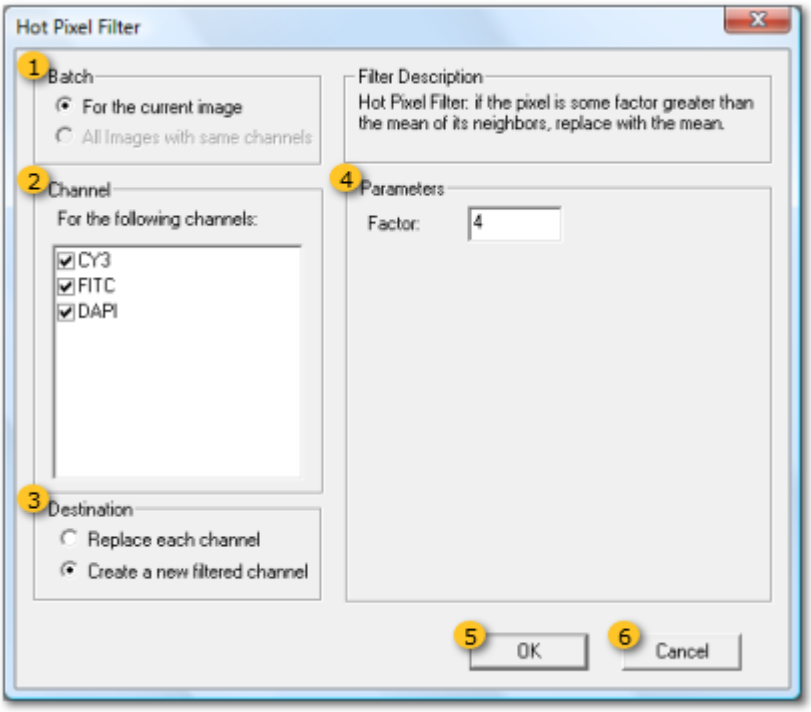


Hot Pixel - Factor = 6:



Access by ***Image > Filter > Hot Pixel***

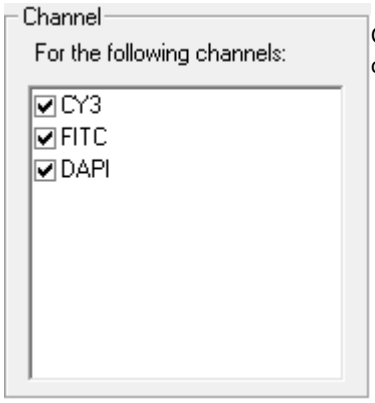
Channel name appended by **hp**.



1 Batch group

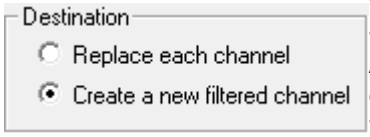
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



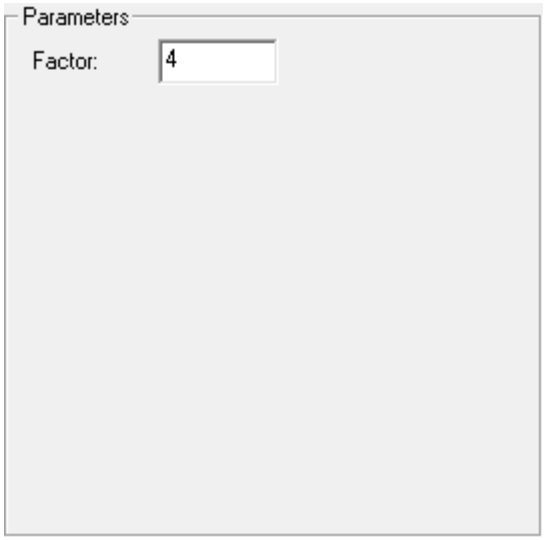
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group

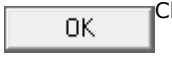


You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **hp** added to the channel name.

4 Parameters group

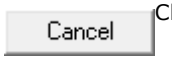


5 OK button



Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button

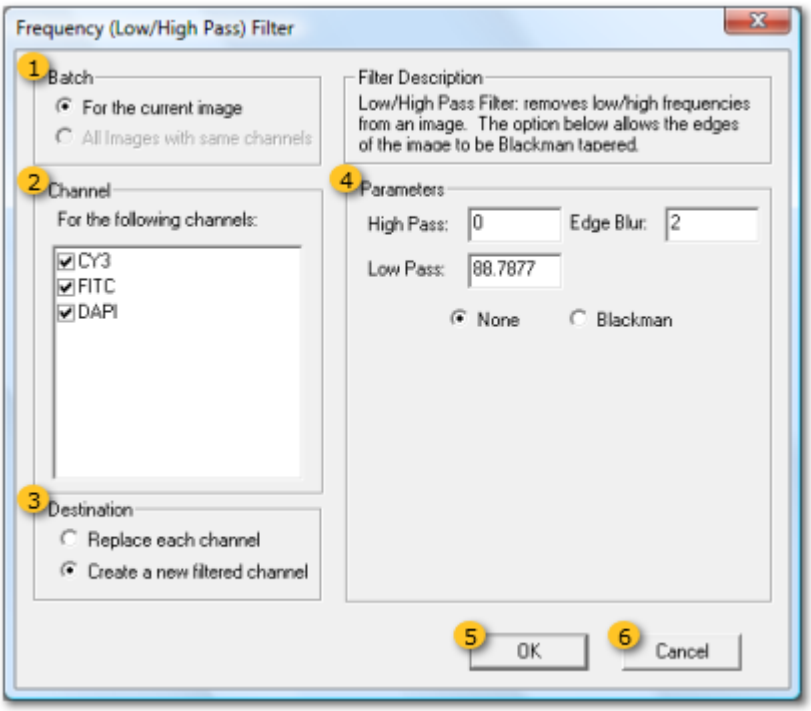


Click ***Cancel*** to abandon the filter and close the dialog.

Frequency (Low/High Pass) Filter window

The FFT bandpass filter removes low/high frequencies from an image and allows the edges of the image to be Blackman tapered.

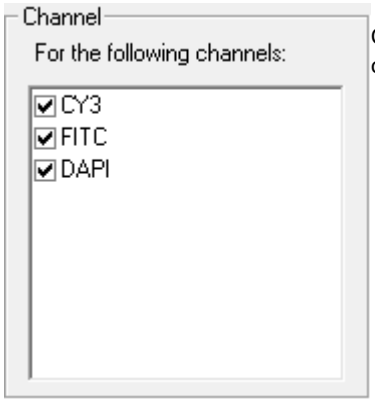
Channel name appended by **fft**.



1 Batch group

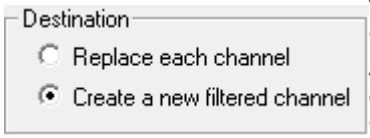
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



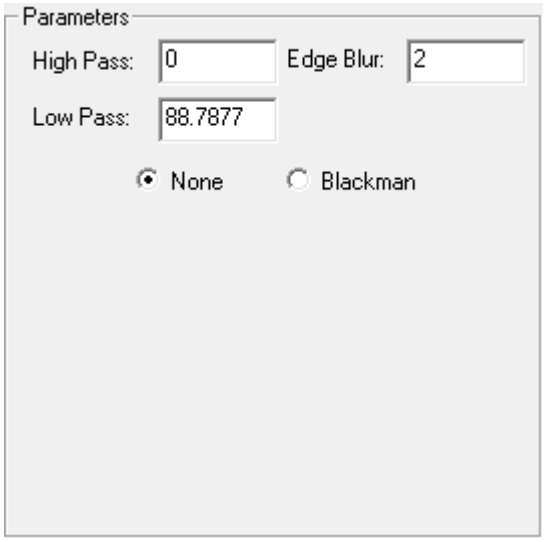
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **fft** added to the channel name.

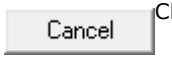
4 Parameters group



5 OK button

Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button

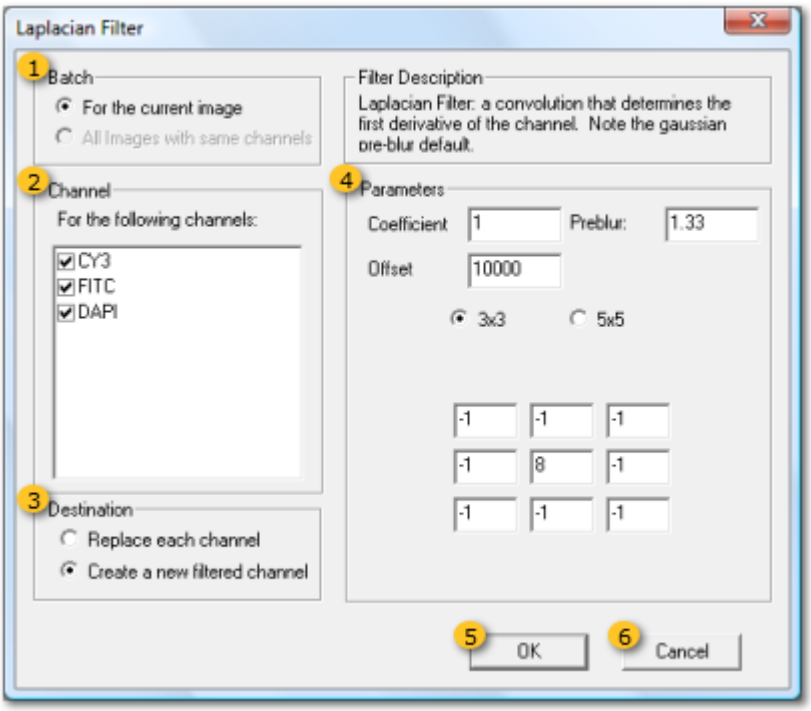


Click ***Cancel*** to abandon the filter and close the dialog.

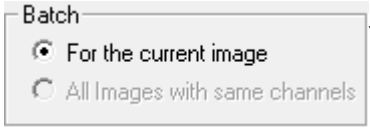
2D Laplacian Filter window

The laplacian filter is a convolution that determines the first derivative of the channel.

Channel name appended by **lap**.

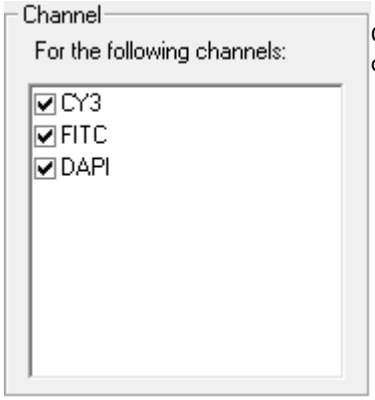


1 Batch group



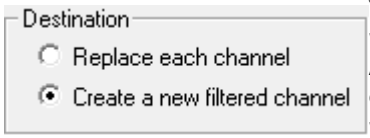
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



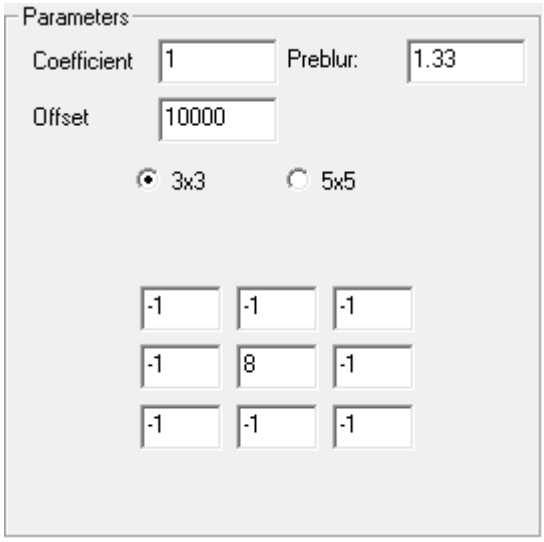
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **lap** added to the channel name.

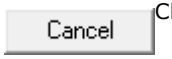
4 Parameters group



5 OK button

Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button

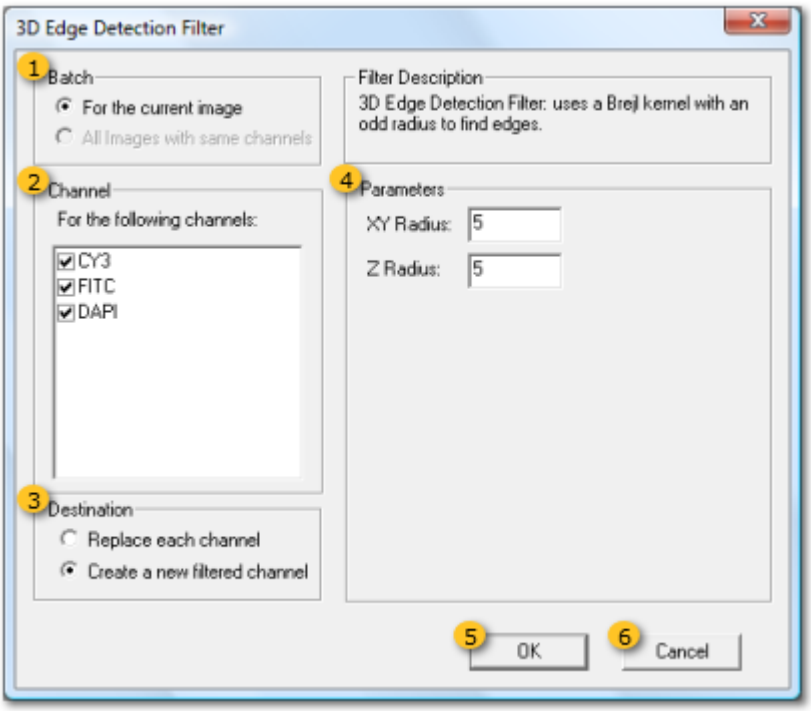


Click ***Cancel*** to abandon the filter and close the dialog.

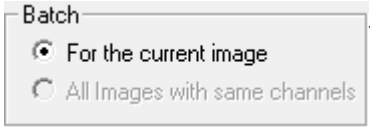
3D Edge Detect Filter window

The 3D edge detection filter uses a Brejl kernal with an odd radius to find edges.

Channel name appended by **br**.

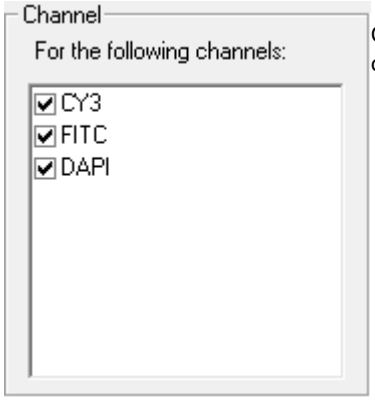


1 Batch group



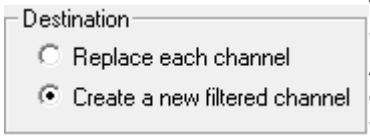
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



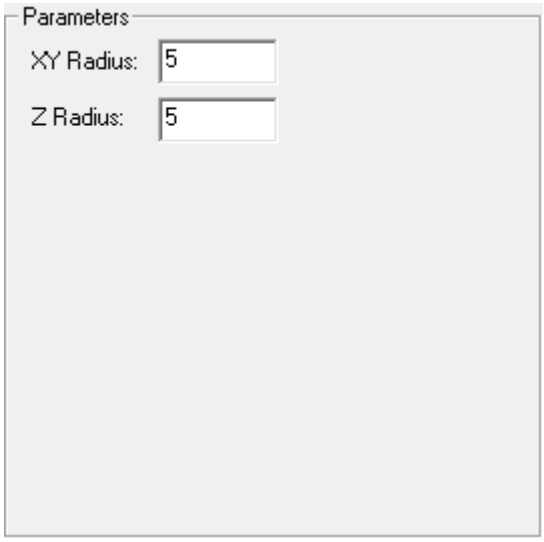
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **br** added to the channel name.

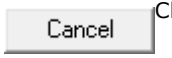
4 Parameters group



5 OK button

Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button

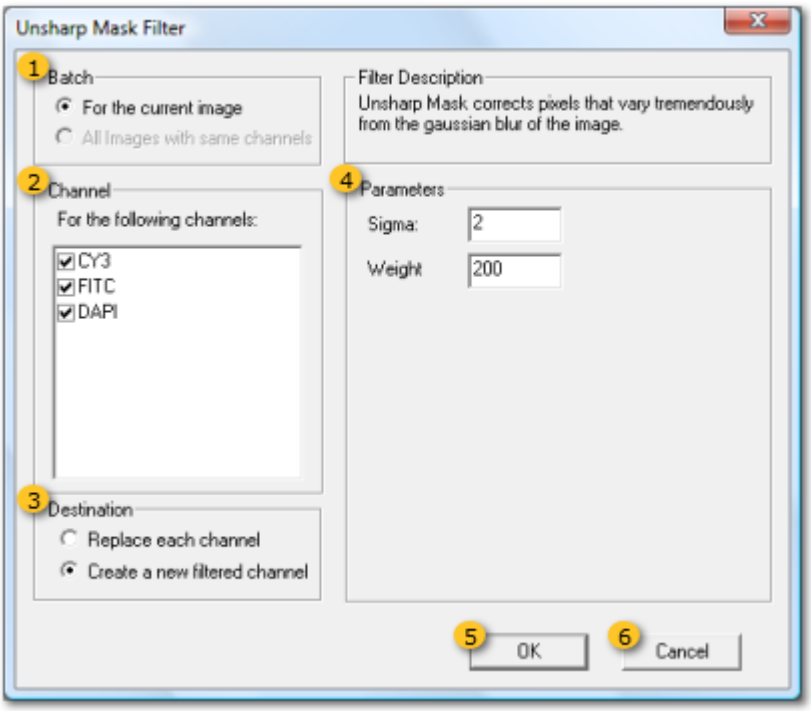


Click ***Cancel*** to abandon the filter and close the dialog.

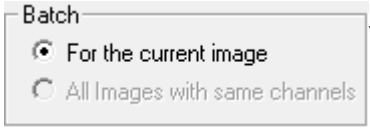
Unsharp Mask Filter window

The unsharp mask filter corrects pixels that vary tremendously from the gaussian blur of the image.

Channel name appended by **usm**.

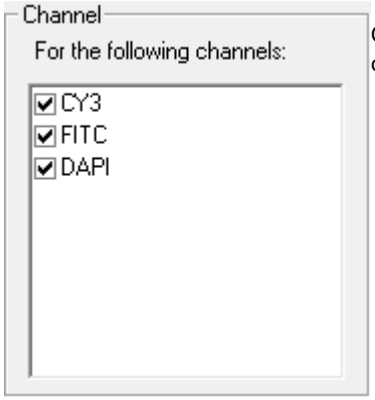


1 Batch group



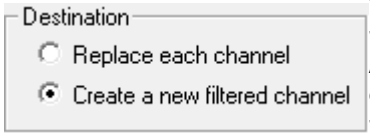
You may choose to run the filter on the current image or all images in the **.sld** that have the same channels.

2 Channel group



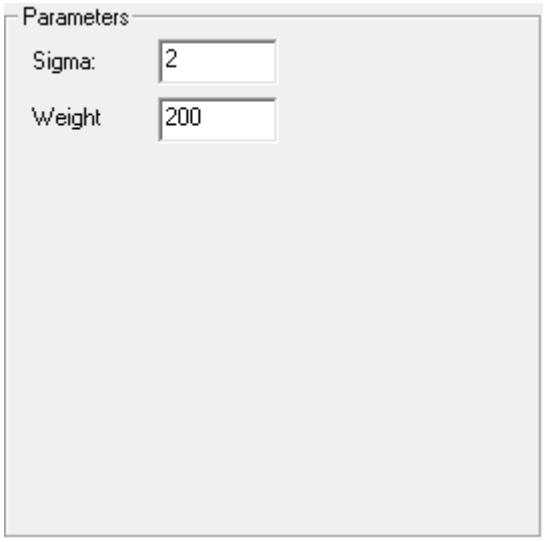
Check the box for each channel you would like to filter. If desired, you may select channels that have already had other filters applied.

3 Destination group



You may choose to replace the existing channel with the filtered channel data. This will overwrite the raw original data so you may wish to backup the data first. Alternatively, you may choose to create a new filtered channel in the image. The channel will be available from the dropdown channel menu in any image view window and will have **usm** added to the channel name.

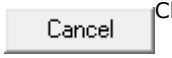
4 Parameters group



5 OK button

Click ***OK*** to run the filter as specified and close the dialog.

6 Cancel button



Click ***Cancel*** to abandon the filter and close the dialog.

Correct Menu

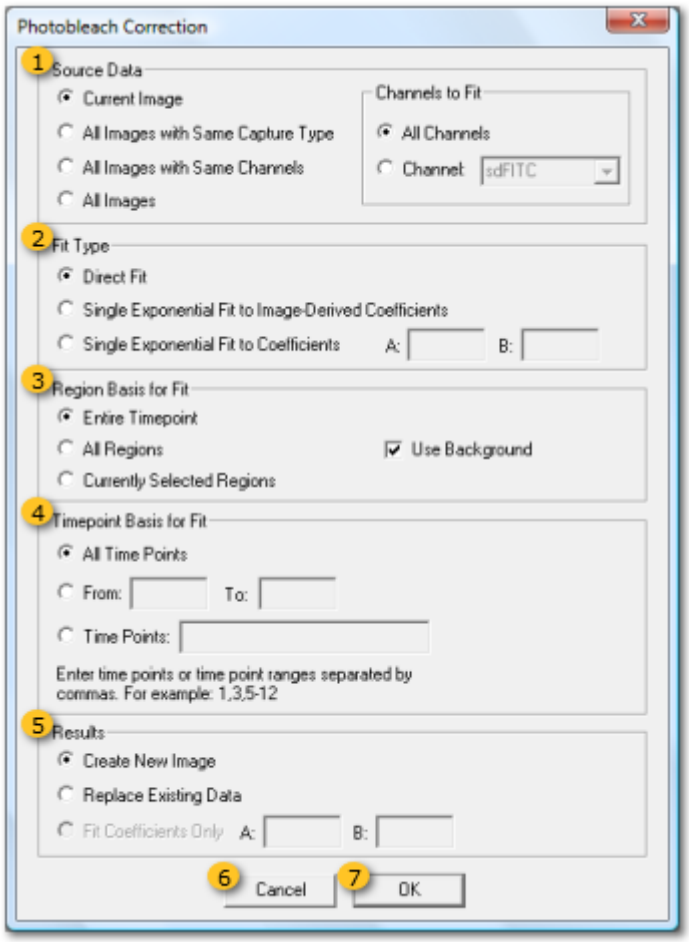
The Correct menu provides tools to correct for photobleaching of the sample. field curvature of lenses (flat field) and dark current from the camera (dark field).

Photobleach Correction Window

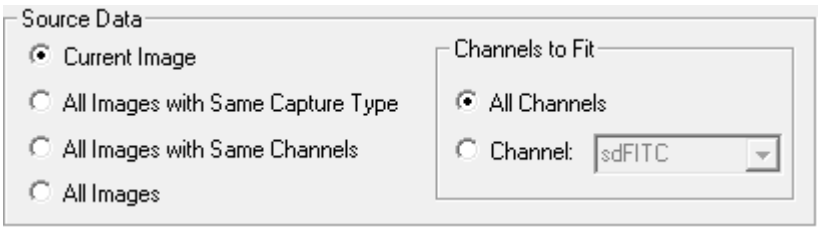
Photobleach correction can be used to correct for photobleaching in 2D timelapse images. The photobleach correction operation takes the mean intensity at each time point and fits a line or curve through the mean intensity versus time data. Then, it determines a multiplication factor for each timepoint that is used to adjust the intensities at each pixel, resulting in constant intensities over time.

Before performing photobleach correction, you should examine your data to determine what type of fit is most appropriate. To do so, you need to view the intensity-time profile of your data set. You can do this by drawing a large region, then selecting ***Graph*** and selecting intensity of the channels you are interested in correcting. If the intensities decrease linearly over time, you may perform a direct fit. If they seem to decrease exponentially, choose exponential fit.

Appends image name with **.pbc**



1 Source Data group



Select the scope of operation as described below:

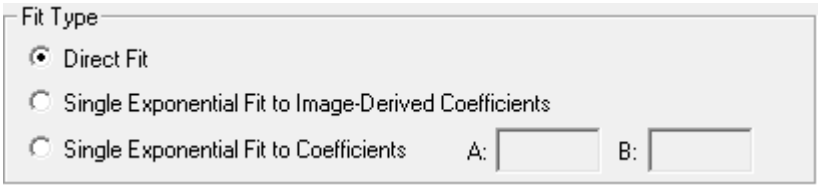
Current Image – Operation will be performed on the selected image only.
All Images with Same Capture Type – Operation will be performed on all images in the slide with the same capture type (2D, 3D, 4D, etc.) as the selected image.
All Images with Same Channels – Operation will be performed on all images in the slide with the same channels as the selected image.

All Images – Operation will be performed on all images in the slide.

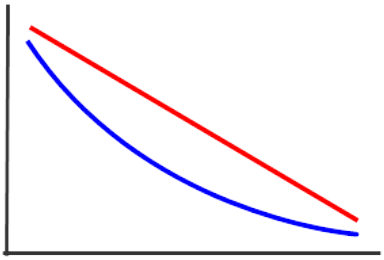
Select the channels to correct as follows:

All Channels - All channels will be corrected as directed
Channel - An individual channel can be selected from the dropdown menu for correction. This is particularly useful when different types of photobleaching occurs in different channels.

2 Fit Type group



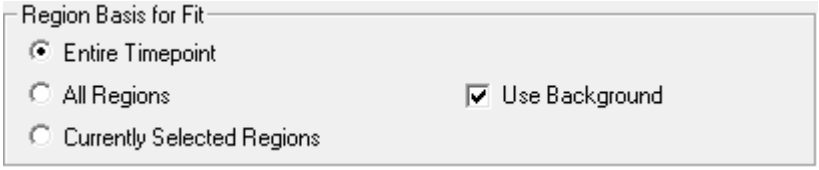
Select the fit type for the bleaching. If you do not know the fit type, close the Photobleach Correction window and draw a large region on the image. Go to ***Graph > Intensity*** and review the shape of the intensity decrease as depicted below. The red line represents a ***Direct Fit***, the blue line represents a ***Single Exponential Fit***.



To allow SlideBook to determine the appropriate coefficients based on the data, select ***Single Exponential Fit to Image-Derived Coefficients***.

If you already know the coefficients, select ***Single Exponential Fit to Coefficients*** and enter the desired values in the ***A*** and ***B*** edit fields.

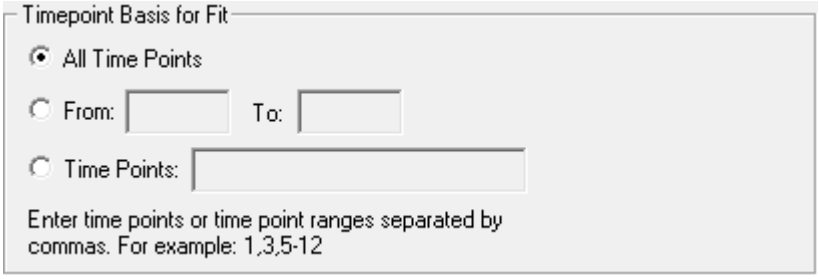
3 Region Basis for Fit group



Select the regions to use for intensity analysis of photobleaching profile as follows:
Entire Timepoint - Uses the full image field
All regions - Uses all regions drawn in the image
Currently Selected Regions - Uses only selected regions, regions where the number is white (see also Selecting Regions of Interest)

Check the ***Use Background*** box to dynamically subtract a mean background intensity before photobleach correction.

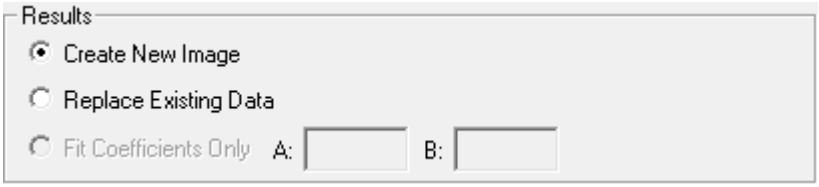
4 Timepoint Basis for Fit group



Select the time points to use for the photobleaching fit calculations as follows:

All Time Points - Use all time points in the image
From: To: - Enter a start and end time point range to use
Time Points: - Enter individual time points or time point ranges separated by commas such as 1, 3, 5-12

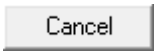
5 Results group



Select how you would like to handle the photobleach correction results as follows:

Create New Image - Creates a new image in the slide with the **.pbc** extension and closes the dialog
Replace Existing Data - Replaces the existing image in the slide and closes the dialog. It is recommended you backup your raw data before proceeding with replacing the data.
Fit Coefficients Only - When ***Single Exponential Fit to Image-Derived Coefficients*** is selected, this option becomes available. The ***OK*** button will turn to a ***Compute*** button below. Click ***Compute*** to have SlideBook return only the image-derived coefficients in the provided fields.

6 Cancel button



Press ***Cancel*** to abandon photobleach correction and close the dialog.

7 OK button

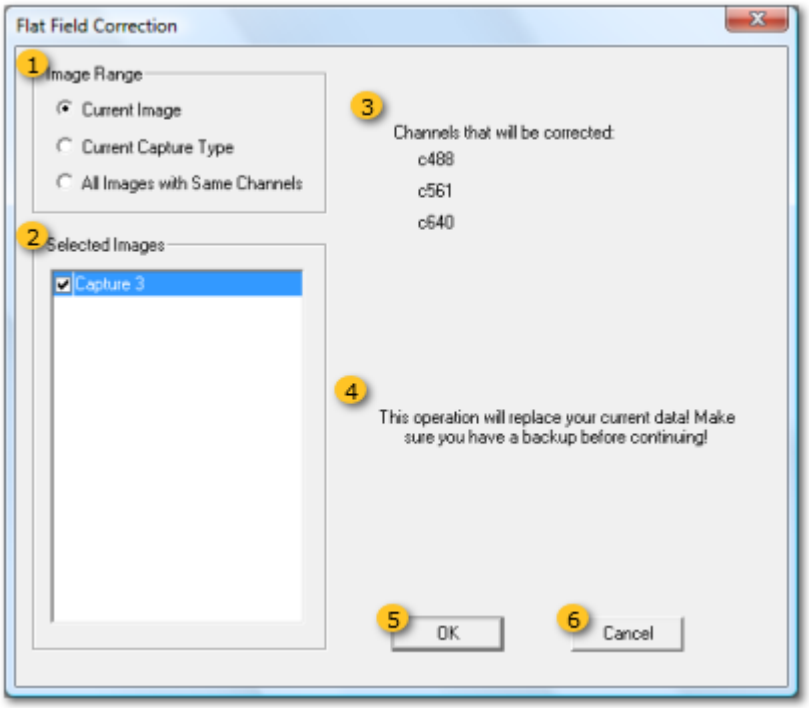
Press ***OK*** to run photobleach correction as designated and close the dialog. An image with extension **.pbc** will be created.

Flat Field Correction window

To apply flat field correction post-acquisition, you may use the ***Flat Field Correction*** window. This window allows you to select an image or images from a **.sld** to apply previously collected flat field calibration files.

See also:

[Flat Field Guide](#) for information on collecting flat field calibrations
[Display Flat Field](#) for information on managing flat field calibrations

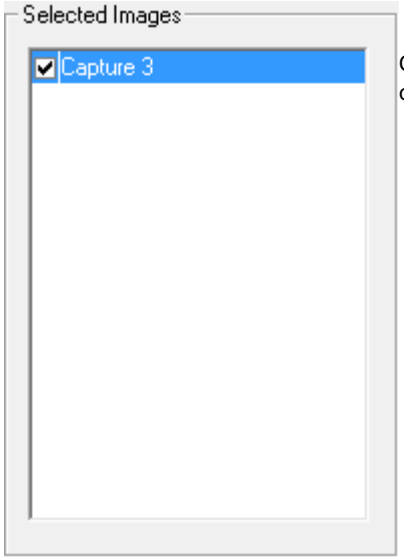


1 Image Range group

Select image range for flat field calibration as follows:

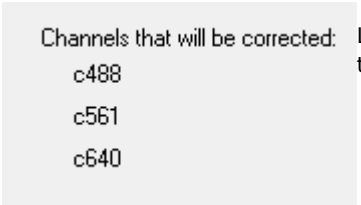
Current Image - Calibrates only the currently selected image
Current Capture Type - Calibrates all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Calibrates all images captured using the same channels as the selected image in the current slide.

2 Selected Images group



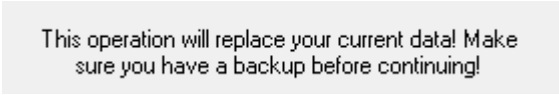
Check the boxes for the images you would like to flat field calibrate. This list will change depending on the ***Image Range*** selected above.

3 Flat Field Correction list



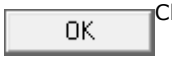
Lists the channels in the image that have flat field calibration files associated with them. Only channels that have previously been calibrated will be listed in this area.

4 Data Overwrite warning



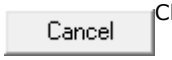
When you apply a flat field calibration, the raw data will be overwritten in the image file. To preserve the raw, un-calibrated data, you should make a copy of the image before calibrating.

5 OK button



Click ***OK*** to apply flat field calibration and overwrite the raw data and close the dialog.

6 Cancel button



Click ***Cancel*** to discard changes and close the dialog.

Collect Flat Field

As with any optical system, specks of dust or pieces of lint will find their way into the microscope's optical path. There may also be some variation in the level of illumination across the field of capture. SlideBook offers an operation called flat field correction, which eliminates many of these constant artifacts from an image. Flat field correction is particularly useful in brightfield and DIC (Nomarski) imaging, where such artifacts are usually more evident than in fluorescence imaging. Flat field correction (or shading correction) is not the same as background correction (background subtraction is strictly subtraction). When flat field correction is applied, the desired image is multiplied by a matrix of coefficients that is determined from a series of flat field images. To generate this matrix, flat field images are collected, and the average of each pixel for the set of images is calculate to generate a single averaged image (flat field image). The average intensity of all the pixels in the resulting averaged image is then calculated. Finally, a coefficient matrix (pixel by pixel map) is calculated so that when the intensity at any pixel location is multiplied by its coefficient, the generated pixel intensity matches the average pixel intensity of the flat field image. If you are planning to perform both background subtraction and flat field correction, perform flat field correction first.

See also:

Creating a Flat Field Calibration Sample
Generating a Spline-Fitted Flat Field Image (recommended for fluorescent imaging)

Collecting Flat Fields

In order to perform flat field correction, SlideBook needs to know what an image with no sample in focus looks like. This image should contain all the constant artifacts, but little that will change from sample to sample. From this image, SlideBook can compute a coefficient matrix that can be applied to an image to eliminate most of its constant artifacts. SlideBook, in fact, can average several "empty images" to better ensure that the flat field contains only constant artifacts of the optical system and not some peculiarity of a single "empty image."

Since constant artifacts might exist anywhere along the optical path, a flat field matrix is necessary for each combination of objective, filter, and magnification changer setting. To keep things simple, SlideBook stores all collected flat fields in a single database and keeps track of the optical parameters for each one.

Adding or Replacing a Flat Field

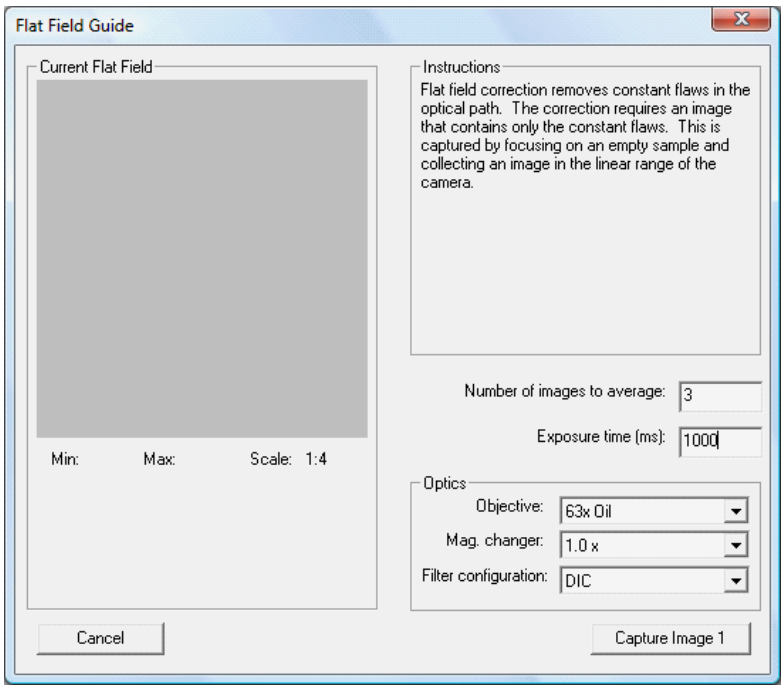
If you want to add a flat field matrix to the database (or replace an existing entry), you will need to capture a series of images on an empty sample using the desired set of optics, including the filter, objective, and magnification changer. To add or replace a flat field, do the following:

1. Place a blank slide containing no sample (or a uniformly fluorescent slide if collecting a fluorescent flat field) on the stage.

2. Bring the desired optics into place using the Focus Controls Window.

   In order to bring the *z* stage of the microscope to the correct position, focus on any irregularities (e.g., dust or scratches) on the blank slide. Then move the *xy* position of the slide to a location where there are few irregularities and adjust the microscope so it is slightly out of focus. This will minimize the contribution of irregularities with the slide to the flat field images.

3. Select ***Correct menu > Flat Field Guide*** to bring up the flat field guide. The following dialog will appear:



4. Specify an ***Exposure time*** and ***Number of images to average***.

   Later, you will be able to adjust the exposure time so that the linear range of the camera is being used. The number of images required depends on the user: the more images, the more accurately the flat field matrix will reflect only constant artifacts, though the collection process will take more time. Typically three will be sufficient.

5. Press ***Capture Image 1***. SlideBook will capture an image and display it in the upper left corner of the guide.

6. Adjust the exposure time such that the maximum intensity shown in the histogram is 3000-3500 for 12 bit CCDs (on a 0-4095 scale) or 48000-56000 for 16 bit CCDs (on a 0-65535 scale)

7. If necessary, make an appropriate adjustment to the exposure time and click ***Recapture Image 1***. Otherwise, move the *xy* position of the slide slightly and capture the next image by clicking ***Capture Image 2***. After the first image, it is unlikely you will need to recapture any of the subsequent images, but you will still have that option.

8. Acquire the remaining images making sure to move the slide slightly between captureS.

9. Once you have captured all the images, click on the ***Finish*** button to complete the process.

10. Repeat the process for other optical combinations.

Creating a Flat Field Calibration Sample

Protocol for creating a thin layer of fluorescence for flat field correction

1. Use your preferred fluorescence solutions (not beads) or secondary antibody conjugated with dye. If you are using your dye conjugated secondary antibody solution, dilute it 1:500 from the primary stock.

2. Pipette a small dot onto the slide (10uL is usually sufficient), cover with glass coverslip, then seal with your preferred method (i.e. nail polish).

Generating a Spline-Fitted Flat Field Image

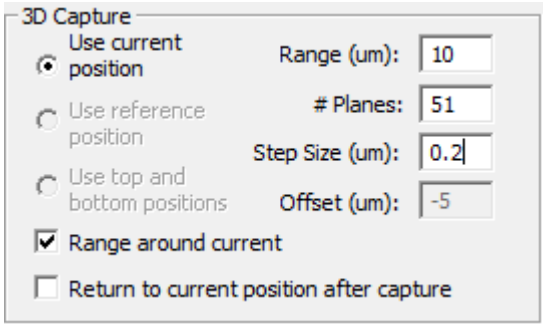
Steps for generating a spline-fitted flat field image for correcting uneven illumination in a Spinning Disc Confocal Image

1. Mount flat field calibration sample onto stage insert. Make sure the glass coverslip side is facing the objective.

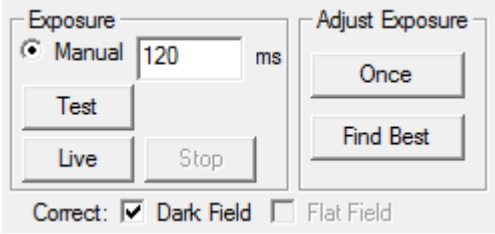
2. Focus with the brightfield or widefield illumination by finding any defects or bubbles in dye.

3. Focus again, this time with confocal imaging mode and the laser line of your choice. Move away from the defect and go to an area that is as even as possible.

4. Go to ***Capture*** (in the Home tab). Take a 3D image stack by using ***Range around current*** with a ***Range*** of 10 microns and sampled at 0.2 micron ***Step Size***. Use an exposure time that will not saturate. Capture the image by clicking ***OK***.



5. Use an ***Exposure*** time that will not saturate. Check the box to ***Correct Dark Field***. Capture the image by clicking ***OK***.



6. Go to Create Projection Image (in the Home tab, View group). Choose ***Maximum*** under ***Operation*** and ***Over Z axis*** under ***Axis***. Select ***OK*** to create a maximum projection image of the 3D image stack.

7. Go to ***Administration*** tab, ***Flat Field*** menu > ***Smooth Flat Field***. This will create a new image in the **.sld** appended with **Smooth FF**.

8. Go to ***Administration*** tab, ***Flat Field*** menu > ***Manage Flat Field***. Select the ***Import*** (in the lower left of the dialog), choose the image name of the smooth flat field you just created.

9. Perform flat field correction of your acquired image in the ***Analyze*** tab, ***Process Image*** group, ***Correct*** menu > ***Apply Flat Field***. Select the flat field you created. For Montage images, perform this step before you ***Generate Montage***.

Manage Flat Fields

When to Update the Flat Field Database

As time passes, it is possible for a flat field matrix to become outdated. Specks may have changed position, or other constant artifacts have either appeared or disappeared. A tell tale sign that it is time to update a flat field is when performing the flat field operation no longer does its job — marked by white specks appearing after applying the flat field.

Flat field calibrations are specific to a set of optics including the objective, filter, and magnification changer. If multiple calibrations are found for identical sets of optics, the most recent calibration will be used.

See also:

Creating a Flat Field Calibration Sample
Generating a Spline-Fitted Flat Field Image

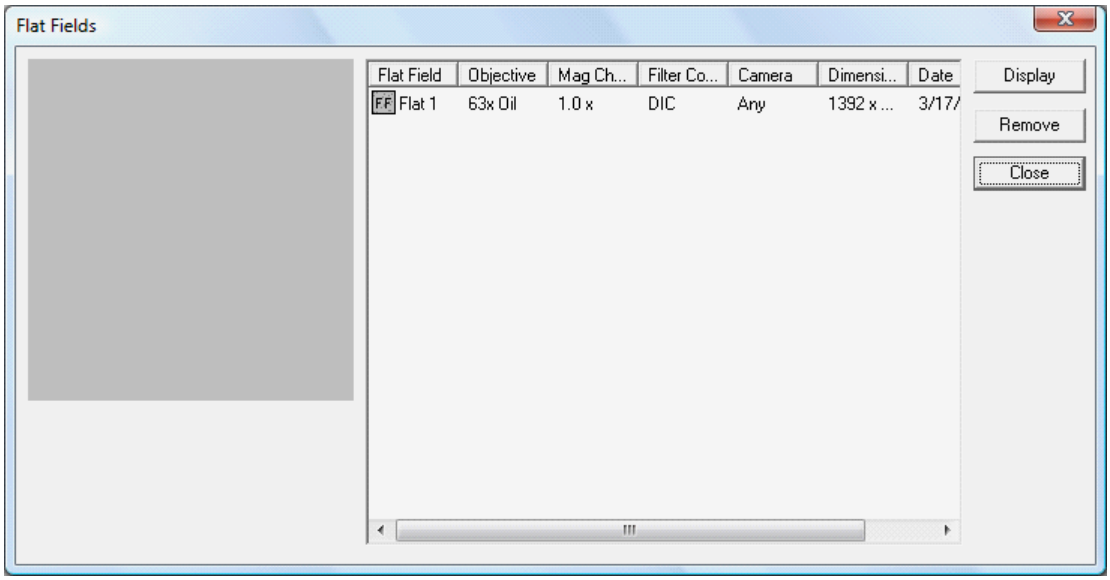
Displaying Existing Flat Fields

You can view of the existing flat fields (and their corresponding optical combinations) by selecting ***Correct menu > Manage Flat Fields***.

To display the flat field, click on the desired flat field icon in the table and press the ***Display*** button.

To remove a flat fields, select the desired file and click ***Remove***.

When finished, click ***Close*** to close the dialog.



Math Menu

The Math menu provides tools to perform basic arithmetic on the pixels values of images. The resulting values are either saved in a new image or a new channel.

Channel Math - opens a dialog to perform arithmetic on different channels within the same image

Image Math - opens a dialog to perform arithemetic on two different images with the same channels and dimensions within the same **.sld**

Average Images - opens a dialog to average different images with the same channels and dimensions within the same **.sld**

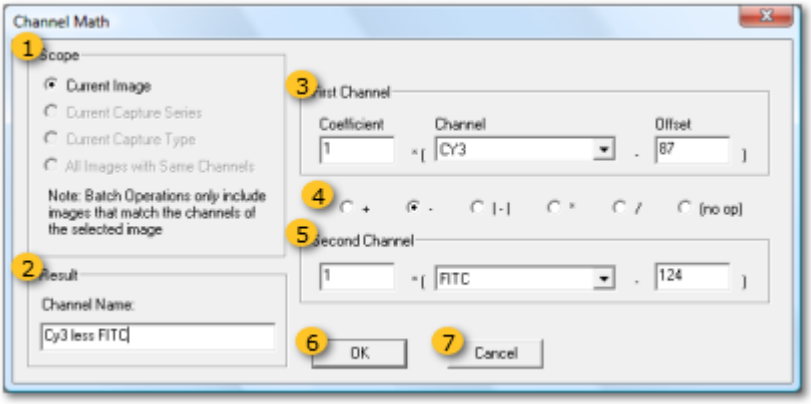
Invert Channel Intensities - performs intensity inversion for all channels does not open a dialog andalters current channel data. This can be easily undone by repeating the inversion.

Subtract Region Background - permanently subtracts the mean intensity of a defined background region (see Region tools, ***Set as background***) from every pixel of the image. This can only be undone by closing and reopening the **.sld** if you do not save after performing the operation. A less permanent solution for background region subtraction would be to define a background region and use the values for analysis only without altering the raw data.

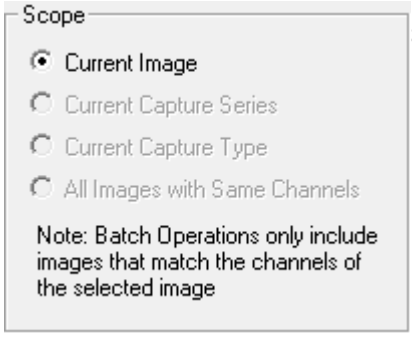
Channel Math Window

Channel math provides an interface to perform per pixel mathematical calculations based on intensity and constants according to the following formula:

a(CHANNEL_1 - b) [+,-,*,/] c(CHANNEL_2 - d)



1 Scope group



Select the scope of the selected operation as follows:

Current Image - Apply the selected operation to only the currently selected image

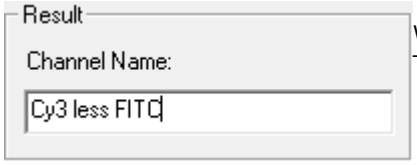
Current Capture Series -

Current Capture Type - Apply the selected operation to all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)

All images with Same Channels - Apply the selected operation to all images captured using the same channels as the selected image in the current slide.

It is important to note that batch operations (operations applied to more than one image) will only include images that match the channels of the selected image.

2 Result group



When channel math is performed, a new channel will be added to the image. Type a name for this new channel in the ***Channel Name*** edit field.

3 First Channel group

Enter the parameters for the first channel in the ***First Channel*** group. Each pixel will be adjusted as directed.

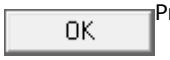
4 Operations group

Select an operation to apply between the first and second channel. Select ***no op*** to perform math on only the first channel.

5 Second Channel group

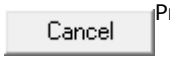
Enter the parameters for the second channel in the ***Second Channel*** group. Each pixel will be adjusted as directed.

6 OK button



Press ***OK*** to perform the selected operation and close the dialog.

7 Cancel button

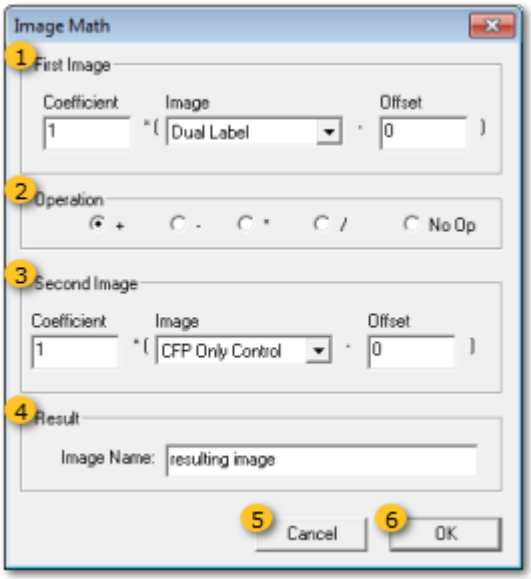


Press ***Cancel*** to discard changes and close the dialog.

Image Math window

Image math provides an interface to perform per pixel mathematical calculations based on intensity and constants according to the following formula:

a(IMAGE_1 - b) [+,-,*,/] c(Image_2 - d)



1 First Image group

Enter the parameters for the first image in the ***First Image*** group. Each pixel will be adjusted as directed using coefficient and offset values.

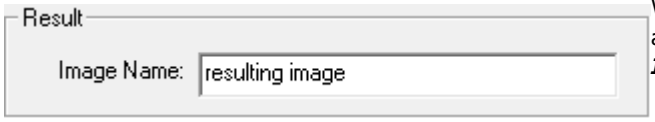
2 Operation group

Select an operation to apply between the first and second channel. Select ***no op*** to perform math on only the first image.

3 Second Image group

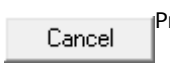
Enter the parameters for the second image in the ***Second Image*** group. Each pixel will be adjusted as directed using coefficient and offset values.

4 Result group



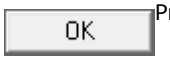
When image math is performed, a new image will be added to the **.sld**. Type a name for this new image in the ***Image Name*** edit field.

5 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

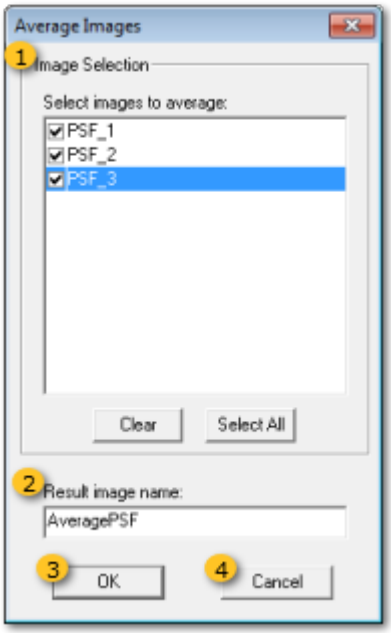
6 OK button



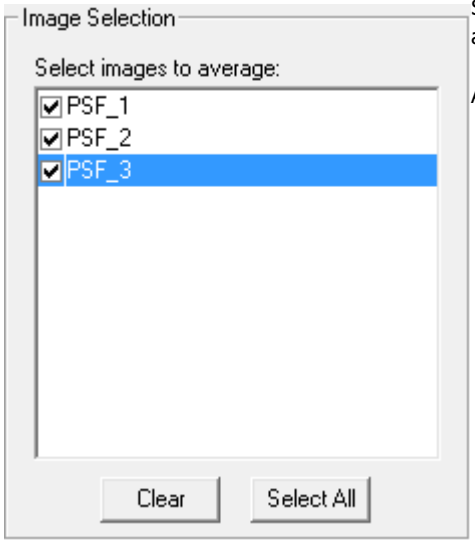
Press ***OK*** to perform the selected operation and close the dialog.

Average Images window

SlideBook includes the ability to average images with identical dimensions and channels.



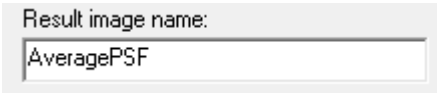
1 Image Selection group



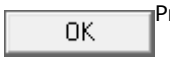
Select the images to average by checking the boxes. There are ***Select All*** and ***Clear*** buttons to assist in image selection.

All images must be in the same **.sld**.

2 Result image name field

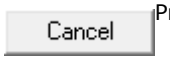


3 OK button



Press ***OK*** to perform the selected operation and close the dialog.

4 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

Align Menu

The Align menu provides options to shift images to improve alignment issues do to sample shift or hardware limitations. This can include shifting channels or time points

Automatic Image Alignment is used to correct homogenous movement of an entire imaging field.

Align Channels is used to shift individual channels due to misregistration.

Compute Channel Registration and ***Apply Channel Registration*** are used to calibrate channels acquired from two different imaging devices and later apply that registration. These tools are explained in TUTORIAL: Channel Registration.

Set Best Planes from Autofocus (4D) uses an autofocus algorithm on 4D data to set the best focal plane at each time point. You can then create a new image from these best planes in the Extract menu.

Align Images window

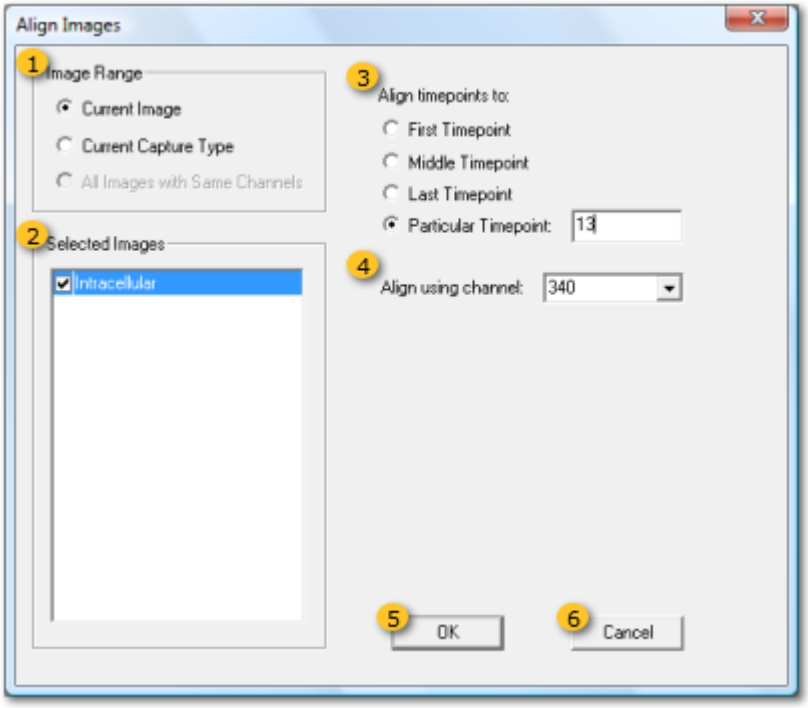
At times, an entire imaging field may drift over time due to unstable temperature or external forces such as a perfusion device or manual disruption when adding reagents. When this occurs, analysis results based on regions may be inaccurate. SlideBook's alignment tool is a helpful solution to re-stabilize the image for analysis.

NOTE: The alignment technique in this tutorial is for correcting homogenous movement of an entire field. If individual areas or cells are moving within the field, you will need to use the Mask Generation for analysis and particle tracking. If individual channels need alignment, you will need to use the Align Channels tool.

Automatic Image Alignment is only available when a 2D Timelapse or 4D Capture type image selected.

See also:

TUTORIAL: Automatic Image Alignment
Ratio Module, TUTORIAL: Aligning Images

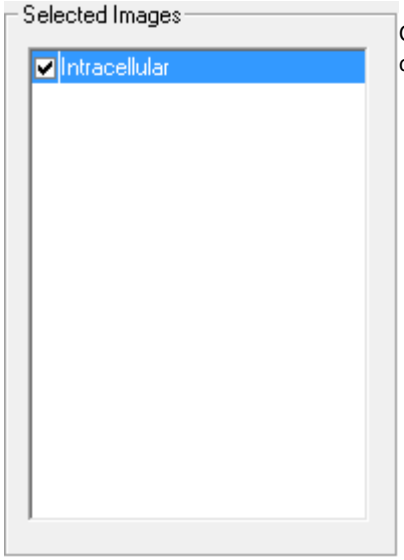


1 Image Range group

Select image range for alignment as follows:

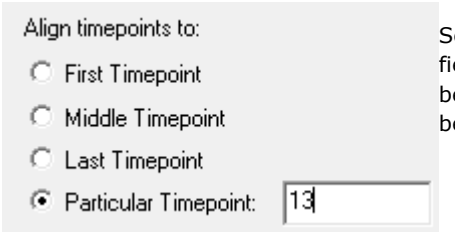
Current Image - Aligns only the currently selected image
Current Capture Type - Aligns all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Aligns all images captured using the same channels as the selected image in the current slide.

2 Selected Images group



Check the boxes for the images you would like to align. This list will change depending on the ***Image Range*** selected above.

3 Timepoint Selection



Select a time point to which the other time points will be aligned. If there field drifted in one direction consistently throughout the capture, you may benefit from selecting Middle Timepoint. If movement was sporadic, you may be better off selecting a time point where there is minimal movement.

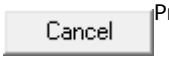
4 Channel Selection

Select a channel to use for running the alignment algorithm. You will want to select the most consistent channel for this action.

5 OK button

Press ***OK*** to run the alignment using the set parameters. A new image will be written to the slide with - **auto aligned** appended to the image name.

6 Cancel button



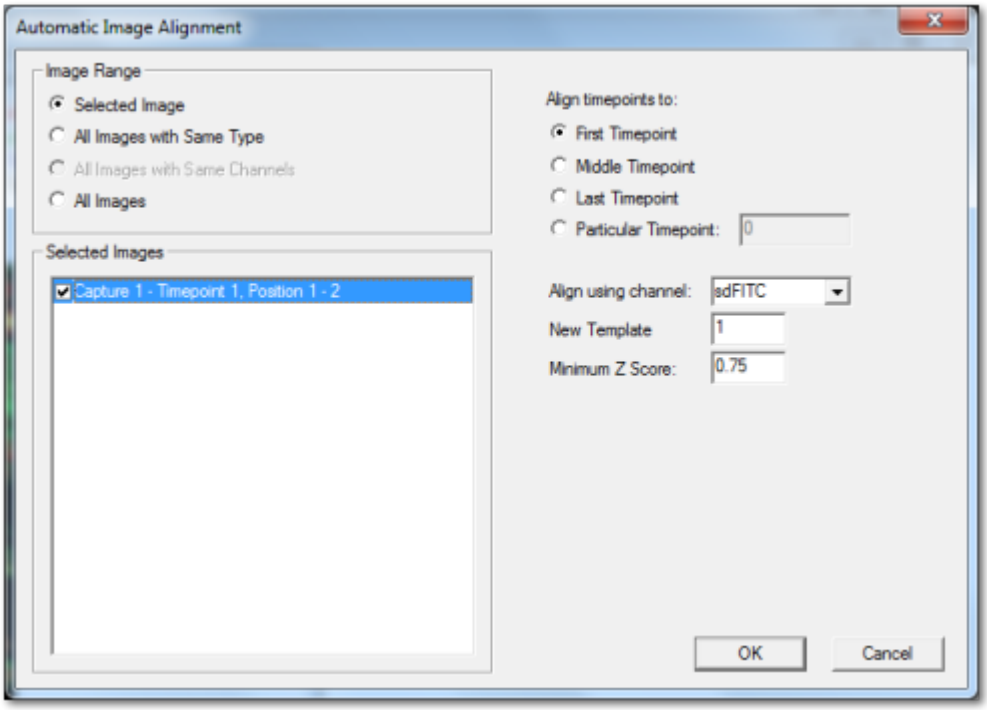
Press ***Cancel*** to close the window without running an image alignment.

TUTORIAL: Automatic Image Alignment

Occasionally, the sample or specimen may shift during image capture. SlideBook includes a tool to automatically align time points.

See also:

Align Channels window
Photobleach Correction window



1. Select the ***Image Range*** for alignment. If multiple images were defined, check the appropriate boxes in the ***Selected Images*** area.

2. Select ***First***, ***Middle***, ***Last***, or a ***Particular Timepoint*** to align all other time points to, that is which time point should be the central time point.

3. Select a channel from the ***Align using channel*** dropdown menu. This should be the channel that has the best contrast and stability and serves as a good frame of reference.

4. Set New Template and Minimum Z Score as follows:

> ***New Template*** - If you set this to something less than 1.0, then when a time point has a Z score above 1.0 it will become the new template that all following time points are matched against. This is useful if the pattern (image morphometry) evolves over time.
>
> ***Minimum Z Score*** - Set the minimum Z score to 0.0 (or perhaps .25). You can also review your data and look at the 'Notes' annotations to see what score each frame gets for it's best match. Unless the score is above the minimum Z score no shift is performed. This feature protects against finding a random offset on time points with no clear match. If alignment is overly aggressive, increase this value, if alignment missed some tinepoints, lower this value.

4. Click ***OK*** to perform the designated alignment.

> NOTE: After alignment, you can turn on the Notes annotation to see z score and any alignment shifts that took place in the format of:
>
> > **score:** z score value, **shift:** x shift (cumulative x shift), y shift (cumulative y shift) **avg:** average x shift to current plane, average y shift to current plane stddev*2: standard deviation in average x shift multiplied by 2, standard deviation in average y shift multiplied by 2

Ex. **score:** 0.65 **shift:** -5.00 (-5.00), 0.00 (0.00) **avg:** 0.00, 0.00 ***stddev*2:*** 0.00, 0.00

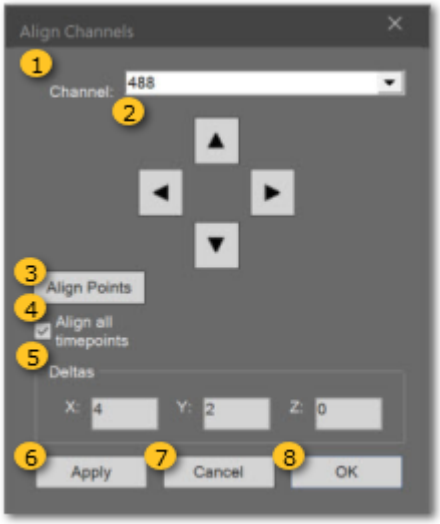
Align Channels window

It is sometimes necessary to shift individual channels of an image to correct for misregistration. The most common cause of misregistration is when separate dichroic mirrors are used. You can test for channel misregistration by collecting images of multi-colored fluorescent beads. Channel misregistration can be corrected by shifting individual channels of an image using the ***Alignment Guide*** or by purchasing specially aligned dichroic mirrors.

Changing channel alignment for an image makes a permanent change to the image itself and will be saved with the image. In other words, every view that you open from that point on will reflect the new alignment. Of course you can re-adjust the alignment later if you wish.

See also:

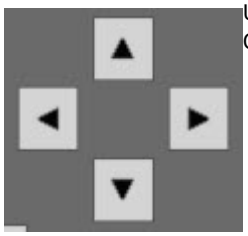
[TUTORIAL: Align Channels Manually]
[TUTORIAL: Align Channels Using Points]



1 Channel Selection

Select the channel to move from the drop down menu. The channel that is selected is the one that will move relative to the other channel(s).

2 Move buttons



Use the up, down, left, and right buttons to shift the selected channel in the desired direction. One click on a button results in a one pixel move in that direction.

3 Align Points button

Press ***Align Points*** to run a procedure where you click on a point in each channel and SlideBook automatically aligns the selected points. Each step of the process will appear next to the button after you click it.

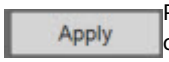
4 Align all timepoints check

Check this box if you would like the channel alignment to apply to all timepoints in the data set.

5 Deltas group

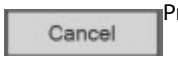
This field reads out the total shift in *x*, *y*, and *z* directions of the selected channel. This information can be recorded and typed in to apply identical channel shifts in future images.

6 Apply button



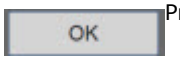
Press ***Apply*** to apply the channel shift to the current channel in the image. This will simply apply the changes without closing the Alignment Guide.

7 Cancel button



Press ***Cancel*** to discard changes and close the window.

8 OK button



Press ***OK*** to apply changes and close the Alignment Guide.

TUTORIAL: Align Channels Manually

The instructions below will instruct on manually shifting the channels pixel by pixel. Alternatively, you may use a point in each channel from Align Points.

See also:

Align Channels window
Align Channels Using Points Tutorial

1. Select the channel that you wish to align from the ***Channel*** dropdown menu.

2. To manually shift channels relative to each other, use the left and right arrows to move the selected channel in the *x* direction, and the up and down arrows to move in the *y* direction, or enter an offset for any axis directly into the corresponding ***Deltas*** edit controls.

> **NOTE:** The view will update automatically when you use the arrow buttons, but you must press the ***Apply*** button if you manually enter the offsets. Clicking ***Cancel*** will ignore any changes to the edit field that were not previously applied.

3. Check the box to ***Align all timepoints*** if you would like to shift the channels in all time points as you did for the current time point.

4. Click ***Apply*** then ***OK*** when you are satisfied with the image alignment.

> **NOTE:** If you find it necessary to align certain filters consistently, your system may need repair. Please contact support@intelligent-imaging.com.

TUTORIAL: Align Channels Using Points

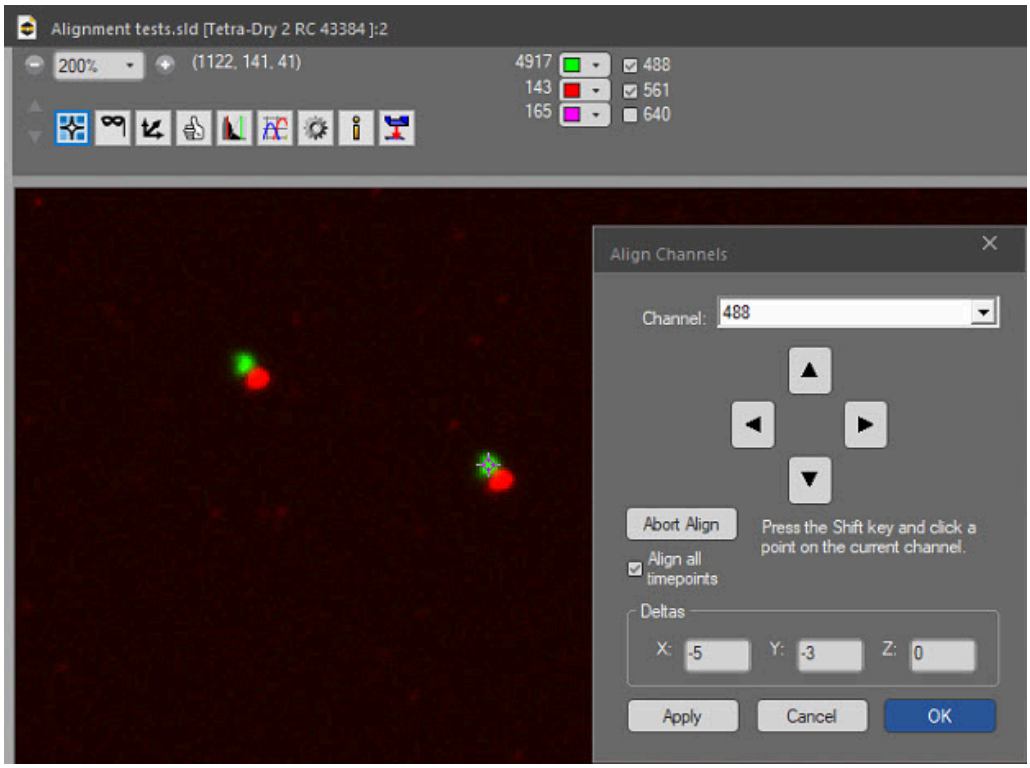
You may find it easier to select a single point in two different channels that you know should be aligned. This is particularly useful when using multicolored beads for system calibration.

See also:

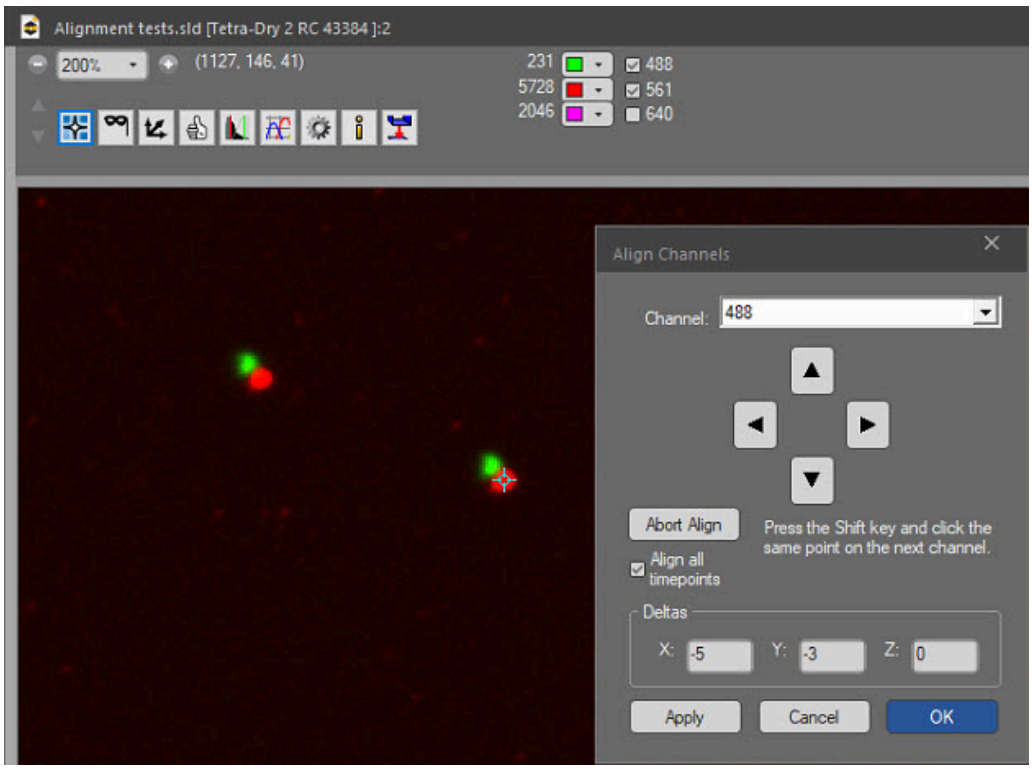
[Align Channels window]
[Align Channels Using Points Tutorial]

1. Select the first channel that you wish to align from the ***Channel*** dropdown menu.

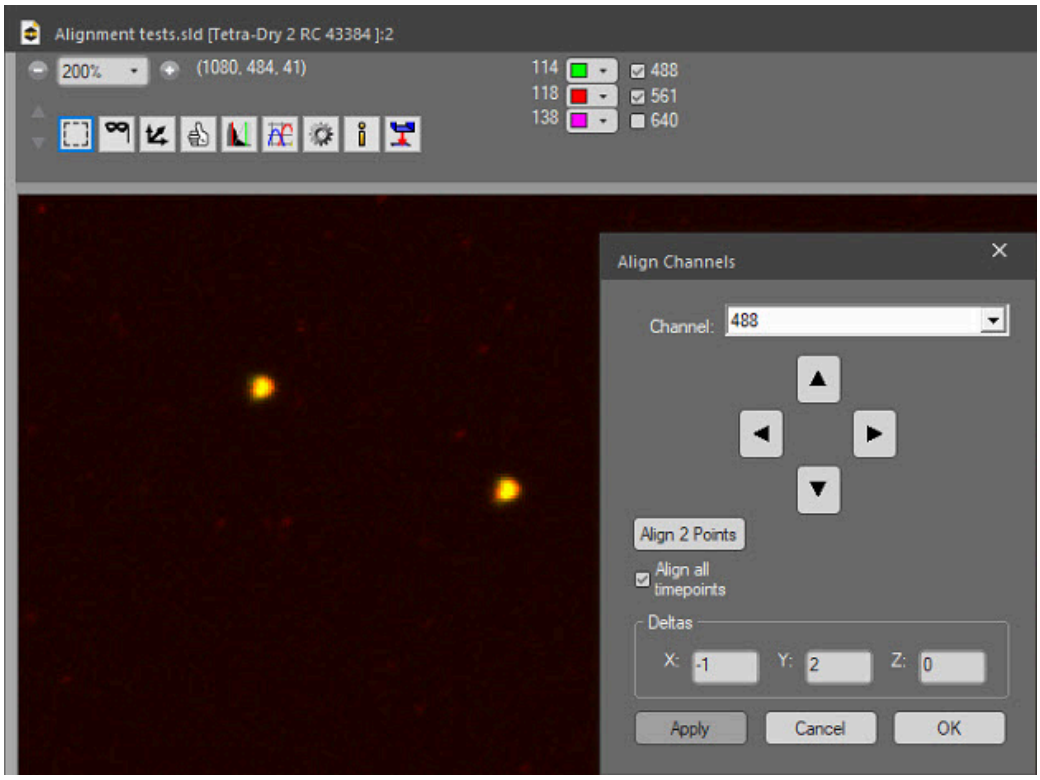
2. Click ***Align Points***. A prompt will appear below the arrow buttons. The prompts reads: ***Press the Shift key and click a point on the current channel***. Do as instructed.



3. The next prompt will read: ***Press the Shift key and click the same point on the next channel***. Do as instructed.



4. The two clicked points will be aligned. If desired, you may use the arrow buttons to fine-tune the adjustment.



5. Click ***Apply*** to apply the changes to the image.

6. Click ***OK*** to close the dialog.

TUTORIAL: Two Channel Registration

When capturing and combining images from two different cameras, alignment issues are critical. SlideBook now has two functions to accommodate any combination of scaling, rotation, translation, and shear between the two channels' coordinates: ***Compute Channel Registration*** and ***Apply Channel Registration***.

Compute Channel Registration finds the affine transformation between the two channels, using three control points defined for each channel. The affine transformation is a common spatial transformation in image processing (e.g., Wolberg 1990):

$$x' = ax + bx + c$$
$$y' = dy + ex + f$$

Affine transformations do not correct for more severe distortions, such as pincushion and barrel distortions or other nonlinear distortions. However, for any misregistration within a pixel or two, it is a reasonable first approximation and easier to determine and faster to compute than higher-order approaches.

Apply Channel Registration uses the transformation to interpolate pixel intensities from one channel into the same coordinates of a different channel, using bilinear interpolation. Bilinear interpolation computes the image value at an intermediate pixel position according to the four nearest neighbors weighted according to their relative distances.

Determining the transform

To compute the parameters for the transform, perform the following steps:

1. Image a slide of sub-resolution beads at high magnification. The beads should be sparse and provide sufficient bleedthrough into the other channel so that the same beads can be easily identified in both cameras' images.

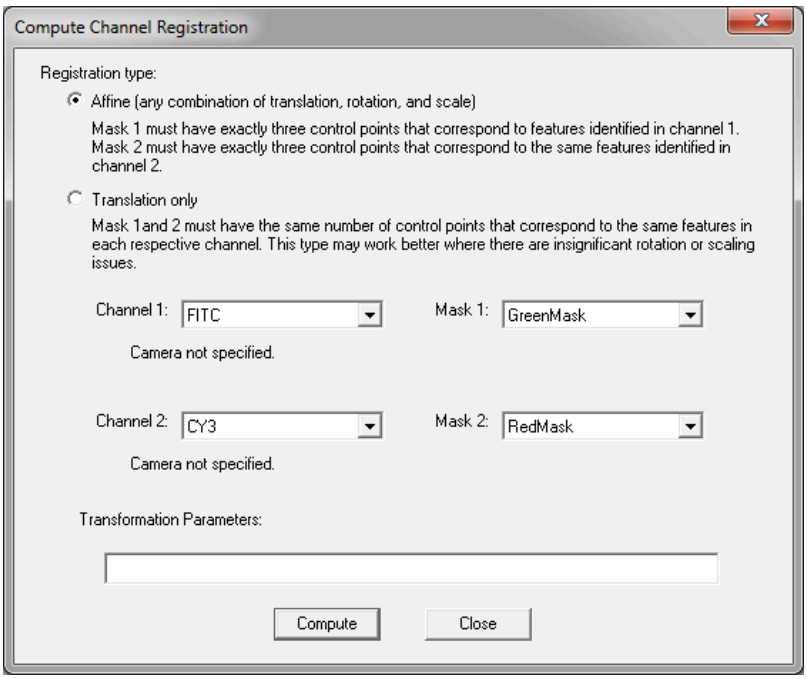
2. Find an arrangement of beads so that three span a good fraction of the camera field.

3. Acquire an image of the beads with two channels, A and B, where A is recorded by one camera and B is recorded by the other.

4. Create a mask and use the small mask tool to mark the three beads as they appear in channel A.

5. Create a second mask and use the small mask tool to mark the same three beads as they appear in channel B.

6. In the Analyze tab, Process Image group, select ***Align menu > Compute Channel Registration***. The following dialog box will appear:



7. Select the channels that correspond to the two cameras' images of the beads and the masks that correspond to each channel. Then click ***Compute***.

8. SlideBook produces a set of parameters that are used later to perform the registration and interpolation, e.g.:

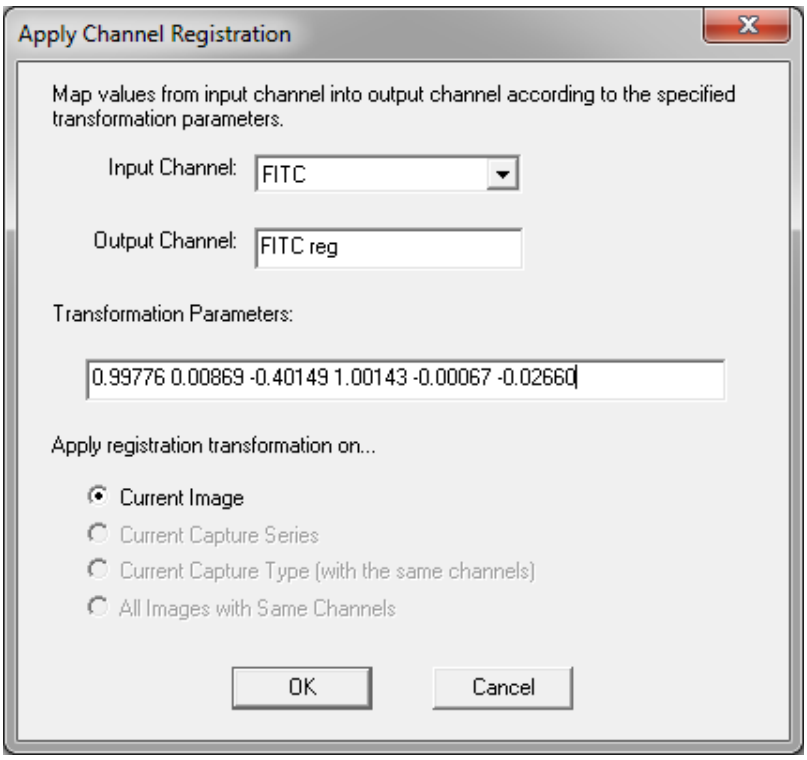
0.99776 0.00869 -0.40149 1.00143 -0.00067 -0.02660

(These correspond to the a, b, c, d, e, and f coefficients described above.)

Using the transform to register images

In order to transform a channel (such as FITC above) into the space of another channel (such as CY3 above) select ***Align menu > Apply Channel Registration*** and enter the ***Transformation Parameters*** copied from the ***Compute Channel Registration*** dialog.

By default, SlideBook will name the new, registered channel by adding **reg** to the end of the channel name.



If the parameters you enter would lead to more than a 10% scale change, SlideBook will warn you, as this means that the parameters are likely to be incorrect.

Storing a default transform

There are two hardware properties (access from the Administration tab) that will automatically determine portions of the ***Apply Channel Registration*** dialog box.

Under the ***Simultaneous Capture*** group, the ***Channel Registration Parameters*** stores the exact parameter string that goes into the ***Transformation Parameters*** field.

The ***Less Magnified Camera*** lets you specify which camera (1 or 2) is less magnified, if there is a consistent scale change across both cameras. Then, the ***Apply Channel Registration*** dialog box will automatically populate the ***Input Channel*** with the first filter configuration that specifies this camera.

As a rule, it is better to expand the less magnified camera's channel into the more magnified camera, so as to reduce the amount of zero valued border in the resulting channel.

References

Wolberg, G. (1990) Digital Image Warping. Washington: IEEE Computer Society Press.

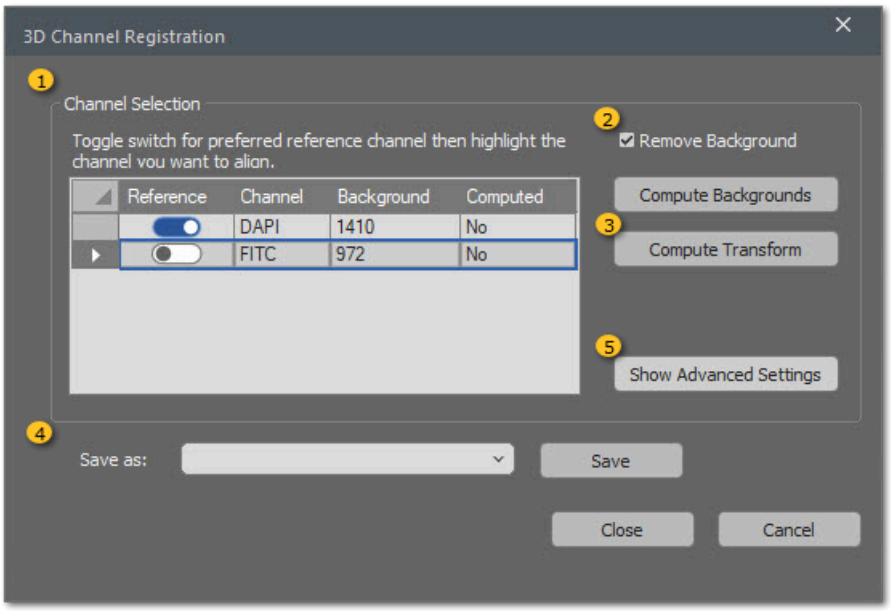
Compute 3D Channel Registration (LXCOR) window

The 3D Channel registration tool is a multi-channel alignment tool to compensate for axial and lateral shifts caused by chromatic aberration. The algorithm is using local cross correlation to compute a system generic registration per channel, magnification and illumination type on a template.
The result will be stored in SlideBook and can then be applied on data in batch using the Apply 3D Channel Registration (LXCOR).

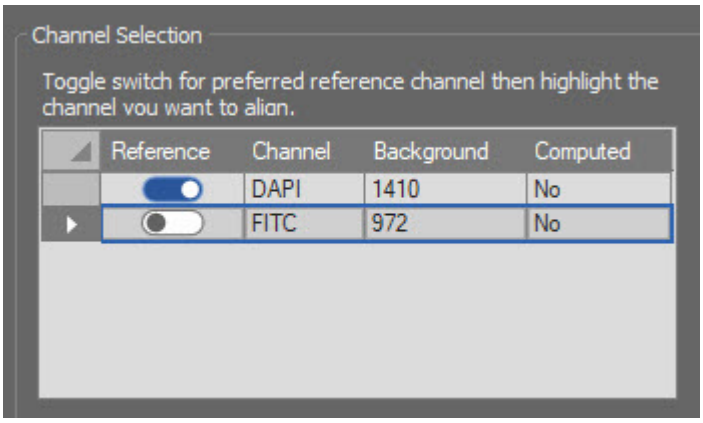
The protocol consists of two steps:

1. Compute Alignment - recommended to be done on a template
   a. Open a **.sld** or **.sldy** file with template image, preferably a TetraSpeck beads 3D capture
   b. Highlight the file and navigate to the Analyze Tab > Align > Compute 3D Channel Registration (LXCOR)

2. Apply the transform to selected channels on sample data
   a. Open one or several **.sld** or **.sldy** file(s) with matching channels and magnifications to apply the saved transforms
   b. Highlight the captures of interest and and navigate to the Analyze Tab > Align > Apply 3D Channel Registration (LXCOR)

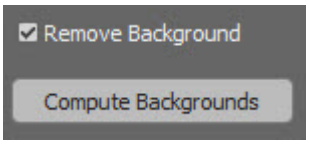


1 Channel Selection



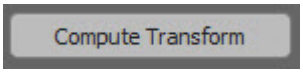
- Toggle the toggle switch to select a ***Reference Channel*** to which all other channels will be aligned to
- One channel can be registered at a time - highlight the channel you want to start with

2 Remove Background



Enable ***Remove Background*** and ***Compute Background*** to enhance the registration accuracy (no effect on resulting image). The background calculation will run for all channels in the image and list the result next to each channel. Alternatively a custom background value can be entered in the Background column of the channel list.

3 Compute Transform

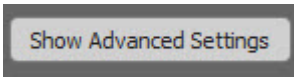


Hit ***Compute Transform*** to run the alignment algorithm
Once a transform is calculated the 3D/ 4D Volume Viewer will open to display the shift for the selected channel. In the ***3D/ 4D Volume Viewer*** tick or un-tick the ***Registration*** tick box then hit ***Refresh*** to switch between transform applied and not applied view.

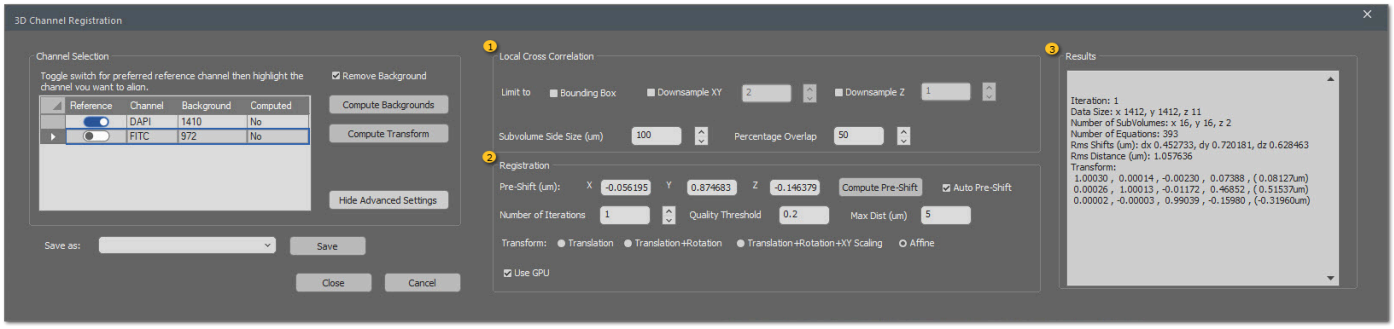
4 Process Controls

• When happy with the result select a previously saved transform name to override or add to or type a new custom name for this set of registration in the ***Save as:*** box. Hit ***Save*** to save the transform to the registration set - the channel name will automatically be specified by the system
• ***Close*** the window when all registrations required are found and saved
• Hit ***Cancel*** to stop the process and close the window

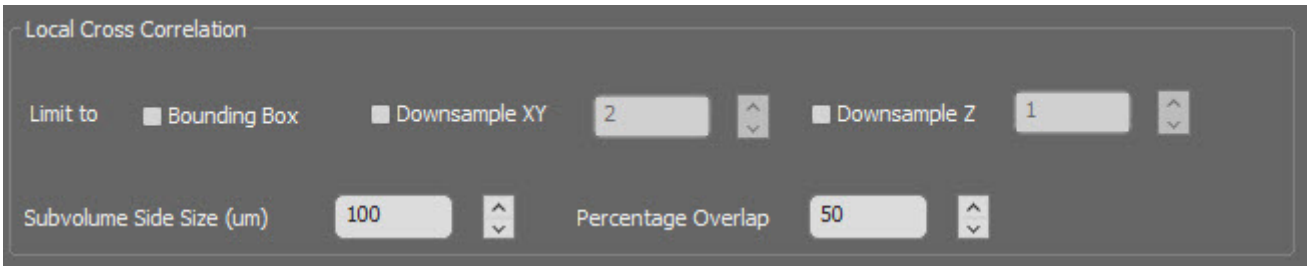
5 Advanced Calculate Registration Parameters



Hit the ***Show Advanced Settings*** button to access the advanced parameters. The window will extent to view and alter advanced settings. The button will automatically turn into a ***Hide Advanced Settings*** button to minimize the window again.



1 Advanced Settings: Local Cross Correlation



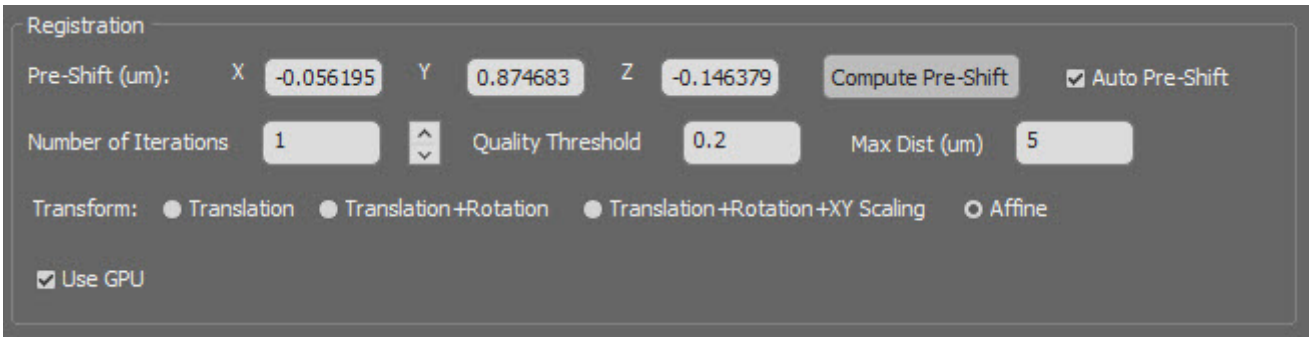
Define the volume and sample size for the cross correlation algorithm to consider.

• To save time, select ***Bounding Box*** if you want to limit the registration to a smaller volume. You need to define this prior to the process in the ***3D/ 4D Volume Viewer*** > ***Bounding Box***

• Select and define a value to ***Downsample*** in ***XY*** or in ***Z*** if the dataset is large

• ***Subvolume Side Size*** is the size of the cubes in um, the image is divided into to find individual transforms. Each subvolume transform is then compared with the neighboring cubes using a ***Percentage Overlap*** in order to find a global registration. This can be set higher or lower - a good estimate is the grid size given when opening the image in the 3D/ 4D Volume Viewer.

2 Advanced Settings: Registration



Define the parameters of the registration process.

• Before the process:

  • Enter a ***Pre-Shift*** for the X, Y and/ or Z coordinates or hit ***Compute Pre-Shift*** to let the algorithm find an initial translation transform to give the alignment procedure a good start

  • If no pre-shift is desired, set the values to 0 and disable ***Auto Pre-Shift.***

• **NOTE:** Once a shift is found the 3D/ 4D Volume Viewer will open to display the shifts - select the channels you are comparing in the ***3D/ 4D Volume Viewer***. The initial shift might be too small to be visible in the 3D/ 4D Volume Viewer.

• For the alignment process select:

  • the ***Number of Iterations*** - usually 1 or 2 are sufficient, more can have the algorithm overshoot

  • the ***Quality Threshold*** - the higher the more accurate but also more time consuming and can lead to the alignment to fail

  • the ***Maximum Distance*** - increase if a bigger shift is expected especially when not using the pre-shift

• Select the type of ***Transform*** the algorithm should apply to the selected channel:

  • ***Translation*** only

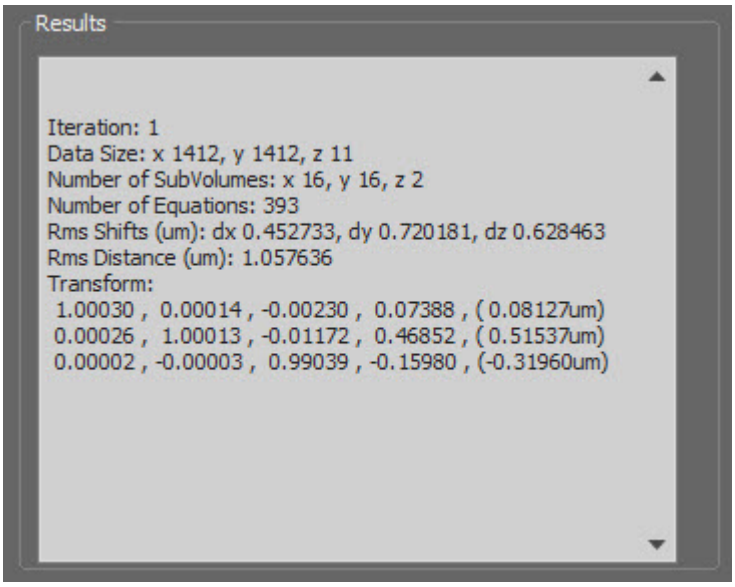
  • ***Translation and Rotation***

  • ***Translation, Rotation and XY Scaling***

  • ***Affine***: Translation, Rotation, XY Scaling and Skew

• Enable ***GPU*** when a graphics card is present to accelerate the process

3 Advanced Settings: Results



The transform matrix and parameters of the current registration is displayed in the ***Results*** field

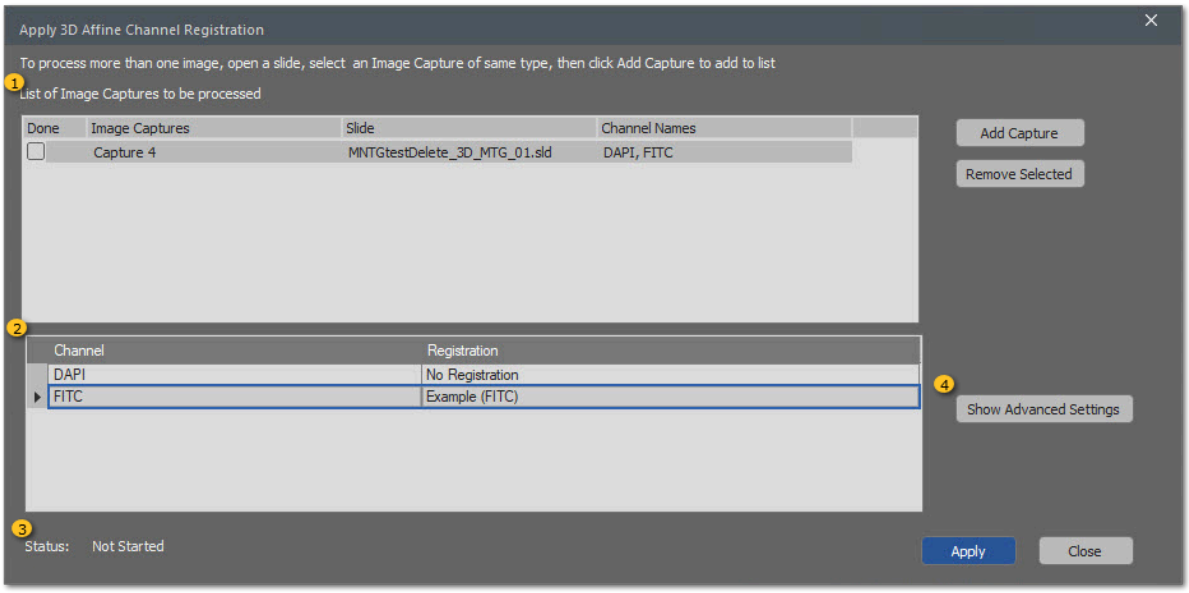
Apply 3D Channel Registration (LXCOR) window

The 3D Channel registration tool is a multi-channel alignment tool to compensate for axial and lateral shifts caused by chromatic aberration. The algorithm is using local cross correlation to compute a system generic registration per channel, magnification and illumination type on a template using the Compute 3D Channel Registration (LXCOR). The result will be stored in SlideBook and can then be applied on data in batch using the Apply 3D Channel Registration (LXCOR).

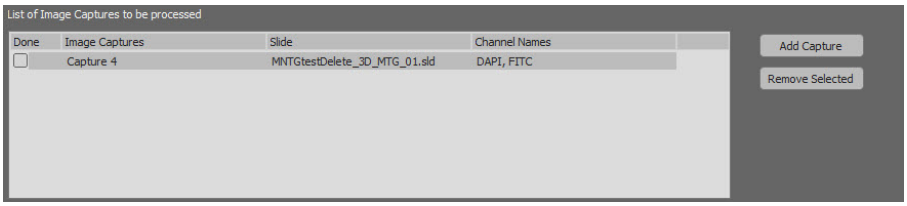
The protocol consists of two steps:

1. Compute Alignment - recommended to be done on a template
   a. Open a **.sld** or **.sldy** file with template image, preferably a TetraSpeck beads 3D capture
   b. Highlight the file and navigate to the Analyze Tab > Align > Compute 3D Channel Registration (LXCOR)

2. Apply the transform to selected channels on sample data
   a. Open one or several **.sld** or **.sldy** file(s) with matching channels and magnifications to apply the saved transforms
   b. Highlight the captures of interest and and navigate to the Analyze Tab > Align > Apply 3D Channel Registration (LXCOR)



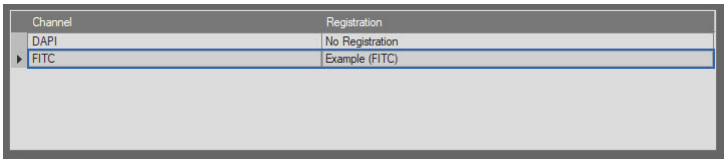
1 Selected Images List

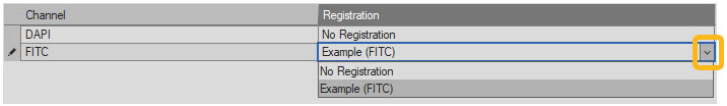


This list displays the images to be processed, the origin slide files and recognized channel names

- This list can be altered, to add images open the **.sld** or **.sldy** file containing the captures of interest. Highlight the capture(s) of interest and hit ***Add Capture*** left to the list.
- To exclude a capture highlight it in the list and hit ***Remove Capture*** left to the list.

2 Channel Registration List



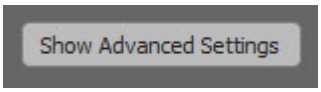


This list displays the detected registrations for the channels of the highlighted capture above.
• If no transform is saved for a specific channel it will show ***No Registration***, the channel will be copied without change into the new capture
• If a transform is saved it will display the name of the registration (like "Example (FITC)" here)
• Other available transforms for the selected channel will be displayed when selecting the dropdown arrow in the Registration column and can be selected independently
• Alternatively ***No Registration*** can be selected if this particular channel should not be aligned for this capture

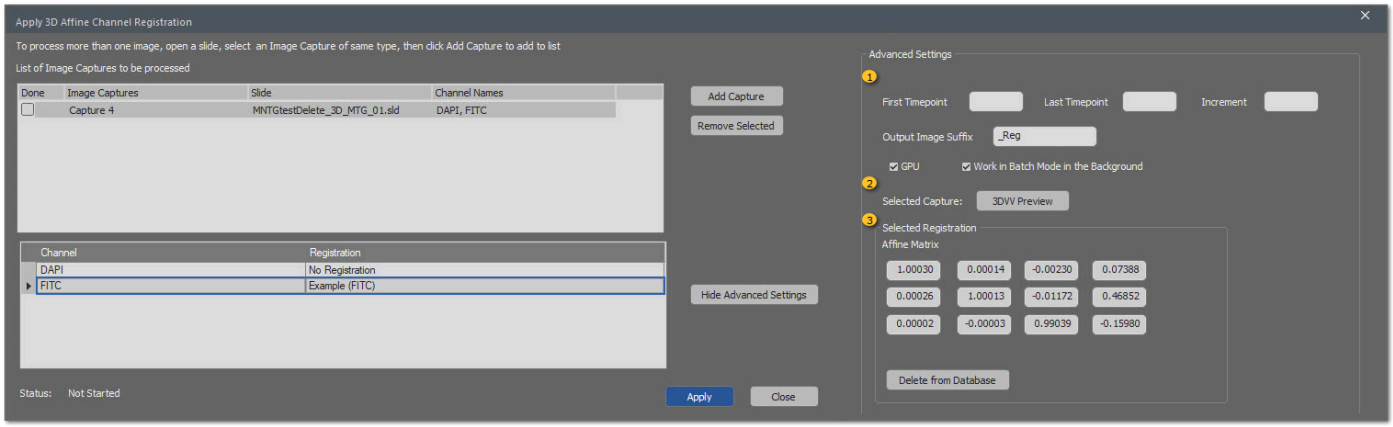
3 Process Control

• ***Status:*** will display the image currently in process, in the Selected Image list the ***Done*** tick box will indicate if this image has already been processed while the batch process is running
• Hit ***Apply*** to start the (batch) process
• Hit ***Close*** to close the window

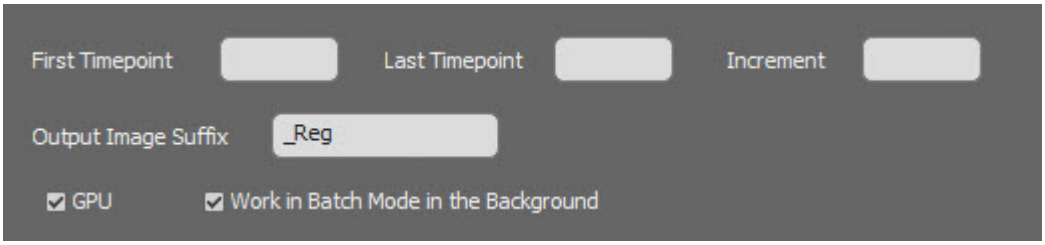
4 Advanced Apply Registration Parameters



Hit the ***Show Advanced Settings*** button to access the advanced parameters. The window will extent to view and alter advanced settings. The button will automatically turn into a ***Hide Advanced Settings*** button to minimize the window again.



1 Advanced Settings: Output & Process Control

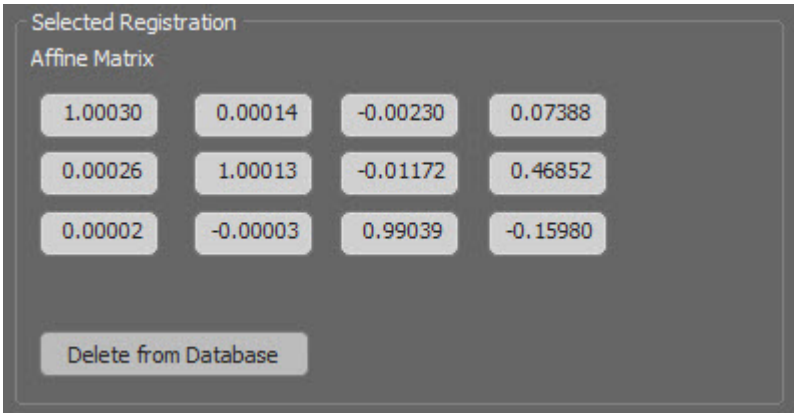


• If you want to limit the output to only a subset of images in a timelapse, select a ***First Timepoint*** and a ***Last Timepoint*** to process and/or enter an ***Increment***
• Enter an intuitive ***Output Image Suffix*** to distinguish the captures with channel registrations applied from the originals
• Enable ***GPU*** when a graphics card is present to accelerate the process
• Enable ***Work in Batch Mode in Background*** when you expect this process to take a while

2 Preview Transform

To preview a specific transform highlight the capture of interest and a channel of interest, then hit the ***3DVV Preview*** button. The Image will be displayed in the ***3D/ 4D Volume Viewer,*** tick or un-tick the ***Registration*** tick box then hit ***Refresh*** to switch between transform applied and not applied view.

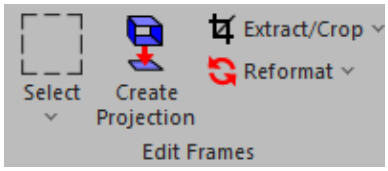
3 Transform Matrix



- The ***Affine Matrix*** (3 by 4 values max) of the selected channel will be displayed in this group
- ***Delete from Database*** will completely erase the selected transform for this channel

Edit Frames Group

The Edit Frames group is used to alter the base data set. Tools included in this group allow you to crop in x, y, z or time as well as extract subsets of data or delete specific frames.



Select Menu

Marquis Tool - Allows you to select an area of the image, useful in the ***Live*** camera window to select an area for imaging or in the ***Main*** view to select an area to crop.

Select all - Selects the entire image in *x*, *y*, *z*, and time.

Select in all planes - uses the marquis selection defined and extends the selection to all planes of the image, useful for cropping an image in *x/y*.

Define selection cube - opens the Define Selection Cube window where you can type in the exact dimensions/coordinates of the selection area.

Create Projection

Opens the Create Projection dialog that will create a new image per the chosen parameters. This method projects a ray through a stack of vowels to determine the maximum, sum, or average intensity pixel then uses that pixel to create a 2D image. This is performed for each channel at each pixel location. Alternatively, you can view a projection without creating a new image by clicking on the projection button in a Main View window.

Extract/ Crop Menu

Crop from Selection Cube in x, y, or z.
Remove Channel from the image.
Create Line Extraction extracts a line of data (drawn with the ruler tool) into a new image.
Extract/ Crop Timepoints extracts a single consecutive time range from a data set.
Generate Image from Best Planes in 4D creates a new image from the best planes in a 4D data set.

These planes are set individually using the thumb icon or by the Set Best Planes from Autofocus function.

Reformat Menu

Subsample opens a dialog that allows you to subsample an image in xy or z, reducing the image size.
Create Interpolated Image allows you to create more voxels by interpolating the original data, this can be useful for smoothing the image in creating figures and renderings.
Process Split Camera Data opens a dialog that allows you to split a data set into two or more channels when a physical device such as a DualView or QuadView was used to acquire data but not configured during acquisition.
Rotate allows you to enter coordinates to rotate data and create a a new images based on the rotation.
Create Blank creates a blank image on the slide.
Merge Timelapse Images opens a dialog to combine multiple images to append a time series.
Insert Channel opens a dialog to insert one channel from image A into image B.
Extract from 4D Stream opens a dialog to extract and reformat data captured using Rapid 4D Stream acquisition.

Crop from Selection Cube window

Images can be cropped in a number of different dimensions: *x*, *y*, *z*, or time. To crop an image in *x*, *y*, and/or *z*, you will use the ***Crop from Selection Cube*** window. To use this, you must first select the area to crop as follows:

1) Open the image in ***Main*** view

2) Use the Marquee tool to select an *x*-*y* area by clicking and dragging a rectangular shape on the open image.

3) To select all *z* planes, press **Ctrl+B** or go to the ***Edit Frames menu > Select >***

Select all

Select in all Planes

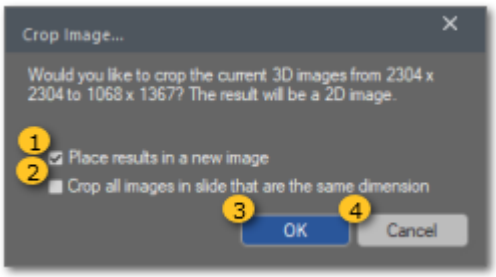
Define Selection Cube to manually enter *x*, *y*, and *z* parameters.

4) Go to ***Extract/Crop menu > Crop from Selection Cube***

NOTE: You can not crop time points from an image using this command. To crop time, you must Extract Subseries to New Image.

See also:

Define Selection Cube
Define Bounding Box
Extract Subseries to New Image



1 Place results in a new image

Place results in a new image

Check the box to copy the cropped data into a new image on the slide. If you do not check this box the existing image will be cropped and overwritten.

2 Crop all images

Crop all images in slide that are the same dimension

Check the box to perform an identical crop on all images in the slide with the same dimensions.

3 OK button

OK

Press ***OK*** to proceed with cropping as designated.

4 Cancel button

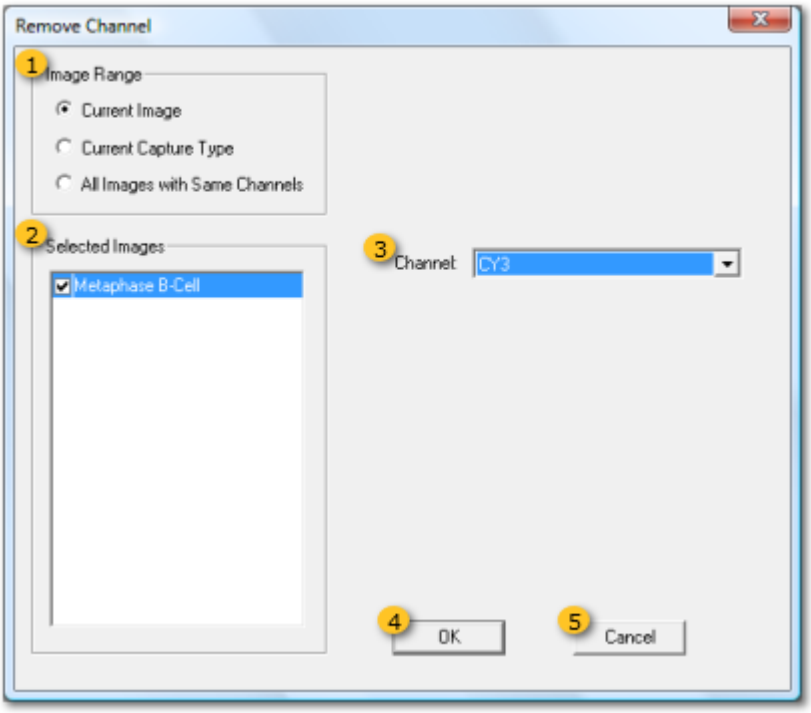
Cancel

Press ***Cancel*** to abandon cropping and close the dialog.

Remove Channel window

Should you wish to remove a channel from an image, you will use the Remove Channel dialog. This may be necessary if you created or added channels post-capture that you would not like to save or if you captured unwanted channels during image acquisition.

Access in the Analyze tab, Edit Images group, ***Extract/ Crop menu > Remove Channel***

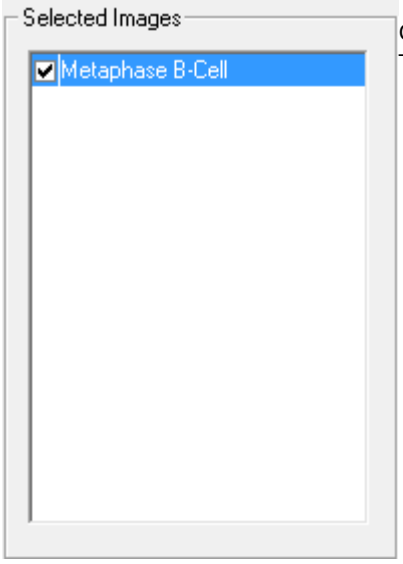


1 Image Range group

Select image range as follows:

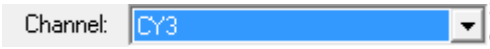
Current Image - Removes channel from only the currently selected image
Current Capture Type - Removes channel from all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Removes channel from all images captured using the same channels as the selected image in the current slide.

2 Selected Images list



Check the box(es) for the images for which you would like to remove a channel. This list will change depending on the ***Image Range*** selected above.

3 Channel Selection drop down

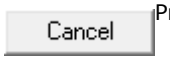


Select the channel you would like to remove from the drop down list. Only channels present in the image will be available.

4 OK button

Press ***OK*** to remove the channel as directed and close the dialog.

5 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

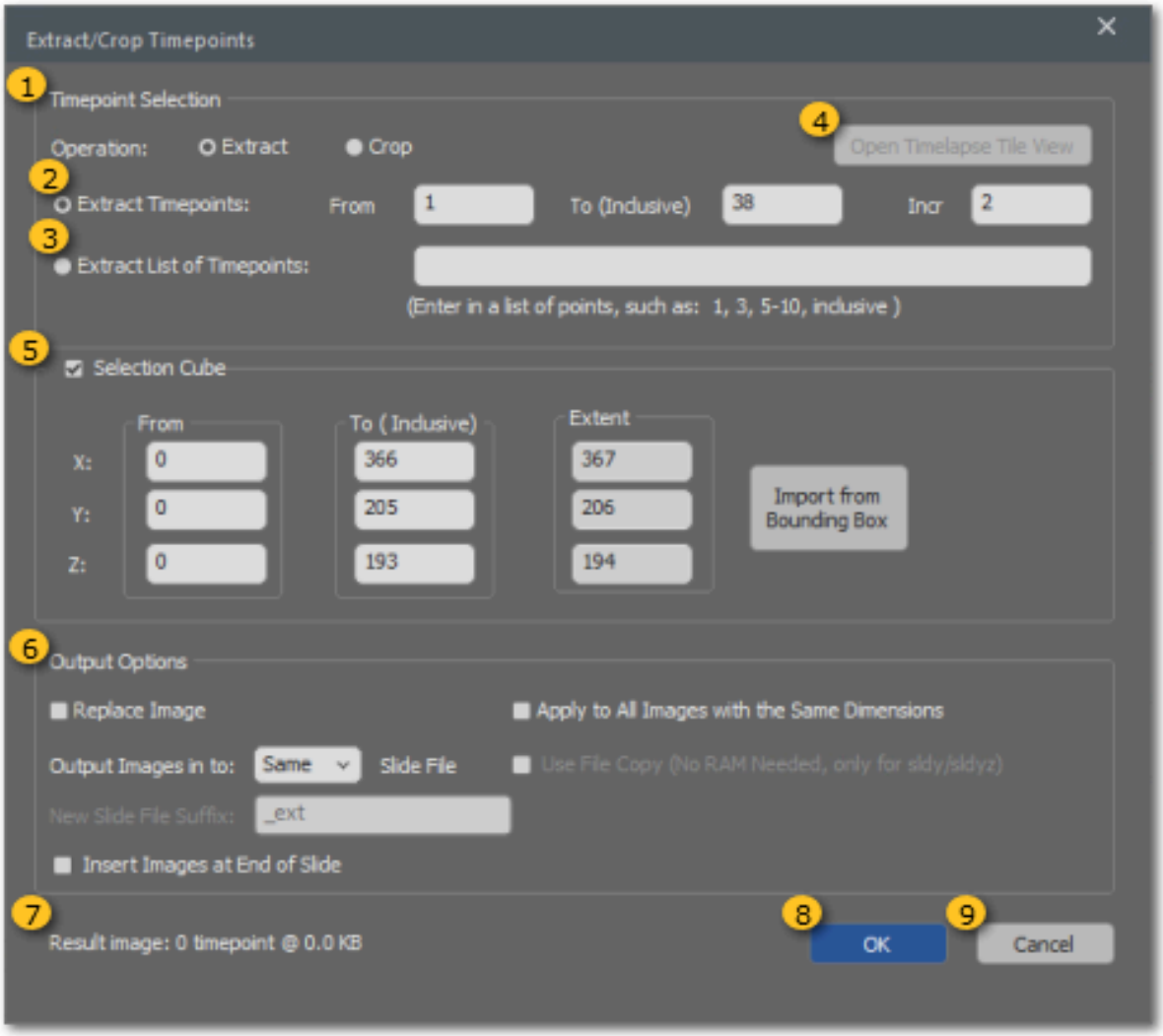
Extract/ Crop Timepoints window

Images can be cropped in a number of different dimensions: *x*, *y*, *z*, or time. To crop data in time, you will use the ***Extract/ Crop Timepoints/ Tiles*** window.

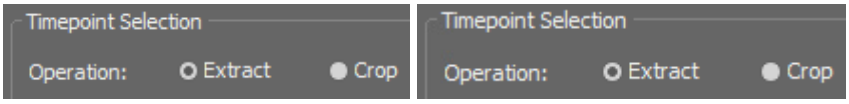
Access in the Analyze tab, Edit Images group, ***Extract/ Crop menu > Extract/ Crop Timepoints***, can also be accessed from an open view of the image from the Settings button.

See also:

[Crop Image from Selection Cube window]
[View Settings window]



1 Timepoint/Tile Operation Selection



Depending on the capture type you can select Timepoints for 2D or 3D timelapses or Tiles for 2D, 3D or 4D montages for the following ***Operation***:

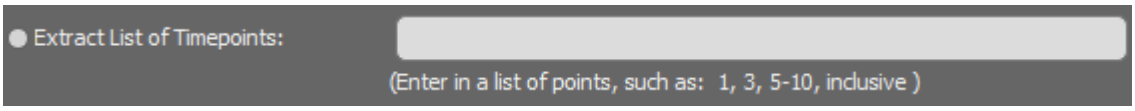
Extract: Keeps only the selected Timepoints/ Tiles

Crop: Removes only the selected Timepoints/ Tiles

2 Select Range

Allows you to define a range and increment (Incr) of timepoints/ tiles to extract or remove. The selected values are inclusive for either operation. From/ To will display the full image extent on opening. Make sure you set the increment to 1 if you require all timepoints between the bounds to be extracted or removed.

3 Define List



Allows you to define specific timepoints/ tiles to extract/remove. The list is inclusive for either operation.

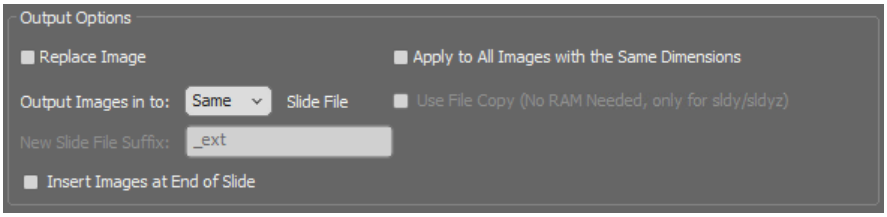
4 Open Timelapse Tile View

The ***Open Timelpase Tile View*** button is enabled for montages only and will display the montage in ***Timelapse Tile View*** including the tile numbers to assess which tiles to remove/ extract. ***Close Timelapse Tile View*** will hide the window again.

5 Selection Cube

For the X, Y and Z dimensions you can define values for the cube/ plane to extract like using the Define Selection Cube window. You can also define a Bounding Box from the ***Home*** tab > ***View*** > ***3D/4D Viewer*** > ***Bounding Box*** and import it here.

6 Output Options



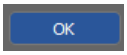
Replace Image: Tick this box if the original image should be replaced
Apply to All Images with the Same Dimensions: Tick this box if you want to apply the extraction to all images with the same dimensions in the slide.
Output Images in to: Same/ New Slide File, select 'New' if you work with large images for better data management
Use File Copy (No RAM Needed, only for sldy/sldyz files)

New Slide File Suffix: When saving the extracted file into a new slide there is the option to change the slide name suffix. Default is *OriginalFileName_ext.*
Use File Copy: Tick for SLDY files for faster copy process, it will not go through RAM.
Insert Images at End of Slide: Forces the image to the bottom of the slide file, otherwise it is placed right under the original capture.

7 Result image

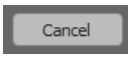
Displays the expected size of the resulting image.

8 OK



Starts the extraction process.

9 Cancel



Closes the window without action.

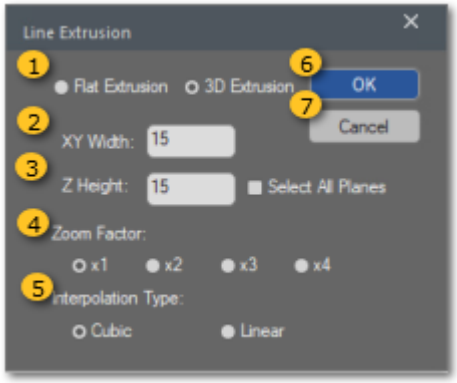
Create Line Extraction window

You may find it useful to extract a line of data from an image for closer inspection. This can be done as a single plane or a 3D stack with a user-defined line thickness.

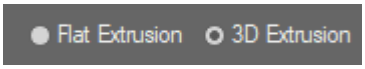
Access with a 2D or 3D image by drawing a line using the ***Ruler tool*** then in the Analyze tab > Edit Images group > ***Extract/ Crop menu > Create Line Extraction***

See also:

[Main View](#)
[Tool Menu](#)
Smooth Curve Analysis



1 Extrusion Options



Select ***Flat Extrusion*** to extract only the current *z*-plane. Select ***3D Extrusion*** to extract a *z* range of the line.

2 Width Selection

Enter a value (in pixels) for the desired width of the extracted image.

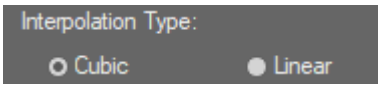
3 Z Selection

When performing a 3D extrusion, enter the desired number of *z*-planes for the resulting image depth. Alternatively, check the box to ***Select All Planes*** to extract the entire 3D stack.

4 Zoom Factor

Select the desired ***Zoom Factor*** from the options provided. You may also zoom in on the resulting image in the ***View*** window.

5 Cubic option

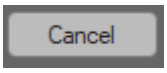


For diagonal lines or when a zoom factor is selected, interpolation will be required between data points. ***Cubic*** interpolation of two points on a line uses those points as well as the next pair of data points. ***Linear*** interpolation of two points on a line uses only the two adjacent values.

6 OK button

Press ***OK*** to create the line extraction image and close the dialog.

7 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

Generate Image from Best Planes in 4D

Generate Image from Best Planes in 4D creates a new image from the best planes in a 4D data set and creates a new 2D data set.

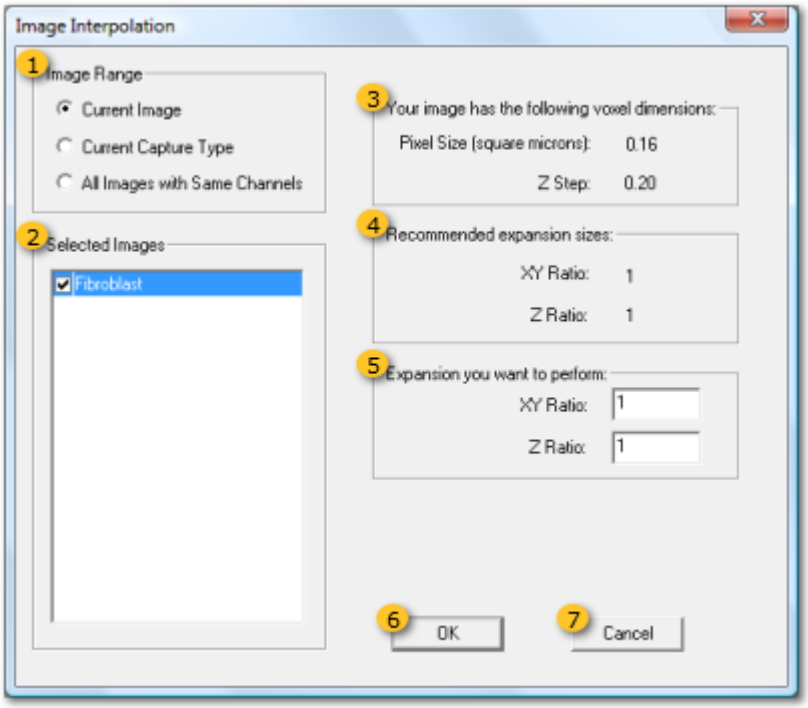
This function is found in the Analyze tab, Edit Frames group, under the ***Extract/ Crop menu***.

. These planes are set individually using the thumb icon or by the Set Best Planes from Autofocus function.

Image Interpolation window

Sometimes, you will not be able to capture an image at the desired *xy* or *z* resolution due to timing or photosensitivity issues. For cases such as these, you may perform ***Image Interpolation*** to generate more voxels and create a smoother looking image. The new image will have **.Interp** appended to the image name.

Access in the Analyze tab, Edit Images group, ***Reformat menu > Create Interpolated Image***

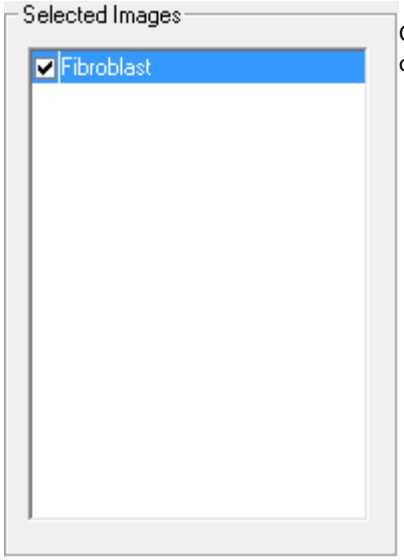


1 Image Range group

Select image range as follows:

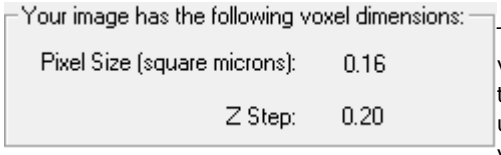
Current Image - Interpolate only the currently selected image
Current Capture Type - Interpolates all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Interpolates all images captured using the same channels as the selected image in the current slide.

2 Selected Images group



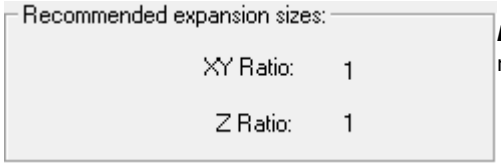
Check the box(es) for the image(s) you would like to interpolate. This list will change depending on the ***Image Range*** selected above.

3 Voxel Dimensions group



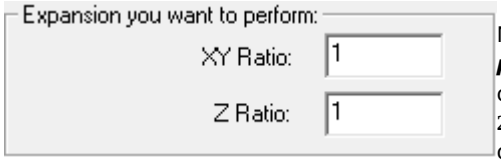
This area provides information about the current dimensions of each voxel of the image. ***Pixel Size*** is obtained from information entered in the configuration of the camera and objectives as well as any binning used during capture. ***Z step*** is designated during image capture. Both values are give in microns.

4 Recommended Expansion Sizes group



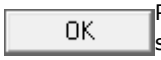
Recommended expansion sizes uses some information to recommend how much the image should be interpolated.

5 Expansion group



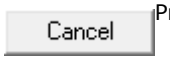
Manually enter in whole integer values for the ***Expansion you want to perform***. You may use the recommended value as a guideline or enter other desired values for expansion. For example, if you enter the value 2 in the ***Z Ratio*** field, a new *z* plane will be created between each pair of existing *z* planes. If you enter the value 3 in the ***Z Ratio*** field, two new *z* planes will be created between each pair of existing *z* planes.

6 OK button



Press ***OK*** to perform the image interpolation and close the dialog. A new image will be written to the slide with **.Interp** appended to the image name..

7 Cancel button

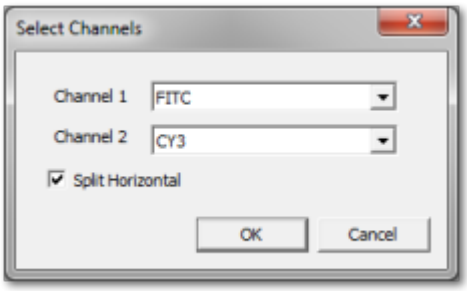


Press ***Cancel*** to discard changes and close the dialog.

Process Split Camera Data window

Some systems include optical devices that split an image by channel. SlideBook includes tools to configure such devices in a way that images are acquired and automatically split and overlayed. In circumstances when images are acquired on a different system, or not split during acquisition for some reason, they can be split post-acquisition in SlideBook.

Access in the Analyze tab, Edit Images group, ***Reformat menu > Process Split Camera Data***



The menu item is enabled at all times a single-channel image is displayed. The result is an image half as wide or half as tall as the original, with the data split down the X or Y axis.

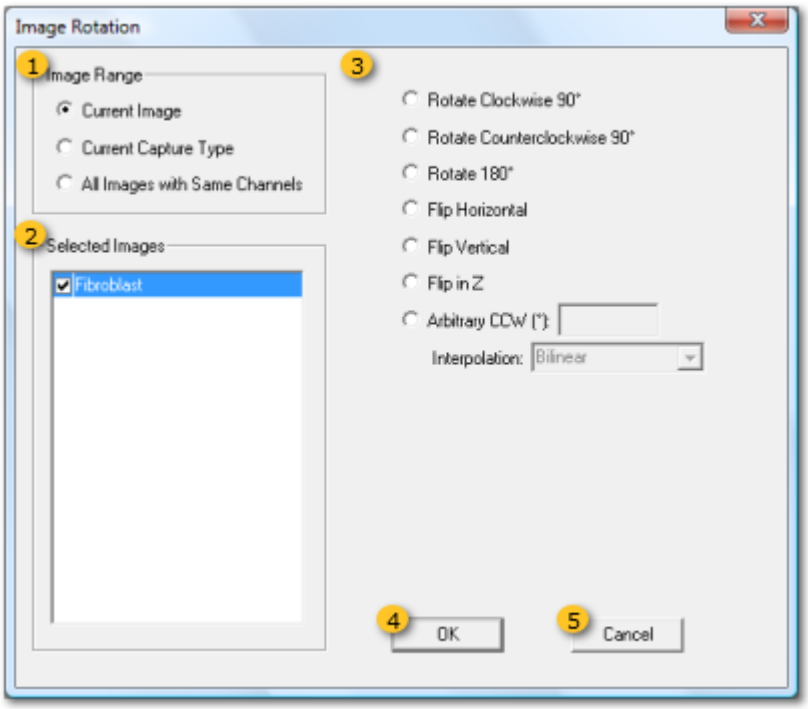
Image Rotation window

At times, you may find it useful to adjust the orientation of your data set. This is a different operation from ***View*** options such as volume rendering or orthographic rendering in that you are actually writing new image to the slide where the voxels are reoriented according to the options specified.

Access in the Analyze tab, Edit Images group, ***Reformat menu > Rotate***

See also:

[Three View]
[3D/ 4D Volume View]

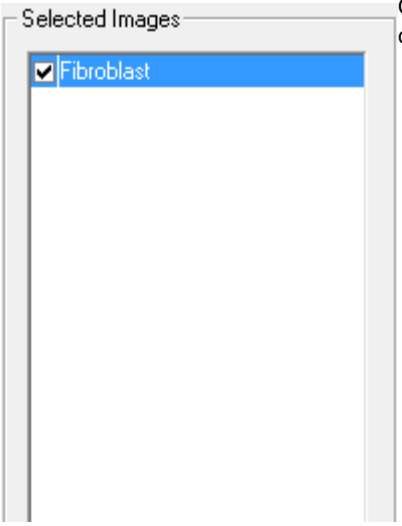


1 Image Range group

Select image range as follows:

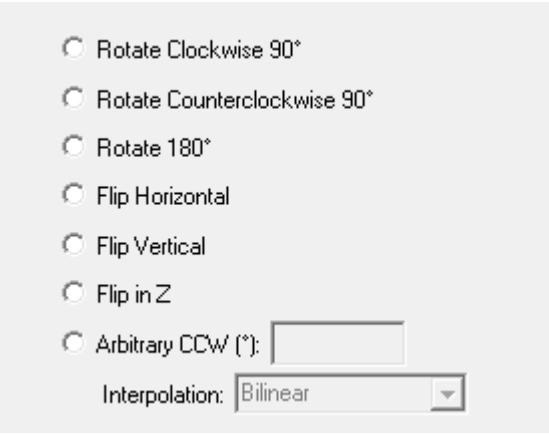
Current Image - Rotate only the currently selected image
Current Capture Type - Rotates all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Rotates all images captured using the same channels as the selected image in the current slide.

2 Selected Images list

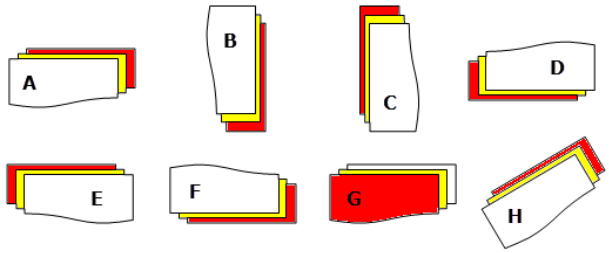


Check the box(es) for the image(s) you would like to rotate. This list will change depending on the ***Image Range*** selected above.

3 Rotation Options



Select rotation options as depicted below:



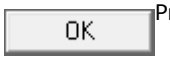
(A) None, (B) Rotate Clockwise 90°, (C) Rotate Counterclockwise 90°, (D) Rotate 180°, (E) Flip Horizontal, (F) Flip Vertical, (G) Flip in Z, (H) Arbitrary CCW*

* ***Arbitrary CCW*** allows a user-defined counterclockwise rotation degree to be manually entered in the edit field. Because the voxels will not be re-oriented squarely, interpolation is required for this process

Bilinear interpolation considers the closest 2x2 neighborhood of known pixel values surrounding the unknown pixel. It then takes a weighted average of these 4 pixels to arrive at its final interpolated value.

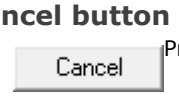
Bicubic goes one step beyond bilinear by considering the closest 4x4 neighborhood of known pixels for a total of 16 pixels. Since these are at various distances from the unknown pixel, closer pixels are given a higher weighting in the calculation. Bicubic produces noticeably sharper images at the cost of higher processing time.

4 OK button



Press ***OK*** to proceed with image rotation and close the dialog.

5 Cancel button

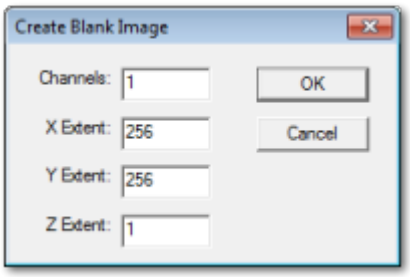


Press ***Cancel*** to discard changes and close the dialog.

Create Blank Image window

The ***Create Blank Image*** window allows you to generate a blank image in the slide. You can specify number of channels, XY dimensions, and number of Z planes. Blank images can be useful for manually assembling imported montages or computing theoretical models.

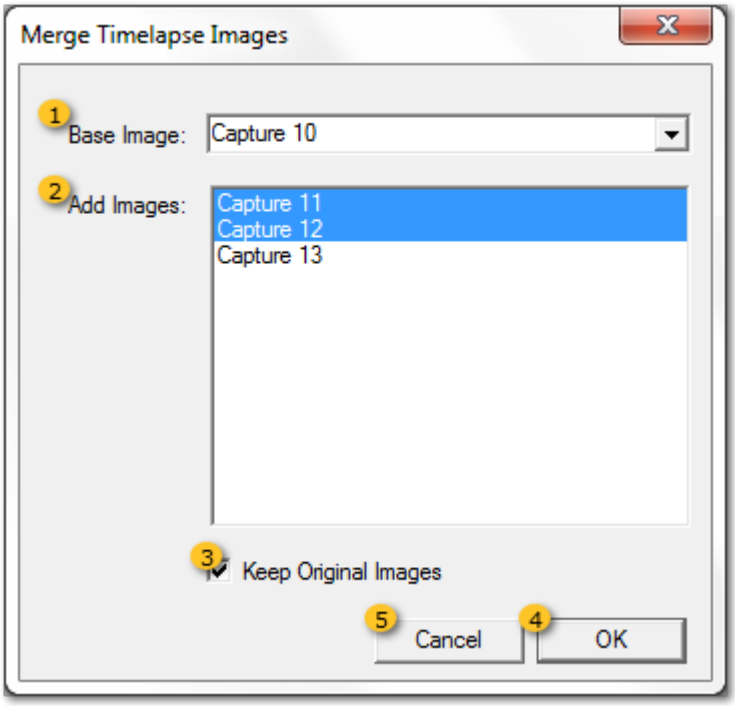
Access in the Analyze tab, Edit Images group, ***Reformat menu > Create Blank***



Merge Timelapse Images window

You may find it useful to add one time series to another time series. To do so, the images must be of type ***2D Timelapse*** o***r 4D Capture*** and contain the same channels and be of the same x/y/z dimensions.

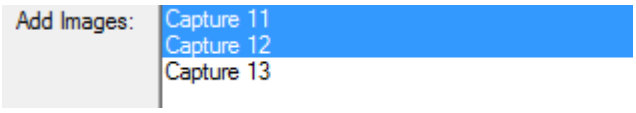
Access in the Analyze tab, Edit Images group, ***Reformat menu > Merge Timelapse Images***



1 Base Image

Select the image that will be the first image in the merged series.

2 Add Images List

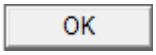


Select the image or images you would like to add. Hold **Ctrl** to select multiple images. Selected images will be highlighted in blue.

3 Keep Original Images check

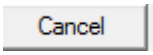
Check the box to save the original images as well as the new merged image.

4 OK button



Click ***OK*** to execute the operation and generate the merged image.

5 Cancel button

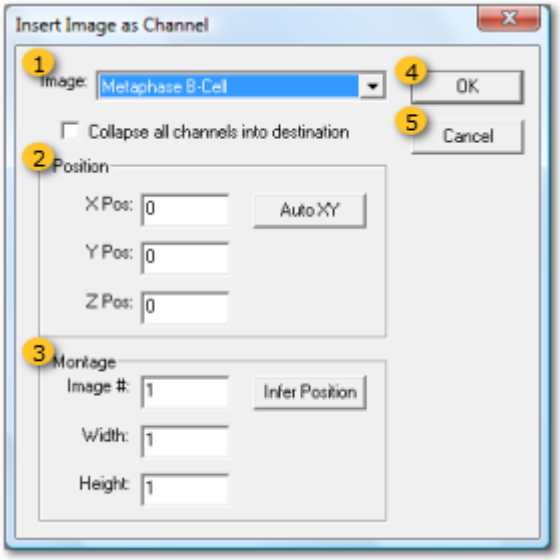


Click ***Cancel*** to discard changes and close the window.

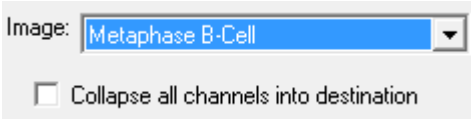
Insert Image as Channel window

You may find it useful to merge the channels from one capture into a different capture.

Access in the Analyze tab, Edit Images group, ***Reformat menu > Insert Channel***



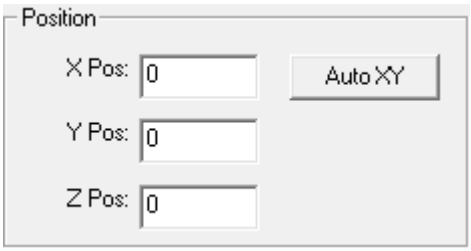
1 Image selection



The ***Image Name*** is the name of the image that will be inserted into the image selected on the slide. By default, the first channel of the image will be inserted.

If you check the box for ***Collapse all channels into destination***, all channels for the image from the drop down selection will be added to the selected image on the slide. The new channels will be merged with the present channels such that the same total number of channels will exist.

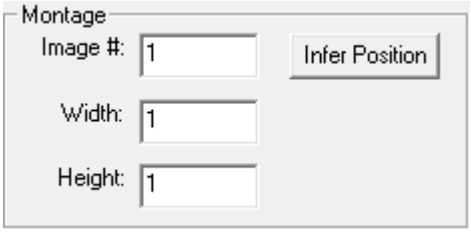
2 Position group



Enter in pixel values to manually offset the inserted channel(s) in ***X***, ***Y***, and/or ***Z***. Use ***Auto XY*** to allow SlideBook to automatically align the images based on common features/ camera pixel information.

Alternatively, you may find it useful to position the channels after inserting them by going to (Analyze tab, Process menu) ***Align > Align Channels*** (note this will not work when the box is checked to collapse all channels into destination).

3 Montage group

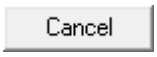


You may wish to create a montage of the current and selected images. To do so, enter a value of 2 for ***Image #***. If the image is already a montage, use a value of ***n+1***. Use ***Infer Position*** to automatically detect where in XY the inserted channel should go.

4 OK button

Press ***OK*** to insert image as directed and close the dialog.

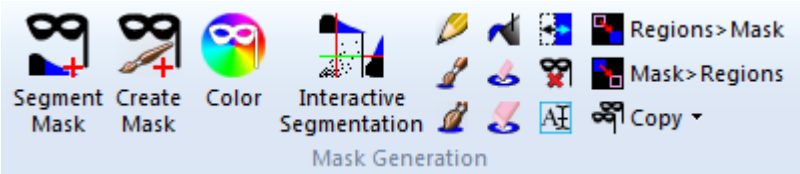
5 Cancel button



Press ***Cancel*** to discard changes and close the dialog.

Mask Generation Group

When performing analysis on images, it is useful to define the specific pixels you wish to analyze by masking. A *mask* is a 2D or 3D set of binary values that has the same extent as the image to which it is assigned. Once created, a mask is permanently stored with its corresponding image. Masks are used for performing advanced selection and analysis of image data. They can either be created and edited manually or generated automatically through threshold segmentation and other techniques such as converting region data. Once masks are generated, you can use the Edit Mask group to define, separate, and track objects, further refining the masks before obtaining Mask Statistics.



Segment Mask opens a dialog to generate a mask automatically by user-defined threshold values (also available from the mask menu in a view window).

Create Mask opens a dialog to generate an empty mask and enables the mask drawing tools (also available from the mask menu in a view window).

Color opens the ***Mask Color*** dialog that allows the user to define the display color for masks, selected or highlighted objects, and mask outline. Use this function to change the default color from royal blue to a color of your choosing.

Interactive Segmentation opens a dialog that allows you to create a mask by thresholding two channels simultaneously. This is a very flexible and powerful tool for advancing thresholding of masks and colocalization analysis.

Single pixel pencil mask drawing tool

Small paint brush mask drawing tool, 4 pixel wide round-shaped brush

Large paint brush mask drawing tool, 12 pixel wide round-shaped brush

Paint bucket mask fill drawing tool, fills areas drawn with the paint brushes. You can also select an area using the marquee tool and type **Ctrl + M** to fill the selected area with mask.

Small eraser mask tool, eliminates pixels that have been masked by drawing or thresholding techniques, 4 pixel wide round-shaped eraser

Large eraser mask tool, eliminates pixels that have been masked by drawing or thresholding techniques, 12 pixel wide round-shaped eraser

Mask outline toggle button alters the way the mask is displayed, either as a filled color or as an outline bounding the shape

Opens the ***Delete Mask*** dialog that allows you to delete one or more masks from the current image or all images in the slide

Opens the ***Rename Mask*** dialog to rename the currently selected mask

Converts all regions to mask areas

Converts masked areas to regions, it is important to note that regions persist through all *z* and time dimensions.

Copy this plane - Opens a dialog to copy the current mask in the current plane to other planes or time points in the image
Copy to all images in slide copies the current mask to all images in the slide with the same capture type and xy dimensions.

A single image can contain multiple masks. Any mask can be created and edited using the mask drawing tools. When you create (or draw) a mask, it will only be generated in the current plane/time point. To extend the mask to other z planes or time points, you will need to use Copy this plane.

Often you may have several distinct objects that will have intensities that are very similar, and thus will segment together. A mask that contains several distinct entities may be divided into *objects*. For instance, if an image contains a field of many cells, performing threshold segmentation of a nuclear stain such as Hoechst will result in a mask that contains distinct "islands." Defining objects permits functions such as counting of objects and generating statistics for each subregion. Defining Objects, Splitting Objects, and Particle Tracking and more (including Boolean operations on masks) are discussed in the Edit Mask group.

> **NOTE:** Mask drawing tools are also found under the tool menu when a mask is displayed or created.

See also:

TUTORIAL: Using Thresholding Techniques to Generate Masks
Configuring Background Subtraction with Mask Statistics
Exporting Statistics from Masks

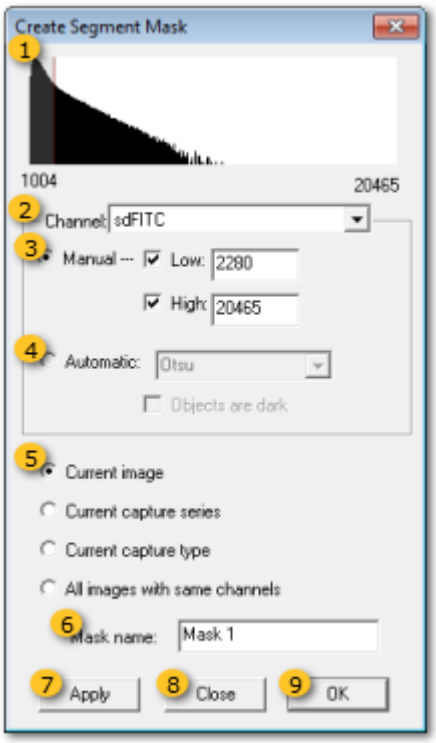
Create Segment Mask window

Thresholding, also known as segmentation, allows you to perform a global selection based on image intensities. SlideBook's segmentation process generates regions of interest that persist throughout the entire image in all dimensions.

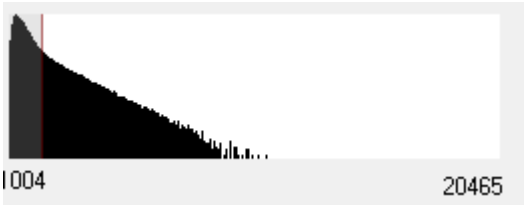
Access in the Process Image tab, Mask Generation Group, or in the image window under the ***Mask*** menu. Once a mask has been created by this technique, you can edit it using the drawing tools as described in Creating a New Mask or other advanced mask processing techniques in the Mask Edit group.

See also:

TUTORIAL: Using Thresholding Techniques to Generate Masks



1 Histogram area



The histogram is a graphical representation of intensity values of the pixels of an image. The x-axis represents intensity value in gray levels; the y-axis represents number of pixels. When you segment a mask, you are selecting the pixels that fall between low and high intensity values. It is important to note the ***Channel*** (2) that is selected as you only segment on one channel at a time.

The red line designates a ***Low*** value for display. You may click and drag on this line to adjust the low intensity value. Pixels with intensity values at or below the low level will not be selected as part of the mask. Alternatively, you may type a value in the ***Low*** edit field.

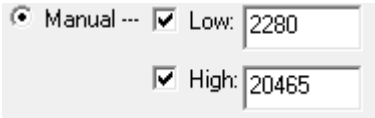
The green line designates a ***High*** value for display. You may click and drag on this line to adjust the high intensity value. Pixels with intensity values at or above the high level will not be selected as part of the mask. Alternatively, you may type a value in the ***High*** edit field.

2 Channel Selection

Select a ***Channel*** from the drop down menu. This channel will be represented in the histogram above.

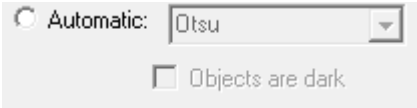
SlideBook will mask any pixel that has intensity values that lie between the red and green bars in all of the three channels. In other words, segmentation is performing an AND operation across the bounds defined in all channels.

3 Manual option



Instead of clicking and dragging the red and green lines in the histogram, you may select ***Manual*** and type a value in the ***Low*** and/or ***High*** fields.

4 Automatic option



For unbiased mask generation, the following algorithms are available:

Ridler-Calvard:

"Picture Thresholding Using an Iterative Selection Method"
T. W. Ridler and S. Calvard
IEEE Transactions on Systems, Man, and Cybernetics
Vol. SMC-8, No. 8, August 1978
pp. 630-632

Otsu:

"A Threshold Selection Method from Gray-Level Histograms"
Nobuyuki Otsu
IEEE Transactions on Systems, Man, and Cybernetics
Vol. SMC-9, No. 1, January 1979
pp. 62-66

5 Mask Span

Current image – Segments the current image only

Current capture series (on OK) – Segments the current capture series only. This applies mainly to point visiting experiments.

Current capture type (on OK) – Segments the current capture type only. For instance, if you have a 2D timelapse image open in a slide with at least one other timelapse image, as well as other capture types, only the 2D timelapse images would be segmented.

All images with same channels (on OK) – Segments images that have the exact same number and type of channels. For instance, if you wish to segment a FITC channel, and one image contains a FITC channel while another contains both DAPI and FITC, both images will NOT be segmented. Only the active image will be segmented.

6 Mask Name

Mask name: Mask 1

Type a name for the mask. You can use the default naming of **Mask 1**, **2**, etcetera or enter a relevant name. This name will be how you select the mask to show in View windows and in analysis. If necessary the mask can later be renamed by going to Mask Generation group, ***Rename***.

7 Apply button

Apply

Applies the mask to the current image. dialog stays open.

8 Close button

Close

Closes dialog, only saving changes once ***Apply*** has been pressed.

9 OK button

OK

Applies the mask parameters to all images designated in ***Mask Span*** and closes the dialog.

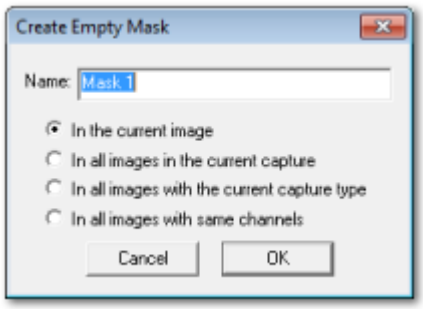
Create Empty Mask window

Masks can be manually drawn to visually select objects for analysis. When you do so, you may create the mask in only the current image or a range of images from the slide. It is important to note that an alternative to drawing masks and copying them to all planes/timepoints is to use regions tools that persist in all planes/timepoints and convert the regions to masks for analysis.

Access in the Process Image tab, Mask Generation Group, or in the image window under the ***Mask*** menu

See also:

[Mask Generation]
[Segment Image window]



Enter a ***Name*** for the mask in the edit field. If necessary the mask can later be renamed by going to the Mask Generation group, ***Rename***.

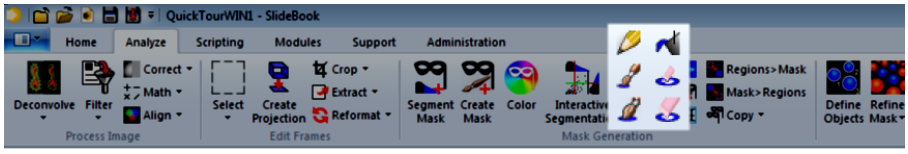
Select the range of images to which you'd like to apply the mask as follows:

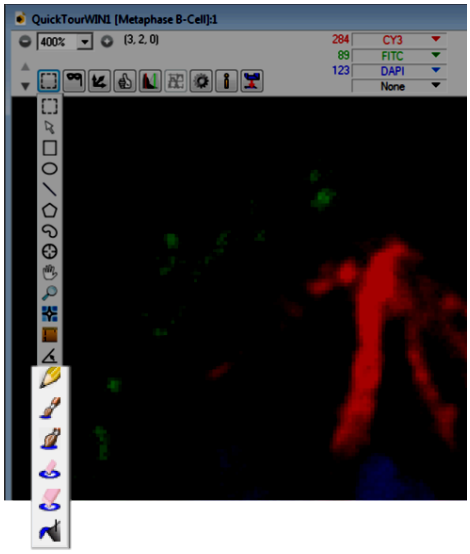
Current Image - Apply the selected operation to only the currently selected image
Current Capture Series -
Current Capture Type - Apply the selected operation to all images with same capture type in current slide (i.e. 2D Timelapse, 4D Capture, etc)
All images with Same Channels - Apply the selected operation to all images captured using the same channels as the selected image in the current slide.

Press ***Cancel*** to abandon the operation and close the window

Press ***OK*** to proceed and create a blank mask.

Once the blank mask is created, you may use the mask drawing tools found in Mask Generation group of the Analyze tab or the Tool menu of the view window to draw a mask as shown below.




QuickTourWIN1 [Metaphase B-Cell]:1
400%
(3, 2, 0)
284
CY3
89
FITC
123
DAPI
None

Interactive Segmentation window

Interactive Segmentation provides a single tool to create a mask based on segmentation of more than one channel, perform binary operations, remove small objects, and links directly to statistical analysis. It also adds novel operations, such as polygon selection in a two channel scatter plot.

You may select up to three different channels to contribute to the mask generation:

- Channel 1
- Channel 2
- Gating Channel

In an image with three channels that are contributing to mask selection, the optional (gating) channel defines a channel whose super-threshold pixels will be ANDed with the results of the pixels selected in the scatter plot. For instance, an image of a cell that has a nuclear marker and two proteins of interest would assign the nuclear marker as the gate channel.

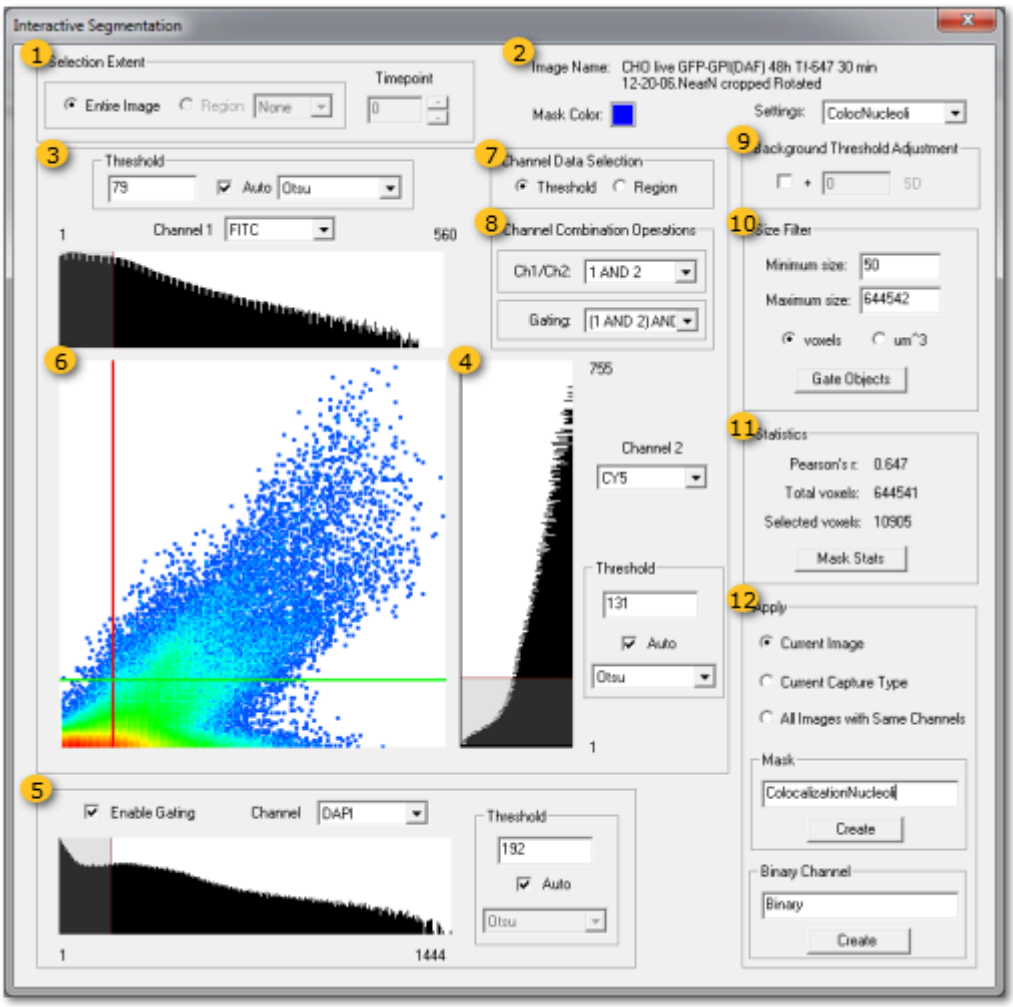
In an image with at least two channels, you may select two channels to participate in the scatter plot. From there, you would independently select thresholds using the per-channel histograms (and optionally perform a Boolean operation on the two), or select a threshold that is based on the interaction of the two channels by selecting a region in the scatter plot.

If there are only two channels in the image, there is no need for the gate channel and it is disabled.
If there is only one channel in the image, there is no value in having the scatter plot and it is disabled.

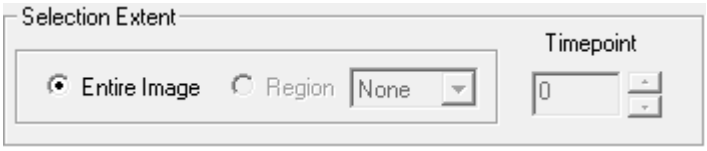
With a main view open, access in the Process tab, Mask Generation group ***Interactive Segmentation***

See also:

TUTORIAL: Interactive Mask Segmentation
Segment Image window
Colocalization window



1 Selection Extent group



You can define the extent of both which voxels are included in the histogram/scatter plot and the voxels that would be marked in a resulting mask or binary channel. If regions are present, the extent can be restricted to a specific ***Region*** or ***All Regions***. For time lapse images, the extent can be either the current time point or a specific time point.

2 Settings

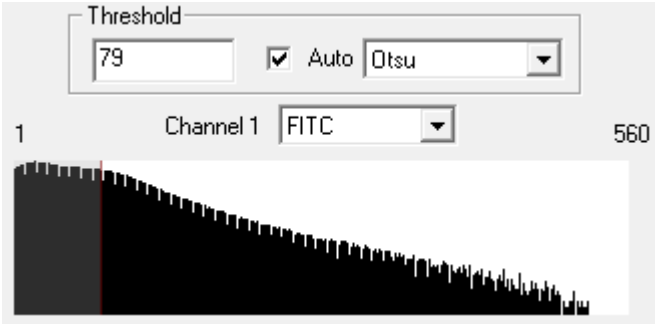
The ***Image Name*** of the current image will be displayed.

The color of the selection, as it appears in the associated view, can be user-selected by clicking on the ***Mask Color*** color block. This color will also dictate the default color of a corresponding mask in a surface plot (or any other views that support user-defined mask color).

Interactive Mask Segmentation settings can be saved for future use (or loaded from previously saved sessions) by using the ***Settings*** drop-down menu. A file with the extension **.int_mask_seg.prefs** will be saved per user in the **User**

Preferences directory (accessible by going to Administration tab, ***More Options*** menu ***> Open User Preferences Folder***).

3 Channel 1 Threshold group

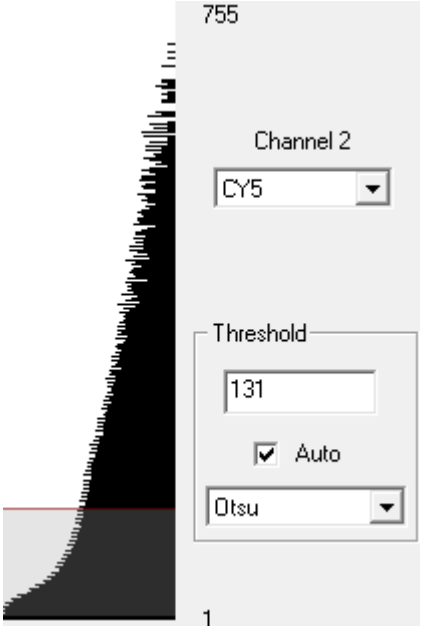


Two different channels may be thresholded to define a binary channel or mask (as well as one Gating Channel). Select a ***Channel*** from the drop down menu. For each channel, the user can set a minimum intensity threshold in one of three ways:

- Drag the red bar on the histogram to the desired position
- Type a value in the ***Threshold*** edit field
- Select ***Auto*** and choose either the ***Otsu*** or ***Ridler-Calvard*** algorithm

If a region has been drawn on the scatter plot, it is cleared when the user changes any of the individual histogram threshold settings, and it is replaced by a rectangular region that corresponds to the thresholds and ***Boolean Operation*** selection.

4 Channel 2 Threshold group

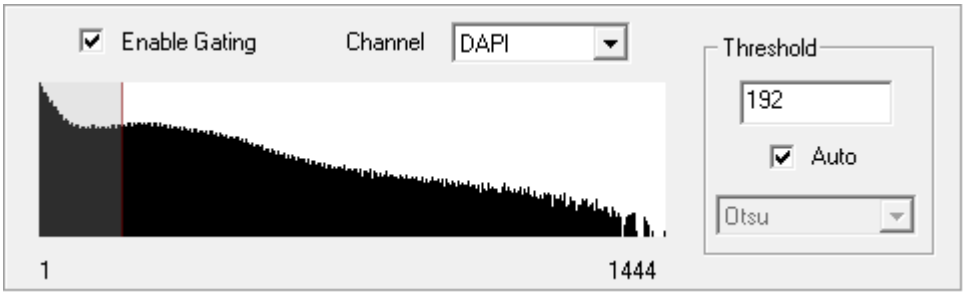


Two different channels may be thresholded to define a binary channel or mask (as well as one Gating Channel). Select a ***Channel*** from the drop down menu. For each channel, the user can set a minimum intensity threshold in one of three ways:

- Drag the red bar on the histogram to the desired position
- Type a value in the ***Threshold*** edit field
- Select ***Auto*** and choose either the ***Otsu*** or ***Ridler-Calvard*** algorithm

If a region has been drawn on the scatter plot, it is cleared when the user changes any of the individual histogram threshold settings, and it is replaced by a rectangular region that corresponds to the thresholds and ***Boolean Operation*** selection.

5 Gating Channel Threshold group



In an image with three channels that are contributing to mask selection, the ***Gating Channel*** defines a channel whose super-threshold pixels will be ANDed with the results of the pixels selected in the scatter plot. For instance, an image of a cell that has a nuclear marker and two proteins of interest would assign the nuclear marker as the gate channel.

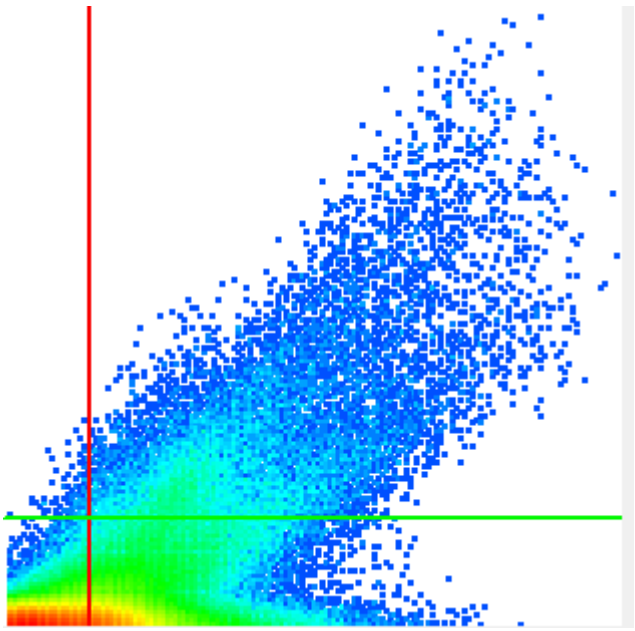
To use a gating channel, check the box for ***Enable Gating*** and select the ***Channel*** from the drop-down menu.

You may set a minimum intensity threshold in one of three ways:

- Drag the red bar on the histogram to the desired position
- Type a value in the ***Threshold*** edit field
- Select ***Auto*** and choose either the ***Otsu*** or ***Ridler-Calvard*** algorithm

If a region has been drawn on the scatter plot, it is cleared when the user changes any of the individual histogram threshold settings, and it is replaced by a rectangular region that corresponds to the thresholds and ***Boolean Operation*** selection.

6 Scatter Plot area



The scatter plot is a graphical representation of the pixels in the image. Each pixel is represented as a dot where the x-axis represents Channel 1 intensity and the y-axis represents Channel 2 intensity.

The red line represents the threshold value for Channel 1 and can be set by dragging the line in the scatter plot or channel histogram, or by manually typing in a value. You may also set the value for this line manually or automatically in the ***Threshold*** area of the ***Channel 1 Threshold Group***.

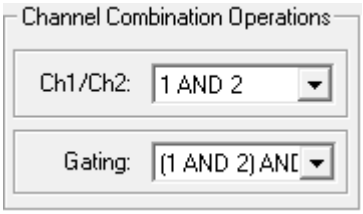
The green line represents the threshold value for Channel 2 and can be set by dragging the line in the scatter plot or channel histogram, or by manually typing in a value. You may also set the value for this line manually or automatically in the ***Threshold*** area of the ***Channel 2 Threshold Group***.

Alternatively, you can click and drag a rectangle on the scatter plot to select a population of pixels for analysis. To do so, select ***Region*** for the ***Channel Data Selection*** type then click and drag on the scatter plot area.

7 Channel Data Selection group

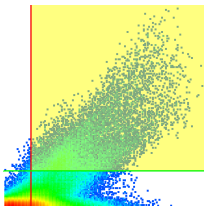
Channel Data Selection refers to the binary channel or mask you are creating, as represented on the scatter plot. ***Threshold*** refers to the area to the right of the red line and above the green line. ***Region*** allows you to click and drag a rectangle in the scatter plot to select a population of voxels.

8 Channel Combination Operations group

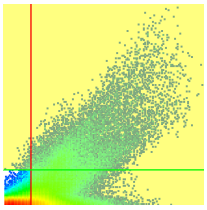


Channel Combination Operations are only relevant when ***Threshold*** is the type of ***Channel Data Selection*** used (see above).

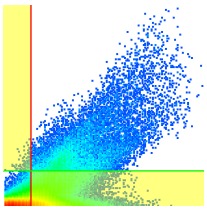
When at least two channels are present, and the user is adjusting individual channel histograms (as opposed to drawing a ***Region*** in the scatter plot), there will be five different ***Ch1/Ch2*** options available: **1 AND 2**, **1 OR 2**, **1 XOR 2**, **1 AND NOT 2**, and **2 AND NOT 1**. The default will be **1 AND 2**. Portions of the scatter plot that are excluded by the Boolean operation will be not be used as part of the binary channel or mask. Boolean operations are defined as follows:



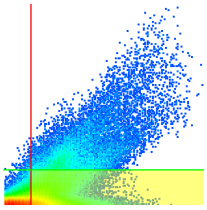
1 AND 2 selects pixels that are above the threshold values of both channels



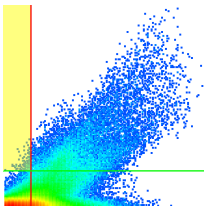
1 OR 2 selects pixels that are above at least one of the threshold values



1 XOR 2 selects pixels that are above one of the threshold values but not above both threshold values



1 AND NOT 2 selects pixels that are above the threshold value of channel 1 but below the threshold value of channel 2



2 AND NOT 1 selects pixels that are above the threshold value of channel 2 but below the threshold value of channel 1

The ***Gating*** options are the same as for individual channels in the scatter plot. The result of the ***Ch1/Ch2*** selection can be combined using Boolean operations with the selected ***Gating Channel***, **G** as follows:

(Ch1/Ch2 Selection) AND G – selects pixels that are above the threshold value of the gating channel and selected by the the Ch1/Ch2 selection

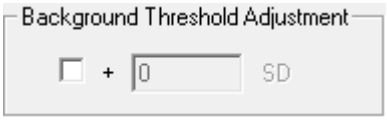
(Ch1/Ch2 Selection) OR G - selects pixels that are either above the threshold value of the gating channel or selected by the Ch1/Ch2 selection

(Ch1/Ch2 Selection) XOR G – selects pixels that are either above the threshold value of the gating channel or selected by the Ch1/Ch2 selection but not both

(Ch1/Ch2 Selection) AND NOT G – selects pixels that are above the threshold value of the gating channel but not selected by the Ch1/Ch2 selection

(Ch1/Ch2 Selection) AND NOT G - selects pixels that are selected by the Ch1/Ch2 selection but below the threshold value of the gating channel

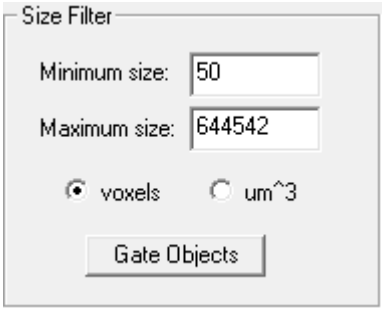
9 Background Threshold Adjustment group



You will have an option of placing an additional constraint on what constitutes a background voxel. If selected, any voxel for which no channel is above the mean intensity for that channel plus a given number of standard deviations will be still considered background.

NOTE: This modified definition of background will only govern which voxels are selected and will not affect any statistics of the selected voxels or the subtraction of a ***Background Region*** if defined.

10 Size Filter group



Size Filter allows you to place a ***Minimum*** and ***Maximum*** object size on the selection display, either in voxels or cubic microns. This will also affect the generated mask or binary channel.

11 Statistics group

A few basic statistics will be displayed directly in the dialog:

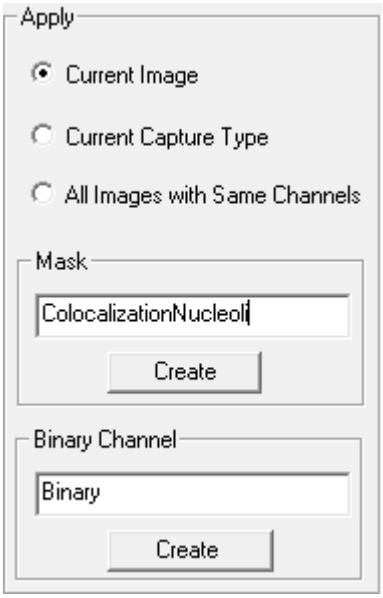
Pearsons r: Pearson's correlation between channel 1 and channel 2 among the selected voxels (when channel 1 and channel 2 are defined)

Total Voxels: Total number of voxels represented by the current image (or region(s) if ***Region*** is selected under ***Selection Extent***)

Selected Voxels: Number of selected voxels that pass either the threshold tests or fall within the scatterplot region

Mask Stats: Opens up the Mask Statistics dialog box

12 Apply group



Once all parameters of ***Interactive Segmentation*** have been configured, you have the option of applying the technique to other images in the slide with the same ***Capture Type*** or ***Same Channels***. You can save the selected pixels as either a ***Mask*** or a ***Binary Channel*** by naming the relevant selection and clicking ***Create***. If you create a mask, you can later rename it by going to ***Mask > Rename***. By creating a binary channel, you will have the ability to do Volume Rendering of the mask; otherwise, you may only render masks in the Surface View.

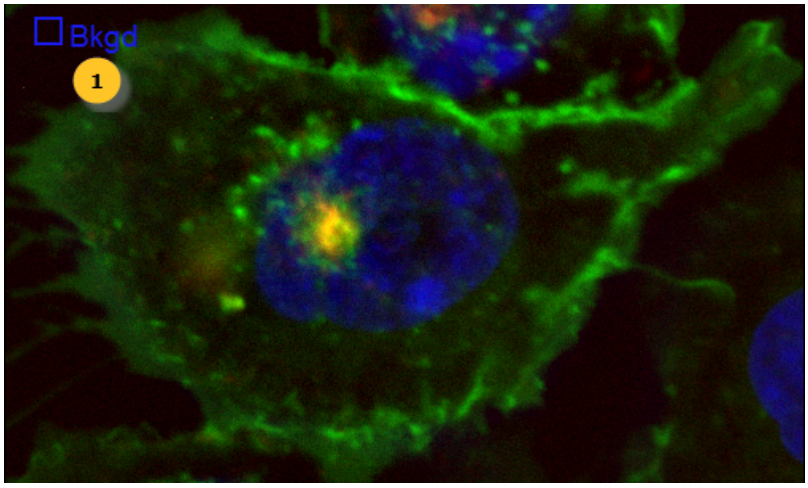
NOTE: You can save all parameters for future use in the Settings area of this window.

TUTORIAL: Interactive Segmentation

Interactive Segmentation provides a single tool to create a mask based on segmentation of more than one channel, perform binary operations, remove small objects, and links directly to statistical analysis. It also adds novel operations, such as polygon selection in a two channel scatter plot. In the following tutorial, we will examine this dialog as it may be utilized to perform 3D colocalization analysis of the following image. For additional information on this dialog, please review the user interface page Interactive Segmentation window.

In this example, our user is interested in quantifying the amount of colocalization of red and green signal within the nucleoli of the cell. We will be using thresholding techniques to segment on the red and green channels and gate them by the blue channel.

1. Open the image in SlideBook. It is recommended that you first set a background region for subtraction. To do so, draw a region in a background area using the Region Tools. Right-click and select ***Set as Background***. You may need to enable background subtraction as discussed in Configuring Background Subtraction with Mask Statistics.

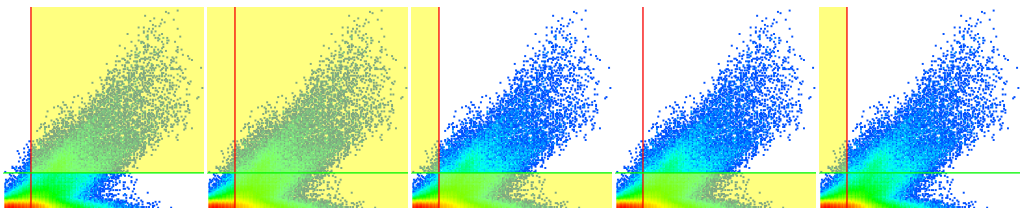


2. Go to Process tab, Mask Generation group, ***Interactive Segmentation***. The ***Interactive Segmentation*** dialog will open.

3. Select the two channels you would like to analyze and the channel you would like to use for gating. In this example, we will set ***Channel 1*** to **FITC**, ***Channel 2*** to **CY5**, and check the box to ***Enable Gating***. The gating channel should be set to **DAPI**.

4. For unbiased thresholding, select ***Auto*** then select the ***Otsu*** method for all channels.

5. Next, you need to define how you would like to utilize the threshold values of each of the channels in the ***Channel Combination Operations Group*** as follows:

> ***Ch1/Ch2*** - When at least two channels are present, and the user is adjusting individual channel histograms (as opposed to drawing a ***Region*** in the scatter plot), there will be five different ***Ch1/Ch2*** options available. Boolean operations are defined as follows:
>
> **1 AND 2** **1 OR 2** **1 XOR 2** **1 AND NOT 2** **2 AND NOT 1**
>
> 
>
> Since the goal is to find colocalized green and red pixels (pixels above the threshold value in both Channel 1 and Channel 2), we will use **1 AND 2** for ***Ch1/Ch2***.

The ***Gating*** options are the same as for individual channels in the scatter plot. The result of the ***Ch1/Ch2*** selection can be combined using Boolean operations with the selected ***Gating Channel***, **G** as follows:

(Ch1/Ch2 Selection) AND G – selects pixels that are above the threshold value of the gating channel and selected by the the Ch1/Ch2 selection

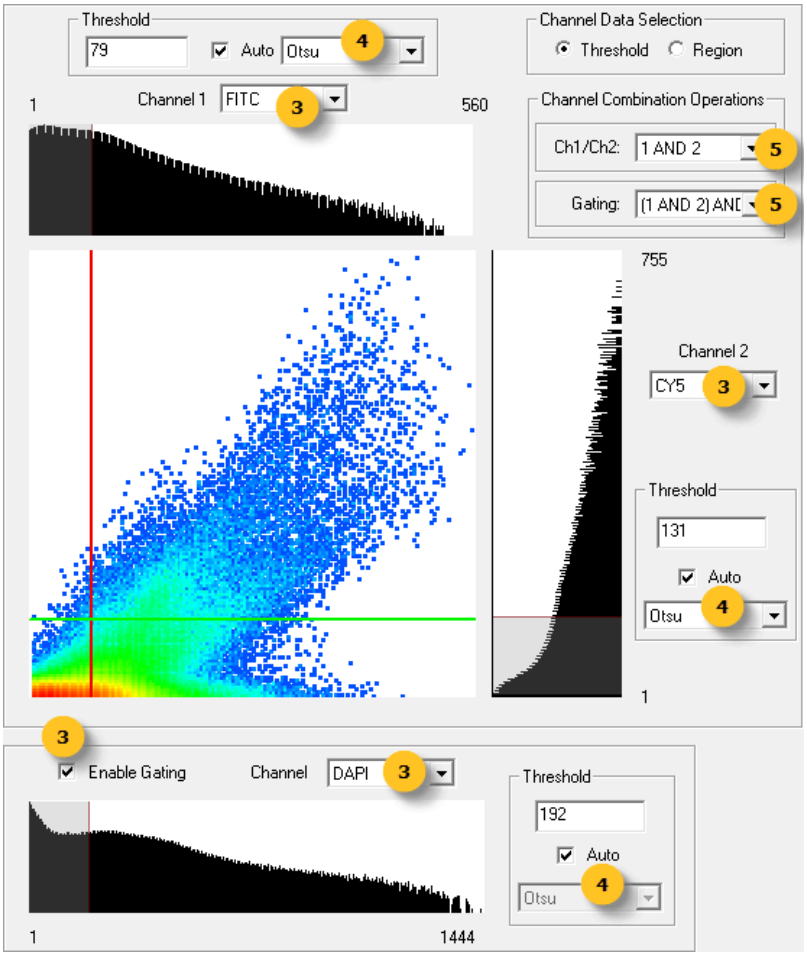
(Ch1/Ch2 Selection) OR G - selects pixels that are either above the threshold value of the gating channel or selected by the Ch1/Ch2 selection

(Ch1/Ch2 Selection) XOR G – selects pixels that are either above the threshold value of the gating channel or selected by the Ch1/Ch2 selection but not both

G AND NOT (Ch1/Ch2 Selection) – selects pixels that are above the threshold value of the gating channel but not selected by the Ch1/Ch2 selection

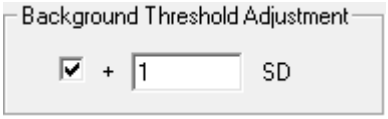
(Ch1/Ch2 Selection) AND NOT G - selects pixels that are selected by the Ch1/Ch2 selection but below the threshold value of the gating channel

We will be gating by the blue channel in a way to select for nucleoli - areas that do not contain the DAPI stain but are over the threshold values for both Channel 1 and Channel 2. The ***Gating*** selection we will make is **2 AND NOT 1**, pixels that are defined by the ***Ch1/Ch2*** definition and are not over the threshold value defined in the gating channel.

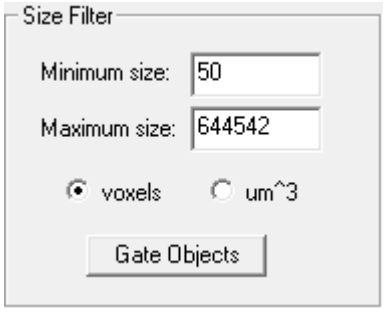


6. When a background region is defined in the image, the mean intensity in that region for each channel is subtracted from each voxel before they are plotted on the scatterplot. You have the option of increasing this value by x number of standard deviations of the mean intensity value calculation. To do so, check the box to enable the adjustment then enter a value for the number of standard deviation values you would like to add to the mean intensity. For example, if the mean background intensity for a channel was 115 and the standard deviation was 15, you could use a value of **1** in this field and subtract 130 or [115 + (1*15)] from each voxel instead of the mean value 115.

NOTE: This modified definition of background will only govern which voxels are selected and will not affect any statistics of the selected voxels or the subtraction of a ***Background Region*** if defined.



7. ***Size Filter*** allows you to place a ***Minimum*** and ***Maximum*** object size on the selection display, either in voxels or cubic microns. This will also affect the generated mask or binary channel.



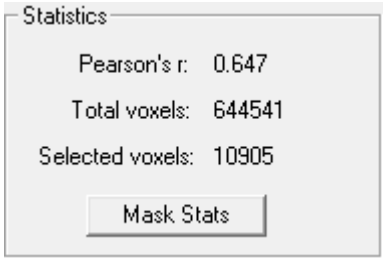
8. A few basic statistics will be displayed directly in the dialog:

Pearsons r: Pearson's correlation between channel 1 and channel 2 among the selected voxels (when channel 1 and channel 2 are defined)

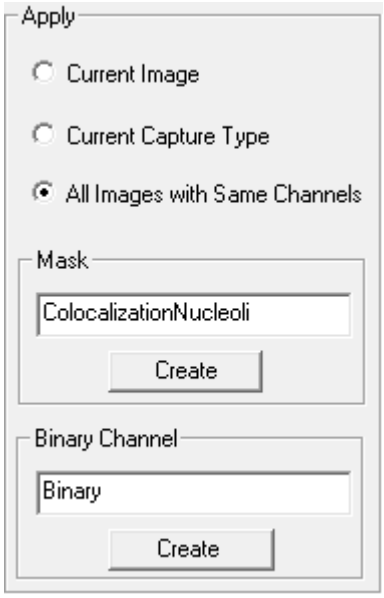
Total Voxels: Total number of voxels represented by the current image (or region(s) if ***Region*** is selected under ***Selection Extent***)

Selected Voxels: Number of selected voxels that pass either the threshold tests or fall within the scatterplot region

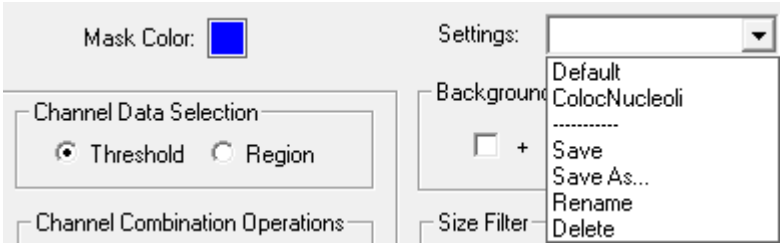
Mask Stats: Opens up the Mask Statistics dialog box



9. Once all parameters of ***Interactive Segmentation*** have been configured, you have the option of applying the technique to other images in the slide with the same ***Capture Type*** or ***Same Channels***. You can save the selected pixels as either a ***Mask*** or a ***Binary Channel*** by naming the relevant selection and clicking ***Create***. If you create a mask, you can later rename it by going to ***Mask > Rename***. By creating a binary channel, you will have the ability to do Volume Rendering of the mask; otherwise, you may only render masks in the Surface View. Both masks andbinary channels will be saved with the image when you resave the **.sld.**

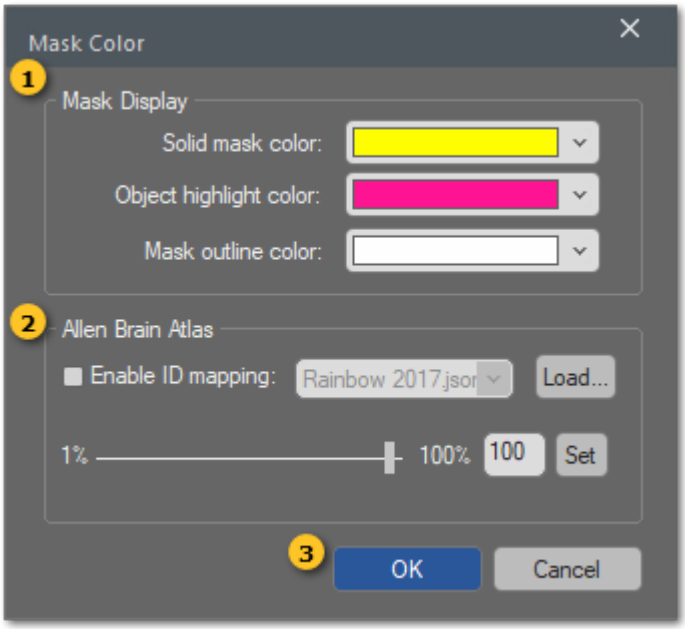


10. If desired, you can adjust the color of the displayed mask and save the Interactive Masking parameters for future use by using the ***Settings*** menu.

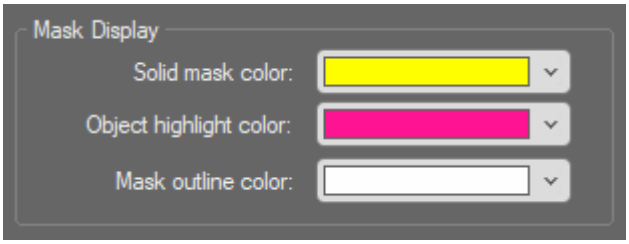


Mask Color window

The Mask Color dialog allows you to assign colors used to show masks, highlighted objects, and mask outlines. It is accessed from the Analyze tab > Mask Generation group.

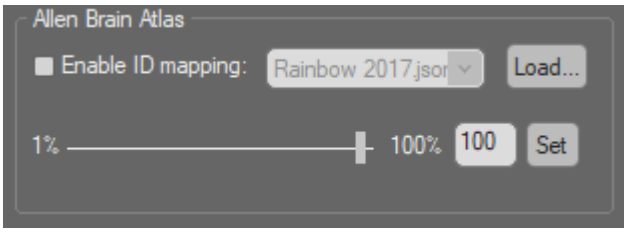


1 General Mask Display



Allows to select the mask colors for best contrast

2 Allan Brain Atlas specific Masks



Allows to load, enable and adjust a mask used in the Allan Brain Atlas tool through the Synergy module.

3 OK/ Cancel

OK saves the settings and closes the window. ***Cancel*** closes the window without saving changes.

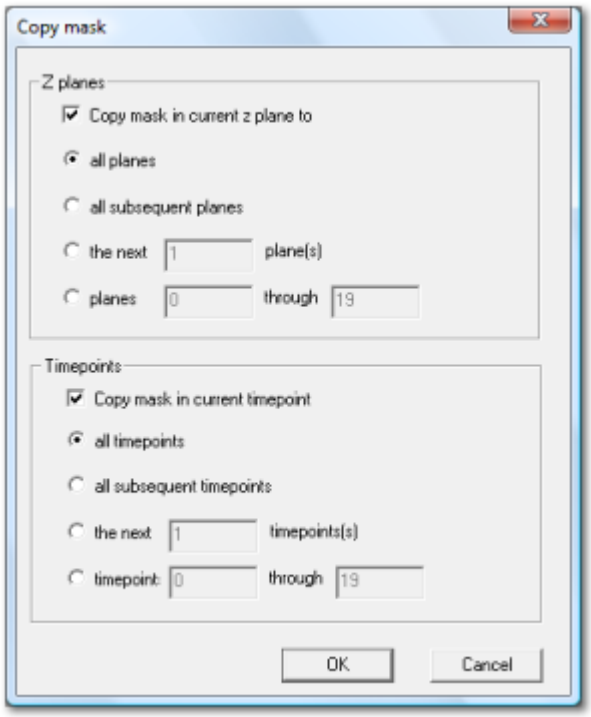
Copy mask window

When drawing a mask manually (via ***Create*** dialog), the mask is only drawn in the current plane. You may wish to copy the drawn mask to other z planes or time points. To do so, you will use the ***Copy mask*** window. It is important to note that an alternative to drawing masks and copying them to all planes/timepoints is to use regions tools that persist in all planes/timepoints and ***Convert Regions to Masks*** for analysis.

Access in Process tab, Mask Generation Group, ***Copy Mask*** menu **>** ***Copy this Plane***

See also:

[Create New Mask window](#)
[Segment Image window](#)



To copy the mask to other ***Z Planes***, check the box for ***Copy mask in current z plane to...***
To copy the mask to other ***Timepoints***, check the box for ***Copy mask in current timepoint to...***

Select the range as follows:

All planes/timepoints - copies the mask to all z planes or timepoints in the image
All subsequent planes/timepoints - copies the mask to all z planes or time points after the one in which the mask was created
The next __ plane(s)/ timepoint(s) - type a value in the edit field, the mask will be copied to that number of planes or timepoints after the one in which the mask was created
Planes/Timepoints __ through __ - type values for start and end z planes or timepoint to create a range to copy the mask to

Press ***OK*** to apply the copying action for the mask and close the window.

Press ***Cancel*** to discard changes and close the window.

Mark or Unmark Selection

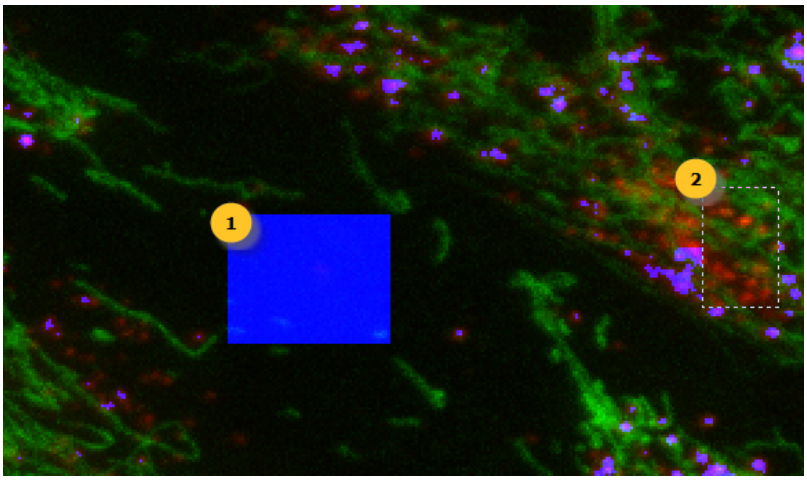
Any time a mask is being displayed (whether generated via Create or Segment), you may draw rectangular regions using the ***Marquee tool*** to either add or subtract from the mask.

Simply select the ***Marquee tool*** and click and drag to draw an area on the image.

In the view window, go to ***Mask > Mark*** or type **Ctrl + M** to fill the area with mask (shown as "1" below).

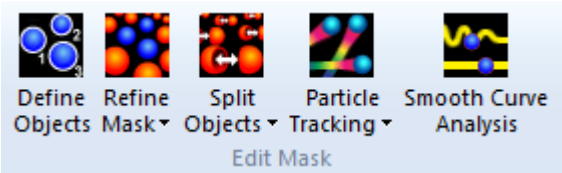
In the view window, go to ***Mask > Unmark*** or type **Ctrl + U** to remove any masking from the area (shown as "2" below).

> **NOTE:** These actions will only apply to the current time point or *z* plane unless subsequent steps are take to copy the mask (see Copy mask window).



Mask Edit Group

Once a mask has been created, it can be further prepared for statistical analysis using the tools available in the Edit Mask group.



Define Objects

The Define Objects dialog allows you to number objects within a mask and exclude objects smaller or larger than a specific size.

Refine Mask

Remove Edge Objects - exclude masked objects that aren't completely within the image bounds
Mask Operations - combine or edit masks using Boolean operations
Select Objects (Bivariate Plot) - plot statistic A vs Statistic B and use the graph to select objects based on defined criteria
Fit Data - used to compute cross-talk coefficients or bleedthrough between channels
Erode - applies a kernel to the entire mask that removes a user-defined number of voxels from the perimeter of the mask. This function can be applied to in only the x, y dimension (2D) or in the z dimension (3D) as well.
Dilate - applies a dilation kernel to the entire mask and thickens features. The user defines a radius of the number of pixels the current mask will be expanded by and whether the mask should be dilated in 2D or 3D.
Skeletonize - reduces the mask to a set of connected junctions. This function can only be applied to single plane, 2D images and is only available when the CellNet module has been purchased.

Split Objects

Split Objects Along Lines - Use the line region tool to draw dividing lines on objects (hold **Ctrl** key to make a multi-segment line), select ***Split Objects Along Lines***. Be sure to update object definitions to confirm object splitting.
Undo Split Objects Along Lines - Reverses the split objects along lines operation.
Split Objects Automatically - Performs a basic watershed operation on the mask.
Split Objects Protocol - Allows you to configure and apply and advanced object splitting protocol.

Particle Tracking

Once you have defined objects via masking (in either 2D or 4D data), you may wish to track them over time by ensuring the same object ID carries between timepoints. SlideBook offers two methods for particle tracking, automated and manual. For visualization of paths, you may wish to use ***Create Time Composite Channel*** (found in the View group of the Home tab).

To perform particle tracking, you may either use the Particle Tracking Protocol or individual dialog boxes. The Particle Tracking Protocol has the advantage of being able to save and reuse your particle tracking parameters, and apply them in a batch fashion. When this is not necessary, you may prefer to use Basic Particle Tracking.

For objects that are particularly difficult to track automatically, SlideBook includes Manual Particle Tracking. Using this approach, you will click through a data set, identifying each object through time.

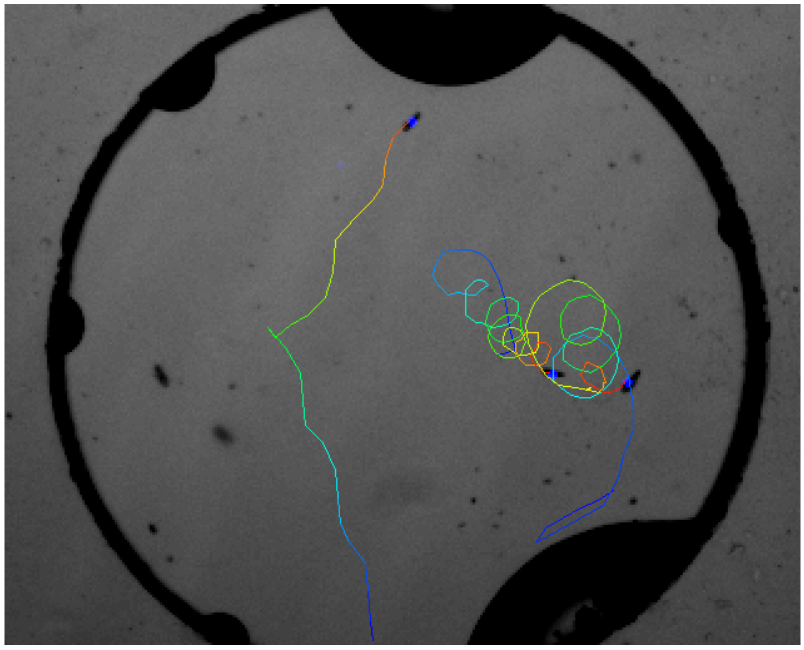
After objects are tracked in software, you can display paths by selecting ***Display Paths*** from the Particle Tracking menu. The paths for each object will be colored from blue to red where blue indicates the first time points and red indicates the end time points as shown in the example image below.

After tracking, you may retrieve information on the individual paths from the Path Statistics window.

Advanced Particle Tracking
Export Paths to MATLAB
Subresolution Particle Tracking

Smooth Curve Analysis

Smooth Curve Analysis allows you to follow time-dependent lateral intensity changes along a static object by straightening it out and calculating velocity and other statistics.



Displayed paths following Particle Tracking

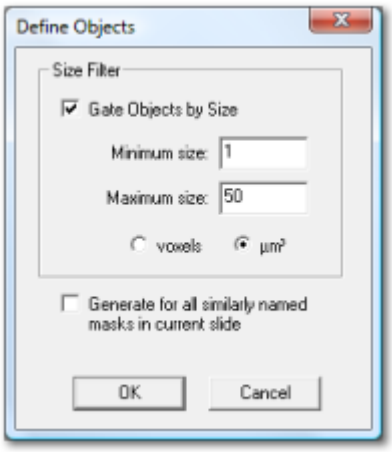
Define Objects Window

When generating statistics based on masks, you may find it useful to exclude objects smaller or larger than a specific size. For example, excluding objects smaller than 10 voxels is a good way to eliminate artifacts from sample preparation. The ***Define Objects*** window is the only place to eliminate objects larger than a certain size but several analysis dialogs (Mask Statistics, Particle Tracking, and Split Objects Protocol) have the option to exclude objects smaller than a specified size.

Access in the Process tab, Edit Mask group, ***Define Objects*** or ***Update Objects***

See also:

Annotation Settings Object IDs tab
Segment Image window
Ratio Module - TUTORIAL: Using Thresholding Techniques to Generate Masks



If you do not want to exclude any objects and simply want to define/count the objects in a mask, simply click ***OK***. A progress dialog will open then a window will display the total number of objects. You can then view the object numbers by going to ***Annotations*** in the Home tab.

Should you choose to exclude some objects based on size, check the box to ***Gate Objects by Size***. Enter values for ***Minimum size*** and ***Maximum size*** as desired. Be sure to select the appropriate unit of ***voxels*** or ***µm^3***.

To define objects for all masks with the same name in the slide, check the box to ***Generate for all similarly named masks in the current slide***.

Click ***OK*** to define objects and close the dialog. A progress dialog will open then a window will display the total number of objects. You can then view the object numbers by going to in the Home tab. Should you wish to exclude more objects from the definitions, this dialog will now be called ***Update Object Definitions***.

Click ***Cancel*** to abandon definition of objects.

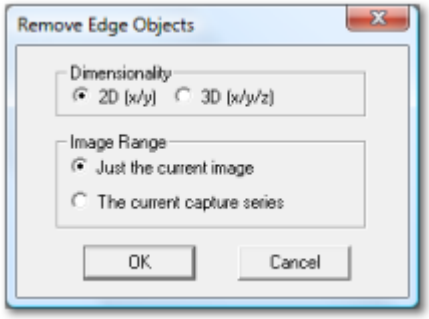
Remove Edge Objects window

When analyzing an image using segmented masks, often times objects will be located on the edge of the image. Depending on the type of analysis, you may wish to exclude objects that are not located entirely within the image. To do so, you may ***Remove Edge Objects*** to eliminate these objects from the mask.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Remove Edge Objects***

See also:

Segment Image window
Define Objects window



Select ***Dimensionality*** as follows:

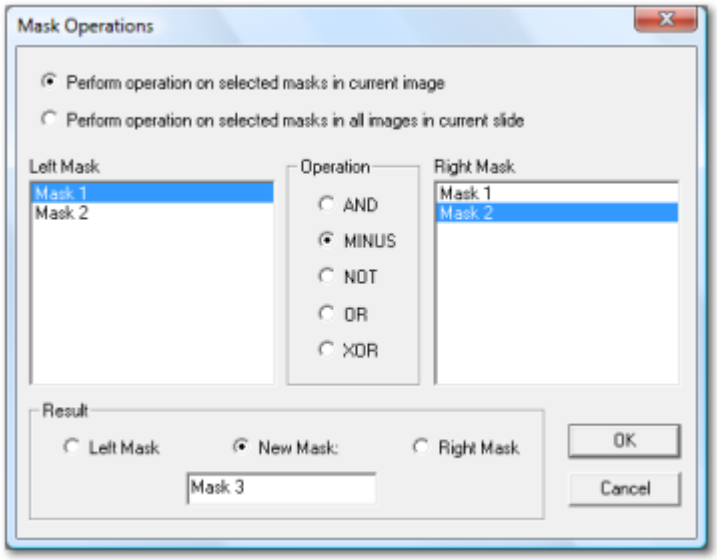
2D (x/y) will only look for and eliminate objects that are located on the x or y borders (not the top and bottom of z stacks).

3D (x/y/z) will eliminate objects occurring on any of the cube of data borders - x, y, or z.

Mask Operations window

Masks can be combined or altered using Boolean operations. For instance, the intersection of two masks can be generated by an AND operation. The ***Mask Operations*** dialog lets you either generate a new mask that contains the result of a Boolean operation or replace an existing mask with the result of an operation.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Mask Operations***



Use the radio buttons to select whether the operation will take place for the current image or all images on the slide.

Select the masks to be used from the ***Left Mask*** and ***Right Mask*** lists.

Select the operation to be performed using the radio buttons. Click here to visualize the operations.

- ***AND*** – selects pixels that are in both masks
- ***MINUS*** – removes any pixels that are present in the right mask from the left mask
- ***NOT*** – selects all pixels that are not marked in the left mask, operation only applies to one mask
- ***OR*** – selects pixels that are in either of the two selected masks
- ***XOR*** – adds both masks and removes any common pixels

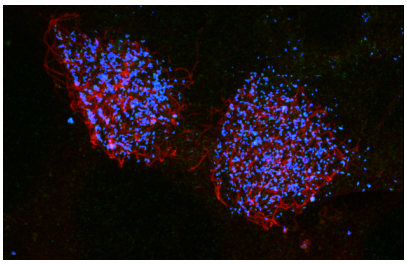
Select the destination for the mask operation using the radio buttons. If you want the ***Result*** to replace the contents of either the left or right mask, select the corresponding radio button for that mask. If you want to generate a new mask, select the ***New Mask*** radio button and give the new mask a name in the edit field.

Click ***OK*** to perform the Boolean operation or ***Cancel*** to discard changes and close the dialog.

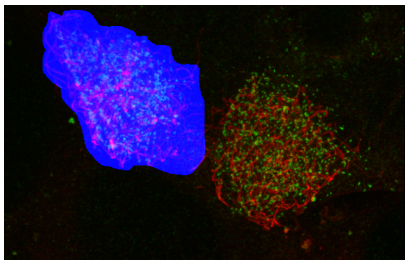
Mask Operations Examples

Below, you will find a guide that illustrates various ***Mask Operations***. We begin with a data set and use segmentation (thresholding) on the green channel generate **Mask 1**. We then manually create a **Mask 2** that selects for one of the cells in the field of view.

Mask 1

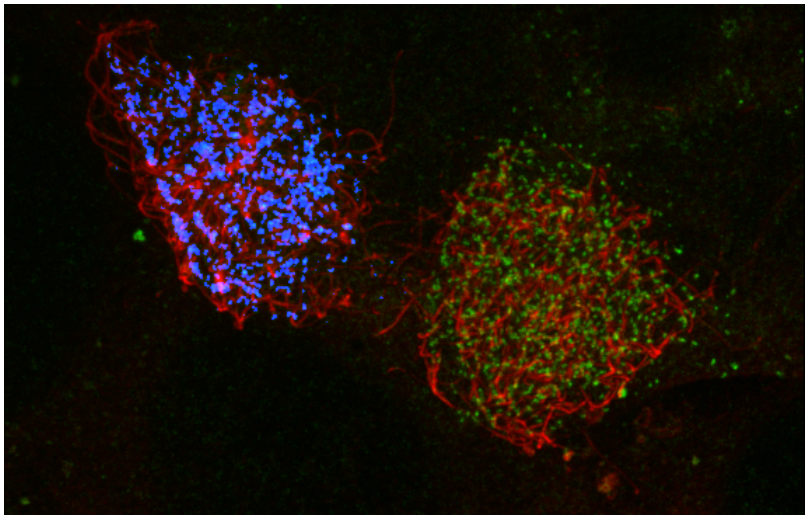


Mask 2

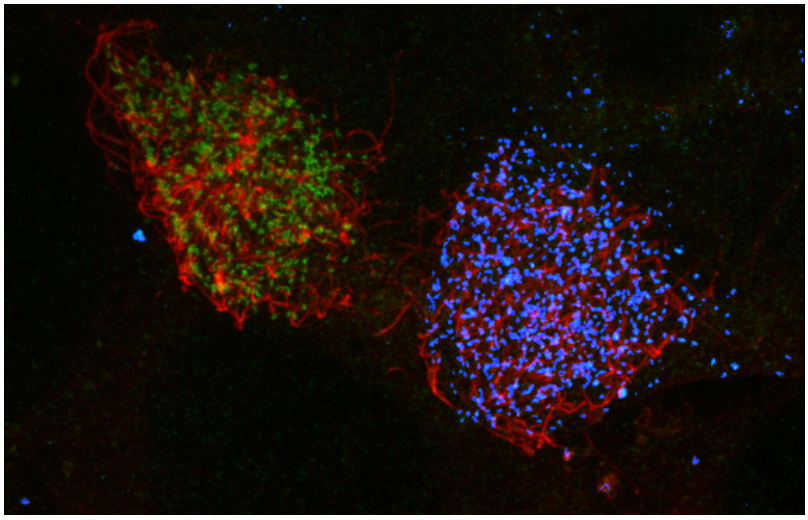


We perform various ***Mask Operations*** and display the results below.

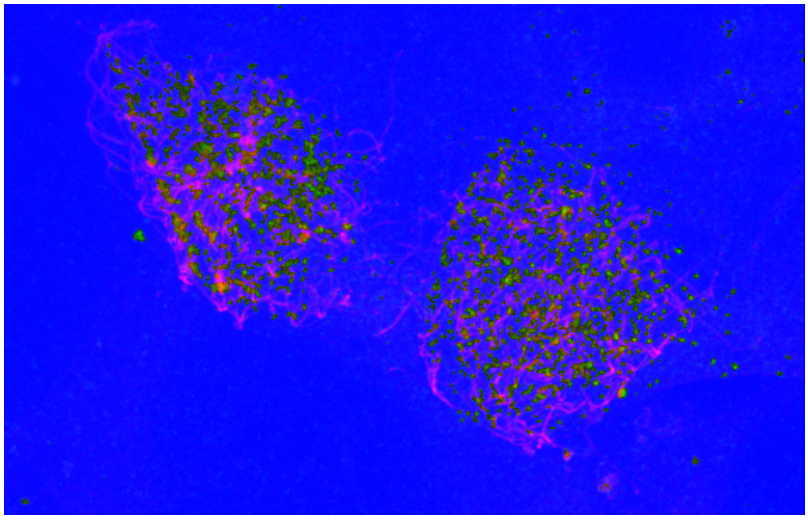
Mask 1 ***AND*** **Mask 2** – selects pixels that are in both masks



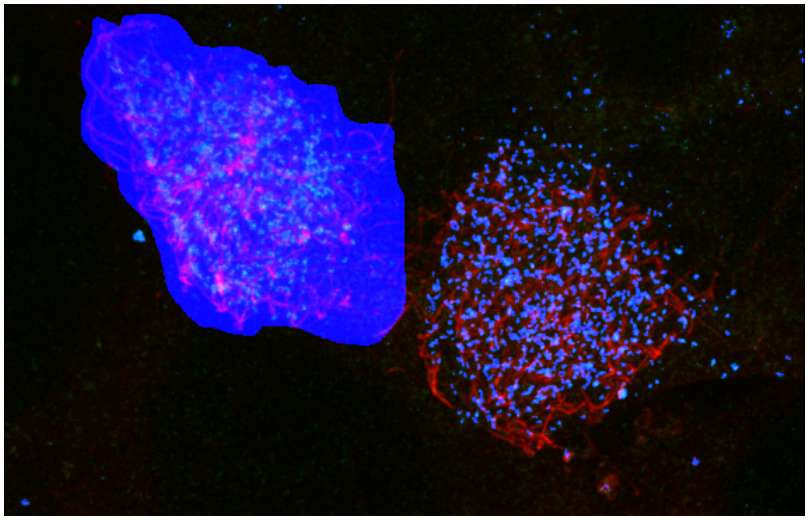
Mask 1 ***MINUS*** **Mask 2** – removes any pixels that are present in the right mask from the left mask



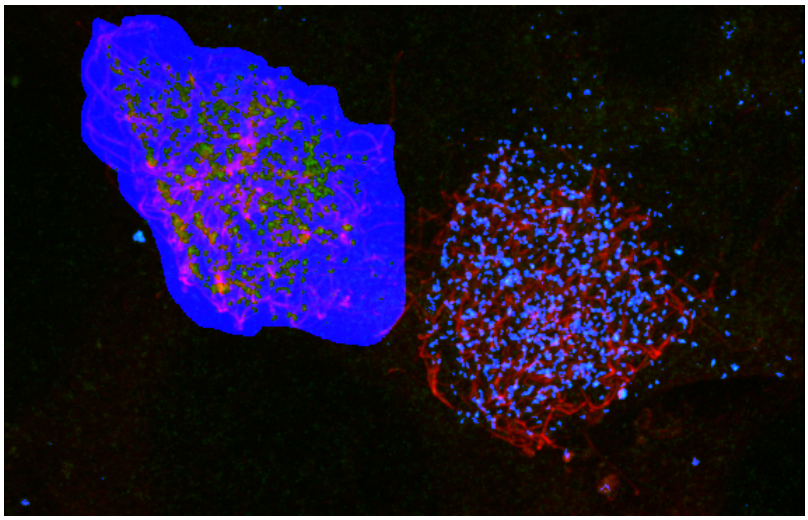
Mask 1 ***NOT*** – selects all pixels that are not marked in the left mask, operation only applies to one mask



Mask 1 ***OR*** **Mask 2** – selects pixels that are in either of the two selected masks



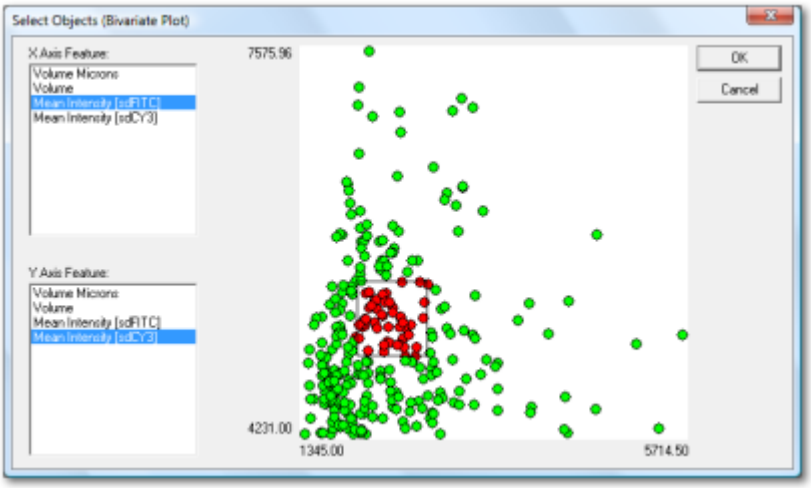
Mask 1 ***XOR*** **Mask 2** – adds both masks and removes any common pixels



Select Objects (Bivariate Plot) window

The ***Bivariate Plot*** allows you to plot statistics against each other and select objects meeting a desired criteria. To use this window, you must first generate a mask and calculate Mask Statistics.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Select Objects (Bivariate Plot)***



Select statistics to plot on the ***X Axis*** and ***Y Axis***. These statistics must have been previously calculated using the Mask Statistics window.

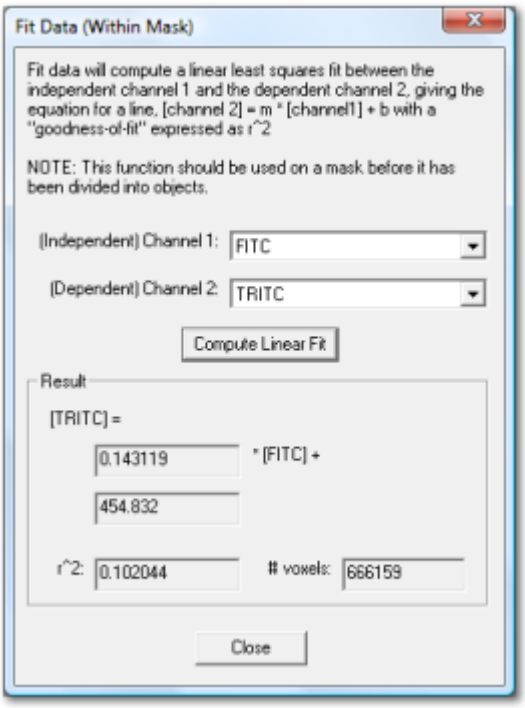
Green dots will populate the graph. Each dot represents an object in the image. You may find it useful to display object IDs (**Ctrl** + **Shift** + **O**).

Click and drag to draw a rectangle on the plot area. The selected objects will turn to red dots on the graph and the masks covering these objects in the image will be a pink color (instead of the standard blue mask color).

Fit Data window

You may find it useful to compute a cross-talk coefficient if you suspect bleedthrough due to filter inefficiencies or incompatibility between filter sets and fluoroprobes. To do so, you will need to prepare single channel control samples and then image in multiple channels. After the linear fit is computed, you may use Channel Math to create corrected channels for analysis.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Fit Data***



Select the ***Independent*** channel and ***Dependent*** channel from the dropdown menus. The independent channel is the control. The dependent channel is the one you are checking for cross-talk. Press ***Compute Linear Fit***.

The linear least squares fit will be displayed in the Results section, giving the equation for a line:

$$Dependent\ channel = m * Independant\ channel + b$$

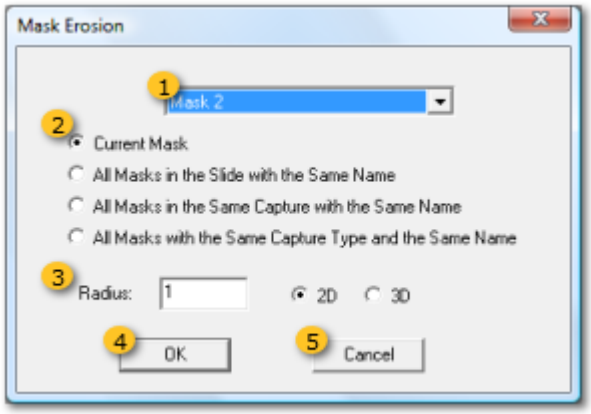
with a "goodness-of-fit" expressed as r^2 and the # of voxels used for analysis displayed.

Manually record the values and press ***Close*** to close the dialog.

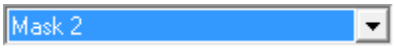
Mask Erosion window

Mask Erosion applies a kernel to the entire mask that removes a user-defined number of voxels from the perimeter of the mask. This function can be applied to in only the x, y dimension (2D) or in the z dimension (3D) as well.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Erode***

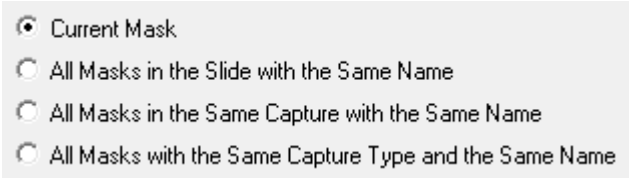


1 Mask Selection dropdown



Select the mask to erode from the dropdown menu. It is important to note that the mask displayed on the image is not necessarily the mask that will be eroded unless selected from the list.

2 Mask Range option



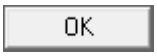
Select the mask range as follows:

Current Mask - operation will be performed only on the mask selected from the dropdown above
All Masks in the Slide with the Same Name - operation will be repeated on all masks with the same name on any image in the slide
All Masks in the Same Capture with the Same Name -
All Masks with the Same Capture type and the Same Name - operation will be repeated on all masks with the same name on only on images with the same ***Capture Type*** in the slide

3 Radius Settings

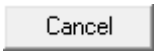
Type a value for the ***Radius*** to erode. This is the number of voxels that will be subtracted from the edges of the mask. Select ***2D*** or ***3D*** to perform the operation on each plane individually or on the entire mask as 3D objects.

4 OK button



Click ***OK*** to apply the erosion and close the dialog.

5 Cancel button

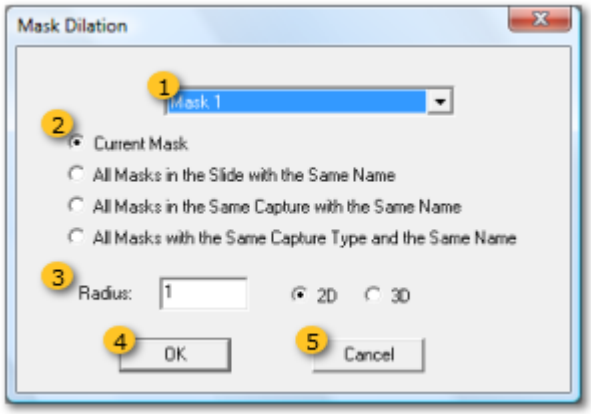


Click ***Cancel*** to discard the changes and close the dialog.

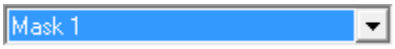
Mask Dilation window

Mask Erosion applies a kernel to the entire mask that removes a user-defined number of voxels from the perimeter of the mask. This function can be applied to in only the x, y dimension (2D) or in the z dimension (3D) as well.

Access in The Edit Mask Group of the Home tab, ***Refine Mask > Dilate***

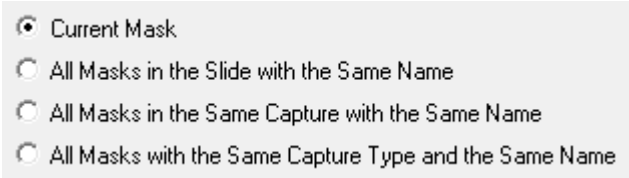


1 Mask Selection dropdown



Select the mask to dilate from the dropdown menu. It is important to note that the mask displayed on the image is not necessarily the mask that will be dilated unless selected from the list.

2 Mask Range option



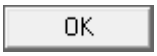
Select the mask range as follows:

Current Mask - operation will be performed only on the mask selected from the dropdown above
All Masks in the Slide with the Same Name - operation will be repeated on all masks with the same name on any image in the slide
All Masks in the Same Capture with the Same Name -
All Masks with the Same Capture type and the Same Name - operation will be repeated on all masks with the same name on only on images with the same ***Capture Type*** in the slide

3 Radius Settings

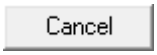
Type a value for the ***Radius*** to dilate. This is the number of voxels that will be added to the edges of the mask. Select ***2D*** or ***3D*** to perform the operation on each plane individually or on the entire mask as 3D objects.

4 OK button



Click ***OK*** to apply the dilation and close the dialog.

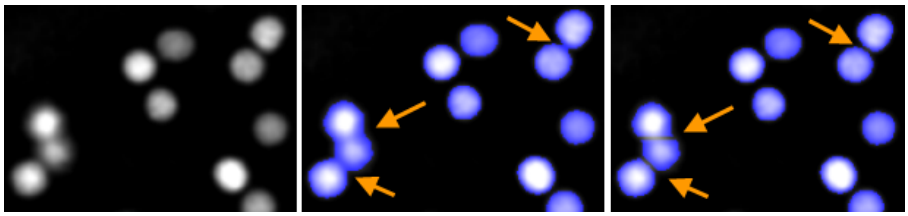
5 Cancel button



Click ***Cancel*** to discard the changes and close the dialog.

Split Objects Automatically window

To split objects automatically, SlideBook employs a "watershed" algorithm. This is useful to separate objects that may be joined by thin connectors as shown below.

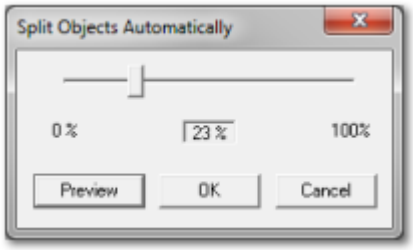


With a segmented mask displayed and objects defined, access in the Edit Mask group of the Process tab, ***Split Objects > Split Objects Automatically***

> **NOTE:** Splitting objects is only available for 2D and 2D Timelapse data types as well as projection images of 3D and 4D data.

See also:

- Segment Image window
- Define Objects window
- Mask Generation



The slider bar controls how aggressive the splitting algorithm will be. A value of **100%** refers to no splitting while **0%** refers to the most aggressive splitting algorithm. Adjust this slider to a position you feel will give you clear object separation.

Click ***Preview***. You will notice that the mask is updated on the image.

If the splitting is adequate, click ***OK***. If the splitting is inadequate, adjust the percentage and ***Preview*** until you are satisfied with the result, then click ***OK***.

Split Objects Protocol

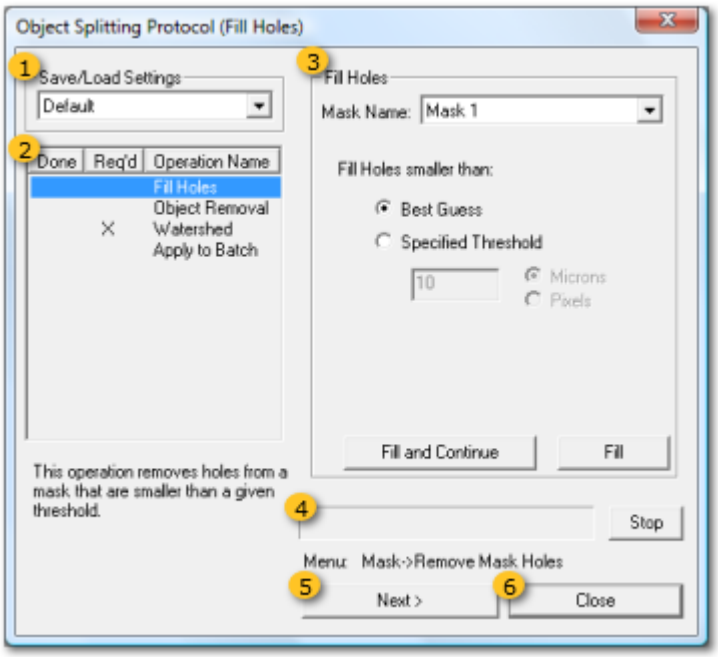
The Split Objects Protocol is a useful tool for batch processing analysis as it allows you to combine several mask processing steps (including watershed separation) and save the parameters for future use. Once a protocol has been created, you can apply it to multiple images on a slide with just a few button clicks.

> **NOTE:** Splitting objects is available for 2D and 2D Timelapse data types as well as projection images of 3D and 4D data.

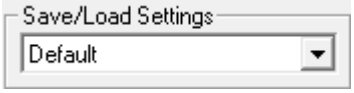
With a segmented mask displayed and objects defined, access in the Edit Mask group of the Process tab, ***Split Objects > Split Objects Protocol***.

Object Splitting Protocol (Fill Holes) window

The first step of the Object Splitting Protocol allows you to select a mask and fill holes, or gaps, in the mask.



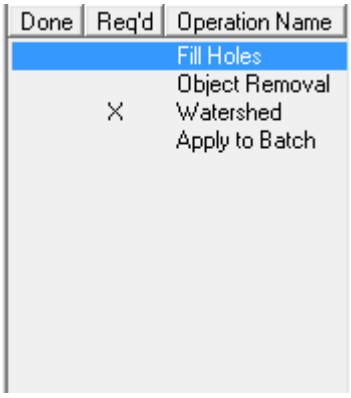
1 Save/Load Settings group



Load an existing ***Object Splitting Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

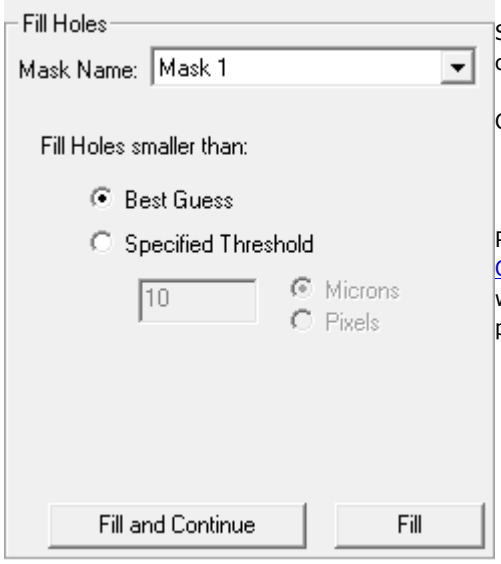
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in object splitting. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process.

3 Tracking Parameters group



Select a mask from the ***Mask Name*** dropdown menu. The default option will be the mask that is displayed.

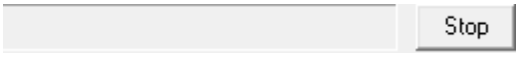
Choose parameter to use for filling holes as follows:

Best Guess –

Specified Threshold –

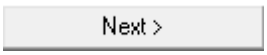
Press ***Fill and Continue*** to fill holes as designated and proceed to Object Removal. Press ***Fill*** to fill holes as designated and remain in this window in case you would like to adjust any parameters before proceeding.

4 Progress bar



The progress bar reports the hole filling progress. You may interrupt the process at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

5 Next button



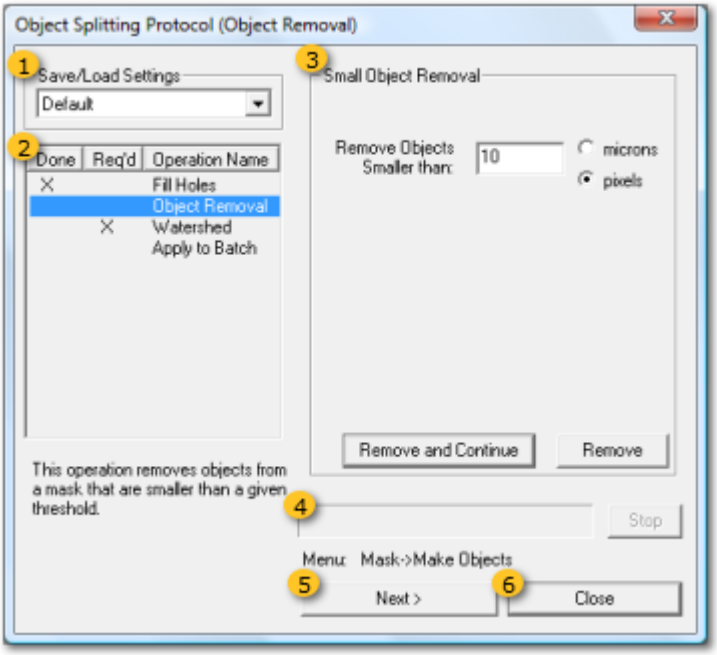
Once tracking is complete, you may press ***Next*** to proceed to Object Removal.

6 Close button

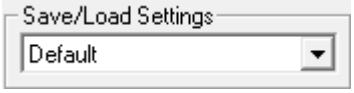
Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Any mask manipulation performed in the process will remain with the mask and must be saved with the image.

Object Splitting Protocol (Object Removal) window

The second step of the Object Splitting Protocol allows you to exclude small objects from the mask.



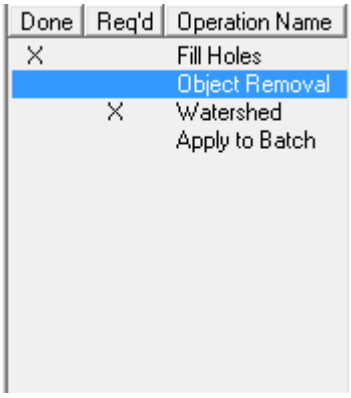
1 Save/Load Settings group



Load an existing ***Object Splitting Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

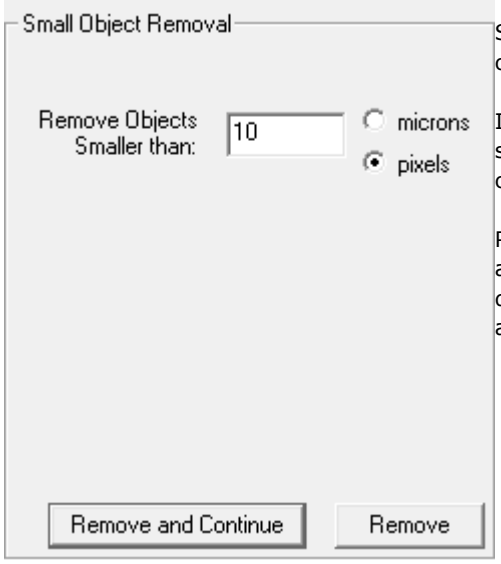
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in object splitting. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process.

3 Small Object Removal group

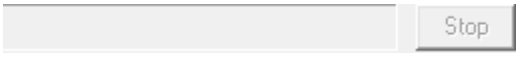


Select a mask from the ***Mask Name*** dropdown menu. The default option will be the mask that is displayed.

If you would like to exclude object smaller than a specific size, enter a size and designate units of the size. Alternatively, you may define objects based on size using the Define Objects window.

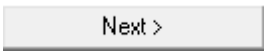
Press ***Remove and Continue*** to remove small objects as designated and proceed to Watershed. Press ***Remove*** to remove small objects as designated and remain in this window in case you would like to adjust any parameters before proceeding.

4 Progress bar



The progress bar reports the object removal progress. You may interrupt the process at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

5 Next button



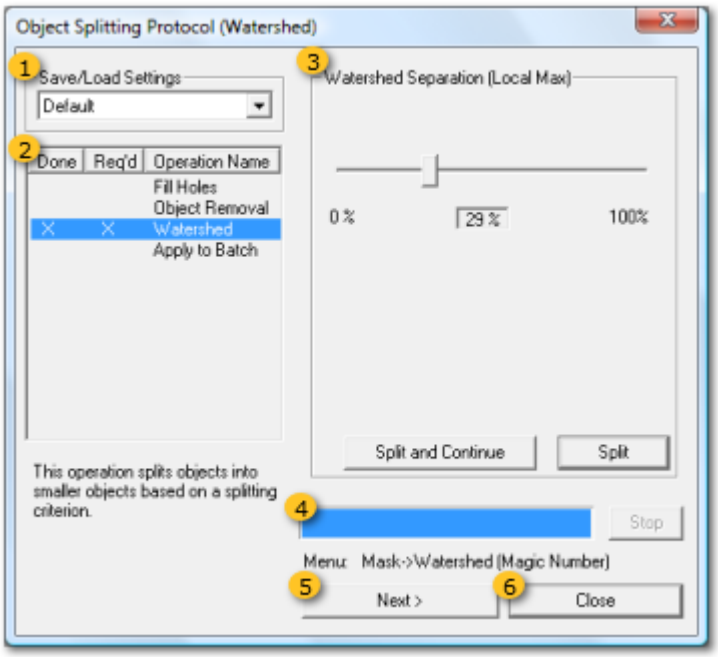
Once object removal is complete, you may press ***Next*** to proceed to Watershed.

6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Any mask manipulation performed in the process will remain with the mask and must be saved with the image.

Object Splitting Protocol (Watershed) window

The third step of the protocol is where the watershed separation takes place. See also Split Objects Automatically (Watershed Separation) window.

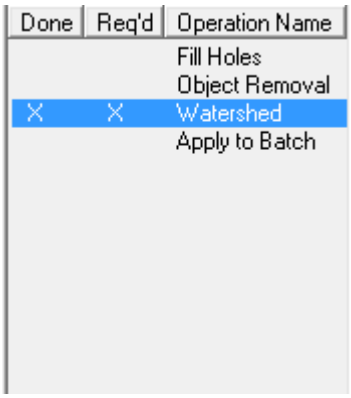


1 Save/Load Settings group

Load an existing ***Object Splitting Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

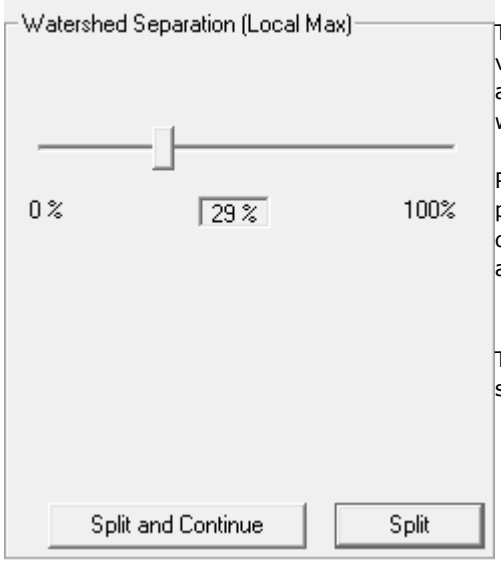
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in object splitting. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process.

3 Watershed Separation group

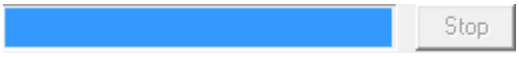


The slider bar controls how aggressive the splitting algorithm will be. A value of **100%** refers to no splitting while **0%** refers to the most aggressive splitting algorithm. Adjust this slider to a position you feel will give you clear object separation.

Press ***Split and Continue*** to remove small objects as designated and proceed to Apply Operation. Press ***Split*** to remove small objects as designated and remain in this window in case you would like to adjust any parameters before proceeding.

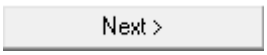
To display the results of object splitting, go to the ***Mask Menu*** and select the mask with **spl** appended to the name.

4 Progress bar



The progress bar reports the splitting progress. You may interrupt the process at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

5 Next button



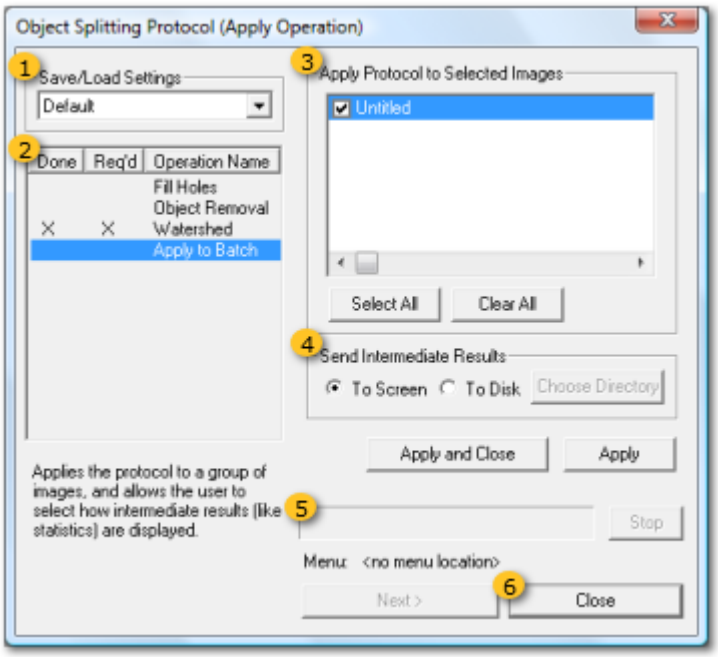
Once splitting is complete, you may press ***Next*** to proceed to Apply Operation.

6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Any mask manipulation performed in the process will remain with the mask and must be saved with the image.

Object Splitting Protocol (Apply Operation) window

In the final step of the Object Splitting Protocol, you can select which images to perform the watershed separation on. Once a protocol is saved, you may simply skip to this step and apply the saved protocol to new images.

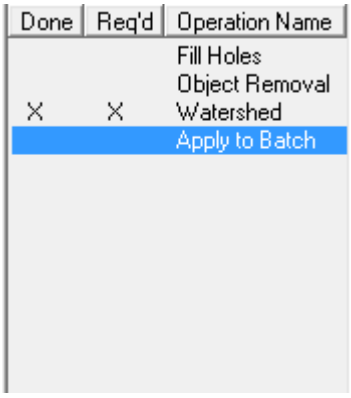


1 Save/Load Settings group

Load an existing ***Object Splitting Protocol*** from the drop down menu.

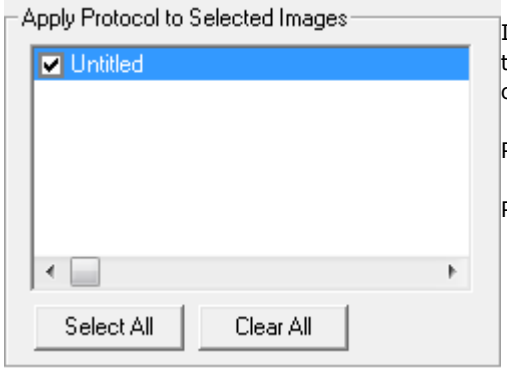
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in object splitting. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process.

3 Apply Protocol to Selected Images group

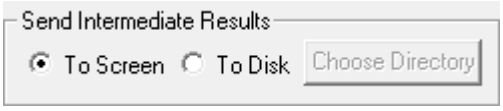


If you have more than one image in the slide, you may choose to apply the protocol to other images in the slide. Simply check the checkboxes of the images you wish to analyze.

Press ***Select All*** to check all boxes.

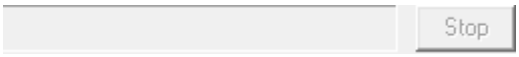
Press ***Clear All*** to uncheck all boxes.

4 Send Intermediate Results group



You may select whether you would like to send results ***To Screen*** for display only or save ***To Disk*** to choose a directory and save the output.

5 Progress bar



The progress bar reports the status of splitting objects. You may interrupt the process at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Any mask manipulation performed in the process will remain with the mask and must be saved with the image.

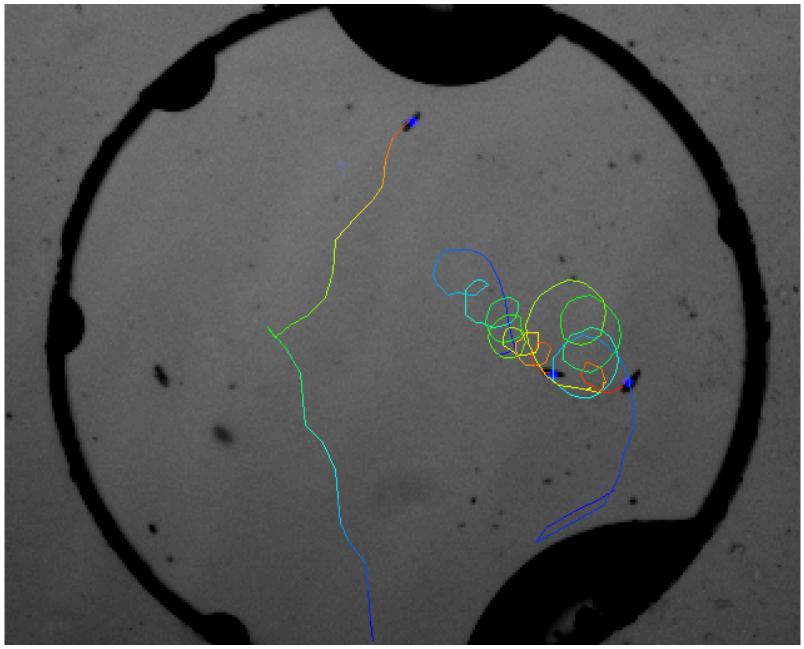
Particle Tracking Menu

Once you have defined objects via masking, you may wish to track them over time (in either 2D or 4D). SlideBook offers two methods for particle tracking, automated and manual.

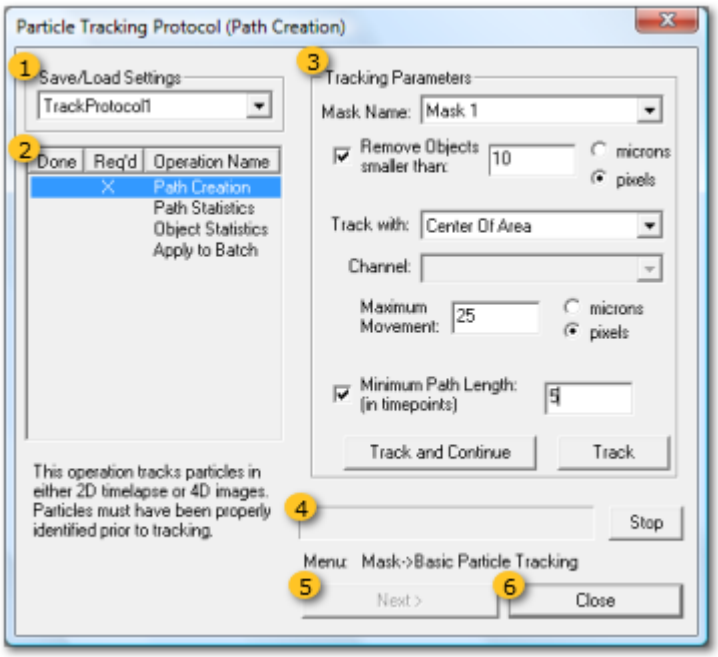
To perform particle tracking, you may either use the Particle Tracking Protocol or individual dialog boxes. The Particle Tracking Protocol has the advantage of being able to save and reuse your particle tracking parameters, and apply them in a batch fashion. When this is not necessary, you may prefer to use Basic Particle Tracking.

For objects that are particularly difficult to track automatically, SlideBook includes Manual Particle Tracking. Using this approach, you will click through a data set, identifying each object through time.

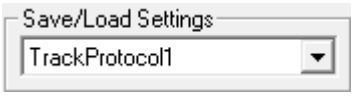
After objects are tracked in software, you can display paths by selecting ***Display Paths*** from the ***Particle Tracking*** menu in the Edit Mask group of Process tab. Paths will be drawn for each object. The paths will be colored from blue to red where blue indicates the first time points and red indicates the end time points as shown in the example below.



Particle Tracking Protocol (Path Creation) window



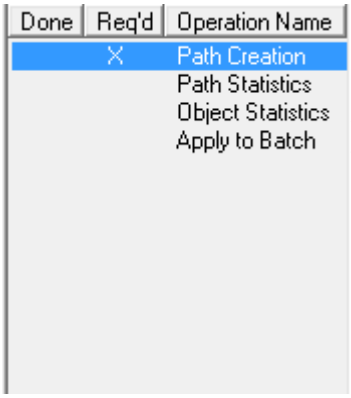
1 Save/Load Settings group



Load an existing ***Particle Tracking Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

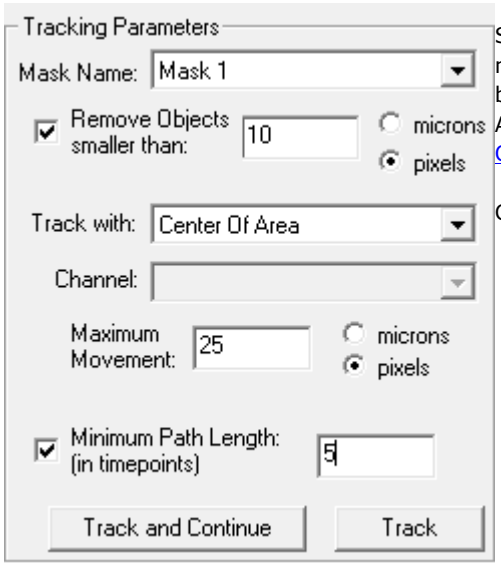
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in particle tracking. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process. Once ***Tracking Parameters*** have been applied for ***Path Creation***, you may close the Particle Tracking Protocol and directly access individual operations from the Particle Tracking Menu.

3 Tracking Parameters group



Select a mask to use for tracking from the ***Mask Name*** dropdown menu. The default option will be the mask that is displayed. Check the box if you would like to exclude object smaller than a specific size. Alternatively, you may define objects based on size using the Define Objects window.

Choose parameter to use for tracking as follows:

Center of Area/Volume – the coordinates of the center of the object, unweighted by intensity values.

Center of Intensity – the coordinates of the center of the object (either in 2D or 3D), weighted by intensity values. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

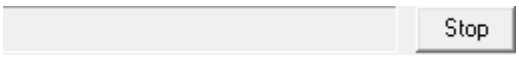
Mean Adjusted Center of Intensity – the coordinates of the center of the objected, weighted by intensity values that are above the mean intensity. This is a useful feature to track if you have delineated your objects by hand. By using values that fall above the mean, any imprecision in your hand delineation does not impact the center of intensity calculation. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

Enter the ***Maximum Movement*** that you expect from any given object from one time point to the next. This will help the particle tracking algorithm distinguish between objects that may have similar centroid values.

Check the box if you would like to set a limit on the minimal number of time points an object must pass through to be counted in analysis. Enter the ***Minimum Path Length (in timepoints)*** that must be tracked in order to determine a path. For instance, if you enter "3," any object that cannot be followed for at least three time points will be removed for the mask.

Press ***Track and Continue*** to track as designated and proceed to Path Statistics. Press ***Track*** to track as designated and remain in this window in case you would like to adjust any tracking parameters before proceeding. To view the paths that are created with tracking, go to ***Mask > Particle Tracking > Display Paths***.

4 Progress bar



The progress bar reports the tracking progress. You may interrupt tracking at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

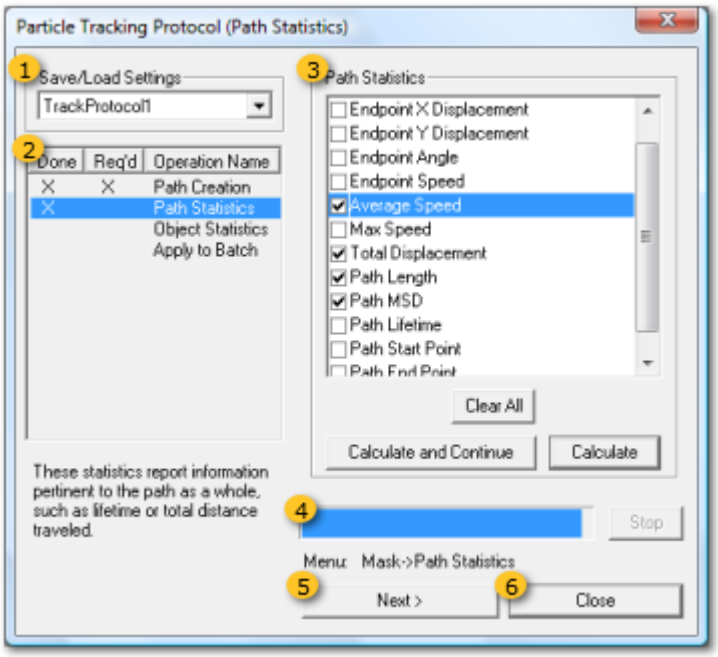
5 Next button

Once tracking is complete, you may press ***Next*** to proceed to Path Statistics.

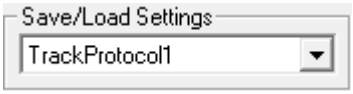
6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Object paths are part of the mask and will not be saved with an image until the slide is saved.

Particle Tracking Protocol (Path Statistics) window



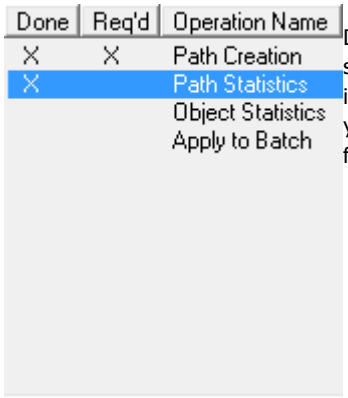
1 Save/Load Settings group



Load an existing ***Particle Tracking Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

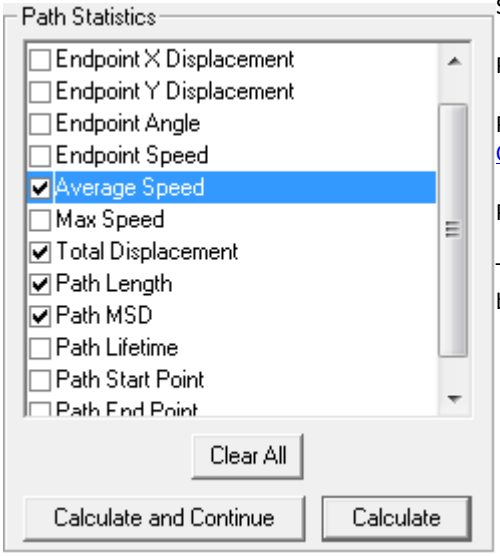
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in particle tracking. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process. Once ***Tracking Parameters*** have been applied for ***Path Creation***, you may close the Particle Tracking Protocol and directly access individual operations from the Particle Tracking Menu.

3 Path Statistics group



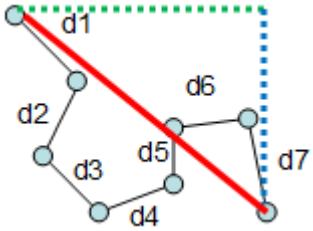
Select the ***Path Statistics*** you would like to calculate for each path.

Press ***Clear All*** to uncheck all boxes.

Press ***Calculate and Continue*** to calculate and move on to Apply Operation.

Press ***Calculate*** to display statistics and remain in this dialog.

The following statistics are available for each path based on the diagram below.



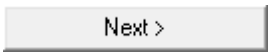
Endpoint Displacement – the distance traveled between the first and last timepoints of the path, as represented by the length of the red line in the figure above.
Endpoint X Displacement – the distance traveled in the x-axis between the first and last timepoints of the path, as represented by the length of the dotted green line in the figure above.
Endpoint Y Displacement – the distance traveled in the x-axis between the first and last timepoints of the path, as represented by the length of the dotted blue line in the figure above.
Endpoint Speed – the distance represented by the length of the red line divided by the time required to travel that distance.
Average Speed – the total distance traveled (the sum of distances d1 to d7 as shown in the figure above) divided by the time required to travel that distance.
Total Displacement – the total distance traveled by the object (the sum of distances d1 to d7 as shown in the figure above).
Path Length – number of timepoints in the path.
Path MSD -
Path Lifetime – the total elapsed time for a given path.
Path Start Point – centroid of the first point in the path.
Path End Point – centroid of the last point in the path.

NOTE: The Path Statistics dialog box can also be found by selecting ***Mask > Particle Tracking > Path Statistics***.

4 Progress bar

The progress bar reports the tracking progress. You may interrupt tracking at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

5 Next > button

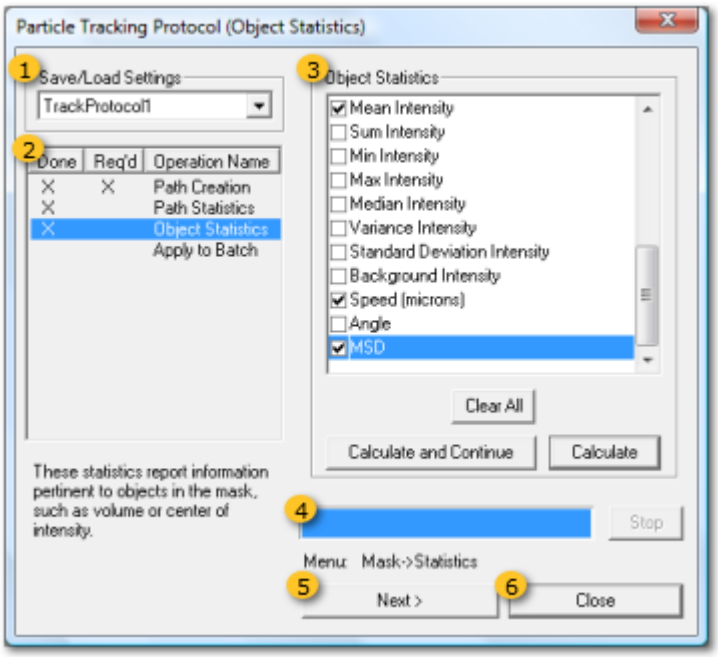


Once tracking is complete, you may press ***Next*** to proceed to Object Statistics.

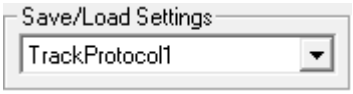
6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Object paths are part of the mask and will not be saved with an image until the slide is saved.

Particle Tracking Protocol (Object Statistics) window



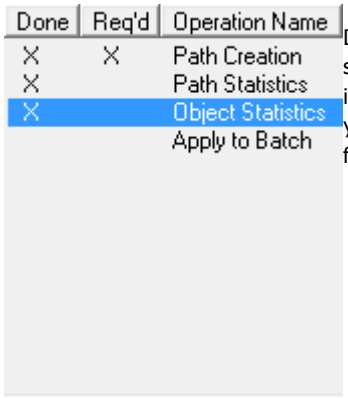
1 Save/Load Settings group



Load an existing ***Particle Tracking Protocol*** from the drop down menu. When you load an existing protocol, you will automatically advance to the Apply Operation window.

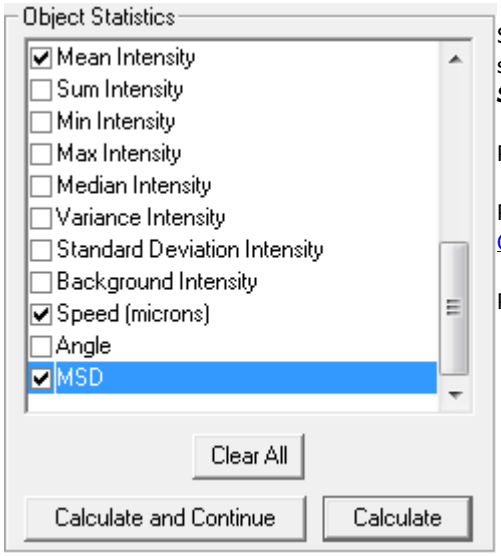
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in particle tracking. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process. Once ***Tracking Parameters*** have been applied for ***Path Creation***, you may close the Particle Tracking Protocol and directly access individual operations from the Particle Tracking Menu.

3 Object Statistics group



Select ***Object Statistics*** to calculate by checking the boxes next to the statistic. These are the same object statistics available in ***Mask Statistics***.

Press ***Clear All*** to uncheck all boxes.

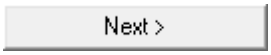
Press ***Calculate and Continue*** to calculate and move on to Apply Operation.

Press ***Calculate*** to display statistics and remain in this dialog.

4 Progress bar

The progress bar reports the tracking progress. You may interrupt tracking at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

5 Next > button

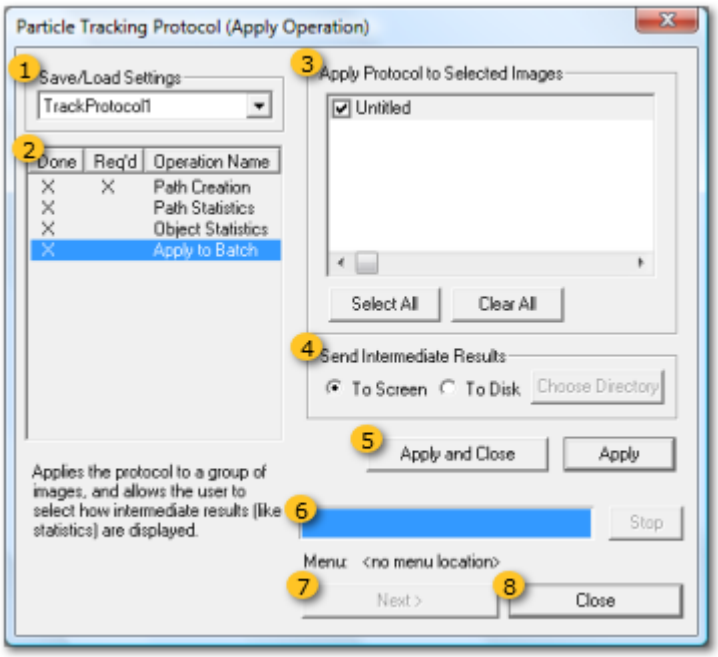


Once tracking is complete, you may press ***Next*** to proceed to Apply Operation.

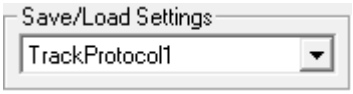
6 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Object paths are part of the mask and will not be saved with an image until the slide is saved.

Particle Tracking Protocol (Apply Operation) window



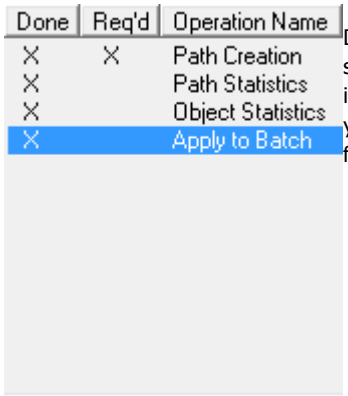
1 Save/Load Settings group



Load an existing ***Particle Tracking Protocol*** from the drop down menu.

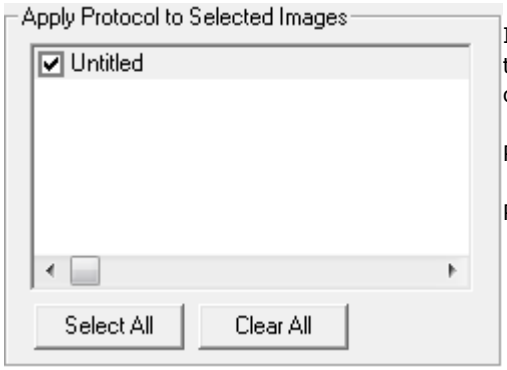
Select ***Save As*** to save a new protocol. Select ***Save***, ***Rename***, or ***Delete*** to save, rename, or delete the loaded protocol.

2 Protocol list



Displays a list of steps involved in particle tracking. The ***Done*** column shows which steps have been completed and the ***Req'd*** column shows which steps are necessary in the process. Once ***Tracking Parameters*** have been applied for ***Path Creation***, you may close the Particle Tracking Protocol and directly access individual operations from the Particle Tracking Menu.

3 Apply Protocol to Selected Images group

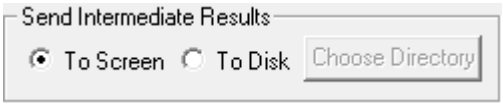


If you have more than one image in the slide, you may choose to apply the protocol to other images in the slide. Simply check the checkboxes of the images you wish to analyze.

Press ***Select All*** to check all boxes.

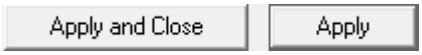
Press ***Clear All*** to uncheck all boxes.

4 Send Intermediate Results group



You may select whether you would like to send results ***To Screen*** for display only or save ***To Disk*** to choose a directory and save the output.

5 Apply buttons



Select ***Apply and Close*** to finish the protocol or select ***Apply*** to perform the batch analysis and keep the window open.

6 Progress bar

The progress bar reports the tracking progress. You may interrupt tracking at any time by pressing ***Stop***. Once the process is complete, the bar will be filled with blue.

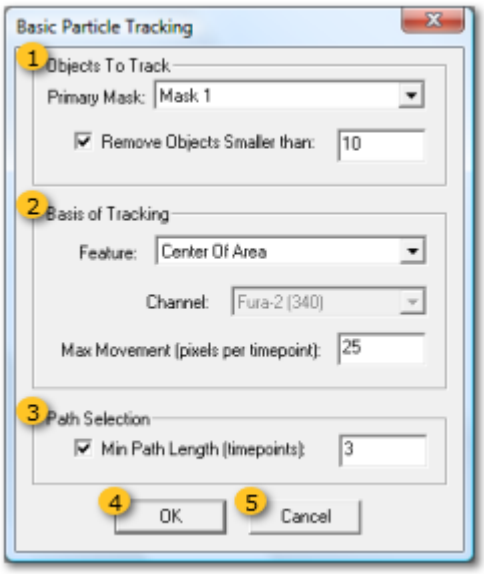
7 Next > button

As this is the last step in particle tracking, ***Next*** is not available.

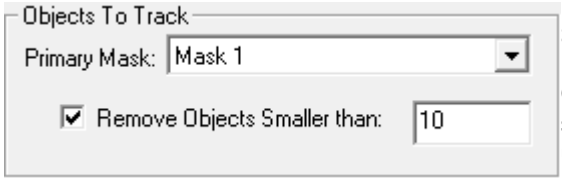
8 Close button

Press ***Close*** to close the dialog. Any completed steps of the process will be applied to the image. Object paths are part of the mask and will not be saved with an image until the slide is saved.

Basic Particle Tracking window

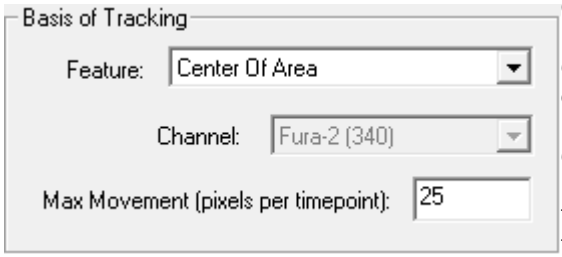


1 Objects To Track group



Select a mask to use for tracking from the ***Mask Name*** dropdown menu. The default option will be the mask that is displayed. Check the box if you would like to exclude object smaller than a specific size. Alternatively, you may define objects based on size using the Define Objects window.

2 Basis of Tracking group



Choose parameter to use for tracking (***Feature***) as follows:

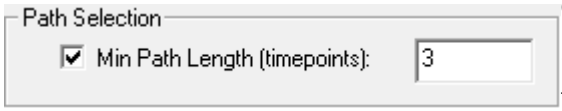
Center of Area/Volume – the coordinates of the center of the object, unweighted by intensity values.

Center of Intensity – the coordinates of the center of the object (either in 2D or 3D), weighted by intensity values. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

Mean Adjusted Center of Intensity – the coordinates of the center of the objected, weighted by intensity values that are above the mean intensity. This is a useful feature to track if you have delineated your objects by hand. By using values that fall above the mean, any imprecision in your hand delineation does not impact the center of intensity calculation. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

Enter the ***Maximum Movement*** that you expect from any given object from one time point to the next. This will help the particle tracking algorithm distinguish between objects that may have similar centroid values.

3 Path Selection group



Check the box if you would like to set a limit on the minimal number of time points an object must pass through to be counted in analysis. Enter the ***Minimum Path Length (in timepoints)*** that must be tracked in order to determine a path. For instance, if you enter "3," any object that cannot be followed for at least three time points will be removed for the mask.

4 OK button

OK

Press ***OK*** to track objects and close the dialog. You may find it useful to display object IDs (**Ctrl** + **Shift** +**O**) or by selecting ***Display Paths*** from the ***Particle Tracking*** menu.

5 Cancel button

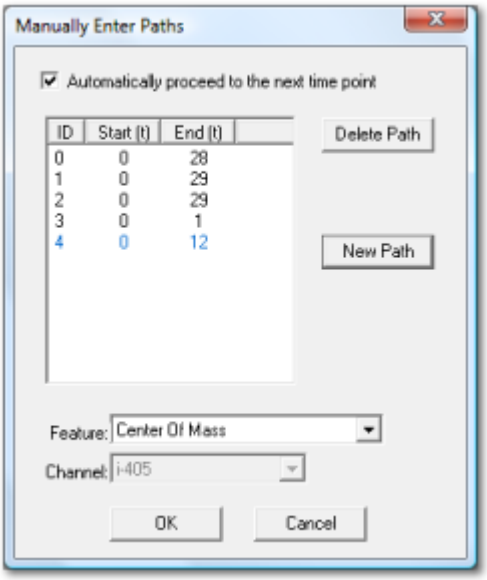
Cancel

Press ***Cancel*** to close the window without tracking objects.

Manual Particle Tracking window

At times, you may find that using thresholding techniques to mask and track objects is not successful due to uneven fluorescence or other sample specific parameters. For situations such as these, ***Manual Particle Tracking*** is available. This approach allows you to visually identify the point to track each object at each time point.

Access by selecting ***Manual Particle Tracking*** from the ***Particle Tracking*** menu in the Edit Mask group of the Process tab.



When paths are manually generated, you will use the mask drawing tools to draw paths by clicking on objects through time. When you open the Manual Particle Tracking dialog, a new empty mask is generated and the medium sized drawing tool is selected from the Tool Menu. Three tools are available for drawing paths and you may switch between these tools during the path creation process:

- Small, single pixel drawing tool

- Medium, 4 pixel in diameter, drawing tool

- Large, 12 pixel in diameter, drawing tool

Manual Particle Tracking works by allowing you to click on an object at every time point. By checking the box to ***Automatically proceed to the next time point***, you are allowing the software to advance the data set to the next time point as soon as you click in the current time point. You may find it useful to display ***Object IDs*** by typing **Ctrl + Shift + O**.

Begin by moving the data set to the first time point and object you would like to track. Click once on the object in the first time point. The data set will automatically advance to the next time point. If the data is 3D over time (4D Capture type), you can adjust the z plane at each step to the desired focal plane. Continue clicking on the object once in each time point. When you finish the object's path, click ***New Path*** to begin tracking a new object. If the object's path continues to the last time point, a new path will automatically be started and the data set will go back to the first time point to begin tracking the next object.

If an error is made in tracking an object, select the ID from the list (will be highlighted in blue) and click ***Delete Path***.

Continue in this manner until paths have been created for all objects as desired. The tracking step will use a Feature for each object to determine the exact location of the object as follows:

Center of Area/Volume – the coordinates of the center of the object, unweighted by intensity values.

Center of Intensity – the coordinates of the center of the object (either in 2D or 3D), weighted by intensity values. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

Mean Adjusted Center of Intensity – the coordinates of the center of the objected, weighted by intensity values that are above the mean intensity. This is a useful feature to track if you have delineated your objects by hand. By

using values that fall above the mean, any imprecision in your hand delineation does not impact the center of intensity calculation. If you select this feature, you will also need to select the ***Channel*** that is used for weighting.

NOTE: Feature selected will be irrelevant if the single pixel drawing tool was used to create the paths.

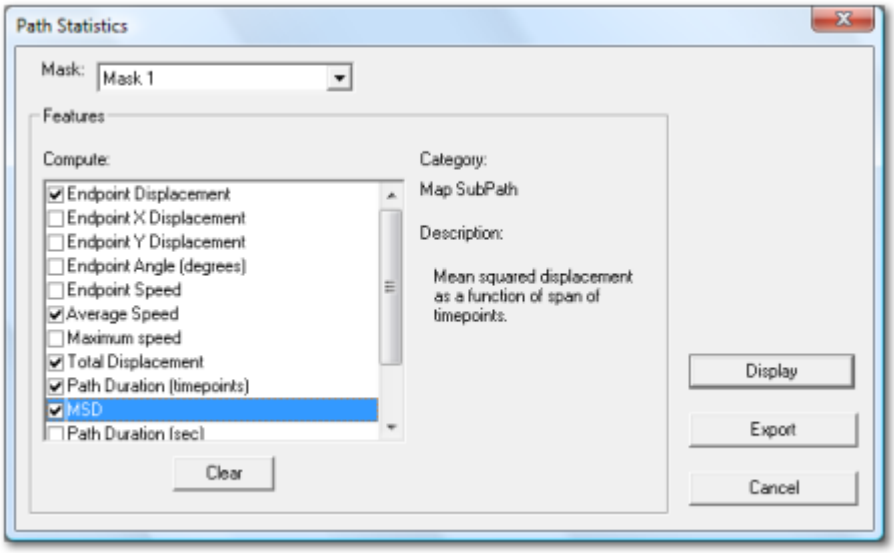
Press ***OK*** to track objects and close the dialog. Press ***Close*** to abandon tracking and close the dialog.

After paths are generated, you may find it useful to display paths by selecting ***Display Paths*** from the ***Particle Tracking*** menu in the Edit Mask group of the Process tab or to retrieve Path Statistics.

Path Statistics window

When Basic Particle Tracking or Manual Particle Tracking is performed, you may retrieve information on the individual paths from the ***Path Statistics*** window.

Access by selecting ***Path Statistics*** from the ***Particle Tracking*** menu in the Edit Mask group of the Process tab (only available after tracking is performed).



Select the ***Mask*** from the dropdown menu, be sure it is a mask that has had tracking performed.

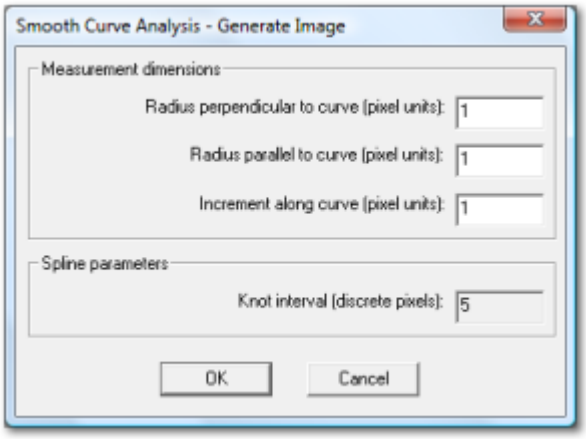
Check the box next to each statistic you would like to ***Compute***. When you select a statistic, the ***Description*** will be displayed to the right of the selection list. Press ***Clear*** to uncheck all boxes.

Press ***Display*** to open a table that will display all path statistic computations. Press ***Export*** to save a tab-delimited **.txt** file. Press ***Cancel*** to close the dialog.

Smooth Curve Analysis - Generate Image window

Smooth curve analysis allows you to follow time-dependent lateral intensity changes along a static object, also known as a kymograph. For example, an action potential moving along a neural spine could be displayed as an x vs. time image using smooth curve analysis. You could then extract velocity and displacement information from this image. SlideBook's Smooth Curve Analysis is a mathematically rigorous and flexible operation with several adjustable parameters.

Access by creating a mask and drawing a single pixel line along an object of interest. Copy the mask to all planes then go to the Process tab, Edit Mask group, ***Smooth Curve Analysis***



Measurement dimensions

Radius Perpendicular to Curve (pixel units) – shown as dimension r1 in the figure below

Radius Parallel to Curve (pixel units) – shown as dimension r2 in the figure below

Increment Along Curve (pixel units) – center to center distance between rectangular areas used to calculate smooth curve. In the below figure, the boxes are drawn every two pixels units along the curve. The resultant smooth curve analysis with have half the resolution of the original image.

Spline parameters

Knot Interval (discrete pixels) - the default is set to 5. A spline is fit between two "knots." Multiple splines are used to fit a given curve. The knots are shown in pink in the figure below.

Once these parameters are defined, the smooth curve algorithm averages the areas at each timepoint and creates a new image in two dimensions (distance in x vs. time in y).

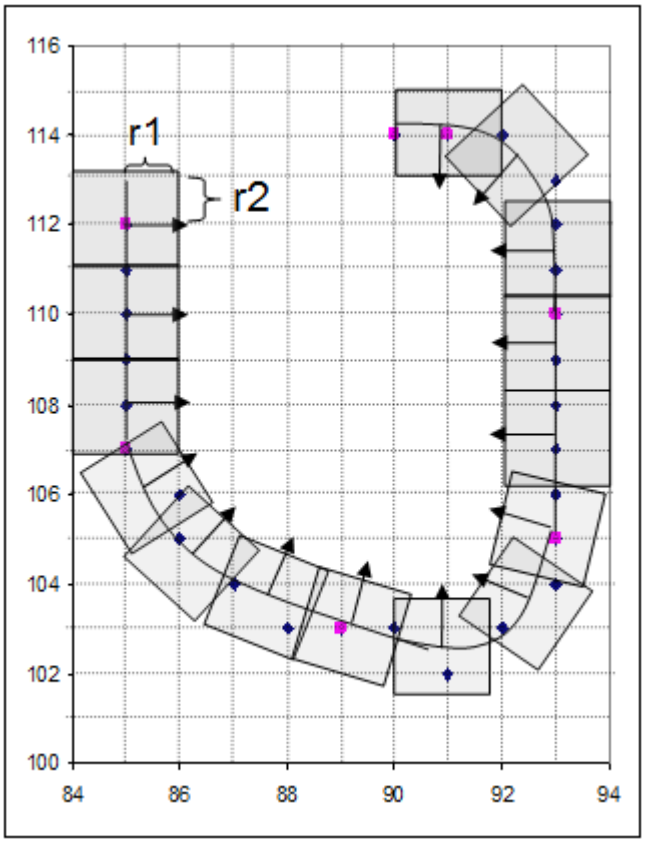
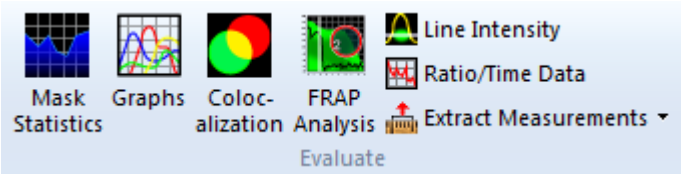


Diagram illustrating ***Smooth Curve Analysis*** terms

Evaluate Group

In order to further analyze your data, you may wish to obtain statistics on masks, regions, or lines or graph ratios or intensity over time. These functions and more can be found in the Evaluate group of the Process tab.



Mask Statistics

When at least one mask is present, opens the Mask Statistics dialog to calculate and display or export a wide variety of statistics about the entire mask, each time point in a time lapse image, and or objects.

Graphs

When regions are present, opens the New Graph dialog where you select what type of information (intensity, intensity changes, or ratio) and which channels you would like to display in a Graph. Also available from the ***Graph*** icon in view windows.

Colocalization

Colocalization analysis can be performed on 2D and 2D time lapse images by using the Colocalization dialog.

3D and 4D data can be analyzed in this manner using the Interactive Mask Segmentation dialog.

FRAP Analysis

When analyzing photomanipulation data, you may find it useful to use the FRAP Analysis window. This window provides an interactive way to set the important variables for calculating values such as half-life and mobile fraction.

Line Intensity

Opens a Line Intensity graph of a line drawn using the ***Ruler*** tool.

Ratio/Time Data

Opens a ratio or time intensity data graph of the entire image or a single point of the image when you have the ***Pixel*** tool selected and hold **Ctrl** when you click on a pixel.

Extract Measurements

Export Line Lengths - Use the line region tool to draw lines on the image then select this command to write a tab-delimited text file (**.txt**) with region number and line length in pixels and microns.

Export Angle Measurements to Excel - Use the angle tool to create a 3-point angle then select this command to open a Microsoft Excel file. Additional drawn angles will be written to the open file.
Export Ratio/Timelapse Data - Use with a drawn mask with objects defined, exports a tab-delimited text file (**.txt**) with average intensity information for each object at each time point.
Intensity Statistics - displays an exportable histogram of pixel intensity values.
Spatial Correlation -
Export Pixel Intensities -

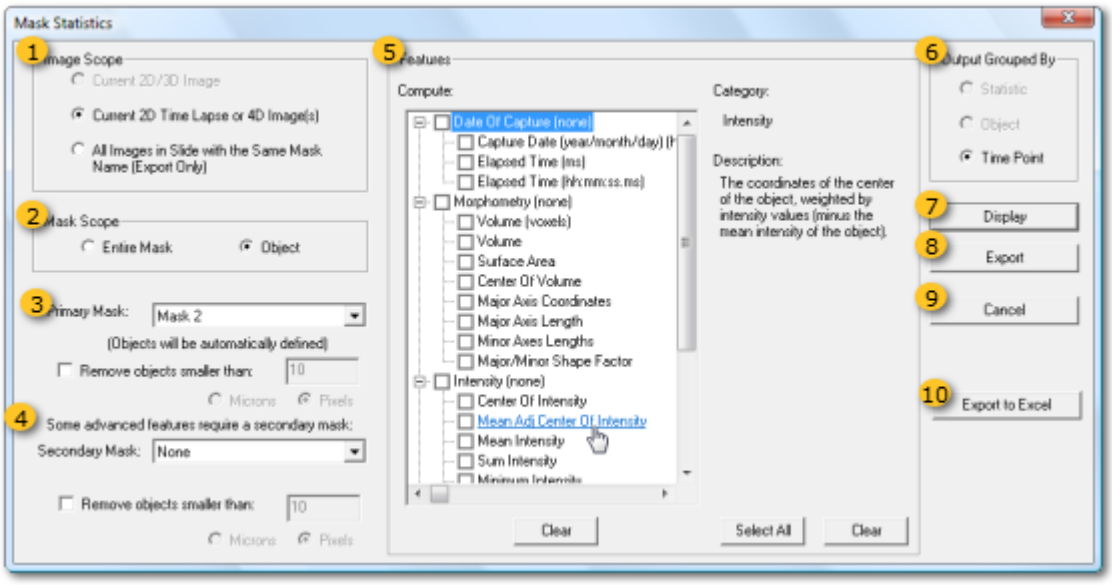
Mask Statistics window

You can answer many quantitative questions about a mask through ***Mask Statistics***. SlideBook lets you compute three types of mask statistics: statistics for the entire mask, statistics for each time point in a time lapse image, and statistics for objects. SlideBook allows for a wide range of statistics to be generated, from simple to sophisticated.

Access in the Evaluate group of the Process tab

See also:

- Create New Mask window
- Segment Image window
- Define Objects window
- Ratio Manual: Exporting Statistics from Masks
- Configuring Background Subtraction with Mask Statistics



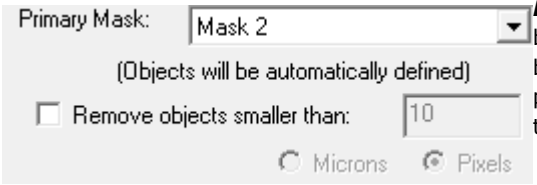
1 Image Scope group

Image Scope allows for generation of statistics in multiple images. If you are working with a 2D or 3D image, the first radio button will be selected by default. If you are working with a 2D time lapse or 4D data set, the second radio button will be selected by default. You may select the third radio button if you have multiple images with the same mask name.

2 Mask Scope group

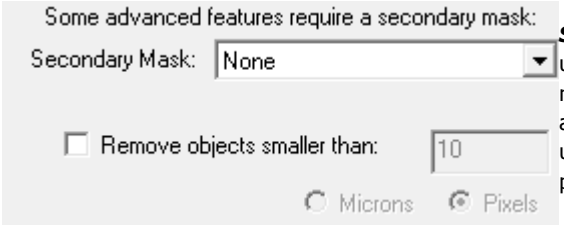
Mask Scope allows you to select whether you want statistics for the entire mask or on a per object basis. If you have chosen to obtain statistics for multiple images, the ***Object*** option will be unavailable.

3 Primary Mask Selection



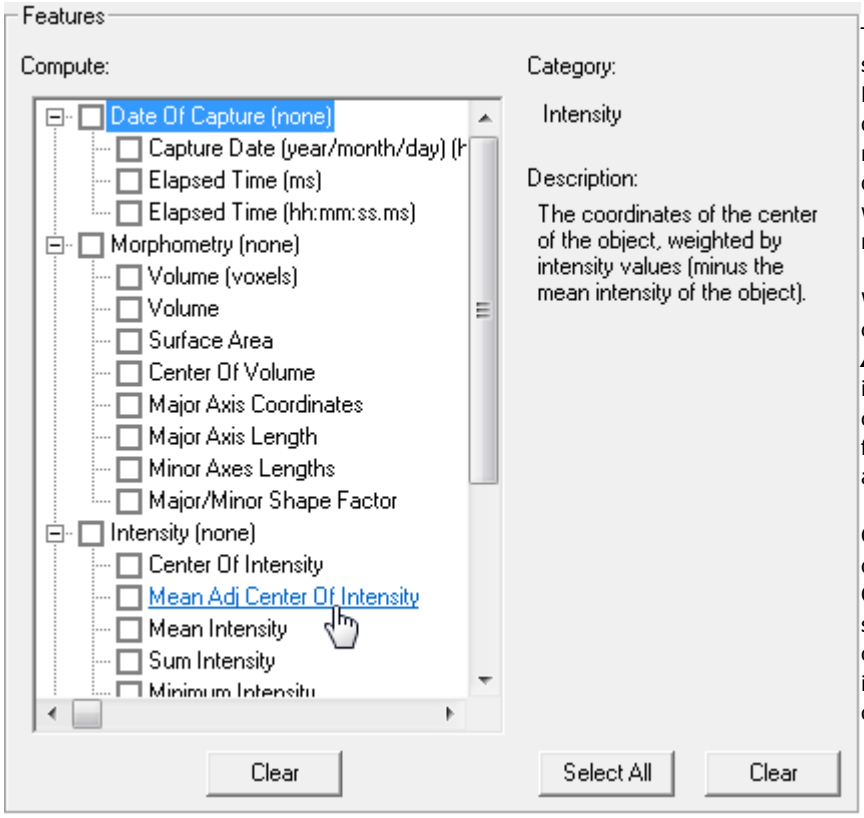
Primary Mask defaults to the mask that is currently displayed but can be altered via the dropdown menu. The primary mask will automatically be made into objects. You may also remove objects under a specified physical or pixel size. To get statistics for all pixels in the mask, leave this box unchecked.

4 Secondary Mask Selection



Secondary Mask can be selected to obtain additional statistics under the ***Cross Mask*** feature set. You may select a secondary mask from the dropdown menu. The secondary mask will automatically be made into objects. You may remove objects under a specified physical or pixel size. To get statistics for all pixels in the mask, leave this box unchecked.

5 Features group



The ***Features*** area lists all available statistics that may be calculated. Note that the list of features will depend on the dimensions of the mask. For instance, area will be displayed as a statistic for 2D masks, while volume will be displayed for 3D masks.

When you click on a statistic to compute (in the image this is ***Mean Adj Center Of Intensity***), information regarding the calculation can be found in the ***Description*** field. For a complete list of features and their descriptions, click here.

Click on the **+/-** to expand or collapse different categories of computations. Check the box next to a category to select all features within the category or check individual boxes to select individual calculations. Press ***Clear*** to clear all checked boxes.

6 Output Grouped By group

These choices are only activated if you are performing object statistics on 2D timelapse or 4D images.

Output Grouped By Statistic will give a table with a drop down menu of statistics calculated at the top. The table will list time points and data will be given per object at each time point (for the selected statistic).

Output Grouped By Object will give a table with a drop down menu of objects at the top. The table will list time points and data will be given per statistic at each time point (for the given object).

Output Grouped By Time Point will give a table with a drop down menu of time points at the top. The table will list objects and data will be given per statistic for each object (at the time point).

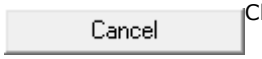
7 Display button

Clicking ***Display*** will display the selected calculations in an interactive table.

8 Export button

Clicking ***Export*** will export the selected calculations into a tab delimited .txt file that you will save to your computer.

9 Cancel button



Clicking ***Cancel*** will discard selections and close the dialog.

10 Export to Excel button

Clicking ***Export to Excel*** will open a Microsoft Excel worksheet with the selected data calculations.

Statistics Feature Descriptions

Available statistics feature descriptions are listed below.

Morphometry

Object Count – number of objects. This statistic is only available when you select Entire Mask in the Mask Scope section of the statistics dialog.

Volume (voxels) / Area (pixels) - total number of voxels. Note that in a 2D or time lapse image, you will always get an area measurement.

Volume (μm³) / Area (μm²) - volume in cubic microns (or square microns in 2D or timelapse images). Note that the image must have the correct objective and optovar selected for these values to be accurate (see Defining Objectives on page 62 and Defining Magnification Changers on page 73).

Surface area (μm²) / Perimeter (μm) – the area of exposed voxel faces for 3D objects (or exposed pixel faces in 2D or time lapse images). Note that these values should only be used as rough estimates, as area or perimeter measurements are highly affected by the resolution of the image.

Center of Area/Volume – the coordinates of the center of the object, unweighted by intensity values.

Major Axis Coordinates – the coordinates of the two edge voxels that lie along the major axis.

Major Axis Length – the length of the longest line that runs through the center of volume, also called the 'axis of minimal inertia'.

Minor Axis Length – the length of the minor axis (in 2D) or both minor axes (in 3D).

Longest Chord Length – the distance between the two furthest voxels in the object.

Major/Minor Shape Factor – The ratio of major axis to first minor axis (i.e. width vs. height).

Intensity

Center Of Intensity – the coordinates of the center of the marked object weighted by intensity, calculated for each channel.

Mean Adj Center Of Intensity – the coordinates of the center of the object, weighted by intensity values (minus the mean intensity of the object).

Mean Intensity – average marked voxel intensity value for each channel.

Sum intensity – sum of all marked voxel intensity values for each channel. Note that this value is equivalent to the product of the mean intensity and the volume or area.

Minimum Intensity – the minimum voxel value in the object.

Maximum Intensity – the maximum voxel value in the object.

Median Intensity – the median voxel value in the object.

Variance – the variance of intensities of the marked voxels.

Standard Deviation – the standard deviation of intensities of the marked voxels.

Background Intensity – the average background intensity in the channel as defined by a background region.

Cross-Channel

Pearson's Correlation – the correlation of intensity in one channel with another; a 0 value signifies no correlation, while a value of 1.0 signifies perfect correlation. A negative value signifies anti-correlation.

This statistic can be used for measuring colocalization. The equation used is shown below. R_i is the intensity in channel 1 for pixel I, and G_i is the intensity in channel 2 for the same pixel. R_{av} and G_{av} are the average (mean) intensity values over all pixels.

$$r = \frac{\sum_i (R_i - R_{av}) \cdot (G_i - G_{av})}{\{\sum_i (R_i - R_{av})^2 \cdot \sum_i (G_i - G_{av})^2\}^{\frac{1}{2}}},$$

Mander's Coefficients – these coefficients indicate the percent of protein 1 colocalized with protein 2 (M1) and the percent of protein 2 colocalized with protein 1 (M2) as given by the equation below. The value T (threshold) is considered to be zero intensity. Thus, adequate background subtraction must be performed.

$$M_1 \cong \frac{\sum_{I_1 > T} I_1}{\sum_{\text{All } I_1} I_1}, \quad \text{and} \quad M_2 \cong \frac{\sum_{I_2 > aT+b} I_2}{\sum_{\text{All } I_2} I_2},$$

SlideBook offers the following statistics to describe relationships between objects that are present in two different masks. You must first set the Mask Scope to Object, then select a secondary mask to view these statistics.

Cross-Mask (available only when multiple masks are generated and a secondary mask is selected)

Mask Overlap (objects) - for each object in the primary mask, this returns a count of the number of overlapping objects in the secondary mask.

Mask Overlaps (voxels) – for each object in the primary mask, this returns the number of overlapping pixels/voxels in the secondary mask.

Mask Overlaps (largest object volume) – for each object in the primary mask, this returns the area or volume of the largest overlapping object in the secondary mask.

Mask Contains Other Masks – returns the IDs of the objects in the secondary mask that overlap with the primary mask.

Motion (available only after particle tracking is performed)

Speed - A measurement of instantaneous speed using the centroid measured during particle tracking. Instantaneous speed is calculated as an average of the speed approaching the current location from the previous time point with the speed the particle approaches the subsequent time point. For the first time point, instantaneous speed is simply the speed from time 0 to the next time point. For the last time point, the instantaneous speed is the speed from the second to last time point to the last. The diagram below depicts the time range of speed calculations per time point.

Angle of Motion - Angle from the last time point to the current time point, in degrees, using the centroid measured during particle tracking.

MSD - Mean squared displacement as a function of span of time points

Configuring Background Subtraction with Mask Statistics

Up to this time, we have discussed setting a background region for calculations. We will now discuss configuring SlideBook to use this background region for background subtraction in statistical analysis of masks.

The method of background subtraction is as follows:

- Mean intensity value is calculated from designated background region.
- This background mean intensity is subtracted from each pixel/voxel of the image.
- Only pixels/voxels with background-subtracted values >0 will be used for statistical analysis

1. Create a background region as described in Selecting Regions of Interest

2. Go to ***Administration tab >*** ***Hardware Properties***.

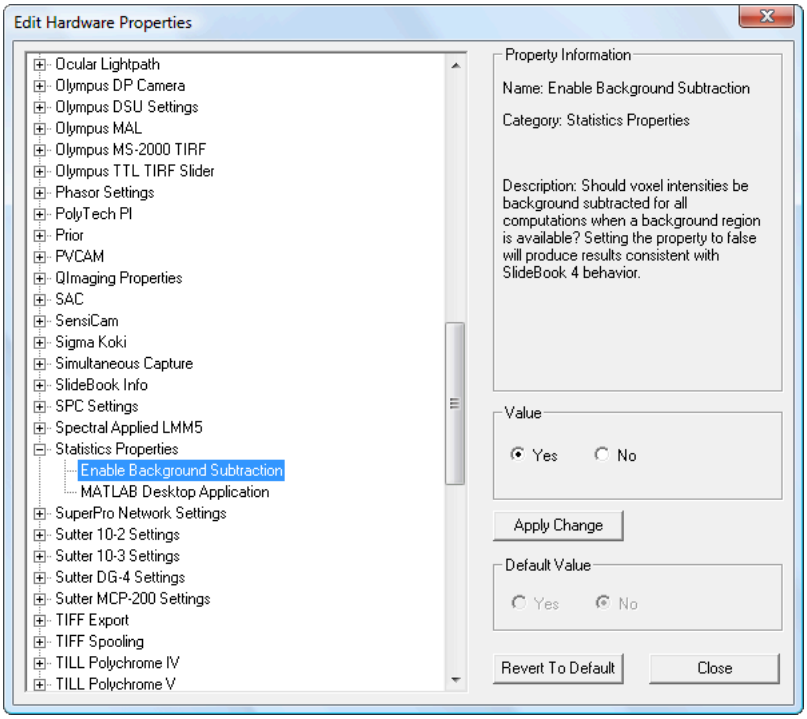
   NOTE: Hardware Properties is not enabled on offline (analysis) workstations. Hold the **CTRL** key down while in the Administration tab to enable clicking on and opening Hardware Properties.

3. Navigate down the alphabetical list to ***Statistics Properties***, click the "**+**" to expand the selection.

4. Set the ***Value*** to ***Yes***.

5. Click ***Apply Change*** then ***Close***.

6. Restart SlideBook.



Graph View window

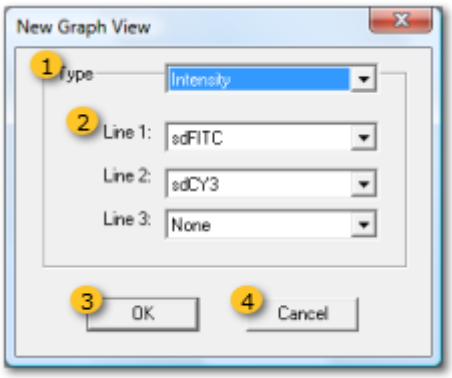
When regions are drawn, you may graph several types of intensity information from the ***New Graph View*** window. All regions will be graphed and the mean background intensity will be subtracted when defined.

Access in the Evaluate group of the Process tab or by the ***Graph*** icon in view windows (when regions are present)

NOTE: When a background region is defined, background subtraction will be performed as follows. The mean intensity of the background will be subtracted from each pixel in the defined regions for graphing. Pixels with a background-subtracted value greater than 0 will be used for the requested analysis.

See also:

Graph window
Ratio Module, Graph Display
Ratio Module, Multiple Region Graphing and Export
Photomanipulation Module, Displaying Region Intensity during Capture
Photomanipulation Module, Displaying Regions and Graphs Post-Capture



1 Drop down

Select the type of data you would like to graph from the drawn regions as follows:

Intensity - Graphs the mean intensity of each region over time

Intensity: Min - Graphs the minimum intensity of each region over time

Intensity: Max - Graphs the maximum intensity of each region over time

Delta F/F - Used to emphasize intensity changes over time point 0. Graphs $F/F_{baseline}$ intensity of the current time point divided by the mean intensity of the baseline (average of the first 10 time points). All regions will start at a value of **1.00**. This mode is particularly useful for analyzing photomanipulation data or other data where the intensity values of regions are drastically different and you would like to view changes on a relative scale.

Ratio- Graphs the ratio when ratio channels are configured, see also Ratio Module.

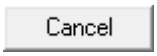
2 Drop down

There are three line types available in the graph. ***Line 1*** is solid, ***Line 2*** is dashed, and ***Line 3*** is dotted. Select the channel to graph per line. Each region will be graphed per the channel selected in the mode assigned. Line colors will correspond to the colors of the region in the image.

3 OK button

Click ***OK*** to open the graph as specified.

4 Cancel button



Click ***Cancel*** to abandon graphing and close the dialog.

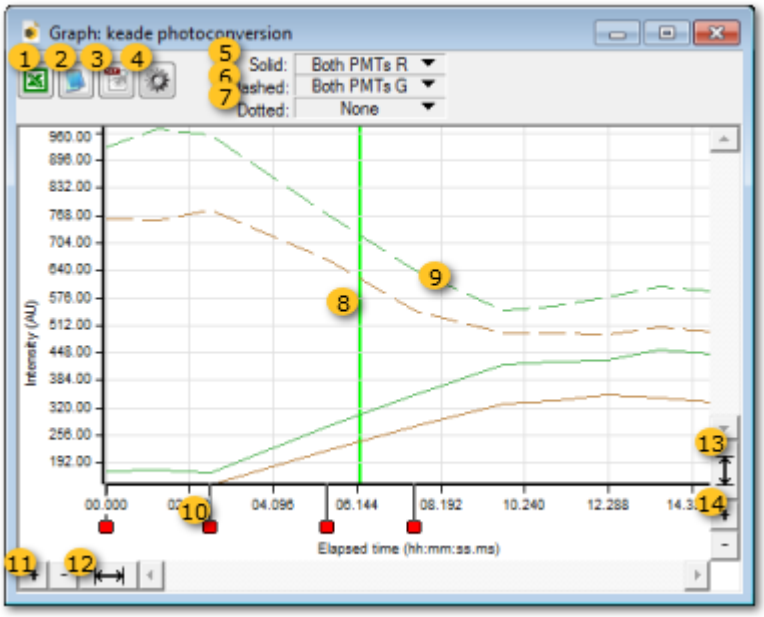
Graph window

The ***Graph*** window displays intensity or ratio data for drawn regions over time. By clicking in the graph, you can navigate to a time point in the image view window. When a background region is defined, mean background intensities will automatically be subtracted.

Access in the Evaluate group of the Process tab or by the ***Graph*** icon in view windows (when regions are present)

See also:

Ratio Module, Graph Display
Ratio Module, Multiple Region Graphing and Export
Photomanipulation Module, Displaying Region Intensity during Capture
Photomanipulation Module, Displaying Regions and Graphs Post-Capture



1 Export to Excel

Click this button to export the data to an Excel spreadsheet.

2 Export Text File

Click this button to export the data to a tab delimited **.txt** file.

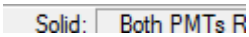
3 Export TIFF

Click this button to export a **.tif** of the graph area exactly as it appears in the window.

4 Graph Settings

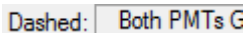
Click this button to turn on and off Clock Time. Clock time will display the elapsed time along the x axis as opposed to timepoint number.

5 Solid Line Channel Selection



This is the selection for the channel that will be displayed as a solid line in the graph. Press the button to select a channel from the drop down menu. The line color corresponds to the region color on the image.

6 Dashed Line Channel Selection



This is the selection for the channel that will be displayed as a dashed line in the graph. Press the button to select a channel from the drop down menu. The line color corresponds to the region color on the image.

7 Dotted Line Channel Selection

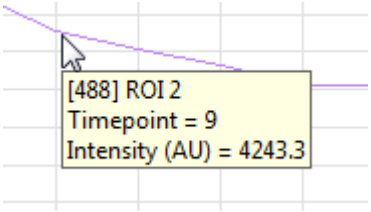
This is the selection for the channel that will be displayed as a dotted line in the graph. Press the button to select a channel from the drop down menu. The line color corresponds to the region color on the image.

8 Current Timepoint

When you click in the white area of the graph, a vertical green line will appear. This line controls the time point displayed in the image view.

9 Cursor over a Line

When you hold the cursor over a region line where it meets a timepoint vertical line, an information window will open as shown below.



10 Note/Event Marker

Red dots along the ***Timepoint*** axis indicate where photomanipulation events took place or notes were added to the image either during or post-capture. Clicking on the dot will move the image to the corresponding time point. Moving the cursor over the dot will display the text of the note.

11 X-axis Zoom Controls

Press these buttons to zoom in and out on the x-axis of the graph.

12 Fit X-axis (Time) to Range

Press this button to set the x-axis range to the number of time points in the associated image.

13 Fit Y-axis (Intensity) to Range

Press this button to set the y-axis range to the intensity range in the associated image.

14 Y-axis Zoom Controls

Press these buttons to zoom in and out on the y-axis of the graph.

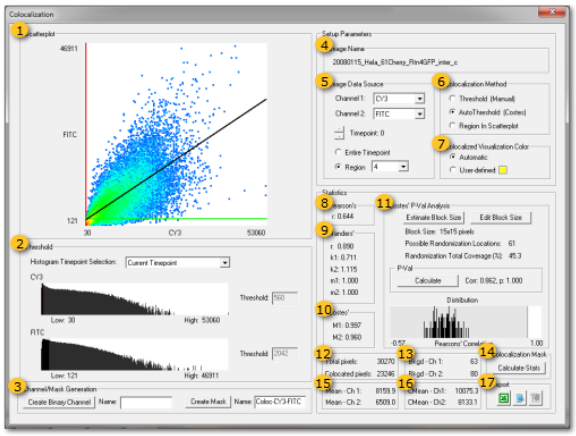
Colocalization window

Colocalization analysis can be performed on 2D and 2D time lapse images by using the ***Colocalization*** dialog. 3D and 4D data can be analyzed in this manner using the ***Interactive Mask Segmentation*** dialog. It is strongly suggested you define a background region before performing this analysis.

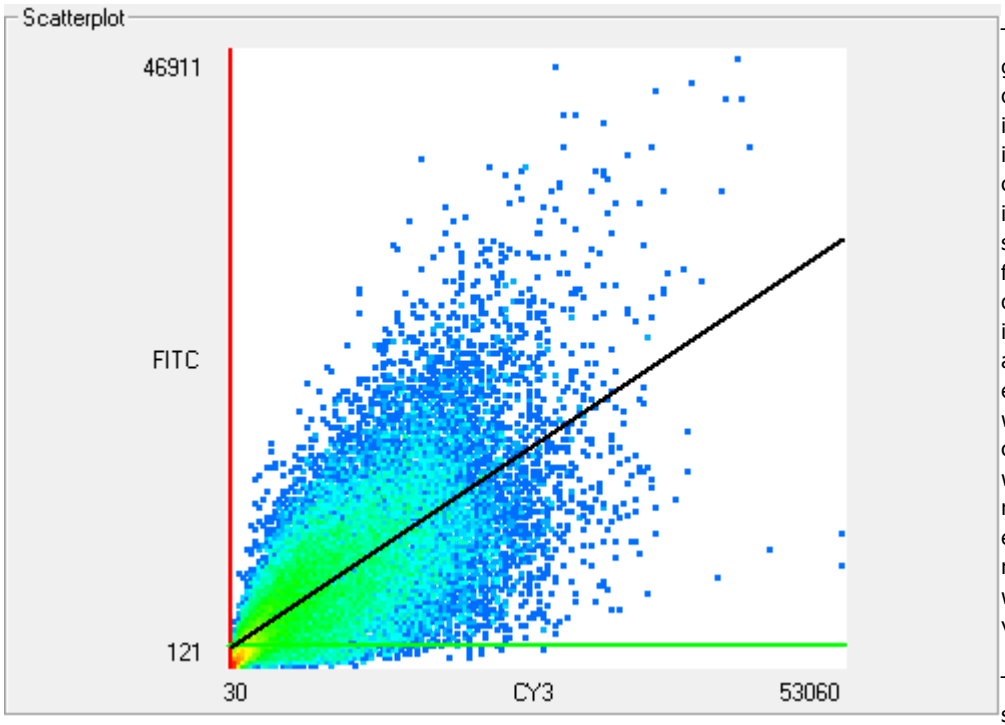
Access in the Evaluate group of the Process tab

See also:

[Interactive Mask Segmentation]



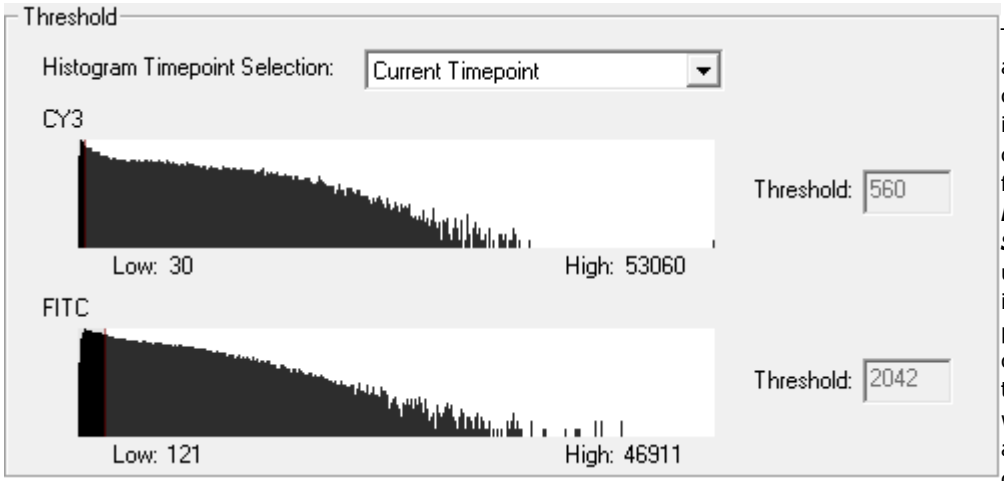
1 Scatterplot group



The ***Scatterplot*** is a graphical representation of the intensity all pixels in channel 1 versus the intensity of all pixels in channel 2 for a whole image, or a sub-selection of pixels from regions. The ordinate is pixels with an intensity value one unit above background in each channel. The points within the scatterplot are colored blue to red where dark blue represents single pixel events and red represents many pixels with identical intensity values for both channels.

The black line in the scatterplot represents a linear regression of the data present in this 2D histogram. The slope of this line is related to the correlation of the two signals. A slope of 1 is defined as perfectly correlated signals where the intensity of both is proportionate in each channel at a given pixel. A slope of -1 is defined as perfectly non-correlated signal where the signals are inversely related. A slope of 0 is defined as random correlation. Thus values between 1 and 0 are positively correlated and signals between 0 and -1 are negatively correlated.

2 Threshold group



The ***Threshold*** panel allows for manual control of the lowest intensity value for each channel to be analyzed for colocalization. ***Histogram Timepoint Selection*** will allow the user to display the intensity histogram profile for either the current time point or all time points. The data within the histograms are background corrected based on the mean intensity within the Bkgd region. The scatterplot will also graph all values in the intensity histograms based on the data selection above. Manual control of the histograms will only be active when the threshold radio button is selected in the data selection option as the colocalization method.

The channel histograms are present below the scatterplot for each channel being analyzed. Its behavior is similar to that for Mask Segmentation.

- Enter the minimum intensity value for colocalization analysis in the ***Threshold*** boxes for each channel.
- The red vertical line in the channel 1 histogram, located below the ordinate of the scatterplot, controls the minimum height of the RED vertical line within the scatterplot.
- The red vertical line in the channel 2 histogram, located parallel to the abscissa of the scatterplot, controls the minimum height of the GREEN horizontal line of the scatterplot.
- The area to be analyzed for colocalization resides in the upper right portion of the scatterplot where the lower limits are defined by the RED and GREEN lines.
- The RED and GREEN lines in the scatterplot can also be individually relocated by a left click and hold on the red vertical line in the histogram for each channel and moving the mouse to redefine the minimum intensity for colocalization analysis to begin. The red lines in each channel histogram will relocate appropriately upon release of the mouse button.

3 Channel/Mask Generation group

The ***Create Binary Channel*** button will add a new channel to the working image. The ***Name*** edit field allows you to name the channel. This channel is binary and is comprised of pixels that have been identified by the ***Colocalization Method*** and will be a binary color based on the ***Colocalized Visualization Color***. This channel is now a part of the working image and can be manipulated as any other channel.

The ***Create Mask*** button will add a new mask to working image mask list. The name of this mask can be entered in the adjacent ***Name*** edit field.

4 Image Name group

Image Name is the working image that is currently being used for colocalization analysis

5 Image Data Source group

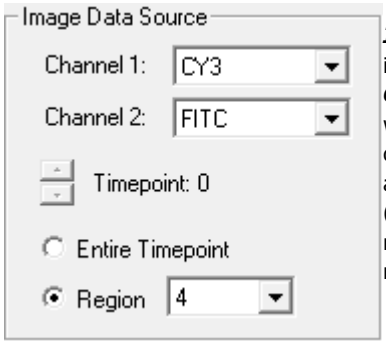
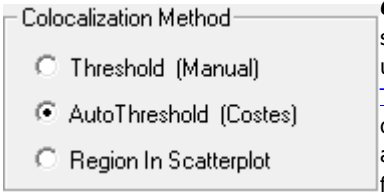


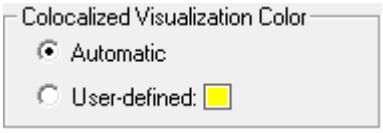
Image Data Source allows the user to determine what properties of the working image are to be utilized for colocalization analysis. The drop down menus for ***Channel 1*** and ***Channel 2*** can be individually changed to the proper channels within the working image for colocalization analysis. The current time point displayed in the scatterplot can be changed by using the up/down ***Timepoint*** arrows. The entire working image (***Entire Timepoint***) or individual regions (***Region***) can be displayed be selecting the correct radio button. Once the ***Region*** radio button is selected, the drop down window will become activated and each region in the working image will be available.

6 Colocalization Method group



Colocalization Method determines the pixels within the scatterplot that will be selected for colocalization analysis. The ***Threshold (Manual)*** button will allow the user to manually identify pixels of interest based on the intensity histogram in the Threshold group. The ***Autothreshold (Costes)*** radio button will use the algorithms described by Costes' et al for the non-biased identification of colocalized pixels, that is an algorithm will set the threshold values automatically. More on this method can be found in Costes' P-Val Analysis group. The ***Region In Scatterplot*** option will allow the user to click and drag to draw a box around points within the scatterplot for colocalization analysis .

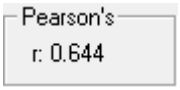
7 Colocalized Visualization Color group



Colocalization Visualization Color allows the user to alter the color of the colocalized pixels in the working image. ***Automatic*** will use the blue mask color. To use a different color, select the ***User-defined*** radio button then press the colored box adjacent to the text. This will open a color table window where you can select any color to represent colocalization.

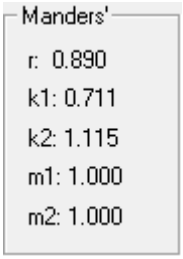
While the color of the individual channels' pixels will remain in a gradient lookup table defined in the working image, those of the highlighted colocalized pixels in the working image will remain binary.

8 Pearson's group



r = Pearson's Correlation Coefficient

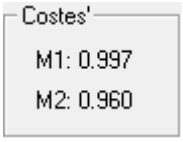
9 Manders' group



r = Manders' overlap Coefficient
k1 = Channel 1 Overlap Coefficient
k2 = Channel 2 Overlap Coefficient
m1 = Channel 1 Colocalization Coefficient

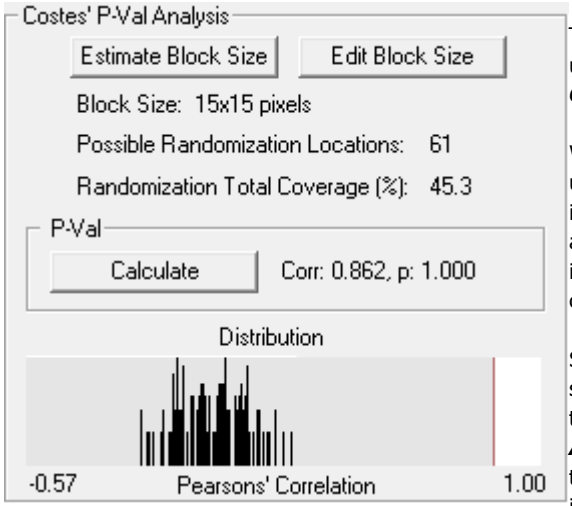
m2 = Channel 2 Colocalization Coefficient

10 Costes' group



M1 = ratio of colocalization of Channel 1:total signal Channel 1
M2 = ratio of colocalization of Channel 2:total signal Channel 2

11 Costes' P-Val Analysis group



The ***Costes' P-Val Analysis*** group is used when ***AutoThreshold (Costes)*** is selected as the ***Colocalization Method***.

When you select the ***AutoThreshold*** radio button, SlideBook will use the algorithms defined by Costes et al to automatically identify all pixels with a ***Correlation Coefficient*** > **0**. This will automatically identify all non-contiguous pixels in the entire image or within a particular region depending on the selection criteria selected in the Image Data Source.

Statistical Significance - In order to calculate the statistical significance of colocalization SlideBook will compare your image to 200 randomized permutations of the pixels identified by the ***AutoThreshold*** and will calculate correlation and P-values of the results. Randomizations are based on block sizes rather than individual pixels as most structures within a specimen encompass a region larger than a single pixel. In fact, diffraction limited point sources smaller than the resolution limit of the microscope are, under most circumstances, larger than a single pixel do to point spread. A logical block size for the randomizations is then related to the point spread function of the optical path utilized. This can be determined by pressing the ***Estimate Block Size*** button. The calculated block size will be displayed and the possible number of rearrangements that can be accomplished with the given size of the data range selected is reported as ***Possible Randomization Locations***.

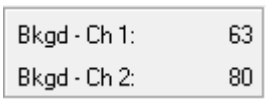
To override the estimated block size or to enter your own block size value, press the ***Edit Block Size*** button. A new window will open. Enter a value for the desired block size, i.e type **10** for a 10x10 block size or type **10x30** for a rectangular block size.

Press the ***Calculate*** button to determine the Correlation and p-Value of the comparison of the original image with the randomizations. A histogram of the measured Pearson's Correlation Coefficients of each randomization will be populated and a vertical red line will represent the Pearson's Correlation Coefficient of the original data.

12 Pixels group

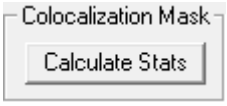
Total pixels – number of pixels in the Image Data Source
Colocalized pixels – number of pixels determined by the Colocalization Method

13 Background group



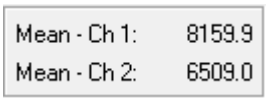
Bkgd – Ch 1 – average intensity of the pixels in the background region for channel 1
Bkgd – Ch 2 – average intensity of the pixels in the background region for channel 2

14 Colocalization Mask group



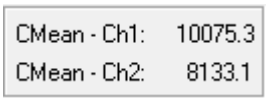
The ***Calculate Stats*** button will automatically generate a mask in the working image encompassing the pixels identified by the ***Colocalization Method***. The Mask Statistics dialog box will open allowing for additional statistics metrics available in SlideBook.

15 Mean group



Mean – Ch 1 – average intensity of the total pixels in channel 1
Mean – Ch 2 – average intensity of the total pixels in channel 2

16 CMean group



CMean – Ch 1 – average intensity of the pixels determined by the colocalization method in channel 1
CMean – Ch 2 – average intensity of the pixels determined by the colocalization method in channel 2

17 Export group

The ***Export to Microsoft Excel™*** button will open an Excel spreadsheet with the data from the colocalization window transcribed.

The ***Export to text file*** button will open a save dialog button and the data from the colocalization window will be saved as an ASCII tab delimited text file upon pressing the save button.

The ***Export as TIFF*** button will save the Colocalization window to a TIFF file

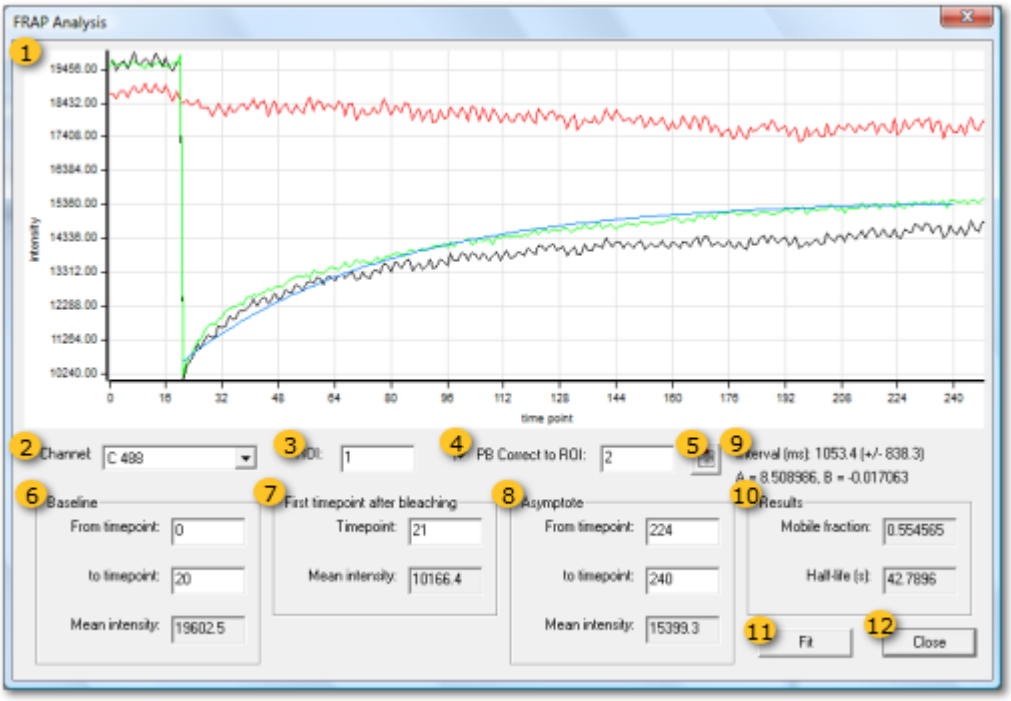
FRAP Analysis window

When analyzing fluorescence recovery after photobleaching, you may find it useful to use the ***FRAP Analysis*** window. This window provides an interactive way to set the important variables for calculating values such as half-life and mobile fraction.

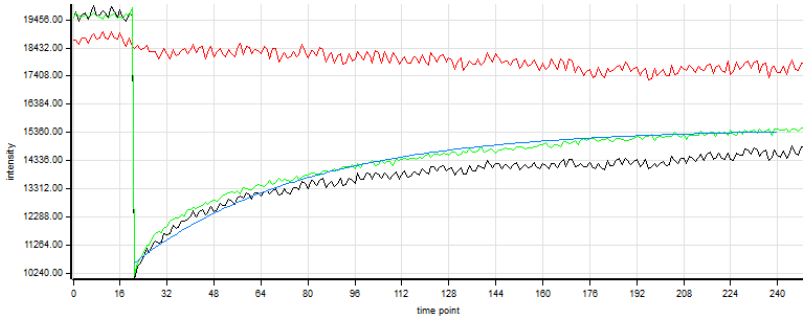
Access in the Evaluate group of the Process tab

See also:

[FRAP Analysis Tutorial]
[Appendix A Concepts of Photobleaching Recovery]



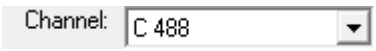
1 Graph area



Graph of background corrected mean intensity over time according to parameters designated in the dialog below. Colored lines are as follows:

Green - Region of interest for analysis designated in [Analysis Region]
Red - Region of interest to be used for photobleach correction as designated in [Photobleach Correction]
Black - Photobleach corrected intensity of analysis region
Blue - Resulting FRAP Analysis, the exponential fit from the first time point after photobleaching to the asymptote.

2 Channel menu



Use the dropdown menu to select the channel used for intensity analysis.

3 Analysis Region

ROI: 1

Enter the value of the region you would like to use for FRAP analyis.

4 Photobleach Correction

☑ PB Correct to ROI: 2

Check the box if you would like to perform photobleach correction on the analysis region. To do so, you must have a region outside of the photomanipulation event designated in the image as shown in the FRAP Analysis Tutorial.

5 Export Tiff

Exports a tiff of the graph as it is currently displayed

6 Baseline Information

Baseline
From timepoint: 0
to timepoint: 20
Mean intensity: 19602.5

Enter the baseline timepoint range. This is start and end timepoints that will be used to calculate intensity before the photomanipulation event took place. When you enter the ***From timepoint*** and ***to timepoint*** values, the ***Mean intensity*** field will be calculated when the ***Fit*** button is pressed. The ***Mean intensity*** will be the mean intensity of the analysis region over the time range specified.

7 First timepoint after bleaching

First timepoint after bleaching
Timepoint: 21
Mean intensity: 10166.4

Enter the first timepoint that was imaged after the bleaching took place. When Fit is pressed, the ***Mean intensity*** of the analysis region will be calculated for this timepoint.

8 Asymptote Information

Asymptote
From timepoint: 224
to timepoint: 240
Mean intensity: 15399.3

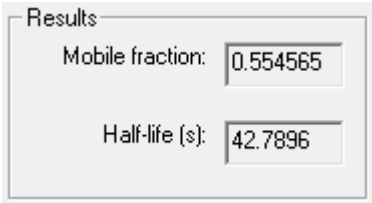
Locate the asymptote where recovery levels off in the graph. Enter a start value for ***From timepoint*** and an end value for ***to timepoint***. When ***Fit*** is pressed, the mean intensity for the analysis region over the time range will be calculated.

9 Analysis Information

Interval (ms): 1053.4 (+/- 838.3)
A = 8.508986, B = -0.017063

When ***Fit*** is pressed, the average timelapse Interval will be calculated along with A and B. A and B are the coefficients determined by the exponential equation of the mobile fraction recovery. Additional information on this can be found in APPENDIX A Concepts of Photobleaching Recovery.

10 Results

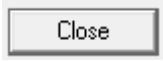


When ***Fit*** is pressed, the Mobile fraction and Half-life will be calculated and displayed here. Refer to APPENDIX A Concepts of Photobleaching Recovery for more information on the calculations used in this analysis.

11 Fit button

Press ***Fit*** to calculate the FRAP Analysis values and draw the blue line on the graph. Refer to APPENDIX A Concepts of Photobleaching Recovery for more information on the calculations used in this analysis.

12 Close



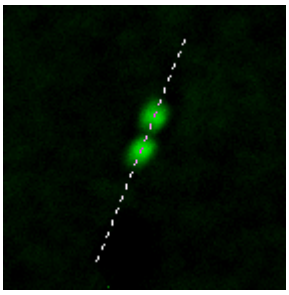
Press the ***Close*** button to close the FRAP Analysis dialog.

Line Intensity window

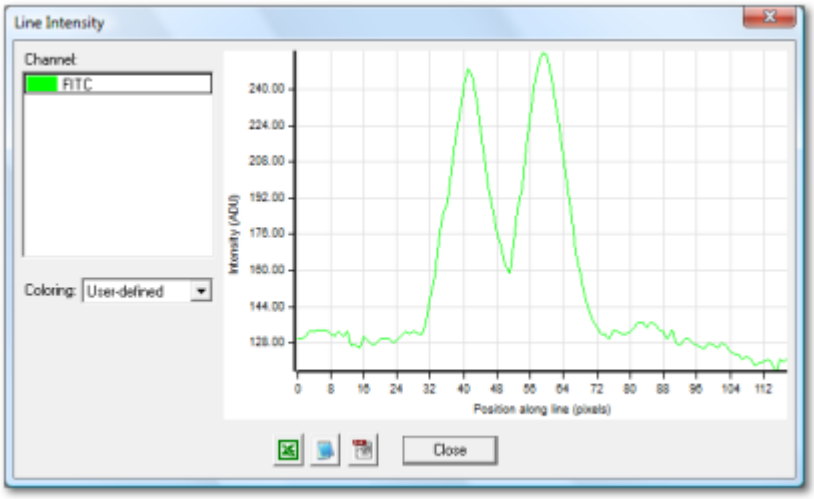
It is often useful to examine intensity information along a single line. This can aid in analysis such as colocalization evaluation or lateral resolution determination.

Access in the Evaluate group of the Process tab.

To view a Line Intensity Profile, open an image and use the Ruler Tool to draw a line for analysis as shown below.



Access by ***Statistics > Line Intensity Profile***



Select the channel to view or export from the ***Channel*** list. To display more than one channel hold the **Ctrl** key while clicking on the desired channels.

Select ***Coloring*** for the graph as desired.

Use the icons to export as follows:

- Opens a Microsoft Excel file containing the graphed data
- Saves a tab-delimited **.txt** file of the graphed data
- Saves a TIFF image file of the graph itself

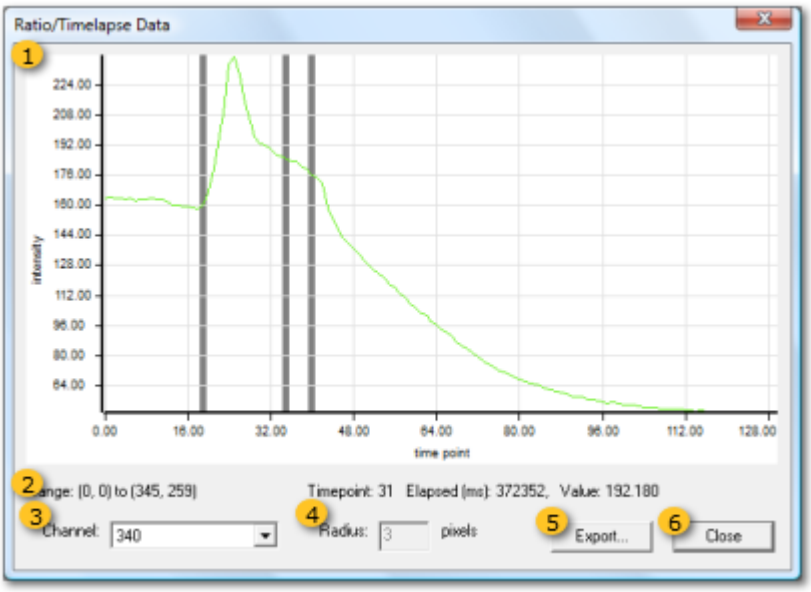
Ratio/Timelapse Data window

A graph of intensity information over time can be brought up independent of marked regions or masks. By accessing the ***Ratio/Timelapse Data*** graph, you will graph the entire image. Alternatively, you can select the ***Point Selection tool*** from the Tool Menu then hold the **CTRL** key while clicking on a cell/pixel. This method will allow you to graph intensity information for a single point with a radius in pixels. Either method will allow you to view and export information for individual channels or a defined ratio.

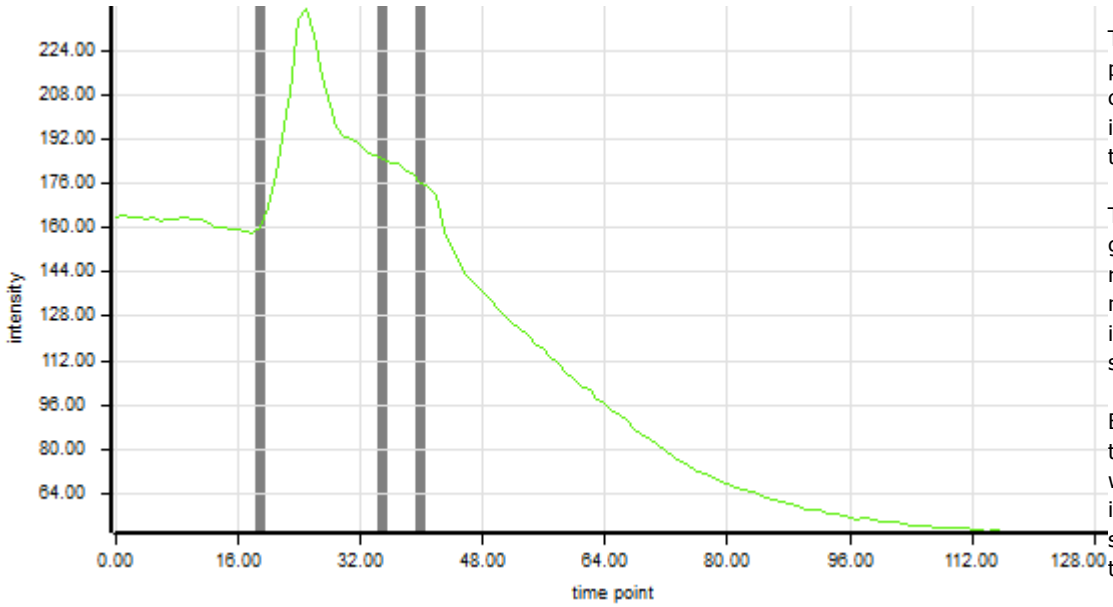
Access in the Evaluate group of the Process tab or ***Point Selection tool*** **+ CTRL +** ***Click*** on image

See also:

Ratio Module: Quick Graphing and Data Export
Photomanipulation Module: Viewing and Analyzing Vector Data



1 Graph area



The graph plots a single channel intensity over time.

The vertical gray lines represents notes in the image data set.

By clicking in the graph, you will move the image to the selected timepoint.

2 Image Information

Range: (0, 0) to (345, 259) Timepoint: 31 Elapsed (ms): 372352, Value: 192.180

Range: Displays the area that is being graphed or the pixel location that is the center of the circle being graphed.
Timepoint: Displays current timepoint selected on the graph and in the image.
Elapsed (ms): Displays the amount of time elapsed until the the current time point.
Value: Displays the intensity or ratio value for the current time point.

3 Channel Selection

Select a channel to graph. If a ratio channel is configured, you may also select **Ratio**.

4 Radius field

When you open the graph using the ***Point Selection tool*** **+** CTRL **+** ***Click*** on image, you may enter a value for the ***Radius*** used to calculate mean intensity plotted on the graph.

5 Export button

Press ***Export*** to export a tab-delimited **.txt** file.

6 Close button

Press ***Close*** to close the window.

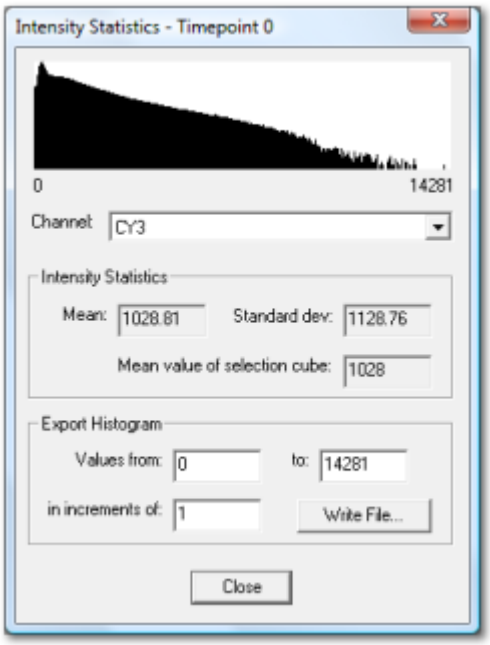
Intensity Statistics

You may find it useful to review or export the overall intensity information for an image. You may quickly get a statistical snapshot of your image using the ***Intensity Statistics*** function. A dialog box will display a histogram of the intensities of the entire image by channel along with the mean intensity and standard deviation. You must first open a view of your image.

Access in the Evaluate group of the Process tab, ***Extract Measurements > Intensity Statistics***

See also:

- 16-bit Tiff Export window
- Renormalize Image window
- Segment Image window



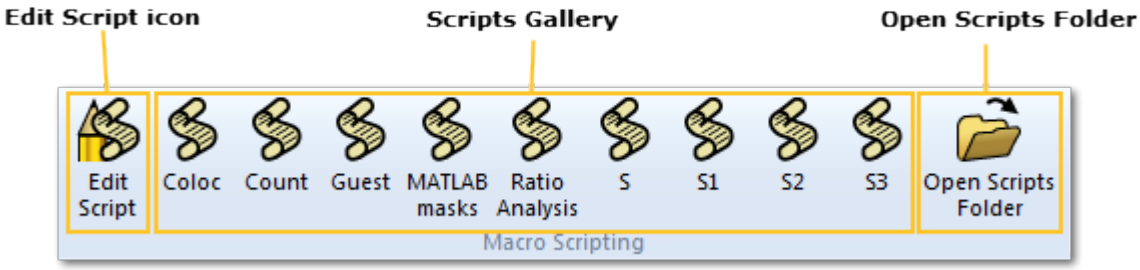
Select a ***Channel*** from the dropdown menu. Observe the histogram and statistics of the selected channel at the displayed time point.

If desired, ***Export Histogram*** values. Enter values for the intensity range to export as well as increments for data grouping. Press ***Write File*** to create a tab delimited **.txt** file. The file will have a voxel count for each pixel value or grouping of pixel values.

When finished, click ***Close***.

Scripting Tab

The Scripting tab allows you to write and edit scripts, execute scripts, and open the scripts folder.



Edit Script icon opens the Edit Script window to edit, create, and test scripts.

Scripts Gallery displays saved scripts. Click on a named script to load it into the Edit Script window and execute.

Open Scripts Folder is a shortcut to the directory where scripts are saved. Use this icon to rename, save, copy, and further manage scripts.

NOTE: Additional information regarding script writing can be find in the Macro Scripting portion of this help system.

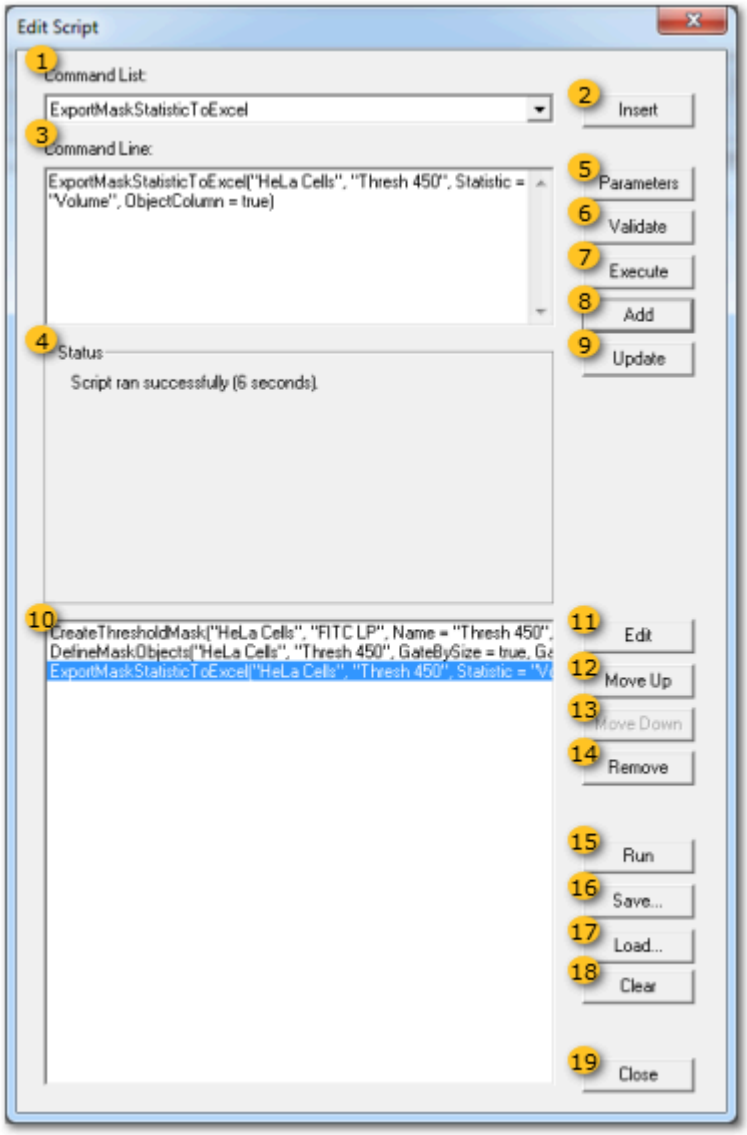
Edit Script window

SlideBook includes a scripting interface for writing command language that automates analysis. Complete information on creating scripts and the SlideBook scripting language can be found in the Macro Scripting section of online help. It is highly recommended that you review that section for a thorough understanding of the features available.

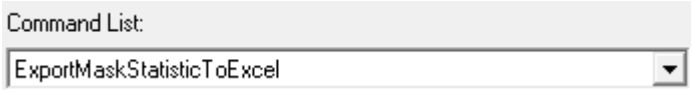
Access in the Scripting tab, ***Edit Script***

See also:

- Using the Edit Script Dialog
- Editing a Command
- Building a Script
- Macro Command Language



1 Command List drop down

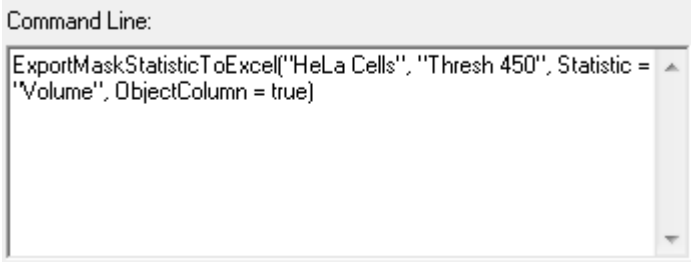


Select a command from the drop down menu. When you click ***Insert***, the command will be entered in the ***Command Line*** edit field. The ***Status*** field will display parameters used in the command.

2 Insert button

Insert will add the macro command to the ***Command Line*** field. You will need to enter parameters for the command, test the command, then add it to the script.

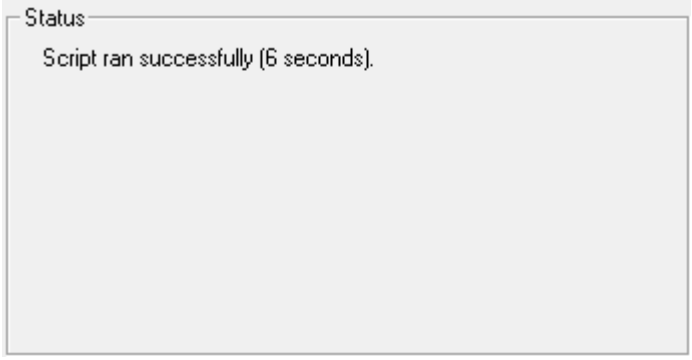
3 Command Line Edit field



This is the field you will use to set up and test the parameters used with a command to create a line of the script. After you ***Insert*** a command, press ***Parameters*** to populate the available parameters. Customize the parameters to suit the data then test the command by pressing ***Validate*** and ***Execute***.

Complete information on commands and parameters can be found in Macro Command Language.

4 Status Display



The ***Status*** display area will give details regarding the active command or script. For example, when you ***Insert*** a command in the ***Command Line*** edit field then press ***Parameters***, a list of available parameters will populate this area. When you ***Execute*** a command or script, the success or failure will be reported in this field.

5 Parameters button

Pressing this button will display a list of available parameters for the selected command in the ***Status*** display area. It will also fill in blank parameters in the ***Command Line*** edit field. You will need to customize these parameters based on the Macro Command Language.

6 Validate button

Validate will test the command line and parameters entered. If an error is found, you can count positions starting at **0** with **0** being the first parameter after the "**(**".

7 Execute button

Execute will run the command line and return either a success or failure value.

8 Add button

Add

Add will add the command line text to the ***Script*** list.

9 Update button

Update

If an existing line in the ***Script*** list has been edited using the command list, you may update the command in the ***Script*** list by pressing ***Update***.

10 Script list

```
CreateThresholdMask("HeLa Cells", "FITC LP", Name = "Thresh 450",
DefineMaskObjects("HeLa Cells", "Thresh 450", GateBySize = true, Ga
ExportMaskStatisticToExcel("HeLa Cells", "Thresh 450", Statistic = "V
```

The ***Script*** list displays all commands that have been added to create an automated process. Commands in this list can be edited and their positions can be changed. The text in this field is what constitutes a macro script. Scripts can be saved and loaded to run at future times as well as linked to icons for simple execution from the main SlideBook interface. Please review Macro Scripting for more information on this.

11 Edit button

Edit

Select a command from the ***Script*** field and press ***Edit*** to add it to the ***Command List*** field for editing.

12 Move Up button

Move Up

Select a command from the ***Script*** field and press ***Move Up*** to move it up the list in the Script field.

13 Move Down button

Move Down

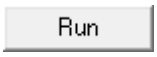
Select a command from the ***Script*** field and press ***Move Down*** to move it down the list in the Script field.

14 Remove button

Remove

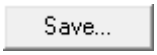
Select a command from the ***Script*** field and press ***Remove*** to remove it from the Script field.

15 Run button



Press ***Run*** to execute the script.

16 Save button



Press ***Save*** to save the script for future use.

17 Load button

Press ***Load*** to load a previously saved script.

18 Clear button

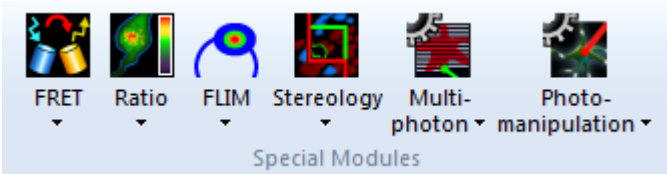
Press ***Clear*** to clear the entire script from the ***Script*** field.

19 Close button

Press ***Close*** to close the dialog.

Modules Tab

In addition to Deconvolution, there are many available advanced software modules available in SlideBook. These include:



FRET

FRET Settings
Compute FRET Bleedthrough
FRET Channel Math
Physical FRET Channel from Virtual

Ratio

Ratio Settings
Intracellular Calibration
Ratio Calibration Guide
Extract from Ratio Stream

FLIM

FLIM Settings
Lifetime Histogram
Create Single-Tau FLI Channel
Compute FLIM-FRET
Gaussian Filter
Compute Phase Flat Field
Apply Phase Flat Field
Calibrate Lifetime
Recalibrate Lifetime
Clear Calibration
Intensifier Settings
Homodyne Demo
Test

Stereology

Setup
Count Offline
Save Results
Delineate Features using CellNet

Multiphoton

Multiphoton Laser Control
Scanning Diagnostics
Calibrate Pockels' Cell

Phasor Setup
Calibrate SLM

Photomanipulation

FRET

SlideBook's FRET Module adds functionality that simplifies a wide variety of quantitative techniques. Measurement of FRET allows you to determine whether or not two molecules are colocalized at a distance less than 100 angstroms, enabling measurements below the optical resolution of a light microscope. FRET is a distance-dependent interaction between the electronic excited states of two dye molecules where the excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon. FRET experiments may be performed on live or fixed cells. SlideBook supports three FRET methods: Sensitized Emission/Direct FRET (both Two-Channel Corrected and Three-Channel Corrected), Acceptor Photobleaching FRET, and Anisotropy FRET. For more information on FRET methods, analysis, and configuration, please review the help sections on the FRET Module. Alternatively, you may review the following user interface pages.

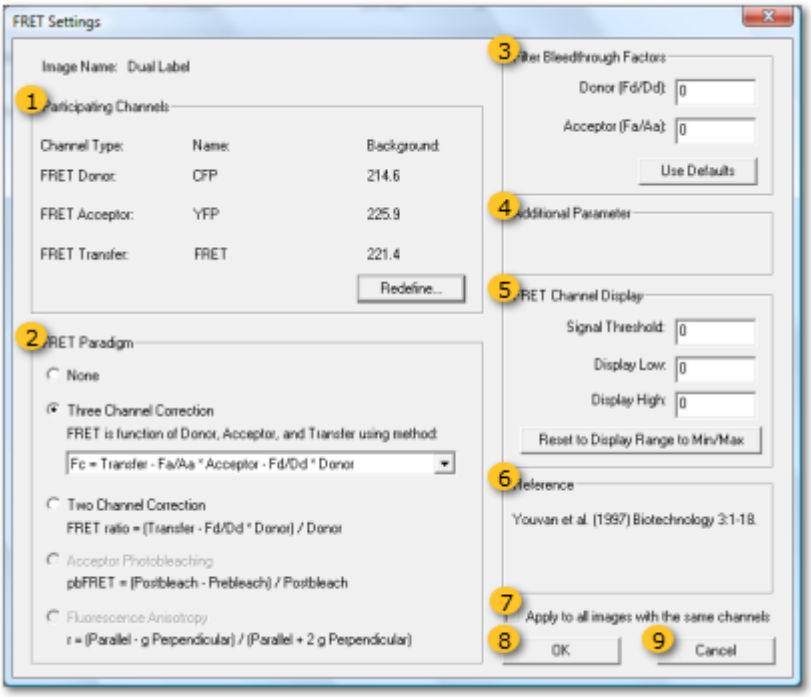
FRET Settings window

The ***FRET Settings*** window is used to select a FRET paradigm and values associated with that paradigm. In this dialog, you can assign channels for calculations, enter bleedthrough factors, choose and equation for calculation, and adjust the display of FRET. You may also view the reference used for the FRET paradigm selected.

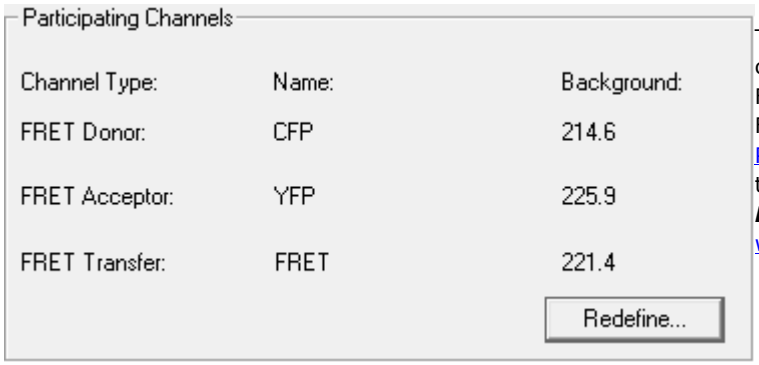
Access in the Modules tab under ***FRET > FRET Settings***

See also:

- Redefine FRET Channels window
- FRET System Configuration
- Three Channel Corrected FRET
- Two Channel Corrected FRET
- Acceptor Photobleaching FRET
- FRET Module: Appendix B: Direct FRET Calculations
- FRET Module: Appendix C: Photobleaching FRET Calculations

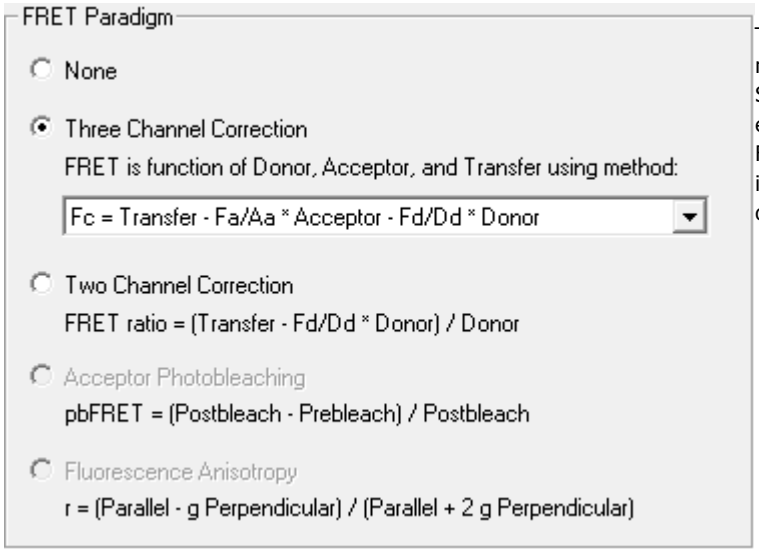


1 Participating Channels group



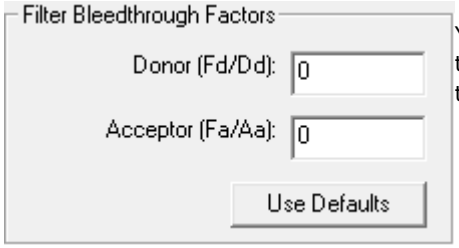
The ***Participating Channels*** group lists channels that have been identified to be used in FRET calculations. Channels can be assigned as FRET channels in the Filter Configuration Parameters Dialog. If you would like to change the participating channels, you may click ***Redefine*** to open the Redefine FRET Channels window.

2 FRET Paradigm group



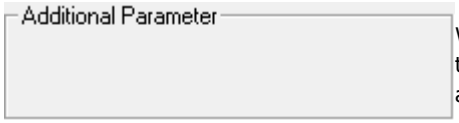
The ***FRET Paradigm*** group lists several methods of FRET that are available in SlideBook. Additional information on these equations can be found in the appendix of the FRET Module portion of help. When a paradigm is selected, the paper about the method is displayed in the Reference group to the right.

3 Filter Bleedthrough Factors group



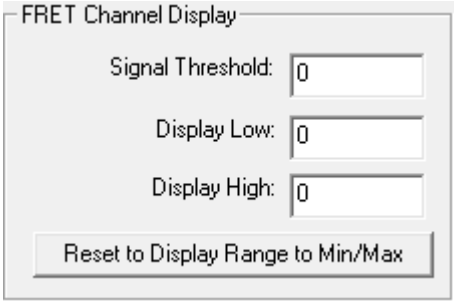
You may manually enter values for channel bleedthrough factors for both the ***Donor*** and the ***Acceptor***. Alternatively, click ***Use Defaults*** to enter the values calculated using the FRET Bleedthrough Computation window.

4 Additional Parameter group



When certain FRET paradigms are selected, additional parameters are in this area. One example is for E-FRET, a G parameter entry field becomes available.

5 FRET Channel Display group



Once FRET calculations have been performed, you may adjust the display of the FRET data in this area by adjusting parameters as listed below:

Signal Threshold – The intensity above which FRET values will be calculated. This threshold is background subtracted. Therefore, if you select a value of 50, any pixel that is above 50 after background subtraction will be used for calculating FRET.

Display Low – The value that corresponds to black in RGB display or blue in pseudocolor display.

Display High – The value that corresponds to saturated color in RGB display or red in pseudocolor display.

6 Reference group

Reference

Youvan et al. (1997) Biotechnology 3:1-18.

The ***Reference*** area cites references used for calculations selected in ***FRET Paradigm***.

7 Apply to all images with the same channels check

☐ Apply to all images with the same channels

Check this box to apply the same FRET settings to all images in the **.sld** with the same channels.

8 OK button

OK

Click ***OK*** to apply settings and close the dialog.

9 Cancel button

Cancel

Click ***Cancel*** to discard changes and close the dialog.

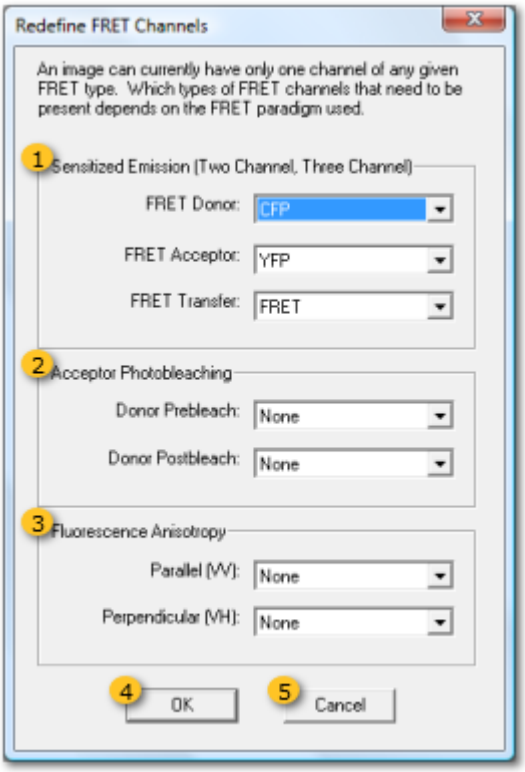
Redefine FRET Channels window

At times, you may wish to analyze FRET on imported imaged or on images acquired with channels that were not previously defined as FRET channels. When you do so, you will need to identify channels for specific FRET techniques using the ***Redefine FRET Channels*** window.

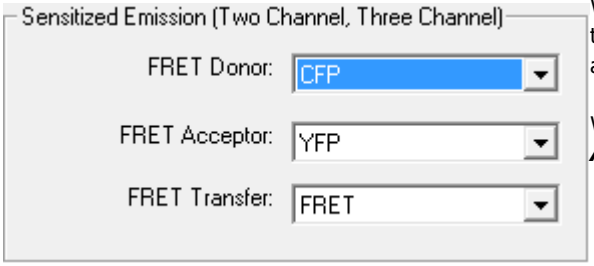
Access in the Modules tab under ***FRET > FRET Settings > Redefine***

See also:

- FRET Settings window
- FRET System Configuration
- FRET Module



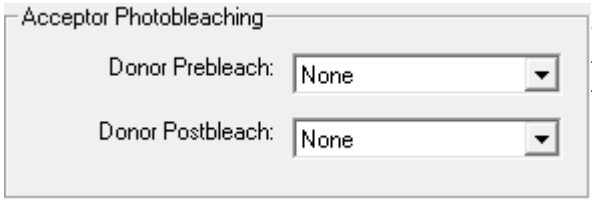
1 Sensitized Emission (Two Channel, Three Channel) group



When computing ***Sensitized Emission FRET***, select the filter from the dropdown menu to use for the ***FRET Donor***, ***FRET Acceptor***, and ***FRET Transfer*** channels.

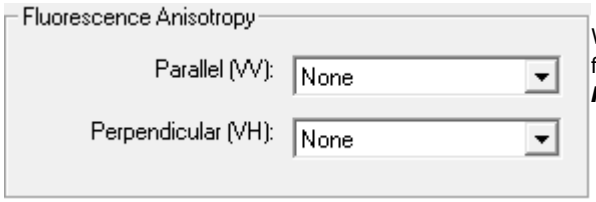
When computing ***Two Channel Corrected FRET***, set ***FRET Acceptor*** to **None**.

2 Acceptor Photobleaching group



When computing ***Acceptor Photobleaching FRET***, select the filter to use for ***Donor Prebleach*** and ***Donor Postbleach*** from the dropdown menus of channels in the image.

3 Fluorescence Anisotropy group

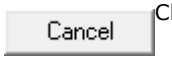


When computing ***Fluorescence Anisotropy FRET***, select the filters from the dropdown menu for ***Parallel (VV)*** and ***Perpendicular (VH)***.

4 OK button

Click ***OK*** to apply changes and close the dialog.

5 Cancel button



Click ***Cancel*** to discard changes and close the dialog.

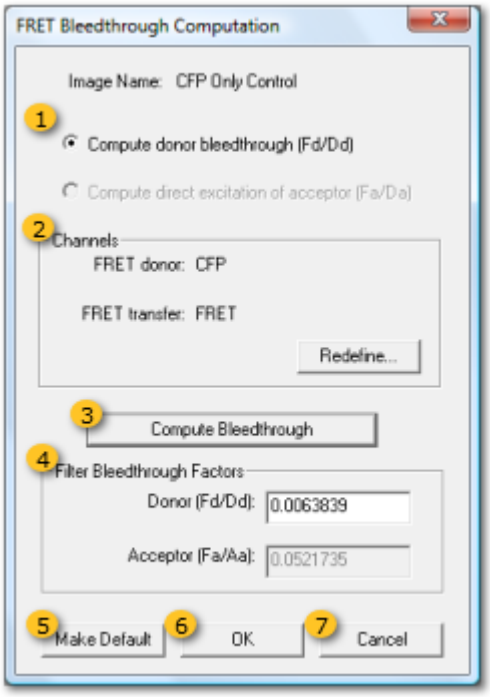
FRET Bleedthrough Computation window

In order to perform direct FRET, you must be able to calculate bleedthrough coefficients, or crosstalk, for your particular filter sets. To calculate bleedthrough coefficients, two control samples must be imaged: one that contains donor only, and one that contains acceptor only. Once these images have been obtained, you may use the ***FRET Bleedthrough Computation*** dialog to set default bleedthrough coefficients.

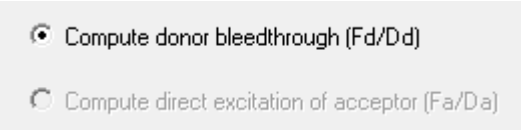
Access by opening an image, segmenting a mask on the desired channel, setting the background, then go to the Modules tab under ***FRET > Compute FRET Bleedthrough***.

See also:

FRET Settings window - Filter Bleedthrough Factors
Performing Bleedthrough Calculations

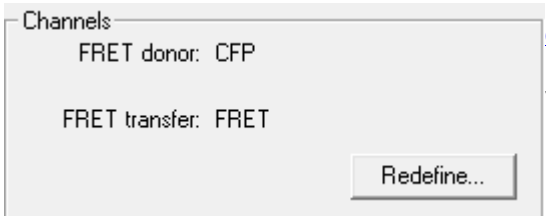


1 Compute Bleedthrough Selection



Select the bleedthrough you would like to calculate. When FRET channel types are assigned in Filter Configuration, the appropriate selection will be made for you depending on whether the donor or acceptor channel is present.

2 Channels group

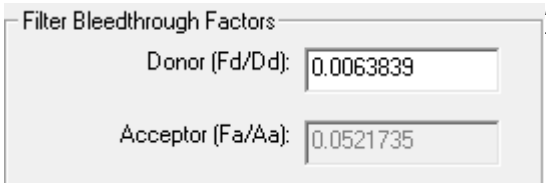


Depending on the associated channel type assigned in Filter Configuration, the relevant channels will be listed for the type of bleedthrough you are calculating. If this information is incorrect, you may press Redefine to manually assign channels.

3 Compute Bleedthrough button

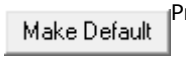
Click ***Compute Bleedthrough*** to calculate the selected bleedthrough.

4 Filter Bleedthrough Factors group



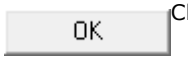
Filter Bleedthrough Factors lists the computed bleedthrough factors. The active computation will have a white background.

5 Make Default button



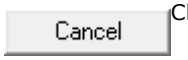
Press ***Make Default*** to use the computed bleedthrough factor as a default value in FRET Settings.

6 OK button



Click ***OK*** to apply bleedthrough factors and close the dialog.

7 Cancel button



Click ***Cancel*** to discard changes and close the dialog.

Ratio

In order for these software functions to be enabled, you must purchase the SlideBook Ratio Module. The Ratio Module enables functionality within SlideBook to create numerator and denominator channel pairs, calibrate using solution based in vitro or intracellular techniques, and perform data analysis and export both real-time and post capture. A complete review of ratio imaging can be found in the Ratio Module portion of this user's guide.

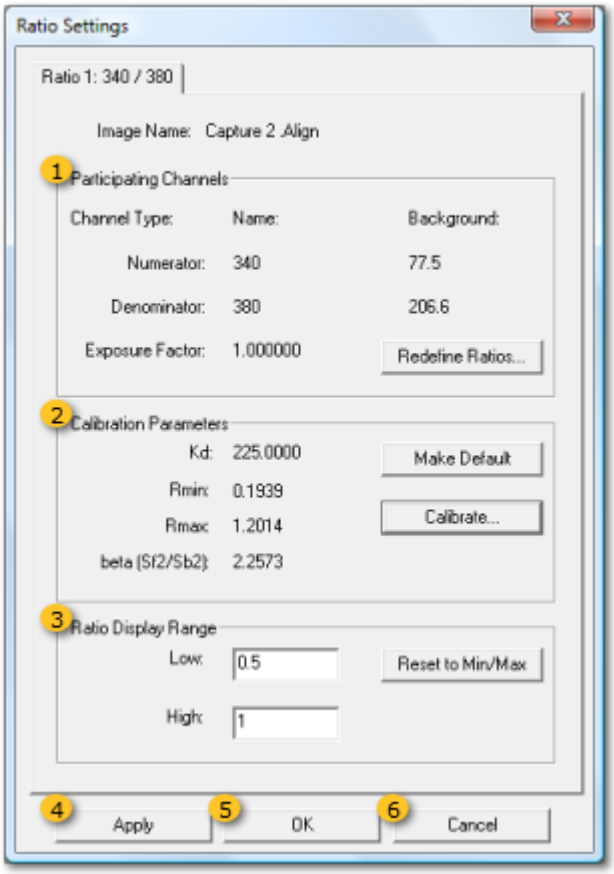
Ratio Settings window

SlideBook lets you adjust, if necessary, many of the settings that go into post-acquisition display and analysis.

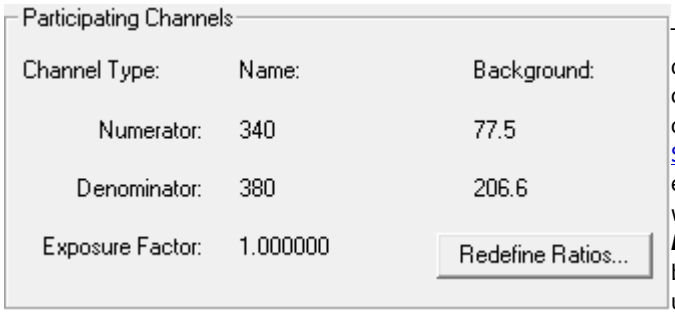
Access in the Modules tab under ***Ratio > Ratio Settings***

See also:

Ratio Module: Ratio Settings
Ratio Module: TUTORIAL: Using Ratio Settings
Ratio Module: Intracellular Calibration
Ratio Module: TUTORIAL: Intracellular Ratio Calibration

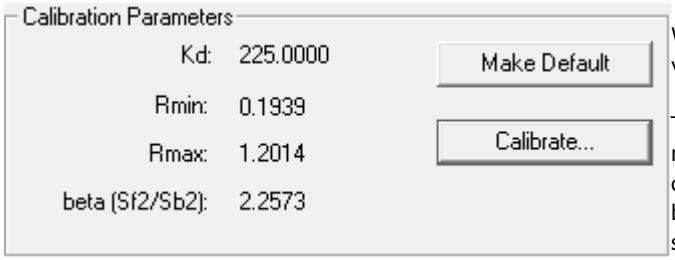


1 Participating Channels group



The ***Numerator*** and ***Denominator*** channels are displayed for a given ratio as designated during system configuration. Background values are shown for each channel when a background region is defined (see Selecting Regions of Interest). Additionally, the ratio of exposure times for the numerator and denominator wavelengths is given; this is called the ***Exposure Factor***. The channels used for calculating the ratio can be edited by selecting ***Redefine Ratios***, which brings up the Add Ratio Information window.

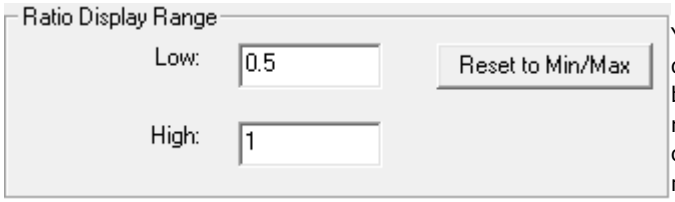
2 Calibration Parameters group



When ratio calibration has been performed calibration values are displayed in this section.

The Ratio Calibration Guide may be used to calibrate or recalibrate an image by selecting ***Calibrate***. For calibrating the ratio, you will need to image the background, ion free indicator solution, and ion saturated indicator solution. Alternatively, Intracellular Calibration is available. Both of these calibration techniques are discussed in greater detail in the Ratio Module, Ratio Calibration.

3 Ratio Display Range group

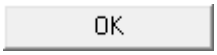


You may select the range of ratio values that are displayed in any view of the ratio image. Any ratios below the ***Low*** value are displayed as blue, and any ratios above the ***High*** value are displayed as red. This operation will not affect the underlying data. Learn more about ratio display in TUTORIAL: Using Ratio Settings.

4 Apply button

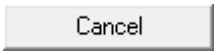
Applies changes to data/display.

5 OK button



Applies changes and closes the window.

6 Cancel button



Discards changes and closes the dialog.

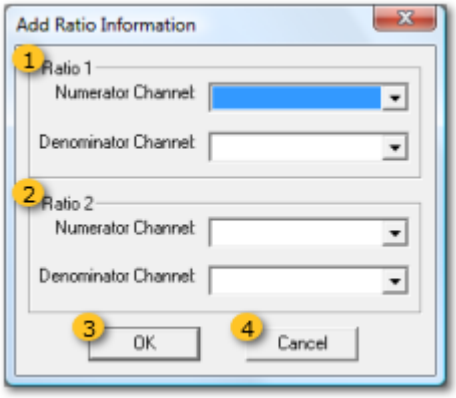
Add Ratio Information window

This dialog is used to manually assign channels to a ratio pair. You may choose to do this if you are analyzing data not captured in SlideBook or if you wish to see ratio information for channels not configured as ratio numerator and ratio denominator.

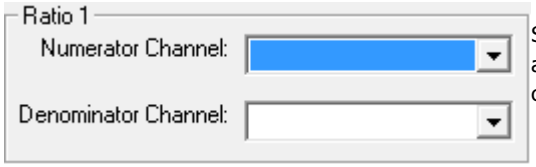
Access in the Modules tab under ***Ratio > Ratio Settings > Redefine Ratios***

See also:

- Ratio Setting window
- Ratio Module: Ratio System Configuration
- Ratio Module: Defining Ratio Channels Tutorial

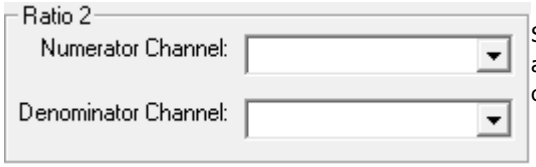


1 Ratio 1 group



Select a channel from the dropdown menu to assign the ***Numerator*** and ***Denominator*** for ***Ratio 1***. These channels will be used in ratio calculations and calibrations.

2 Ratio 2 group



Select a channel from the dropdown menu to assign the ***Numerator*** and ***Denominator*** for ***Ratio 2***. These channels will be used in ratio calculations and calibrations.

3 OK button

Press ***OK*** to apply changes and close the dialog.

4 Cancel button

Press ***Cancel*** to discard changes and close the dialog.

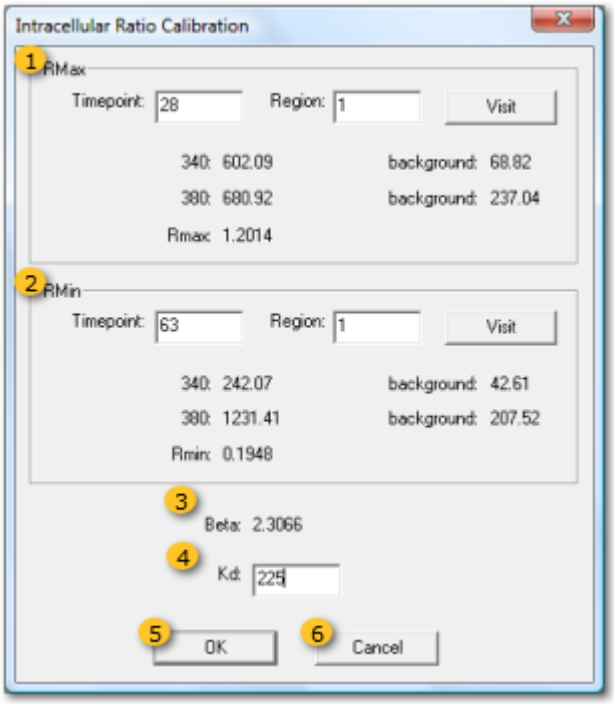
Intracellular Ratio Calibration window

The ***Intracellular Calibration Guide*** in SlideBook allows you to select representative regions in a time series and use them as the basis for determining the maximum and minimum ratios that are observed within cells.

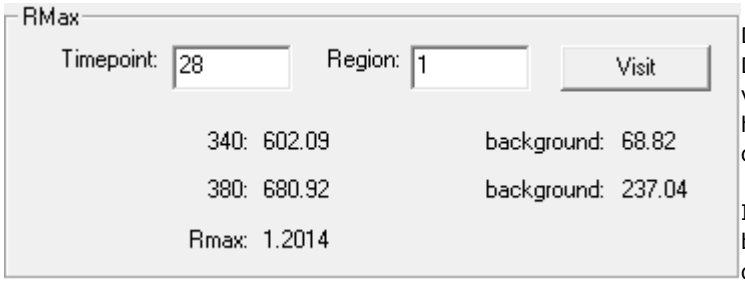
Access in the Modules tab under ***Ratio > Intracellular Calibration***

See also:

Ratio Manual: Intracellular Calibration
Ratio Manual: TUTORIAL: Intracellular Ratio Calibration
Ratio Manual: Solution-based in vitro Calibration
Ratio Manual: Ratio Settings



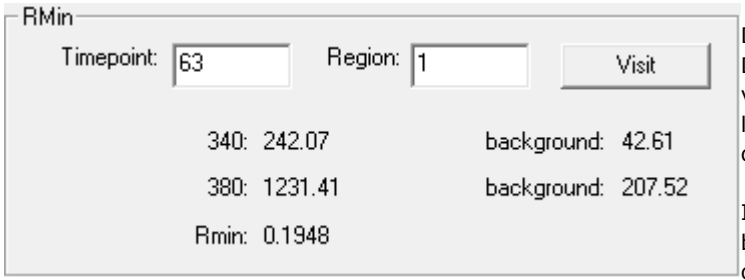
1 RMax group



Determine the highest ratio value in the data set. Draw a region encompassing this area. Enter a value for the ***Timepoint*** where the ratio is highest. Enter the number of the ***Region*** you drew to encompass the area.

Intensity of each channel with the associated background value will be calculated and displayed. The R_{max} will also be calculated.

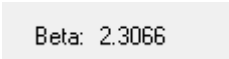
2 RMin group



Determine the lowest ratio value in the data set. Draw a region encompassing this area. Enter a value for the ***Timepoint*** where the ratio is lowest. Enter the number of the ***Region*** you drew to encompass the area.

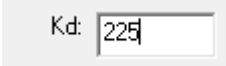
Intensity of each channel with the associated background value will be calculated and displayed. The R_{min} will also be calculated.

3 Beta Readout



Beta is calculated as the ratio of the denominators of the minimum and maximum conditions.

4 Kd Field



Enter the K_d in this field. K_d is the dissociation constant of the fluoroprobe.

5 OK button

Press ***OK*** to apply the calibration and begin reading concentration information instead of ratio values.

6 Cancel button

Press ***Cancel*** to discard changes and close the dialog.

Ratio Calibration Guide

When performing solution-based in vitro ratio calibration, SlideBook provides a guide to walk you through capturing images of buffer solutions. For more on this topic, please review one of the following topics in the Ratio Module help files:

Ratio Calibration - General overview on types of calibration available in SlideBook
Solution-based in vitro Calibration - Information regarding setup and samples required for solution-based in vitro calibration
Generating Ratio Calibration Data - A walkthrough of using the Ratio Guide

FLIM

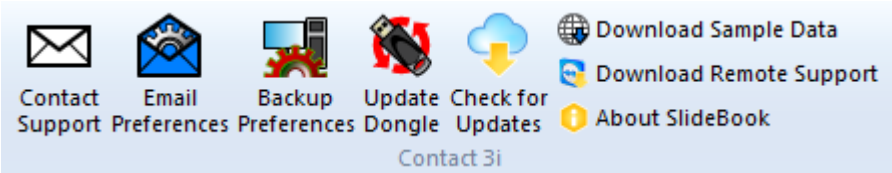
Please review the FLIM Module section for information on using FLIM user interface dialogs.

The following topics are available:

Lifetime Acquisition
Notation and Units
Lifetime Data Format
FLI Hardware Configuration in SlideBook
Control and Calibration of FLIM
Acquiring and Viewing a Lifetime Image
Creating a Single-τ Channel
The Lifetime Histogram
Calculating FRET using Lifetime
Graphing Lifetime

Support Tab

The Support tab provides access to the most common technical support related functions.



Contact Support Opens the web form to contact 3i technical support

Email Preferences Sends the system configuration files to 3i for cloud backup and/or technical support and troubleshooting. For more info, review Working with 3i Support.

Backup Preferences Opens a directory browser to backup the system preferences on your computer or local backup system

Update Dongle Updates the software license key (or dongle) when support contracts or additional features are purchased through the Update Dongle interface.

Check for Updates Check for SlideBook software version updates

Download Sample Data Download sample .sld files used in help tutorials

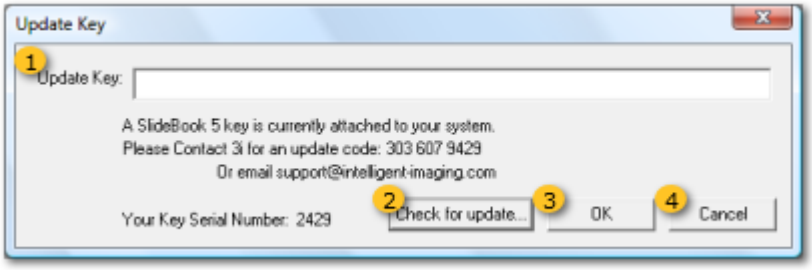
Download Remote Support Download TeamViewer software for 3i support sessions

About SlideBook View information about SlideBook license, version, support contract dates, and author information as well as access patent and legal information

Update Dongle window

When software support has been extended or modules have been added to the license, an update of the software key (also called a dongle) needs to be performed. To do so, you may manually copy and paste the update string sent by 3i Support or you can obtain the string directly from the 3i database if the computer is connected to the internet.

See also:



1 Edit field

Update Key:

If the computer is not connected to the internet, copy and paste the update string provided in this field. It is important that all characters are copied with no additional spaces or characters.

2 Check for update... button

Check for update...

If the computer is connected to the internet, simply click ***Check for update***. The update string will be copied from the 3i server and pasted into the ***Update Key*** field.

3 OK button

OK

Click ***OK*** to apply the update and close the window.

4 Cancel button

Cancel

Click ***Cancel*** to discard changes and close the window.

Working with 3i Support

When working with 3i's Technical Support group, you may be asked to allow remote access of your computer or send important configuration files. Instructions for these tasks are below.

NOTE: Internet connection is required for the operations described below.

Prepare for Remote Access Session

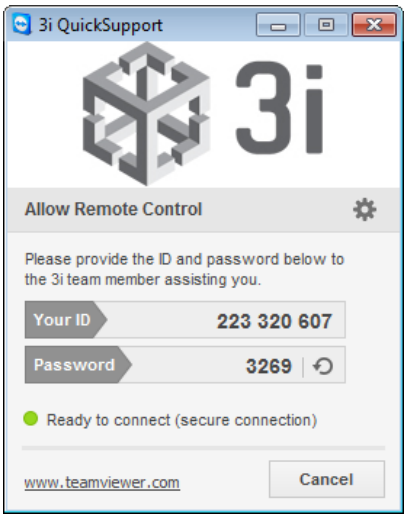
1. Go to ***Support tab > Download Remote Support***.*

2. The TeamViewer **.exe** file download will begin. Double click to start the installation then click ***Run*** when prompted.

3. ***3i QuickSupport*** will launch.

4. Copy and paste the codes for ***Your ID*** and ***Password*** into a reply email to your support case when you are ready to begin the remote access session.

*To download TeamViewer directly, please visit https://www.intelligent-imaging.com/teamviewer.php



Send Files to Support

1. Go to ***Support tab > Email Preferences***.*

2. The ***3i Technical Support*** dialog will open

3. Fill in ***Your email address***. Enter the ***Case ID*** if you know it. The case ID can be found in parentheses in the subject line of your support email. Add any ***Additional comments*** as desired. These may include specific dates of crashes or other information about your system or case.

4. Check the boxes as follows:

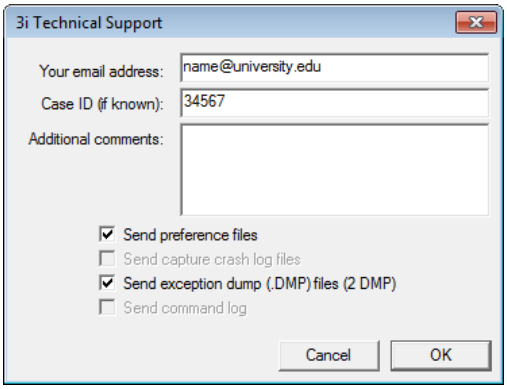
   Send preferences files - sends configuration files to 3i cloud for system backup and support processes.

   Send capture crash log files - sends information about captures that crashed before completion; only available if capture crash log files exists.

   Send exception dump - sends crash logs from the previous 60 days. Only available if **.dmp** files exists.

   Send command log - ***Hardware Properties > SlideBook > Enable Command Log*** enables SlideBook's logging feature. It logs commands and errors and sending this file can aid the developers in tracking down issues. Only available if the log file exists.

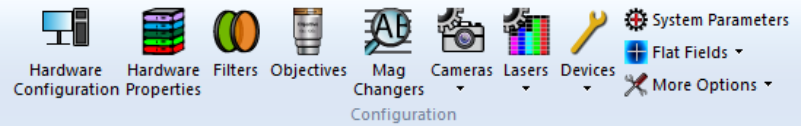
5. Click ***OK*** to send requested files.



*In SlideBook 5.5 and 5.0, access this dialog from ***Help > Email Support***.

Administration Tab

The Administration tab provides access to system configuration tools and other advanced administrator tasks.



Hardware Configuration

Opens Hardware Configuration dialog to designate the hardware to be controlled by SlideBook.

Hardware Properties

Open Hardware Properties dialog to specify details regarding system configuration.

Filters

Open Filter Configuration dialog where imaging and viewing channels are configured.

Objectives

Opens the Configure Objectives dialog where specifications are entered for the system objective lenses.

Mag Changers

Enter parameters for Magnification Changers, either physical lenses or effective magnification changers included in other hardware.

Camera Settings

Compute Camera Registration
Configure Andor iXon
Reset PICAM Camera
Configure PVCAM

Laser Settings

Calibrate Laser Power
Calibrate Pockels' Cell

Devices

AOTF Configuration Information
Calibrate Polychrome
Configure Optical Metadata
Set Yokogawa Disk Speed

System Parameters

System Parameters includes general system settings not covered by other configuration dialogs including system password, automatically check for updates, and automatically backup preferences.

Flat Fields menu

Collect Flat Fields
Manage Flat Fields
Smooth Flat Field

More Options menu

Open User Preferences Folder - Opens directory where the user configuration files are stored.
Open System Preferences Folder - Opens directory where the system configuration files are stored.
Open Documentation Folder - Obsolete command, help documentation is access by F1 or the help icon.
Compute Optimal InFocus Setting - Tests InFocus Settings on sample.
Compute Autofocus Metric - Tests Autofocus Settings on sample.
Get Slide File Version - Determines SlideBook version used to capture or save .sld file.

Recover SLD File - Used to recover data in case of fatal error.
Analyze Log File - Displays statistics about SlideBook use, only available when logging is enabled. Provides access to open log file for viewing. Contact support for more information.
Reset Registry - Resets all window positions to the default,
Copy Optics Information - Copies optical information (accessed in Image Info/Edit Info windows) from current image to all images in **.sld**.
Compare Property Files - C0mpares configuration files to track changes.
Compare Property File Against Current - Compares configuration file to current configuration to detect changes.

Hardware Configuration

The ***Hardware Configuration*** dialog is where SlideBook is directed to communicate with hardware. It is important not to modify this configuration unless specifically directed to by 3i personnel. Should you have any questions about this dialog, please contact support@intelligent-imaging.com.

Access in the Administration tab

See also:

- Preferences Options
- System Administration
- Backing up and Restoring Preferences

Hardware Properties

The ***Hardware Properties*** dialog is where SlideBook can be customized with regards to how it communicates with specific hardware. It is important not to modify this configuration unless specifically directed to by 3i personnel. Should you have any questions about this dialog, please contact support@intelligent-imaging.com.

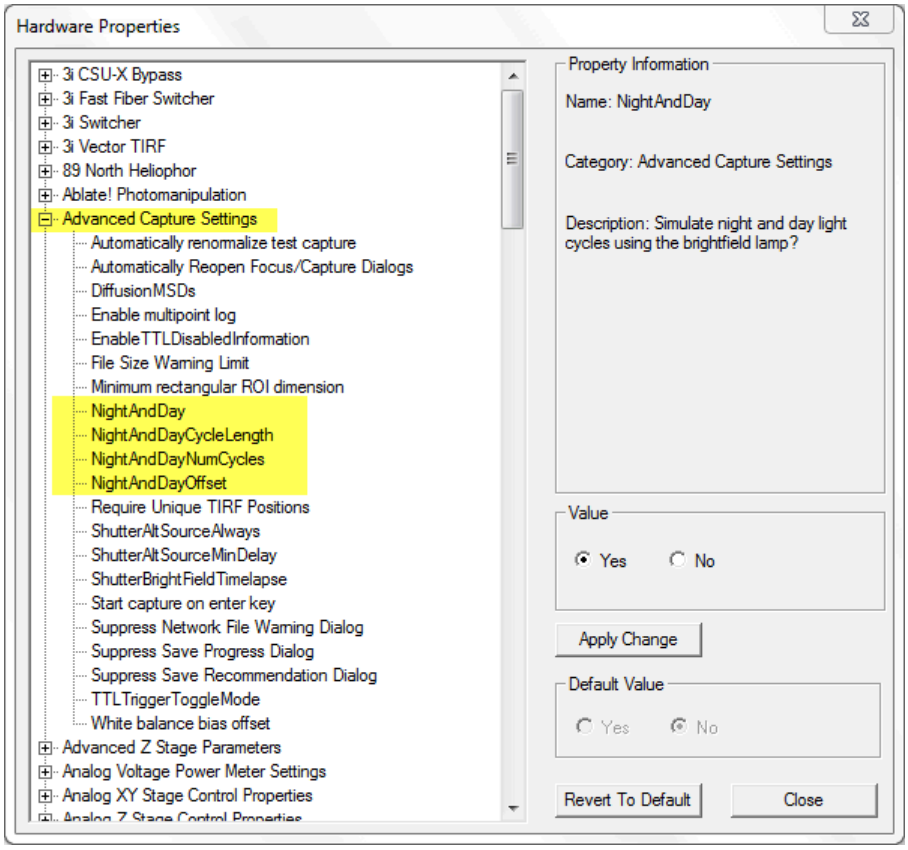
Access in the Administration tab

See also:

- Preferences Options
- System Administration
- Backing up and Restoring Preferences

Night and Day Capture

Night and Day capture turns on the brightfield lamp between specified time point acquisitions for long-term capture. This mode is enabled and customized by setting hardware properties under the ***Advanced Capture Settings*** group (accessed by selecting ***Administration tab > Hardware Properties***):



The following properties are available:

NightAndDay	turns the mode on and off
NightAndDayCycleLength	determines the total number of time points that participate in a single daily cycle (both "day" and "night"). Currently, SlideBook divides this length by two to set the "day" and "night" length in terms of time points, so it is important that this value is even. For instance, if NightAndDayCycle is set to 24, SlideBook will turn on the brightfield lamp for the first twelve intervals and off for the second twelve.
NightAndDayNumCycles	sets the number of daily cycles that are run for the capture. After this number of daily cycles has been ran, the brightfield lamp will remain off between captures.
NightAndDayOffset	allows a given number of time points to be captured before the first cycle begins.

So, for instance, if you set:

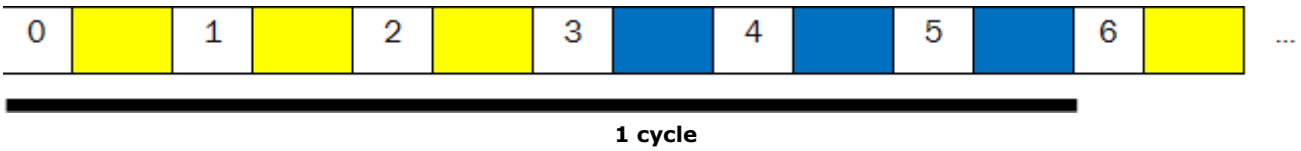
NightAndDayCycleLength = 24
NightAndDayNumCycles = 3
NightAndDayOffset = 0

and specify a capture with 84 timepoints, you would get this behavior:

time points 0 – 11	light after timepoint is captured
time points 12 – 23	no light on after capture
time points 24 – 35	light
time points 36 – 47	no light
time points 48 – 59	light
time points 60 – 83	no light

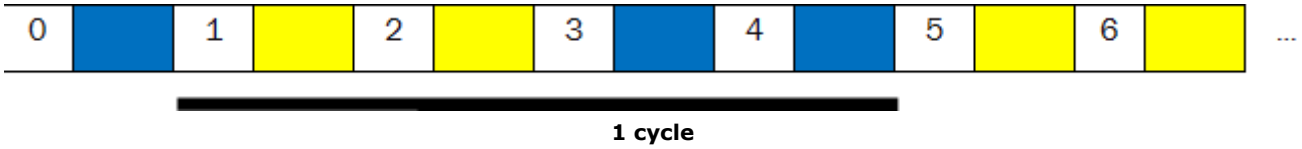
Example 1:

NightAndDayCycleLength = 6
NightAndDayNumCycles = 2 (or more)
NightAndDayOffset = 0



Example 2:

NightAndDayCycleLength = 4
NightAndDayNumCycles = 2 (or more)
NightAndDayOffset = 1



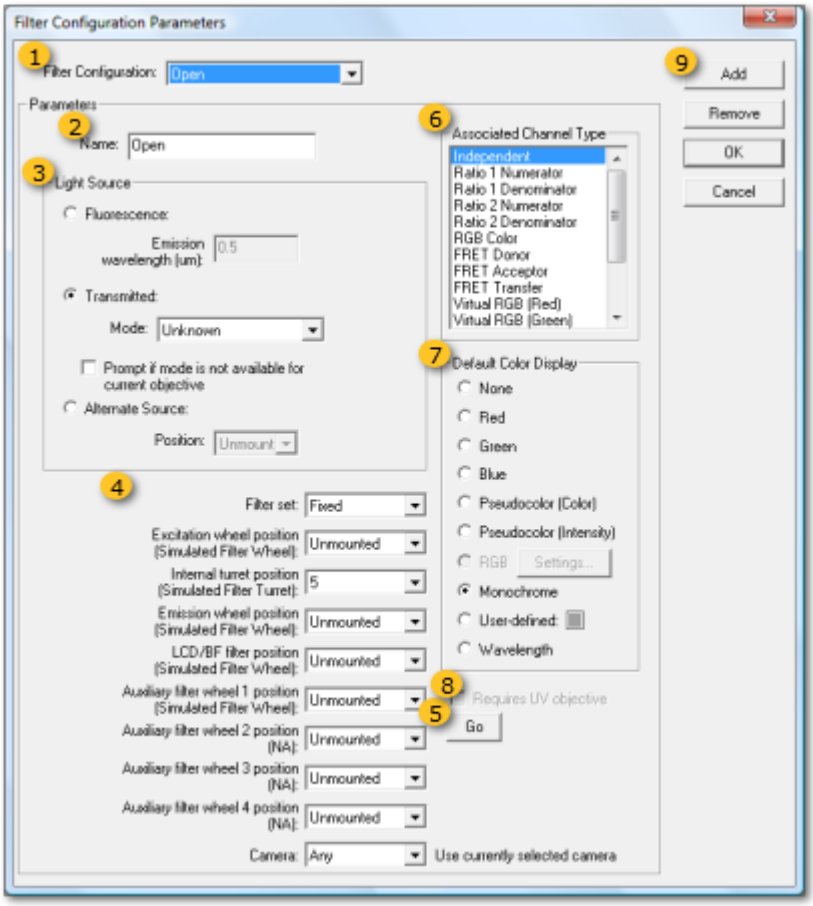
Filter Configuration Parameters Dialog

The **Filter Configuration Parameters** dialog allows you to configure imaging channels on your system.

Access in the Administration tab

For specific instructions on using this window, please refer to:

Defining Ratio Channels
Defining FRET Channels



1 Filter Configuration

Filter Configuration: Open

Displays the ***Name*** of the channel displayed. When a new fluoroprobe is created, this field will display **New Fluoroprobe** until the settings have been applied by restarting SlideBook.

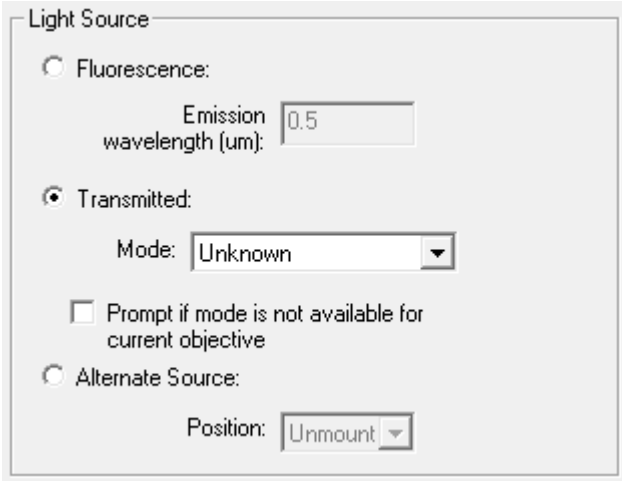
It is important to note that the filter buttons in the ***Focus Controls*** window will be in the same order as this list (on a per ***Filter Set*** basis). That is, if the list reads **GFP** above **RFP**, the **GFP** button will appear to the left of the **RFP** button and **GFP** will be captured before **RFP** by default. It is possible to change channel capture order from the ***Capture*** dialog but users should remember they are also setting up the default channel capture order when configuring filters.

2 Name

Name: Open

Type the desired ***Name*** for the channel in this field. This is the label that will appear in the corresponding filter button in ***Focus Controls*** and the channel selection area in the ***Capture*** window. It is important to note that no special characters can be used in this field and only the first 8 characters will appear in the button in ***Focus Controls***.

3 Light Source



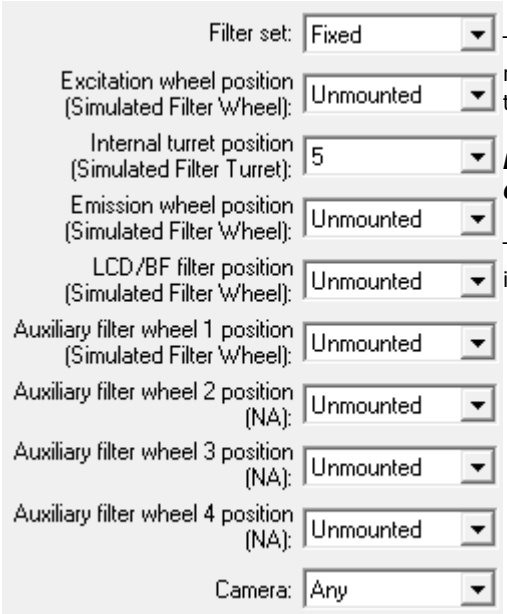
Select the appropriate ***Light Source*** as follows:

Fluorescence: Should be used when a standard fluorescent light source is used in conjunction with excitation and emission filters. Select the radio button control and enter a value for the emission wavelength in µm. For example, GFP has an emission peak wavelength at 509 nm so you would enter **.509** in this field. This value will be used in some methods of deconvolution as well as when ***Wavelength*** is selected for ***Default Color Display***.

Transmitted: Should be used when the standard transmitted (halogen) light source is used. Select the radio button to enable this light source then select the ***Mode*** for the channel you are configuring. Available modes include ***Unknown***, ***Brightfield***, ***Darkfield***, ***DIC (Nomarski)***, and ***Phase***. These refer to transmitted light imaging techniques that may or may not have relevant condenser positions or objective properties associated with them. Certain modes will only be available for some objectives based on the objective configuration (see also Objective Configuration). If you would like to be notified of these compatibilities, check the box to ***Prompt if mode is not available for current objective***. It is important to note that, when you configure a transmitted light channel such as DIC and image it with other

Alternate Source: Should be used when controlling a laser light source via TTL. Select the radio button to enable this light source then select the ***Position*** number from the drop-down list to correspond to the appropriate TTL line.

4 Hardware Configuration

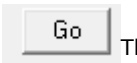


The ***Hardware Configuration*** section consists of a series of dropdown menus used to direct hardware movement. Use these dropdown menus to select the appropriate position on various configured hardware.

Filter Set can be used to help organize groups of channels in the ***Focus Controls*** and ***Capture*** window (See also Working with Filter Sets).

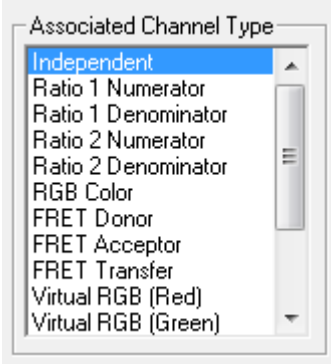
To test a specific filter configuration, simply press ***Go*** and observe to see if the correct movements are occurring.

5 Go button



The ***Go*** button will move all of the hardware to the positions configured in this dialog. This button is used to test the configuration to ensure the channel is transmitting.

6 Associated Channel Type



For each configured channel, you must select the appropriate Associated Channel Type. The options available include:

Independent -defines an independent channel used for basic image acquisition and analysis

Ratio 1 Numerator - defines the numerator channel for the first ratio pair configured, will identify a channel used in the display and analysis of ratio data (see also Ratio System Configuration)
Ratio 1 Denominator - defines the denominator channel for the first ratio pair configured, will identify a channel used in the display and analysis of ratio data (see also Ratio System Configuration)
Ratio 2 Numerator - defines the numerator channel for the second ratio pair configured, will identify a channel used in the display and analysis of ratio data (see also Ratio System Configuration)
Ratio 2 Denominator - defines the denominator channel for the second ratio pair configured, will identify a channel used in the display and analysis of ratio data (see also Ratio System Configuration)

RGB Color - defines a color channel acquired using a color camera

FRET Donor - defines a donor channel in FRET acquisition and analysis (see also FRET System Configuration)
FRET Acceptor - defines an acceptor channel in FRET acquisition and analysis (see also FRET System Configuration)
FRET Transfer - defines a FRET transfer channel in FRET acquisition and analysis (see also FRET System Configuration)

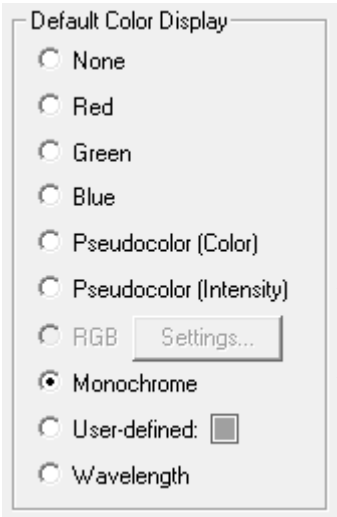
Virtual RGB (Red) - defines the red channel when using a color filterwheel or LCD color filter to create a virtual RGB image (see also Performing Auto-White Balance for Color Capture)
Virtual RGB (Green) - defines the green channel when using a color filterwheel or LCD color filter to create a virtual RGB image (see also Performing Auto-White Balance for Color Capture)
Virtual RGB (Blue) - defines the blue channel when using a color filterwheel or LCD color filter to create a virtual RGB image (see also Performing Auto-White Balance for Color Capture)

FRET Donor Prebleach - defines a prebleach donor channel in FRET acquisition and analysis (see also FRET System Configuration)
FRET Donor Postbleach - defines a postbleach donor channel in FRET acquisition and analysis (see also FRET System Configuration)

Fluorescence Lifetime - defines a channel used for FLIM

Photomanipulation - defines a channel used for photomanipulation such as photoactivation or photobleaching

7 Default Color Display



Select the the radio button to designate the appearance of the channel in an image. Channels may be set to standard RGB, pseudocolor, RBG, monochrome, user-defined, or set to the emission wavelength.

NOTE: The color assigned here may not be the color displayed during acquisition but will be the default color displayed when the image is opened. To change color assignments on an open image, go to the ***View*** menu and select the preferred display.

None may be used for channels that do not need to be viewed, no color will be assigned to the channel when the image is opened.

Red, ***Green***, or ***Blue*** allows channels to be assigned to the standard RGB display.

Pseudocolor (Color) and ***Pseudocolor (Intensity)*** together determine the look of a pseudocolored image. A filter configuration that has ***Pseudocolor (Color)*** as its default will set the hue value of the display according to the normalized (i.e., scaled from minimum to maximum) value of each voxel. A filter configuration that has ***Pseudocolor (Intensity)*** as its default will gate the intensity of the pseudocolored image according to the normalized value of each voxel. If a captured image contains a channel with a filter configuration set to ***Pseudocolor (Color)*** but not ***Pseudocolor (Intensity)***, the corresponding view will show a range of hues at maximum intensity (i.e., ranging from bright blue to bright red). If the image also contains a channel with a filter configuration set to ***Pseudocolor (Intensity)***, the corresponding view will show a range from dark blue (black) to bright red. If the image only contains a channel with a filter configuration set to ***Pseudocolor (Intensity)*** and no ***Pseudocolor (Color)*** counterpart, the default setting will be treated as if it were set to ***None***.

Pseudocolor color types cannot be displayed along with ***Red***, ***Green***, or ***Blue*** channels. In multiple channel images, the pseudocolor setting will override any red, green, or blue settings. Red, green, or blue settings will in turn override a monochrome setting.

RGB is for color image acquisition either using a color camera or ***Virtual RGB*** channels. When RGB is selected, you will need to complete the Settings dialog to adjust gain settings for red, green, and blue.

Monochrome may be used to display a channel in monochrome or for a background channel when displayed with RGB or pseudocolored channels.

User-defined may be used to assign a specific color to a channel. This is particularly useful when you routinely collect images in more than three channels. To define the color, click on the color block to open the ***Color*** dialog. Select a color then click ***OK***.

Wavelength may be used to to automatically assign a color based on the emission wavelength of the channel as assigned in the ***Light Source*** configuration. This is another approach that will be helpful if you routinely capture more than three channels or if you wan the image to be displayed realistically according to fluorophore.

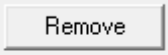
Again, you can view any image using any color display option at any time, this configuration simply determines what will be visible initially when an image opens.

8 Requires UV objective

Select this check box if the channel you are configuring requires that the objective lens passes UV light. For example, you may select this box if you are configuring a channel for Fura-2. Checking this box will enable SlideBook to open a warning window when you are using this channel with an objective that will not pass UV.

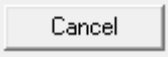
9 Finishing Buttons

Click ***Add*** to add a new channel to the configuration. A new channel called **New Fluoroprobe** will be created.



Click ***Remove*** to remove the current channel from the configuration.

Click ***OK*** to apply the changes and close the dialog. You will be prompted to restart SlideBook.



Click ***Cancel*** to discard changes and close the dialog.

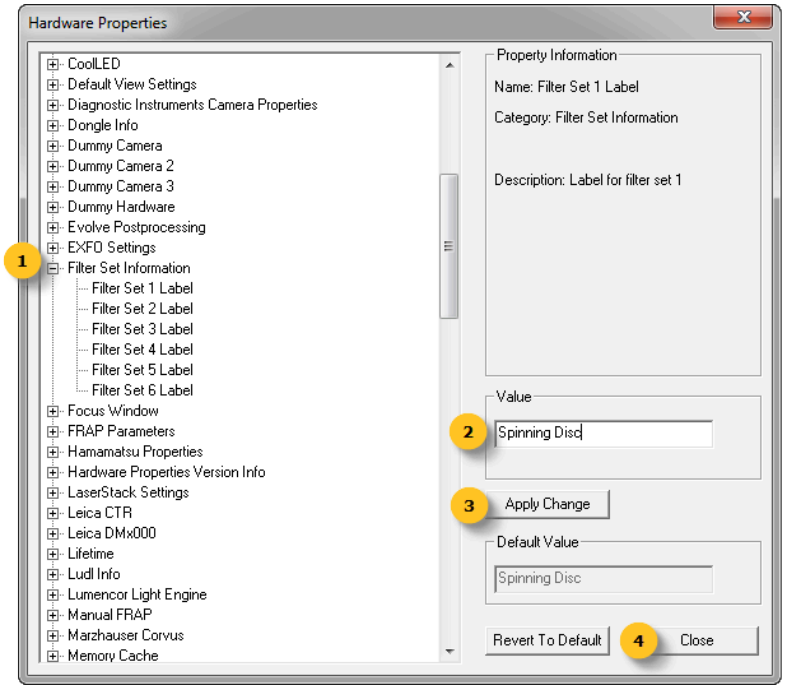
Working with Filter Sets

When you have many optical configurations or imaging techniques on one system, it is helpful to utilize ***Filter Sets*** in organizing the modalities. ***Filter Sets*** determine which imaging "channels" are available together in the ***Focus Controls*** and ***Capture*** windows.

Name Filter Sets in Hardware Properties

Configuring custom filter set names is a simple process that can be accomplished quickly. Once the configuration is complete, you should be sure to Backup System Preferences.

1. Go to ***Administration tab >*** ***Hardware Properties***. Navigate to the section called ***Filter Set Information*** and click the **+** to expand the selection. Select **Filter Set 1 Label**.



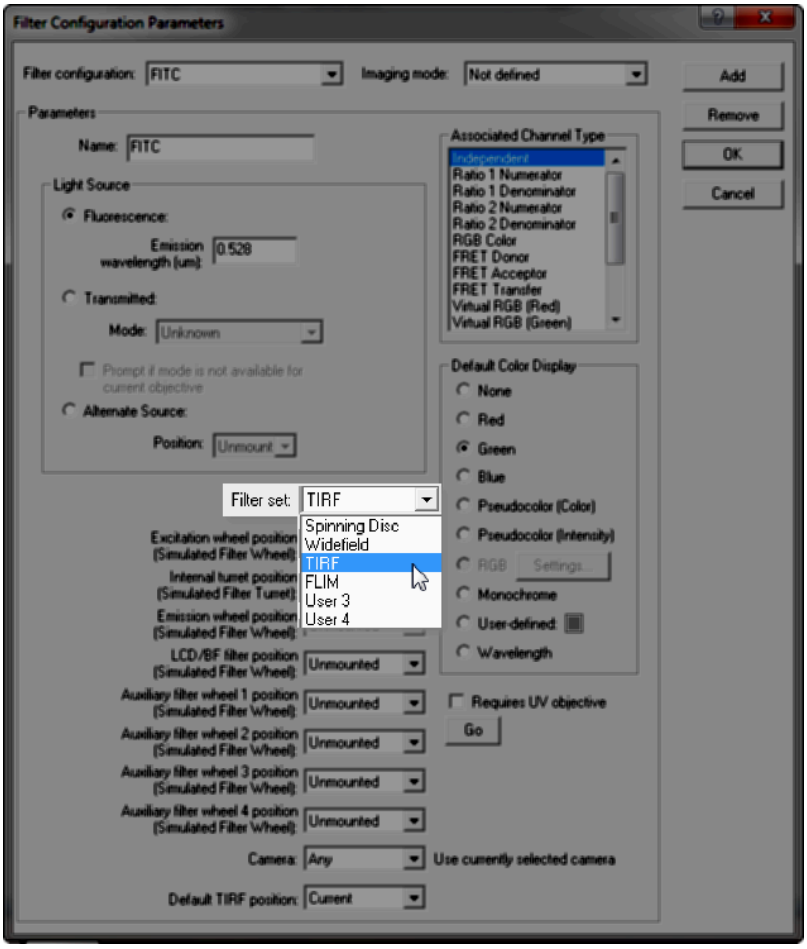
2. Type the desired filter set name/ imaging modality in the ***Value*** field.

3. Click ***Apply Change***.

4. Repeat steps 1-3 for all desired filter set labels. Press ***Close*** when finished. When prompted, restart SlideBook.

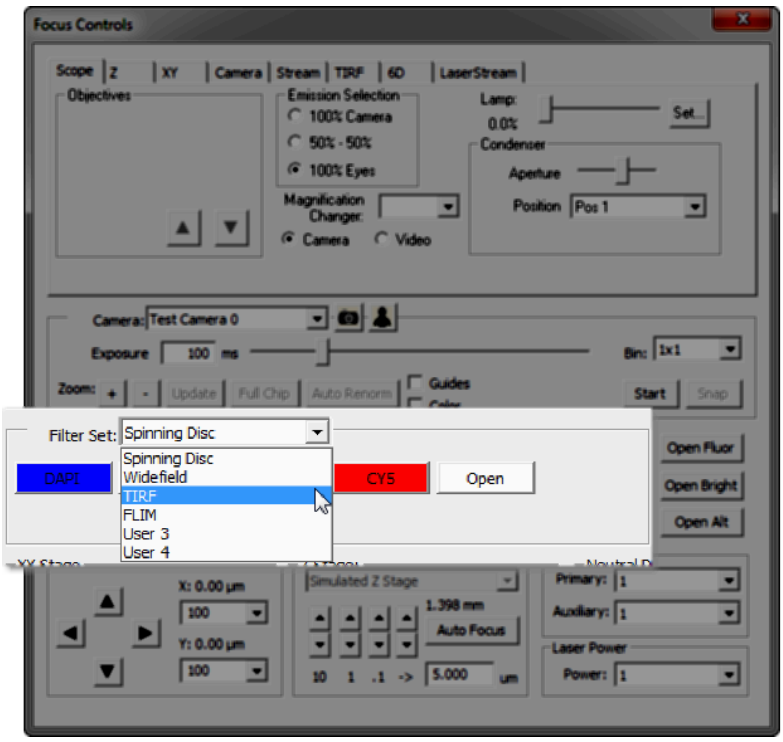
Designating Filter Sets for Channels

When configuring channels, you may then assign the appropriate ***Filter Set*** in ***Administration tab >*** ***Filters***. More information on this dialog can be found in Filter Configuration Parameters Dialog.



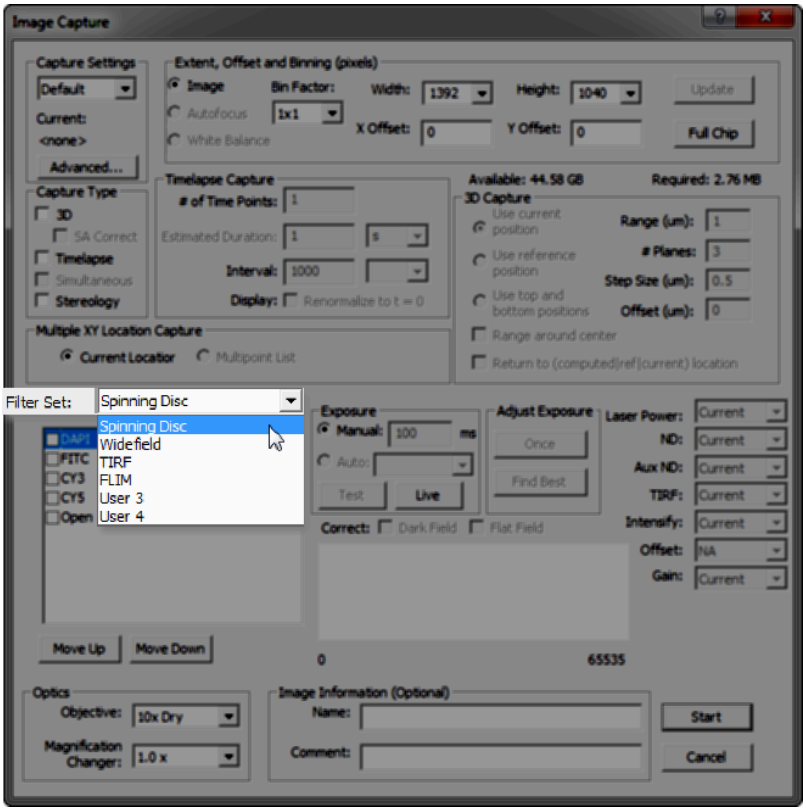
Using Filter Sets in Focus Controls

The ***Filter Sets*** can be accessed in ***Focus Controls*** as shown below. More information on this dialog can be found in Focus Controls Window.



Using Filter Sets in Capture

When setting up channels in image capture, you will access the ***Filter Sets*** as shown below. More information on this dialog can be found in Capture Window.



Performing Auto White Balance for Color Capture

If you are performing a color capture with an LCD filter, you may first wish to perform a white balance procedure (you must first define your filters as discussed in the Filter Configuration Parameters Dialog). This will yield the truest color rendering. To do so:

1. In the focus window, find a clear portion of your slide. Alternatively, use a piece of white lens paper.

2. Make sure that the halogen lamp is set to 3200K and the microscope is set for Köhler illumination.

3. Select the green filter and check that your exposure time is at least 25ms (this yields the best white balance results). If you are saturating the camera at 25ms, you should use neutral density filters.

4. Once you have achieved an adequate exposure time, go to select the image capture icon .

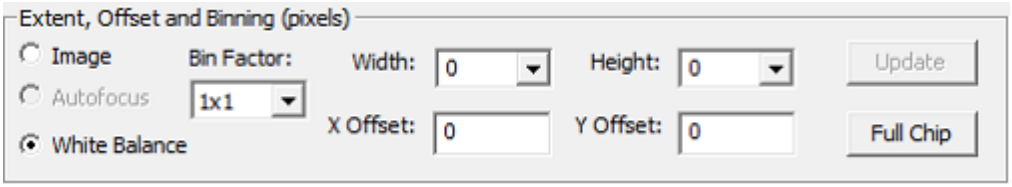
5. Select the filter configuration checkbox corresponding to the color channel and then ***Test***.

6. Select the area to be imaged and any binning desired.

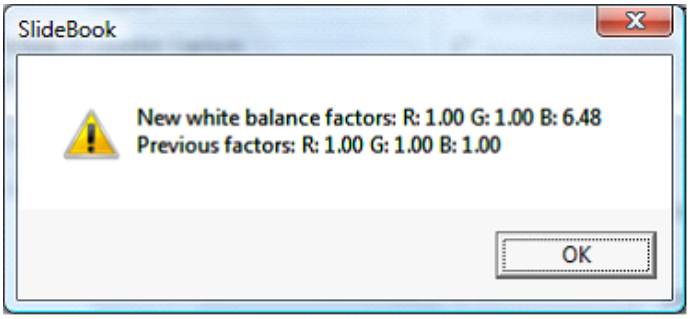
7. Adjust the exposure time as described above.

8. Select the area of the test view that you would like to use for performing white balance by clicking and dragging over the region.

9. Select ***White Balance*** from the radio buttons and then click ***Update***.



10. A white balance operation is performed and a dialog box similar to the following will appear.



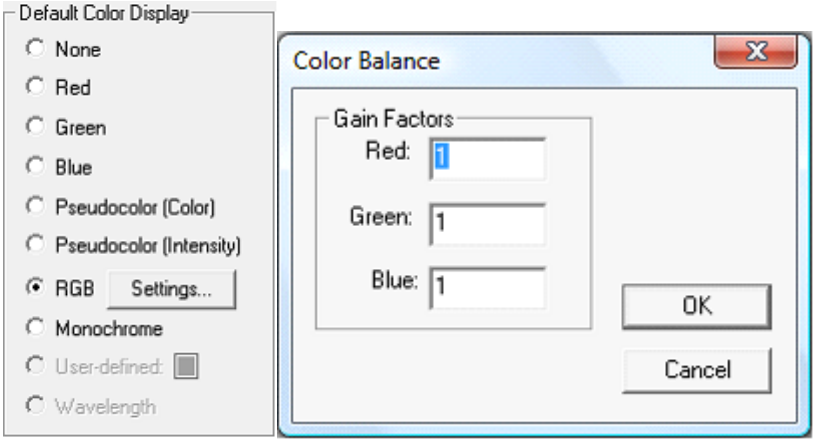
NOTE: If you are getting inconsistent color maximums when performing capture and the RGB factors are varying significantly each time you perform a white balance, then you should adjust your illumination. Bright, dim or inconsistent lighting may cause this phenomenon.

11. Select ***OK*** to close the dialog box.

12. You may now capture an image by selecting ***OK*** in the ***Capture*** dialog box. The white balance settings will remain in the system memory until the slide file is closed.

13. If you would like to manually enter white balance settings, go to Administration tab **>** ***Filters*** and select the red filter configuration for an LCD filter, or to the color filter configuration for a color camera.

14. In the ***Default Color Display*** section, select the ***Settings*** button. The following dialog box will be displayed:



15. Enter the white balance gain factors. In general, the green factor will be the largest.

16. Click ***OK*** to save the settings.

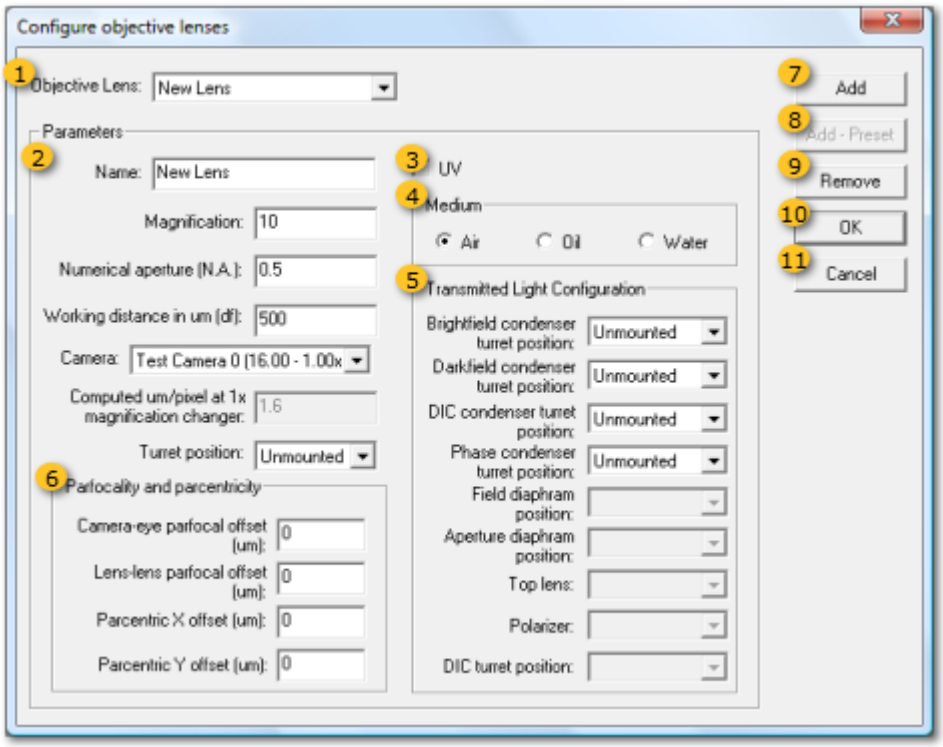
Configure Objective Lenses window

Objective lenses can be defined and set up in the ***Configure Objective Lenses*** window. Once an objective is defined, it can be controlled (if the objective turret is motorized) from the Focus Controls and Capture window. If the objective turret is not motorized, it may be selected in the capture window and the objective definition will automatically be stored with the image information. If the objective was used to capture an image that has been imported, the objective information can be added to the image information.

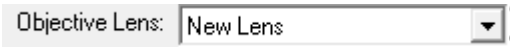
Access through the Administration tab

See also:

Measuring Pixel Size
Per-Camera Magnification Factor
TUTORIAL: Adding an Objective

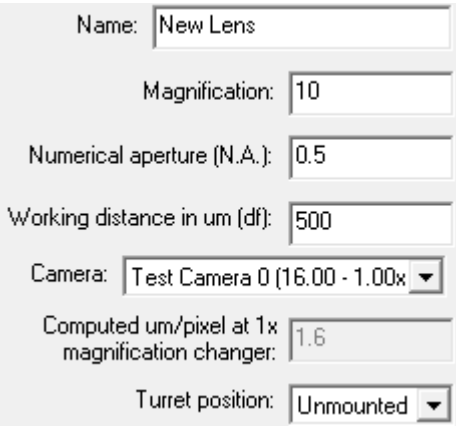


1 Objective Lens Name



Objective Lens is a dropdown menu containing a list of configured objective lenses. When you click ***Add***, a new option will be selected and labeled **New Lens**. You may edit the name and other details of the magnification changer in the ***Parameters*** group. To edit an existing objective lens, select it from the menu, edit the parameters, and click ***OK***.

2 Edit field



Enter the objective name, magnification, numerical aperture (N.A.), working distance, and turret position. You must give the lens a name and fill out all parameter fields listed in this section.

NOTE: For optimal results, objective pixel size should be measured by traditional means using a stage micrometer. If this is not possible, a close estimate can be calculated by dividing the camera pixel size by the magnification of the objective. Please note that this will not take into account any optics that alter magnification such as a spinning disc confocal or Spherical Aberration Correction device by 3i. When pixel sizes are measured, refer to Measuring Pixel size for instructions.

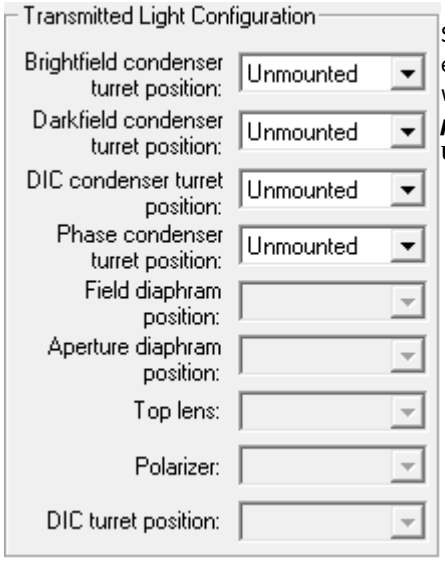
3 UV check

Some objective lenses have improved performance in the UV range of light. Check this box when you configure an objective that passes UV light. This information will be used to notify users of incompatible channel/objective combinations.

4 Medium group

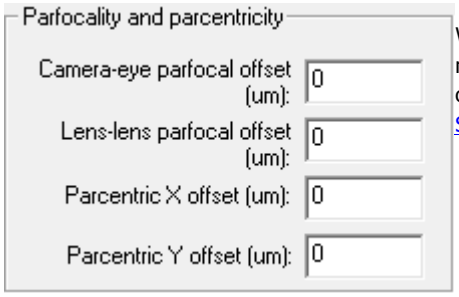
Select the imaging medium for which the objective was designed. This information is necessary for some advanced functions such as deconvolution.

5 Transmitted Light Configuration group



Select any transmitted light functionality associated with the objective. For example, if there is a DIC prism in the motorized condenser turret, you would want to select the correct position from the ***DIC condenser turret position*** dropdown menu. If the condenser is not motorized, select **Unmounted**.

6 Parfocality and parcentricity group



When the image in the eyepieces and the camera are not the same, you may use the parfocality and parcentricity parameters to correct for any offsets. It is suggested that you first review system alignment with 3i Support.

7 Add button

Add Click ***Add*** to add a new objective to the microscope configuration.

8 Add - Preset button

Add - Preset Functionality coming soon!

9 Remove button

Remove Select an objective from the ***Objective Lens*** dropdown menu then click Remove to delete it from the configuration.

10 OK button

OK Click ***OK*** to apply changes and close the dialog.

11 Cancel button

Cancel Click ***Cancel*** to discard changes and close the dialog.

Measuring Pixel Size

A straightforward formula can be used to calculate approximate pixel size:

pixel size (µm) = physical pixel size of the camera/magnification of objective/any other auxiliary magnification

Thus, a camera with a 16 µm pixels and no auxiliary magnification would have a calculated size of 0.2540 µm/pixel at 63X and 0.016 µm/pixel at 100X. It is important to keep in mind that many external devices can alter the magnification and, therefore, pixel size. Some examples of such devices include relay lenses, certain spinning disc confocals, and the 3i SAC device. If you are unsure of your peripheral devices, please contact the company that sold you the hardware.

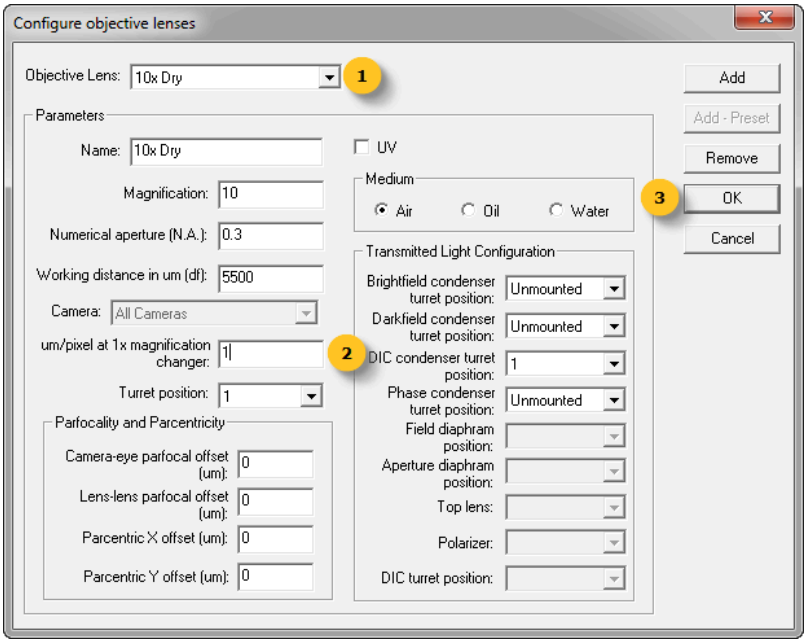
While calculated pixel size can give good results, the most accurate results are obtained by measuring pixel size. Measuring pixel size also eliminates the need to know magnification factors of peripheral devices. The only special equipment you will need is a metric slide micrometer also known as a stage micrometer. This is a glass microscope slide with a ruler you will image through the microscope.

In SlideBook, the workflow for measuring pixel size consists of setting all pixel sizes to 1 (in order to measure the number of pixels for a known distance), capturing images at each desired magnification, performing calculations, and configuring the measured pixel sizes. Detailed instructions follow.

1) Open SlideBook. Go to ***Administration tab >*** ***Objectives***. More information on this dialog can be found in the Configure Objective Lenses window.

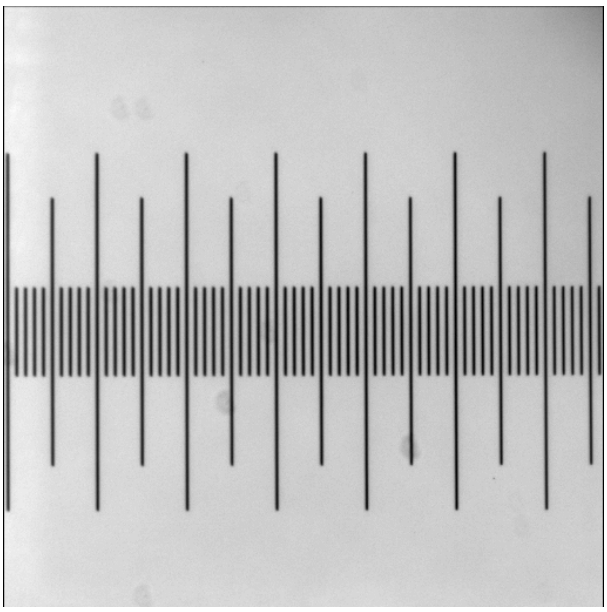
2) Refer to the image below. 1 Select an objective you will be using to measure pixel size from the ***Objective Lens*** dropdown menu. 2 Set the ***um/pixel at 1x magnification changer*** value to 1. Repeat for each objective you will be measuring.

3 Click ***OK*** to apply changes and close the dialog. You will be prompted to restart SlideBook, do so at this time.



3) Open the Focus Controls window. Select the first objective you would like to use to measure pixel size. Confirm that your micrometer slide is clean and dry. Arrange the slide on the microscope using the required immersion media when necessary. Use the focus controls to bring the sample into focus and get a live image on the monitor. You may use fluorescence or brightfield.

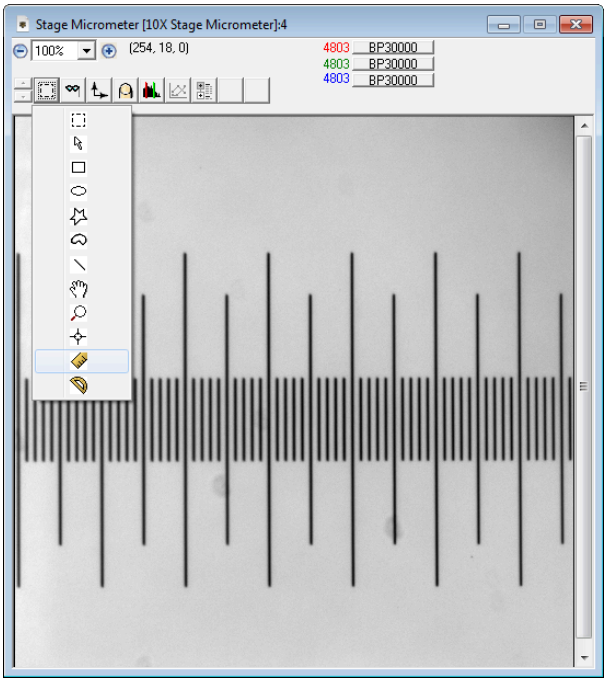
4) Adjust the slide on the stage so that the ruler image is as horizontal as possible. Press ***Snap*** to capture a single 2D image. A sample image of a slide micrometer at 10X is shown below.



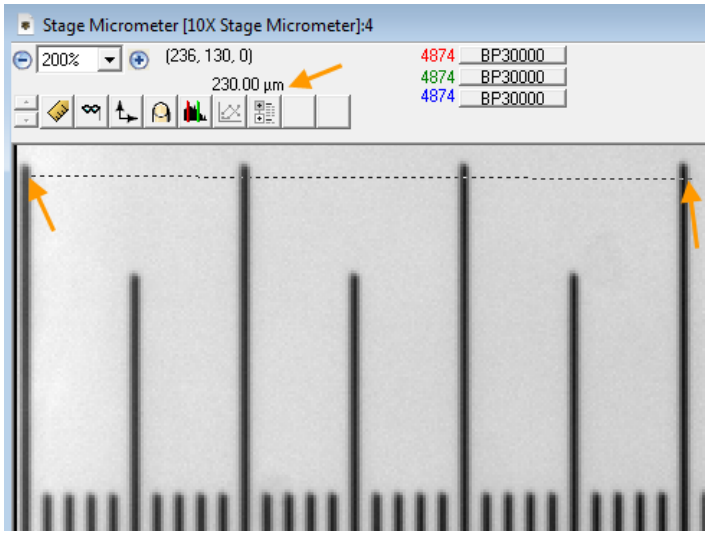
5) In the **.sld**, select the image you just captured by clicking on it. Right-click and select Rename from the menu. Name the image according to the objective used.

6) Repeat steps 3-5 for any additional objective you would like to use to measure pixel size. If you have multiple cameras, please refer to Per-Camera Magnification Factor for information on how to configure pixel sizes for either identical or different optical paths.

7) Once all images have been acquired, you are ready to make the measurements. Open the first image. Select the ruler tool from the tool dropdown menu.



8) Click and drag on the image to draw a straight line between two measuring points. Be careful to place the cursor on the same side of the line to improve accuracy. A measurement will be shown above the ***View Settings*** icon in the View window. Even though the measurement in given in microns, it is actually the number of pixels as each pixel is set to 1 µm.



9) Record the measurement for the known distance. Repeat the measurement at least three times to reduce measurement error. Now you have the number of pixels for a known number of microns. To determine pixel size, take the number of microns divided by the number of pixels. In the example above, it would be 300 microns (each long tic marks 100 microns) divided by 230 pixels = 1.3043 µm/pixel.

10) For each image at different objectives, repeat the measurements and calculations.

11) Go to ***Administration tab >*** ***Objectives***. For each objective you measured, select the objective from the dropdown menu and set the ***um/pixel at 1x magnification changer*** to the calculated value.

12) After all pixel sizes have been set, click ***OK*** and restart SlideBook when prompted.

Per-Camera Magnification Factor

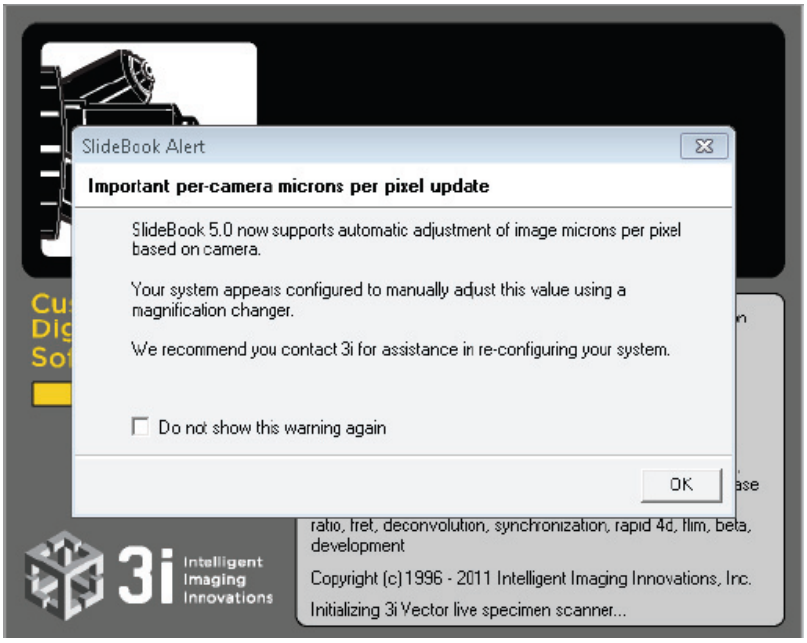
Previously, when systems had multiple cameras, the user would measure pixel sizes for **Camera1** and **Camera2**. They would then enter pixel sizes for **Camera1** for each individual objective. Next, the user would calculate a conversion factor between **Camera1** and **Camera2** and create a manual magnification factor for **Camera2**. When operating the systems, all users would need to remember to manually select the appropriate magnification changer when capturing images using **Camera2**.

In SlideBook 5.0.0.24 and later, this process has been simplified by automatically calculating pixel sizes for multiple cameras as well as providing per-camera adjustment factors for true measured pixels sizes. This approach will eliminate user error by removing any manual steps to assign the appropriate pixel size to an image.

Your system is likely to benefit from this software change if one or more of the following is true:

- Multiple cameras are detected and these cameras are compatible with new ability for SlideBook to query a camera for its pixel size
- A manual magnification changer is configured

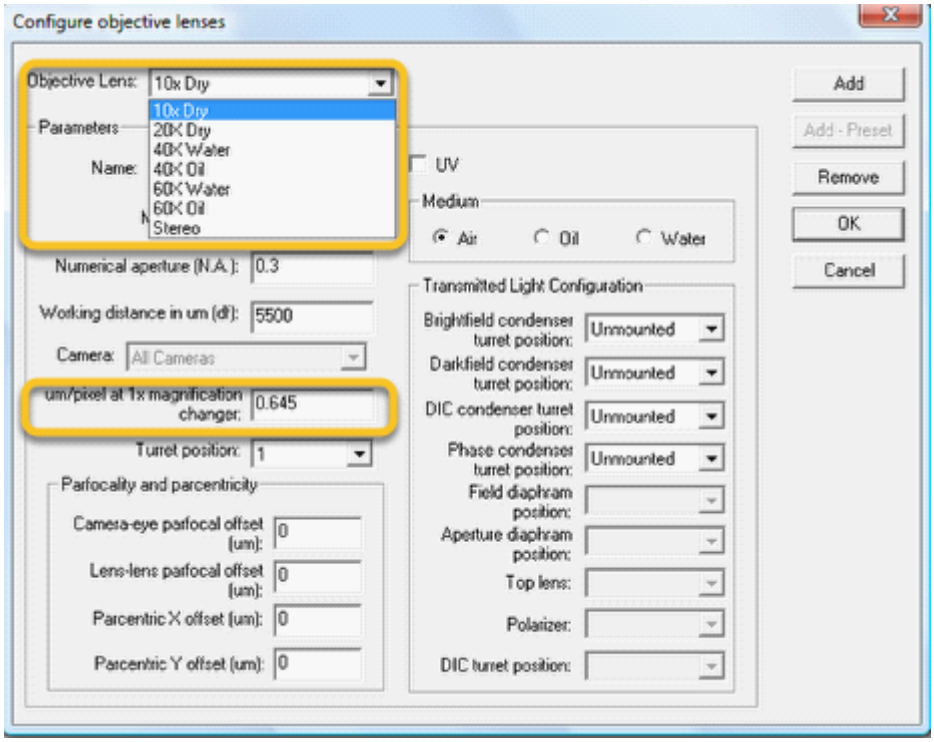
In the case where your system may benefit from this software change, the following dialog will appear at startup (after installing SlideBook 5.0.0.24 or later).



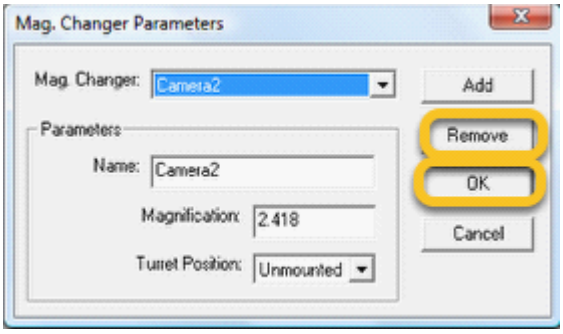
There are two different procedures available depending on the physical configuration of your system. That is, you must first determine whether the two cameras share an optical path or have different optical paths. If you are unsure which category your system falls into or have any questions during this process, please consult support@intelligent-imaging.com.

Configuring Pixel Size, Multiple Cameras Identical Optical Paths

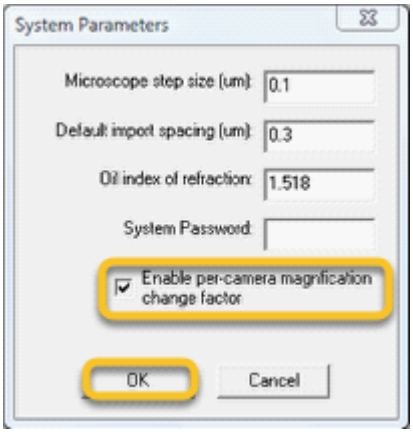
1. Record current pixel size for each objective by going to ***Administration tab >*** ***Objectives***. Write down the value for each objective on the system.



2. Go to ***Administration tab >*** ***Mag Changer***. Press ***Remove*** then ***OK***. This factor is simply a ratio of the physical pixel size of the detectors. With your new configuration, SlideBook will automatically read these values off the CCDs.



3. Go to ***Administration tab >*** ***System Parameters***. Check the box to ***Enable per-camera magnification change factor*** then click ***OK*** when finished.



4. Compute the per-camera magnification factor. This is the amount of magnification introduced between the objective lens and the camera mount and can be calculated as follows:

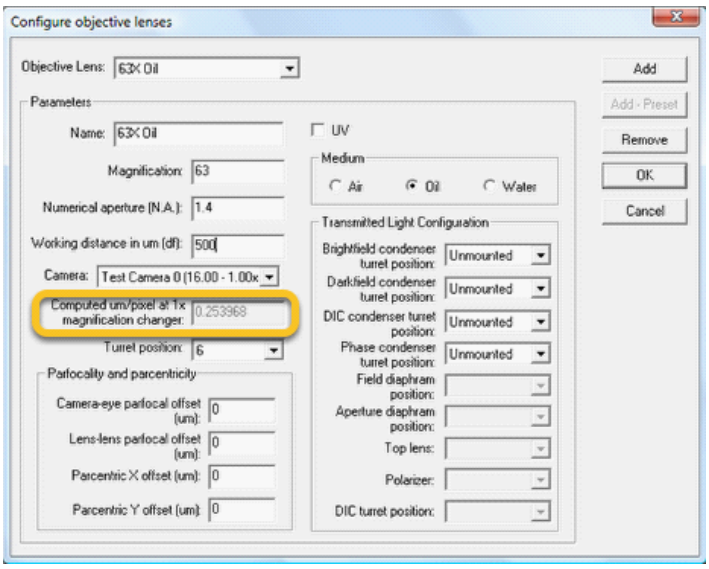
$$\frac{measuerd\ um/pixel}{theoretical\ um/pixel} = magnifcation\ factor\ for\ optical\ path$$

Where:

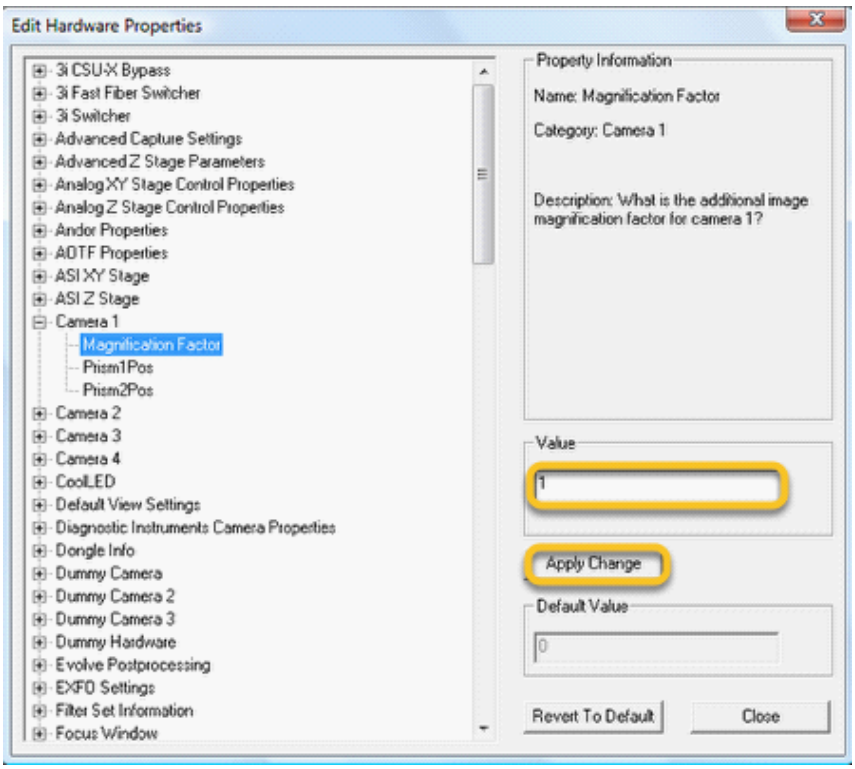
Measured um/pixel is the value obtained from step #1

Theoretical um/pixel is calculated by dividing the physical pixel size of the CCD by the magnification of the objective. For example, the theoretical µm/pixel for a CoolSNAP HQ at 63X is 6.45/63 = 0.1024 µm/pixel. Once the per-camera magnification factors have been enabled, you can find theoretical pixel size by going to ***Administration tab > Objectives > Computed um/pixel at 1x magnification changer*** for **Camera1** at each ***Objective Lens***.

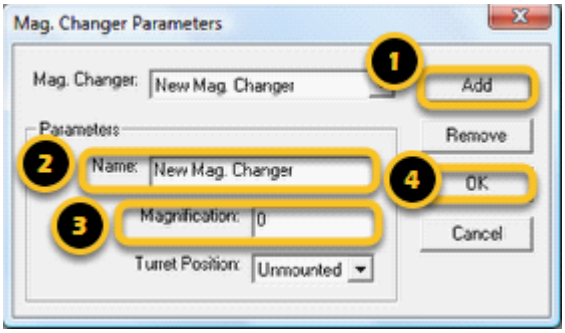
You may find it useful to average the magnification factor across several objective lenses.



5. Enter the per-camera magnification factor under ***Administration tab > Hardware Properties > Camera 1,2***, etc. The ***Magnification Factor*** will be identical for each of the cameras on the same optical path. Click ***Apply Change*** for each value entered.



6. Create ***Magnification Changer***(s) to represent the physical magnification changer slider (sometimes known as an optovar) on your microscope by going to ***Administration tab >*** ***Mag Changer***. Click ***Add***, ***Name*** the mag changer, and enter the ***Magnification*** value. When finished, click ***OK***.

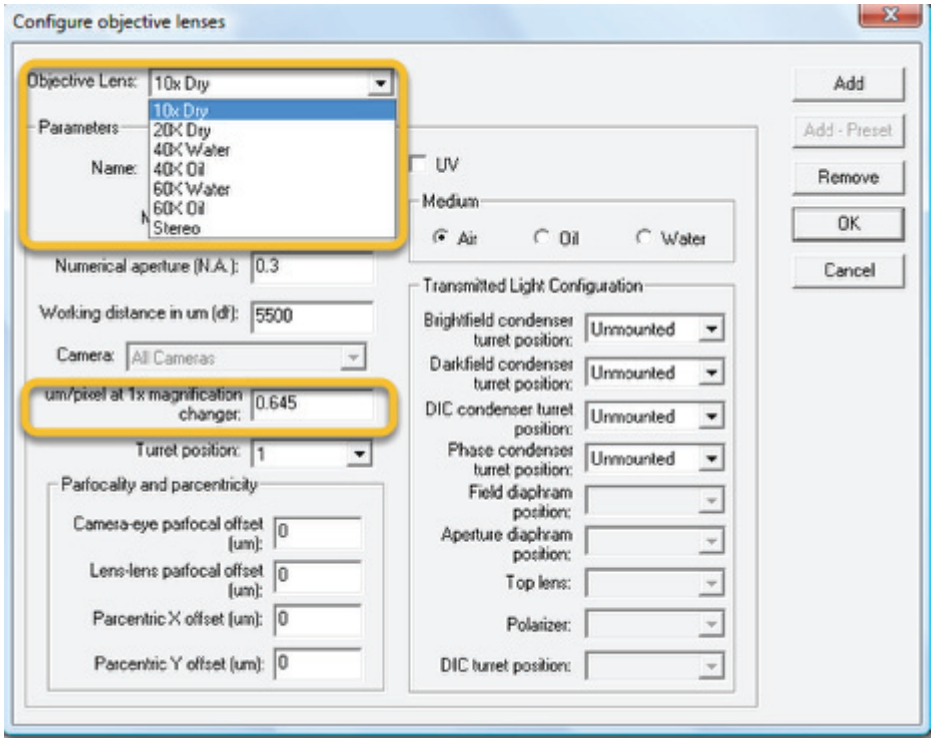


7. Save backup configuration files in a secure location. To do so, go to ***Support tab >*** ***Backup Preferences***.

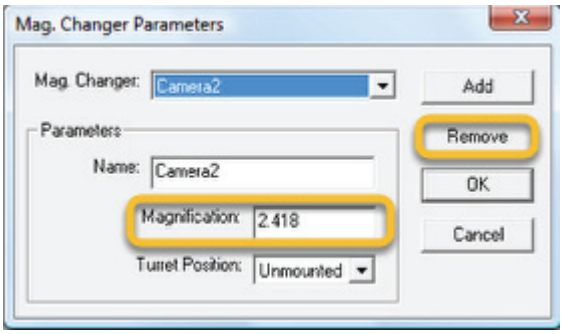
Once this configuration change has been made, pixel size adjustments will be made automatically each time a user selects a camera. For older images that require pixel size adjustment, select the image and go to ***Get Info > Edit*** to select a camera or manually enter a value for pixel size. For additional assistance, please contact support@intelligent-imaging.com.

Configuring Pixel Size: Multiple Cameras, Different Optical Paths

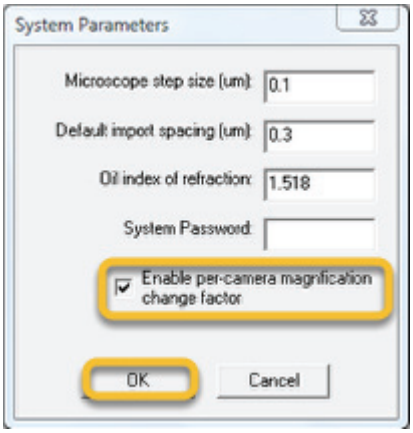
1. Record current pixel size for each objective by going to ***Administration tab >*** ***Objectives***. Write down the value for each objective on the system.



2. Go to ***Administration tab >*** ***Mag Changer***. Record manual magnification factor then remove it as you will no longer need to select a manual magnification changer for a secondary camera.



3. Go to ***Administration tab > System Parameters***. Check the box to ***Enable per-camera magnification change factor*** then click ***OK*** when finished.



4. Compute the per-camera magnification factors and true objective magnification values based on the following equation:

um/pixel = physical pixel size of detector/magnification of objective/per-camera magnification factor/magnification changer

Where:

Physical pixel size of detector is a defined value based on the camera type. If your camera is compatible with this system of pixel size application, SlideBook will read the pixel size from the CCD.

Magnification of objective is the nominal amount an objective magnifies an object. While manufacturers strive to make this the true magnification, there is often some amount of error in the true magnification of the objective. The true magnification of the objective can be calculated by directly coupling a camera to a microscope body and measuring the um/pixel using a stage micrometer. Some systems may already be configured so that you may measure the true magnification of objectives. If your system is not configured in such a way, it is acceptable to assume the nominal value assigned by the lens manufacturer.

Per-camera magnification factor takes into account many factors that can alter the um/pixel value for each camera. These factors may include:

- CSU-X, introduces a 1.2X factor
- old-style SAC introduces either a 2X or 1.6X factor depending on style
- additional relay optics in light path
- "true" magnification factor for the objective when not measured directly

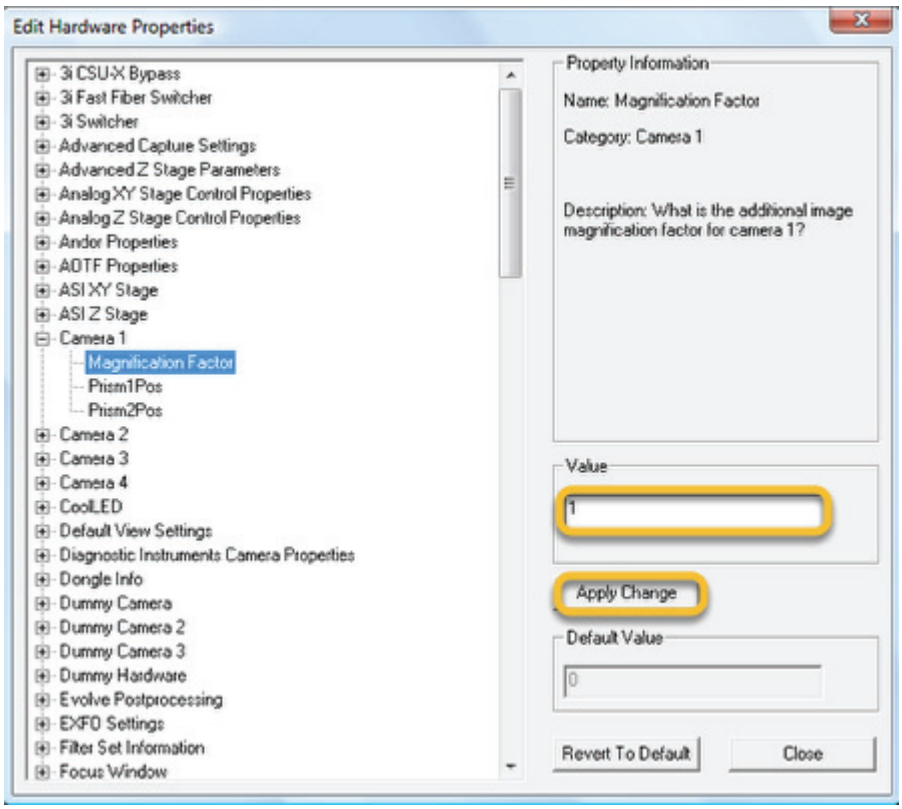
Magnification changer (sometimes called an optovar or referred to as auxiliary magnification) is a part of the microscope body that provides additional magnification in the form of a manual or motorized slider. All measurements and calculations should be done with the optovar in the 1X (or bypass) position.

It is advised that you measure pixel size for each camera at each magnification. If you have measured pixel sizes and an old manual magnification factor to accommodate a secondary camera, you can use these values as follows:

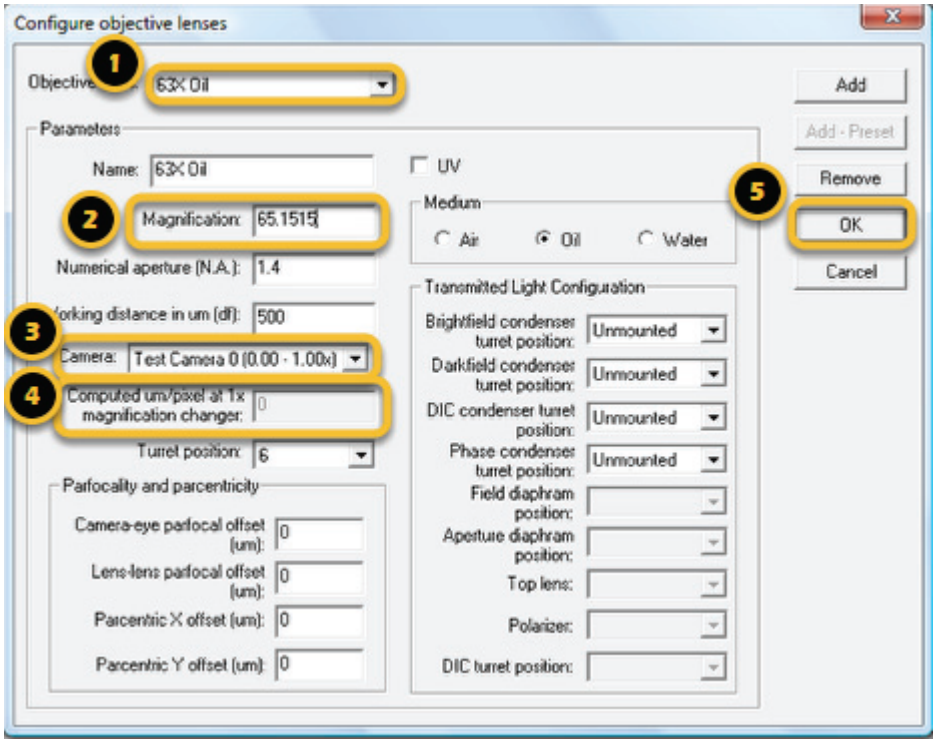
measured pixel size camera 1 = values found in Step #1
measured pixel size camera 2 = (values found in Step #1) (value found in Step #2)

For your convenience, a PIXEL SIZE WORKSHEET to compute these values can be downloaded from our support site. Please go to http://www.slidebook.com and download **Pixel Size Worksheets.xlsx**.

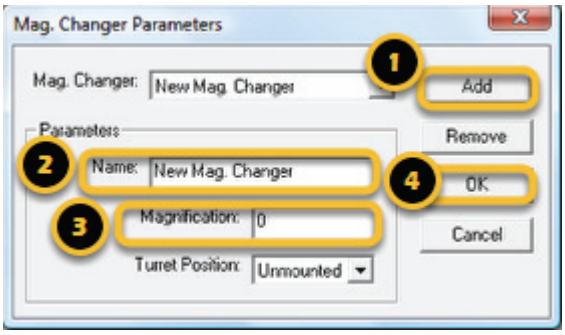
5. Enter the per-camera ***Magnification Factor*** under ***Administration tab >*** ***Hardware Properties > Camera X***. Click ***Apply Change*** for each value entered.



6. Go to ***Administration tab >*** ***Objectives***. Select an ***Objective Lens*** and enter the true ***Magnification*** value from step #4. Check to make sure the ***Computed um/pixel at 1X magnification changer*** value is correct for each ***Camera***. Click ***OK*** when finished.



7. Go to ***Administration tab >*** ***Mag Changer***. Create a magnification changer to represent the physical magnification changer slider (sometimes known as an optovar) on your microscope. Click ***Add***, ***Name*** the mag changer, and enter the ***Magnification*** value. When finished, click ***OK***.



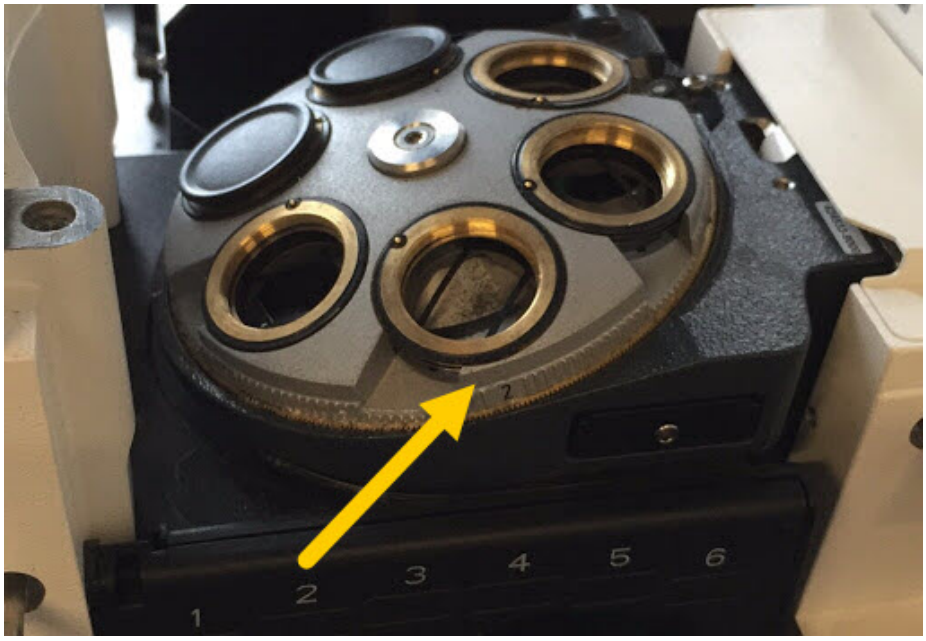
8. Save backup configuration files in a secure location. To do so, go to ***Support tab >*** ***Backup Preferences***. Once this configuration change has been made, pixel size adjustments will be made automatically each time a user selects a camera. For older images that require pixel size adjustment, select the image and go to ***Get Info > Edit*** to select a camera or manually enter a value for pixel size.

For additional assistance, please contact support@intelligent-imaging.com.

TUTORIAL: Adding an Objective

When you add a new objective to your system, it must be configured properly in SlideBook. The following tutorial will walk you through the process.

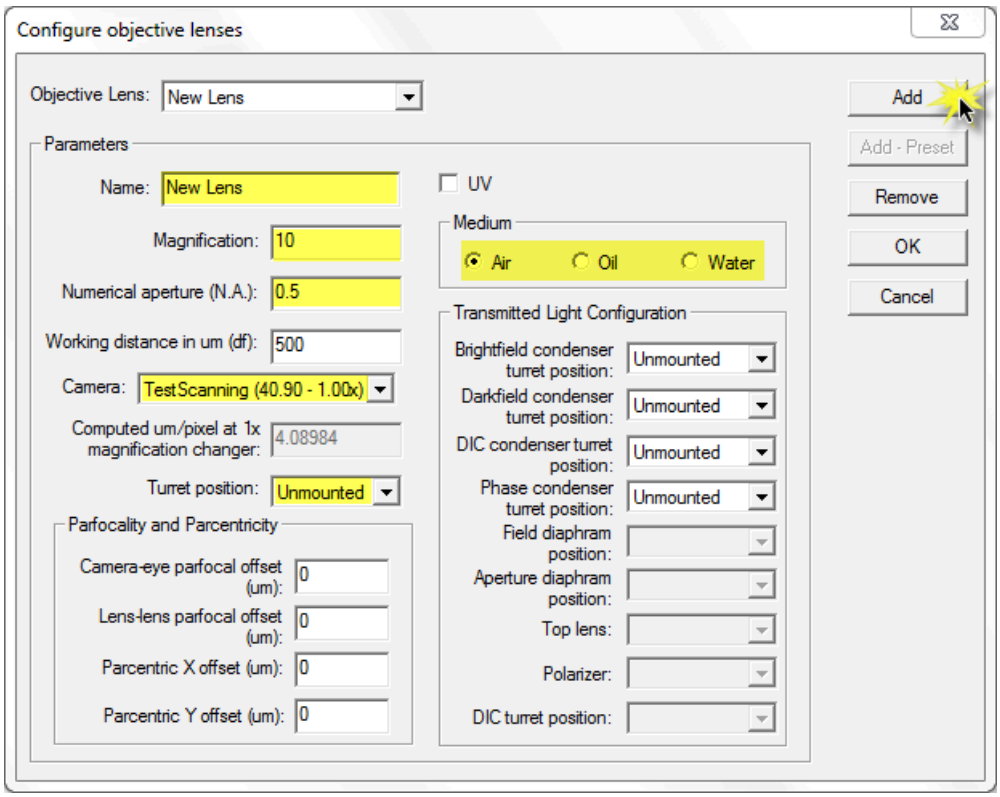
1. Locate an open space in the microscope objective turret. If you have a motorized objective turret (meaning you use SlideBook to physically change objective lenses), note the position you are using. In the example below, the arrow is pointing to position 2 as depicted on the outer edge.



2. Screw the objective into the turret, ensuring it is secure while not over tightening.

3. Within SlideBook, go to ***Administration tab >*** ***Objectives***. The ***Configure objective lenses*** window will open.

4. Click ***Add***. Complete the fields highlighted below. Refer to the Configure Objective Lenses window page for more details on the fields available. You may also wish to measure the pixel size for the most accurate analysis and image processing results. Refer to Measuring Pixel Size for more details.



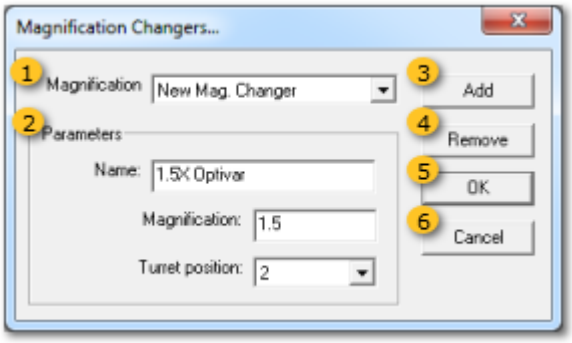
5. If you purchased additional optics such as a DIC prism or phase ring, load these into the condenser noting the positions used. If you have a motorized condenser, select the relevant condenser position for the associated technique from the dropdown menus in the ***Transmitted Light Configuration*** group.

6. Click ***OK***. Restart SlideBook to complete adding an objective.

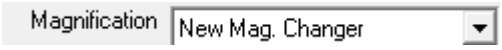
Magnification Changer Parameters window

A magnification changer, or optovar, is a component which may or may not be part of your imaging system. These optical components act to increase or decrease the effective magnification of an objective. If you do not have a magnification changer, you should use the predefined **1.0 x** definition.

Access in the Administration tab

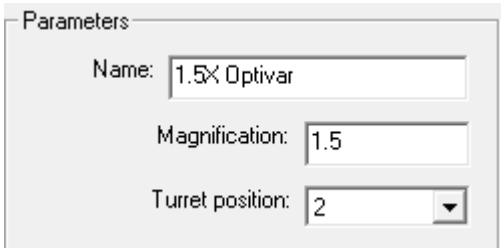


1 Mag. Changer Selection



Magnification is a dropdown menu containing a list of configured magnification changers. When you click ***Add***, a new option will be selected and labeled **New Mag. Changer**. You may edit the name and other details of the magnification changer in the ***Parameters*** group. To edit an existing magnification changer, select it from the menu, edit the parameters, and click ***OK***.

2 Parameters group



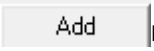
Enter ***Parameters*** as follows:

Name - Enter the name as it will appear for selection in the ***Focus Controls*** and ***Capture*** window.

Magnification - Enter the factor of magnification that the changer introduces. This value will be multiplied times the um/pixel value for an objective to assign pixel size.

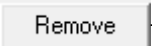
Turret Position - Select the turret position of the microscope when a motorized magnification turret is available. If the selection is manual, you must still assign a turret position.

3 Add button



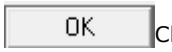
Press ***Add*** to add and configure a new magnification changer.

4 Remove button



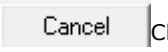
To remove a magnification changer, select it from the dropdown menu then click ***Remove***.

5 OK button



Click ***OK*** to apply changes and close the dialog.

6 Cancel button

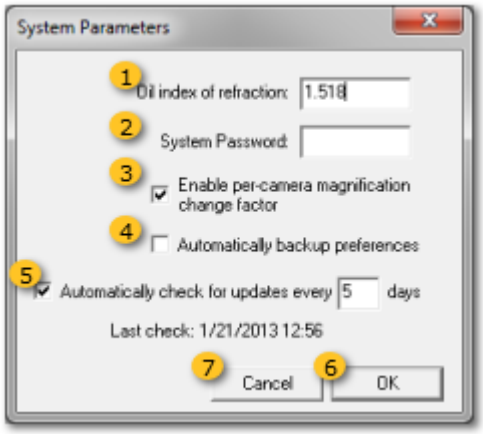


Click ***Cancel*** to discard changes and close the dialog.

System Parameters window

System Parameters includes general system settings not covered by other configuration dialogs.

Access in the Administration tab



1 Oil Index of Refraction

Oil index of refraction: 1.518

Enter the refractive index of the immersion oil for the microscope you are using. It is important to note that different manufacturers use different immersion oil and that you should always use the manufacturer's recommended immersion oil to maintain the optical integrity of the objective lenses.

2 System Password

System Password:

Enter a system password to password protect system configuration files. This is recommended at multi-user facilities. To remove a system password, simply delete it from this field and click ***OK***.

3 Enable per-camera magnification change factor check

☑ Enable per-camera magnification change factor

A per-camera magnification change factor is recommended when systems meet one of the following two conditions:

- Multiple cameras are detected and these cameras are compatible with new ability for SlideBook to query a camera for its pixel size
- A manual magnification changer is configured

Please review Configuring Pixel Size for complete details on how to measure and configure pixel sizes for your system.

4 Automatically Backup Preferences

☐ Automatically backup preferences

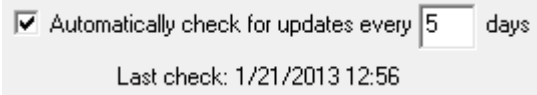
Check this box to automatically send copies of vital system configuration files (**SlideBookHardwarePropeties.dat** and **SlideBookPrefs.dat**) to 3i every time they are modified. This backup will only be successful if the computer is connected to the internet. Backup files will be created when ***OK*** is pressed in any the following dialogs:

Filter Configurations
Magnification Changers
Objectives

System Parameters
Hardware Configuration
Hardware Properties

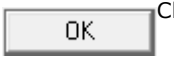
NOTE: The initial backup file will not be created until you open one of the relevant dialogs and click ***OK***. After initially setting up the automatic backup, you may find it useful to open ***Hardware Configuration*** and click ***OK*** then open ***Hardware Properties*** and click ***OK***. Because nothing is changing in this particular instance, it will not be necessary to restart SlideBook.

5 Check for Updates



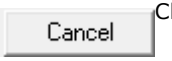
Check this box if the computer is connected to the internet (with anti-virus protection) and you would like SlideBook to automatically check for a more recent version every ***N*** days.

6 OK button



Click ***OK*** to apply changes and close the window.

7 Cancel button



Click ***Cancel*** to discard changes and close the window.

Preferences Options

SlideBook has several important files that contain configuration information. These are called ***Preferences*** files. Preferences are saved for the general system configuration and for specific user accounts. You can open folders containing these files or perform a backup by using menu items as described below.

See also:

[Suggestions for System Administrators]

Open User Preferences Folder

Opens the ***SlideBook > Users*** folder. This folder contains file for user-specific settings such as saved capture profiles, multiwell capture setups, custom user toolbar, etc.

Open System Preferences Folder

Opens the ***SlideBook > Global Preferences*** folder. This folder contains the essential configuration files: **SlideBookPrefs.dat** and **SlideBookHardwareProperties.dat**. Also contained here are system calibrations such as **PSFs.dat**, **Photomanipulation Calibrations.dat**, and **FlatFields.dat**.

Backup Preferences

Opens a dialog in which you can either create a new folder or select an existing folder. The selected folder will contain backups of the two directories listed above (**Users** and **Global Preferences**). It is suggested that you create backup files periodically and any time system configuration changes are made. These files can be used to completely restore a system should there be a catastrophic failure of the computer.

An additional insurance to back up your system includes sending your configuration files to 3i for free secure off-site backup. To do so, simply go to ***Support tab > Email Preferences***.

Exceptions

The **Exceptions** directory can be accessed by going to ***Administration tab >*** ***More options > Open System Preferences Folder*** then navigating up a level to the **SlideBook** directory. For example, in Windows 7, you may locate the **Exceptions** directory in **C:\ProgramData\Intelligent Imaging Innovations\SlideBook**. In the unexpected event of a software crash, **ExceptDump** files are created. These files contain important information about what was happening before the crash and, on the rare occasion when data was not saved, may be helpful in recovering data. Should you experience a software crash, please contact support@intelligent-imaging.com for instructions on providing the necessary files. System administrators may find it useful to routinely check this folder to delete unnecessary files.

Backing up and Restoring Preferences

Below, you will find useful information on backing up vital system configuration files locally. In addition to these tools, you may also want to explore the option to backup preferences files on 3i's servers (free of charge). More information can be found on automatically backing up preferences in the System Parameters window.

Backing Up SlideBook Configuration

1. To back up your SlideBook configuration, go to ***Support tab >*** ***Backup Preferences***.

2. Select where you would like the backup files to be located. If desired, create and name a new folder.

3. Click ***OK***.

4. Three folders containing system configuration will be placed in the directory designated. The folders are as follows:

   Exceptions – This folder contains backup data from interrupted sessions and will be discussed later in this section.

   Global Preferences – This folder contains the system configuration files such as **SlideBookHardwareProperties.dat**, **SlideBookPrefs.dat**, **PSFs.dat**, and **FlatFields.dat**.

   Users – This folder will contain files specific to individual users or the default user. This is where saved capture settings, point lists, and analysis protocols are saved.

5. As an added precaution, send your **SlideBookHardwareProperties.dat** and **SlideBookPrefs.dat** to 3i for offsite backup. Go to ***Help > Email Preferences***. Enter your email address and click ***OK***. The system preferences will be stored in our database with your system information. Simply contact support@intelligent-imaging.com should you need to retrieve these files in the future.

Restoring SlideBook Configuration

1. In order to restore your system preferences, you must first open the backup configuration folder.

2. Double-click on the ***Global Preferences*** folder and select all of the files and folders. Copy the data by right-clicking and selecting ***Copy*** from the menu.

3. Now, return to SlideBook and go to ***Administration tab >*** ***More options > Open System Preferences Folder***.

4. Click inside of the Windows Explorer folder view that opens. Right-click and select ***Paste*** from the menu.

5. You will be informed that there is already a file in this location with the same name. Select the option to overwrite all files or folders and click the box to apply to all files and continue.

6. You have now replaced the active system configuration files with the backup copies. Now you will restore the user preferences.

7. Go back to the backup configuration from Step 1.

8. Double-click on the ***Users*** folder and select all of the files and folders. Copy the data by right-clicking and selecting ***Copy*** from the menu.

9. Now, return to SlideBook and go to ***Administration tab >*** ***More options > Open System Preferences Folder***.

10. Click inside of the Windows Explorer folder view that opens. Right-click and select ***Paste*** from the menu.

11. You will be informed that there is already a file in this location with the same name. Select the option to overwrite all files or folders and click the box to apply to all files and continue.

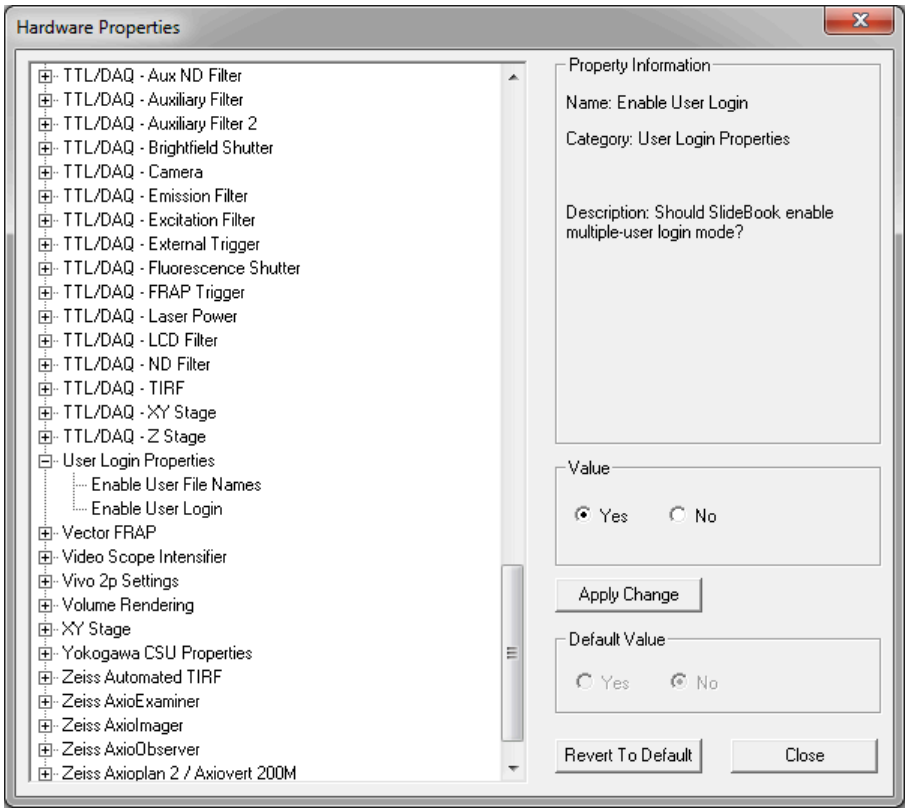
12. Close all folder views and restart SlideBook.

Creating Individual User Accounts

You may define multiple user logins to separate user preferences. The user login will allow individual users to access their capture preferences, various protocols, user toolbars, and other user-specific preferences. Hardware, filter, and objective configurations are shared among all users.

To configure user logins:

1. Go to ***Administration tab >*** ***Hardware Properties*** and scroll down until ***User Login Properties*** is visible. Click on the **+** next to ***User Login Properties*** to expand the section, and then click on ***Enable User Login***.



2. Click ***Yes*** and then ***Apply Change***.

3. Restart SlideBook. Upon startup, you will see the following screen:

4. To add a user, click ***Add User***. The following dialog will appear:

5. Enter the desired user name in the ***User Name*** edit field and click ***OK***.

6. Upon future logins, choose your user name from the drop-down list and then click ***Login***.

> **NOTE:** To delete a user login, from the ***Login*** window, select the desired user. Click ***Delete User,*** the required password is **microscope**.

Setting Up Multiple Configurations of SlideBook

Often, it is useful to have access to multiple sets of configuration files for SlideBook. This document will walk you through configuring shortcuts that will access different configuration files for a single installation of SlideBook 6.

How to Create Two Different Configurations of SlideBook

1. Right-click on the SlideBook desktop icon and select ***Copy***.

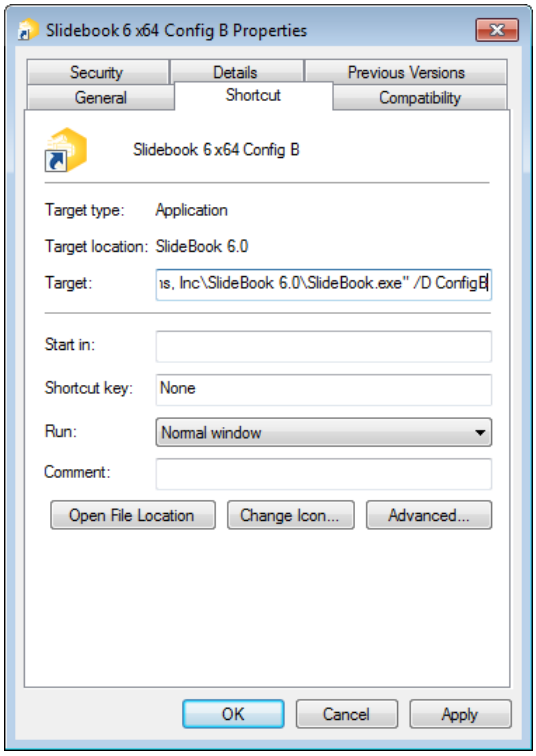
2. Left-click on an open area on the desktop. Right-click and select ***Paste***.

3. Right-click on the copy of the icon and select ***Rename***. Name the new icon something that will indicate the alternate configuration. For demonstration purposes, call this **SlideBook Config B**.

4. Right-click on the Config B icon again and select ***Properties***. Locate the field titled ***Target***. It should read something similar to **C:\Program Files\Intelligent Imaging Innovations, Inc\SlideBook 6\SlideBook.exe**.

> **NOTE:** If the ***Target*** field is not available, confirm that only one icon is selected and that the selected icon is the new icon and not the original icon.

5. In this step, we will create a folder that will contain the Configuration B preferences files. Append the ***Target*** field with **/D foldername**. Using our previous example, the field will read **"C:\Program Files\Intelligent Imaging Innovations, Inc \SlideBook 6.0\SlideBook.exe" /D ConfigB**. It is important that there are no spaces in the folder name.



6. Click ***Apply*** then ***OK***.

7. Depending on how you would like to create the alternate configuration, follow one of the options below:

   a) To use preferences files you have saved in a different location:

      i) Open SlideBook (using the new shortcut), decline to offer to migrate preferences from the previous version of SlideBook.

ii) Go to Administration tab***, More Options > Open System Preferences Folder***.
iii) Copy the other preferences files and paste them into this location.

b) To modify the preference files of the other installation of SlideBook 6:

i) Open SlideBook using the original shortcut, go to Administration tab***, More Options > Open System Preferences***.
ii) Copy **SlideBookHardwareProperties.dat** and **SlideBookPrefs.dat** from this location.

iii) Open SlideBook using the new (Config B shortcut), go to Administration tab***, More Options > Open System Preferences***.
iv) Paste **SlideBookHardwareProperties.dat** and **SlideBookPrefs.dat** to this location.

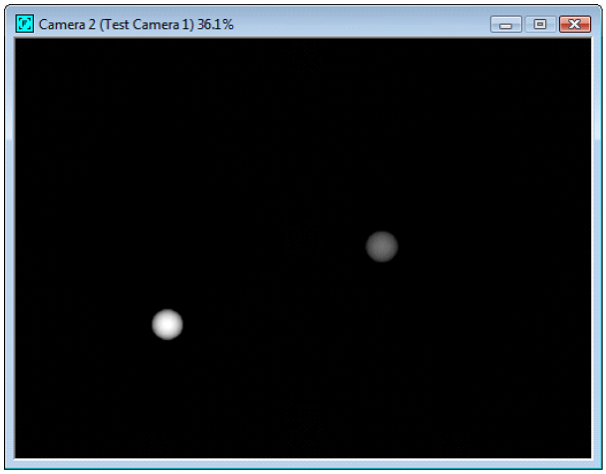
c) To create a completely new configuration:

i) Open SlideBook (using the new shortcut), decline to offer to migrate preferences from previous verisons of SlideBook.
ii) Configure SlideBook as usual.

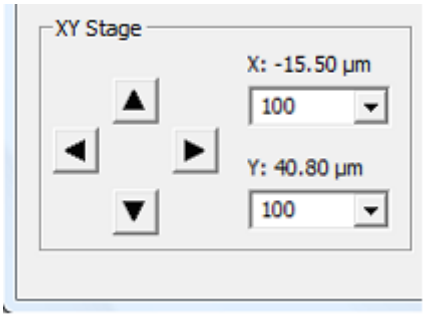
Configuring Camera Orientation for Motorized Stages

An essential part of system configuration is setting camera orientation with regards to the motorized XY stage. One way to tell if your camera orientation is configured correctly is to double-click on an object of interest in a live window. Double-clicking should result in the object of interest being centered in the window. If double-clicking does not result in this behavior, either pixel size or camera orientation has not been set properly. For instructions on setting pixel size, please refer to Configuring Pixel Size. Read below to learn more about configuring camera orientation on your system.

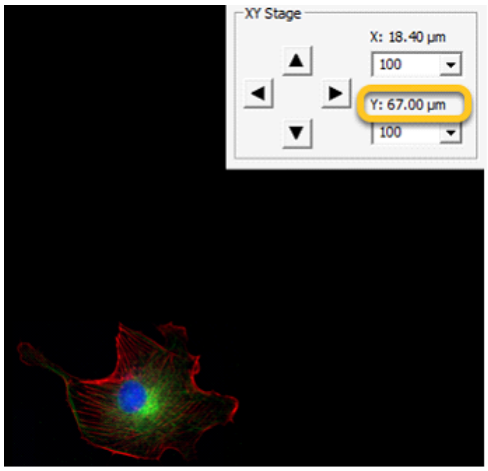
1. Begin by placing a slide on the microscope and bringing it into focus using the ***Focus Window***.

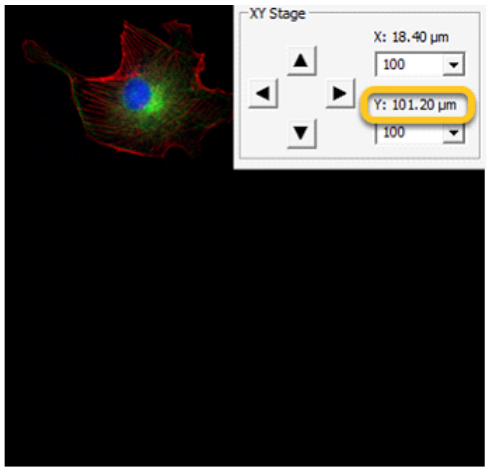


2. In the lower left corner of the ***Focus Controls***, note the ***XY Stage*** control area.

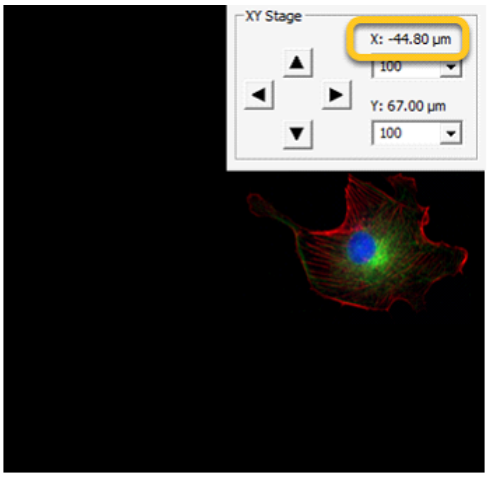


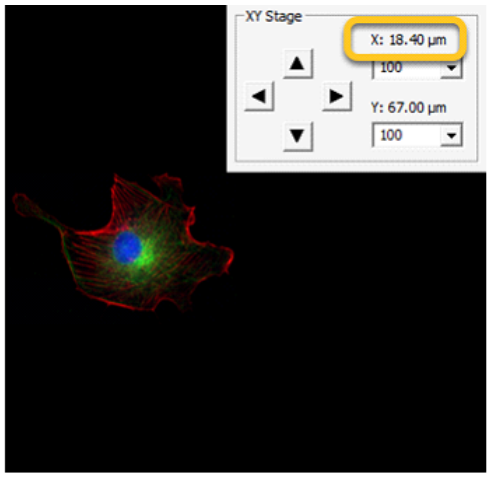
3. Using the stage controller, move the stage such that the field in the live ***Camera*** window moves down (object moves up). Note whether the ***Y*** value in the ***Focus Controls*** increases or decreases when you move the field down.



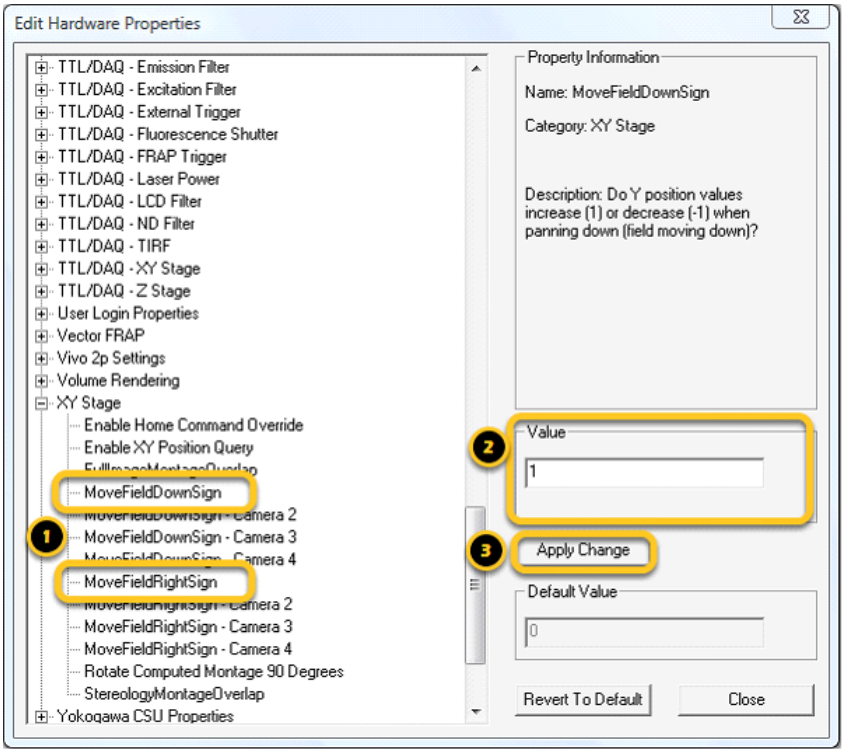


4. Using the stage controller, move the stage such that the field in the live ***Camera*** window moves to the right (object moves left). Note whether the ***X*** value in the ***Focus Controls*** increases or decreases when you move the field to the right.





5. Go to ***Administration tab >*** ***Hardware Properties***. Navigate to the ***XY Stage*** properties and expand the category. The ***MoveField*** properties are the ones you will edit.



MoveFieldDownSign - Camera x - This property refers to the Y axis. If the Y value increased when moving the field of view down, enter "**1**" in the ***Value*** field and click ***Apply Change***. If the Y value decreased, enter "**-1**" and click ***Apply Change***.

MoveFieldRightSign - Camera x - This property refers to the X axis. If the X value increased when moving the field of view right, enter "**1**" in the ***Value*** field and click ***Apply Change***. If the X value decreased, enter "**-1**" and click ***Apply Change***.

6. Click ***Close*** and restart SlideBook to apply changes.

7. To test the camera orientation properties, bring up a live window. Select the ***Select Tool*** from the ***Regions*** group. Double-click on an object of interest in the live view; the object should become centered in the live ***Camera*** window.

8. Repeat this process for each camera on the system. Backup system configuration by going to ***Support tab >*** ***Backup Preferences***.

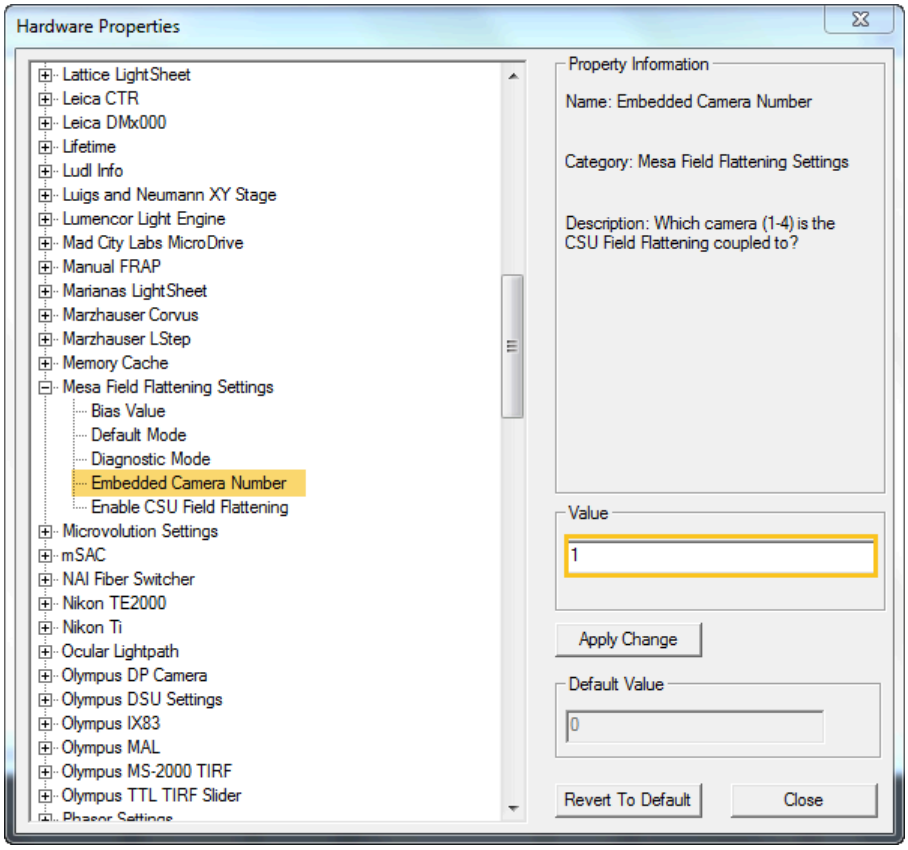
If you have properly set the MoveField properties and objects are still not centering properly, check the pixel size settings for the objective or consult support@intelligent-imaging.com.

Using Mesa in SlideBook

Proper Mesa operation requires a combination of hardware and software. The Mesa optics provide better excitation throughput and field uniformity for the Yokogawa CSU-X and CSU-W scan heads. Additional processing in SlideBook further smooths the effect of predictable local fluctuations in illumination intensity. It does this by automatically applying flat field correction to all frames collected by the designated camera, even in the live window.

For Mesa to work correctly, you must designate a specific camera for which Mesa flat field correction will be applied and collect a flat field for every filter configuration that may be used with that camera.

In Hardware Properties:
This camera is designated with the ***Mesa Field Flattening Settings > Embedded Camera Number*** hardware property. Note that this number matches the number in the Cameras section of Hardware Configuration, namely it should be 1 - 4.



The flat fielding is turned on when ***Mesa Field Flattening Settings > Enable CSU Field Flattening*** is set to ***Yes*** and when there is a matching flat field in the list of flat fields for the active filter configuration.

Conventional flat field correction in SlideBook involves using a captured dark frame for subtraction in each image capture. Mesa field flattening uses an estimated dark frame according to the value set in ***Mesa Field Flattening Settings > Bias Value***. It is important that this value is as close to the actual bias value of the camera as possible. The bias value can be estimated by taking an image with no light going to the camera and determining the average pixel value.

NOTE: There should be no noticeable gradient to the dark image used for bias value estimation image in modern cameras. If there is a gradient, there is a problem that needs investigation and you should contact 3i Support.

It is possible to temporarily change the bias value (and whether the field flattening is enabled or not) by selecting ***Administration > More Options > Mesa Field Flattening Diagnostics***. Note that to make any change permanent, you will need to edit the hardware properties and restart SlideBook.

System Administration

For any imaging system, it is best if one or two primary people are responsible for the care and upkeep of the equipment. Upkeep ranges from ensuring the system is clean and aligned to maintaining the most up to date software available. Please read on to learn what you can do to keep your system operating at its best.

SlideBook Maintenance

It is recommended that you use the most recent version of SlideBook available. From within SlideBook, go to ***Support tab >*** ***Check for Updates*** to confirm you are running the latest version. Free software updates are available for the first year after installation and can be maintained after that time for a small fee. We put out quarterly user newsletters with the option to receive an email every time a new version of SlideBook is released. You may subscribe to our newsletters here.

Always maintain current backup files. More information on this can be found in Backing up and Restoring Preferences for local backup or in System Parameters window for remote backup through 3i.

3i Support (support@intelligent-imaging.com or https://www.intelligent-imaging.com/contact) can answer any questions you have with regards to software support contracts and updates. The team of application specialists and engineers can also assist with any questions you may have about SlideBook that aren't answered in this online help. Please also contact support if you have a lost or broken software dongle (key) or are interested in purchasing additional offline licenses for post-acquisition analysis.

Hardware Maintenance

System Maintenance Agreements are available for purchase after the initial 1 year system warranty. There are a variety of plans to fit individual needs and budgets. For more information contact support@intelligent-imaging.com.

In addition to 3i-provided hardware maintenance, there are a number of things a system administrator can do to ensure the equipment is performing optimally. These include:

- Fluorescent light source bulb maintenance
- Transmitted light source bulb replacement
- Transmitted lightpath alignment (Koehler Illumination)
- Cleaning objective lenses (an excellent resource is the Molecular Expressions website http://micro.magnet.fsu.edu/primer/anatomy/cleaning.html)
- General system cleanliness (an excellent resource is the Molecular Expressions website http://micro.magnet.fsu.edu/primer/anatomy/cleaning.html)
- Laser care and maintenance
- System power cycling

Computer Maintenance

Just as with any personal computer, the computer running your imaging system requires regular care. It is recommended that you regularly do the following:

- Maintain an up-to-date anti-virus program
- Run recommended operating system (Windows) updates
- Backup the hard drive
- Remove unused data/files
- Empty trash/recycling bin
- Defragment computer
- Check ***Exceptions*** directory and delete unnecessary files (more information here)
- Delete Users who are no longer active (simply delete the entire user folder from **C:\ProgramData\Intelligent Imaging Innovations\SlideBook 6.0\Users**) and purge old saved capture settings (**.prefs** files).

Setting Up Koehler Illumination

Introduction

Koehler illumination is a critical determinant of optical performance in light microscopy. When a microscope has been properly adjusted for Koehler, each source point from a lamp contributes equally to illumination in the specimen plane. Thus, variations in intensity in the image can be attributed to the object and not to irregular illumination from the light source. The following tutorial describes the process of setting Koehler illumination on a Zeiss 200M research microscope.

Adjusting the microscope for Koehler illumination

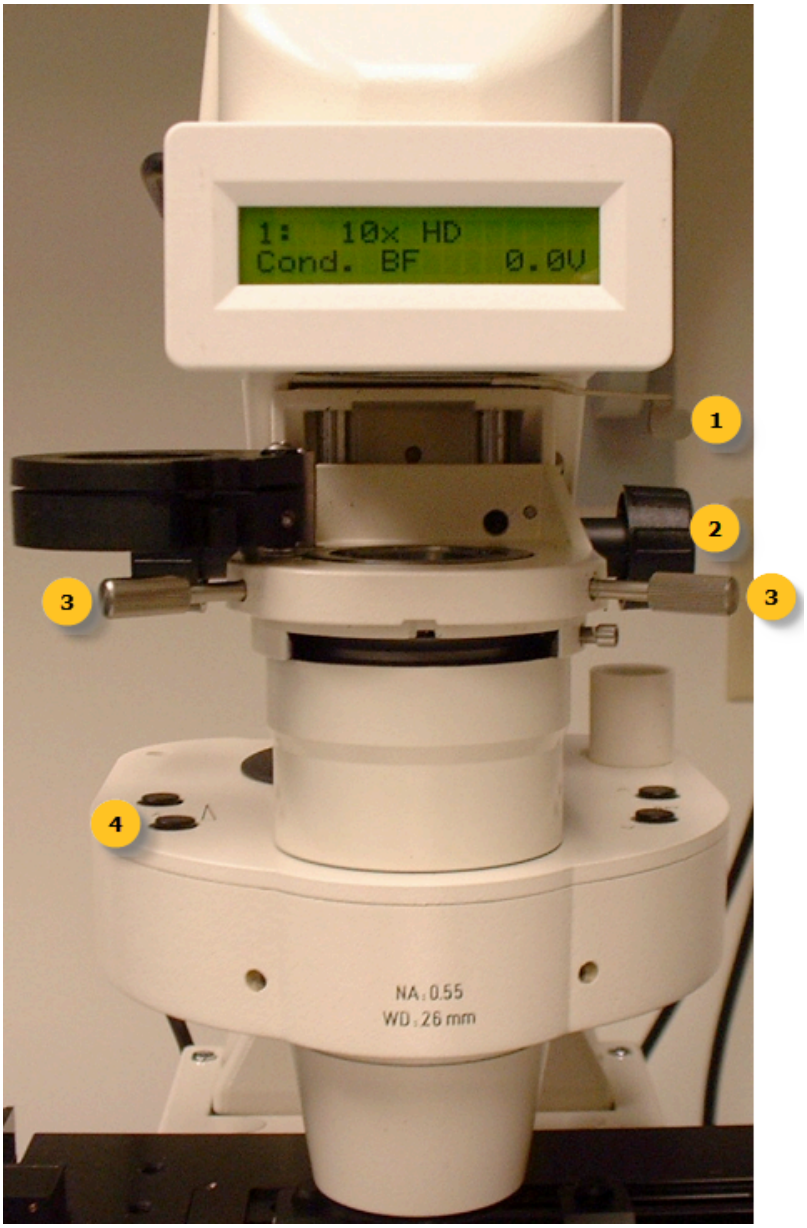


Figure 1	
1	Field diaphragm adjust lever
2	Condenser focusing knob
3	Condenser positioning screws
4	Condenser numerical aperture adjust buttons (front aperture diaphragm)

1. Place a specimen slide on the stage of the microscope.

2. Turn on the microscope lamp and focus on the specimen. At this point, the field diaphragm and the condenser front aperture diaphragm (figure 1) can be fully opened to ensure that the sample is illuminated.

3. Focus and center the condenser. With the specimen in focus, close down the field diaphragm completely. Now while examining the specimen through the eyepiece, focus on the angular outline of the diaphragm using the condenser's focusing knobs. If there is no light after closing the field diaphragm, the condenser diaphragm may be out of the field of view. In this case use the condenser positioning screws to move the condenser diaphragm into the field of view (it may be helpful to open the field diaphragm slightly thereby producing a larger target to find). After focusing the image of the field diaphragm in the eyepiece, bring it into the center of the field of view using the condenser positioning screws. Figure 2 shows a picture of a properly focused and centered field diaphragm.

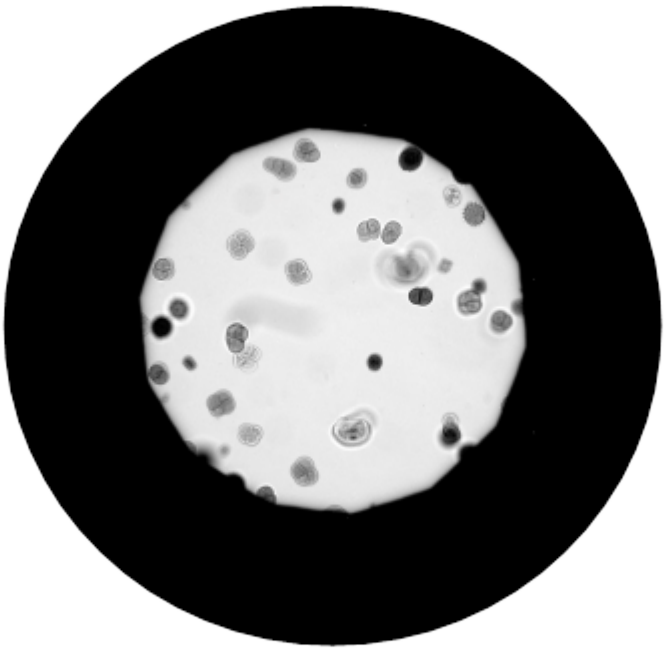


Figure 2

4. Open the field diaphragm until it is almost as large as the field of view in the eyepiece. By constantly keeping a small amount of the field diaphragm visible during imaging, the degree of Koehler illumination can be easily determined. If the image of the field diaphragm appears blurry of off centered at any time, simply readjust the condenser position as described above to achieve even illumination. When imaging through the camera, opening the field diaphragm to a point where it is only slightly larger than the field of view of the camera may yield the best results.

5. The microscope is now adjusted for Koehler illumination. As a final adjustment, the numerical aperture of the condenser can be changed using the two buttons located on the left side of the condenser diaphragm. This adjustment will affect the resolution of the microscope, change the contrast of the image, and establish the depth of field. It is not possible to optimize the condenser aperture position for both contrast and resolution and the final setting is usually based on the inherent contrast of the specimen.

Useful references

Inoué, S., and Spring, K. R. (1997). Video microscopy : the fundamentals. (New York, Plenum Press).

Murphy, D. B. (2001). Fundamentals of light microscopy and electronic imaging. (New York, Wiley-Liss).

"Molecular Expressions Optical Microscopy Primer" website at Florida State University. http://micro.magnet.fsu.edu/primer/index.html

SlideBook Permissions

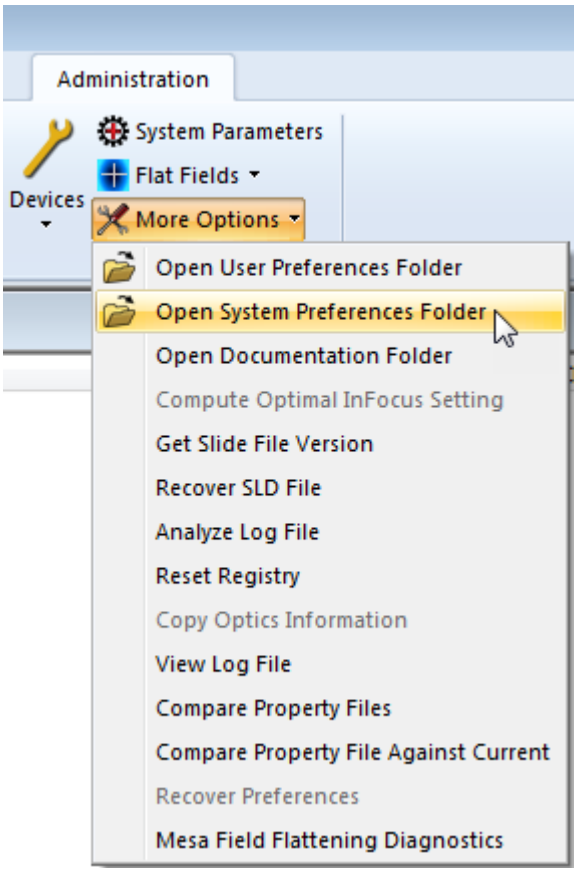
Often facilities will limit administrative privileges to IT professional. This page will explain when SldieBook needs specific permissions.

At Installation

Administrative privileges are required for initial installation of SlideBook. You may also require these permissions to download and install upgrades. When adding hardware upgrades, you may require these privileges to install DLLS.

Preferences Directory

SlideBook needs full read/write access to the Global Preferences directory. The Preferences directory can be located by opening SlideBook and going to the Administration tab > ***More Options > Open System Preferences Folder***.



Command Logging

At times, 3i personnel may ask you to enable command logging. If this is the case, SlideBook will need access to the log file. The log file can be found byby opening SlideBook and going to the Administration tab > ***More Options > Analyze Log File***. Then click ***Show Log*** to see the location.

Email Preferences / Backup Preferences

These options will contact the 3i server dbks.intelligent-imaging.com using regular HTTPS.

Network Permissions

In cases where specific network permissions need to be identified for a firewall, SlideBook requires network access to the following sites:

www.slidebook.com
www.intelligent-imaging.com
support.intelligent-imaging.com
spaces.hightail.com/uplink/3isupport
get.teamviewer.com

In addition to these sites, when a network key is used, SlideBook will require access to the IP address / domain name for the key.

Deconvolution Module

Conventional widefield, spinning disc, and point scanning confocal systems all introduce some amount of blur to an image. This is the result of having a limited aperture of the objective lens. Though the blur is greatly reduced when confocal techniques are used, image contrast and resolution can often be improved using deconvolution algorithms.

The Deconvolution Module adds advanced deconvolution tools to the SlideBook architecture. SlideBook's Deconvolution module is a powerful tool for removing or reassigning out-of-focus information from a fluorescence image set. The Deconvolution module adds the following functionality:

Point Spread Function (PSF) Guide

Constrained Iterative deconvolution allows the use of PSFs that are either measured directly using fluorescent beads or approximated computationally. The PSF Guide allows you to generate a database of measured point spread functions for each optical configuration. This allows you to refer to stored data automatically when performing Constrained Iterative deconvolution. The Points Spread Function Guide summarizes the process of collection or import of a PSF as well as walks you through adding the PSF to your library. SlideBook also provides the ability to Manage PSFs.

Nearest Neighbors Deconvolution

This algorithm is more computationally intensive and accurate than the No Neighbors algorithm. An image must have at least three planes in order to be deconvolved with the Nearest Neighbors algorithm.

Constrained Iterative Deconvolution

The constrained iterative approach is the most computationally expensive form of deconvolution and, if performed properly, can result in the best final image. All statistical analysis should be performed on images that have been deconvolved with the constrained iterative algorithm. Constrained iterative deconvolution can be performed using calculated or theoretical PSFs (point spread functions) but better results can be obtained when a true measured PSF is used.

Autoquant Blind for Widefield or Confocal

Blind deconvolution is a class of constrained iterative in which not only the resultant image but also the PSF is computed by algorithm. Blind Deconvolution can be used with Widefield Fluorescence, Point Scanning Confocal, and Spinning Disk Confocal.

Nearest Neighbor Deconvolution

Nearest neighbors deconvolution is a deblurring technique that uses a simplifying assumption that most of the out of focus blur in an image is due to the planes directly neighboring the plane of interest (i.e. directly above and below). To perform nearest neighbors deconvolution, one must start with a data set that is at least three planes deep. The algorithm computes how much blur the neighboring planes contribute to the captured image and subtracts the blur out. Since the algorithm is purely subtractive, it tends to delete very low intensity features. It is considerably less sensitive than constrained iterative deconvolution to low intensity image defects such as dust in the optical path. The largest single factor that affects the result of nearest neighbor deconvolution is the distance between neighboring planes. A smaller distance between neighboring planes will cause the algorithm to subtract more from the plane of interest.

More information regarding the user interface and settings can be found in the Nearest Neighbors Deconvolution window.

Constrained Iterative Deconvolution

The constrained iterative approach is the most computationally expensive form of deconvolution and, if performed properly, can result in the best final image. All statistical analysis of intensities should be performed on images that have been deconvolved with the constrained iterative algorithm. Essentially, the algorithm makes sequential guesses about the true distribution of fluorescence in an unblurred three dimensional data set. It then compares its computationally blurred guess with the original data and modifies its next guess appropriately. Sequential guesses should converge on a unique solution. The user must determine how many iterations or guesses the algorithm makes.

Because constrained iterative deconvolution considers all of the data to perform its guesses, the approach is very sensitive to the condition of the original data. If performed improperly, the constrained iterative approach will result in a highly artifactual data set. To ensure good results with this approach the following conditions should be met:

- The original data set should span the sources of fluorescence in the z dimension. If there are significant sources of fluorescence that reside above or below the original data set, the algorithm will not be able to properly assign their source within its next guess.

- The data should be flat field corrected. If there are any constant artifacts within each plane of the original data (e.g. dust specks), the algorithm must guess 3D structures which will account for them. This usually results in fictitious tube structures in the final image set.

- The data, as always, should reside in the linear range of the camera (not saturated) and should be well above the noise floor of the camera. Very low intensity data sets have a poor signal to noise ratio. This will result in a “scratchy looking” final guess.

- While calculated or estimates PSFs (point spread functions) will give valid results, the result will almost always be improved with true measured PSFs. Measured PSFs are like the custom fingerprints of an imaging system. See also the Point Spread Function Guide.

Constrained iterative deconvolution was historically very time consuming as well as memory intensive. With the advances of computer technology, data sets that used to take hours to deconvolve now deconvolve in seconds. That being said, you may still wish to save particularly large 4D data sets to deconvolve at night in order to free up the computer.

More information regarding the user interface and settings can be found in the Constrained Iterative Deconvolution window.

Autoquant Blind Deconvolution

3D Blind Deconvolution is an iterative and constrained algorithm. It is iterative in the sense that it repeats the same computational operations many times while converging to the enhanced image solution. It is constrained in the sense that it only accepts deconvolved images that have the correct mathematical properties of nonnegativities, (that is, it does not allow the tracer concentration to have negative values) and smoothness (suppresses snowy-like noise due to low-light levels). 3D Blind Deconvolution is a method of deconvolution that adapts itself to the real PSF of the microscope system (which can be significantly different from the theoretical PSF and from the previously measured PSFs) due to specimen and instrument variations. The Autoquant Blind Deconvolution system is able to adapt to PSF changes within a specimen itself. Thus, the deconvolved results are superior to those methods which utilize theoretical or previously measured PSFs.

More information regarding the user interface and settings can be found in the Autoquant Blind Confocal Deconvolution window and the Autoquant Blind Widefield Deconvolution window.

Tips for Deconvolution

There are several keys to ensuring that your deconvolution runs smoothly and produces optimal results.

- Make sure that the image information is correct as described in the Get Info/ Image Info window. You must have the emission wavelengths and all objective information correct.

- Do not allow pixels to become saturated. If data seems vastly over-processed or deconvolution results in **0** intensity values the raw data may have saturated pixels. It is essential that images do not contain any saturated pixels.

- Capture images at optimal z section spacing. Capturing too planes too close together adds excess file size and image processing time. Capturing planes too far apart excludes information and may result in lost features.

- Ensure the entire area is imaged. In an ideal world, you would be able to capture 3 planes on either side of the actual object you are imaging.

- If at all possible crop the image before performing constrained iterative or blind deconvolution. This will increase the speed of the operation and prevent your operating system from crashing.

- If you are deconvolving a large image, be sure that no other images or programs are open.

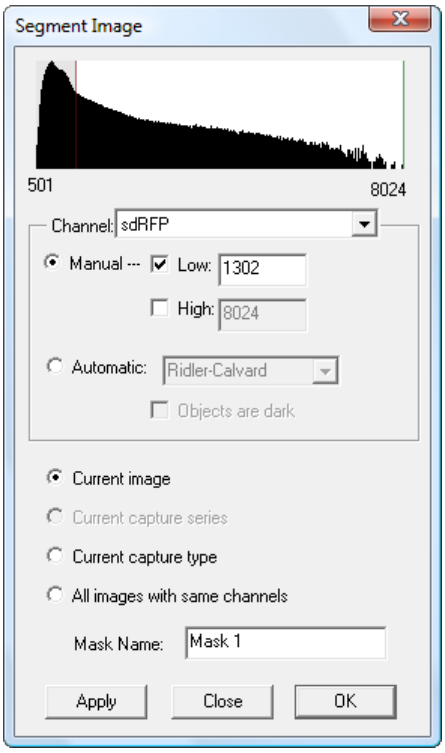
- An error message of "The ETA is greater than the lens NA". This means that the objective lens was not configured properly in the Configure Objective Lenses window.

Macro Scripting

SlideBook™ includes a macro scripting system to enhance the flexibility and power for advanced users. Many image and mask processing commands now have a macro command counterpart. This lets you automate sequences of operations on images and masks and ensure that all images in each slide are processed identically.

The scripting language is very simple - it is a sequence of text commands that let SlideBook know what operation to do, on which image (or images) to do it, and what the parameters are.

Take, for instance, creating a mask through threshold segmentation. Interactively, you would open up the ***Segment Mask*** dialog box:



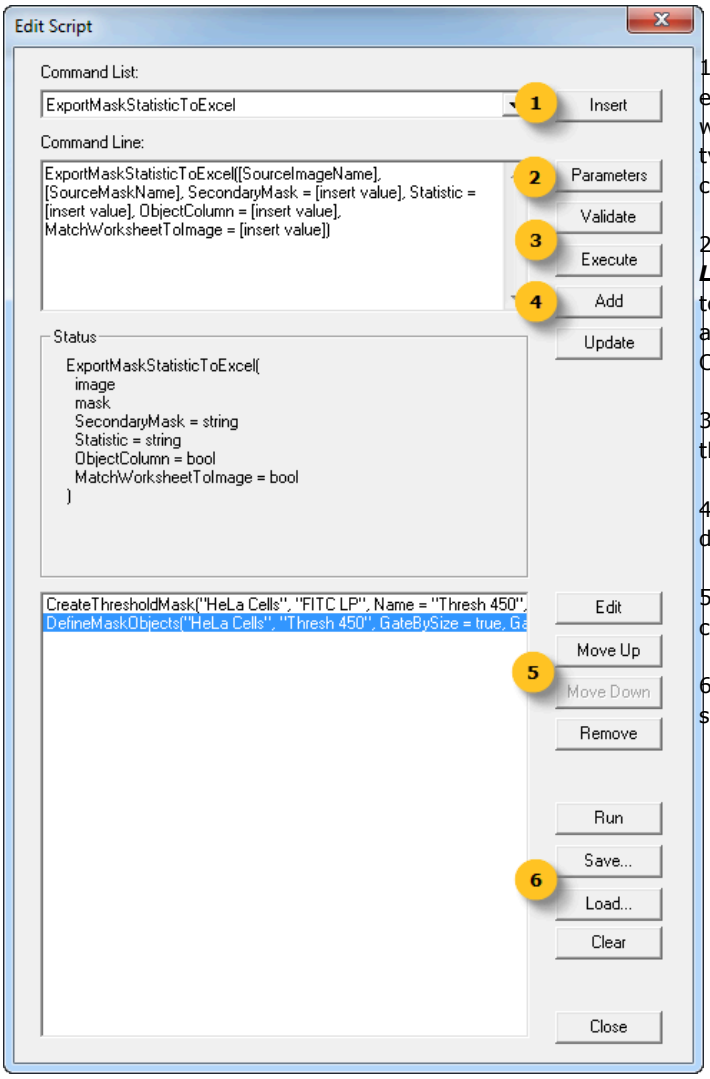
In the macro scripting language, the equivalent command would be:

CreateThresholdMask(<current image>, "sdRFP", Name = "Mask 1", LowThreshold = 1302)

The command language syntax was designed to be simple, readable, and extensible. For instance, the name of an image can be either the name, enclosed in double quotes, like **"He La Cells"** or a placeholder, such as **<current image>** or **<all images>**. All but the most common parameters are named, so when you are reading a script, you immediately see what the values mean. Any optional parameters can be omitted. Finally, even if the command is expanded significantly in the future, an old script is likely to still work without requiring any modification.

Using the Edit Script Dialog

All macro scripting control is done through the ***Edit Script*** dialog, which you can open from the Scripting tab. This dialog will be explained in greater detail on the following pages. You can also review the specific functions of each field in the dialog in the Edit Script window page. Below, you will find a general overview of the use of this dialog.



1. Select a command and press ***Insert***. The command will be entered in the ***Command Line*** field and parameters available will be displayed in the ***Status*** field. Alternatively, you may type directly in the ***Command Line*** field if you know the command (see also Macro Command Language).

2. Press the ***Parameters*** button to populate the ***Command Line*** field with parameters available. Refer to the ***Status*** field to see the type of variable required. Each available command and associated parameters are detailed within the Macro Command Language section of online help.

3. Use ***Validate*** and ***Execute*** to test the command and run the command.

4. Press ***Add*** to add the command to the script. The script is displayed in the lower large edit field.

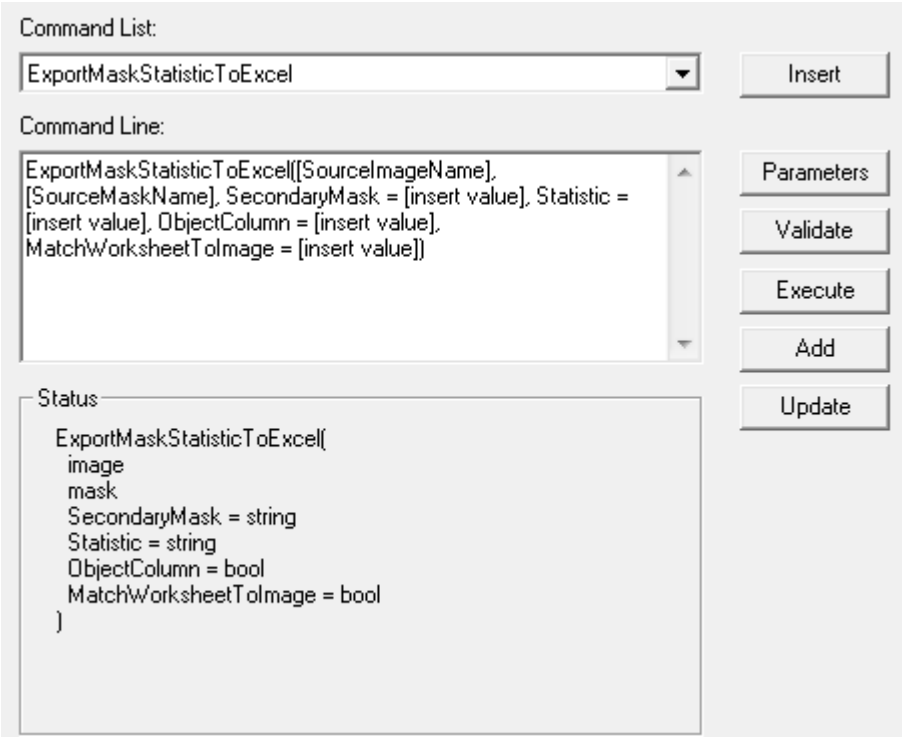
5. Use the ***Edit***, ***Move***, and ***Remove*** buttons to alter the commands and the command order in the script.

6. Use the ***Run***, ***Save***, ***Load***, and ***Clear*** buttons to manage scripts.

Editing a Command

The ***Command List*** is a dropdown list that contains all of the available commands. The commands are currently sorted so that similar operations appear together, rather than alphabetically.

If you click on the ***Insert*** button, the command will appear in the ***Command Line*** edit field and the parameters will be listed in the ***Status*** text box. Each parameter describes the type of parameter and whether it is required or not:



Clicking on the ***Parameters*** button will fill out the ***Command Line*** text field with a template that you can overwrite with actual parameter values. You may refer to the Macro Command Language section for a detailed description of each command and the associated parameters.

> **NOTE:** It is not necessary to enter commands using the ***Insert*** and ***Parameters*** buttons. You can also simply type in the command line directly.

The ***Validate*** button will perform an initial syntax check to see if the command line is properly formed. It does not check the values to see if they are appropriate for the command (e.g., whether an image parameter indeed corresponds to an image in the current slide, etc). In other words, clicking ***Validate*** will help catch some basic errors in the script command, but by no means guarantee that it will work properly.

The ***Execute*** button will run the command in the ***Command Line***. If you have used a placeholder such as <**all images**> it will run the command on all images in the current slide.

The ***Add*** button adds the command to the script (list of commands) at the bottom of the dialog.

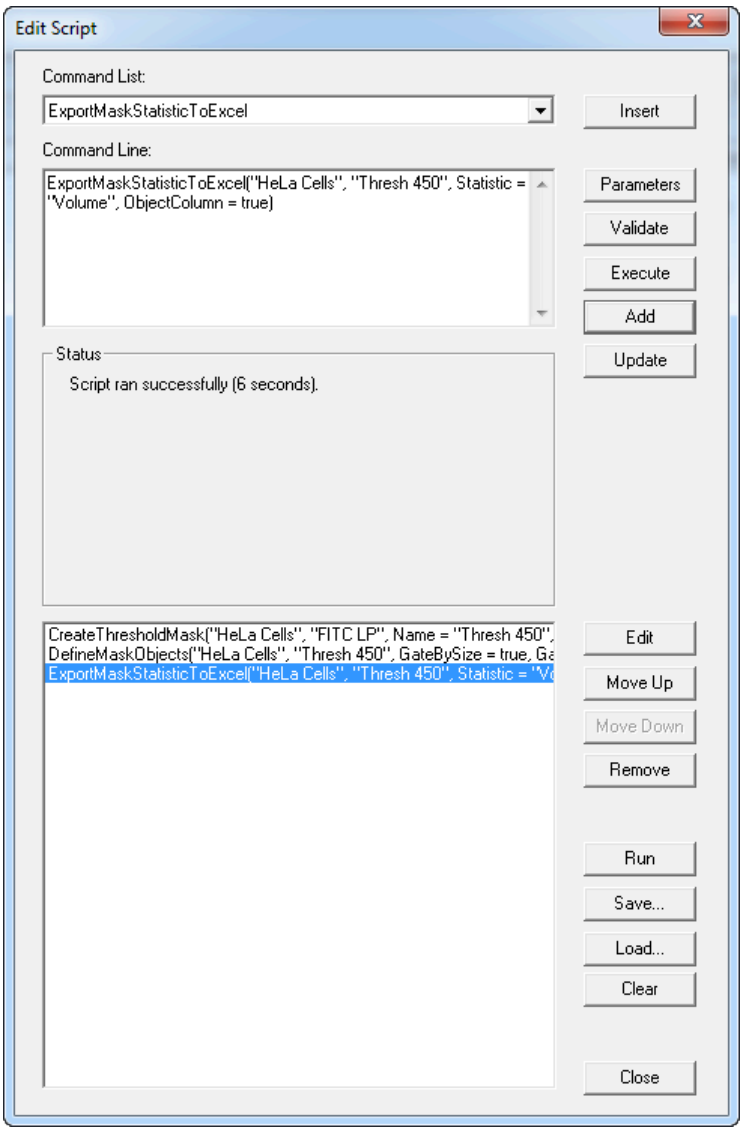
Building a Script

Assembling a script can be done by simply entering individual command lines and adding them to the script one by one. This interface is interactive, so you can try out each command line using ***Execute*** and then using ***Add*** to append it to the script. You have full access to the rest of the SlideBook user interface while the dialog is open, so you can also perform other operations that do not end up in the script.

For instance, the following script takes an image in the **QuickTourWIN2.sld** slide, creates a mask by thresholding the **FITC LP** channel, removing small objects, and then exporting the areas to Excel:

CreateThresholdMask("HeLa Cells", "FITC LP", Name = "Thresh 450", ThresholdLow = 450)
DefineMaskObjects("HeLa Cells", "Thresh 450", GateBySize = true, GateMinimum = 4, SizeAsVoxels = true)
ExportMaskStatisticToExcel("HeLa Cells", "Thresh 450", Statistic = "Volume", ObjectColumn = true)

You can try entering each of these commands one by one, and then adding them to the script:



You can run the entire script at once by selecting the ***Run*** button.

If you want to edit one of the command lines, select the command line and click the ***Edit*** button. The ***Command Line*** edit field will be updated with that line. After editing, click the ***Update*** button to replace the corresponding line in the script.

If you want to change the order of commands, you can use the ***Move Up*** and ***Move Down*** buttons. ***Remove*** deletes the selected command line from the script.

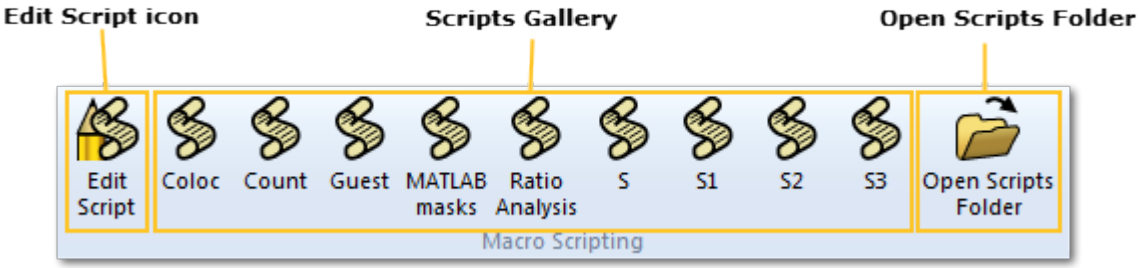
SlideBook macro scripts can be saved as files with the default extension **.sbs**. You can click ***Save*** to save a script file to disk and ***Load*** to load a script file from disk.

> **NOTE:** It is also possible to create and edit a script using any text editor. The only restriction is that you must save it as a pure text file, and not in rich-text format (RTF) or some other enhanced format.

Clear erases the entire macro script and lets you start over.

Run Macro Scripts

SlideBook displays the first 10 scripts as icons in the script gallery (shown below). You can manage scripts through ***Open Scripts Folder***. The displayed scripts are the first 10 **.sbs** files listed in alphabetical order.



Macro Command Language

Version 1 of the SlideBook macro scripting language is intended to provide an underlying basis for specifying sequences of operations on slides, images, masks, and regions.

In version 1, there are no explicit flow control operators. A macro script consists of a simple sequence of commands.

Commands can have a combination of required and optional arguments. Most arguments, besides a few expected for most all commands (e.g., which image the command is working on) are named. In other words, the argument is comprised of a pair — the name of the argument and its value. This allows for easier readability as well as forward compatibility if the argument list is modified in future versions of SlideBook. An example command follows:

Convolve2DChannel("Image 1", GFP, InPlace = true, KernelSize = 3, KernelElements = [0 -1 0 1 0 1 0 -1 0])

While there are no flow control operators, there are reserved identifiers that stand in place of an image and instruct the script processor to run the command on either the current image, <**current image**>, or all images, <**all images**>.

In version 1, these identifiers are evaluated on a line by line basis right before each line is executed. In other words, if line 1 in the script adds an image to the slide, and line 2 works on all images, it will work on all the pre-existing images as well as the one created due to the execution of line 1.

The following sections list the macro commands, grouped by category:

General Control Commands
Image Processing Macro Commands
Mask Commands
Annotation Commands
Deconvolution and Advanced Image Processing Commands
Import and Export Macro Commands
Particle Tracking Macro Commands
MATLAB Interface Commands
Miscellaneous Commands

The description for each command gives a template for the command and a description of each argument. A typeface guide is provided at the top of each command page to assist you in fully understanding each argument.

Each command has an implied return value, which is whether it succeeded or failed. Depending on how a script processor is operating, a command failure may terminate the entire script or merely append the failure and its cause to a log file.

Scope

Images in SlideBook can have one of the following five types:

- 2D
- 2D timelapse (2DTL)
- 3D
- 4D
- Montage

The current macro command scripting system does not support montage images.

Argument Types

Macro commands can have arguments of the following general types:

- bool – true or false
- enum – enumerated type
- int – a signed integer between -32767 and 32767
- float – a floating point value
- int array – a vector of signed integers, as in [0 1 2]
- float array – a vector of floating point values, as in [0.0 0.5 1.0]
- string – text delimited by double quotes

and one of three specific types:

- image – text or a string that corresponds to an image that already exists (or <all images>)

- mask – text or a string that corresponds to a mask that already exists
- channel – text or a string that corresponds to a channel that already exists

General Control Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

OpenSlide

OpenSlide (Filename = *"filename"*)

Opens the specified slide from disk. A complete location string is required unless the slide file is located in the directory where SlideBook is installed.

Example:
OpenSlide(filename="C:\Users\CoreLab\Desktop\SampleImages\QuickTourWIN2.sld")

CloseSlide

CloseSlide (Filename = *"filename"*)

Opens the specified slide from disk. A complete location string is required unless the slide file is located in the directory where SlideBook is installed.

Example:
CloseSlide(filename="C:\Users\CoreLab\Desktop\SampleImages\QuickTourWIN2.sld")

Image Processing Macro Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

ApplyFlatField

ApplyFlatField (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"***)**

Finds the flat field in the flat field database that corresponds to channel and applies it to image. Fails if the flat field cannot be found. To create a new channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true.**

Example:
ApplyFlatField("Capture 1", "CY3", InPlace = false, AppendString = "FF")

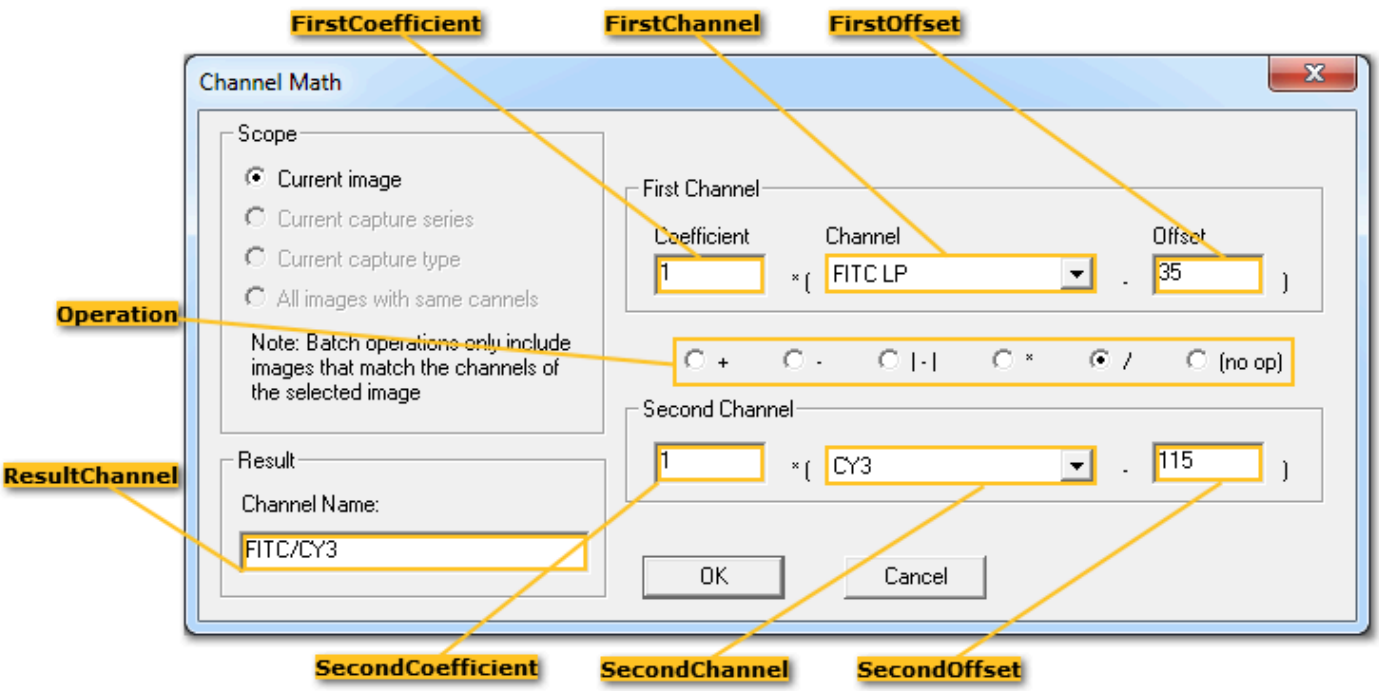
Parameter	Type	Required	Default
InPlace	bool	yes	n/a
AppendedString	string	no	n/a

ChannelMath

ChannelMath (*"SourceImageName"*, Operation = *enum*, FirstChannel = *"ChannelName"*, FirstCoefficient = *float*, FirstOffset = *int*, SecondChannel = *"ChannelName"*, SecondCoefficient = *float*, SecondOffset = *int*, **ResultChannel = *"ResulantChannelName"*)**

Performs channel math on the specified channels according to:

ResultChannel = FirstCoefficient × (FirstChannel – FirstOffset) [operation] SecondCoefficient × (SecondChannel – SecondOffset)



where **operation** is one of the following: **Add**, **Subtract**, **AbsoluteDifference**, **Multiply**, **Divide**, **NoOp**. If operation is **NoOp**, all of the second channel's arguments are ignored.

Example:
ChannelMath("HeLa Cells", Operation = Divide, FirstChannel = "FITC LP", FirstCoefficient = 1, FirstOffset = 35, SecondChannel = "CY3", SecondCoefficient = 1, SecondOffset = 115, ResultChannel = "FITC/CY3")

Parameter	Type	Required	Default
Operation	enum	yes	n/a
FirstChannel	channel	yes	n/a
FirstCoefficient	float	no	1.0
FirstOffset	int	no	0
SecondChannel	channel	no	n/a
SecondCoefficient	float	no	1.0
SecondOffset	int	no	0
ResultChannel	string	yes	n/a

ConvolveFilter2D

ConvolveFilter2D (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendedString = *"AppendedString"*, **KernelSize = *int*, KernelElements = *[float array]*,** Coefficient = *float*, Offset = *float***)**

Convolves the specified **channel** with the provided kernel. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true. KernelElements** is a float array which represents the row-major "flattening" of the kernel matrix. For instance, to convolve the **cGFP** channel in "**Image 1**" with the following matrix:

0	-1	0
1	0	1
0	-1	0

you would issue the command:
ConvolveFilter2D("Image 1", "cGFP", InPlace = true, KernelSize = 3, KernelElements = [0 -1 0 1 0 1 0 -1 0])

Each pixel in the output channel is the result of summing the element-wise product of the pixel's neighbors and the kernel, then multiplying that sum by the specified coefficient and adding the specified offset. For examples and additional information, see Convolution Filter window.

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
AppendedString	string	no	"conv"
KernelSize	int	yes	n/a
KernelElements	float array	yes	n/a
Coefficient	float	no	1.0
Offset	float	no	0.0

GaussianBlur2D

GaussianBlur2D (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"*, **Sigma = *float*)**

Performs a Gaussian blur on the specified channel, using a kernel whose standard deviation is **Sigma**. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true**. See also Gaussian Filter window.

Example:
GaussianBlur2D("Capture 3", "FITC", InPlace = false, AppendString = "_gb2D", Sigma = 2)

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
Sigma	float	yes	n/a
AppendedString	string	no	"gb"

HotPixelFilter

HotPixelFilter (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"*, **Factor = *float*)**

Any pixels whose value is **Factor** times as high as the mean of the surrounding pixels is set to the mean of the surrounding pixels. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true**. See also: Hot Pixel Filter window

Example:
HotPixelFilter2D("Capture 2", "FITC", InPlace = false, AppendString = "_HotPix", Factor = 4)

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
Factor	float	yes	n/a
AppendedString	string	no	"hot"

MeanFilter2D

MeanFilter2D (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"*, **Radius = *int*)**

Performs a mean filter on the specified channel with a kernel size of **Radius** $\times 2 + 1$. In other words, this replaces the current pixel with an average of the pixels in the given radius. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true**. See also Mean Filter window.

Example:
MeanFilter2D("Capture 1", "FITC", InPlace = false, AppendString = "_mean", Radius = 2)

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
Radius	int	yes	n/a
AppendedString	string	no	"mf"

MedianFilter2D

MedianFilter2D (*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"*, **Radius = *int*)**

Performs a median filter on the specified channel with a kernel size of **Radius** $\times 2 + 1$. In other words, this replaces the current pixel with the median of the pixels in the given radius. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true**. See also Median Filter window.

Example:
MedianFilter2D("Capture 1", "FITC", InPlace = false, AppendString = "_median", Radius = 2)

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
Radius	int	yes	n/a
AppendedString	string	no	"med"

RemoveChannel

RemoveChannel (*"SourceImageName"*, *"SourceChannelName"*)

Removes specified channel from the specified image.

Example:
RemoveChannel("Capture 1", "DAPI")

RegisterChannel

RegisterChannel (*"SourceImageName"*, InPlace = bool, TransformType = enum, TransformMatrix = float array, AppendedString = string)

Interpolates channel in image according to the specified transform. Currently, the only permitted value for **TransformType** is **Affine**. To create a new filtered channel, use **InPlace = false;** a new channel will be created with the same name and the **AppendString** added to the end. To overwrite the existing channel, use **InPlace = true.**

Parameter	Type	Required	Default
InPlace	bool	yes	n/a
TransformType	enum	yes	n/a
TransformMatrix	float array	yes	n/a
AppendedString	string	no	"reg"

RotateImage

RotateImage (*"SourceImageName"*, InPlace = *bool*, RotationAngle = *float*, Flip = *bool*, FlipType = *enum*, InterpolationType = *enum*)

Rotates (and optionally flips) all channels in the specified image. **RotationAngle** can be **1** through **360** degrees, it is the amount the image will be rotated counterclockwise. **FlipType** can be **Horizontal**, **Vertical**, or **Z**. **InterpolationType** is used when a **RotationAngle** other than **0, 90, 180, 270,** or **360** is used. **InterpolationTypes** available are **Bilinear** or **Bicubic**. To overwrite the existing image, use **InPlace = true**; otherwise, a new rotated image will be created with "**Rotated**" appended to the image name.

Examples:
RotateImage("Capture 1", InPlace = false, RotationAngle = 90)
RotateImage("Capture 1", InPlace = false, Flip = true, FlipType = Vertical)
RotateImage("Capture 1", InPlace = false, Flip = true)
RotateImage("Capture 1", InPlace = false, RotationAngle = 30, Flip = true, FlipType = Vertical, InterpolationType = Bicubic)

Parameter	Type	Required	Default
InPlace	bool	no	false
RotationAngle	float	no	0
Flip	bool	no	false
FlipType	enum	no	Horizontal
InterpolationType	enum	no	Bilinear

Interpolate

Interpolate(*"SourceImageName"***,** InPlace = *bool*, RatioXY = *float*, RatioZ = *float***)**

Interpolates planes in between existing z planes and/or pixels in the xy dimension. By default, **InPlace = false**, so a new image will be created appended with **.Interp**. The **RatioXY** and **RatioZ** values refer to desired end ratio of the number of voxels/the original number of voxels. For example, if **RatioZ = 2**, a new *z* plane will be created between each pair of existing *z* planes. If **RatioZ = 3**, two new *z* planes will be created between each pair of existing *z* planes. See also: Image Interpolation window.

Example:
Interpolate("Capture 1", RatioXY = 2, RatioZ = 3)

Parameter	Type	Required	Default
InPlace	bool	no	false
RatioXY	float	no	1
RatioZ	float	no	1

Mask Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

CreateMask

CreateMask (*"SourceImageName"*, MaskName = *"MaskString"*)

Creates a new empty mask, named by the **MaskName** parameter, in the specified image.

Example:
CreateMask("Capture 1", MaskName = "Mask1")

Parameter	Type	Required	Default
MaskName	string	yes	n/a

CreateMaskAutomaticSegmentation

CreateMaskAutomaticSegmentation (*"SourceImageName"*, *"SourceChannelName"*, MaskName = *"MaskString"*, Algorithm = *enum*)

Create a mask by performing either the Ridler-Calvard or Otsu automatic threshold determination algorithm on the designated channel in the designated image. The resulting mask is named according to **MaskName**. **Algorithm** can be set to either **Ridler** or **Otsu**.

Example:
CreateMaskAutomaticSegmentation("HeLa Cells", "CY3", MaskName = "Mask1", Algorithm = Ridler)

Parameter	Type	Required	Default
MaskName	string	yes	n/a
Algorithm	enum	yes	n/a

CreateMaskInteractiveSegmentation

CreateMaskInteractiveSegmentation(*"SourceImageName"*, MaskName = *"MaskString"*, ChanOne = [insert value], ChanTwo = [insert value], ChanGate = [insert value], ImageDataSource = [insert value], RegionNumber = [insert value], ChanOneTwoSubSelect = [insert value], ChanOneTwoBounds = [insert value], ChanOneAutoThreshAlgorithm = [insert value], ChanTwoAutoThreshAlgorithm = [insert value], ChanGateAutoThreshAlgorithm = [insert value], ChanOneThreshold = [insert value], ChanTwoThreshold = [insert value], ChanGateThreshold = [insert value], ChanOneTwoOp = [insert value], GateOp = [insert value], UseBkgd = [insert value], BkgdStdDevCoeff = [insert value])

Parameter	Type	Required	Default
MaskName	string		
ChanOne	string		
ChanTwo	string		
ChanGate	string		

ImageDataSource	enum		
RegionNumber	int		
ChanOneTwoSubSelect	enum		
ChanOneTwoBounds			
ChanOneAutoThreshAlgorithm	enum		
ChanTwoAutoThreshAlgorithm	enum		
ChanGateAutoThreshAlgorithm	enum		
ChanOneThreshold			
ChanTwoThreshold			
ChanGateThreshold			
ChanOneTwoOp			
GateOp			
UseBkgd			
BkgdStdDevCoeff			

CreateThresholdMask

CreateThresholdMask (*"SourceImageName"*, *"SourceChannelName"*, MaskName = *"MaskString"*, ThresholdLow = *int*, ThresholdHigh = *int***)**

Create a mask named according to **MaskName** by performing threshold segmentation. That is, selecting pixels whose value in channel lie between **ThresholdLow** and **ThresholdHigh**. Omitting either the low or high threshold will use the image low or high values respectively.

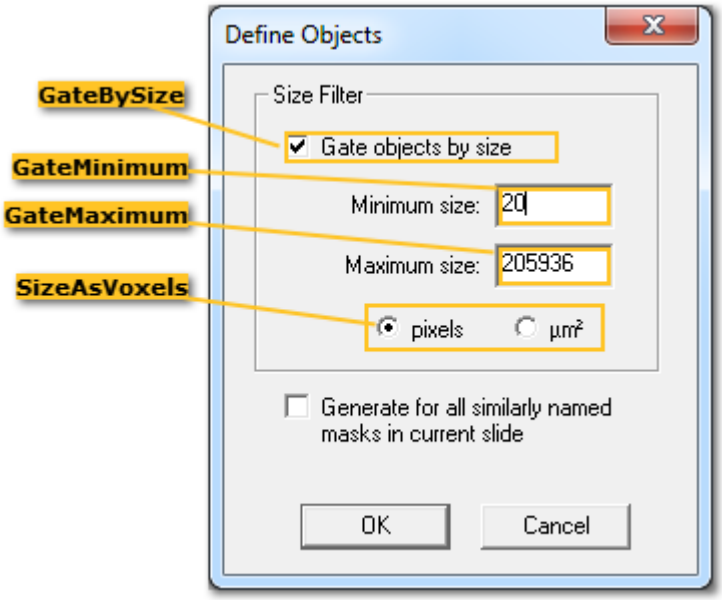
Examples:
CreateThresholdMask("HeLa Cells", "CY3", MaskName = "Mask1", ThresholdLow = 285, ThresholdHigh = 1195)
CreateThresholdMask("HeLa Cells", "CY3", MaskName = "Mask2", ThresholdHigh = 1195)
CreateThresholdMask("HeLa Cells", "CY3", MaskName = "Mask3", ThresholdLow = 285)

Parameter	Type	Required	Default
MaskName	string	yes	n/a
ThresholdLow	int	no	minimum pixel value
ThresholdHigh	int	no	maximum pixel value

DefineMaskObjects

DefineMaskObjects (*"SourceImageName"*, *"SourceMaskName"*, GateBySize = *bool*, GateMinimum = *int*, GateMaximum = *int*, SizeAsVoxels = *bool***)**

Builds mask objects in the specified mask. If **GateBySize** is set to **true**, any objects below or above the **MinimumSize** and **MaximumSize** parameters, respectively, will be eliminated. If **SizeAsVoxels** is set to **true**, the gate sizes will be interpreted as voxel counts, otherwise they will be interpreted as square microns or cubic microns, depending on whether the image is 2D or 3D.



Example:
DefineMaskObjects("HeLa Cells", "Mask 1", GateBySize = true, GateMinimum =20, SizeAsVoxels = true)

Parameter	Type	Required	Default
GateBySize	bool	no	false
MinimumSize	float	no	0
MaximumSize	float	no	max possible
SizeAsVoxels	bool	no	true

DeleteMask

DeleteMask (*"SourceImageName"*, *"SourceMaskName"*)

Delete mask from specified image.

Example:
DeleteMask("HeLa Cells", "Mask 1")

DilateMask

DilateMask(*"SourceImageName"*, *"SourceMaskName"*, Radius = *int*, TreatAs2D = *bool*)

Dilate designated mask by the assigned **Radius** number of pixels in XY. When **TreatAs2D = false**, the mask will also be dilated in the z direction on 3D images; this parameter has no effect on 2D or 2D Timelapse images.

Example:
DilateMask("Capture 1", "Mask 1", Radius = 2, TreatAs2D = false)

Parameter	Type	Required	Default
Radius	int	yes	n/a
TreatAs2D	bool	no	true

ErodeMask

ErodeMask(*"SourceImageName"*, *"SourceMaskName"*, Radius = *int*, TreatAs2D = *bool*)

Erode designated mask by the assigned **Radius** number of pixels in XY. When **TreatAs2D = false**, the mask will also be eroded in the z direction on 3D images; this parameter has no effect on 2D or 2D Timelapse images.

Example:
ErodeMask("Capture 1", "Mask 1", Radius = 2, TreatAs2D = false)

Parameter	Type	Required	Default
Radius	int	yes	n/a
TreatAs2D	bool	no	true

ExportCrossMaskStatistic

ExportCrossMaskStatistic (*"SourceImageName"*, *"SourceMaskName"*, SecondaryMask = *"SecondaryMaskName"*, Statistic = *"StatisticString"*, Directory = *"DirectoryString"*, Filename = *"Filename.txt"*)

Export specific cross-mask statistic to a tab-delimited text file. Available cross-mask statistics include: **Mask Overlaps (objects), Mask Overlaps (voxels), Mask Overlaps (largest object volume),** and **Mask Contains Other Masks**. If **Directory** is omitted, the file will be written to the current working directory. If **Filename** is omitted, the filename will be generated from the image and mask names, e.g., **Image1_Mask1_MaskOverlaps.txt**.

Example:
ExportCrossMaskStatistic("Capture 1", "GreenMask", SecondaryMask = "RedMask", Statistic = "Mask Overlaps (objects)", Directory = "C:\Users\CoreLab\Desktop", Filename = "Colcalization Object Overlaps.txt")

Parameter	Type	Required	Default
SecondaryMask	string	yes	n/a
Statistic	string	yes	n/a
Directory	string	no	(current working directory)
Filename	string	no	(image name + statistic name)

ExportMaskIntensityStatistic

ExportMaskIntensityStatistic (*"SourceImageName"*, *"SourceMaskName"*, SecondaryMask = *"SecondaryMaskName"*, **Statistic = *"StatisticString"*,** Directory = *"DirectoryString"*, Filename = *"Filename.txt"*)

Export specific intensity statistic to a tab-delimited text file. Available intensity statistics include: **Center of Intensity, Mean Adj Center Of Intensity, Mean Intensity, Sum Intensity, Minimum Intensity, Maximum Intensity, Median Intensity, Variance, Standard Deviation,** and **Background Intensity**. If **Directory** is omitted, the file will be written to the current working directory. If **Filename** is omitted, the filename will be generated from the image and mask names, e.g., **Image1_Mask1_Ch1_MeanIntensity.txt**.

Example:
ExportMaskIntensityStatistic("Capture 1", "GreenMask", Statistic = "Mean Intensity", Directory = "C:\Users\CoreLab\Desktop", Filename = "MeanIntensityExport.txt")

Parameter	Type	Required	Default
SecondaryMask	string	no	n/a
Statistic	string	yes	n/a
Directory	string	no	(current working directory)
Filename	string	no	(image name + statistic name)

ExportMaskIntensityStatistics

ExportMaskIntensityStatistics (*"SourceImageName"*, *"SourceMaskName"*, SecondaryMask = *"SecondaryMaskName"*, Directory = *"DirectoryString"*, Filename = *"Filename.txt"*)

Export all intensity statistics to a tab-delimited text file. Available intensity statistics include: **Center of Intensity, Mean Adj Center Of Intensity, Mean Intensity, Sum Intensity, Minimum Intensity, Maximum Intensity, Median Intensity, Variance, Standard Deviation,** and **Background Intensity**. If **Directory** is omitted, the file will be written to the current working directory. If **Filename** is omitted, the filename will be generated from the image and mask names, e.g., **Image1_Mask1_Ch1_MeanIntensity.txt**.

Example:
ExportMaskIntensityStatistics("Capture 1", "GreenMask", Directory = "C:\Users\CoreLab\Desktop", Filename = "IntensityStatisticsExport.txt")

Parameter	Type	Required	Default
SecondaryMask	string	no	n/a
Directory	string	no	(current working directory)
Filename	string	no	(image name + statistic name)

ExportMaskMorphometryStatistic

ExportMaskMorphometryStatistic (*"SourceImageName"*, *"SourceMaskName"*, SecondaryMask = *"SecondaryMaskName"*, **Statistic** = ***"StatisticString"*,** Directory = *"DirectoryString"*, Filename = *"Filename.txt"*)

Export specific morphometry statistic to a tab-delimited text file. Morphometry statistics available include: **Volume Microns** (volume given in microns/cubic microns), **Volume** (volume given in voxels), **Surface Area, Center of Mass, Center Of Volume, Major Axis Coordinates, Major Axis Length, Major Axes Lengths, XY Aspect Ratio,** and **Major/Minor Shape Factor**. If **Directory** is omitted, the file will be written to the current working directory.

Example:
ExportMaskMorphometryStatistic("Capture 1", "GreenMask", Statistic = "Surface Area", Directory = "C:\Users\CoreLab\Desktop", Filename = "SurfaceAreaExport.txt")

Parameter	Type	Required	Default
SecondaryMask	string	no	n/a
Statistic	string	yes	n/a
Directory	string	no	(current working directory)
Filename	string	no	(image name + statistic name)

ExportMaskMorphometryStatistics

ExportMaskMorphometryStatistics(*"SourceImageName"*, *"SourceMaskName"*, SecondaryMask = *"SecondaryMaskName"*, Directory = *"DirectoryString"*, Filename = *"Filename.txt"*)

Export mask's morphometry statistics to a file in the specified directory. Morphometry statistics available include: **Volume, Volume (voxels), Surface Area, Center Of Volume, Major Axis Coordinates, Major Axis Length, Minor Axes Lengths,** and **Major/Minor Shape Factor**. If **Directory** is omitted, the file will be written to the current working directory.

Example:
ExportMaskMorphometryStatistic("Capture 1", "GreenMask", Directory = "C:\Users\CoreLab\Desktop", Filename = "SurfaceAreaExport.txt")

Parameter	Type	Required	Default
SecondaryMask	string	no	n/a
Directory	string	no	(current working directory)
Filename	string	no	(image name + statistic name)

ExportMaskStatisticToExcel

ExportMaskStatisticToExcel(*"SourceImageName"***, *"SourceMaskName"***,** SecondaryMask = *"SecondaryMaskName"*, **Statistic** = ***"StatisticString"*****,** ObjectColumn = *bool*, MatchWorksheetToImage = *bool***)**

Export specific statistic to a worksheet in Microsoft Excel. If **MatchWorksheetToImage** is **true**, it will place the results in the worksheet that has the same name as the image, if it exists, or create a new worksheet otherwise. If **ObjectColumn** is **true**, it will fill a column of data with the object IDs, and place the results in the adjacent columns to the right. Otherwise, it will just place the results in the next available column.

Example:
ExportMaskStatisticToExcel("Capture 1", "GreenMask", Statistic = "Mean Intensity", ObjectColumn = true, MatchWorksheetToImage = true)
ExportMaskStatisticToExcel("Capture 1", "GreenMask", Statistic = "Mean Intensity")

Parameter	Type	Required	Default
SecondaryMask	string	no	n/a
Statistic	string	yes	n/a
ObjectColumn	bool	no	true
MatchWorkSheetToImage	bool	no	true

FillMaskHoles2D

FillMaskHoles2D(*"SourceImageName"***, *"SourceMaskName"***,** SizeThreshold = *float*, SizeAsVoxels = *bool***)**

Fills in "holes" in mask objects as seen on a plane-by-plane basis . If **ThresholdSize** is provided, any holes of the specified size or smaller will be filled in. If **SizeAsVoxels** is set to **true**, the threshold size will be interpreted as voxel counts, otherwise it will be interpreted as square microns in the 2D image or single plane of the 3D image. If **SizeThreshold** is not provided, SlideBook will make a best guess about the appropriate size, given the distribution of mask object sizes in the image.

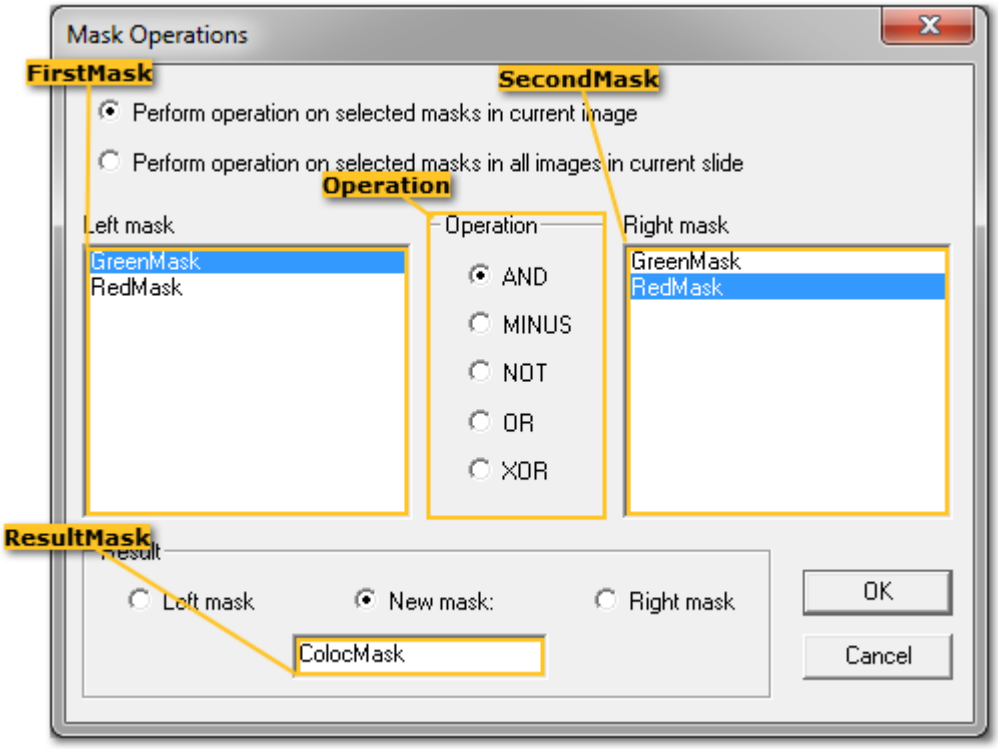
Example:
FillMaskHoles2D("Capture 3", "Mask 2", SizeThreshold = true, SizeAsVoxels = 30)

Parameter	Type	Required	Default
SizeThreshold	float	no	best guess
SizeAsVoxels	bool	no	true

MaskOperation

MaskOperation(*"SourceImageName"***, FirstMask =** ***"MaskName1"*****, SecondMask =** ***"MaskName2"*****, ResultName =** ***"ResultantMaskName"*****, Operation =** ***enum*****)**

Perform the specified operation between **FirstMask** and **SecondMask** (unless the operation is **Not**, in which case the second mask does not need to be specified). The result creates a mask named by the parameter **ResultName**. If a mask of that name does not already exist, one is created. Operation can be **And**, **Or**, **Xor**, **Subtract**, or **Not**.



Example:

MaskOperation("Capture 1", FirstMask = "GreenMask", SecondMask = "RedMask", ResultName = "ColocMask", Operation = And)

Parameter	Type	Required	Default
FirstMask	mask	yes	n/a
SecondMask	mask	yes*	n/a
ResultName	string	yes	n/a
Operation	enum	yes	n/a

*SecondMask is required unless the operation is Not.

Annotation Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

CreateLineAnnotation

CreateLineAnnotation (*"SourceImageName"*, Coordinates = *[int array]*)

Adds a new line annotation to the specified image. The **Coordinates** parameter is expected to have four values: **[x1 y1 x2 y2]**. No *z* or time coordinates are necessary because line annotations persist through all *z* and time.

Example:
CreateLineAnnotation("Capture 1", Coordinates = [88 89 188 189])

Parameter	Type	Required	Default
Coordinates	int array	yes	n/a

CreateRectangleROI

CreateRectangleROI (*"SourceImageName"*, Coordinates = *[int array]*, SetAsBackground = *bool*)

Adds a new rectangle region to the specified image. The **Coordinates** field is expected to have four values: **[x1 y1 x2 y2]**. If **SetAsBackground** is true, the created annotation will become the new background region. No z or time coordinates are necessary because regions persist through all z and time.

Example:
CreateRectangleROI("Capture 1", Coordinates = [230 6 248 16], SetAsBackground = true)

Parameter	Type	Required	Default
Coordinates	int array	yes	n/a
SetAsBackground	bool	no	false

DeleteAllROIs

DeleteAllROIs (*"SourceImageName"*)

Removes all line annotations and regions from the specified image.

Example:
DeleteAllROIs("Capture 1")

Deconvolution and Advanced Image Processing Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

DeconvolveNoNeighbors

DeconvolveNoNeighbors(*"SourceImageName"***,** ***"SourceChannelName"***,** SubtractionConstant = *float*, EdgePadding = *[int array]***)**

Deconvolves the selected channel (or **<all channels>**) using the no neighbors algorithm, according to the supplied parameters, and creates a new image, with **.NoN** appended to the name. Channels that were not specified for deconvolution will be copied into the new image undeconvolved. **EdgePadding** expects and array of 3 numbers, the number of pixels in x, y, and z.

Example:
DeconvolveNoNeighbors("Metaphase B-Cell", "DAPI", SubtractionConstant = 0.7, EdgePadding = [30 30 3])

Parameter	Type	Required	Default
SubtractionConstant	float	no	0.9
EdgePadding	int array	no	[0 0 0]

DeconvolveBlind

DeconvolveBlind("Capture 1", "SourceChannelName", ResultChannel = "ResultChannelName", ScopeMode = enum, NumIterations = int, EdgePaddingXY = int, EdgePaddingZ = int, SubPixelXY = int, SACorrect = bool)

Deconvolves the selected channel (or **<all channels>**) using the blind algorithm, according to the supplied parameters, and creates a new image, with **.Blind** appended to the name. Channels that were not specified for deconvolution will be copied into the new image undeconvolved. **ScopeMode** refers to the imaging modality used to capture the data; available modes include: **Widefield**, **SpinningDiskConfocal**, **TwoPhoton**, and **PointScanningConfocal**. **NumIterations** is the number of guesses the software will make in approximating the deconvolved image. The deconvolution algorithm operates by generating a guess, blurring the guess with the point spread function, and then comparing the blurred guess with original image. If the comparison determines that the image is not improving, the algorithm is stopped, regardless of the number of iterations specified. **EdgePaddingXY** and **EdgePaddingZ** expect a single integer for the number of pixels in XY or Z to add to the image before deconvolving. **SubPixelXY** allows you to include interpolation of the image in XY before performing deconvolution.

Example:
DeconvolveBlind("Capture 1", "FITC", ResultChannel = "FITC_BLIND", ScopeMode = Widefield, NumIterations = 10, EdgePaddingXY = 15, EdgePaddingZ = 3, SACorrect = false)

Parameter	Type	Required	Default
ResultChannel	string		
ScopeMode	enum		
NumIterations	int		10
EdgePaddingXY	int		
EdgePaddingZ	int		
SubPixelXY	int		

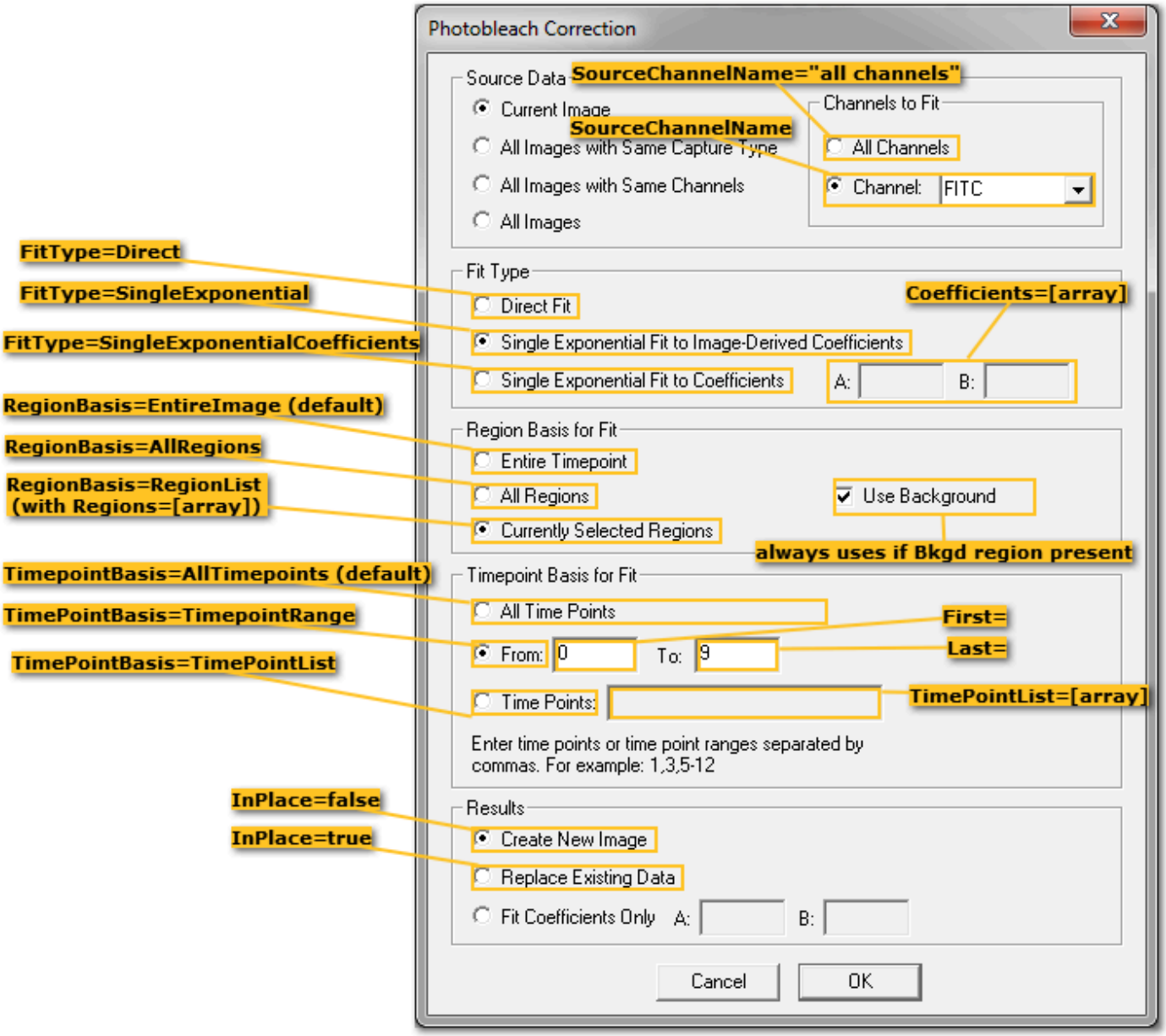
SACorrect	bool		

PhotobleachCorrection

PhotobleachCorrection(*"SourceImageName"*, *"SourceChannelName"*, InPlace = *bool*, AppendString = *"AppendedString"*, **FitType = *enum*,** Coefficients = *[float array]*, RegionBasis = *enum*, Regions = *[int array]*, TimepointBasis = *enum*, First = *int*, Last = *int*, Timepoints = *[int array]*)

Performs photobleach correction on a 2DTL or 4D image. If a background region is present, fit is based on background-corrected intensities. **FitType** can be **Direct, SingleExponential,** or **SingleExponentialCoefficients**. If **FitType** is **SingleExponentialCoefficents**, then the **Coefficients** argument must be provided. **RegionBasis** can be **EntireImage, AllRegions,** or **RegionList**. If **RegionBasis** is **RegionList**, then the **Regions** argument must be provided. **TimepointBasis** can be **AllTimepoints, TimepointRange,** or **TimepointList**. If **TimepointBasis** is **TimepointRange**, the correction will be based on a range determined by the **First** and **Last** arguments. If **First** is missing, timepoint **0** is assumed. If **Last** is omitted, the final timepoint is assumed. If **TimepointBasis** is **TimepointList**, the correction will be based on the specific timepoints in the list provided for the **Timepoints** argument.

Example:
PhotobleachCorrection("Capture 1", "CY3", InPlace = false, AppendString = "_pbc", FitType = DirectFit, RegionBasis = EntireImage, Regions = "2", TimepointBasis = AllTimepoints)



Parameter	Type	Required	Default
InPlace	bool	yes	n/a
AppendString	string	no	.pbc
FitType*	enum	yes	n/a
Coefficients	float array	*if SingleExponentialCoefficients	n/a
RegionBasis**	enum	no	EntireImage
Regions	int array	**if RegionsList	n/a
TimepointBasis***	enum	no	AllTimePoints
First	int	no	0
Last	int	no	(last timepoint)
Timepoints	int array	***if TimePointList	n/a

SubtractBackground

SubtractBackground(*"SourceImageName"*, InPlace = *bool*)

Subtract the background intensity of a 2DTL or 4D image, as determined by the background region, on a timepoint-by-timepoint basis. If InPlace is true, this is a "destructive" operation and permanently changes image values. Either way, the background region is deleted in the resulting image, as it no longer applies.

Note: This operation is only recommended for advanced applications which may require it. In general, it is far better to preserve original intensities, and let SlideBook perform background correction "on the fly."

Example:
SubtractBackground("Capture 1", InPlace = false)

Parameter	Type	Required	Default
InPlace	bool	yes	n/a

Import and Export Macro Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

"SourceImageName" may be:
<**current image**>, <**all images**> or **"example name"**

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

ExportImageAsTIFF

ExportImageAsTIFF(*"SourceImageName"***,** Directory = *"DirectoryString"*, FilenameFormat = *"FormatString"*, ExportOMEXML = *bool*, SeparatePlanes = *bool*, WriteLog = *bool***)**

Exports the specified image as 16-bit TIFF file(s). The directory is assumed to be the working directory unless provided in the command. The filename format is assumed to be %N_XY%P_Z%Z_T%T_C%C unless provided in the command. Use **ExportOMEXML = true** to include OME metadata in the tiff files (encoded in XML). If **SeparatePlanes** is **true**, a separate TIFF file will be generated for each timepoint or *z* location, depending on whether the image is 2D timelapse or 3D. The parameter has no effect on a 2D image. If **WriteLog** is true, a **.txt** file will be written that contains information about the image(s) being exported.

Example:
ExportImageAsTIFF("Capture 1", Directory = "C:\Users\Monica\Desktop\eraseme", FilenameFormat= "%N_Z%Z_T%T_c%c", ExportOMEXML = true, WriteLog = true)

Parameter	Type	Required	Default
Directory	string	no	(current working directory)
FilenameFormat	string	no	"%N_XY%P_Z%Z_T%T_C%C"
ExportOMEXML	bool	no	false
SeparatePlanes	bool	no	false
WriteLog	bool	no	false

ExportToMatlab

ExportToMatlab(*"SourceImageName"***,** *"SourceChannelName"***,** First = *int*, Last = *int*, **Matrix =** *"MatrixName"***)**

Export the specified channel to MATLAB as a matrix named by the parameter **Matrix**. If **First** is specified, this is the zero-based index of the first plane to be exported of a 3D image or first timepoint of a 2D timelapse image. If **Last** is specified, this is the zero-based index of the last plane to be exported of a 3D image or the last timepoint of a 2D image.

A 2D image (or a single plane of a 3D image or a single timepoint of a 2D timelapse image) is exported as a 2D matrix. A 3D image or 2D timelapse image is exported as a 3D matrix. Note that in both cases the indices of the MATLAB matrix will be one higher than in the corresponding SlideBook image.

Example:
ExportToMatlab("Capture 1", "FITC", First = 2, Last = 5, Matrix = "FITC_Capture1")
ExportToMatlab("Capture 1", "FITC", Matrix = "FITC_Capture1")

Parameter	Type	Required	Default
First	int	no	0

Last	int	no	(last plane/timepoint)
Matrix	string	yes	n/a

ExportMaskToMatlab

ExportMaskToMatlab(*"SourceImageName"*, *"SourceMaskName"*, First = *int*, Last = *int*, **Matrix = *"MatrixName"*,** Binarize = *bool***)**

Export the specified mask to MATLAB as a matrix named by the **Matrix** parameter. If **First** is specified, this is the zero-based index of the first plane to be exported of a 3D image's mask or first timepoint of a 2D timelapse image's mask. If **Last** is specified, this is the zero-based index of the last plane to be exported of a 3D image's mask or the last timepoint of a 2D image's mask. By default, if the mask has been divided into objects, the value output for a particular voxel will be the corresponding object's ID plus 1 if the mask is set in that voxel, and **0** otherwise. If **Binarize** is set to true, however, all values will be **0** or **1**, depending on whether the corresponding pixel is set in the mask or not, regardless of the object ID.

A 2D image (or a single plane of a 3D image or a single timepoint of a 2D timelapse image) is exported as a 2D matrix. A 3D image or 2D timelapse image is exported as a 3D matrix. Note that in both cases the indices of the MATLAB matrix will be one higher than in the corresponding SlideBook image.

Example:
ExportMaskToMatlab("Capture 1", "GreenMask", Matrix = "GreenMask_Capture1", Binarize = true)

Parameter	Type	Required	Default
First	int	no	0
Last	int	no	(last plane/timepoint)
Matrix	string	yes	n/a
Binarize	bool	no	false

ImportChannelFromMatlab

ImportChannelFromMatlab(*"SourceImageName"*, Matrix = *"MatrixName"*, Channel = *"ChannelName"*)

Import the designated channel from Matlab into the designated image in SlideBook with assigned channel name.

Example:
ImportChannelFromMatlab("HeLa Cells", Matrix = "FITC_Capture1", Channel = "FITC")

Parameter	Type	Required	Default
Matrix	string	yes	n/a
Channel	string	yes	n/a

ImportMaskFromMatlab

ImportMaskFromMatlab(*"SourceImageName"*, Matrix = *"MatrixName"*, Mask = *"MaskName"*)

Import the designated mask from Matlab into the designated image in SlideBook with assigned channel name.

Example:
ImportMaskFromMatlab("HeLa Cells", Matrix = "ProteinX_Mask", Mask = "Mask1")

Parameter	Type	Required	Default
Matrix	string	yes	n/a
Mask	string	yes	n/a

Particle Tracking Macro Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

ImageTrackParticles2D

ImageTrackParticles2D(*"SourceImageName"*, *"SourceMaskName"*, CentroidStatistic = *"StatisticString"*, CentroidChannel = *"ChannelName"*, SmallObjectSizeThreshold = *float*, SizeAsVoxels = *bool*, MaximumMovement = *float*, MinimumPathDuration = *int***)**

Performs basic 2D particle tracking according to each object's designated **CentroidStatistic** parameter. Available statistics include **"Center of Area"**, **"Center of Intensity"**, and **"Mean Adjusted Center of Intensity"**. If one of the later two intensity-based statistics is used, the **CentroidChannel** parameter must be provided. If **"Center of Area"** is used, this parameter may be omitted.

If an object is smaller than **SmallObjectSizeThreshold**, it is removed from the mask before tracking. Object size is given in voxels when **SizeAsVoxels** is omitted (**true**) or in microns^2 when **SizeAsVoxels=false**. Objects in successive timepoints whose distance exceeds **MaximumMovement** microns are not considered the same. Objects whose paths are shorter than **MinimumPathDuration** timepoints are eliminated.

Example:
ImageTrackParticles2D("ParticleTrackDemo", "Mask 1", CentroidStatitstic= "Mean Adjusted Center of Intensity", CentroidChannel="i-485", SmallObjectSizeThreshold = 25, SizeAsVoxels = true, MaximumMovement = 15, MinimumPathDuration =3)

Parameter	Type	Required	Default
CentroidStatistic*	string	yes	n/a
CentroidChannel	string	*if intensity-based CentroidStatistic	n/a
SmallObjectSizeThreshold	float	no	0
SizeAsVoxels	bool	no	true
MaximumMovement	float	no	(unlimited)
MinimumPathDuration	int	no	1

MATLAB Interface Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

RunMatlabCommand

RunMatlabCommand(Command = ***"CommandString"*****)**

Run the specified MATLAB command (or script). If the command is a script, it should exist as a **.m** file in the work directory of the MATLAB installation.

Parameter	Type	Required	Default
Command	string	yes	n/a

Miscellaneous Commands

TypeFace	indicates
Typewriter Bold	required parameter
Typewriter Bold Red	required parameter, user-defined value
Typewriter	optional parameter
Typewriter Blue	optional parameter, user-defined value

Value	indicates
bool	- true or false
enum	- enumerated type, values provided
int	- signed integer between -32767 and 32767
float	- floating point value
int array	- vector of signed integers, as in **[0 1 2]**
float array	- vector of floating point values, as in **[0.0 0.5 1.0]**
string	- text delimited by double quotes, as in **"Capture 1"**

"SourceImageName" may be:
<current image>, **<all images>** or **"example name"**

CompareChannels

CompareChannels(*"SourceImageName"***, FirstChannel =** *"FirstChannelName"***, SecondChannel =** *"SecondChannelName"***,** Directory = *"DirectoryString"*, **Logfile =** *"LogfileName"***)**

Compares the pixel values of **FirstChannel** against the values of **SecondChannel**. Appends to the **Logfile** whether there are any differences, and if so, the maximum absolute value of the difference.

Example:
CompareChannels("Capture 1", FirstChannel = "FITC", SecondChannel = "CY3", Directory = "C:\Users\CoreLab\Desktop", Logfile = "FITCvsCY3")

Parameter	Type	Required	Default
FirstChannel	string	yes	n/a
SecondChannel	string	yes	n/a
Directory	string	no	default working directory
Logfile	string	yes	n/a

EnforceUniqueImageNames

EnforceUniqueImageNames()

Renames any images in slide as necessary to ensure that each image has a unique name.

SetBestPlane

SetBestPlane(*"SourceImageName"***, Plane =** ***int*****,** First = *int*, Last = *int***)**

Sets the best (thumbnail) plane of a 3D or 4D image to **Plane** value. If **First** is specified, this is the zero-based index of the first timepoint of a 4D image for which to set the best plane. If **Last** is specified, this is the zero-based index of the last timepoint of a 4D image for which to set the best plane.

Parameter	Type	Required	Default
Plane	int	yes	n/a
First	int	no	0
Last	int	no	(last plane/timepoint)

Ratio Module

The Ratio Module adds functionality to SlideBook that simplifies a wide variety of quantitative techniques. Ratio imaging is a live cell imaging technique that measures pairs of wavelengths. Quantification of ratio pairs is relatively unaffected by issues such as photobleaching, uneven dye loading, and dye leakage. Thus, it is an excellent technique for performing quantitative measurements. Commonly, ratio imaging techniques employ ratiometric dyes that indicate ion concentration. Examples of such dyes are Fura-2 (for Ca2++) and BCECF (for pH).

The Ratio Module adds several components to the SlideBook base package that allow users to designate numerator and denominator channels and use these designations in several types of analysis and calibration procedures. Included in this manual are several tutorials that utilize sample data as well as some basic concept review on general operations in SlideBook. To obtain the sample images, download **RatioTutorial.sld** from www.intelligent-imaging.com.

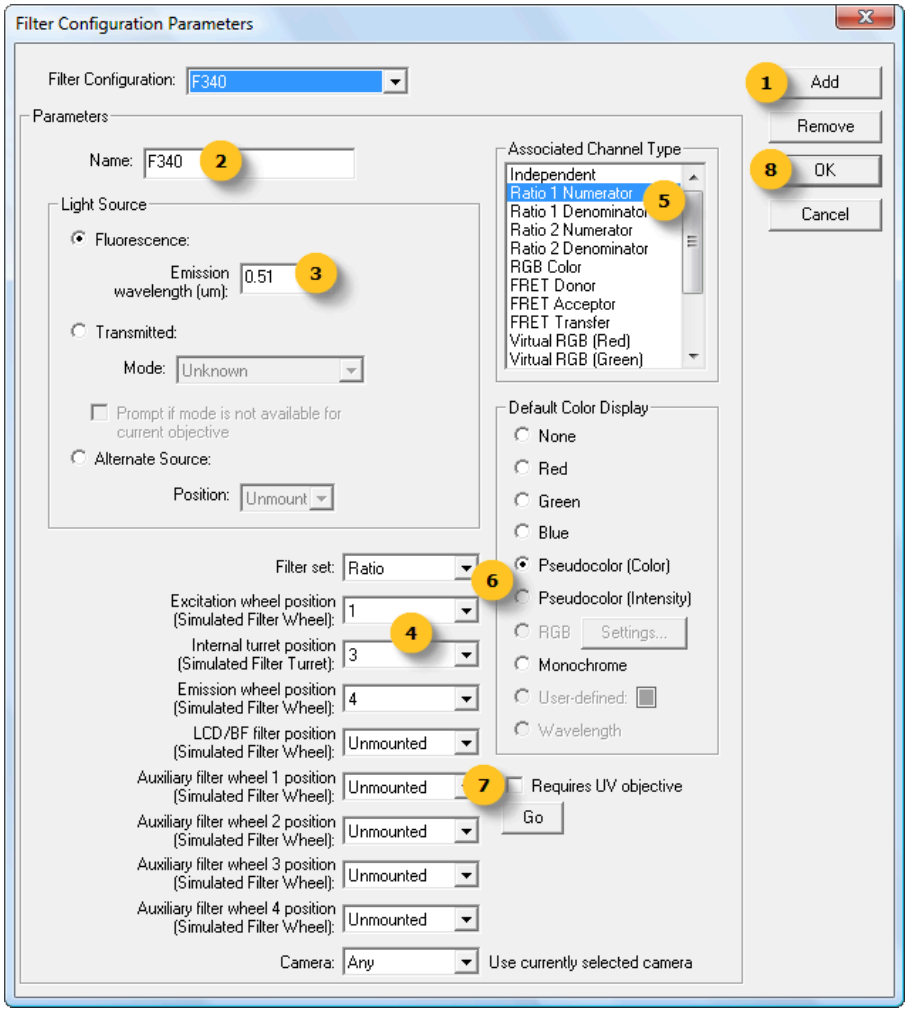
Ratio System Configuration

In order for SlideBook to be able to capture ratio images, the filter configurations must be set properly. One filter configuration should be defined as a ***Ratio Numerator*** and one filter configuration should be defined as a ***Ratio Denominator***. These two filter configurations determine a ratio channel.

SlideBook lets you define two ratio channels: ***Ratio 1*** and ***Ratio 2***. If you wish to view ratio values real-time, you must set ratio channel definitions before initiating capture. Alternatively, you may add ratio information on imported data or post-capture as discussed in Ratio Settings.

TUTORIAL: Defining Ratio Channels

Select ***Administration tab >*** ***Filters*** . The ***Filter Configurations Parameters*** dialog box will appear. A detailed explanation of all the settings for this dialog is found in the Filter Configuration Parameters Dialog. Relevant settings are discussed in this section.



1. Add a new channel by clicking ***Add***.

2. ***Name*** the new channel.

3. Select the appropriate ***Light Source*** information.

4. Select a ***Filter Set*** and configure the hardware to move to the appropriate positions for the channel.

5. Choose the appropriate ratio configuration under ***Associated Channel Type***. Make sure that the numerator and denominator configurations for a particular indicator are associated with the same ratio channel (***Ratio 1*** or ***Ratio 2***).

6. Select the appropriate ***Default Color Display***. For numerator, assign ***Pseudocolor (Color)***; for denominator, assign ***Pseudocolor (Intensity)***.

7. If the channel requires UV excitation, check the box for ***Requires UV objective***.

8. Click ***OK***.

9. When prompted, close and restart SlideBook to update the configuration.

Real-Time Display

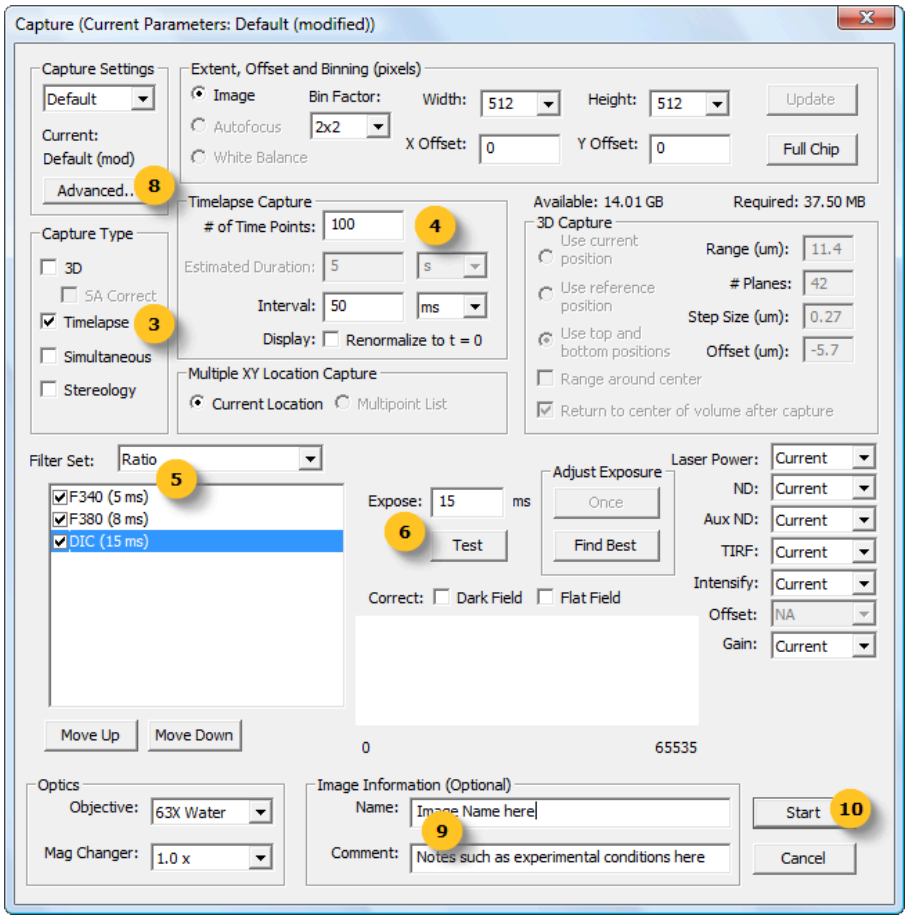
During capture, SlideBook permits you to delineate multiple regions of interest (including a background region) and graph ratio changes over real-time. Simultaneously, SlideBook provides a pseudocolored image of ratio information across the entire field. This section will discuss setting up a ratio acquisition and displaying the data during capture.

Setting Capture Parameters for Timelapse Ratio Imaging

Ratio capture works much like other image capture paradigms in SlideBook. For a more detailed explanation of image capture, please see Capture Window . To capture a new time lapse ratio image:

1. Bring your sample into view and focus using the ***Focus Controls*** (see also Focus Controls Dialog).

2. Open the ***Capture*** window. The ***Capture*** dialog box will appear (see also Capture window).



3. Select the ***Timelapse*** check box; once you select ***Timelapse*** in the ***Capture Type*** section, you will activate ***Timelapse Capture*** options.

4. In the ***Timelapse Capture*** section, specify the ***Interval*** between time points as well as the total ***# of Time Points***.

> **NOTE:** The time interval refers to the amount of time from the start of one time point to the start of the next time point. When the interval is less than the actual time required to capture a time point, capture will continue at the fastest rate possible with the given exposure times and time required to move hardware. Thus, if you desire to capture images as quickly as possible, set ***Interval*** to **0** ms.
>
> If desired, check the box for ***Display: Renormalize to t = 0***. This uses the minimum and maximum pixel intensity values for the first time point in a time lapse capture series to determine the renormalization values of all subsequent time points. This option only affects the image display during capture and will not affect the captured image or data.

5. Specify a ratio capture by selecting the appropriate ***Filter Set*** and clicking on the checkboxes for both the ratio numerator and denominator channels.

> It is easy to collect other data (such as a **GFP** or **DIC** channel) during ratio capture. Simply select additional channels to capture as in normal time lapse imaging. If desired, you may capture a channel at a slower rate than your ratio data by

selecting the ***Periodic*** tab found in the ***Capture Preferences*** dialog (use the ***Advanced*** button in the the the upper left of this dialog to access ***Capture Preferences***).

6. Test the exposure duration for each channel. When setting ***Expose*** value, know that identical exposure times are not necessary as an exposure time factor is figured into analysis. However, it is necessary that you do not saturate the image in either channel as this will jeopardize quantification later.

> **NOTE:** It is often advantageous to use binning for timelapse ratio capture. Not only will this permit better temporal resolution, but it will also dramatically decrease the total size of the collected data. Exposure times should be adjusted after binning is set.

7. Once you have captured a test image, you may use the region tools to draw areas to monitor during capture. To learn more about region tools, please see Selecting Regions of Interest.

8. If you would like to mark events, such as reagent addition, set up ***Notes*** using the ***Advanced*** button as discussed in Creating Event Markers (Notes).

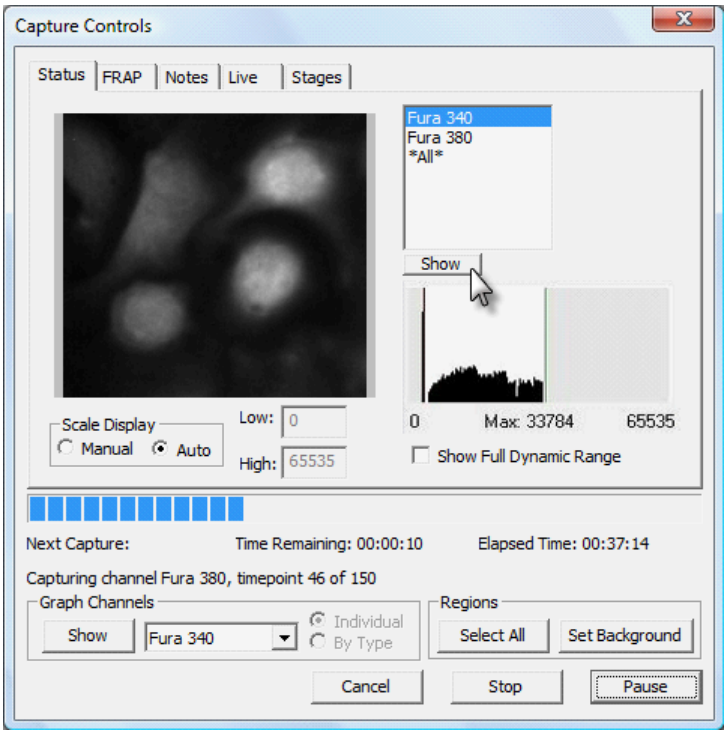
9. ***Name*** the image and write ***Comments*** such as experimental parameters.

10. Select ***Start*** when you are ready to begin capture.

Live Display

SlideBook can display a live pseduocolored image of ratio values for each time point as well as update a graph of ratios for multiple regions of interest. Additionally, you may wish to adjust the focus of the microscope or create event markers.

During capture, the image in the live ***Camera*** window will display a pseudocolored ratio image. To display an individual channel, go to the ***Status*** tab of the ***Capture Controls*** dialog. In the upper right portion, you will find a list of channels. Simply select an individual channel to display and click ***Show***. A live window displaying a monochrome image of the selected channel will open. In this manner, it is possible to watch a live pseudocolor display of the ratio as well as live monochrome displays of the numerator and denominator.



Selecting Regions of Interest

You may select regions of interest for graphing either before capture has started or during capture. Region tools may be used on test images generated before capture or on the live image display during capture. If you set a background region, the average value of this region will be subtracted from the average values in the other regions of interest before ratio computation. The background region will also be stored in the ***Ratio Settings*** dialog. To draw regions before capture, perform the following:

1. Make sure that the ***Camera*** window is displaying the specimen. You can do so from the ***Focus Controls*** or by pressing ***Test*** in the ***Capture*** dialog box.

2. Choose one of the region tools, which are located on the SlideBook toolbar at the top of the application window.

Rectangle tool - click and drag to draw a rectangle; alternatively, click once on the image to open a dialog to define the region dimensions in pixels or microns or to define a rotation angle for the region

Ellipse tool - click and drag to draw an ellipse; alternatively, click once on the image to open a dialog to define the region dimensions in pixels or microns or to define a rotation angle for the region

Polygon tool - click to draw a series of straight lines, double-click to close the shape

Free Hand tool - click and drag to draw freehand, release mouse button to close the shape

Line tool - click and drag to draw a line; hold **Ctrl** and click for a multi-segment line, double click to complete multi-segment line

Point tool - click to draw a point

Region Selection tool - use to select a region. Holding **Shift** and clicking on multiple regions will allow you to select more than one region. Clicking and dragging on a selected region(s) will move the selected region(s). If the region number is displayed in white, this indicates that the region is selected.

3. Continue to draw regions as desired. The following operations can be performed by selecting the region with the ***Region Selection*** tool then right-clicking to bring up the region menu. A region is selected when the region number appears in white. Unselected regions have numbers that match the color or the region outline.

Set as background – uses the selected region for background subtraction during graphing (must first be turned on under ***Administration tab > Hardware Properties > Statistics Properties > Enable Background Subtraction > Yes*** see also Configuring Background Subtraction with Mask Statistics).

Unselect – de-selects the selected region(s)

Duplicate – creates a copy of the selected region(s) that can be dragged to a new location

Move – allows the region(s) to be moved

Copy – copies the region(s) to allow for pasting, specifically when copying to a different image

Paste – pastes the copied region(s) in the same location on a different image

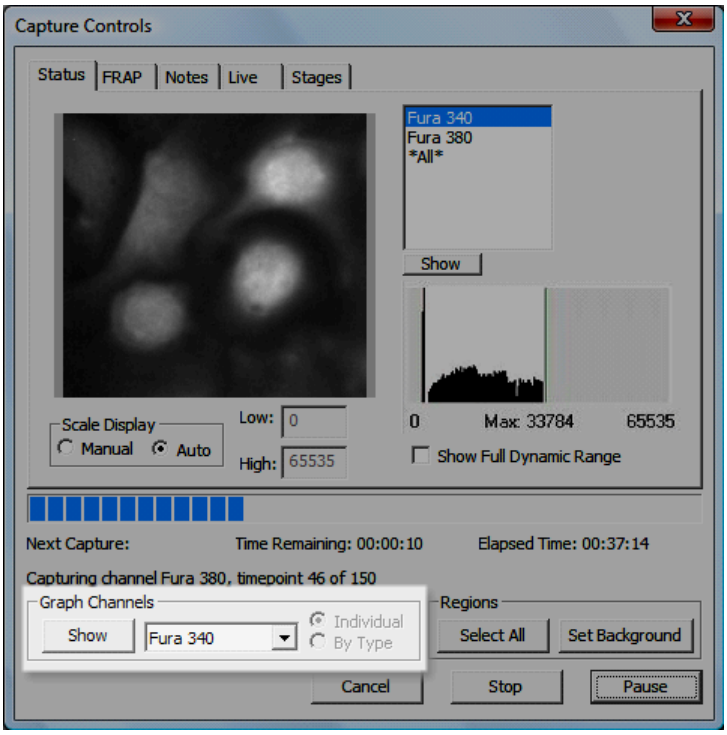
Delete – removes the selected region(s)

4. Once you have created the desired regions of interest and begun capture, you can display a graph by selecting the desired channel in the ***Graph Channels*** section at the bottom left of the ***Capture Controls*** and then clicking on the ***Show*** button as discussed in Graph Display.

5. The regions of interest that you have created can also be displayed after capture by selecting any of the region drawing tools or by checking the box for ***Regions*** in the Annotations group of the Home tab (shortcut key is ***Ctrl + Shift + G***). To recreate the graph post capture, please see Data Graphing and Export.

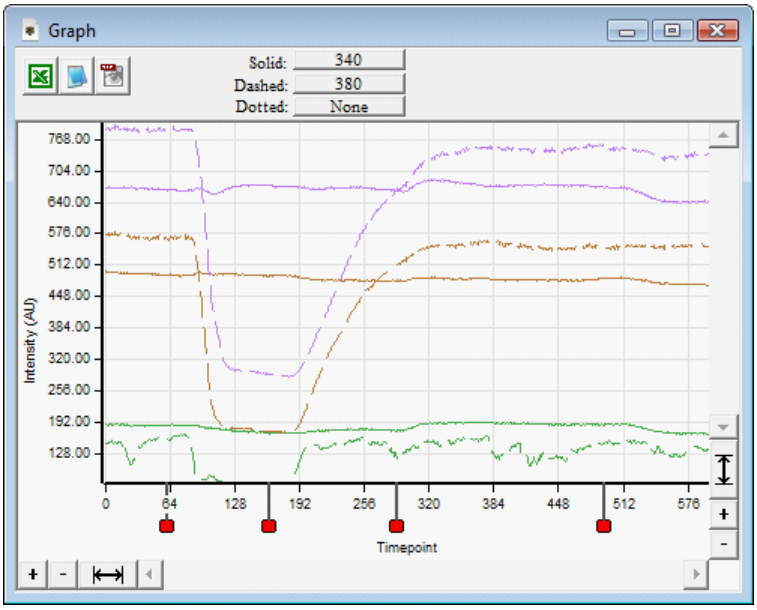
Graph Display

To display the ratio graph during capture, select the channel that you wish to graph from the drop-down list then press the ***Show*** button in the ***Graph Channels*** section of the ***Capture Controls*** dialog.



Repeat for any additional channels you wish to display, or simply select the channels from the channel menus on the graph.

NOTE: Underlying intensities and ratios cannot be shown in the same window because they have different vertical ranges. Thus, you must have one graph open to display individual channel intensity information and a second graph open to display the ratio values.

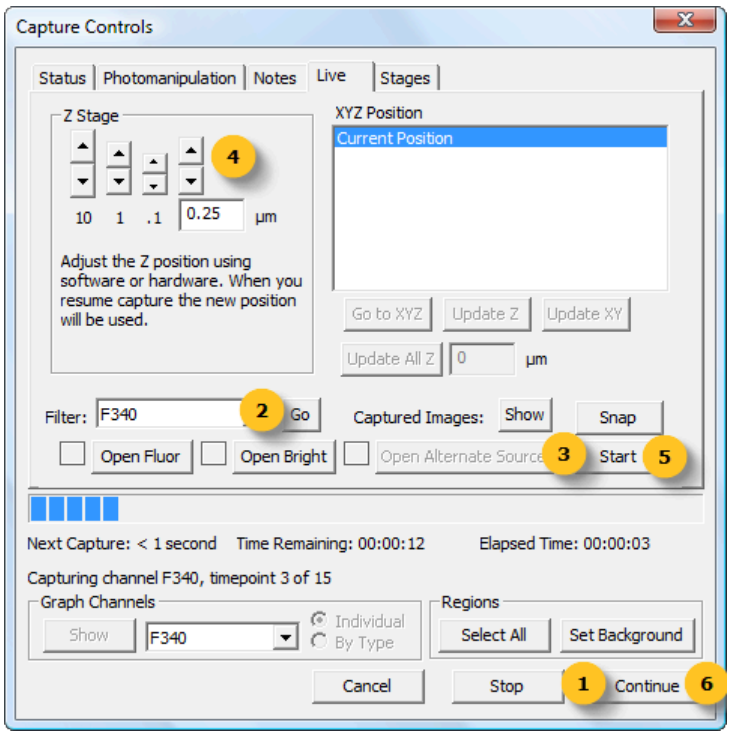


For more information on this view, please refer to Graph window. Once the capture is finished, the graph will remain open for viewing and the export tools will become available.

To recreate this graph post capture, see Data Graphing and Export.

Focusing During Capture

You may adjust the focus of your image during capture. To do so, go to the ***Live*** tab of the ***Capture Controls*** window. The ***Live*** tab works similarly to the ***Focus Controls***. To learn more about these controls, please review the Live Tab of Capture Controls window.



1. ***Pause*** the experiment to stop writing images to the file.

2. Select the desired ***Filter*** from the dropdown menu, press ***Go*** to move the appropriate hardware into place.

3. Press ***Start*** to begin snapping images. (Alternatively, you could press ***Snap*** to take a single image if you are concerned about photobleaching or press ***Open Fluor/Bright/Alt*** to direct light to the eyepieces to focus).

4. Adjust the focus using the ***Z Stage*** controls or the focus knob on the microscope or stage.

5. Press ***Stop*** to stop snapping images.

6. Press ***Continue*** to continue image acquisition and writing the images to file.

Creating Event Markers (Notes)

Event markers can be set up prior to capture or during capture. You can use an event marker to note an experimental condition change that you instigate or observe. For example, you may use an event marker to note when you add a reagent or when something interesting occurs. Event markers show as a ***Notes*** annotation in the image and are marked on graphs and statistical export.

Configuring Notes prior to Capture

1. Click on ***Advanced*** in the upper left-hand corner of the ***Capture*** window.
2. Navigate to the ***Notes*** tab. (See also Notes Tab of Capture Preferences window)
3. Type the text you would like to appear to mark the event(s) then select ***OK***.
4. Start experiment as usual.

Configuring and Using Notes during Capture

1. Set up an experiment as desired and begin acquiring images.
2. Navigate to the ***Notes*** tab of ***Capture Controls***. (See also Notes Tab of Capture Controls window)
3. To mark events set up prior to capture, simply click on the ***Note - #*** next to the marker you would like to use. To add new notes, click in an empty field and type a new note. The associated ***Note - #*** will become activated for use.

Post-Acquisition Display

Ratio data is generally best displayed using pseudocolor settings. This will be the default setting if you define filters used for ratio imaging as discussed in Defining Ratio Channels. We will now explore displaying ratio data using the example slide, **RatioTutorial.sld**. This slide is available for download from www.intelligent-imaging.com. If you are a new user, please familiarize yourself with the Main View before beginning the tutorials in this section.

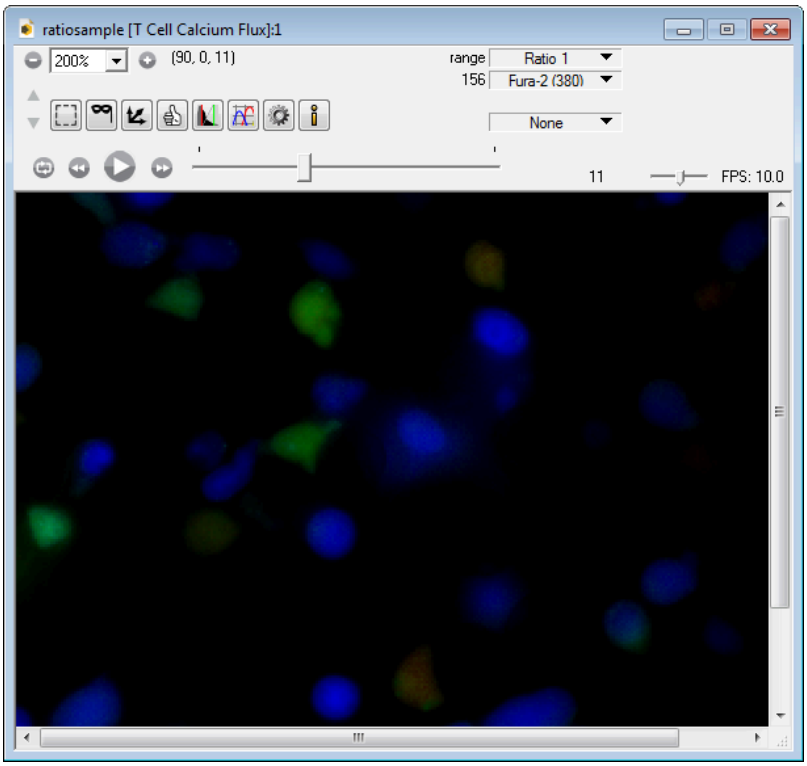
TUTORIAL: Viewing Ratio Data in SlideBook

To begin this demonstration:

1. Open SlideBook and select ***Open Slide*** from the Quick Access Toolbar.

2. Navigate to **RatioTutorial.sld** and select ***Open***. This file is courtesy of Dr. Jackson Egan of the University of California, Berkeley.

3. Double-click on the **T Cell Calcium Flux** image to generate a ***Main View***. The following image will appear. For additional information on data views, see the View Menu section of User Interface.

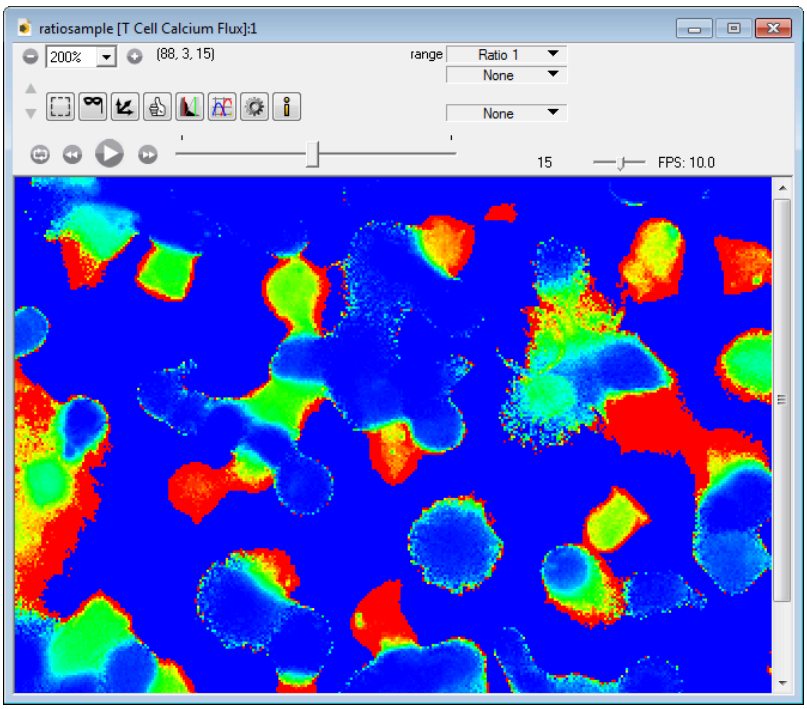


4. Notice that the image is displayed in pseudocolor, with the **Ratio 1** channel appearing in the first channel menu. Also note that SlideBook stores both the numerator and denominator time lapse image. The ratio values are computed when the image is opened and are not stored with the image.

> **NOTE:** If you have not defined ratio channels as discussed in Ratio System Configuration, you may view your data in pseudocolor by selecting ***Mode >*** ***Pseudocolor*** in the View group of the Home tab.

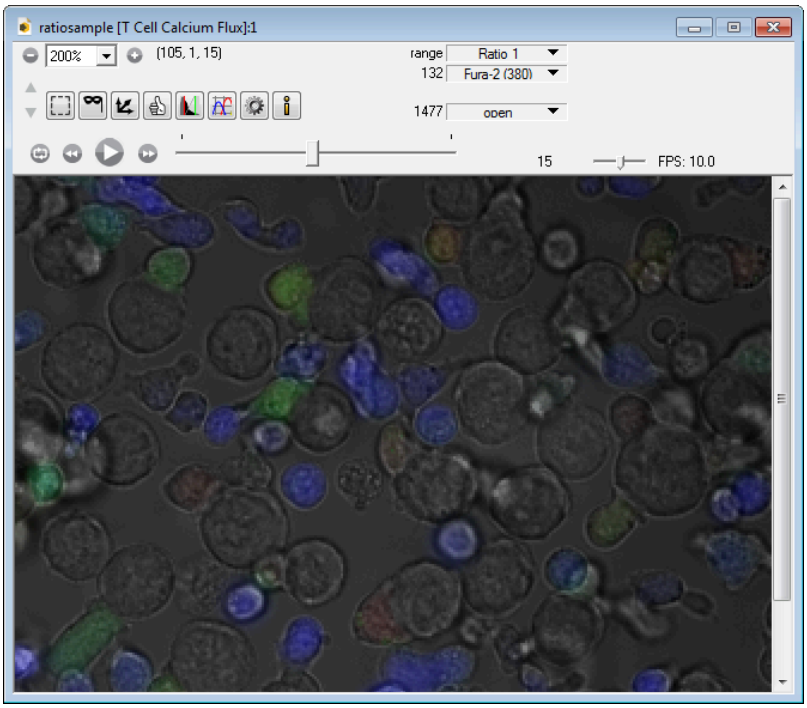
5. In the pseudocolor view mode, there are two channel selections. The first channel selects the hue of the display and should be set to the ratio channel. The second channel selects what determines the intensity, or brightness, of the displayed colors. If it is set to the numerator or denominator channel, the pseudocolored display will be scaled by the selected fluorescence intensity, such as in the example above. Thus, in the example above, an area with high pixel intensity for the ratio channel and low pixel intensity on denominator channel will appear as dim red. An area with low pixel intensity for the ratio channel and high pixel intensity on the denominator channel will appear as bright blue. This helps distinguish areas with real data from background. This is often desirable as computed ratio values for regions of the image that are just slightly above background can vary greatly.

6. Set the second channel menu selection to **None**. The image is now displayed as a range of low (blue) to high (red) ratio values. The brightness/dimness of the hue is not scaled.



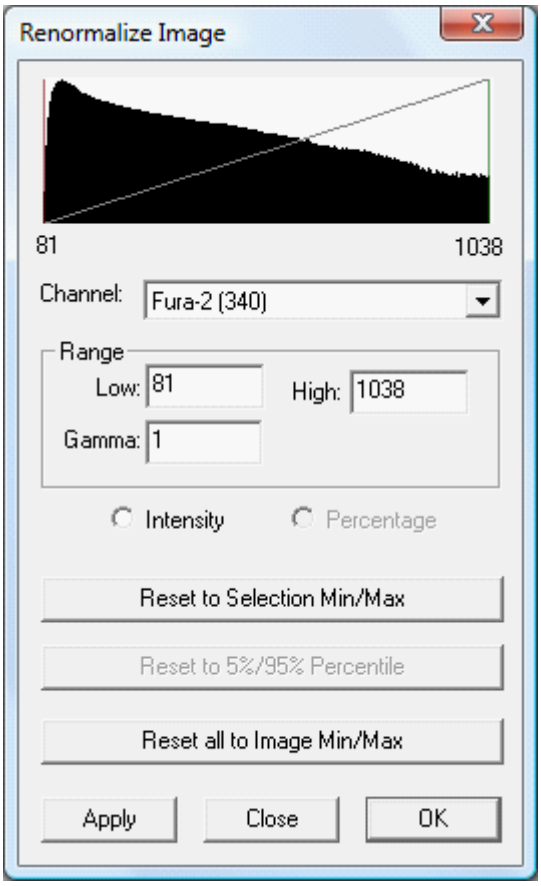
7. The large amount of noise you observe in this image is the result of displaying a straight ratio, not scaled by intensity data. Return the second menu to **Fura-2 (380)** to scale the ratio by intensity.

8. Set the ***Background channel menu*** to **Open**. A DIC image is now displayed along with the ratio data. To improve the display, blend the DIC image with the fluorescence image by selecting ***View Settings > Blend Background*** either in the View group of the Home tab or in the image window.



9. Return the background channel to **None**.

10. Open the ***Renormalize Image*** dialog box from the View group of the Home tab or in the image window. If this is a new dialog to you, please review the Renormalize Image window.



11. Move the red bar back and forth. Note that the display does not change. **Fura-2 (340)** is not a displayed channel in this image.

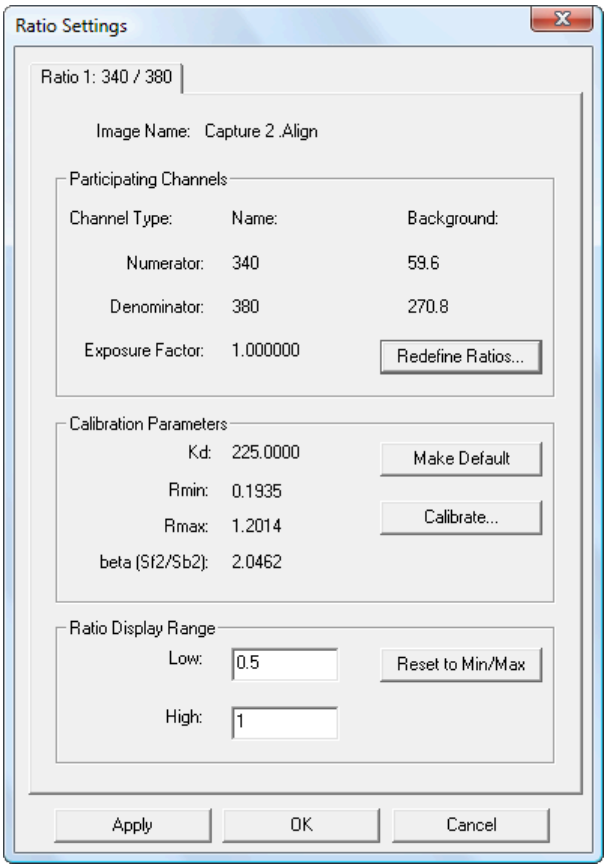
12. Select **Fura-2 (380)** from the channel drop-down menu and move the red bar back and forth. Note that the hue, or color, of the display does not change. Note also that the intensity of the color becomes dim as you move the red bar to the right and becomes bright as you move the red bar to the left.

> **NOTE:** Hues that are displayed cannot be changed using the renormalization dialog; only the intensity of color may be changed. To change the hues that are displayed, you must alter the Ratio Settings, as discussed in the following section.

Ratio Settings

SlideBook lets you adjust, if necessary, many of the settings that go into both post-acquisition display as well as analysis. These settings are accessed in the ***Ratio Settings*** dialog box available from the Modules tab, ***Ratio > Ratio Settings*** or by clicking on the icon in the image window then selecting ***Ratio Settings***.

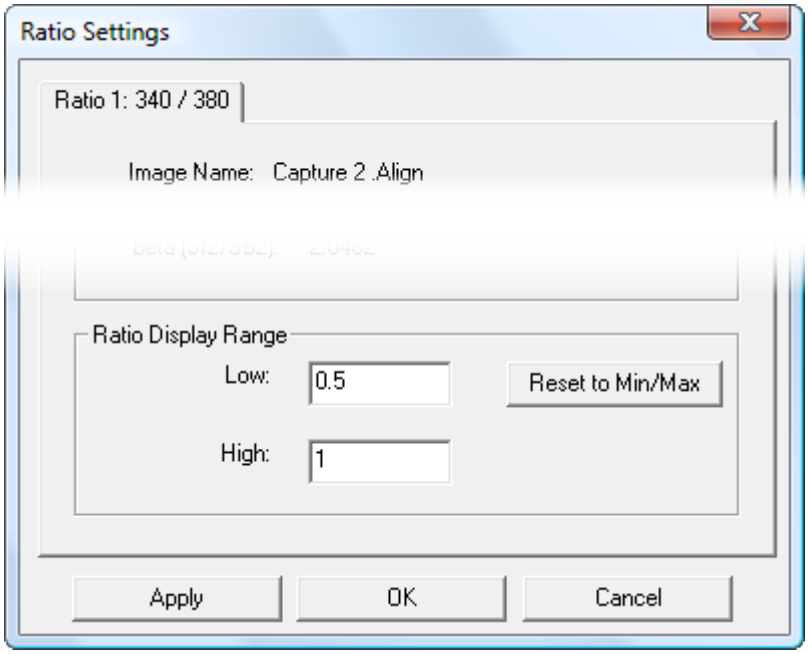
The dialog is divided into three main sections: ***Participating Channels***, ***Calibration Parameters***, and ***Ratio Display Range***. The dialog is detailed in User Interface section: Ratio Settings window. You can also read more about calibration in Ratio Calibration or follow the TUTORIAL: Using Ratio Settings to learn how to adjust the display of ratio images.



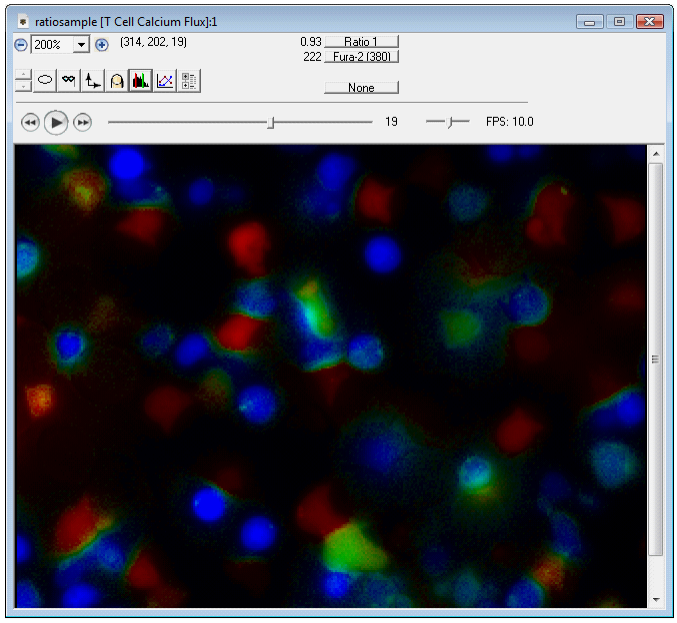
TUTORIAL: Using Ratio Settings

We will now walk through a tutorial to learn how to adjust ***Ratio Settings*** on our sample image.

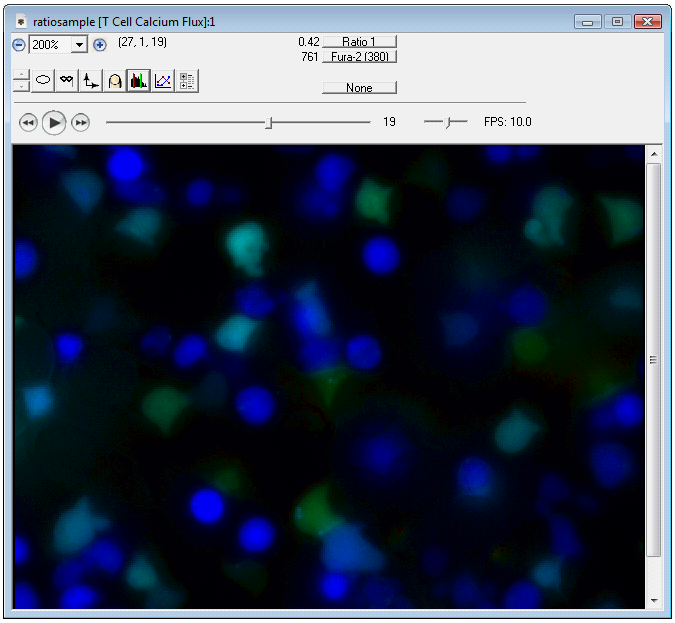
1. Make sure a view of **T Cell Calcium Flux** is active and then click on the icon in the image window and select ***Ratio Settings*** (also accessed from Modules tab, ***Ratio > Ratio Settings***).



2. In the ***Ratio Display Range*** section, change the ***High*** edit field to **0.5** and click ***Apply***. Note that the display hues have changed and more cells are red.



3. Change the ***Ratio Display Range High*** edit field to **2.0** and click ***Apply***. Note that display hues are all blue/green.

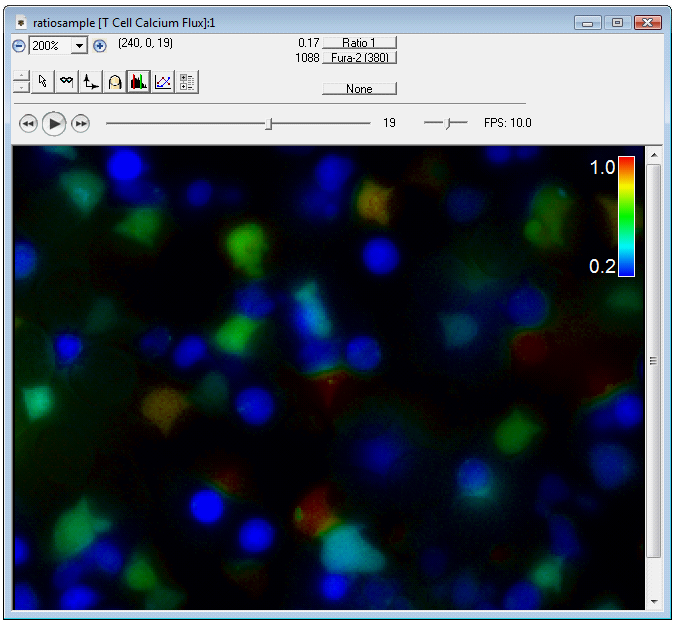


4. Return the ***Ratio Display Range High*** edit field to **1.0** and click ***Apply***.

5. Click ***OK*** to close the ***Ratio Settings*** dialog.

6. Select ***File > Save Slide*** to save your new settings. Next time you open this image, these display settings will be applied by default.

7. Go to the Home tab, Annotations group and check the box ***Lookup Tables*** or hit **Ctrl** + **Shift** + **L**. Notice the lookup table reflects the designated ratio display range.



8. Add additional annotations from the ***Annotations*** group or by using shortcut keys; additional annotations available include: scale bar, timestamps, notes, object IDs, and regions.

9. Adjust the appearance of these annotations under ***Annotations Settings***.

10. Select ***View >*** ***Timelapse Tile View***. In this view, each time point is displayed in an individual panel. For more information on this view, see Timelapse Tile View window.

11. If desired, export a figure as it is displayed by going to ***Export > Qualitative Export*** options (see also Qualitative Image Export).

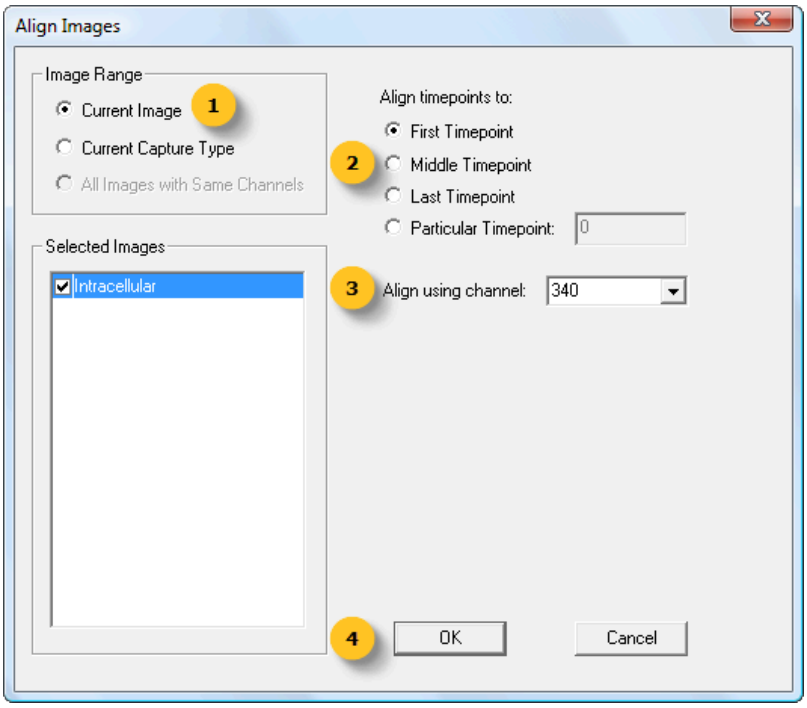
TUTORIAL: Aligning Images

At times, an entire imaging field may drift over time due to unstable temperature or external forces such as a perfusion device or manual disruption when adding reagents. When this occurs, analysis results based on regions may be inaccurate. SlideBook's alignment tool is a helpful solution to re-stabilize the image for analysis. More information on this window can be found in User Interface: Align Images window.

NOTE: The alignment technique in this tutorial is for correcting homogenous movement of an entire field. If individual areas or cells are moving within the field, you will need to use masks for analysis as discussed in Using Thresholding Techniques to Generate Masks.

1. Open the image with a field that shifts over time. Scroll through the image and observe the movement of the imaging field.

Go to Analyze tab, Process Image group, ***Align > Automatic Image Alignment***. Under ***Image Range***, select ***Current Image***. You would use ***Current Capture Type*** if you had multiple time lapse images in one **.sld** that required alignment.



2. Select the timepoint you would like to use as the alignment timepoint. All other images will be shifted to align with the alignment timepoint.

3. Select a channel to use for the alignment.

4. Click on ***OK***. A new image will be written to the slide with - **auto aligned** appended to the image name.

Ratio Calibration

SlideBook provides an easy-to-follow guide for calibrating a fluoroprobe such as Fura-2 using the solution-based in vitro calibration technique outlined in Grynkiewicz et al (1985). This section also teaches intracellular calibration for use when a sample has enough representative data in a time series.

Ratio channels can be calibrated so that ratio values correspond to concentrations. SlideBook supports two different methods of calibration. The first is a solution-based in vitro method that may be performed pre- or post-acquisition. This calibration may be applied to multiple experiments. The second is an intracellular calibration that is performed post-acquisition for each experiment. This method performs calibration within a single cell and then applies those values to the entire data set. With either method, a ratio calibration guide assists you in determining three important numbers for correctly calibrated concentration display:

R_{min} - the minimum ratio value (e.g., Fura-2 with no free Ca++ ions present)

R_{max} - the maximum ratio value (e.g., Fura-2 in an ion-saturated solution)

β - the ratio of the denominators of the minimum and maximum conditions

The ratio calibration guide is also where you specify three other parameters:

K_d - the dissociation constant of the fluoroprobe

R_{low} - the ratio value below which a view displays the color corresponding to the minimum value

R_{high} - the ratio value above which a view displays the color corresponding to the maximum value

From these parameters, calcium concentration can be calculated from the following equation:

$$[CA^{++}] = K_d * \frac{R - R_{min}}{R_{max} - R} * \beta$$

Solution-based in vitro Calibration

This type of calibration requires you to capture images of buffer solutions. When imaging buffer solutions, you may wish to add a small amount of non-fluorescent polystyrene beads to aid in obtaining focus. Alternatively, you may mark or scratch the surface of the coverslip or dish used for imaging. Example buffer solutions for calibrating calcium concentrations when using Fura-2 may be found in Appendix A: Buffers for Ratio Calibration.

NOTE: Do not attempt calibration using an AM ester derivative of an intracellular probe. AM ester derivatives have little to no response to ion concentration differences. You must use a salt form of your fluorophore for calibration.

Background Image

The first ratio image that you must collect (consisting of both the numerator and denominator channels) is a background image. This is usually an image (or average of a set of images) of the experimental setup with a buffer solution that has no fluoroprobe present. Prior to starting the ***Ratio Calibration Guide***, you should place a buffer solution slide on the stage and adjust any microscope parameters in the ***Focus Window***.

Ion-free Image

The second ratio image that you must collect is an ion-free image. This is usually an image (or average of a set of images) of the experimental setup with a buffer solution that has fluoroprobe present. The concentration of fluoroprobe should be approximately equal to the concentration of fluoroprobe present in loaded cells.

Ion Saturated Image

The third and final ratio image that you must collect is an ion saturated image. For this image, you will need a ion saturated buffer solution. The buffer should contain the same concentration of fluoroprobe as the buffer used to capture the ion-free images.

Beginning In Vitro Calibration

Once you have the sample on the microscope for the background image and the ion-free and ion saturated samples ready, you can proceed to Generating Ratio Calibration Data.

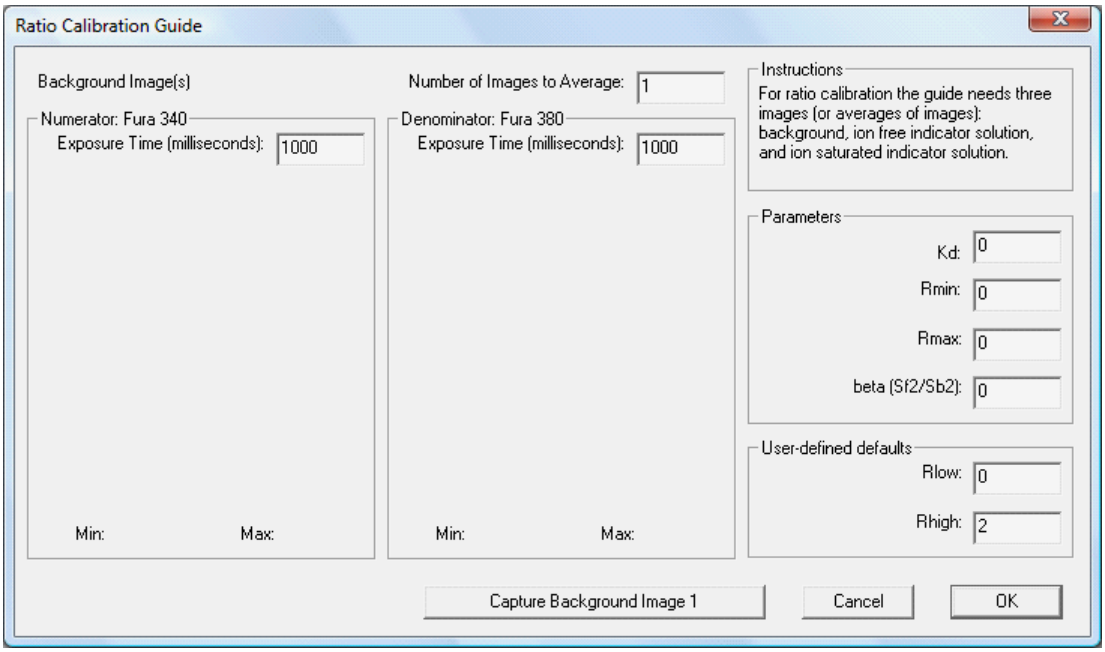
Generating Ratio Calibration Data

To generate in vitro ratio calibration data:

1. Place the background buffer solution in your experimental setup and bring the sample into view and focus.

2. Bring up the ***Ratio Calibration Guide*** by selecting ***Ratio > Ratio Calibration Guide*** in the Modules tab.

Alternatively, you may bring up the calibration guide from an open ratio image by selecting ***View Settings > Ratio Settings*** and selecting ***Calibrate***.



3. Enter the number of images that you wish to average in the ***Number of Images to Average*** data entry field. In general, three images will be sufficient.

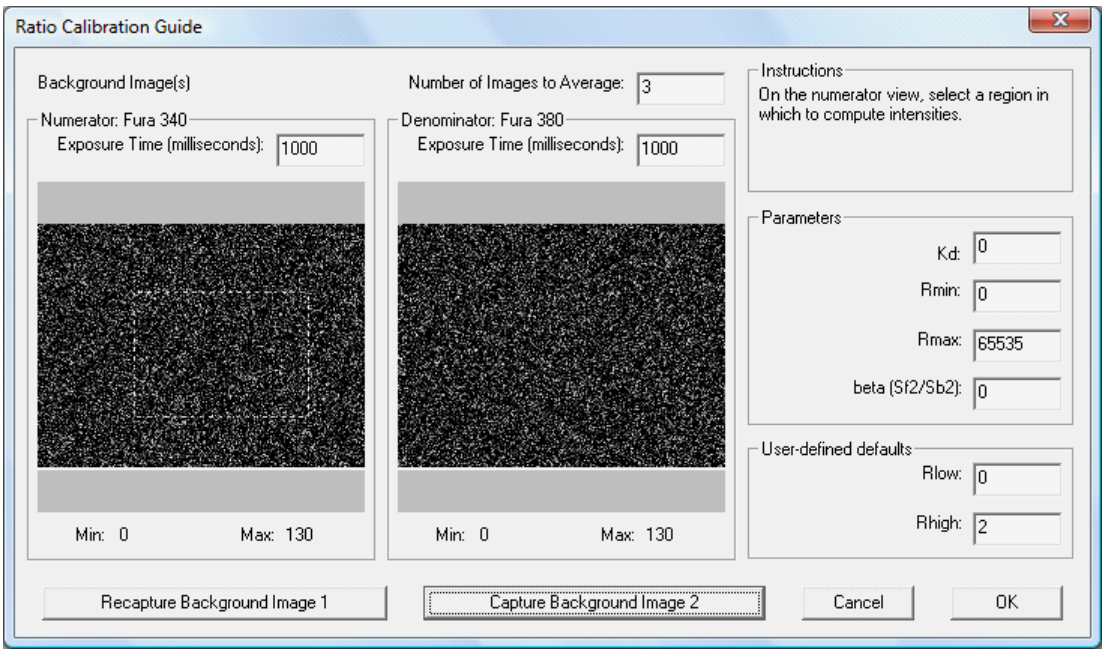
4. Enter the ***Exposure Time*** in milliseconds for both the numerator and denominator in the data entry fields.

> **NOTE:** You should aim for an exposure length that yields only moderate intensity values, since there is no fluoroprobe present. Otherwise, the subsequent capture of ion-free and ion saturated images will certainly exceed the dynamic range of the camera.

5. Generate a test image by selecting ***Capture Background Image 1*** and observe the resulting intensity values reported beneath the image. You may recapture the image if the maximum intensity is too high by selecting ***Recapture Background Image 1***.

6. After capturing the first background ratio image, select a region of interest by clicking and dragging on the numerator image view in order to determine the area over which the average background intensity (as well as ion-free and ion saturated intensities) will be determined.

7. In general, try to select the largest region possible, as it means the ratio calibration values will be computed from a larger sample. However, if there are obvious anomalies in the field, the region of interest selection permits you to exclude them.



8. Continue collecting background images by selecting ***Capture Background Image***.

9. When you have finished collecting the background image or images, click on ***Proceed to Ion-Free Image***.

10. Place your ion-free buffer solution (with fluoroprobe) in your experimental setup and bring your sample into view and focus using the ***Focus window***.

11. Select ***Capture Ion-Free Image***. The region of interest that you selected in the background image collection will be used for these captures.

12. Continue collecting ion-free images by selecting ***Capture Ion-Free Image***.

13. When you have finished collecting the ion-free image or images, click on ***Proceed to Ion-Saturated Image***.

14. Place your ion-saturated buffer solution (with fluoroprobe) in your experimental setup and bring your sample into view and focus.

15. Select ***Capture Ion-Saturated Image***. The region of interest that you selected in the background image collection will be used for these captures.

16. Continue collecting ion-saturated images by selecting ***Capture Ion-Saturated Image***.

17. When you have finished collecting the image(s), click on ***Compute Parameters***.

The guide will automatically compute the R_{min}, R_{max}, and ***beta*** values (see definitions). For the exact mathematical equations used to compute these parameters, please refer to Appendix B: Ratio Calibration Calculations.

18. Enter the dissociation constant, K_d, for your fluorophore. This value is specific for your fluorophore and available from the manufacturer.

NOTE: Enter the K_d value in units of nM concentration.

19. Enter the ***User-defined defaults*** for R_{low} and R_{high} values. This will designate the default display scaling for ratio images as discussed in Ratio Settings.

In general, you should set R_{low} and R_{high} to be equal to R_{min} and R_{max} initially. After running experiments, you may wish to revise the R_{low} and R_{high} values to reflect the actual range observed. You may access these values post-capture by selecting ***Image > Ratio > Ratio Settings***.

20. Click ***OK*** to complete calibration and exit the Ratio Calibration Guide. Your ratio calibration data will be used to generate concentration data for ratio images as discussed in Data Graphing and Export.

Intracellular Calibration

As described earlier in this chapter, SlideBook provides an in vitro calibration tool for ion concentration indicators. We have been discussing Ca^{++} imaging using Fura-2 and will continue to do so in this section. Often cellular physiology interacts with Fura-2 such that the dye's behavior inside a cell does not match the dye's behavior in calibration solutions. For more accurate measurement of absolute $[Ca^{++}]$, it is best to measure the range of ratios observed in vivo, or within the cells themselves.

The ***Intracellular Calibration Guide*** in SlideBook allows you to select representative regions in a time series and use them as the basis for determining the maximum and minimum ratios that are observed within cells.

Typically, intracellular Ca^{++} calibration is done as an "end-step" in a live cell experiment. After observing meaningful Ca^{++} dynamics during an experiment, a calcium ionophore, such as ionomycin, is introduced into a cellular buffer solution that has a high free $[Ca^{++}]$. This raises the intracellular $[Ca^{++}]$ dramatically and, after the cytosol reaches equilibrium, will allow us to measure a maximum intracellular ratio ($\boldsymbol{R_{max}}$) as the 340 nm excitation signal will be at its highest and the 380 nm excitation signal will be at its lowest.

After that, a calcium chelator, such as EGTA, is added to drop the intracellular $[Ca^{++}]$. Again, after equilibrium, we can measure a minimum ratio ($\boldsymbol{R_{min}}$). A typical experimental protocol is outlined below:

1. Perform experiment.

2. Once the desired effect is observed, add solution so that final concentration in media is 1 μM ionomycin and 1.8 mM $[Ca^{++}]$ and wait for ratio to stabilize using live ratio display. This will yield a maximal Fura-2 ratio.

3. Add solution of 1 μM ionomycin and 2 mM EGTA and wait for ratio to stabilize. This will yield a minimal Fura-2 ratio.

This is a guideline; you may need to experiment to find out what concentration of calcium and EGTA is appropriate for your cells. The $\boldsymbol{R_{max}}$ and $\boldsymbol{R_{min}}$ from the intracellular calibration must be higher and lower than that which you observe during your experiment. If this is not the case, you must adjust the calibration solutions.

TUTORIAL: Intracellular Ratio Calibration

A tutorial for intracellular ratio calibration follows. Please use **RatioTutorial.sld** for the tutorial.

Set Background Region and Region for Calibration

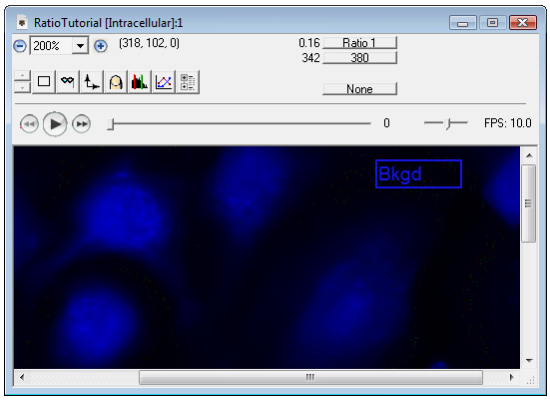
1. Open a ***Main View*** of **Intracellular**.

2. Draw a region in an area with no signal using the square region tool.

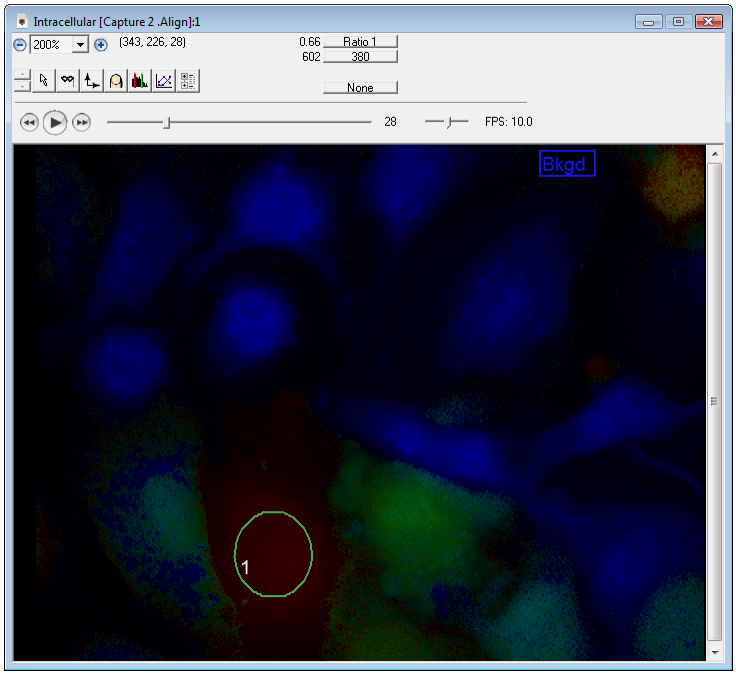
3. Select the region by clicking on it, then right click and select ***Set as Background***.

It is a good idea to scroll through time and make sure that no debris or floating cells enter the proposed background region during some later time point, as the background level is computed for each time point. If you want to change the background region, simply select the region then click and drag it to a different spot or delete it and redraw.

NOTE: The size of a region for calibration purposes does not need to be large. Even a small region contains well over 100 pixels, which is plenty to remove the effects of signal noise in determining a reliable background level.

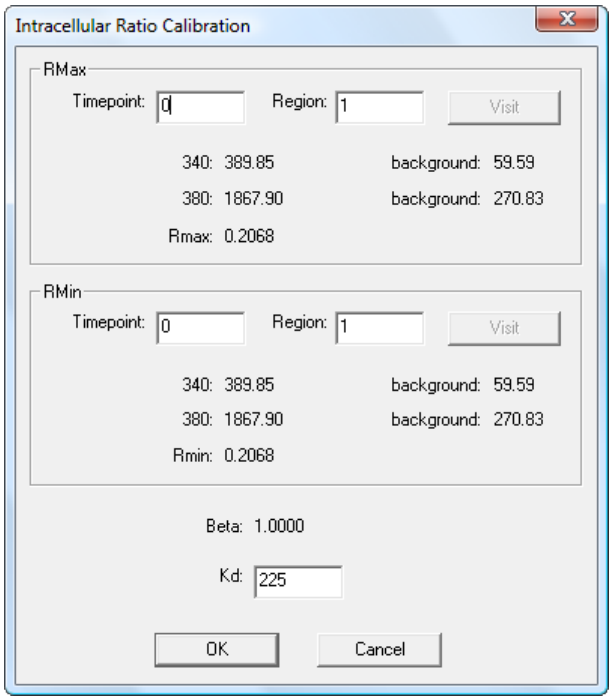


4. Draw a region within a representative cell. It may be helpful to scroll through time and find a cell that has a maximum ratio among the highest in the field of cells.



Find R_{max} and R_{min}

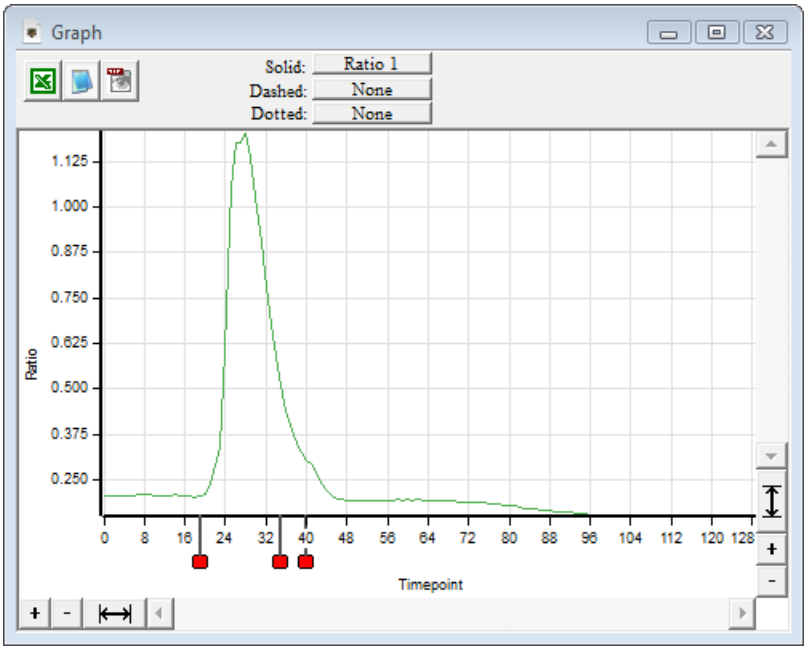
1. Select ***Ratio > Intracellular Calibration*** (from the Modules tab) to open the intracellular calibration dialog box.



2. Initially, the dialog box shows the computed R_{max} and R_{min} based on the first timepoint and the first region.

3. Graph the ratio data of the region by selecting ***Graph*** in the image view window. Set ***Type*** to ***Ratio*** and ***Line 1*** to ***Ratio 1***, click ***OK***.

4. A graph similar to the following will appear:



5. Click on the highest ratio value in the graph, the main view will update to timepoint 28. The vertical green line in the graph indicates the timepoint selected.

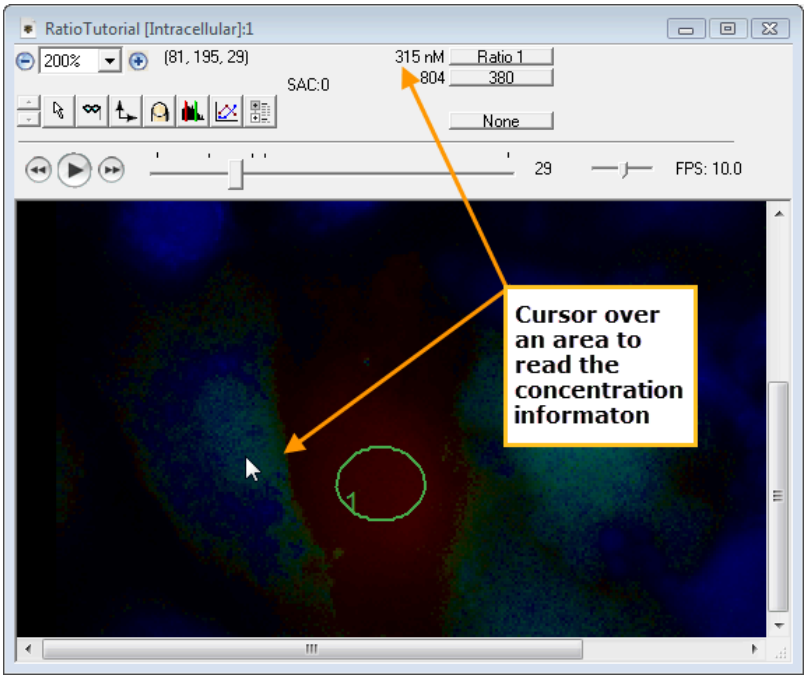
6. In the Intracellular Ratio Calibration dialog, type 28 into the ***Timepoint*** edit field for R_{max}. If the main view is not currently displaying timepoint 28, you may visit that timepoint by pressing ***Visit*** after entering the timepoint in the edit field.

7. Ensure the correct region is listed in the ***Region*** edit field. If region 1 is not the region you would like to use for calibration, update this field accordingly.

8. Now repeat steps 4 - 6 for R_{Min}. In this example, R_{Min} occurs between timepoints 50 and 65. After timepoint 65, the sample begins to photobleach.

9. Enter the dissociation constant, K_d, for your fluorophore. This value is specific for your fluorophore and biological system and can be obtained from the manufacturer of the fluorophore. Please enter the K_d value in units of nM concentration. In this example, we will use 225 nm.

10. Click ***OK*** to complete the calibration. Move the cursor over the main view and observe that calcium concentrations are displayed next to the ratio channel menu.



11. To set your display back to ratio values (not concentration information), re-open the ***Intracellular Ratio Calibration*** dialog and enter 0 in the K_d field and then click ***OK***.

Data Graphing and Export

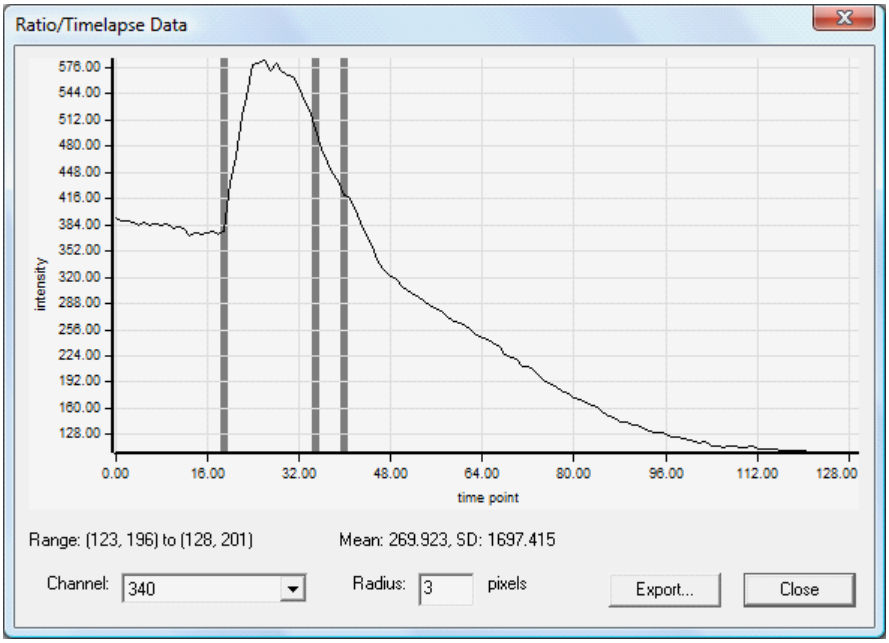
There are three ways to mark areas for graphing and exporting ratio data from an image: single area export from a point-and-click selection, multiple area export from regions, and multiple area export from a mask created manually. You may wish to use the sample files to experiment with data graphing and export.

Quick Graphing and Data Export

To rapidly generate ratio data:

1. Select a location on the image by selecting the ***Point Selection tool*** from the Tool Menu.

2. Hold the **CTRL** key while clicking on a cell. A graph similar to the following will appear. You may select an individual channel or the ratio you wish to display using the drop-down ***Channel*** menu. You may increase the ***Radius*** to increase the area that is being graphed.



3. To export the data, press the ***Export*** button.

4. Type in the desired name of the text file where your data will be written.

5. Open the data file using Excel. You may wish to repeat steps 2 and 3 for different fluorophores and for the ratio.

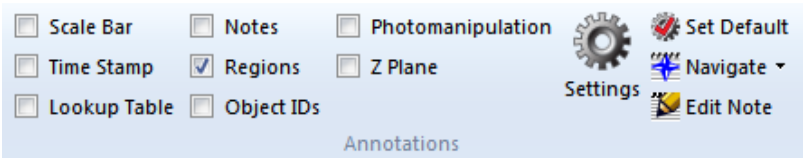
Multiple Region Graphing and Export

You may also export data from multiple regions that you selected during capture. You may add or delete regions post-capture.

Working with Regions

You may display regions that you created during capture, edit selections, and select a background region post-acquisition.

Display Region(s) – select any of the region tools from the Region group or select ***Annotations > Regions***

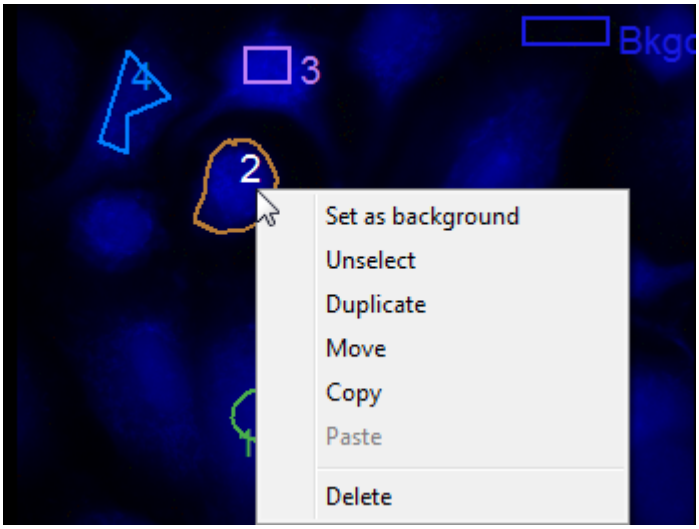


Select Region – click on the region or the number using the ***Selection tool***, the number of a selected region will be white

Create New Region - use the region tools to draw regions (please see Selecting Regions of Interest)

Delete Region – select the region, tap the **Delete** key on the keyboard or right-click and select ***Delete*** from the menu

Select Background Region – click on a region to select it, then right-click and select ***Set as Background*** from the menu



Graphing and Exporting Data

To graph your region data, select ***Graph*** from the image view window and then the channels or ratio you wish to display. For more information on how to use the graph, refer to Graph Display or the User Interface page Graph window. To export a TIFF of the graph, click on the ***Export TIFF icon*** .To export the ratio or concentration data, click on the ***Excel Export icon*** or the ***Text File Export icon***.

The resultant file will include the following information about the image:

- ***Image Name*** - name of the individual image (can be edited from ***Get Info***)
- ***Slide Name*** – name of the **.sld** file
- ***Capture Date*** – date on which the image was acquired
- ***Capture Time*** – time of day at which the acquisition was initiated
- ***Ratio 1 information present*** – header for ratio configuration information
- ***Ratio 1 numerator channel*** – the filter name for the channel identified as Ratio 1 numerator
- ***Exposure Length (ms) or (s)*** – exposure time for Ratio 1 numerator channel
- ***Ratio 1 denominator channel*** – the filter name for the channel identified as Ratio 1 denominator
- ***Exposure Length (ms) or (s)*** – exposure time for Ratio 1 denominator channel
- ***Exposure factor*** – calculated as the exposure time numerator divided by exposure time denominator

There will also be a table of data that lists the following information:

- ***Plane*** – plane number, also known as the timepoint number
- ***Time*** – time of day image was captured
- ***Elapsed (ms) or (s)*** – elapsed time from time 0 to current time point in milliseconds or seconds
- ***[Channel] Bkgd*** – background intensity for each channel/timepoint when a background value is set from a region where **[channel]** is the actual channel name
- ***[Channel] [region#] – Bkgd*** –this value will be the background subtracted (if applicable) intensity for each channel, at each region, for each time point where **[channel]** is the channel name, **[region#]** is the number of the region for the calculation, and **- Bkgd** is added when a background region has been set.
- ***Ratio 1 [region#]*** – the calculated ratio for each region at each time point
- ***Conc1 [region#]*** – computed concentration if calibration data is present (see also Ratio Calibration)

NOTE: The exact mathematical equations used for computing ratio data can be found in Appendix C: Ratio Data Export Calculations.

In order to export additional statistical information, you may convert regions to mask objects then select statistics to export as discussed in Creating a Mask from Existing Regions.

Multiple Region Export from a Mask

Sometimes, region data export from the graph is insufficient because you require additional statistical information or because the specific area you wish to track is moving. If this is the case, you will need to utilize masks for data analysis and export.

You can configure SlideBook to use a background region for background subtraction in mask statistics. Masks can be created from existing regions or can be created using thresholding techniques. Once the masks are generated, a plethora of statistical export options are available.

> **NOTE:** It is important to understand that masks created using thresholding techniques should only be used for analysis of ratio data when a specific area or cell is moving. If the entire field is moving due to drift or disruption of the specimen, you should correct for the movement using image alignment algorithms and proceed with analysis using regions. Instructions on this correction can be found in TUTORIAL: Aligning Images.

Creating a Mask from Existing Regions

When you require data for export that was not included when exporting from a graph (see list of data types in Graphing and Exporting Data), you can convert regions to masks for a full menu of statistical export options. To do so, go to the Analyze tab, ***Mask Generation group >*** ***Convert Regions to Mask Objects***. Continue to Exporting Statistics from Masks for further instructions.

TUTORIAL: Using Thresholding Techniques to Generate Masks

Sometimes, you will find that the region you would like to use for analysis moves over time. When this occurs, static regions will not yield the analysis results desired. Instead, you may need to use thresholding techniques to select pixels for analysis by intensity. To demonstrate, please follow the tutorial below using the example image in **RatioTutorial.sld**.

> **NOTE:** If the entire field is moving, you should align images and use regions for analysis. The techniques discussed in this section are only applicable when individual objects or cells are moving within a field. Instructions for aligning images can be found in TUTORIAL: Aligning Images.

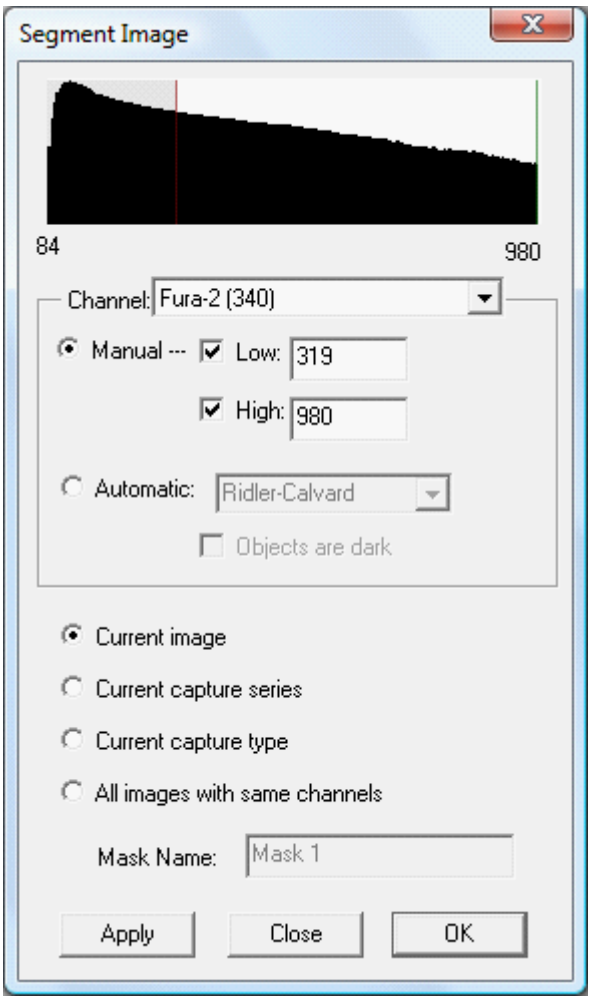
1. Open **RatioTutorial.sld**. Open image **T Cell Calcium Flux**.

2. Go to the Home tab, View group, ***Mode >*** ***Monochrome***. This will change the display to black and white.

3. You will notice that there is only one channel menu available because only one channel can be displayed in monochrome. Select the numerator **Fura-2(340)** from the menu. Scroll through the data and observe the fluorescence level across the field. Select the denominator **Fura-2(380)** from the channel menu. Scroll through the data again. Choose the channel that has the best contrast throughout the time series. In this example, select **Fura-2(340)**.

> **NOTE:** Alternatively, a wavelength at the isosbestic point of the fluorophore can be used. This wavelength will not be reactive to the ion concentration and, therefore, makes an ideal channel for thresholding. If you frequently require thresholding to select analysis regions, you may want to incorporate acquiring and isosbestic channel during imaging.

4. Select the ***Mask icon*** in the image window ***> Create Segment Mask*** or use the Mask Generation group in the Analyze tab ***Segment Mask***. The Segment Image window will open.



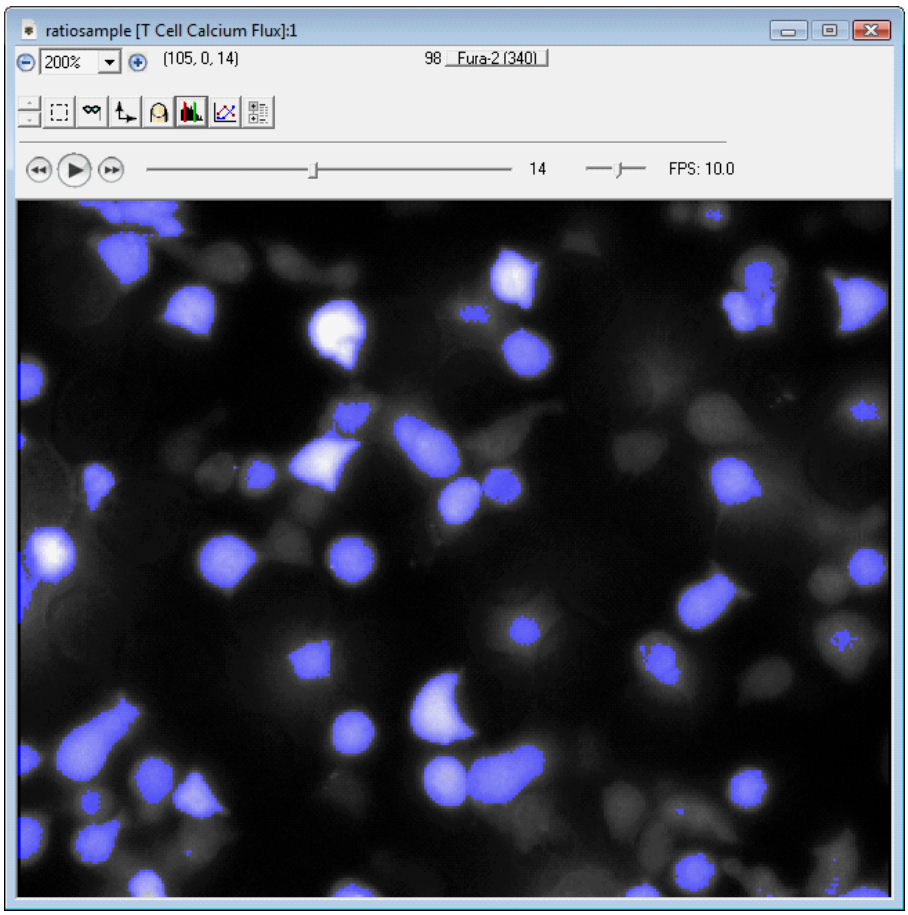
5. Select the channel you would like to use (from step #3 above) to create the mask from the ***Channel*** menu.

6. Use the low selection red line or text edit field to select a low threshold. Pixels with intensities below this value will be excluded from the mask.

7. Use the high selection green line or edit field to select a high threshold. Pixels with intensities above this value will be excluded from the mask. For most applications, the high threshold will be left at the maximum intensity level for the image. However, if you wish to exclude saturated objects from your analysis, you may wish to utilize the high threshold to do so.

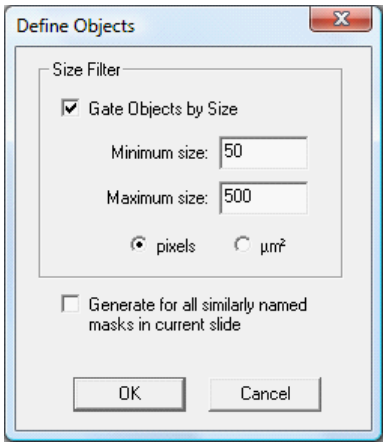
8. As you adjust the threshold values, you will notice blue pixels appear on the image. These pixels represent the mask, the area selected for analysis. You may find it useful to scroll to another time point and click ***Apply*** to see how the threshold values work throughout the time series.

> **NOTE:** When an image is displayed as pseudocolor (instead of monochrome, RGB, etc.), masks will be displayed as white outlines, not as blue highlighted areas.



9. Click ***OK***. It is necessary to click ***OK*** to generate the mask in all planes of the image.

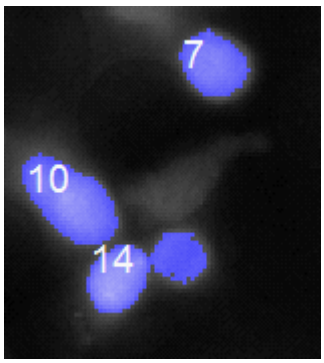
10. Next, we will eliminate objects that are too small or too large for analysis. Go to ***Mask > Define Objects in Mask***. For more information on this dialog, refer to Define Objects window.



11. Check the box for ***Gate Objects by Size***. Set a ***Minimum size*** and ***Maximum size*** in either ***pixels*** or ***µm²***.

12. Click ***OK***. Any objects that are outside of the size range defined will be eliminated from the mask.

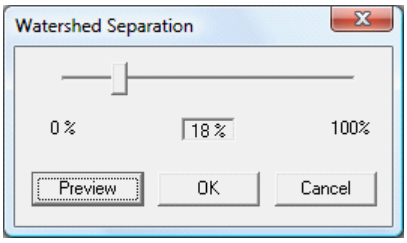
13. Go to the Home tab, Annotation group, select ***Object IDs*** (or type **Ctrl + Shift + O**). A number will appear next to each defined object in the mask, this is the Object ID. At times, two objects may be identified as one as shown with object **14** below:



14. To separate these objects, go to Analyze tab, Edit Mask group, ***Split Objects > Split Objects Automatically*** (see also Split Objects Automatically window).

NOTE: If you perform this operation frequently, you may find it useful to set up a Split Objects Protocol.

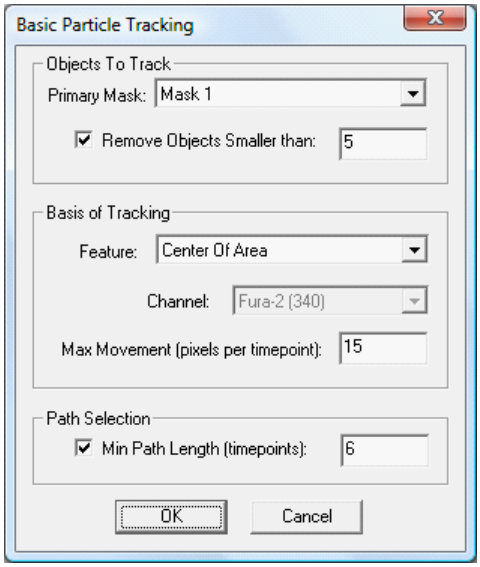
15. Select a percentage of separation in the ***Watershed Separation*** tool then click ***Preview***.



16. Adjust to desired separation then click ***OK***.

17. As you scroll through time in the image, you may notice the ID on an object changes over time. The next step is to track the objects so they maintain their identity throughout the time series. Go to Analyze tab, Edit Mask group, ***Particle Tracking > Basic Particle Tracking*** (see also Basic Particle Tracking window)

NOTE: If you perform this operation frequently, you may find it useful to set up a Particle Tracking Protocol.



18. Select ***Tracking Parameters*** as described below:

Primary Mask – Select the mask that contains the objects you would like to track. The menu will default to the mask that is currently displayed on your open view.

Remove Objects smaller than – Check this box to selectively remove objects smaller than a given size. Enter the size in the edit field and choose either microns or pixels as the unit.

Feature – Select the parameter that you wish to track from the dropdown list:

Center of Area – The coordinates of the center of the object based on perimeter without regard to the intensity values.

Center of Intensity – The coordinates of the center of the object, weighted by intensity values. If you select this feature, you will also need to select the channel that is used for weighting.

Mean Adjusted Center of Intensity – The coordinates of the center of the object, weighted by intensity values that are above the mean intensity of the object. This is a useful feature to track if you have delineated your objects by hand. By using values that fall above the mean, any imprecision in your hand delineation does not impact the center of intensity calculation. If you select this feature, you will also need to select the channel that is used for weighting.

Maximum Movement – Enter the maximum movement that you expect from any given object from one time point to the next. This will help the particle tracking algorithm distinguish between objects that may have similar centroid values.

Min Path Length – Enter the minimum number of timepoints that must be tracked in order to determine a path. For instance, if you enter 3, any object that cannot be followed for at least three time points will be removed for the mask.

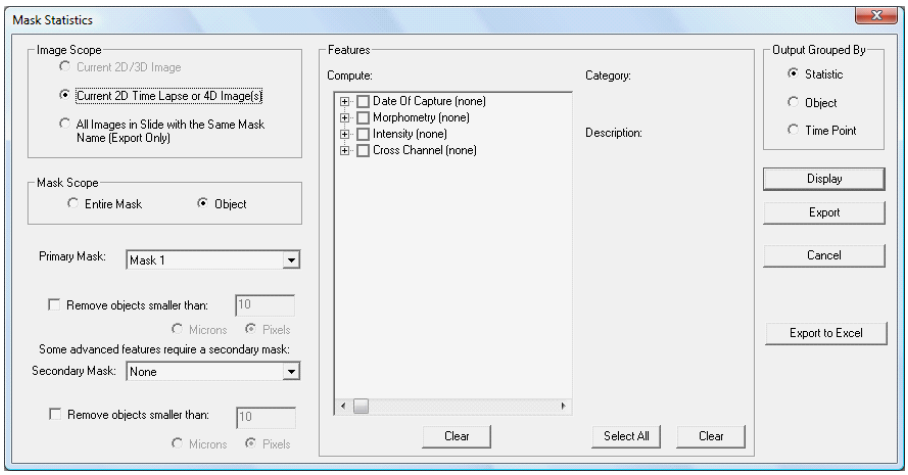
19. Click ***OK***. The mask is complete and refined.

Exporting Statistics from Masks

At times, the data export options described earlier in this chapter will be insufficient because you are interested in additional statistical information. This may include details regarding the morphometry of the regions or masks or intensity information other than mean intensity. Now we will discuss obtaining this statistical information.

1. Create a mask by one of the methods discussed earlier in this chapter. If the image has a mask but it is not displayed, you can display the mask by selecting the ***Mask icon*** in the image window and selecting the mask of interest from the list.

2. Go to Analyze tab, Evaluate group, ***Mask Statistics***. For more on this dialog, please refer to Mask Statistics window. The following dialog will appear:



3. The dialog has the following sections:

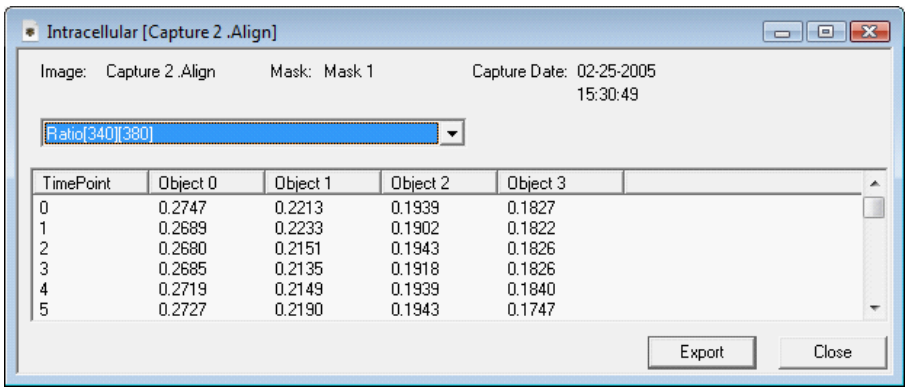
- ***Image Scope*** – allows for generation of statistics in multiple images. Because you are working with a 2D timelapse or 4D data set, the second radio button will be selected by default. You may select the third radio button if you have multiple images with the same mask name.

- ***Mask Scope*** – allows you to select whether you want statistics for the entire mask or on a per object basis. If you have chosen to obtain statistics for multiple images, the ***Object*** option will be unavailable.

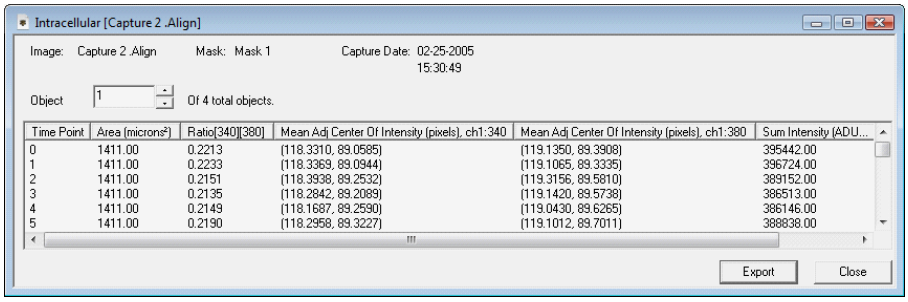
- ***Primary Mask*** – defaults to the mask that is currently displayed but can be altered via the dropdown menu. The primary mask will automatically be made into objects. You may also remove objects under a specified pixel size. To get statistics for all objects in the mask, leave this box unchecked.

- ***Features*** – lists all available statistics that may be calculated. Note that the list of features will depend on the dimensions of the mask. For instance, area will be displayed as a statistic for 2D masks, while volume will be displayed for 3D masks. When a feature is selected, the description of its calculation method will appear beneath the ***Description*** heading.

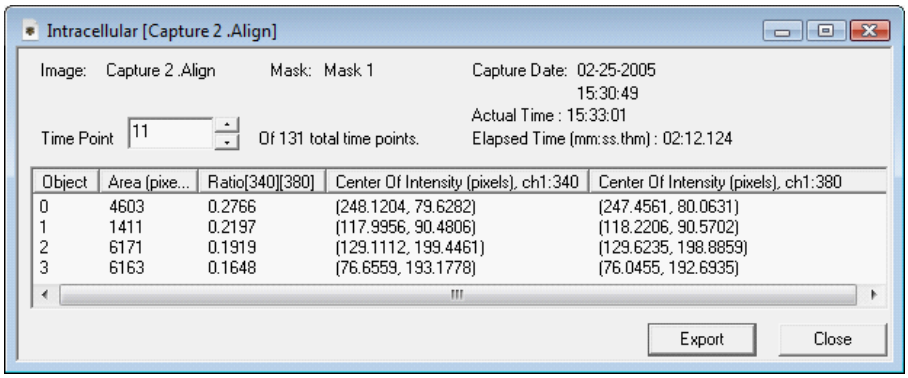
- ***Output Grouped By*** – these choices are only activated if you are performing object statistics on 2D timelapse or 4D images. Sample output is shown below:



Output Grouped by Statistic



Output Grouped by Object



Outpput Grouped by Time Point

4. Check all the statistics you would like to compute. For more information on a statistic computation, please refer to the ***Description*** field.

5. Select how you would like to display or export the data by pressing one of the following buttons:

- ***Display*** – displays the data as shown in the figures above.

- ***Export*** – exports a tab-delimited **.txt** file that can be opened by a spreadsheet program. You will be prompted to name the file.

- ***Export to Excel*** – opens the spreadsheet in Microsoft Excel.

- ***Cancel*** – Closes the ***Mask Statistics*** dialog without displaying or exporting statistics.

Exporting Images and Movies

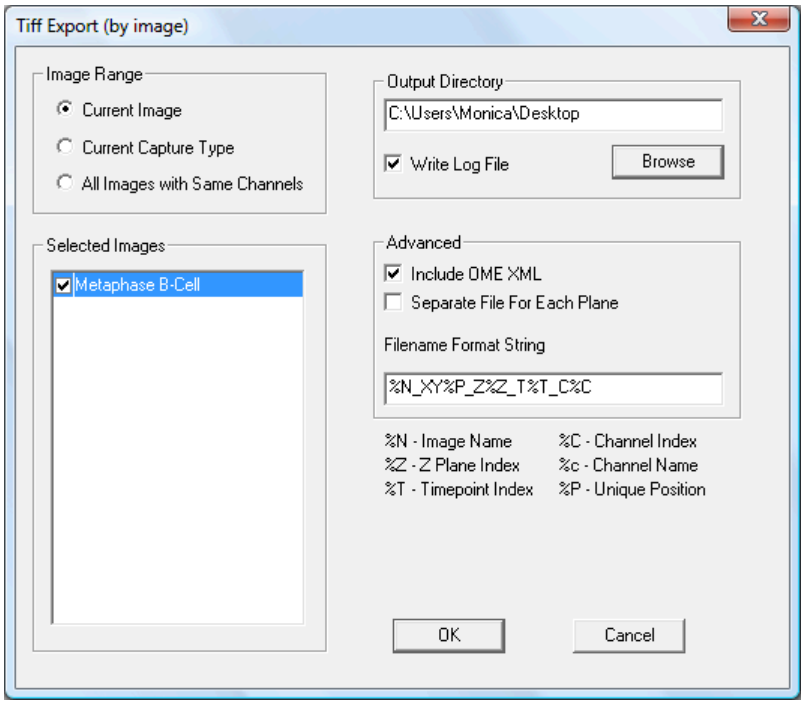
To complement the data export discussed in the previously, we will now review exporting the images. There are three main types of image export available in SlideBook. Quantitative image export enables export of image as data for further analysis or for archival purposes. Qualitative image export allows export of the image exactly as it appears in SlideBook, including and renormalization and annotations. Movie export involves creation of QuickTime or Microsoft AVI files from timelapse data.

Quantitative Image Export

Sometimes, you will wish to share data with colleagues or back up data in a format that can be read by programs other than SlideBook. In order to do this, you will want to maintain the true intensity values of each pixel of the image. For this reason, there is a 16-bit tiff export option available (see also 16-bit Tiff Export window). This export option is compatible with the OME (Open Microscopy Environment) format.

1. Select the image that you would like to export by clicking on it. You may also start from an open view of the image.

2. In the Home tab, Acquire group, select ***Export > 16-bit TIFF***. A dialog box similar to the following will appear.



3. Select the ***Image Range*** as described below. You may also select and deselect images using the checkboxes in the ***Selected Images*** list.

- ***Current Image*** – Operation will be performed on the selected image only.
- ***Current Capture Type*** – Operation will be performed on all images in the slide with the same capture type (2D, 3D, 4D, etc.) as the selected image.
- ***All Images with Same Channels*** – Operation will be performed on all images in the slide with the same channels as the selected image.

4. Select the ***Output Directory*** where the tiffs will be saved by clicking on **Browse** then navigating to the desired location.

5. Check the box for ***Include OME XML*** to include OME metadata in the tiff file (encoded in XML).

6. Check the box for ***Separate File For Each Plane*** if you would like a tiff file for each plane for each channel of the image. If you do not check this box, a multi-plane tiff file will be created for each channel that will include all timepoints? Z Points??

7. If desired, format the naming of the tiffs in the ***Filename Format String*** field.

8. Press ***OK***.

Qualitative Image Export

Qualitative image export is useful when you want to use an image exactly as it appears in SlideBook. Any display adjustments as well as annotations will be preserved during qualitative image export.

1. Open a Main View, Three View, Tile View, or Channel View of the image and adjust the look of the data as desired. This may include altering the ratio settings and renormalization parameters, cropping the image, or adjusting the zoom factor.

2. Add any desired annotations from the ***Annotations*** menu or by using the shortcut keys. Adjust the look and location of the annotations by going to ***Annotations > Settings***.

3. In the Home tab, Acquire group, select ***Export > RGB TIFF*** to export the view as a single TIFF (**.tif**) file or ***> TIFF series*** to export a tiff of each time point of the image.

4. Enter the file name and select ***Save***. The TIFF image can now be used in other programs.

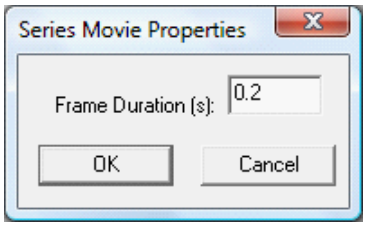
Movie Export

Because ratio imaging is dynamic, it is often useful to create movies from your data that can be used in presentations and shared with colleagues. Two movie types are available, Apple QuickTime (**.mov**) and Microsoft AVI (***.avi***).

1. Open a Main View, Three View, Tile View, or Channel View of the image and adjust the look of the data as desired. This may include altering the ratio settings and renormalization parameters, cropping the image, or adjusting the zoom factor.

2. Add any desired annotations from the ***Annotations*** group or by using the shortcut keys. Adjust the look and location of the annotations by using ***Annotation Settings***.

3. In the Home tab, Acquire group, select ***Export > Create Series Movie***. The ***Series Movies Properties*** dialog box will appear.



4. Enter a value in the ***Frame Duration*** field. This is the amount of time that a single time point will be displayed during the movie playback. Use smaller values for faster playback. For example, a value of **0.2** will play back at a rate of 5 frames per second.

5. A standard ***Save As*** dialog box will open. Name the file then click ***OK***.

Appendix A: Buffers for Ratio Calibration

You may wish to purchase a Fura-2 Calcium Imaging Calibration Kit from Molecular Probes (Cat# F-6774). Sample buffer recipes are shown below. R_{min}, R_{max}, and β values will vary depending on your particular setup, but typical values for Fura-2 may be R_{min} ~ 0.25, R_{max} ~ 2.5 and β ~ 5.

Background Buffer

10X stock solution

Dissolve in 90 ml H2O:

- 8.95 g KCl (1.2 M)
- 0.29 g NaCl (50 mM)
- 0.1 g KH2PO4 (7.4 mM)
- 0.42 g NaCO3 (50 mM)
- 4.77 g HEPES (200 mM)

Do not adjust pH

Bring volume to 100 ml with H2O

For 1X solution dilute 1:9 in H20, pH to 7.4

Ion-Free Buffer

Add 25-50 μM (final concentration) fluorophore (not AM ester derivative, must be salt form) to the Background Buffer. Note that this value may vary based on your fluorophore and loading conditions. You will want to approximate the final concentration of fluorophore in your loaded samples.

Ion-Saturated Buffer

10X stock solution CaCl2

Dissolve in 90 ml H2O:

- 0.22 g CaCl2 (20mM)

Do not adjust pH

Bring to 1X using 1 part CaCl2 10X solution, 1 part 10X background buffer solution, 8 parts H2O, pH to 7.4 and add 25-50 μM (final concentration) fluorophore.

Appendix B: Ratio Calibration Calculations

Ratios are computed as the quotient of a numerator divided by a denominator:

$$R = \frac{num}{den}$$

where, for example, the numerator is Fura-2 signal at 340 nm excitation and the denominator is Fura-2 signal at 380 nm excitation. In order to convert a ratio into a calcium concentration ($[Ca^{++}]$) it is necessary to incorporate the minimum and maximum values of the ratiometric indicator as well as its dissociation constant.

Extracellular (in vitro) calibration of $[Ca^{++}]$ values given ratios is performed by collecting a sequence of N background images (where no dye is present), N images where dye is present with no free calcium, and N images where the dye is present and there is a saturating concentration of calcium.

Terms

$B_{num}(n; x, y)$	Intensity of numerator background image n at pixel x, y
$B_{den}(n; x, y)$	Intensity of denominator background image n at pixel x, y
$F_{num}(n; x, y)$	Intensity of numerator ion-free image n at pixel x, y
$F_{den}(n; x, y)$	Intensity of denominator ion-free image n at pixel x, y
$S_{num}(n; x, y)$	Intensity of numerator ion-saturated image n at pixel x, y
$S_{den}(n; x, y)$	Intensity of numerator ion-saturated image n at pixel x, y
R	Set of all pixels x, y in the calibration region
$\|R\|$	Number of pixels in calibration region
γ	Exposure time correction factor: $num\ exposure\ time / den\ exposure\ time$

Background

The background is computed by averaging both num and den intensities of N images over the region:

$$\bar{B}_{num} = \frac{1}{N}\sum_{n=1}^{N}\frac{1}{|R|}\sum_{R} B_{num}(n; x, y)$$

$$\bar{B}_{den} = \frac{1}{N}\sum_{n=1}^{N}\frac{1}{|R|}\sum_{R} B_{den}(n;x,y)$$

R_{min}

$R_{min}(n)$ is computed by finding the ratio of the average ***num*** intensity divided by the average ***den*** intensity over the region of each of N ion-free image pairs and choosing the overall minimum.

$$\bar{F}_{num}(n) = \frac{1}{|R|}\sum_{R} F_{num}(n;x,y)$$

$$\bar{F}_{den}(n) = \frac{1}{|R|}\sum_{R} F_{den}(n;x,y)$$

$$R_{min}(n) = \frac{\bar{F}_{num}(n) - \bar{B}_{num}(n)}{\gamma(\bar{F}_{den}(n) - \bar{B}_{den}(n))}$$

$$R_{min} = \min_{n} R_{min}(n)$$

R_{max}

Similarly, $R_{max}(n)$ is computed by finding the ratio of the average ***num*** intensity divided by the average ***den*** intensity over the region of each of N ion-saturated image pairs and choosing the overall maximum.

$$\bar{S}_{num}(n) = \frac{1}{|R|}\sum_{R} S_{num}(n;x,y)$$

$$\bar{S}_{den}(n) = \frac{1}{|R|}\sum_{R} S_{den}(n;x,y)$$

$$R_{max}(n) = \frac{\bar{S}_{num}(n) - \bar{B}_{num}(n)}{\gamma(\bar{S}_{den}(n) - \bar{B}_{den}(n))}$$

$$R_{max} = \max_{n} R_{max}(n)$$

Beta

β is computed as the quotient of the average denominator intensity in the ion-free images over the average denominator intensity in the ion-saturated images:

$$\beta = \frac{\frac{1}{N}\sum_{n=1}^{N}\bar{F}_{den}(n) - \bar{B}_{den}}{\frac{1}{N}\sum_{n=1}^{N}\bar{S}_{den}(n) - \bar{B}_{den}}$$

Appendix C: Ratio Data Export Calculations

Currently there are two ways of defining a region for post-acquisition analysis:

- Using region(s) across all time points
- Using a mask (divided into submasks) for contiguous isolated user-delineated areas across all time points included in the mask

Each method uses the same ratio and concentration calculations.

Terms

λ_1	Excitation wavelength used in ratio numerators
λ_2	Excitation wavelength used in ratio denominators
$I_{\lambda 1}(t; x, y)$	Intensity of numerator image of time point t at position x, y
$I_{\lambda 2}(t; x, y)$	Intensity of denominator image of time point t at position x, y
$region(n)$	Region of interest n: a subset of all possible x, y positions
$\lvert region(n) \rvert$	The number of positions in the region of interest n
γ	Exposure time correction factor: $\text{exposure time at } \lambda 1 / \text{exposure time at } \lambda 2$
$\theta_{\lambda 1}$	Minimum threshold intensity for ratio numerator
$\theta_{\lambda 2}$	Minimum threshold intensity for ratio denominator
$\omega_{\lambda 1}$	Maximum threshold intensity for ratio numerator
$\omega_{\lambda 2}$	Maximum threshold intensity for ratio denominator

Background

The background is computed for each time point for both the numerator and denominator images by averaging intensities over the background region of interest, $region_b$:

$$\underline{b}_{\lambda_1}(t) = 1/\lvert region_b \rvert \sum_{region_b} I_{\lambda_1}(t; x, y)$$

$$\underline{b}_{\lambda_2}(t) = 1/\lvert region_b \rvert \sum_{region_b} I_{\lambda_2}(t; x, y)$$

Regions and Thresholding

When mask regions are created using thresholding methods, individual regions of interest for analysis are restricted by the minimum and maximum thresholds for each wavelength. This means that a delineated region of interest region(n; t) actually yields two regions, $region_{l1}(n; t)$ and $region_{l2}(n; t)$, which may not necessarily be identical:

$$region_{\lambda_1}(n; t) = \cup_{x,y} \text{ such that } (x, y) \in region(n; t) \wedge \theta_{\lambda_1} \leq I_{\lambda_1}(t; x, y) \leq \omega_{\lambda_1}$$

$$region_{\lambda_2}(n; t) = \cup_{x,y} \text{ such that } (x, y) \in region(n; t) \wedge \theta_{\lambda_2} \leq I_{\lambda_2}(t; x, y) \leq \omega_{\lambda_2}$$

Ratio Computation

The ratio $r(n; t)$ is computed for region(n; t) by first computing average intensities for the numerator and denominator:

$$\underline{i}(n; t)\lambda_1(n) = 1/|region\lambda_1(n; t)| \Sigma_{region}\ \lambda_1(n; t)\ I\lambda_1(t; x, y)$$

$$\underline{i}(n; t)\lambda_2(n) = 1/|region\lambda_2(n; t)| \Sigma_{region}\ \lambda_2(n; t)\ I\lambda_2(t; x, y)$$

and then computing the background and exposure time corrected ratio:

$$r(n; t) = (\underline{i}(n; t)\lambda_1(n) - \underline{b}\lambda_1(t)) / (\gamma(\underline{i}(n; t)\lambda_2(n) - \underline{b}\lambda_2(t)))$$

Ratio Error Conditions

The following conditions will prevent $r(n; t)$ from being computed:

Concentration Computation

The concentration $c(n; t)$ is computed using the calibration parameters:

$$c(n; t) = K_d \beta\ (r(n; t) - R_{min}) / (R_{max} - r(n; t))$$

Concentration Error Conditions

The following conditions, in addition to those that prevent $r(n; t)$ from being computed, will prevent $c(n; t)$ from being computed:

$$r(n; t) < R_{min}$$

$$r(n; t) \geq R_{max}$$

Terms

l1 Excitation wavelength used in ratio numerators

l2 Excitation wavelength used in ratio denominators

I1(t; x, y) Intensity of numerator image of time point *t* at position *x*, *y*

I2(t; x, y) Intensity of denominator image of time point *t* at position *x*, *y*

region(n) Region of interest n: a subset of all possible *x*, *y* positions

|region(n)| The number of positions in the region of interest *n*

g Correction factor: exposure time at #1 / exposure time at #2

ql1 Minimum threshold intensity for ratio numerator

ql2 Minimum threshold intensity for ratio denominator

wl1 Maximum threshold intensity for ratio numerator

wl2 Maximum threshold intensity for ratio denominator

Background

The background is computed for each time point for both the numerator and denominator images by averaging intensities over the background region of interest, *regionb*:

$$\underline{b}1(t) = 1/|regionb| \sum_{regionb} I1(t; x, y)$$
$$\underline{b}2(t) = 1/|regionb| \sum_{regionb} I2(t; x, y)$$

Regions and Thresholding

When mask regions are created using thresholding methods, individual regions of interest for analysis are restricted by the minimum and maximum thresholds for each wavelength. This means that a delineated region of interest *region(n; t)* actually yields two regions, *region#1(n; t)* and *region#2(n; t)*, which may not necessarily be identical:

region1(n; t) = È x,y such that (x, y) Î region(n; t) ^ ql1 £ I1(t; x, y) £ wl1
region2(n; t) = È x,y such that (x, y) Î region(n; t) ^ ql2 £ I2(t; x, y) £ wl2

Ratio Computation

The *ratio* $r(n; t)$ is computed for $region(n; t)$ by first computing average intensities for the numerator and denominator:

$$\underline{i}(n; t)1(n) = 1/|region1(n; t)| \sum region1(n; t) I1(t; x, y)$$
$$\underline{i}(n; t)2(n) = 1/|region2(n; t)| \sum region2(n; t) I2(t; x, y)$$

and then computing the background and exposure time corrected ratio:

$$r(n; t) = (\underline{i}(n; t)1(n) - \underline{b}1(t)) / (g(\underline{i}(n; t)2(n) - \underline{b}2(t)))$$

Ratio Error Conditions

The following conditions will prevent $r(n; t)$ from being computed:

$|region1(n; t)| = 0$
$|region2(n; t)| = 0$
$i(n; t)1(n) < b1(t)$
$i(n; t)2(n) \leq b2(t)$

Concentration Computation

The concentration $c(n; t)$ is computed using the calibration parameters:

$$c(n; t) = Kd\,b\,(r(n; t) - Rmin) / (Rmax - r(n; t))$$

Concentration Error Conditions

The following conditions, in addition to those that prevent $r(n; t)$ from being computed, will prevent $c(n; t)$ from being computed:

$r(n; t) < Rmin$
$r(n; t) \geq Rmax$

References

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Grynkiewicz, G. et al. (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. Journal of Biological Chemistry 260: 3440-3450.

Moore, E. D. W. et al. (1990) Ca2+ imaging in single living cells: Theoretical and practical issues. Cell Calcium 11: 157-179.

FRET Module

SlideBook's FRET Module adds functionality that simplifies a wide variety of quantitative techniques. Measurement of FRET allows you to determine whether or not two molecules are colocalized at a distance less than 100 angstroms, enabling measurements below the optical resolution of a light microscope. FRET is a distance dependent interaction between the electronic excited states of two dye molecules where the excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon. FRET experiments may be performed on live or fixed cells. SlideBook supports three FRET methods: Sensitized Emission/Direct FRET (both Two-Channel Corrected and Three-Channel Corrected), Acceptor Photobleaching FRET, and Anisotropy FRET. For more information on FRET methods, please see Appendix A: FRET Introduction and Theory.

The FRET Module adds several components to the SlideBook base package that allow you to configure hardware for streamlined FRET acquisition and analysis. Included in this section of help are several tutorials that utilize sample data. To obtain the sample images, download **directFRETsample.sld** from www.intelligent-imaging.com.

FRET System Configuration
We will first explore the dialog for configuring FRET channels in SlideBook. This section includes tables for common types of FRET analysis and how the channels for each type of FRET analysis will be configured.

Three Channel Corrected FRET
Three channel corrected FRET is a subclass of direct FRET or sensitized emission FRET. It includes traditional FRETc as well as some of the newer calculations such as E-FRET, NFRET, and FRETN (see Appendix B: Direct FRET Calculations for more information on these calculations). In this section, we will discuss the entire experimental process for imaging three channel corrected FRET from image capture through analysis and bleedthrough calculations.

Two Channel Corrected FRET
Two channel corrected FRET is a subclass of direct FRET or sensitized emission FRET. This method is acceptable when donor and acceptor are present in a fixed, stoichiometric ratio. When performing two channel FRET, you are essentially taking the ratio of the transfer channel over the donor. In this section, we will discuss the entire experimental process for imaging two channel corrected FRET from image capture through analysis.

Acceptor Photobleaching FRET
In acceptor photobleaching FRET, you will bleach the acceptor and examine the effects on the donor signal. This section will walk you trough determining bleaching parameters, and evaluating your images.

Anisotropy FRET

FRET System Configuration

SlideBook allows you to configure many different types of imaging channels. Standard techniques include independent channels. In order to streamline FRET analysis, specialized FRET channels will be identified in system configuration. Channel definitions for Direct FRET and Acceptor Photobleaching FRET will be discussed in this section. Once channels are defined correctly, they will maintain definitions for all analysis and bleedthrough calculations.

Channel Configuration

Regardless of the type of FRET analysis you will be performing, channels will all be configured using the ***Filter Configuration Parameters*** dialog. Please refer to Filter Configuration Parameters Dialog for a complete review of the user interface.

To create FRET channels:

1. Go to the Administration tab, ***Filters***. The ***Filter Configuration Parameters*** dialog will open.

2. Click ***Add*** to add a new channel.

3. For each channel you will configure, you will need to complete the following fields:

   Name - The name of the channel. This will appear when you select a filter for imaging.

   Fluorescence: Emission wavelength (µm) – For fluorescent channels, select the radio button for fluorescence and enter the wavelength of the emission in µm.

   Filter set and wheel/turret positions – Enter in the appropriate information to ensure the appropriate filters move into position when the channel is selected.

   Associated Channel Type – Select the appropriate channel type (see tables below).

   Default Color Display – Select the default display mode for the channel.

THREE CHANNEL CORRECTED FRET (TRADITIONAL FRETc)			
Type	**Example Name**	**Associated Channel Type**	**Default Color Display**
Donor	CFP	FRET Donor	Blue
Acceptor	YFP	FRET Acceptor	Green
Transfer	FRET	FRET Transfer	Red

THREE CHANNEL CORRECTED FRET (EFRET)			
Type	**Example Name**	**Associated Channel Type**	**Default Color Display**
Donor	CFP	FRET Donor	None
Acceptor	YFP	FRET Acceptor	Pseudocolor (Intensity)
Transfer	E-FRET	FRET Transfer	Pseudocolor (Color)

TWO CHANNEL CORRECTED FRET			
Type	**Example Name**	**Associated Channel Type**	**Default Color Display**
FRET	FRET	FRET Transfer	Pseudocolor (Color)
Donor	CFP	FRET Donor	Pseudocolor (Intensity)

ACCEPTOR PHOTBLEACHING FRET			
Type	**Example Name**	**Associated Channel Type**	**Default Color Display**
Donor Prebleach	CFP-pre	FRET Donor Prebleach	Green
Acceptor Prebleach	YFP-pre	Independent	None
Photobleach	440 bleach	Independent	None

Donor Postbleach	CFP-post	FRET Donor Postbleach	Blue
Acceptor Postbleach	YFP-post	Independent	None

ANISOTROPY FRET			
Type	**Example Name**	**Associated Channel Type**	**Default Color Display**

4. Once parameters are configured for a channel, click ***Add*** to add the next channel to your filter list.

5. Repeat steps 3-4 until all required FRET channels are added.

6. Click ***OK*** to close the dialog and apply the changes.

7. When prompted, restart SlideBook to update the configuration.

Three Channel Corrected FRET

Three channel corrected FRET is a subclass of direct FRET or sensitized emission FRET. It includes traditional FRETc as well as some of the newer calculations such as E-FRET, NFRET, and FRETN (see Appendix B: Direct FRET Calculations for more information on these calculations). In this section, we will discuss the entire experimental process for imaging three channel corrected FRET from image capture through analysis and bleedthrough calculations.

Note: Before you proceed, it is important to have channels configured as described in FRET System Configuration.

Capturing Three Channel FRET Images

In order to perform direct FRET, you must be able to calculate bleedthrough coefficients, or cross-talk, for your particular filter sets. To calculate bleedthrough coefficients, two control samples must be imaged: one that contains donor only, and one that contains acceptor only.

In this section, we will discuss using the Focus Window and Capture Window in SlideBook. If you are a new user and are unfamiliar with these controls, please review the user interface pages before continuing below.

Capturing Control Images

Control images should be acquired for each FRET experiment design. When you change a step in your experiment design, updated FRET control images should be acquired.

1. Mount the donor sample on the stage and open the ***Focus Controls*** in SlideBook.

2. Select the donor filter set (for CFP/YFP FRET, the donor is the CFP channel), and bring the sample into focus.

3. Close the fluorescent shutter and then close the ***Focus Controls***.

4. Open the ***Capture*** window.

5. Set the donor exposure time by selecting the appropriate filter checkbox and then pressing ***Test***. Adjust the exposure time as desired or use the ***Adjust Exposure*** buttons to aid in setting the appropriate exposure time. For additional information on adjusting exposure duration, including setting regions for capture and utilizing binning, refer to the Capture Controls window.

6. Set the FRET transfer exposure time by selecting the FRET filter checkbox and manually entering the same ***Expose*** duration used step 5. It is important that the exposure times of both filters are equal to each other.

7. In the ***Image Information*** field (in the lower right corner of the ***Capture*** dialog), type a ***Name*** to name the image. For example, you may want to name this image **Donor Control**.

8. Click ***OK*** to capture your image.

9. Repeat steps 1-7 with a sample containing only the acceptor, capturing with the acceptor and FRET transfer channels.

Imaging the Experimental Sample

Now bring your experimental sample into view and focus using the ***Focus Controls***. Use the FRET transfer filter when focusing your sample. Then, image your sample(s), capturing the donor, acceptor, and FRET transfer channels. Make sure that all exposure times are equal to each other. If you are performing a live FRET experiment, you may wish to take advantage of the live graphing features that are discussed later in Live Display.

1. Mount the experimental sample on the stage and open the ***Focus Controls*** in SlideBook.

2. Select the FRET transfer filter and bring the sample into focus.

3. Close the fluorescent shutter and then close the ***Focus Controls***.

4. Open the ***Capture*** window.

5. Set the exposure time for the donor, acceptor, and FRET transfer channels. You may find the ***Adjust Exposure*** buttons useful in setting the appropriate exposure time.

> **Note:** The experiment requires the same exposure time for all three channels so be sure to use the brightest channel to set exposure times.

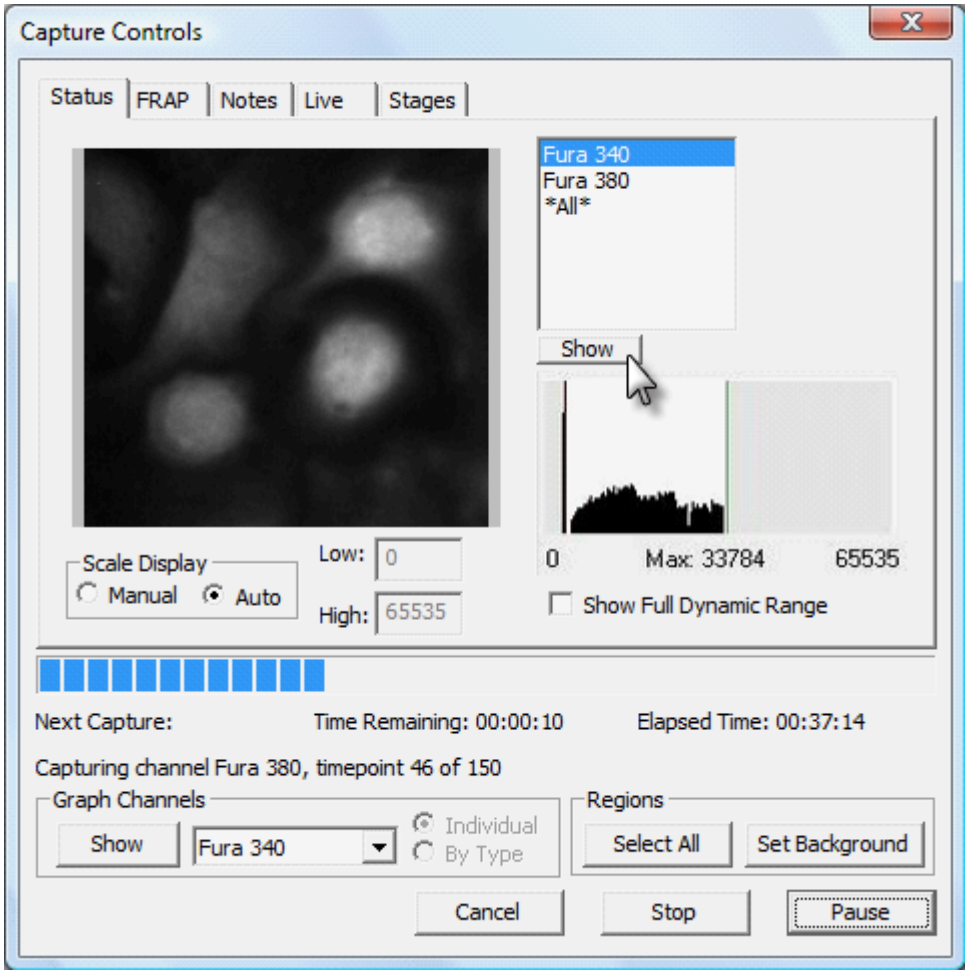
6. Set up timelapse capture if desired.

7. Click ***OK*** to capture the experiment.

Live Display During Three Channel FRET

SlideBook will display a live composite image of the donor, acceptor, and FRET channels for each time point. Additionally, you may wish to graph the intensity of regions of interest, adjust the focus of the microscope, or create event markers.

During capture, the live window will display a composite image as individual channels are captured. To display an individual channel, go to the ***Status*** tab of the ***Capture Controls*** dialog. In the upper right portion, you will find a list of channels. Simply select an individual channel to display and click ***Show***. A live window displaying a monochrome image of the selected channel will open. In this manner, it is possible to watch a live composite display as well as displays of the individual channels.



Other available functions during live capture include:

- Selecting Regions of Interest
- Graph Display
- Focusing During Capture
- Creating Event Markers (Notes)
- Exposure Time Tips

TUTORIAL: Post Capture Analysis

After FRET images are captured, you can perform background subtraction, calculate bleed-through coefficients, and generate the corrected FRET signal. You may follow this procedure step-by-step using the example file **directFRETsample.sld**.

Performing Bleedthrough Calculations

Bleedthrough (also known as crosstalk) is calculated using the two control images (see Capturing Control Images for instructions on capturing control images).

1. Open the sample slide by selecting ***Open Slide*** and navigating to **directFRETsample.sld**.

2. Open a main view of **CFP Only Control** by double-clicking on the thumbnail.

3. Next, select the mask icon in the image window **> *Create segment mask*** or use the Mask generation group in the Analyze tab and select ***Segment Mask***. The Segment Image dialog will open.

4. Select the donor channel from the ***Channel*** menu.

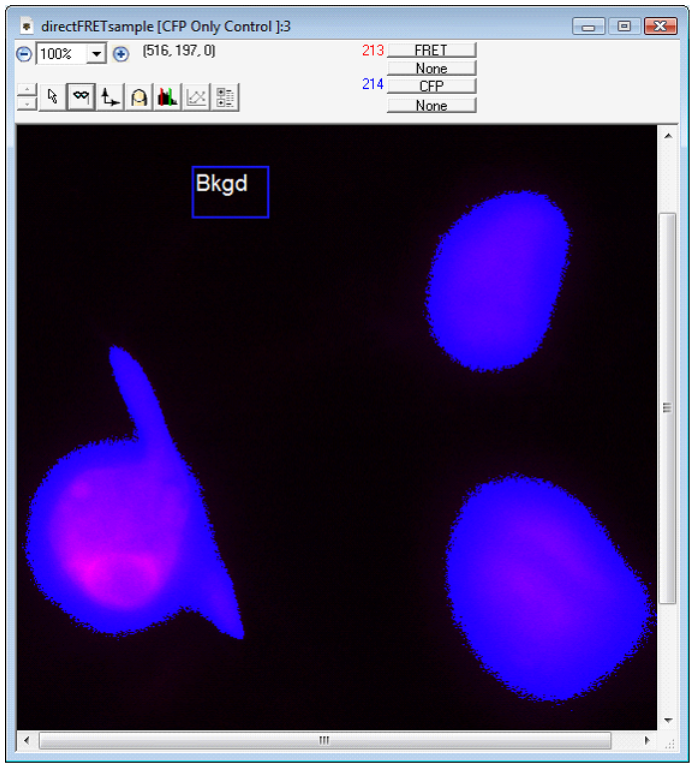
5. Next, you will select the pixels to create the mask. Pixels included in the mask are any pixels with intensity values that fall between the ***Low*** and ***High*** values in the ***Segment Image*** dialog. You can control these thresholds using the red line for low value selection and the green line for high value selection.

6. Move the red bar on the histogram to the right to exclude low intensity value pixels. You should select a significant amount of sample to increase the accuracy of the bleed-through calculation. For this example, a low value near **240** can be used.

7. Click ***OK*** to complete the mask.

8. Now, you will create a background region for background-corrected intensity calculations. Draw a region in an area with no signal using a region tool. The region tools are accessed in the tool menu of an image viewing window or in the Home tab.

9. Select the region by clicking on it, the number will turn white. Then right-click on the region and select ***Set as background***.



10. In the Modules tab, select ***FRET > Compute FRET Bleedthrough***. For more information on this dialog, please review FRET Bleedthrough Computation window.

11. Click on ***Compute Bleedthrough***. A value will populate the appropriate field in ***Filter Bleedthrough Factors***.

> **Note:** If the channels information is not appropriate, you may ***Redefine*** the channels as necessary. This may be useful when you are using images that were not captured using SlideBook.

12. Select ***Make Default*** to use this bleedthrough coefficient for subsequent calculations and then click ***OK*** to close the dialog box.

13. Open a main view **YFP Only Control**. Create a mask and select a background region as you did for the donor sample in steps 3 -6 above. A low value of about **300** is appropriate.

> **Note:** Be sure to select the YFP channel from the ***Channel*** menu in the ***Segment Image*** dialog.

14. Set a background region (as you did in step 9 above).

15. In the Modules tab, select ***FRET > Compute FRET Bleedthrough***.

16. Click on ***Compute Bleedthrough*** to compute the direct excitation of acceptor.

17. Select ***Make Default*** to retain the bleedthrough values you have generated.

18. Click on ***OK*** to close the dialog. Close both control images.

Using FRET Settings

Now, you will determine FRET settings and generate a corrected FRET, or FRETc, channel for viewing your experimental sample images. FRET settings will also be used to select the type of FRET calculation you wish to perform.

1. Open a ***Main View*** of the **Dual Label** image in **directFRETsample.sld**.

2. Select a background region as you did when calculating bleedthrough coefficients.

3. In the Modules tab, select ***FRET > FRET Settings***, the FRET Settings dialog box will appear.

4. Set ***Filter Bleedthrough Factors*** in the upper right corner of the dialog. Click ***Use Defaults*** to use default bleedthrough factors calculated previously. Alternately, you may manually type a value into these fields.

5. Select the ***FRET Paradigm*** from the lower left quadrant of the dialog. For this example, we will use ***Three Channel Correction***. Select the ***Three Channel Correction*** radio button. From the drop down menu below, choose the FRET calculation method you would like to utilize. For this example, we will select ***Fc/Donor*** to normalize the corrected FRET channel with the donor intensity.

6. Next, we will adjust the settings for ***FRET Channel Display***. You may wish to adjust these settings after you perform the FRET calculation and examine your data. For the sample data set, enter **50**, **0.8**, and **1.3** in the ***Signal Threshold***, ***Display Low*** and ***Display High*** edit fields, respectively.

   Signal Threshold – the intensity above which FRET values will be calculated. This threshold is background subtracted. Therefore, if you select a value of 50, any pixel that is above 50 after background subtraction will be used for calculating FRET.

   Display Low – the value that corresponds to black in RGB display or blue in pseudocolor display. See Viewing FRET Data for further explanation of pseudocolor display.

   Display High – the value that corresponds to saturated color in RGB display or red in pseudocolor display.

7. Check the box to ***Apply to all images with the same channels***. Checking this box will calculate FRET for all images in the slide that utilize the ***Participating Channels*** listed in the upper left quadrant of this dialog. In this example, FRET will be calculated for the **Mutant** image as well as the **Dual Label** image.

   Note: While FRET will be calculated for all images in the slide automatically, you will still need to set background regions individually for accurate results. Background regions can be defined before or after completing ***FRET Settings***.

8. Click ***OK*** to calculate FRET and close the dialog box.

Viewing FRET Data

It is very easy to view your three channel corrected FRET data with SlideBook's pseudocolored ***Main View***.

1. Open a ***Main View*** of the **Dual Label** image in **directFRETsample.sld**.

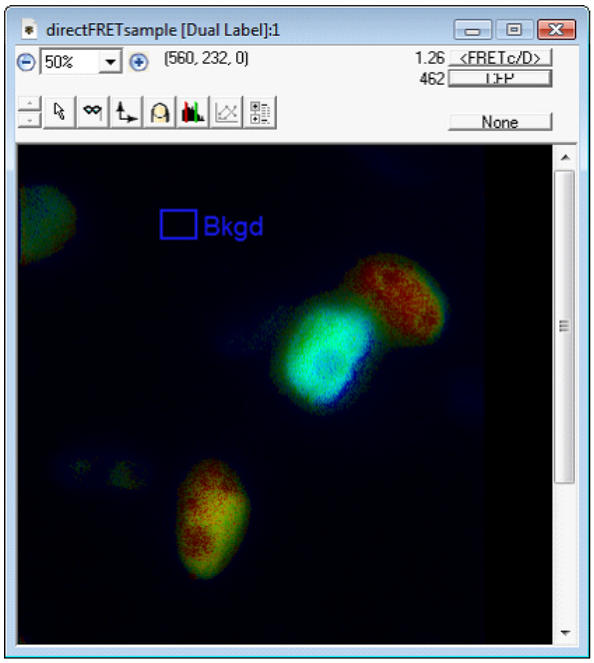
2. In the Home tab, View group, select ***Mode >*** ***Pseudocolor***.

3. Two channel menus will be available. The first channel will be displayed as a pseudocolored image ranging from saturated red to saturated blue, known as ***Pseudocolor (Color)***.

4. Select **<FRETc/D>** from the Hue (top) drop-down channel menu.

5. The second channel menu will act to gate the pseudocolor image based on intensity. This second channel is called ***Pseudocolor (intensity)***. Thus, an area with a high pixel intensity for the first channel and a low pixel intensity on the second channel will appear as dim red. An area with a low pixel intensity for the first channel and a high pixel intensity on the second channel will appear as bright blue. This helps distinguish areas with high intensity from background.

6. Select **CFP** (the donor) from the second channel drop-down menu. Your image will appear similar to the following.



7. Repeat steps 1-4 for **Mutant**. In pseudocolor, red denotes high ratios and blue denotes low ratios. Therefore, red regions have higher normalized FRET relative to blue regions.

8. Compare the two images again. Decide which sample is a positive FRET control and which is a negative FRET control.

Obtaining FRET Statistics

You can now generate FRET information using masks and statistics.

1. Open a ***Main View*** of your sample image.

2. Go to **> *Create Segment Mask*** and generate a mask on the regions you wish to measure FRET (review Mask Generation for detailed information on masking and masking functions). Alternatively, you may generate a mask manually or convert regions of interest to masks.

3. In the Analyze tab, Evaluate group, select ***Mask Statistics***. Select the mask you have just created from the ***Primary Mask*** drop-down menu, and click on the "**+**" next to ***Cross Channel*** to expand the selection category. For more information, please review the Mask Statistics window.

4. Select the Three Channel FRET statistic that you wish to calculate. You may eliminate objects below a certain size threshold by selecting the ***Remove objects smaller than*** checkbox and entering in a value. Available options include:

   Three Channel FRET – FRETc for three channel corrected direct FRET

   Three Channel FRET (Donor Norm) – FRETc normalized by the donor channel for three channel corrected direct FRET

   Three Channel FRET (Acceptor Norm) – FRETc normalized by the acceptor channel for three channel corrected direct FRET

5. Select how you would like to handle the data from the options below:

   Display – displays the data in an interactive table where clicking on an object will highlight the object in the image

   Export – exports the data as a tab-delimited **.txt** file

   Export to Excel – opens the data as a spreadsheet in Microsoft Excel where you can edit and save it as desired

   Cancel – closes the ***Mask Statistics*** dialog and does nothing

Two Channel Corrected FRET

Two channel corrected FRET is a subclass of direct FRET or sensitized emission FRET. This method is acceptable when donor and acceptor are present in a fixed, stoichiometric ratio. When performing two channel FRET, you are essentially taking the ratio of the transfer channel over the donor. In this chapter, we will discuss the entire experimental process for imaging two channel corrected FRET from image capture through analysis and bleedthrough calculations. For more information on the calculations used in this technique, please refer to Appendix B: Direct FRET Calculations.

Note: Before you proceed, it is important to have channels configured as described in FRET System Configuration.

Capturing Two Channel FRET Images

In order to perform direct FRET, you must be able to calculate the bleedthrough coefficient, or cross-talk, for your particular filter sets. To calculate the bleedthrough coefficient, you must image a control sample of the donor signal where no FRET is occurring.

In this section, we will discuss using the Focus Window and Capture Window in SlideBook. If you are a new user and are unfamiliar with these controls, please review the user interface pages before continuing below.

Capturing Control Images

Control images should be acquired for each FRET experiment design. When you change a step in your experiment design, updated FRET control images should be acquired.

1. Mount the donor sample on the stage and open the ***Focus Controls*** in SlideBook.

2. Select the donor filter set (for CFP/YFP FRET, the donor is the CFP channel), and bring the sample into focus.

3. Close the fluorescent shutter and then close the ***Focus Controls***.

4. Open the ***Capture*** window.

5. Set the donor exposure time by selecting the appropriate filter checkbox and then pressing ***Test***. Adjust the exposure time as desired or use the ***Adjust Exposure*** buttons to aid in setting the appropriate exposure time. For additional information on adjusting exposure duration, including setting regions for capture and utilizing binning, refer to the Capture Controls window.

6. Set the FRET transfer exposure time by selecting the FRET filter checkbox and manually entering the same ***Expose duration*** used step 5. It is important that the exposure times of both filters are equal to each other.

7. In the ***Image Information*** field (in the lower right corner of the ***Capture*** dialog), type a ***Name*** to name the image. For example, you may want to name this image **Donor Control**.

8. Click ***OK*** to capture your image.

Imaging the Experimental Sample

Now bring your experimental sample into view and focus using the ***Focus Controls*** . Use the FRET transfer filter when focusing your sample. Then, image your sample(s), capturing the donor, acceptor, and FRET transfer channels. Make sure that both exposure times are equal to each other. If you are performing a live FRET experiment, you may wish to take advantage of the live graphing features that are discussed later in Live Display.

1. Mount the experimental sample on the stage and open the ***Focus Controls*** in SlideBook.

2. Select the FRET transfer filter and bring the sample into focus.

3. Close the fluorescent shutter and then close the ***Focus Controls***.

4. Open the ***Capture*** window.

5. Set the exposure time for the donor, acceptor, and FRET transfer channels. You may find the ***Adjust Exposure*** buttons useful in setting the appropriate exposure time.

   Note: The experiment requires the same exposure time for both channels so be sure to use the brightest channel to set exposure times.

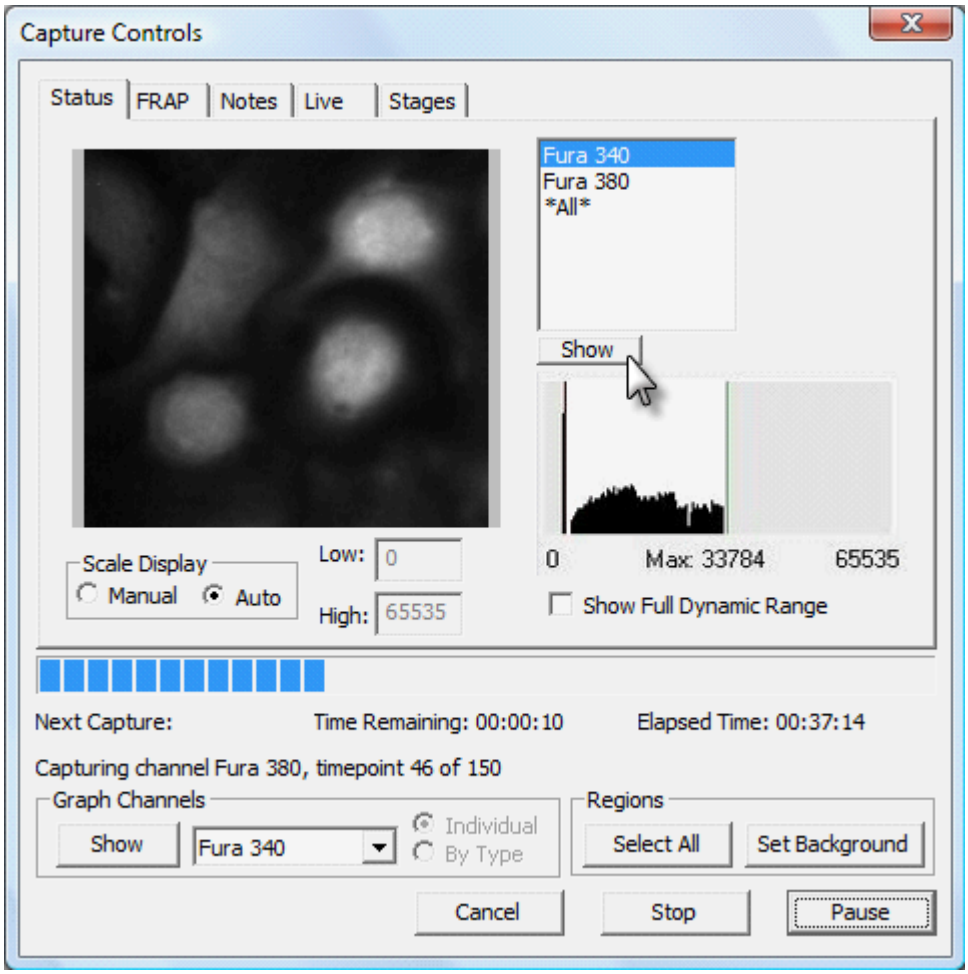
6. Set up timelapse capture if desired.

7. Click ***OK*** to capture the experiment.

Live Display During Two Channel FRET

SlideBook will display a live composite image of the donor and FRET channels for each time point. Additionally, you may wish to graph the intensity of regions of interest, adjust the focus of the microscope, or create event markers.

During capture, the live image window will display a composite image as individual channels are captured. To display an individual channel, go to the ***Status*** tab of the ***Capture Controls*** dialog. In the upper right portion, you will find a list of channels. Simply select an individual channel to display and click ***Show***. A live window displaying a monochrome image of the selected channel will open. In this manner, it is possible to watch a live composite display as well as displays of the individual channels.



Other available functions during live capture include:

- Selecting Regions of Interest
- Graph Display
- Focusing During Capture
- Creating Event Markers (Notes)
- Exposure Time Tips

Post Capture Analysis

After the images are captured, you may perform background subtraction, calculate bleedthrough coefficients, and generate the two channel corrected FRET signal. It may be useful to follow the Post Capture Analysis Tutorial for three channel corrected FRET using the sample data provided. The only differences between this tutorial and the analysis process for Two Channel Corrected FRET are as follows:

1. In Performing Bleedthrough Calculations, you will only calculate bleedthrough for CFP. Omit steps 13-17.
2. In Using FRET Settings step 5, you will select ***Two Channel Correction*** as the ***FRET Paradigm***.
3. In Viewing FRET Data, the image will be automatically be displayed in pseudocolor if you defined channels as advised in FRET System Configuration.
4. In Obtaining FRET Statistics step 4, you will select ***Two Channel FRET*** as the statistic.

Acceptor Photobleaching FRET

In order to perform acceptor photobleaching FRET, you must define five channels: “donor prebleach”, “acceptor prebleach”, “photobleach”, “donor postbleach”, and “acceptor postbleach”. Full details on configuring your system can be found in FRET System Configuration. For more information on the calculations used in this technique, please refer to Appendix C: Photobleaching FRET Calculations.

Capturing Images

For acceptor photobleaching FRET, there are two main steps. First, you will determine bleach time and then you will capture the experimental images. Before you begin this section, you should be familiar with using the Focus Controls Window and the Capture Window.

Determining Photobleach Time

1. Mount the sample on the stage and bring it into view and focus using the ***Focus Controls***.

2. Use the microscope field diaphragm to create a small area of illumination on the sample. You will be using this small area to determine an appropriate photobleaching exposure time.

3. Select ***Capture***. The ***Capture*** dialog box will appear.

4. Now, you must determine an appropriate exposure time for photobleaching. Select the **Photobleach** channel and enter an approximate time for photobleaching.

5. Select ***Test*** to generate a test exposure. Increase the illumination area (by opening the field diaphragm) and observe the amount of photobleaching that has occurred via the oculars. Repeat the testing procedure on fresh areas of sample until an appropriate photobleaching exposure time is determined.

Capturing Experimental Images

1. Use the ***Focus Controls*** to move to an area of interest and, if desired, open the field diaphragm so that entire sample area is illuminated.

2. In the ***Capture*** dialog box, determine exposure times for the donor prebleach and acceptor prebleach channels by generating test exposures.

> **Note:** The exposure time should be the same for the donor and acceptor

3. Select the following channels for capture:

> **Donor pre** – using the exposure time determined above
> **Acceptor pre** – using the exposure time determined above
> **Photobleach** – using the exposure time determined in the previous section
> **Donor post** – using the Donor pre exposure time
> **Acceptor post** – using the Acceptor pre exposure time

4. Set camera parameters and other settings and select ***OK***.

5. Five channels will be captured in succession. It is important that the channels are captured in the order shown in tstep 3 above. You may alter the channel order by selecting a channel and using the ***Move Up*** or ***Move Down*** buttons located below the channel listbox.

Performing Post-Capture Analysis

1. Open a ***Main View*** of the image you wish to analyze.

2. Use the region tools to mark a background area with no signal.

3. Select the region by clicking on it (the number will turn white), then right-click on the region and select ***Set as background***.

4. In the Modules tab, select ***FRET > FRET Settings***.

5. Select ***Acceptor Photobleaching*** and then ***OK***.

6. Return to the ***Main View*** of your image and select <**pbFRET**> from any of the channel menus to display and view your acceptor photobleaching FRET values.

7. To alter the display values in your image, alter the edit fields in the ***FRET Channel Display*** section of the ***FRET Settings*** dialog box.

> ***Signal Threshold*** – the intensity above which pbFRET values will be calculated. This threshold is background subtracted. Therefore, if you select a value of 50, any pixel that is above 50 after background subtraction will be used for calculating pbFRET.
>
> ***Display Low*** – the pbFRET value that corresponds to black in RGB display or blue in pseudocolor display
>
> ***Display High*** – the pbFRET value that corresponds to saturated color in RGB display or red in pseudocolor display.

Generating Photobleaching FRET Statistics

To obtain Photobleaching FRET statistics:

1. Create a mask using either segmentation or manual techniques. Please refer to the Mask Generation for information on creating masks.

2. In the Analyze tab, Evaluate group, select ***Mask Statistics*** and select ***Photobleaching FRET*** from ***Cross-Channel*** statistics.

3. This will generate statistics on a region basis using the following formula where the intensities in parentheses are first averaged for each region before performing division.

$$PB\ FRET = [(Donor\ Post) - (Donor\ Pre)] / (Donor\ Post)$$

Note: Confirm that the appropriate channels are being used for the calculation by checking the ***FRET Settings*** dialog box (in the Modules tab, select ***FRET > FRET Settings***).

4. Select how you would like to handle the data from the options below:

Display – displays the data in an interactive table where clicking on an object will highlight the object in the image

Export – exports the data as a tab-delimited **.txt** file

Export to Excel – opens the data as a spreadsheet in Microsoft Excel where you can edit and save it as desired

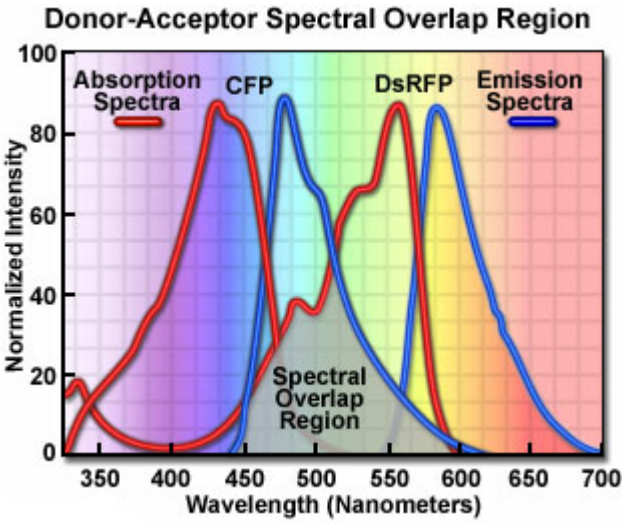
Cancel – closes the ***Mask Statistics*** dialog and does nothing

Anisotropy FRET

Coming soon!

Appendix A: FRET Introduction and Theory

Fluorescence Resonance Energy Transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules where the excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon. More specifically, FRET is the radiationless transmission of energy from its site of absorption to the site of its utilization by resonance between chromophores (figure below, courtesy of Molecular Expressions Optical Microscopy Primer).



There are four main types of FRET, all of which SlideBook™ currently supports.

- Acceptor Photobleaching Recovery
- Direct FRET/Sensitized Emission FRET
- Fluorescence Lifetime Measuring
- Anisotropy FRET

As stated above, FRET involves the non-radiative transfer of energy from a fluorophore in an excited state to a nearby acceptor fluorophore. In order for this transfer to take place, the donor and acceptor molecules must be within 10-50Å (Förster distance) and the emission spectra of the donor fluorophore must overlap the excitation spectra of the acceptor fluorophore. In principle, this overlap will allow the exchange of energy through a non-radiative dipole-dipole transfer. The energy transfer manifests itself by quenching the donor fluorescence in the presence of the acceptor and increased (sensitized) emission of the acceptor fluorescence. The figure above illustrates the spectral overlap of the corresponding fluorophores.

The distance dependent characteristics of FRET make it an excellent tool for investigating a variety of biological phenomena that produce changes in molecular proximity. Further, this distance dependency also allows FRET to distinguish between two fluorophores that are less than 200 nanometers apart.

Acceptor Photobleaching Recovery

The underlying principle of Acceptor Photobleaching Recovery takes advantage of statistical probabilities. Whenever a molecule is excited by a photon, there is a statistical probability of either of three things happening. These three things are:

A. The specimen under questions fluoresces and emits the exciter energy as the electrons drop back to ground state.
B. The specimen becomes photobleached
C. The specimen FRETs – or rather it loses (in the case of the donor) energy through a dipole transfer.

Knowing these three outcomes, it is possible to calculate FRET through a subtractive process. Prior to photobleaching, the defining equation for the donor is as follows:

$$A + B + C = 100\% \text{ before photobleaching} \qquad (1)$$

Once the acceptor is photobleached, FRET cannot occur and C is set to 0. The equation is then reduced to:

$$A + B = 100\% \text{after photobleaching} \qquad (2)$$

Subtracting equation 2 from equation 1, and assuming the probability of spontaneous photobleaching of the donor remains the same, yields:

$$A + C \text{ before photobleaching} = A \text{ after photobleaching} \qquad (3)$$

Thus, after photobleaching, the amount of A is increased by C, the amount of FRET occurring prior to bleaching. The process of photobleaching, a destructive process, eliminates the possibility of FRET occurring in the acceptor. Therefore, if the donor cannot FRET to the acceptor then the acceptor emission is increased.

In practice, there are three steps to calculating FRET using Acceptor Photobleaching Recovery. These steps are

1. Capture an image of the donor
2. Bleach the acceptor
3. Capture an image of the donor

After taking these two images, FRET can be calculated by subtracting image one from image three.

Direct FRET/Sensitized Emission FRET

Direct FRET/Sensitized Emission FRET is a faster, and less intrusive method of FRET imaging than Acceptor Photobleaching FRET, at the cost of computational accuracy. As the name of the technique indicates, FRET is measured directly by imaging the sample through donor excitation and acceptor emission filters, or FRET filters. In theory, this would be the only image necessary if the donor excitation and acceptor emission spectra were perfectly separated. In practice, the spectra do overlap, and corrections for spectral bleedthrough must be performed. There are two techniques for calculating Direct FRET:

Three-Channel Corrected FRET - separately corrects for donor bleedthrough and direct excitation of the acceptor, appropriate when donor and acceptor are not present in a fixed ratio

Two-Channel Corrected FRET – applicable when donor and acceptor are present in a fixed, stoichiometric ratio (e.g. Cameleon imaging)

Three-Channel Corrected FRET Method

For Three-Channel Correct FRET, three images are necessary:

1. Control containing donor only imaged with donor and FRET filters.
2. Control containing acceptor only imaged with acceptor and FRET filters.
3. Experimental sample containing both donor and acceptor imaged with donor, acceptor, and FRET filters

For demonstration purposes, let us assume CFP is the donor and YFP is the acceptor. When CFP excites, a large portion will emit normally (corresponding to the CFP emission spectrum). If the CFP FRETs, a radiationless energy transfer will occur that causes YFP to emit. The CFP emission spectrum overlaps with the YFP emission spectrum, therefore some of the normal CFP emission will be visible through the YFP emission filter. This cross-talk confounds the signal due to FRET and must be corrected. The first image, the CFP control, is used to calculate the spectral bleedthrough of CFP emission visible through FRET filter set.

Additional corrections must be made for non-FRET signal due to the overlap of CFP and YFP excitation spectra.. This overlap causes normal YFP fluorescence to be visible through the FRET filter set. The second image, the YFP control, is used to calculate the spectral bleedthrough of normal YFP emission through the FRET filter set..

The third image is the raw FRET image. Corrected FRET, or FRETc, is calculated with consideration of all three images. The following equation is used to subtract the non-FRET portions from the raw FRET signal:

$$FRETc = FRETraw - Df/Dd[CFP] - Df/Da[YFP] \qquad (4)$$

where FRETraw, [CFP], and [YFP] are the signals visualized through the FRET, CFP, and YFP filter sets, respectively. The constants Df/Dd and Df/Da are the transmissivity, or bleed-through, constants describing donor emission visible in FRET channel and direct excitation of acceptor, respectively [Sorkin, et al.]. These constants are calculated using images 1 and 2. Failure to account for these intensities in raw FRET will result in erroneous data.

Two-Channel Corrected FRET Method

For Two-Channel Corrected FRET, a single two-channel image of the sample is required. The two channels that must be captured are the donor (or acceptor) channel and the FRET channel.

Continuing with the CFP/YFP example in Three-Channel Corrected FRET, if the concentration of CFP equals that of YFP, equation (4) reduces to:

$$FRETc = FRETraw + (Df/Da - Df/Dd)[CFP] \qquad (5)$$

Dividing through by [CFP] yields:

$$FRETc/[CFP] = FRETraw/[CFP] + (Df/Da - Df/Dd) \qquad (6)$$

Thus, the normalized, uncorrected values will differ from the normalized corrected values by a constant offset. Further, for two-channel FRET, if the donor and acceptor bleedthrough factors (Df/Dd and Df/Da) are unknown, changes in normalized, corrected FRET, FRETcN, can still be measured:

$$DFRETcN = D\{FRETc/[CFP]\} = D\{FRETraw/[CFP]\} \qquad (7)$$

Therefore, simply measuring the ratio of the raw FRET channel to the donor channel allows for comparison of two-channel corrected FRET values in various regions of the experimental sample.

Appendix B: Direct FRET Calculations

Terms

l_D	FRET Donor emission wavelength
l_A	FRET Acceptor emission wavelength
l_F	FRET Transfer emission wavelength
F_d/D_d	Donor emission bleedthrough
F_a/A_a	Direct excitation of acceptor
$I_{lD}(t; x, y)$	Intensity of image measured through FRET Donor filter set of time point t at position x,y
$I_{lA}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
$I_{lF}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
$ROI(n)$	Region of interest n: a subset of all possible x, y positions
$\lvert ROI_{(n)}\rvert$	The number of positions in the region of interest n

Background

The background is computed for each time point for FRET donor, acceptor, and transfer images by averaging intensities over the background region of interest ROI_b:

$$b_{lD}(t) = 1/|ROI_b|\ S_{ROIb}\ I_{lD}(t; x, y)$$
$$b_{lA}(t) = 1/|ROI_b|\ S_{ROIb}\ I_{lA}(t; x, y)$$
$$b_{lF}(t) = 1/|ROI_b|\ S_{ROIb}\ I_{lF}(t; x, y)$$

Three Channel Corrected FRET - FRETc Computations

$FRET_C$ (n; t) is computed for ROI(n; t) by first computing average intensities for the donor, acceptor, and transfer channels:

$$i(n; t)_{lD}(n) = 1/|ROI_{lD}(n; t)|\ S_{ROIlD}(n; t)\ I_{lD}(t; x, y)$$
$$i(n; t)_{lA}(n) = 1/|ROI_{lA}(n; t)|\ S_{ROIl2}(n; t)\ I_{lA}(t; x, y)$$
$$i(n; t)_{lF}(n) = 1/|ROI_{lF}(n; t)|\ S_{ROIlF}(n; t)\ I_{lF}(t; x, y)$$

and then computing the background corrected FRETc:

$$FRETc(n; t) = (i(n; t)_{lF}(n) - b_{lF}(t)) - F_d/D_d*(i(n;t)_{lD}(n) - b_{lD}(t)) - F_a/A_a*(i(n;t)_{lA}(n) - b_{lA}(t))$$

Two Channel Corrected FRET - FRETc Computations

$FRET_C$ (n; t) is computed for ROI(n; t) by first computing average intensities for the donor and transfer channels:

$$i(n; t)_{lD}(n) = 1/|ROI_{lD}(n; t)|\ S_{ROIlD}(n; t)\ I_{lD}(t; x, y)$$
$$i(n; t)_{lF}(n) = 1/|ROI_{lF}(n; t)|\ S_{ROIlF}(n; t)\ I_{lF}(t; x, y)$$

and then computing the background corrected FRETc:

$$FRETc(n; t) = (i(n; t)_{lF}(n) - b_{lF}(t)) - F_d/D_d*(i(n;t)_{lD}(n) - b_{lD}(t))$$

Appendix C: Photobleaching FRET Calculations

Terms

l_{pre}	Donor emission wavelength measured before photobleaching
l_{post}	Donor emission wavelength measured after photobleaching
$I_{lpre}(t; x, y)$	Intensity of image measured through FRET Donor filter set of time point t at position x,y
$I_{lpost}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
$ROI(n)$	Region of interest n: a subset of all possible x, y positions
$\|ROI_{(n)}\|$	The number of positions in the region of interest n

Background

The background is computed for prebleach and postbleach images by averaging intensities over the background region of interest ROI_b:

$$b_{lpre} = 1/|ROI_b|\ S_{ROIb}\ I_{lD}(x, y)$$
$$b_{lpost} = 1/|ROI_b|\ S_{ROIb}\ I_{lA}(x, y)$$

Photobleach FRET - pbFRET Computation

pbFRET (n) is computed for ROI(n) by first computing average intensities for the prebleach and postbleach channels:

$$i(n; t)_{lpre}(n) = 1/|ROI_{lpre}(n; t)|\ S_{ROIlpre}(n; t)\ I_{lpre}(t; x, y)$$
$$i(n; t)_{lpost}(n) = 1/|ROI_{lpost}(n; t)|\ S_{ROIl2}(n; t)\ I_{lpost}(t; x, y)$$

and then computing the background corrected pbFRET:

$$pbFRET(n) = [(i(n)_{lpost}(n) - b_{lpre}) - (i(n)_{lpre}(n) - b_{lpost}(t))\ ] / (i(n)_{lpost}(n) - b_{lpre})$$

Photomanipulation Module

Intelligent Imaging Innovations (3i) designs and manufactures a family of photomanipulation hardware that includes **Vector™** and **Ablate!™**. These products are precisely controlled using the integrated photomanipulation software module in SlideBook. This module streamlines everything from calibration to advanced multi-region, complex event photomanipulation experiments and analysis. In addition to our products, several third party devices such as the Andor Mosaic and Micropoint systems can be controlled using the SlideBook Photomanipulation Module.

Vector™

The majority of photomanipulation in SlideBook is done with Vector™. Vector is a diffraction limited high-speed x,y scanner that enables a number of investigative methods, including the following techniques:

- FRAP (Fluorescence Recovery After Photobleaching)
- Photoactivation
- Photostimulation/Uncaging
- Photoconversion
- Photoablation

Ablate!™

For photoablation techniques, Ablate!™ may be directly coupled to either the microscope for single point ablation or to Vector for motorized control of the laser beam. Ablate! is a pulsed laser system (532nm) used for the following photoablation techniques:

- Tissue damage
- Cellular damage
- Organelle ablation
- DNA damage
- Vascular tissue damage for laser injury model

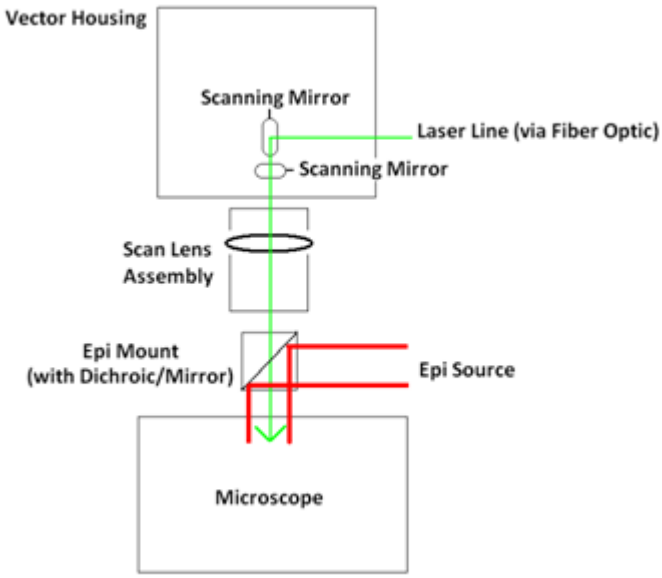
What is Vector

Vector is 3i's patented high-speed laser scan head that utilizes galvanometers to move a laser beam very quickly across a field of view. SlideBook software provides seamless control and calibration of the galvanometers for almost effortless operation by the end-user. Vector can be integrated with LaserStack, the 3i laser launch, as well as a fiber switcher; the end result is that a user can quickly switch from full field confocal, TIRF, or widefield imaging to create a dynamic photon event on a region then return to the original imaging mode within milliseconds.

The most prolific application of photomanipulation techniques is known as FRAP (Fluorescence Recovery After Photobleaching). Other common applications include PA (PhotoActivation of fluorescent proteins) and FLIP (Fluorescence Loss In Photobleaching). Regardless of the specific technique, Vector provides users with a toolbox for applying a laser beam to a specific region (or regions) of an imaging field to create some type of photomanipulation or dynamic photon event.

How does Vector work

The basic components of Vector (mounted at the epi port) are displayed in the diagram below.



A laser line is delivered to Vector's kinematic input via fiber optic or direct coupling. The beam is incident upon the first of two scanning mirrors, which are steered by two analog voltages (x- and y-axis) with 16-bit precision within SlideBook. The second scanning mirror reflects the beam out of the Vector housing, where it is collected and translated by the scan lens (f=35 mm). The scan lens relays the beam into the light train of the microscope, where it is directed to the back aperture of the objective via dichroic/mirror.

Based on the analog voltage values provided by SlideBook, the scanning mirrors can direct the beam to photomanipulate nearly any requested region. The photomanipulation event may include photobleaching, photoactivation, photostimulation/uncaging or photoablation, depending on the sample and experiment set up. Once the area(s) for the event is defined, the next step is to decide how the data should be acquired. Sometimes, a user may prefer to create an event at a single point repeatedly during an experiment while other times a user may want to create an event "on the fly" during acquisition. Once the data is captured, it can be viewed and analyzed in SlideBook.

Vector Specifications

Input Voltage	110 VAC 60 Hz	
	220 VAC 50 Hz	
Optical Transmission Efficiency	400 nm ≤ λ ≤ 660 nm	80%
	660 nm < λ	Not measured
Input Control	Analog Voltage	
Operating Temperature	Ambient (20° - 29° C, 68° - 84° F)	

Vector Performance		
Inputs	Range (Volts)	10
	Steps (mV)	10000
	Degrees	15
	Degrees/mV	0.0015
Angular Resolution	Radians	0.261799388
	Radians/mV	0.000026
	Resolution (mRad)	0.026179939
	Resolution (uRad)	26.17993878
Speed	Steering Speed [ms]/mV	4.25E-01
	Analog Voltage Update Rate (DAQ)	1.11111E-06
Resolution @ Detector	theta (rad)	0.000026
	tan(theta)	2.61799E-05
	35tan(theta)	0.000916298
	micron step	0.916297858
	steps / pixel EMCCD	17.4615709
	steps / pixel Sony 285	7.039195767

Wavelength Range	Reflective Efficiency
300 - 600	> 87%
600 - 1050	> 82%
1050 - Infrared	> 91%

What is Ablate

Ablate! is an add-on device to most microscope systems used for target ablation (such as damage) of regions within a specimen. Ablate!™ uses an attenuatable 355nm or 532nm pulse laser (>60μJ pulses at 200Hz) to inflict localized damage to a diffraction-limited spot in intracellular structures for precise laser ablation and laser wounding. Full software control with SlideBook features automated intensity control and allows users to define one or more regions of interest as single diffraction limited spots or as user-defined shapes.

Directed ablation, or motorized control of the laser beams target location is achieved by coupling Ablate! with Vector (see What is Vector). Using the galvanometer controlled mirrors within Vector, the pulsed laser can be targeted to any location or used to fill regions of interest within the specimen limited only by the steering range of Vector.

Ablate: Scientific Background

There are several reasons and theories behind photoablation. Some are wavelength dependent and some are energy dependent. Temperature and various types of force play the greatest role in causing the ablation.

An excellent reference on photoablation in biology follows:

Mechanisms of pulsed laser ablation of biological tissues
Author(s): Vogel, A; Venugopalan, V
Source: Chemical Reviews Volume: 103 Issue: 2 Pages: 577-644 Published: FEB 2003

Ablate: Specifications

Wavelength	355nm or 532nm options
Pulse Energy for 355nm	60 uJ @ 200 Hz
Pulse Energy for 532nm	E/p > 65 uJ @ 1 Hz (single pulse per second) E/p> 60 uJ @ 200Hz (rapid pulsing)
Max Pulse Rate	1kHz
Pulse Width (FWHM)	<3ns
Polarization ratio	100:1
Power stability (6hrs)	<5% RMS (with <3 deg C temperature variation)
Spatial mode	TEM_{00}
Beam Divergence	3.5mrad
Beam diameter	1500um
Power consumption (max)	30W (80W)
Interface	USB, TTL rising edge
Laser Classification	3B
Warm-up time	5 minutes
Operating Temperature	18-38 deg C

Calibrating Vector in SlideBook

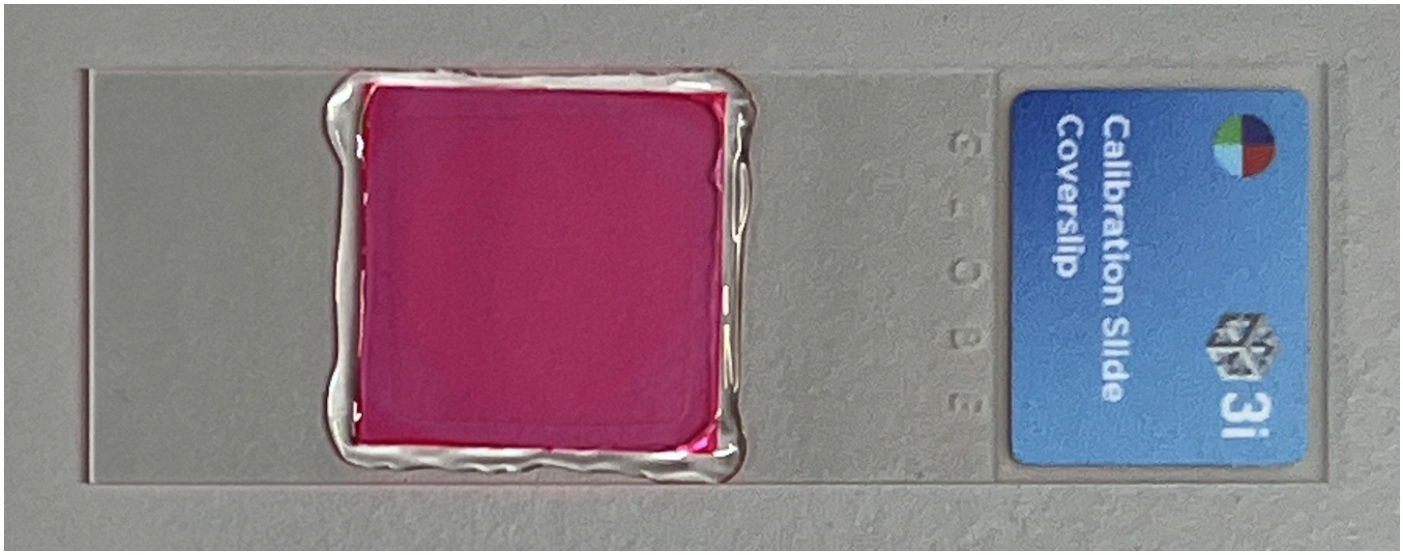
Calibration of Vector is a simple technique performed in SlideBook. This process calibrates laser movement to the spatial coordinates of the CCD. Calibration should be performed for each objective at each auxiliary magnification available and tested during routine maintenance. If you haven't already, first change ***Hardware Properties*** to Enable 4-Point Calibration Technique; otherwise, the older 16-point method will be used.

Once installed and initially calibrated, Vector calibration should not change. It is recommended that calibration be checked regularly or after any type of hardware move or unusual amount of vibration due to construction, earthquake, or other abnormal event.

Preparing for Calibration

Turn on the entire system, including all lasers and light sources, at least 45 minutes prior to beginning the calibration process. This will ensure a stable operating temperature for the lasers and stable room conditions.
Good practice is to run SlideBook as administrator (right click the icon and select 'Run as administrator') from start.

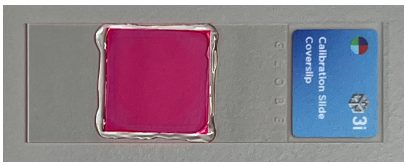
For the calibration process, you will use the Vector Calibration Slide pictured below. If you do not have a Vector Calibration Slide, please contact 3i Support or +1 303.607.9429 ext.2. Alternatively, you can make your own slide by using a standard green Sharpie®. Simply draw directly on the coverslip and mount the coverslip to a slide using your preferred fixing technique.



TUTORIAL: 4-Point Vector Calibration

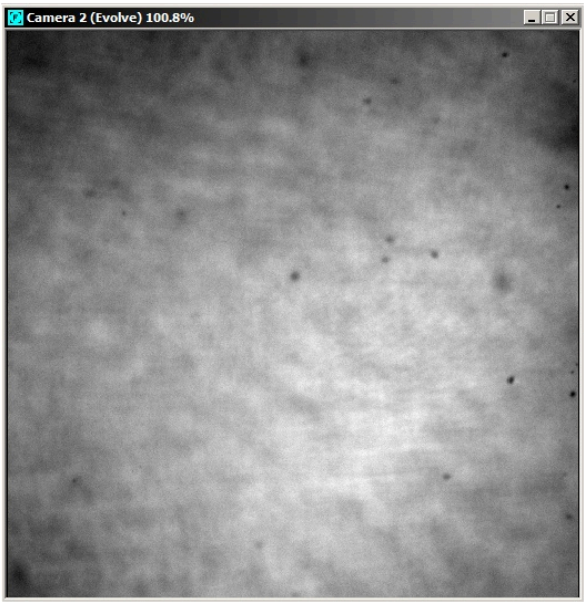
Photomanipulation systems need calibration so that your regions are accurately bleached. The calibration procedure links regions of the FOV with galvanometer positions. Camera alignment, objective, and magnification changers can affect the calibration. The calibration is not affected by excitation wavelength.
This procedure should be used to calibrate Vector when it is directly mounted to the epifluorescent lightpath, with or without a fiber switcher.

1. 3i makes a calibration slide using a fluorescent matrix. It is thin, uniform, and bleaches well in most wavelengths. Alternatively, you can make a calibration slide by marking a coverslip with green sharpie and mounting it on a slide.

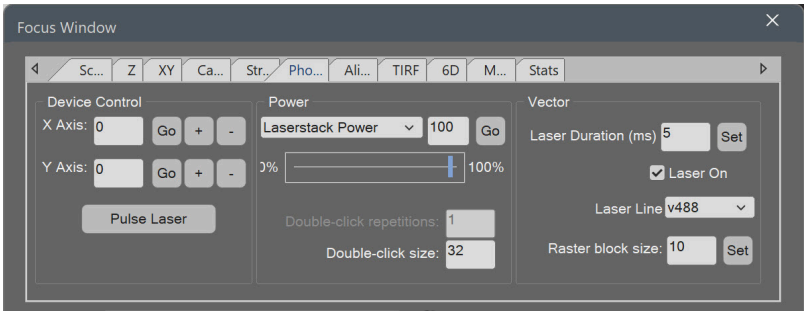


1. To make sure the calibration file is accessible across multiple user accounts on your PC run SlideBook on an administrator account and start it with right click on the icon and select "run as administrator".

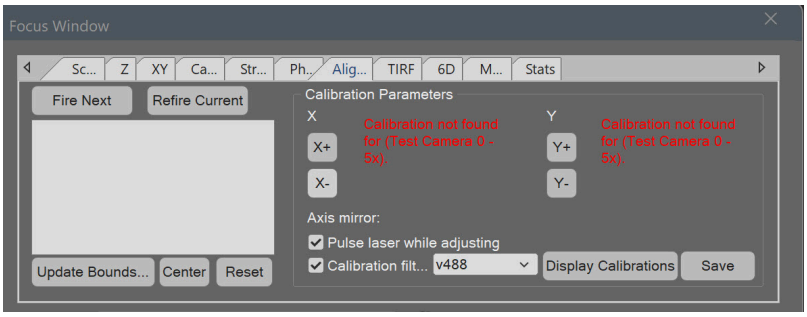
2. Place the Vector Calibration Slide on the microscope. Open the Focus Controls and use a widefield or confocal channel (best GFP/ FITC) to bring the sample into focus at the eyepieces. Focusing on scratches and imperfections in the calibration slide (as shown below) will help with this process. Switch to a widefield or confocal filter set, directing the image over to the camera. Adjust focus as necessary. Click ***Close Fluor*** (or ***Close Alt***).



3. Click the ***Photomanipulation*** tab of the ***Focus window*** dialog, and check the ***Laser On*** checkbox. This will leave the laser running between calibration points. You can start with the default laser power (100%), Laser Duration (5ms) and Raster Block Size (10 px), or adjust these settings as needed. For example, you can decrease the laser power if your calibration spots are very large.



4. Select the ***Align Photomanipulation*** tab. Check ***Pulse Laser*** and ***Calibration Filter***. Select a channel that you want to use for calibration. For example, v488 and v561 work well with the 3i calibration slide.
NOTE: When there is no calibration set for the selected camera and objective combination it will be indicated in red, once a calibration is done the values will be displayed in black. The calibration process will override the existing values.



5. Vector calibration will involve SlideBook moving the illumination spot to the fours corners of the imaging field, which is defined through the values given under ***Update Bounds...***. Contact 3i Support if you have reason to believe those bounds don't fit within the current field of view.

6. Prior to starting calibration, you will need to select the pointer tool from the Home tab.

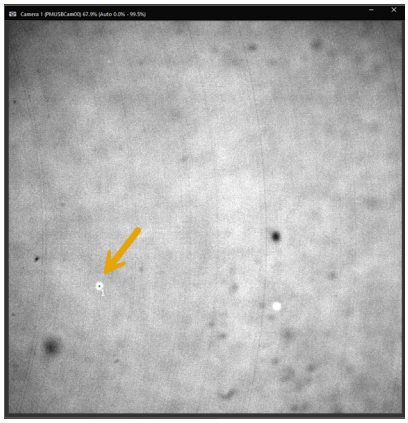
7. Click ***Fire Next*** to begin the calibration process:

a. The Vector galvanometers will move to the first point on the calibration square, a laser spot near a corner of the field should be visible.

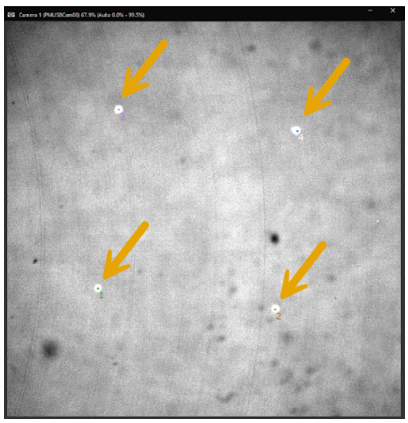
b. ***Double-click*** in the center of the laser spot in the live ***Camera*** window. This will mark it as region 1 and automatically fire the next calibration spot.

c. If you do not see the second spot, then you can click ***Refire Current*** to try bleaching this spot again.

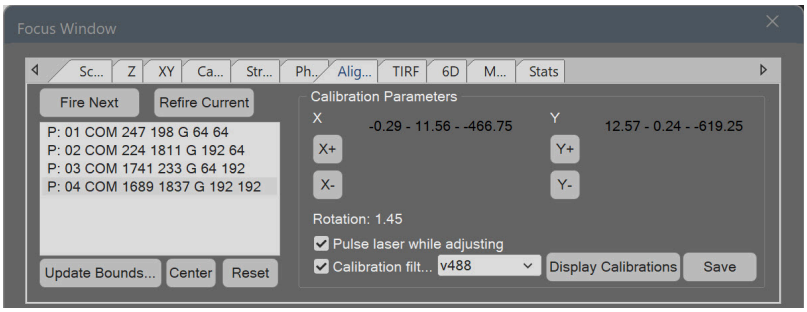
d. ***Double-click*** the second spot.



e. Repeat until all 4 calibration spots have been marked.



NOTE: that the calibration log is updated with Vector X and Y values.

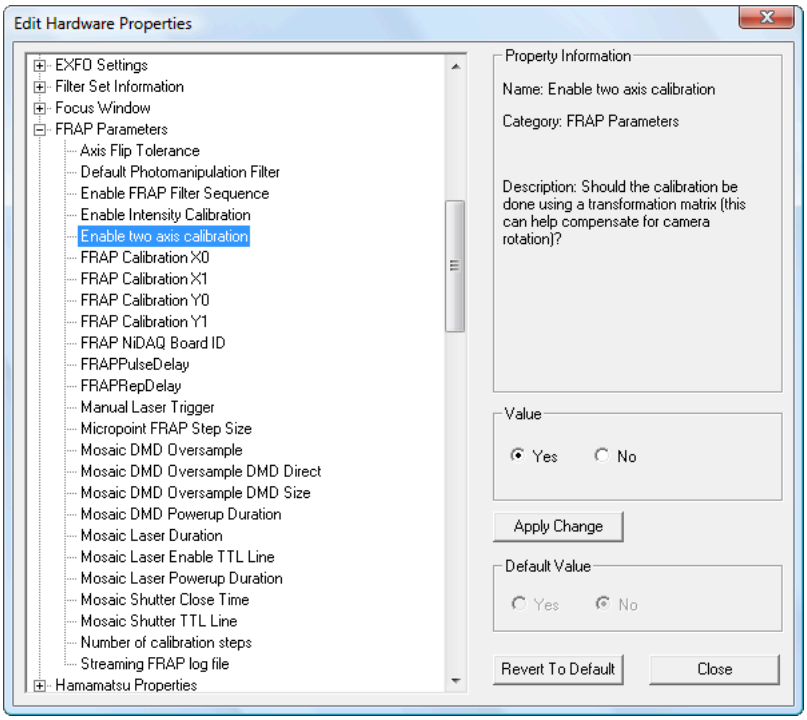


8. Click ***Save*** below the calculated equations relating Vector position values to XY coordinates.

Enable 4-Point Calibration Technique

A new calibration routine has been developed to save you time and improve accuracy of photomanipulation device calibration. To use this routine instead of the older 16-point technique, it must first be enabled in ***Hardware Properties***.

1. Go to ***Administration tab >*** ***Hardware Properties***.
2. Navigate to ***FRAP Parameters > Enable two axis calibration***.
3. Set property to ***Yes***, click ***Apply Change***.
4. Close the dialog and restart SlideBook when prompted.

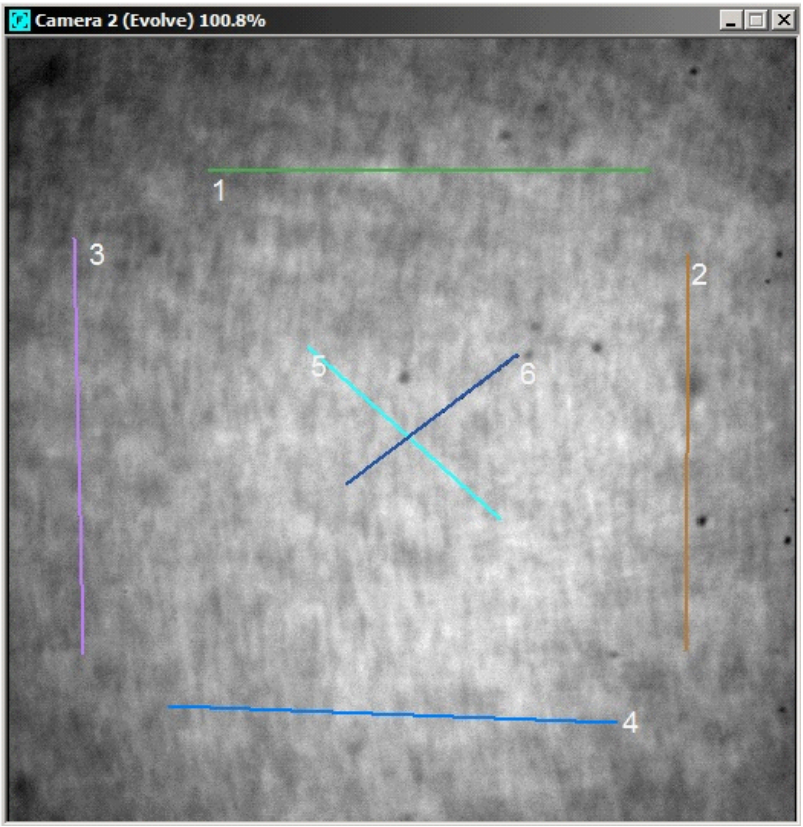


Vector Calibration Evaluation

1. Place the Vector Calibration Slide on the microscope. Open the ***Focus Controls*** and use a widefield or confocal green channel such as **GFP** or **c488** to bring the sample into focus at the eyepieces. Focusing on scratches and imperfections in the calibration slide (as shown below) will help with this process. Switch to a widefield filter set, direct the image over to the camera. Adjust focus as necessary.

2. Select the ***Line*** tool from the ***Region*** group.

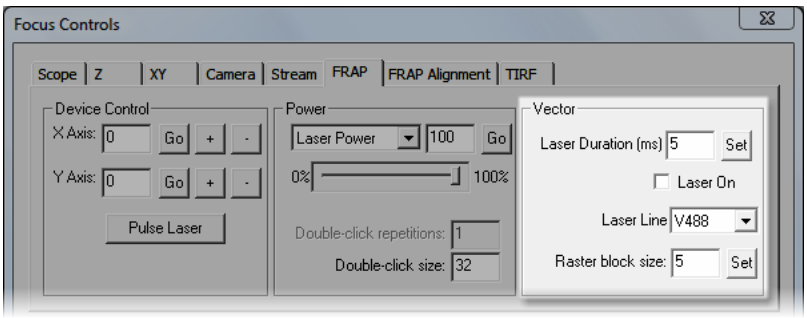
3. Draw the following regions in the live ***Camera*** window:
   - Horizontal line across the top of the field of view
   - Horizontal line across the bottom of the field of view
   - Vertical line down the left side of the field of view
   - Vertical line down the right side of the field of view
   - Two small lines forming an "X" in the center of the field of view



3. In the ***Focus Controls*** click the ***FRAP*** tab, and configure the parameters for evaluation:

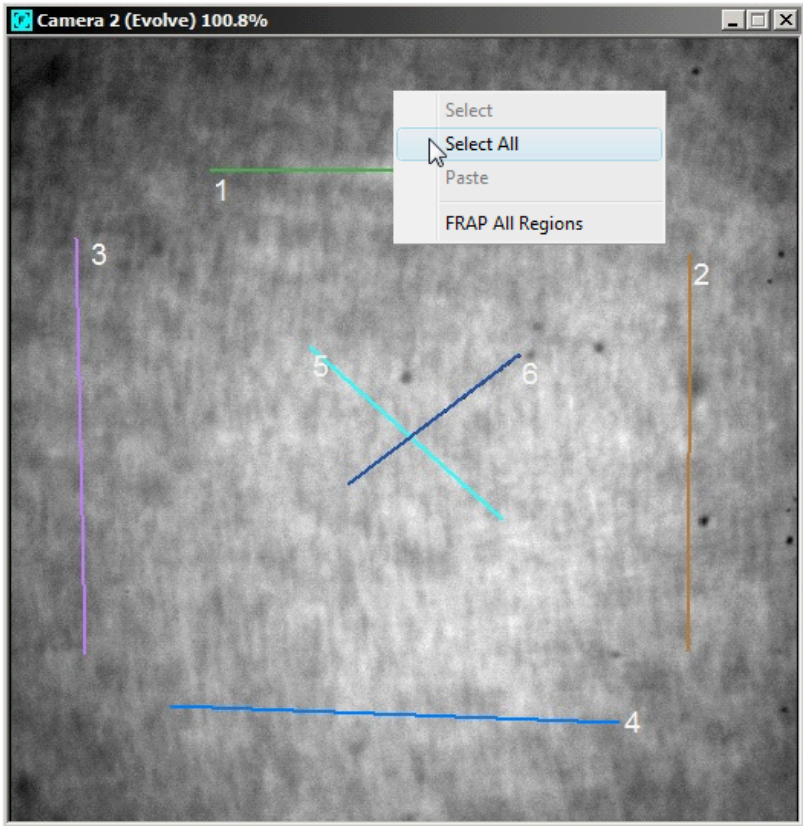
   - ***Laser Line*** - **488** (broadly useful)
   - ***Laser Duration (ms)*** - **5** ms (a good starting value for easily bleachable samples)
   - ***Raster Block Size*** - **5** (a good starting value for easily bleachable samples)

Remember to click ***Set*** after setting ***Laser Duration*** and ***Raster Block Size***.



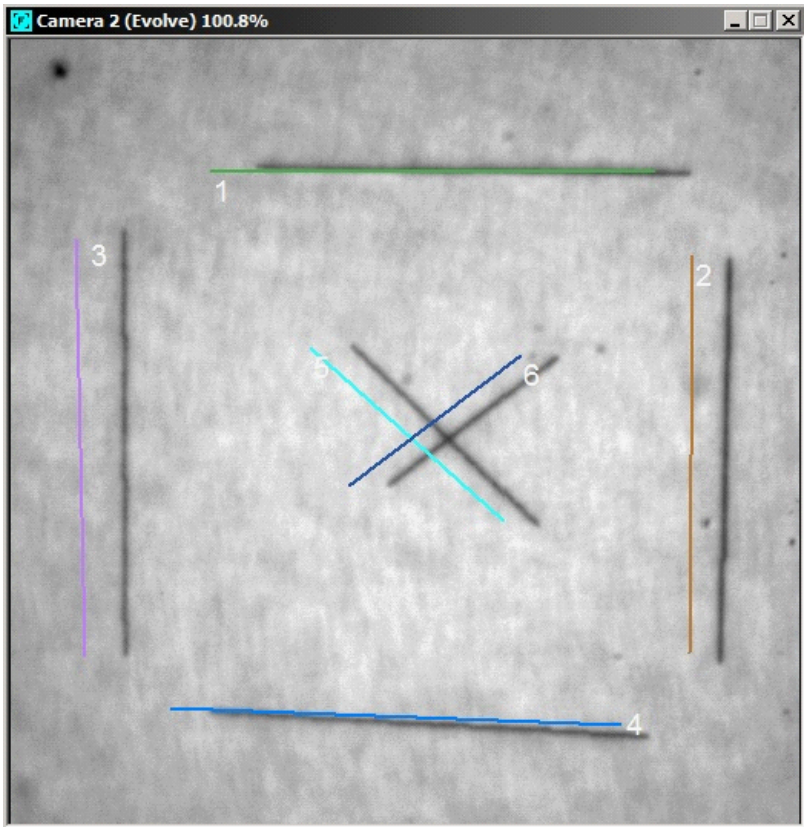
4. From the ***Focus Controls***, select a Vector channel and quickly click the ***Close Alt*** button.

5. Right-click on the live ***Camera*** window, and select ***Select All Regions***.



6. Right-click on the live ***Camera*** window again and select ***FRAP All Regions***. The readout will pause while the Vector bleaches the line regions using the laser parameters designated in Step 3.

7. After the readout resumes, use the ***Focus Controls*** to select a confocal 488 channel (such as **c488**) to review the accuracy of the bleaching. You may find it useful to move the sample a small amount using the XY stage to evaluate the bleached pattern.



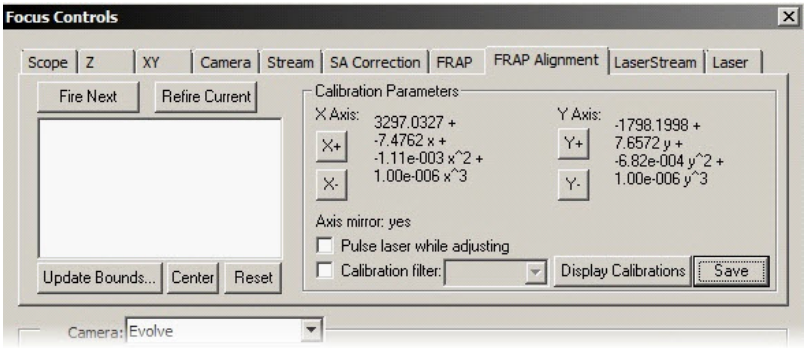
Note: Sample above has been moved to display bleached regions

8. It is possible to make minor translational changes to the calibration via the ***FRAP Alignment tab*** in the ***Focus Controls***:

   a. Underneath the each calibration equation, "**+**" and "**-**" buttons allow for editing of the equation coefficients.

   b. Holding the ***Ctrl key*** will allow for larger steps in editing the coefficients.

   c. Click the ***Save*** button to store changes to the calibration equations.



9. Evaluate edits to the calibration by repeating Steps 1-7. Further update the calibration equations as required.

About Saved Calibration Files

Once the calibration is complete, it is a good idea to create backup files. The easiest way to backup the system is to open SlideBook and go to ***Support tab >*** ***Backup Preferences***.

The specific Vector calibration file is called **Photomanipulation Calibrations.dat** and is located in the User Directory. To access this file, go to ***Administration tab >*** ***More Options > Open System Preferences Folder***. Please note that these calibration files are shared across multiple users with individual logins. If you require additional information on this topic or need to save calibrations on a per-user basis, please contact technical support at support@intelligent-imaging.com.

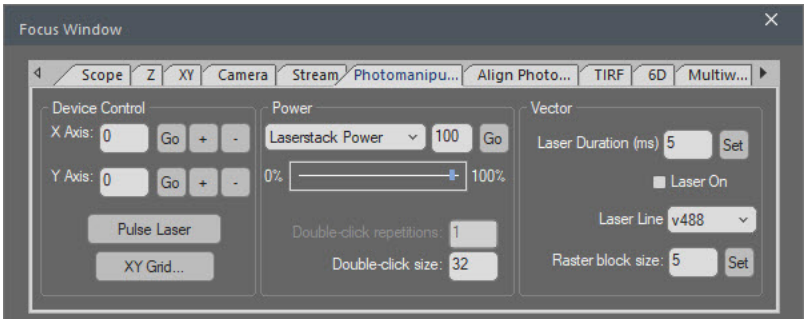
Testing Vector with Samples

Once Vector is calibrated, you may want to test various parameters such as the laser duration (or dwell time) and power level with your sample. This chapter will discuss how to adjust these parameters to obtain optimal results in the final experiment.

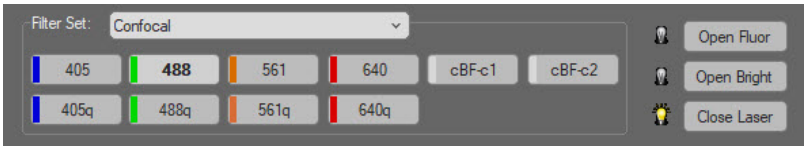
Determining Appropriate Laser Settings

You may want to prepare an extra sample for setting up the laser parameters for your specimen. Each specimen will react differently to different laser exposure durations and laser power. Follow the steps below to determine the settings you will use when designing the experiment later.

1. Place the test sample on the microscope and bring it into focus using the Focus Controls in SlideBook. Navigate to the ***Photomanipulation tab*** and select the ***Laser Line*** (e.g. v488) you would like to use for photomanipulation.



2. Using the ***Filter Set*** and filter (such as **c488**) for the desired imaging technique, find an area of the specimen where you would like to introduce focused laser light.



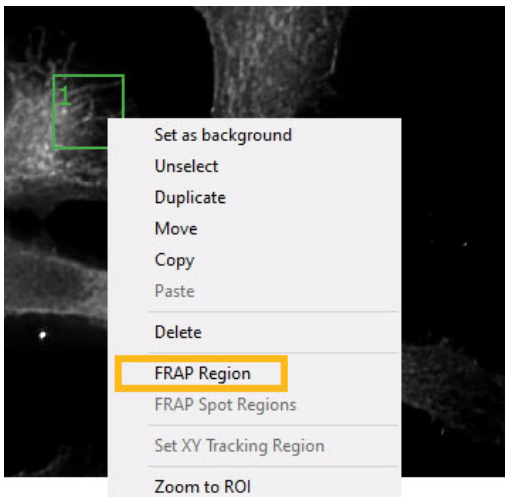
3. Select an region tool from the main user toolbar.

4. Draw an region in the desired location for the dynamic photon event to occur.

5. The region will automatically be selected when drawn. A selected region is indicated by the region number appearing in white.

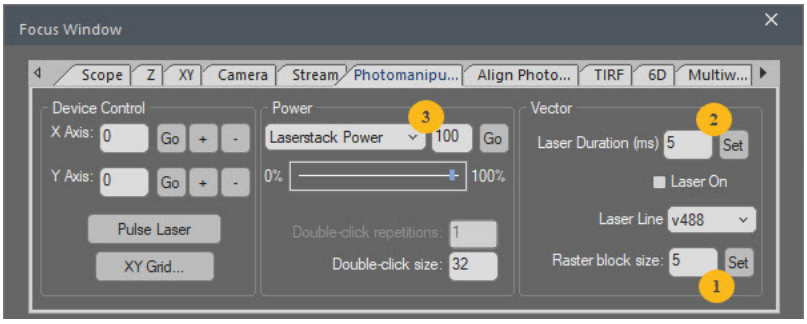
6. Go to the FRAP filter set and select the appropriate laser line you would like to use for the dynamic photon event.

7. Right-click on the selected region and choose ***FRAP Regio***n from the menu as shown below.



8. In the ***Focus Controls***, return to the ***Filter Set*** and filter for the desired imaging conditions. Examine the image. Did the beam produce the desired result?

- If Vector produced the desired results, continue to Designing Experiments Using Vector to design an experiment.
- If the beam produced a pattern of dots, reduce the value of the ***Raster block size*** then click ***Set***()1.
- If the beam was not strong enough or was too strong to produce the desired result, adjust the ***Laser Duration*** (2) and/or ***Laser Power*** (3). Do not forget to click either ***Set*** or ***Go*** depending on the parameter adjusted.
- If you are using Ablate!, be sure to select ***Ablate! ND*** for the ***Power*** (3) dropdown menu and set it appropriately.



9. Repeat steps 5-10 until desired result is achieved.

HINT: When an region is selected (number shows in white) it can be moved to a new area by clicking and dragging it to a new place. This can be useful when testing new Vector FRAP conditions.

Proceed to Designing Experiments Using Vector.

Designing Experiments Using Vector

Now that Vector is calibrated and the laser parameters have been determined, it is time to design an experiment utilizing Vector. There are three basic options for designing an experiment:

- During a time series image capture, right-click over a region to initiate an event.
- During a time series image capture, initiate a single point dynamic photon event by double clicking.
- Create one or more regions and design a complex experiment that will direct the beam to the region(s) with the opportunity to repeat the events over time.

Depending on the science you wish to accomplish, you may find any of these approaches useful. When using Ablate!, is will behave as one of the Vector lasers in SlideBook. Be sure to select Ablate! as a channel or laser selection when appropriate. Please take a moment to familiarize yourself with the different approaches in this chapter.

Right-Click to Initiate an Event

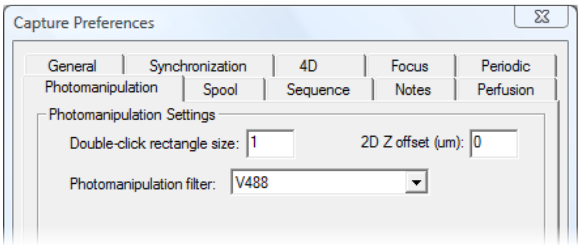
A basic way to begin introducing a focused laser beam to the sample is very similar to the testing approach described in Testing Vector with Samples. Before you begin this process, you must first set the appropriate laser parameters described in Determining Appropriate Laser Settings.

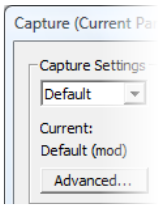
1. Place the specimen on the microscope and bring it into focus.

2. Open the Capture dialog by clicking on the icon in the main user toolbar. Select an imaging filter and take a test exposure.

3. Use the region tools to create an region in the image. Ensure the region is selected by confirming the region number is white.

4. Click on the ***Advanced*** button to open the Capture Preferences dialog. Navigate to the Photomanipulation tab.





5. Select the ***Photomanipulation filter*** from the drop down menu. This filter is usually named by the schema *wavelength*FRAP, f*wavelength*, or V*wavelength*. For example, the ablation filter selection for 488 would be called **488FRAP**, **f488**, or **V488**.

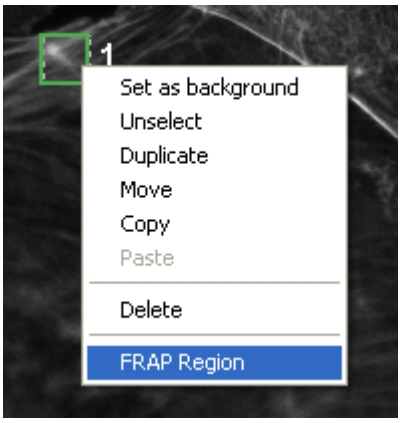
6. Click ***OK***. This will close the ***Capture Preferences*** window and take you back to the ***Capture*** dialog.

7. Set up the capture parameters as desired. If you are unclear on how to do this, please refer to the Capture window.

8. Click ***Start*** to begin image capture.

9. To initiate the event, right-click on the region and select ***FRAP Region***.

NOTE: It may be useful to pause capture when you perform this step.



10. Create events in alternate locations or repeat events as desired.

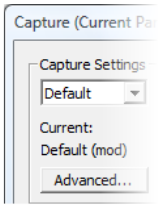
Double-Click to Initiate an Event

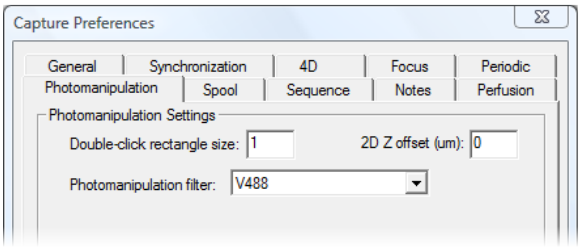
Sometimes, it is necessary to initiate a small event very quickly. For example, you may want to watch FRAP of a rapidly moving dynamic biological sample such as a centrosome in yeast. In this approach, the object you would like to hit with laser light may be moving quickly in the field of view. For this reason, it is important to be able to quickly initiate an event in any desired location. Before you begin this process, you must first set the appropriate laser parameters described in Determining Appropriate Laser Settings.

1. Place the specimen on the microscope and bring it into focus.

2. Open the ***Capture*** dialog by clicking on the icon in the main user toolbar.

3. Click on the ***Advanced*** button to open the Capture Preferences dialog. Navigate to the Photomanipulation tab.





4. Enter a value for the desired ***Double-click rectangle size***. A good starting point to test is the value of the raster block size used in the ***Focus Controls***. This is the number of spots that will be hit by the laser in a square pattern.

5. Select the ***Photomanipulation filter*** from the drop down menu. This filter is usually named by the schema *wavelength*FRAP, f*wavelength*, or V*wavelength*. For example, the ablation filter selection for 488 would be called **488FRAP**, **f488**, or **V488**.

6. Click ***OK***. This will close the ***Capture Preferences*** window and take you back to the ***Capture*** dialog.

7. Set up the capture parameters as desired. If you are unclear on how to do this, please refer to the SlideBook 5.0 User's Manual.

8. Click ***Start*** to begin image capture.

9. At the appropriate time during image capture, double-click on the image where you would like the event to occur. A small region will be created in the image when the double-click event is initiated.

10. Create additional events or allow capture to complete.

Advanced Event Protocols

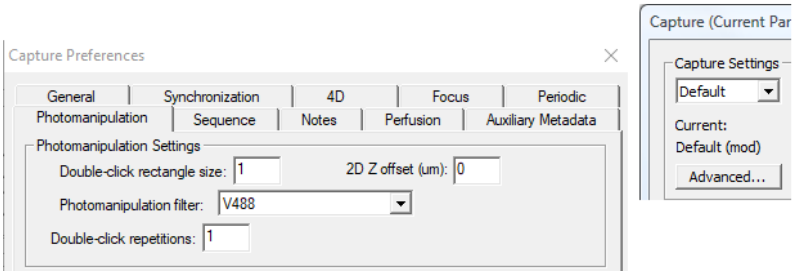
In certain circumstances, it may be desirable to create repeat events or execute events at different times throughout an experiment. All of this is available under an intuitive user interface. Before you begin this process, you must first set the appropriate laser parameters described in Determining Appropriate Laser Settings.

1. Place the specimen on the microscope and bring it into focus.

2. Open the ***Capture*** dialog by clicking on the icon in the main user toolbar. Select an imaging filter and take a test exposure.

3. Use the region tools to create an region or multiple regions in the image. These regions are where the photomanipulation will occur.

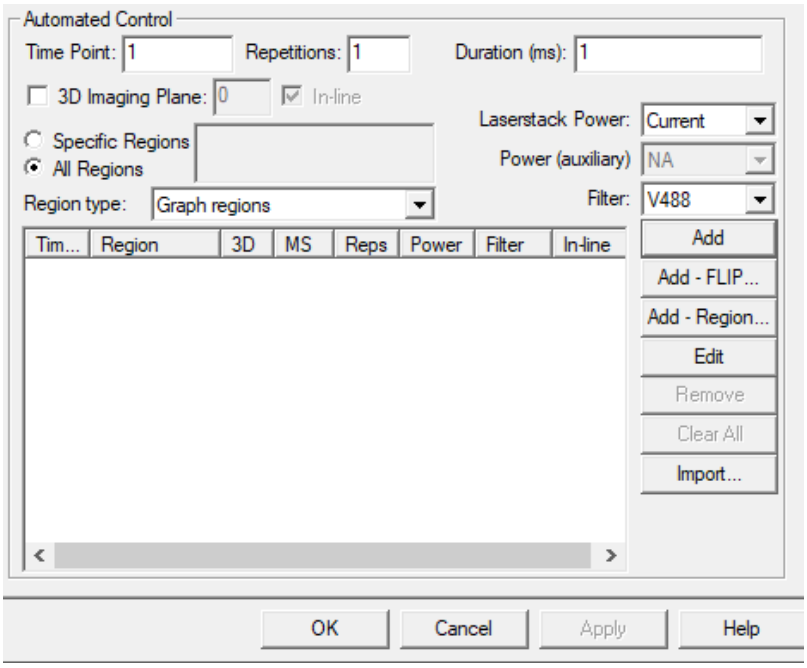
4. Click on the ***Advanced*** button to open the Capture Preferences dialog. Navigate to the Photomanipulation tab.



5. For on-the-fly experiments using the double click option, select the ***Double-click rectangle size*** (in px) the ***2D Z offset***, the desired ***Photomanipulation filter*** from the drop down menu and the Number of ***Double-click repetitions*** required.

> ***NOTE:*** This filter is usually named by the schema *wavelength*FRAP, f*wavelength*, or V*wavelength*. For example, the ablation filter selection for 488 would be called **488FRAP**, **f488**, or **V488**.

6. For a setting an auomated sequence of photomanipulation experiments, move to the lower section titled ***Automated Control***. Begin creating events by entering the ***Time Point***, ***Repetitions***, and either ***Specific regions*** or ***All regions*** to be effected by the event.



7. Click ***Add*** to save the first event.

8. Create additional events as desired by repeating steps 6-7. When all events are entered, click ***OK***. This will close the ***Capture Parameters*** dialog and take you back to the ***Capture*** controls window.

9. Set up the capture parameters as desired. If you are unclear on how to do this, please refer to Capture Window.

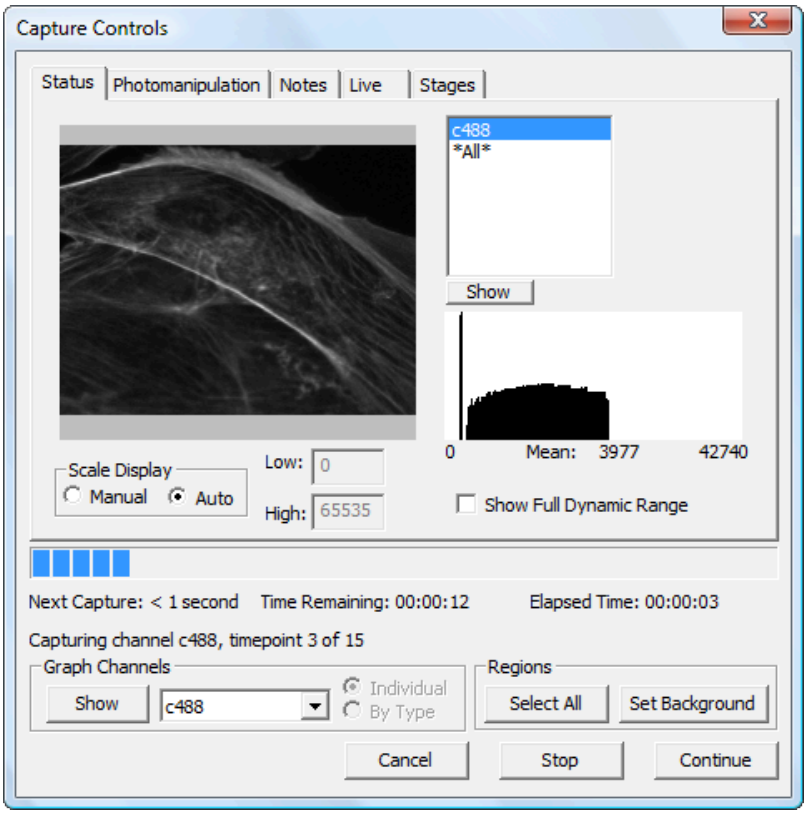
10. Click ***Start*** to begin acquisition.

Displaying Region Intensity during Capture

Whether you set up a basic right-click or double-click event or an advanced event protocol, regions will be drawn where an event or events will take place. It is often useful to graph these events during image capture to determine an experiment's success. During any of the methods described earlier in this chapter, it may be useful to view a graph of the intensity level within the region(s) of the image. Any number of region intensities can be displayed by following the instructions below.

1. Begin image capture using one of the techniques described above.

2. Navigate to the ***Status*** tab of the ***Capture Controls*** dialog.



3. In the ***Graph Channels*** section of the tab, select the channel from the drop down menu for which you would like to plot intensity. Click ***Show***.

4. A graph will open displaying mean intensity on the y-axis and time on the x-axis. Events are marked by red dots along the time axis.

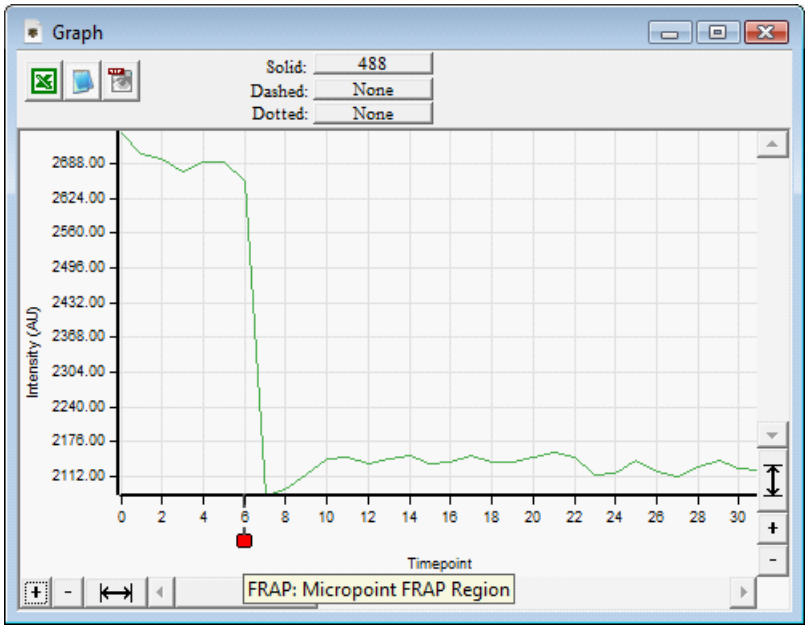
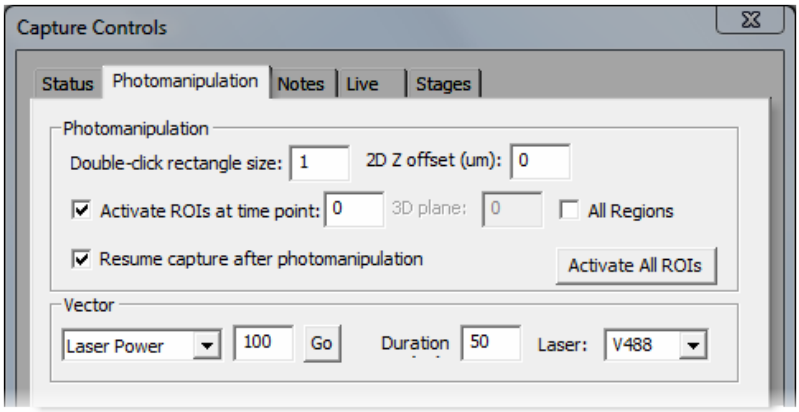


Figure 21 - Intensity Graph of a single region **during capture**

5. Each region in the image will be represented by a corresponding colored line on the graph. If you cursor over an event indicator, the event information will show.

Adjusting Vector Settings during Capture

You may find it useful to access certain Vector settings while an experiment is in progress. The Photomanipulation tab of ***Capture Controls*** allows you to do the following:



- Adjust the ***Double-click rectangle size***
- Adjust ***Laser Power*** or ***Duration*** for right-click and double-click event initiation
- Add an event to ***Activate All Regions***
- Activate a new right-click region using a different ***Laser*** than configured prior to capture

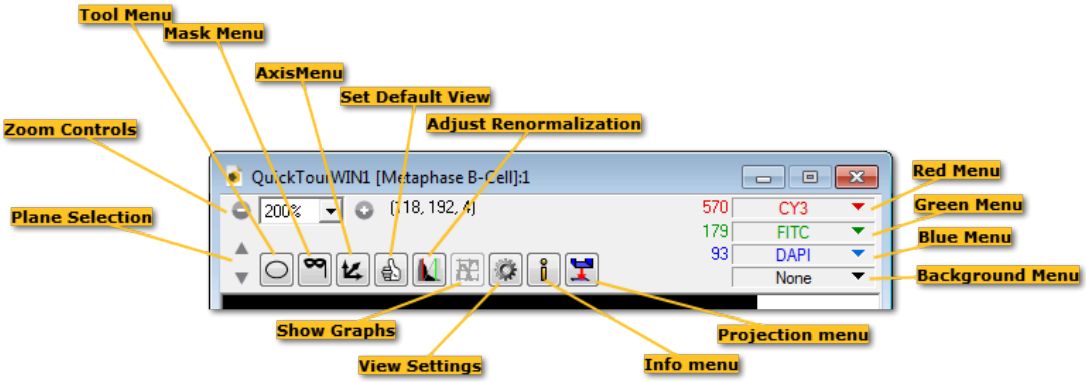
For a complete description of controls, please review the Photomanipulation Tab of Capture Controls.

Viewing and Exporting Timelapse Images

Now that the data has been captured, you will want to visually inspect the images. SlideBook contains numerous tools that are helpful in displaying various aspects of your data. Viewing and exporting tools are described in this chapter. For additional information on viewing and analysis tools, please refer to the View Menu.

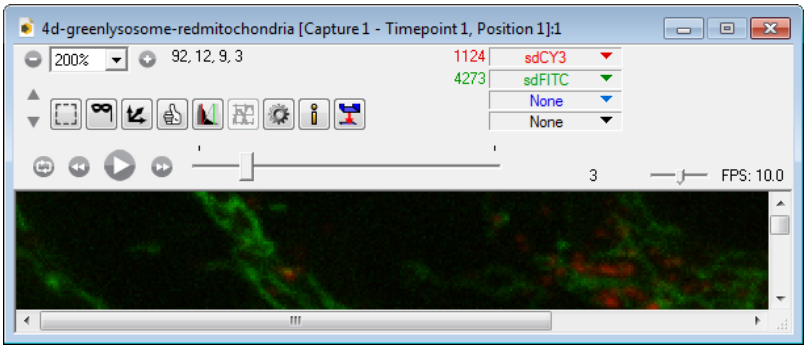
The Main View

When you open an image by double–clicking on the thumbnail in a slide, a Main View will open. Alternatively, you may open a Main View or a Three View from the View Menu. All views contain many useful tools outlined below.



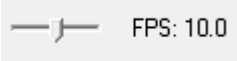
In any open view, the middle mouse scroll wheel will scroll through either *z*-planes or time. In a 4D series the scroll wheel will control time or you may hold down the **CTRL** key while scrolling the mouse wheel to change *z*-planes.

When an image consists of multiple time points, additional tools will appear in the ***Main View***. We will briefly review the tools associated with playing back time lapse images.



The ***Playback Controls*** allow you to toggle looping/single playthrough, move back one time point, play/pause, or move forward one time point.

The ***Time Slider*** displays the current time point both with the small gray box and with a numerical display to the right of the slider bar. In the example above, the user is at time point 3, about 20% of the way through the entire time series. A user can move to a desired time point by clicking on the small gray box and dragging it to the desired time point. When the time slider control is selected, it will be surrounded by a dotted line box. Once selected, you can move forward and backward through time by using the middle scroll button on the mouse.



The ***Playback Speed Slider*** works in a manner similar to the time slider. Instead of adjusting the time point, this slider controls the speed of the image looping in ***FPS*** or frames per second.

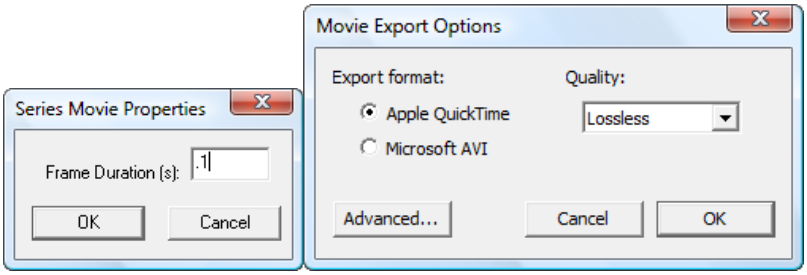
Exporting Images from the Main View

Now that you are viewing your data, there are several ways to export images and data.

A. To export the raw data of the image itself as 16-bit TIFFs for quantitative analysis, go to Home Tab, ***Acquire group > Export > (Quantitative Export)16 bit-TIFF file***. The 16-bit tiffs produced will be compatible with many programs. They are OME (Open Microscopy Environment) certified.

B. To export an individual TIFF of the image exactly as it appears in the image window; go to Home Tab, ***Acquire group > Export > (Qualitative Export) RGB TIFF***. This will create a 24-bit RGB TIFF that utilizes the same intensity scaling and zoom factor as the image displayed in the main view. Any annotations displayed will show up in the TIFF. For more information on image annotations, please refer to Displaying and Formatting Annotations.

C. To create a time series movie of the data set, go to Home Tab, ***Acquire group > Export > (Movie Export) Create Series Movie***. Enter a value for Frame Duration. If you like the playback speed of 10 FPS, you would use a value of **0.1** here. Click ***OK***. Select the type of movie export you prefer and the quality of the movie. Click ***OK***, enter a ***File name*** and ***Save*** the file. A status bar will appear while the export proceeds.



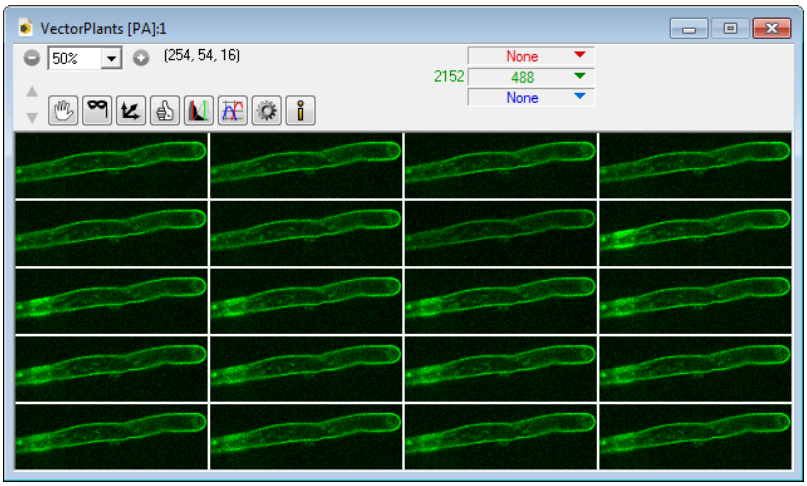
The Timelapse Tile View

It is often useful to view individual time point images in an array rather than in a movie format. This display can be accomplished using the ***Timelapse Tile View***.

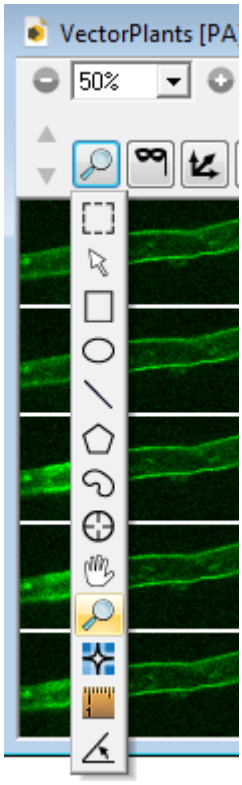
1. Open a **.sld** containing the image you would like to view.

2. Click on the image once to select it. The image will be highlighted in the ***.sld***.

3. Go to Home tab, View Group, ***View >*** ***Timelapse Tile View***. A view window will open showing each individual time point as a tile.

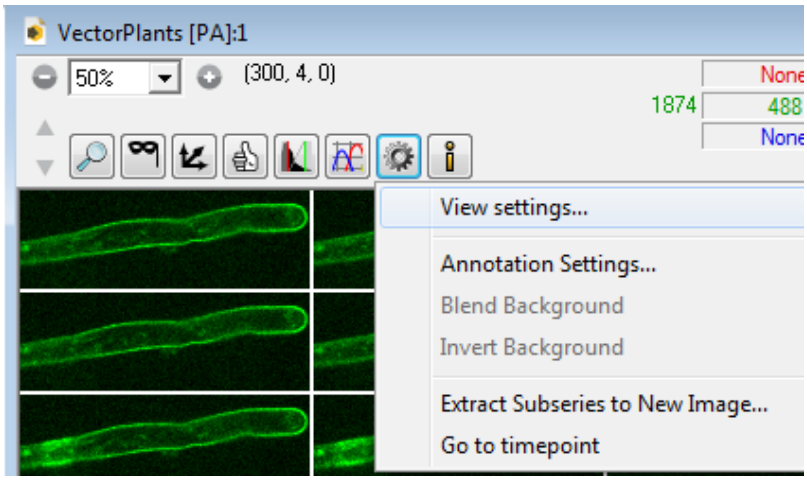


4. To zoom in to a specific region on the image in all tiles, select the magnifying glass from the tool icon drop down menu in the image window.



5. Click on an object or area of interest to zoom in. Holding the ***Shift*** button while clicking with the magnifying glass will zoom out. A "-" sign will appear next to the tool when zooming out. Alternatively, you may use the zoom tools at the top of the view window.

6. Several options are available for adjusting how the tiles are displayed. To access these options, go to ***Settings > View Settings*** in the view window.

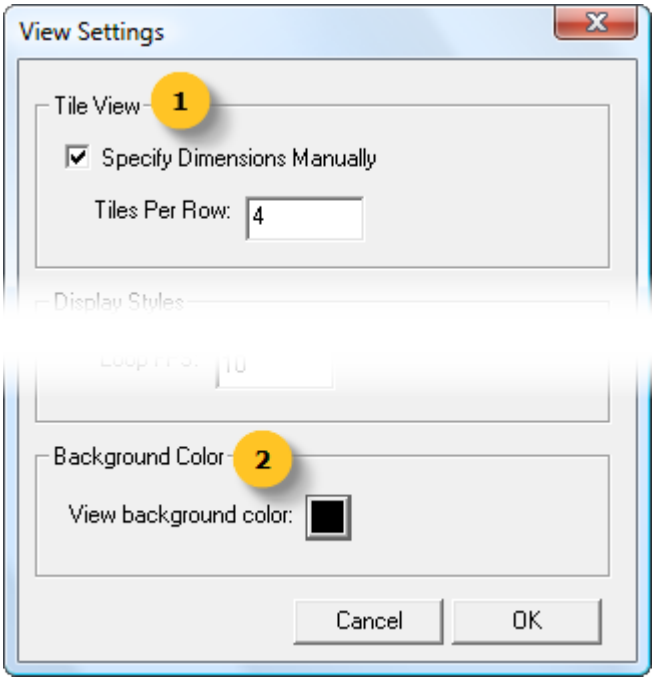


7. The ***View Settings*** dialog will open.

- The ***Tile View*** section allows you to manually specify the number of tiles, or frames, that are displayed across the image window. Simply check the box and enter a value for ***Tiles Per Row***.

- You can change the background color that is displayed between tiles by clicking on the color box next to ***View Background Color***. Simply select a color from the ***Color*** window then click ***OK***.

- Review the View Settings window to learn more about capabilities in this dialog.



8. Once the desired changes have been made, click ***OK***. The changes will be applied to the view window.

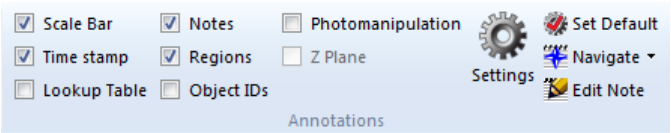
Exporting Images

Displaying and Formatting Annotations

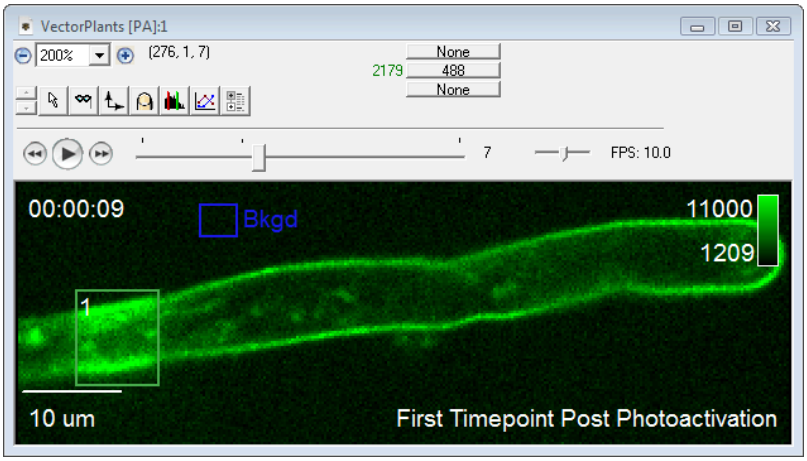
In any of the image views available, SlideBook provides tools to add annotations to displayed images. When annotations are shown in an image, they will be exported with the image any time an export of the image is performed under ***View > Export***. Annotations are not displayed when images are exported quantitatively under ***Image > Export***.

1. Open a view of the image you would like to annotate.

2. Go to the ***Annotations*** group.

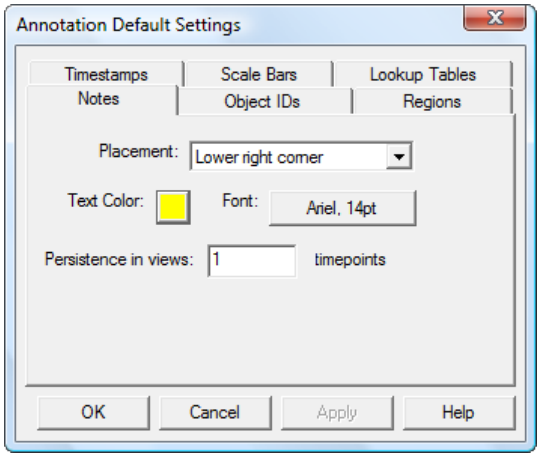


3. Select the annotation(s) you would like to display by clicking boxes. Once selected, a check mark (ü) will appear next to the annotation. Alternatively, you can use the shortcut keys.



4. Once the annotations are displayed, you may find it desirable to adjust the positioning or appearance of the annotations.

To do this, you will need to access the ***Annotation Settings*** dialog. This dialog can be accessed from ***Annotations > Annotation Settings*** or in the view window from the ***Settings > Annotation Settings***.



5. This dialog consists of a number of tabs that allow for formatting of each individual annotation. Users can adjust the color, placement, and font used for each annotation. Some annotations have annotation specific settings such at the length setting for scale bars. Adjust all annotations as desired then click ***OK***. The changes will be made in your image window. It may be necessary to click once on the image for the updates to take place.

6. If you are only interested in adjusting annotations for the image you are working with, continue to the next section of the manual. Should you choose apply these annotation settings to every image you open, it is possible to set the default behavior for annotations. To do so, select ***Set Default Settings***.

NOTE: ***Set Default Settings*** will only set the default annotation settings for the current SlideBook session. If you restart SlideBook, the default annotations will have to be reset.

Working with Notes

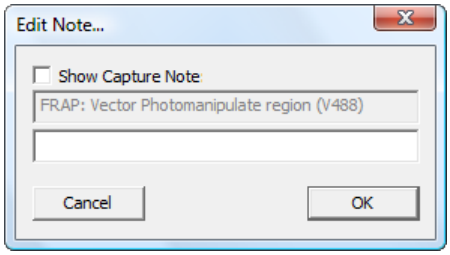
When you capture a photomanipulation event, SlideBook will use the following default naming schema to create Notes in the data set:

- Right-click Events: **FRAP: Vector Photomanipulate Regions {**laser**}**
- Double-click Events: **FRAP {**# repetitions**} time(s) at {**location**} with radius {**N**}**
- Advanced Event Protocols: **FRAP timelapse timepoint: {**N**} {**duration**} ms ROI:{**region(s)**} ({**vector line**})**

You may find it useful to later edit the names of the Notes or how long the annotations persist when reviewing your data or exporting views or movies.

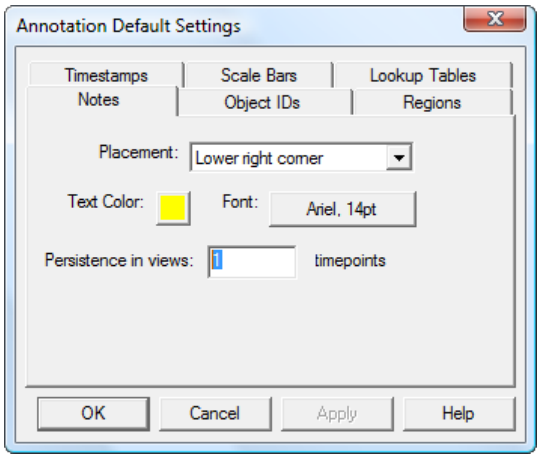
To edit or add a note:

1. Open the image.
2. Navigate to the desired time point.
3. Go to ***Annotations >*** ***Edit Note***.



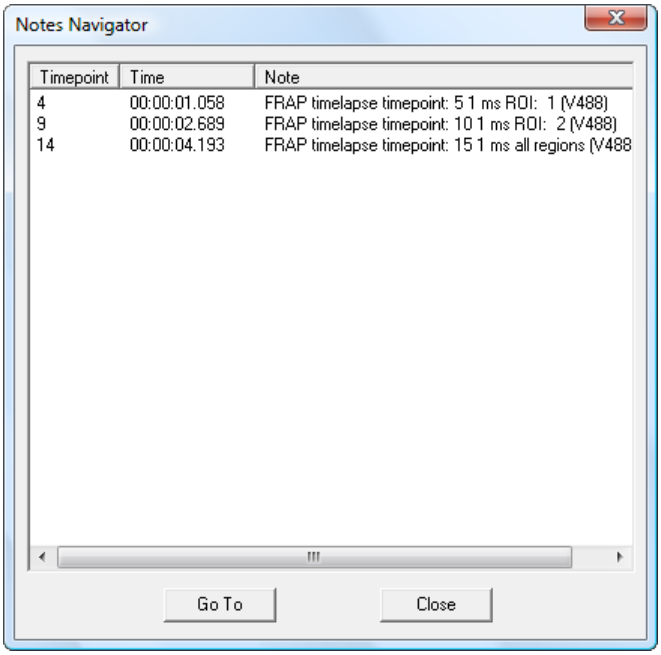
To display notes for more than one time point:

1. Go to ***Annotations Settings***.
2. Navigate to the ***Notes*** tab.
3. Edit the field ***Persistence in views:*** *N* ***timepoints.***



To assist in navigating through notes:

1. Open the image.
2. Go to ***Annotations >*** ***Navigate > Notes Navigator***.
3. Select a note then press ***Go To***.


Notes Navigator
Timepoint
Time
Note
4
00:00:01.058
FRAP timelapse timepoint: 5 1 ms ROI: 1 (V488)
9
00:00:02.689
FRAP timelapse timepoint: 10 1 ms ROI: 2 (V488)
14
00:00:04.193
FRAP timelapse timepoint: 15 1 ms all regions (V488
Go To
Close

The 2D Timelapse Intensity Plot

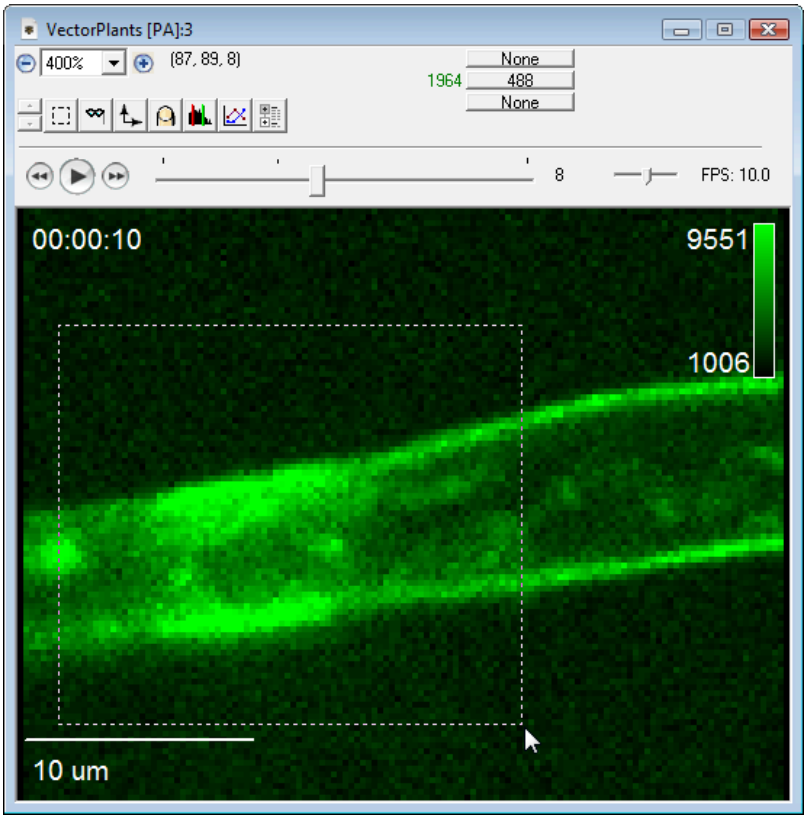
One of the more advanced ways to view timelapse data is the ***2D Timelapse Intensity Plot***. This uses the integrated 3D rendering engine to render a single channel in pseudocolor with the extra dimension of height to further emphasize changes in intensity. This view can help create stunning movies for presentation purposes.

1. Begin with an open view of a 2D timelapse image.

2. You may choose the render the entire image, to do so continue to the next step. To select a subregion of the image to render,

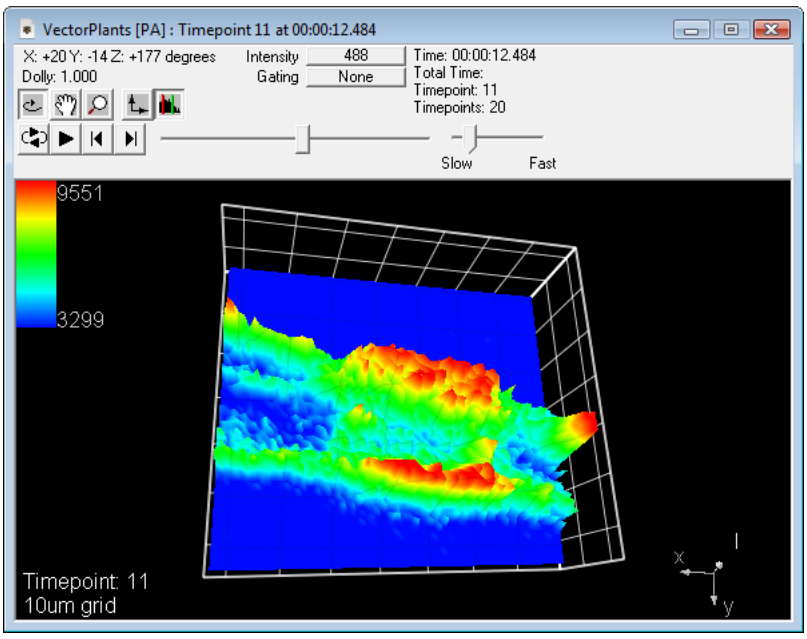
2a. Select the Marquis Select tool from the Region group.

2b. Draw the subregion you would like to render on the image

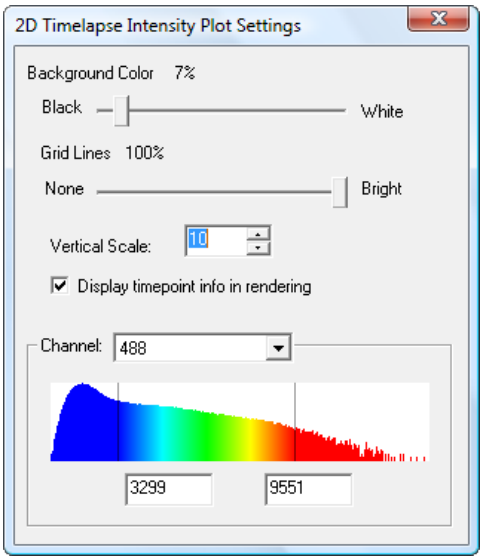


2c. Type ***Ctrl +B*** or go to ***Select >*** ***Select in All Planes*** to render the subregion at all time points.

3. Go to ***View >*** ***2D Timelapse Intensity Plot***. A new image will open. Read more about the tools in this window in [2D Timelapse Intensity Plot window](#).



9. Clicking on the renormalization icon will allow you to adjust the intensity scaling for the lookup table as well as other display properties of the image. Read more about these tools in [2D Timeplapse Intensity Plot Settings window](#).



Exporting from the 2D Timelapse Intensity Plot

Now that you are viewing your data interactively using the 2D Timelapse Intensity Plot, you may find it desirable to export images or movies from this view. Instructions for these exports are found in this section.

- To export an individual TIFF of the image exactly as it appears in the viewer window, go to Home tab, Acquire group ***Export > (Qualitative Export) TIFF***. Selecting the option for ***Include Tool Elements*** will include the time point and grid size information in the lower left corner of the display. A color 24-bit RGB TIFF will be generated.

- It is also possible to export the 2D timelapse intensity plot as a movie over time. To do this, go to Home tab, Acquire group ***Export > (Movie Export) Movie***. Complete the movie option dialog as preferred. Be sure to specify ***None (Timelapse only)*** for the ***Axis for Rotation Options***. Other options you will want to complete are highlighted below.

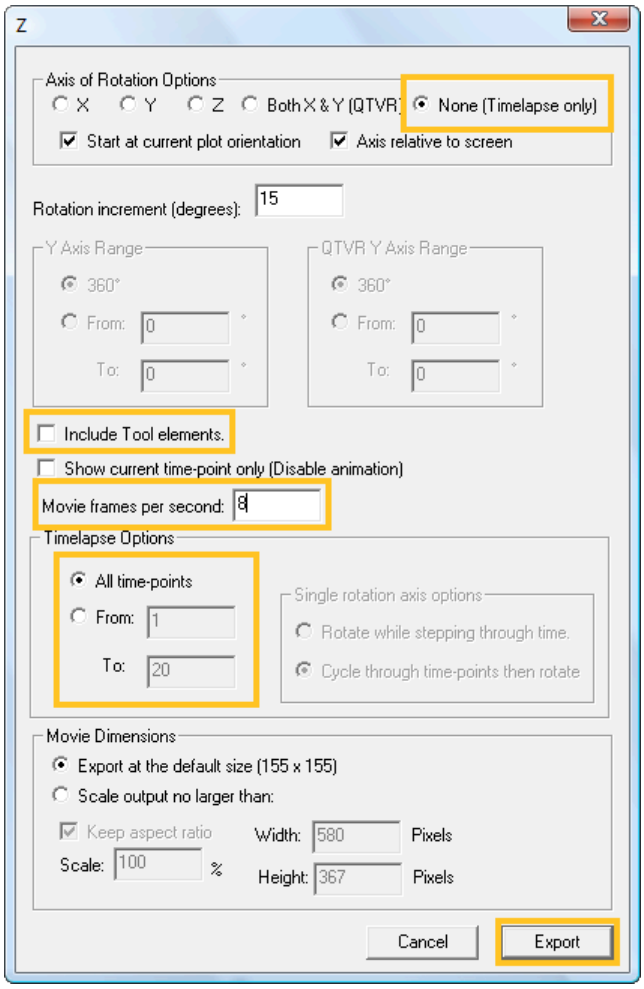


Figure 33 - 2D Timelapse Intensity Plot movie export options

Viewing and Analyzing Vector Data

Now that you have acquired date using Vector, it will be important to view and analyze the results. This chapter will discuss common viewing and analysis techniques for this type of data.

Displaying Regions and Graphs Post-Capture

In the previous chapter, you learned how to draw regions for dynamic photon events to occur. We also discussed graphing the intensity of the regions during capture. In this section, you will learn how to view the regions and graphs once the data set has been saved and closed.

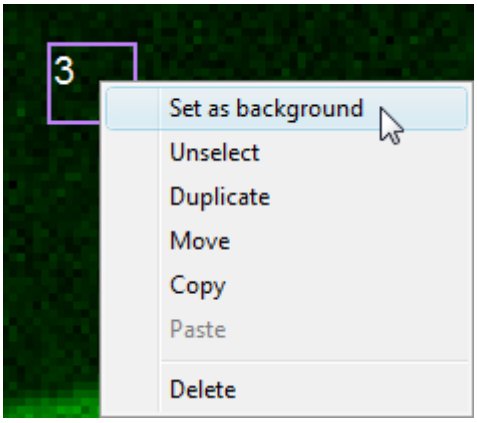
1. Open a **.sld** that contains data from the Vector experiment.

2. Open an image on the slide by double clicking on the thumbnail.

3. You can scroll trough time using the middle mouse scroll wheel. Alternatively, you can click and drag the time slider bar.

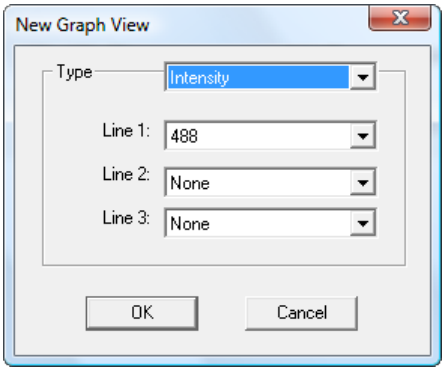
4. There are three way to display previously drawn regions:
   - Select any of the region tools from the region group
   - Go to ***Annotations > Regions***
   - Type **Ctrl** + **Shift** + **G**

5. If you did not draw a background region, you can do so at this time. First draw a region where you would like to define the background. Next, right click on the region and select ***Set as Background***.

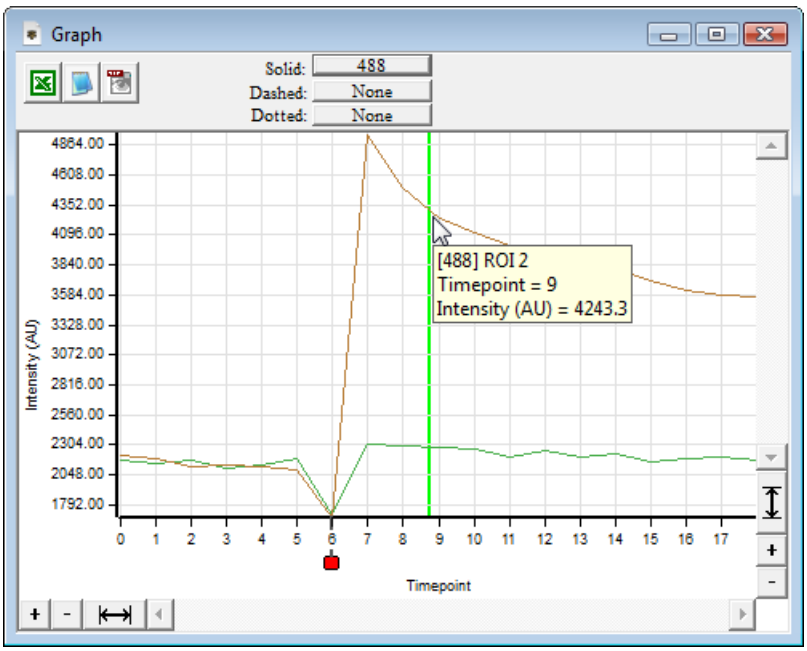


NOTE: In order for SlideBook to automatically subtract the background from values shown in the graph and other analysis, you must first configure the property. To do so, go to ***Administration tab >*** ***Hardware Properties***. Navigate to the section titled ***Statistics Properties***. Click the **+** to expand the selection. For the property ***Enable Background Subtraction***, set the value to ***Yes***. Click ***Apply Change*** then ***Close***. SlideBook will have to be restarted for the change to take effect. The background subtraction configuration change will apply to all future SlideBook uses.

6. To graph the intensity over time in regions, go to the Analyze tab, ***Evaluate group >*** ***Graph*** or click on the ***Graph*** icon in the view window. Select the ***Type*** of graph you would like to display (intensity is a common way to view this type of data) and the channel(s) you would like displayed on the graph. Click ***OK***.



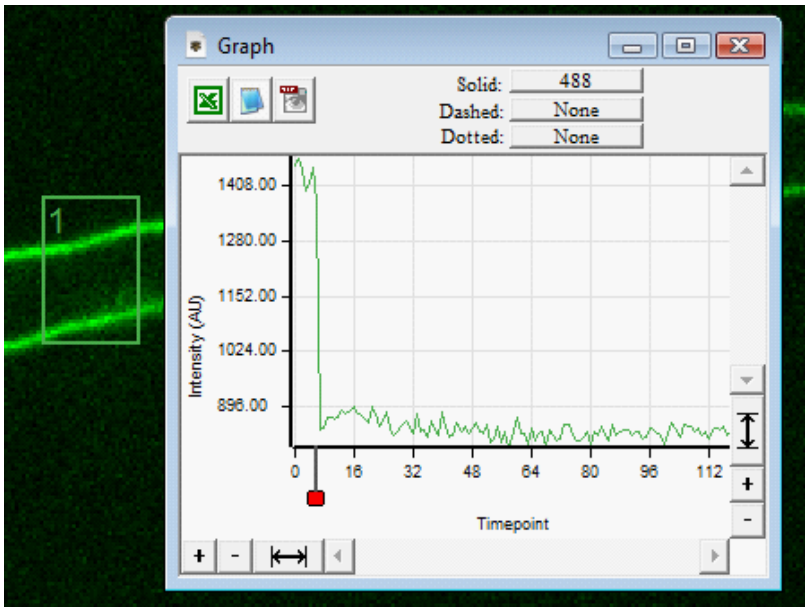
7. A new ***Graph*** window will open. Refer to the Graph window section for more information on using the graph tools and exporting data or images from the graph.


Graph
Solid: 488
Dashed: None
Dotted: None
4864.00
4608.00
4352.00
4096.00
3840.00
3584.00
3328.00
3072.00
2816.00
2560.00
2304.00
2048.00
1792.00
Intensity (AU)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Timepoint
[488] ROI 2
Timepoint = 9
Intensity (AU) = 4243.3

Editing regions

In Displaying Regions and Graphs Post-Capture, you learned how to create and define a background region. Sometimes, it is useful to examine regions in other areas of the image or to look at specific areas within the area where the dynamic photon even occurred. This section will teach you how to work with regions.

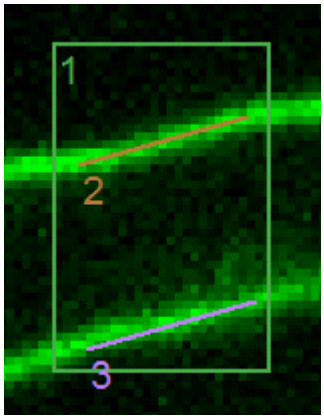
For demonstration purposes, we will use the example shown below. In this example, a somewhat large area of the sample was photobleached. The goal of the experiment is to show recovery after photobleaching. When we graph only the background intensity subtracted region where the event occurred, it appears there was no recovery after the photobleaching event. If we were to refine the region(s) for analysis, the results may improve.



1. Begin with an open image depicting a dynamic photon event. Bring up the region(s) and graph using the skills you learned in Displaying Regions and Graphs Post-Capture.

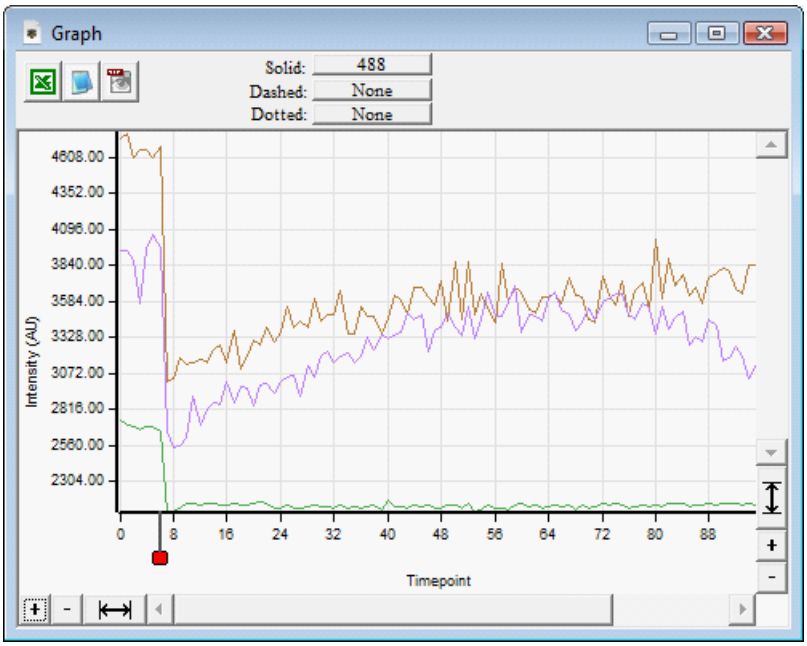
2. Click on the image to select it. With the image selected, choose a region tool from the region group.

3. Draw regions as desired in the image. For this example, we will draw line-style regions.



4. When a region is selected, the number is highlighted in white. To select a region, click inside the region or on the border of the region. Alternatively, right-click outside of a region and ***Select All*** regions if desired. Selected regions (individual or groups) can be moved by clicking and dragging to a new location. They can also be deleted by using the ***Delete*** button on the keyboard.

5. If a graph is open, the new regions will be graphed immediately.

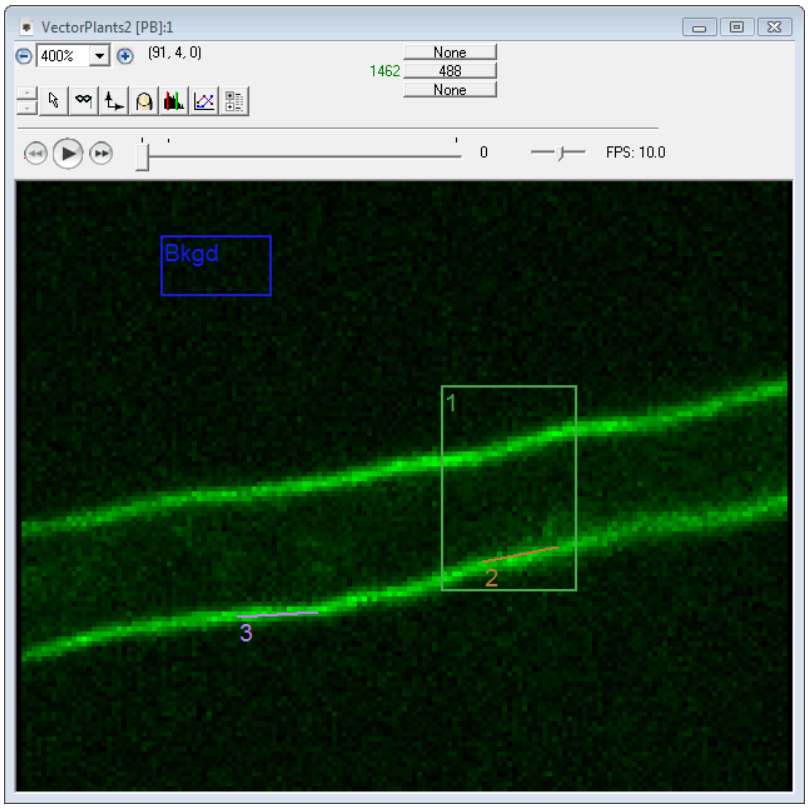


TUTORIAL: FRAP Analysis

Vector includes the SlideBook module for integrated FRAP analysis. The FRAP Analysis module utilizes regions to calculate the mobile fraction and half life of a sample. Users can define unaffected regions to normalize photobleaching that occurred during data capture. When background regions are defined, the mean intensity of the background is subtracted from all data before calculations are made.

For this example, we will use the same sample data we used in previous sections of this manual. We will include our background region for background subtraction. We will also draw a small, specific region inside the area where FRAP occurred for analysis. In addition to this, we will draw a region over a part of the image that was unaffected by the event. This last area will be used to normalize overall photobleaching due to image capture. Our starting image is shown below with the following regions drawn:

- ***Bkgd*** - Background region used for mean intensity background subtraction
- ***Region 1*** - Region used to create the photomanipulation event
- ***Region 2*** - Region drawn post capture to evaluate recovery in a specific area where the event occured
- ***Region 3*** - Region outside of photomanipulation are used to normalize photobleaching due to general exposure during image capture.

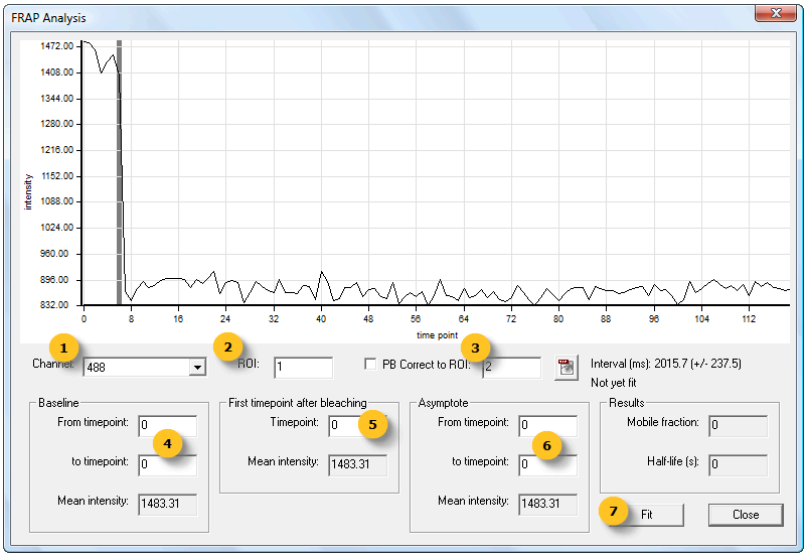


1. Begin by opening the image for analysis. Draw all necessary regions as described above.

2. Go to Analyze tab, ***Evaluate group >*** ***FRAP Analysis***.

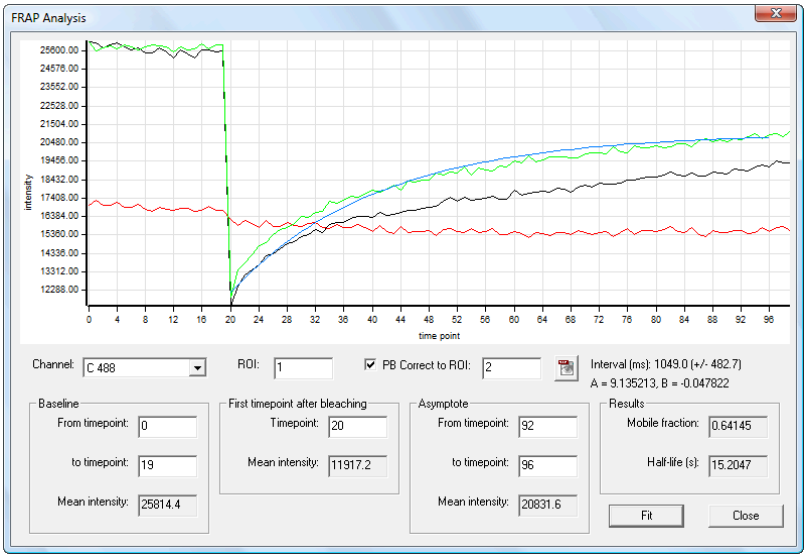
3. The ***FRAP Analysis*** window will open. Use this window to enter information for the FRAP analysis as follows. For additional information on this window, please refer to FRAP Analysis window.

    1. Select the imaging channel for analysis.
    2. Enter the region to use for analysis.
    3. If desired, check the box to use a region outside of the photomanipulation event are to correct for photobleaching. Enter the region to be used in the edit field.
    4. Enter the start and end time points for baseline images.
    5. Enter the first time point after photobleaching.
    6. Enter the start and end time points for the asymptote where recovery levels off.
    7. Press ***Fit*** to run the analysis.



4. The analysis window will update with the corrected curves and calculated results for mobile fraction and half-life as shown below. To learn more about the results, review the FRAP Analysis window or APPENDIX A Concepts of Photobleaching Recovery. Colored lines are as follows:

Green - Region of interest for analysis designated in Analysis Region
Red - Region of interest to be used for photobleach correction as designated in Photobleach Correction
Black - Photobleach corrected intensity of analysis region
Blue - Resulting FRAP Analysis, the exponential fit from the first time point after photobleaching to the asymptote.



5. Adjust any parameters and recalculate as desired.

6. To export a tiff of the resultant graph, click on the ***Tiff*** icon .

7. When finished, click ***Close*** to close the dialog.

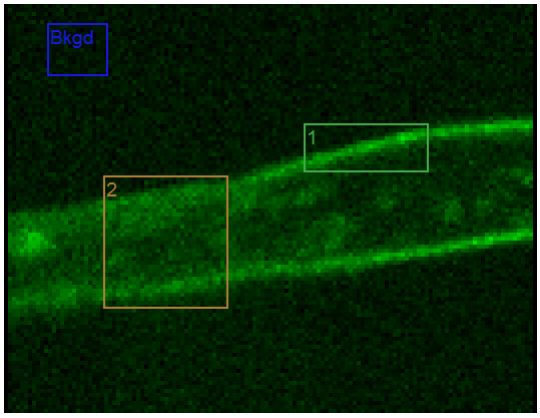
Photoactivation Analysis

As discussed earlier, Vector can be used for various types of experiments where a focused laser beam is directed at a sample. In the previous section, we discussed analyzing FRAP data. In this section, we will discuss photoactivation experiments. For this example, we will use a sample data set from a photoactivation experiment that utilized a 405nm laser to activate a GFP-like fluorochrome.

1. Begin by opening the image from the **.sld**.

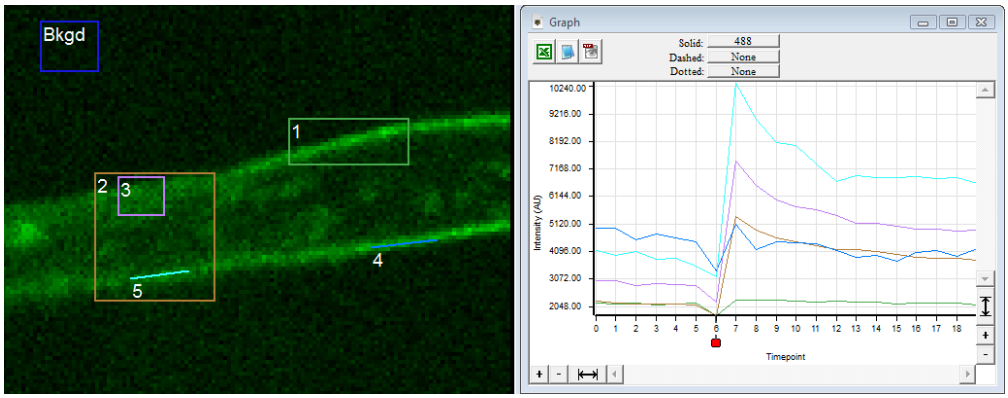
2. There are three way to display previously drawn regions:
   - Select any of the region tools from the region group
   - Go to ***Annotations > Regions***
   - Type **Ctrl + Shift + G**

3. If a background region is not already defined, define one by using the region drawing tools then right-clicking on a region and selecting ***Set as Background***.



4. Go to the Analyze tab, ***Evaluate group >*** ***Graph*** or click on the ***Graph*** icon in the view window. This will open a graph of the background subtracted mean intensity of each of the regions.

5. Draw additional region(s) as desired. The graph will automatically update. You may choose to draw regions to show an area where no photoactivation occurred or to further examine a smaller area of the region where the dynamic photon event occurred.



6. You can interact with the graph as described in Graph window.

7. For photoactivation experiments, the main data used in analysis is the intensity information from individual regions over time. You can export this information in any of the following ways:

• - Perform a direct data export to Excel. If Excel is on the computer, this will open a spreadsheet that contains some basic image information as well as background subtracted intensities for each region at each time point. There will also be markers where the dynamic photon events took place.

• - Export a tab delimited .txt file for later use with Excel or an alternate spreadsheet program.

• - Export a TIFF of the graph portion of the graph window. This can then be used directly as an image in posters, presentations, etc.

APPENDIX A Concepts of Photobleaching Recovery

Fluorophores are molecules that are excited by higher energy light (shorter wavelength) and, as a result, emit lower energy light (longer wavelength). When a fluorophore is exposed to too much excitation light, it will cease to emit light or fluoresce. This phenomenon is called bleaching. Bleaching can result from overexposing a sample when setting up an image capture or can result from the image capture itself.

Bleaching can also be intentionally caused by directing large doses of high energy light at the sample. ***F****luorescence* ***R****ecovery* ***A****fter* ***P****hotobleaching (FRAP)* is a technique that uses bleaching to examine how molecules move in a sample. By depleting the fluorescence in an area of the sample and watching to see if the fluorescence recovers after the event, scientists are provided with additional details of cellular dynamics.

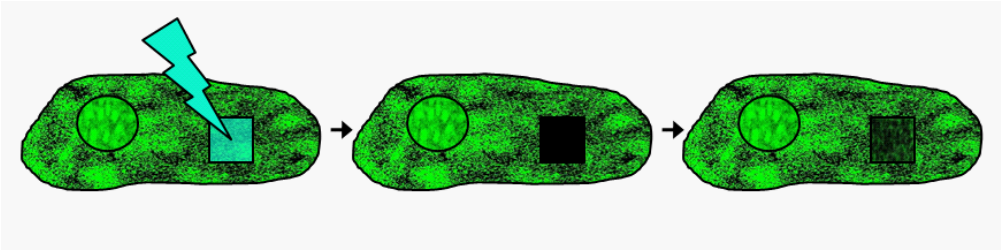


Figure 1 - Overview of FRAP

When we examine FRAP data, it is important to note that the fluorophores themselves are not "recovering" but rather that undamaged fluorophores are moving to occupy the space where fluorescence was depleted. The image below helps to illustrate this concept.

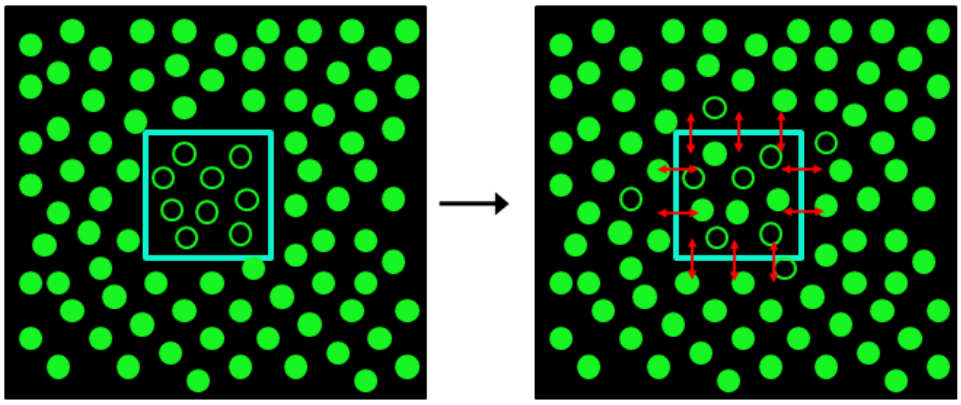


Figure 2 - Diagram showing the movement of molecules responsible for "recovery" of fluorescence

In Figure 2, we notice that some of the bleached molecules stay in the location where they were bleached. The molecules that do not move are referred to as the *immobile fraction*. The molecules that move throughout the sample and are therefore replaced by fluorescing molecules are referred to as the *mobile fraction*.

By imaging the sample over time, we are able to graph the intensity of the fluorescence over time. It is very important to obtain some baseline images to study the fluorophore behavior before the bleaching event. It is also important to image long enough to gain the necessary information about the recovery. There are several common analysis values extracted from the data. These values tell us how many of the molecules are moving (the mobile fraction) and at what rate (the half-life). The graph below illustrates common terms used in reference to FRAP analysis.

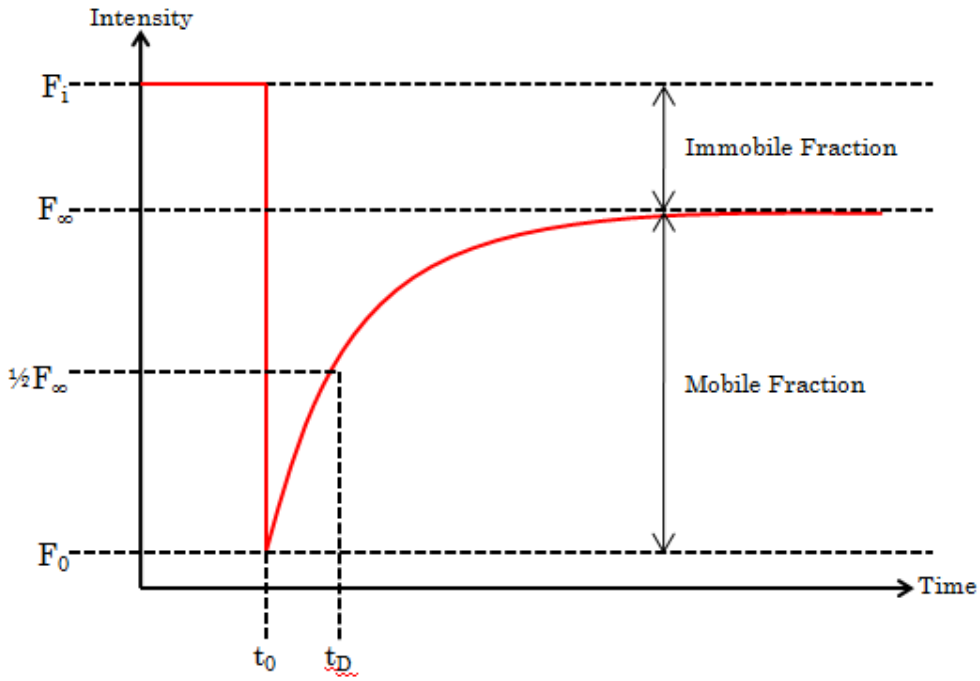


Figure 3 - Graph illustrating common terms used in FRAP analysis

In the graph above, the following variables will be used in FRAP analysis:

F_i - The initial fluorescence is the mean intensity of the ROI across all time points of the baseline images

F_∞ - The infinity fluorescence is the average of the mean intensity of the ROI across all time points in the asymptote range.

½ F_∞ - The half fluorescence is half of the fluorescence value of F_∞ minus F_0.

F_0 – The zero fluorescence is the intensity of the region at the first time point following the bleaching event.

t_0 – Time zero is the time point immediately following the bleaching event.

t_D – Time D is the time it takes to reach ½ F_∞.

The *mobile fraction* is determined by dividing the difference between the average intensity in the asymptote range and the intensity of the first time point after bleaching by the difference between the average intensity of the baseline range and the intensity of the first time point after bleaching. We can mathematically represent the *mobile faction*, or *f*, as follows:

$$f = \frac{F_\infty - F_0}{F_i - F_0}$$

The *half-life* is determined by fitting an exponential function to the background corrected intensities between the first time point after bleaching and the last time point of the asymptote range, inclusive. If ***PB Correct*** is checked in the ***FRAP Analysis*** dialog, the fit is made to the photobleach corrected intensities. Otherwise it is made to the original intensities, after background correction.

The fitted equation is of the form:

$$I(t - t_o) = M - Ae^{-B(t-t_B)}$$

where t is the time point, t_0 is the first time point after bleaching, and M is the maximum background subtracted intensity in the given time point range.

The half-life is determined by the fitted B parameter:

$$\tau = -\frac{1}{B} d$$

where τ is the half-life in seconds (t_D minus t_0) and d is the interval between time points (in seconds). It is necessary that the interval between time points be constant throughout the given time point range.

Motorized Spherical Aberration Correction Device

This section of help describes all of the steps necessary for utilizing 3i's patented Spherical Aberration Correction device (SAC). The sections are outlined below. If you are new to SlideBook™, you may wish to start by reviewing the User Interface pages of help, specifically the Focus Controls Window and the Capture Window. This will familiarize you with basic elements and commands in SlideBook™.

Overview of Spherical Aberration

Spherical aberration is the most prevalent aberration in optical microscopy. This section will review common causes of spherical aberration and explain how the SAC unit corrects this aberration.

Installing SAC

If your system was installed by 3i or a 3i reseller, then you will not need to install hardware on your system. However, if it was not installed by 3i or a 3i reseller, or you need reinstall the unit, you can learn how to do this on these pages.

Configuring SAC in SlideBook

If your system was installed by 3i or a 3i reseller, then you will not need to configure SlideBook™ to control the SAC. If, for any reason, you need to reconfigure the software to recognize the SAC, this section will teach you how to do this.

Using SAC in Image Acquisition

Once your unit is installed, you will need to understand the software control of the SAC. These pages will outline all steps required in setting up the SAC for use in image acquisition.

Troubleshooting

This section contains information on troubleshooting.

Overview of Spherical Aberration

What is Spherical Aberration?

Spherical aberration is the largest monochromatic artifact in modern microscopy. It is the natural result of multiple glass\oil \water\air interfaces. One primary example is that spherical aberration prevents deep imaging using an oil objective into water. The Spherical Aberration Correction device (SAC) is a motorized lens assembly that allows spherical aberration to be corrected with complete software control. Intelligent Imaging Innovations, Inc. (3i) has an exclusive license to this patented technology.

Using high NA objectives can very easily introduce spherical aberration (SA) into your image. Spherical aberration will be introduced if the coverslip and target do not match exactly the specification of the objective. Usually this means imaging a target just on the other side of a coverslip of 0.17 mm thickness.

Imaging conditions different from the objective's specifications can lead to a significant loss of intensity and resolution in the z-axis. The SAC device will improve and can even eliminate spherical aberration in an acquired image. Manufacturers of coverslips give the following specifications for a given coverslip thickness:

Coverslip Type	Min. Thickness	Max. Thickness
#0	0.08mm	0.13mm
#1	0.13mm	0.17mm
#1.5	0.16mm	0.19mm
#2	0.17mm	0.25mm

NOTE: These broad ranges mean that #1, #1.5, and #2 thickness coverslips can all have the possibility of having the correct 0.17 mm thickness.

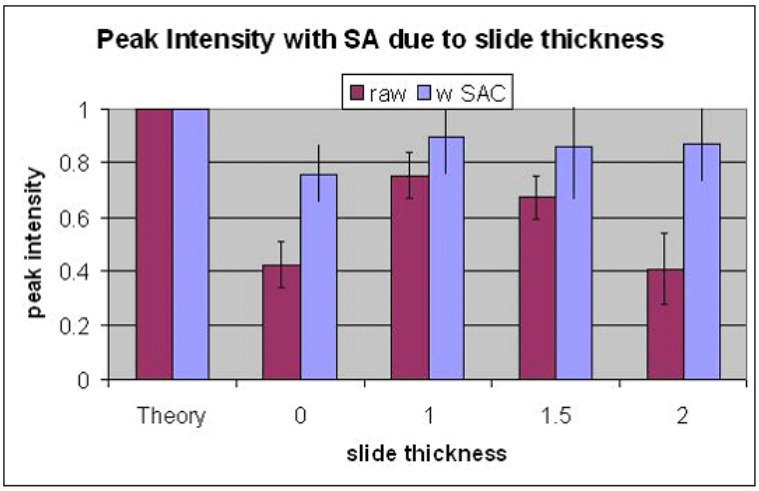


Figure 1 - Peak Intensity vs. Slide Thickness

The chart above (Figure 1) shows how differences in slide thickness can affect the measured intensity. It also shows how the SAC can correct the intensity to near theoretical levels.

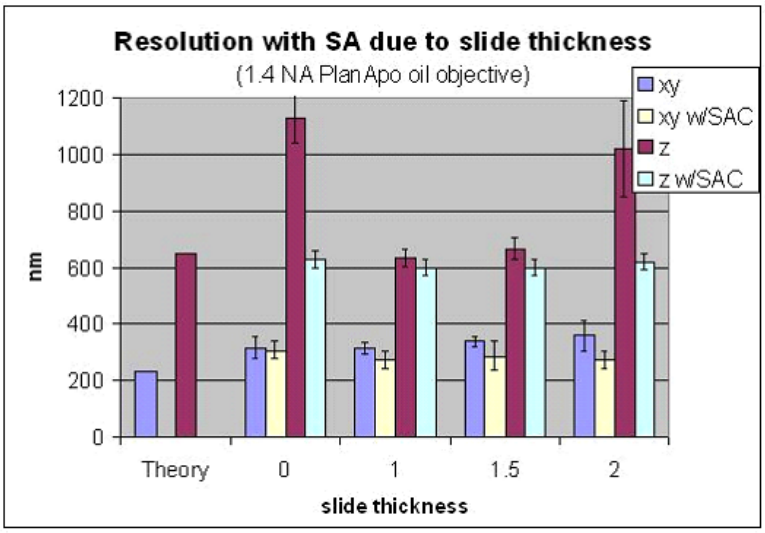


Figure 2 - Spatial Resolution vs. Slide Thickness

The second chart (Figure 2) shows the resolution lost due to slide thickness variation and how the SAC corrects this.

How Does the SAC Correct for Spherical Aberration?

The SAC is designed to be mounted between the camera and microscope on any converging documentation port. There are two lenses in the SAC. The front lens (closest to microscope) is a compound negative lens and the focusing lens is a F15 mm plan achromat. Both lenses are AR coated and the total system loss is about 3% of the light due to reflections. This minor light loss is far outweighed by the intensity gained due to eliminating spherical aberration.

The SAC operates by moving the front lens over a range of 4 cm towards and away from the microscope. Adjustment of the lens position will correct the overall microscope spherical aberration for different imaging conditions. It will also change the focus and may shift the image slightly in x and y. Careful alignment of the SAC in the x- and y-axis can eliminate this shift. Due to the lens system, there is a 10% magnification shift of the system over the complete range or the SAC. This magnification shift is accommodated by post capture re-scaling.

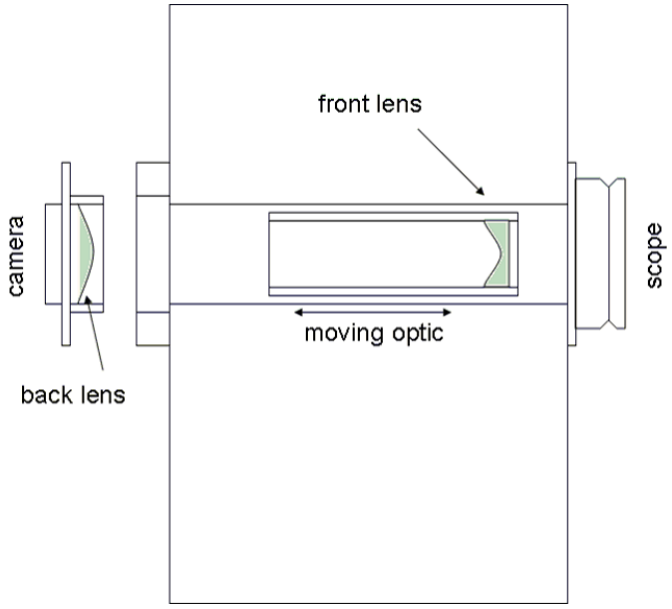
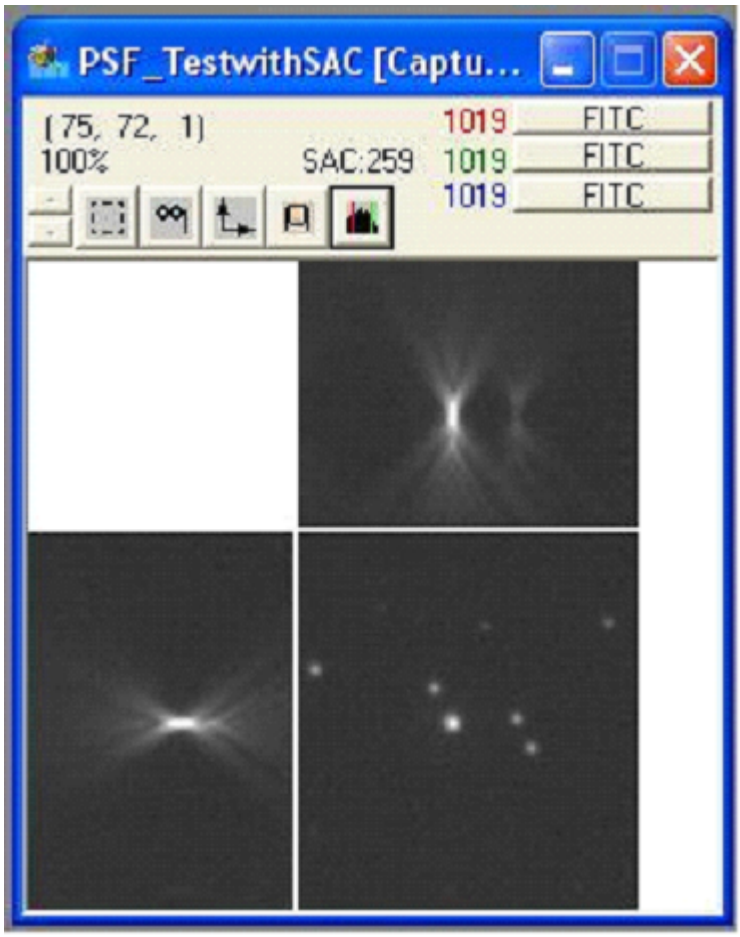


Figure 3 - Diagram of the SAC and its Optics

Notes on Spherical Aberration

When there is spherical aberration in an image, the point spread function (PSF) is distorted, especially in *z*. As the spherical aberration increases you will get an asymmetry between the top and bottom of the PSF. More light will shift from one side of the PSF to the other. In addition the central peak of the PSF will spread out more. As spherical aberration increases you lose spatial resolution primarily in z but also in x / y.

You should notice that as you move the SAC position from one side of the optimal position to the other, the asymmetry will shift from the top of the PSF to the bottom (or bottom to top). At the point where there are equal amounts of light above and below the central peak of the PSF, the spherical aberration is corrected (see figure below). This optimal location will change as you move deeper into the sample.



Sample 3D image of a point spread function with little or no spherical aberration.

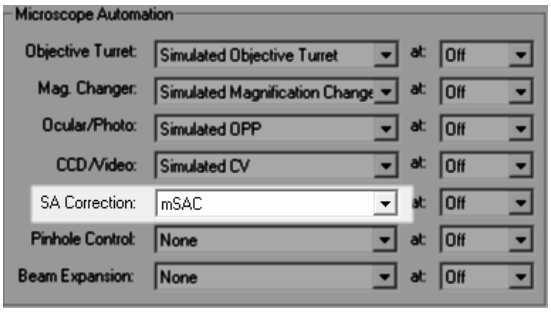
Configuring New mSAC Interface

Our newest spherical aberration correction technology. The mSAC is a 1x high-speed spherical aberration correction optical system compatible with any microscope. To compliment the new hardware, we have a new interface and calibration technique.

Identify mSAC for Usage

To activate the new mSAC interface there are two things that must be set up:

1) Confirm **mSAC** is selected in ***Hardware Configuration***



2) Change ***Harware Properties*** to utilize the new mSAC interface. Open ***Hardware Properties***, scroll to ***mSAC***, expand section, locate **mSAC V2 Interface**. Set property to ***Yes*** and ***Apply*** changes.

3) Restart SlideBook.

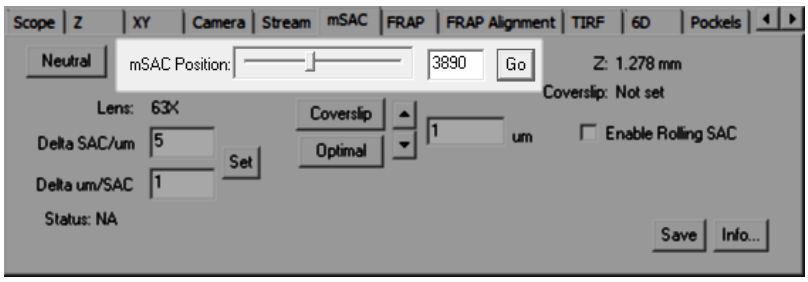
Determine and Set Neutral Position

Before proceeding with the new mSAC interface, you must determine the "neutral" position for the device. Neutral is the setting on the mSAC that yields the same results as a system without the mSAC device. This value can be determined by the following process:

1) Open the ***Focus*** window, direct light to the eyepieces. Confirm the eyepieces are set to the "0" position. Focus on a sample through the eyepieces of the microscope. If necessary, adjust the eyepiece of the non-dominant eye.

2) Redirect the image to the camera.

3) Go to the ***mSAC*** tab of the ***Focus*** window. Without adjusting focus or z-stage position, move the mSAC positon until the live image in the ***Camera*** window matches the focal plane seen through the eyepieces. You will adjust the slider or type a value in the field then press ***Go*** until the desired focal plane is found. Make note of the value shown (below, the value to note is **3890**).



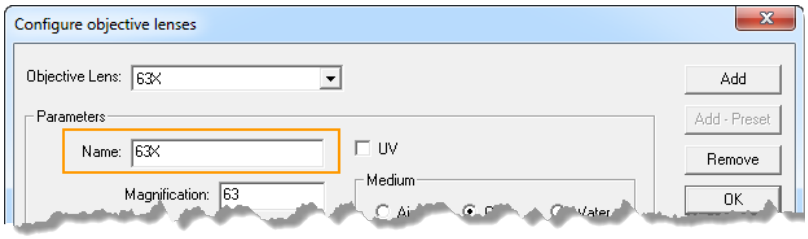
4) Open ***Hardware Properties***, navigate to ***SAC*** and expand the section (note ***SAC***, not ***mSAC***). Select ***Default Position***. Enter the value obtained in step 3. Click ***Apply Change*** then ***Close***. Restart SlideBook or continue to the next section.

Configure mSAC Properties

One part of the new approach includes a entering two calibration factors. Our research has shown that these calibration factors are directly related to the objective specifications and are consistent across identical objectives from the same manufacturer. We are working on a publishable table of these calibration factors but, in the meantime, please contact us through support@intelligent-imaging.com or https://www.intelligent-imaging.com/contact for assistance on calibration factors.

1) Go to ***Administration*** tab > ***Objectives***.

2) Make note of ***Name*** field for the objectives you will be calibrating. It is important that this text is entered exactly as it appears.



3) Open ***Hardware Properties***. Navigate through the alphabetical list to ***mSAC***, expand the section. There are 3 values you will need to enter for each objective:

Lens *N* mSAC micron / mSAC position - obtain from 3i support
Lens *N* mSAC position / micron - obtain from 3i support
Lens *N* Name - obtain from ***Configure Objective Lenses*** window (step 2 above)

NOTE: You can calculate mSAC micron / mSAC position and mSAC position / micron by following the Calculating mSAC Coefficients procedure.

4) After entering each parameter, click ***Apply***. When you have completed the process, ***Close*** the ***Hardware Properties*** dialog and restart SlideBook.

continue on to Using New mSAC Interface...

Calculating mSAC Coefficients

As previously discussed, mSAC coefficients Δ µm/SAC and Δ SAC/µm values are identical for all objective lenses of the same make and manufacturer. Should you wish to calibrate these values yourself, there is a sample you can prepare and a protocol you can follow.

Sample Preparation

1.Make a preparation of 0.1 µm fluorescent microspheres at a 1:100,000 dilution in warm 1% agarose. Be sure to shake the beads well before using. We like Life Technologies' FluoSpheres - Catalog #F-8801.

2. Add the agarose/microsphere mixture to a glass bottom dish with No. 1.5 coverslip.

3. Place dish in freezer and allow agarose to solidify. Bring dish to room temperature before using.

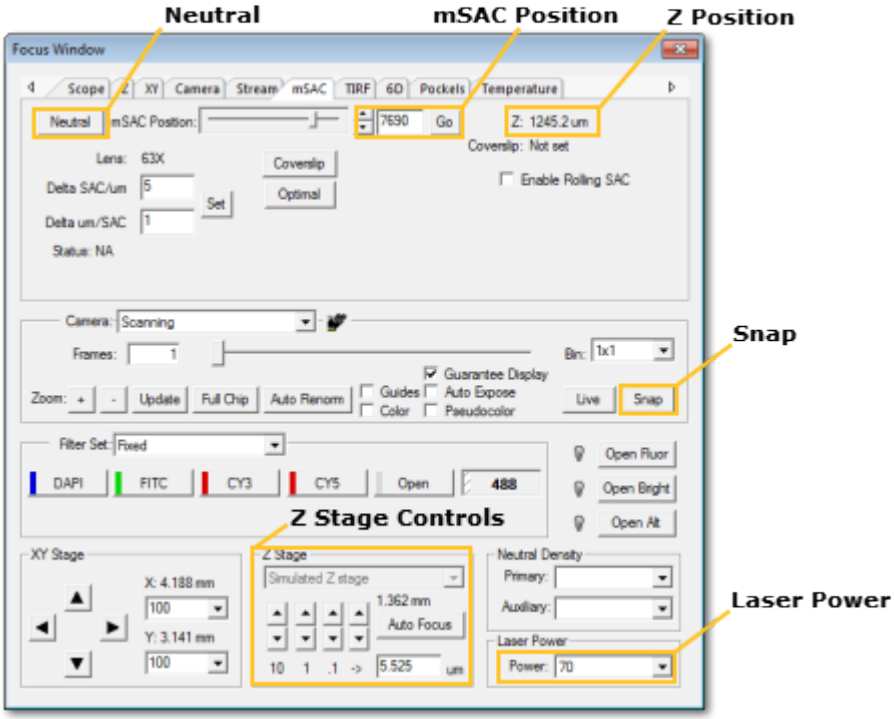
Make sure there is not state saturation

1. Set the mSAC to ***Neutral*** position (see also Setting Neutral Position) using the button in the mSAC tab of Focus Controls.

2. Image the beads at a known ***Laser Power*** using the ***Snap*** function.

3. Double the ***Laser Power*** and take another image using the ***Snap*** function.

4. Open the two images and compare the maximum intensity values using the ***Pixel Intensity*** tool. The values should double. If the values do not double, there could be state saturation. Reduce the ***Laser Power*** and repeat steps 2-4.



Δ µm/SAC

1. Focus on a bead located on the coverslip with the mSAC lens in the ***Neutral*** position.

2. Record the ***Z Position*** of the piezo Z stage.

3. Displace mSAC by + **4000** units by typing in the ***mSAC Position*** field and clicking ***Go***.

4. Refocus using the ***Z Stage Controls*** and record the new ***Z Position***.

5. Calculate $(Z_1-Z_2)/4000$ = Δ µm/SAC. Use for the Hardware Property ***Lens N mSAC micron / mSAC position***.

Δ SAC/µm

1. Focus on a bead located on the coverslip with the mSAC lens in the ***Neutral*** position.

2. Move the ***mSAC Position*** a known distance from the neutral position (around 2000).

3. Bring the coverslip back into focus. Set up 3D capture using current position and traveling 50 um.
   NOTE: It is best to use widefield fluorescence if possible.

4. With the image selected, click ***Create Projection*** (in the Home tab). Choose ***Maximum Operation*** and ***Over Y Axis***.

5. Locate the best corrected beads in the projection, and measure how far they are from the coverslip using the ***Ruler*** tool, the coverslip should be at the top of the Y projection image.

6. Calculate $(SAC_2-SAC_{neutral})/Z$ = Δ SAC/ µm. Use for the Hardware Property ***Lens N mSAC position / micron***.

Using New mSAC Interface

Before proceeding with using the new mSAC interface, it is imperative that you complete all steps in Configuring New mSAC Interface. Once calibrated and configured, the workflow for use is quite simple. There are two modes of operation to choose from as descibed below.

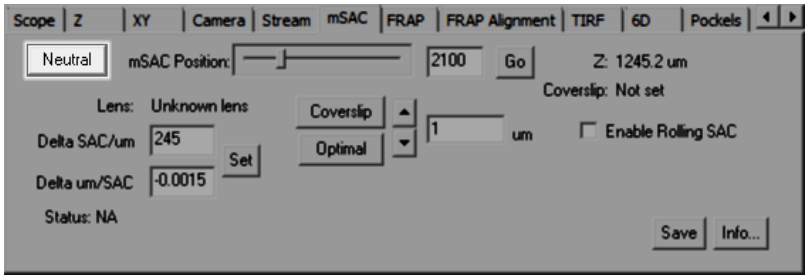
> **NOTE:** Regardless of the approach used, the coverslip will need to be found each time a new sample is placed on the microscope.

Setting Up Optimal Position mSAC

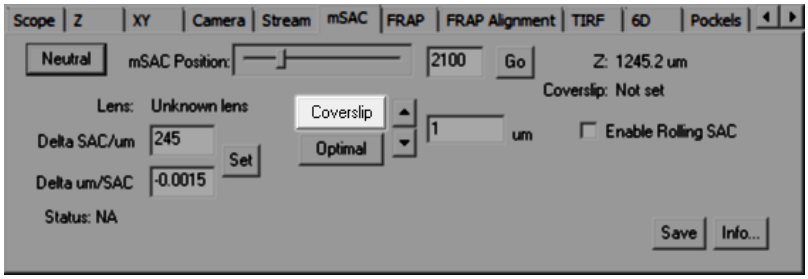
This mode is preferred when you need to image quickly or are imaging a relatively thin specimen. In this mode, the mSAC device operates like a finely tuned correction collar in that you set a position then continue with your experiment with the position static.

1) Have specimen on the microscope and ***Focus*** window open. Click on the ***mSAC*** tab.

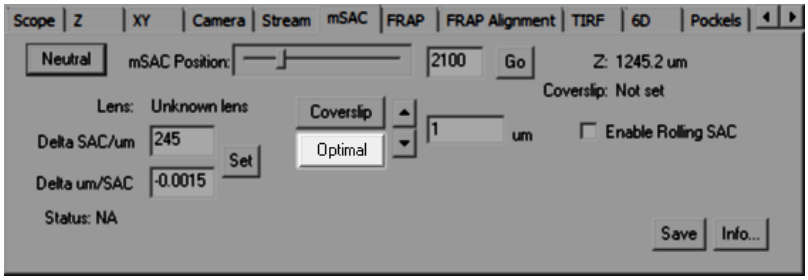
2) Click ***Neutral***.



3) Focus on the coverslip of the specimen. Click ***Coverslip***.



4) Focus into the specimen to desired focal plane for imaging or a central plane if imaging in 3D. Click ***Optimal***. The mSAC will move to the optimal position for this focal plane and remain in position.



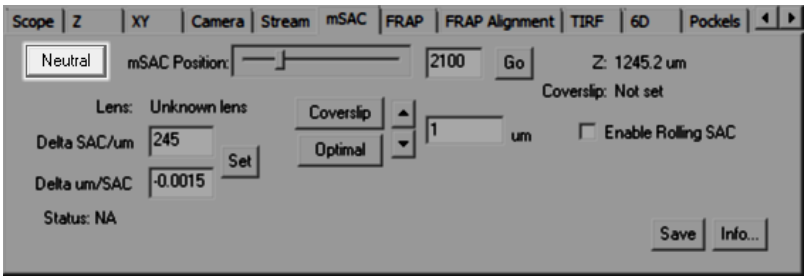
5) Continue setting up your experiment and capturing images as usual.

Setting up Rolling SAC

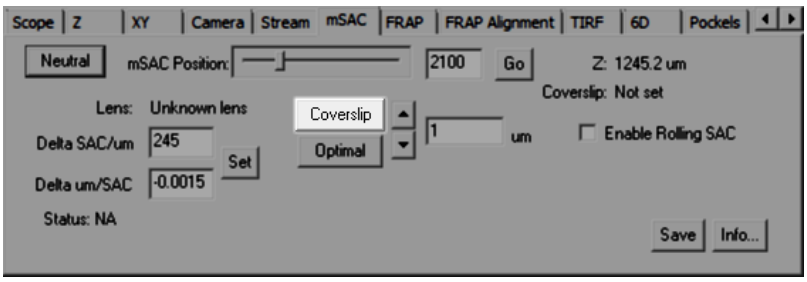
This mode takes full advantage of the innovation and optical technology of the mSAC device. This mode is preferred when you want the best axial resolution and contrast possible at each focal plane. In this mode, the mSAC lens position shifts to provide complete spherical aberration correction at each plane captured.

1) Have specimen on the microscope and ***Focus*** window open. Click on the ***mSAC*** tab.

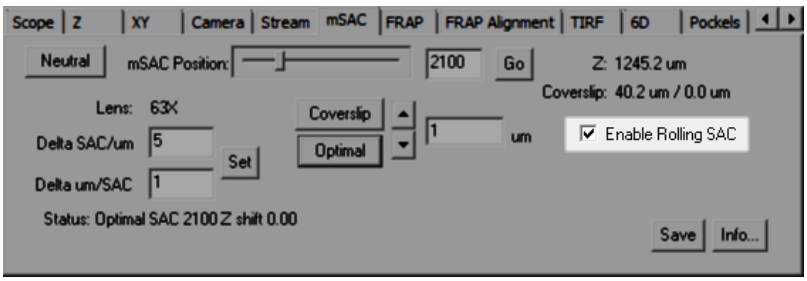
2) Click ***Neutral***.



3) Focus on the coverslip of the specimen. Click ***Coverslip***.



4) Check the box to ***Enable Rolling SAC***. This will initiate a SAC lens movement every time you change the z plane of imaging, thus correcting for spherical aberration for every image taken.



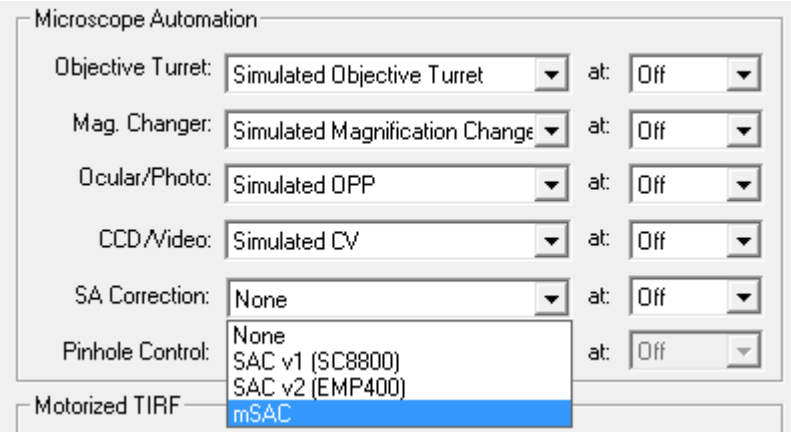
5) Continue setting up your experiment and capturing images as usual.

Configuring SAC in SlideBook™

Now that the hardware is installed, it is time to configure SlideBook to operate the SAC.

Software Configuration for Control of SAC

Open SlideBook™ and go to ***Administration tab >*** ***Hardware Configuration***. In the lower right corner of this dialog, you will find the ***Microscope Automation Configuration*** field. Select the appropriate SAC from the drop down menu and enter the appropriate serial port on the computer that the unit will be using for communication.



If you are unsure which version of SAC you own, as a general rule, follow the rule that units delivered in 2008-2010 are ***SAC v2 (EMP400)*** and that units delivered before 2008 are ***SAC v1 (SC8800)***. Another way to tell the difference between the two units is the color of the serial cable running from the control box to the computer. ***SAC v2 (EMP400)*** will have a tan colored cable that is wired directly into the box while ***SAC v1 (SC8800)*** will have a black serial cable that can be disconnected from the control box. Units delivered in 2010 or later are ***mSAC*** and will be labeled as such.

Next, go to ***Administration tab >*** ***Hardware Properties***, navigate to the section labeled ***SAC***, and expand the selection. Go to ***Maximum Position***, enter the value **1100**, and click ***Apply Change***. You can then close the window and restart SlideBook™ so the changes take effect.

Alignment of SAC

If the SAC is not aligned correctly you will get a pixel shift in x / y when the SAC position changes. To align the SAC, place a fixed sample on the microscope and begin imaging it in the ***Focus Window***. Switch to the ***SAC*** tab. You can shift the SAC position by clicking in different x locations on the chart and re-focusing the microscope. Observe the x / y pixel shift as the SAC moves. Shift the SAC body left, right, up, or down until the pixel shift is minimized. Once an optimal position is determined, secure the unit by bolting the positioning wedges to the antivibration table.

The SAC's front lens position, and thus the operating SAC range, can be shifted by the addition of spacer rings between the microscope and the SAC body. Three spacers should be provided with every unit (15mm, 20mm and 35mm). These can be locked in place with a 0.5mm hex tool. Locking is usually unnecessary. The addition of space sets the possible correction more towards imaging deeper into a less refractive medium than specified by the lens design (i.e. deeper into water using an oil immersion objective). Since most correction in biological imaging is of this variety, it is best to set zero point of the system, where the SAC is providing no change in correction from the objective itself, near the limit of front lens travel proximal to the microscope. For most installations, additional spacers are not necessary.

1) Align the condenser in Kohler illumination.

2) Close down the field diaphragm for transmitted light all the way so you can see the aperture on the camera.

3) Use an objective that just barely will fit the image of the field diaphragm in to field of view.

4) Make sure the entire imaging train is tightened to itself (Sac + camera or sac + camera + CSU). Move the whole imaging train with respect to the microscope until the field diaphragm is centered in the focus window.

5) Test rolling SAC as usual.

6) Adjust fine tuning using conventional method.

7) Determine and set the ***Optimal SAC*** position as described in Finding the Optimal SAC Position.

Using SAC in Image Acquisition

Now that the SAC is installed, configured in SlideBook™, and aligned, it is time to start using the unit. In this section you will learn the following:

- Finding the Optimal SAC Position
- Calibrating the SAC in 3D
- Notes on Spherical Aberration

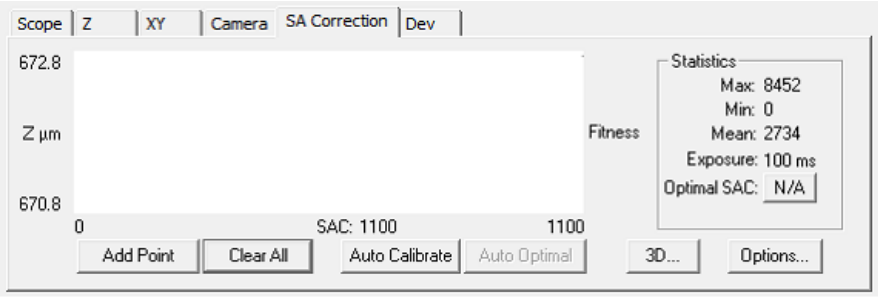
Please note that working knowledge of SlideBook™ is required to proceed with this portion of the manual. If you are not already familiar with the operation of SlideBook™, please review the Focus Controls Window and Capture Window or contact your 3i representative.

Finding the Optimal SAC Position

The SAC functions as an intelligent, computer operated correction collar. You can calibrate the SAC to use a single correction setting to eliminate spherical aberration in the center of your sample or plane of interest as described below.

If you wish to calibrate a neutral position for the SAC unit, prepare a slide using 0.1µm fluorescent microspheres mounted to a #1.5 coverslip. Perform steps #1-5 below and record the ***Optimal SAC*** position in the ***Statistics*** region of the tab. You can then enter this value under ***Administration tab >*** ***Hardware Properties > SAC > SAC Neutral***.

1. Open the ***Focus Controls*** window in SlideBook™ and navigate to the ***SA Correction*** tab. Focus on your sample and make sure the area of interest is in view.



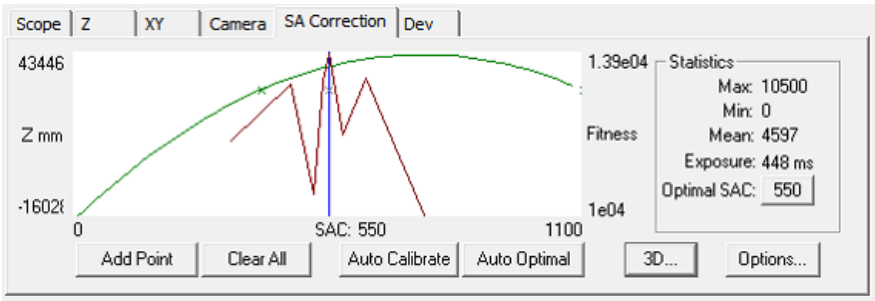
2. If previous data is displayed on the graph, click ***Clear All***. Confirm that your sample is in focus then click ***Add Point***.

3. There should be a vertical line which indicates the current SAC position on the x-axis of the graph (***SAC: setting***). Click on the graph at a different SAC position. You should hear the SAC move. Adjust the focus of your sample so that you are looking at the same image as in step #2. Click ***Add Point***.

4. Repeat step #3 several more times by adding more points. If you need to replace a point, click the point, adjust focus, and click ***Replace Point***. You should notice the formation of a green line. The green line shows the curve of focus vs. SAC position. Please note that each of the points on the green line represents a certain *z*-focus position and SAC position that will produce identical images.

> **NOTE:** As an alternate approach, you can start by clicking ***Auto Calibrate***. ***Auto Calibrate*** will go through a series of steps to estimate what the green line should look like. When this process completes, you can visit points along the line, adjust the focus to fine tune the image, and click ***Replace Point***. Continue this process until you have an accurate green curve.

5. Click ***Auto Optimal***. This will use the green curve you created to look for the highest quality SAC position. A red line will appear. The red line represents the image quality for each position on the green line. The SAC position that eliminates spherical aberration will have the best contrast. When you click ***Auto Optimal***, the SAC will automatically move to this position and create a blue vertical line that represents the optimal SAC position. The "Optimal SAC" position will also be updated in the ***Statistics*** region of the tab.



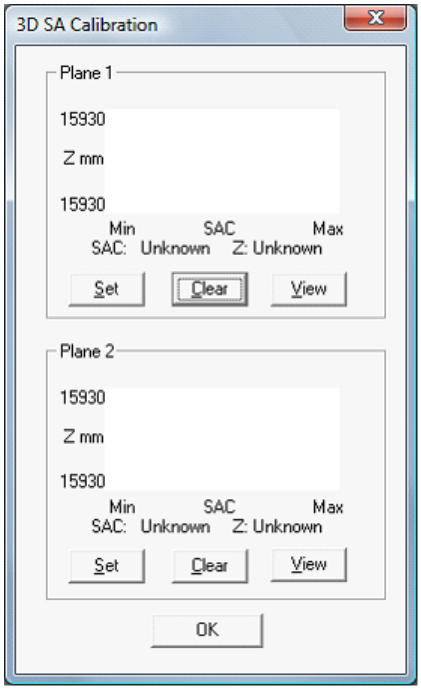
6. If your goal is to eliminate spherical aberration in a single plane, you can now proceed to the ***Capture Window*** and set up the experiment as you normally would. If your goal is to correct in 3D, continue on to Calibrating the SAC in 3D.

Calibrating the SAC in 3D

Now that you have learned how to correct spherical aberration in a single plane, it is time to learn how to apply this to 3D space. Below, you will learn how to calibrate the SAC to correct for spherical aberration at every imaging plane.

1. Follow steps #1-5 from Finding the Optimal SAC Position.

2. Click ***3D***. The following dialog will appear:



3. Click ***Set*** in the ***Plane 1*** region. The ***Plane 1*** graph will update to the calibration you just performed.

4. Return to the ***Focus Controls*** window. Click ***Clear All*** to clear the calibration graph.

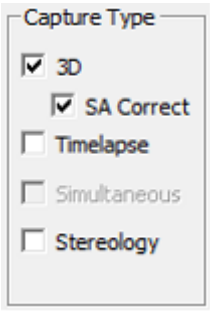
5. Focus on a different *z*-plane in the sample and repeat the calibration steps #1-5 from Finding the Optimal SAC Position.

6. Return to the ***3D SA Calibration*** dialog. Click ***Set*** in the ***Plane 2*** region. The ***Plane 2*** graph will update to the calibration you just performed.

7. Click ***OK***.

8. Proceed to the ***Capture*** window. Set up your experiment as desired (3D, timelapse, point visiting, etc.)

9. In the ***Capture Type*** region on the left side of the dialog, be sure to select ***3D*** and ***SA Correct***. Continue with standard acquisition.



Troubleshooting SAC

Below, you will find several tips for troubleshooting issues with your SAC. If these do not help alleviate the issues or if you are experiencing other issues that are not described below, please contact support@intelligent-imaging.com.

When SlideBook initializes, SAC makes a continuous loud noise

Solution 1: The version of SAC may be wrong in the hardware configuration. Change it to the correct version and restart SlideBook™.

Solution 2: The motor may be stuck. Close SlideBook, turn off the SAC, turn on the SAC, and restart SlideBook™. If this does not fix the problem, turn off the SAC and contact support at 3i.

Optimize button does not produce consistent results

Solution 1: Sometimes different samples require different algorithms to best calculate the quality. You can change the algorithm under the Settings button. For example, beads are best calculated using the max intensity algorithm. Actin strands would be best calculated using weighted high frequency. Try different algorithms until you find one you like.

Solution 2: If you are using the microscope z drive as your primary z, you will get hysteresis (meaning the z will not return to the same position every time). There is an option to use a linear search method for finding the green z vs. SAC curve and for finding the optimum position for the SAC, but it is much slower. Try this option.

SAC is silent on startup and will not initialize

Solution 1: It is IMPERATIVE that the appropriate ***Maximum Position*** is set under ***Administration tab >*** ***Hardware Properties > SAC***. This value should be **1100**. Please see Software Configuration for Control of SAC for additional information.

FLIM Module

Introduction

Fluorescence lifetime imaging (abbreviated as either FLI or FLIM) provides an entirely different dimension with which to characterize a sample's fluorescence apart from emission intensity when excited at a particular wavelength.

Long the domain of spectroscopy and biophysics labs, SlideBook's FLI module strives to make fluorescence lifetime an accessible tool for "mainstream" molecular biology research involving live cell imaging. In measuring a different physical property of a fluorophore, fluorescence lifetime imaging (FLI) has advantages in measuring protein proximity, polymerization, relative concentration of different molecules, separation of different labels with spectral overlap, ion concentration, and removal of autofluorescence.

SlideBook's FLIM module has been designed to work seamlessly with all types of acquisition supported by SlideBook: 2D, 2D timelapse, 3D, 4D, multi-channel, and multi-location.

Much like its fluorescence spectra, a fluorophore's lifetime serves as a molecular "fingerprint." As a molecule's lifetime is largely unrelated to its spectral properties, two molecules that may be nearly indistinguishable through conventional fluorescence measurement, such as FITC and GFP, can be easily separated using lifetime.

One of the most interesting properties of fluorescence lifetime images is that the lifetime measurement is concentration-independent (as long as there is sufficient concentration to detect a signal). Concentration issues frequently plague Förster resonance energy transfer (FRET) measurement using conventional microscopy, as precise bleedthrough correction measurements must be made to fluorescence channels that may have widely varying acceptor concentrations. Minor error in the correction measurement can have a large impact on reported FRET.

Using the fluorescence lifetime to measure FRET is convenient, because only the donor needs to be imaged. The presence of acceptor shortens the lifetime of the donor, and the FRET efficiency can be easily calculated directly from the change in lifetime:

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

where τ_{DA} is the donor lifetime in the presence of the acceptor and τ_{DA} is the donor lifetime when no acceptor is present.

Lifetime Acquisition

A frequency domain lifetime system measures the phase shift and demodulation of a fluorescent sample's emission, by modulating both the light source and the emitted signal at high frequencies (typically around 50-100 MHz).

A single lifetime plane (either in *z* or time) is constructed from taking N exposures at equally spaced phase shifts between the excitation modulation and the camera's intensifier modulation. For instance, if N = 4, four exposures are taken at 0, 90, 180, and 270 degrees, respectively.

The multiple phase-shifted exposures allow SlideBook to estimate the inherent phase shift and demodulation of the sample using what is called homodyne detection theory.

Notation and Units

The following will be explained in more detail and used throughout the documentation:

#	Lifetime (ns)
$\#_{phase}$	Lifetime (ns) as determined by a measurement of phase shift
$\#_{mod}$	Lifetime (ns) as determined by a measurement of demodulation
φ (#)	Phase measurement of a fluorescent signal with lifetime #
M(#)	Modulation measurement of a fluorescent signal with lifetime #
x	x-axis component of polar plot: $x = M(\#) \cos \varphi (\#)$
y	y-axis component of polar plot: $y = M(\#) \sin \varphi (\#)$

Lifetime Data Format

Rather than store the original phase-shifted images, SlideBook computes the best-fit phase shift and demodulation on a pixel-by-pixel basis and then transforms these to x and y locations on a polar plot described in Redford and Clegg (2005):

$$x = M(\tau)\cos\varphi(\tau)$$

$$y = M(\tau)\sin\varphi(\tau)$$

For each FLI exposure, SlideBook creates three channels:

- lifetime x component, designated as (X) following channel name
- lifetime y component, designated as (Y) following channel name
- mean intensity, designated as (Int) following channel name

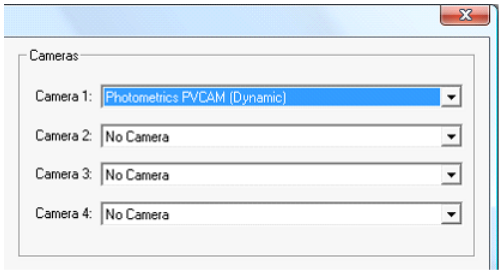
SlideBook stores a lifetime acquisition in three consecutive channels. The first two channels are the x and y locations of a pixel's value after the polar coordinate transform of the fitted phase and modulation lifetimes. The third channel is the mean intensity of the series of phase-shift exposures.

FLI Hardware Configuration in SlideBook

FLI hardware can be configured in the ***Administration tab***, ***Hardware Configuration*** and ***Hardware Properties*** dialog boxes.

Camera

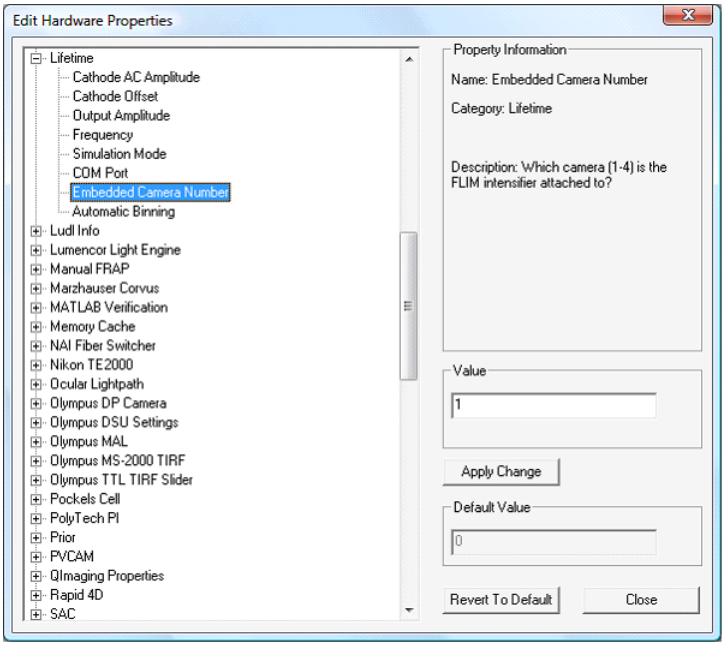
SlideBook controls the intensifier and camera combination as a logical "FLIM camera" that will have different properties than the camera itself. In the ***Administration tab***, ***Hardware Configuration***, select the camera driver that corresponds to the camera connected to the intensifier. Typically, this will be **Photometrics PVCAM (Dynamic)** if the camera connected to the intensifier is a CoolSNAP™ EZ.



If you have multiple cameras in the system, you must take note of which camera (1, 2, 3, or 4) corresponds to the one connected to the FLIM intensifier.

Embedded Camera Number

Under in the ***Administration tab***, ***Hardware Properties***, open the ***Lifetime*** group and set the ***Embedded Camera Number*** to which camera in ***Hardware Configuration*** is attached to the FLIM intensifier.



> **NOTE:** If you set ***Embedded Camera Number*** to **0** or **-1**, you are effectively turning off the FLIM component of the system, and SlideBook will not try to initialize the intensifier.

Automatic Binning

This is also found in the ***Lifetime*** section of ***Hardware Properties***. Set this to **Yes** to have SlideBook automatically bin 2x2 the FLIM camera. Since the intensifier only has a resolution of about 510 x 510, for many cameras (such as the CoolSNAP EZ) this is a good option as it will allow the attached camera to run faster with no real loss of resolution.

Intensifier

Most of the remaining ***Lifetime Hardware Properties*** have to do with configuring the intensifier.

Com Port

Set this to the virtual COM port that corresponds to the intensifiers USB connection to the computer.

Cathode AC Amplitude

This number sets the modulation depth of the intensifier. It has the following values: **0** = none, **1** = low, **2** = med, **3** = high. This should usually be set to **3**.

Cathode Offset

This number is in the range of **1** to **1200** and sets the offset of the modulation. Usually this is set around **593**. If this number is too high, then you will get nice bright images, but low modulation. If this number is too low, you will get little or no signal. This number should be set for a specific intensifier in the factory. Only change it if you have changed intensifiers, computers, etc. To find the optimal setting: change this number by **10**, go through the Pockels' cell optimization procedure below, raise this number until you get the brightest image while maintaining a good modulation level.

Output Amplitude

This is a number between **0** and **10** which determines the peak voltage of the output signal to the Pockels' cell. The default is **7**. Occasionally this number is reduced to **6** if the Pockels' cell is overheating.

Frequency

This is a number in MHz between **10** and **100**. Default is **50**. You can change this number if you are mainly working with long or short wavelengths. 50MHz is good for general use between 1 and 8ns. 80MHz is good for lifetimes at or under 1ns. 20MHz would be good for lifetimes at 10ns or greater. Once this number is changed, the whole calibration procedure must be redone.

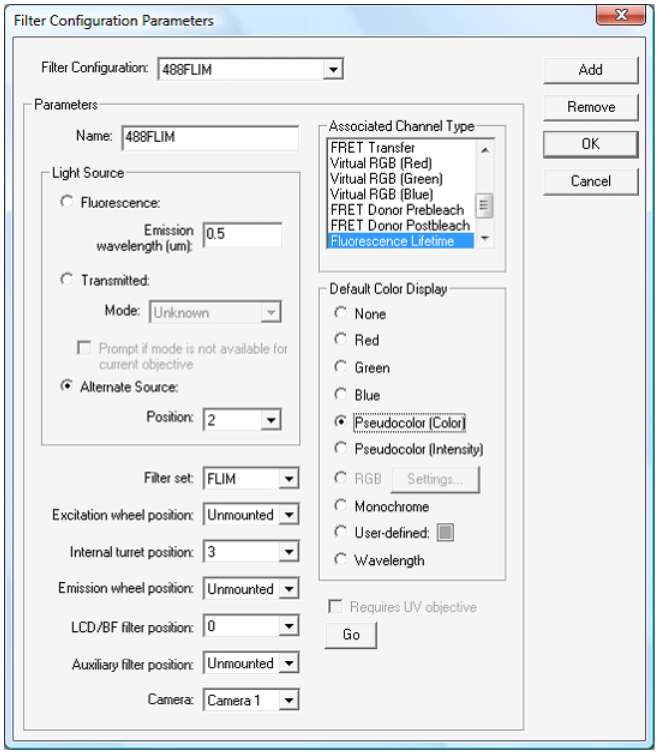
Simulation Mode

If you want to demonstrate the functionality of the system without having the hardware attached, in ***Hardware Properties***, you can set the ***Simulation Mode*** property to **Yes**. You will also need to set the ***Embedded Camera Number*** property to **1** (or whichever other test camera you want to assign it to). When you go to collect a FLIM image, you will get a field that has four distinct vertical bands. If you calibrate the left band to 1 ns, then the remaining bands will fit to 2, 3, and 4 ns respectively.

Defining Filters

Define a filter for each laser line that will be used for lifetime imaging (***Administration tab >*** ***Filters***). The ***Associated Channel Type*** should be set to **Fluorescence Lifetime** and the ***Default Color Display*** to **Pseudocolor (color)**. The turret, shutter, and alternative source channel need to be set correctly just as with any other filter configuration.

NOTE: It is important that you set the ***Camera*** field to the specific camera that is attached to the FLIM system.



Control and Calibration of FLIM

The next sections will walk through hardware control and calibration of the system. Control of the system is available in the ***Focus Controls***, and complete calibration consists of the following steps:

- Dark value correction
- Flat field correction
- Lifetime calibration

Focus Controls

Most of the FLIM-related control and feedback is available in the FLI tab in the Focus Controls. There are a few other components of the ***Focus Controls*** outside this tab that you will also need to use, however.

The FLIM camera works in three different modes:

- Modulation and intensification off – The intensifier's state acts as an electronic shutter for the attached camera. If the intensifier is off, no light is reaching the camera.

- Intensification on, modulation off – With the intensification on, but high-speed modulation off, the FLIM camera acts like any other intensified camera, with the intensification dictated by the ***Intensification*** slider on the ***Camera*** tab of the ***Focus Controls***. This is a useful mode to find your sample and ensure that the intensification is not set too high.

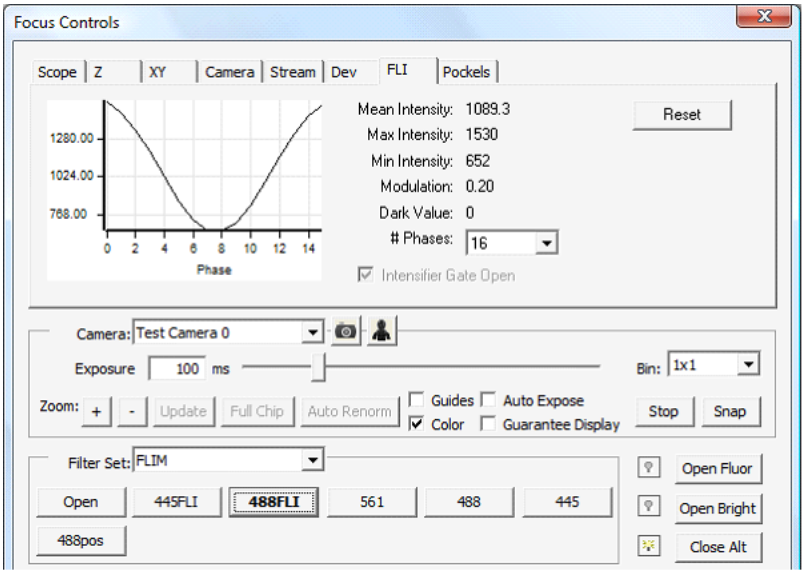
- Intensification and modulation on – This is the mode where a sequence of phase-shifted images are acquired and assembled into a lifetime image.

To turn on the intensification, click on the ***Intensifier Gate Open*** checkbox in the ***FLI*** tab.

To turn on the modulation, click on the ***Color*** checkbox in the main part of the ***Focus Controls***. Note that if you select ***Color*** (modulation) then SlideBook will automatically lock the ***Intensifier Gate Open*** state to on.

> **WARNING:** The intensifier is a sensitive device and can be damaged by too much light or intensification. There is a built-in shutoff circuit but please take care to turn the ***Intensifier Gate Open*** or ***Color*** checkboxes on with the ***Intensification*** set to a minimum level. Slowly raise the ***Intensification*** value until the mean intensity of the sample is approximately 2/3 of the dynamic range of the CCD, and no more. If you do accidentally overload the intensifier, a red light will appear on the control box and you will need to reset the intensifier (and restart SlideBook).

When modulation is on (i.e., ***Color*** is checked), the ***FLI*** tab will display a graph of the mean intensity of the phase-shifted exposures, as well as relevant statistics. ***Mean Intensity*** reports the mean intensity across all phase exposures. ***Max Intensity*** is the maximum of mean intensities of the phase exposures and ***Min Intensity*** is the minimum of the mean intensities of the phase exposures. ***Modulation*** represents the depth of modulation, and a good target value is around 0.2. ***Dark Value*** will be described later.



Dark Value Correction

SlideBook handles dark correction slightly differently for FLIM than for other types of capture.

When you acquire an image using ***Capture*** and select ***Dark Field*** for a FLIM channel, SlideBook will first take an exposure with no illumination and compute the minimum value of the result. SlideBook then stores this value as the ***Dark Value*** (which will be subsequently visible in the ***FLI*** tab of the ***Focus Controls***). All future FLIM acquisitions will subtract this value from each phase shift exposure. While the camera offset of the camera typically attached to the intensifier (a CoolSNAP EZ) has a relatively low offset (a value around **90** out of **4095**) it can still impact proper modulation measurements particularly with low signal levels.

Phase Flat Field Correction

It is common for there to be some variation in phase across the intensifier cathode. This is referred to as phase rollover. To correct for this, SlideBook can take an image of a sample with uniform, finite lifetime (such as fluorescein) and create a phase flat field, which can be stored and applied to images just like intensity-based flat fields.

Ideally, phase flat fielding should be performed even before calibrating the system with an image of a sample with known lifetime.

To create a phase flat field, place a homogenous sample on the microscope. You can monitor the unmodulated fluorescence signal by opening the live ***Camera*** window and ***Focus Controls*** and selecting the appropriate lifetime filter configuration. Click on the ***FLI*** tab and check ***Intensifier Gate Open***.

Make sure the signal is well below saturation by clicking on the ***Camera*** tab and looking at the histogram. You should adjust exposure time and lifetime intensifier intensification so that the brightest pixel is about 2/3 the dynamic range of the camera.

Then, run a ***Test*** exposure in ***Capture*** and make sure to check the ***Dark Field*** option.

After that, collect a full-chip FLIM image at 1x1 binning by going to ***Capture***. Select only one FLIM channel for capture. Select the ***Dark Field*** checkbox but leave the ***Flat Field*** option unchecked.

Once captured, select the image in the slide view in which it appears and select ***Modules tab >*** ***FLIM > Compute Phase Flat Field***.

If you want to verify that it was created, and looks reasonable, select ***Administration tab >*** ***Flat Fields > Manage Flat Fields***. You should see an entry that corresponds to the FLIM filter configuration. A proper phase flat field should present a smooth image. If the FLIM camera's chip size is larger than the intensifier screen, you may see the signal as a clipped circle within a larger rectangle of background. Depending on the level of phase rollover present in the system, it may look like either smoothly varying concentric rings or a flat disc.

Lifetime Calibration

Now, in order to calibrate the system for lifetime computation, you need image a homogenous sample with either a known single lifetime or known #phase and #mod components. Typically, this is a thin layer of fluorescein (with a known lifetime around **4.0**).

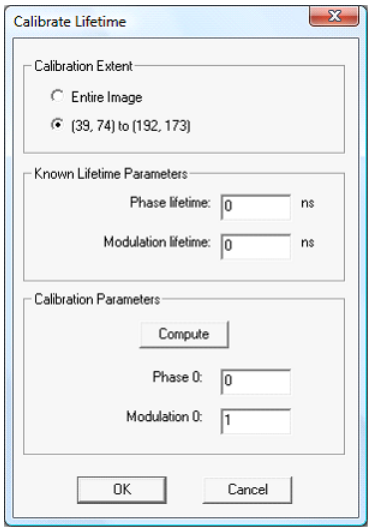
If the system has been previously calibrated, you must first clear the existing calibration from SlideBook. Do this by selecting

Modules tab > ***FLIM > Clear Calibration***.

Once you capture the image and open a view of it, select a homogenous region of the sample with the marquee tool, and then

choose ***Modules tab >*** ***FLIM > Calibrate***.

The following dialog box will appear:



If the known sample has a single lifetime, enter the lifetime for the sample in both the ***Phase lifetime*** and ***Modulation lifetime*** edit fields. Otherwise, enter the known #phase and #mod components in the corresponding edit fields.

Click on the ***Compute*** button.

SlideBook will determine the calibration parameters.

When you click ***OK***, SlideBook will use these new calibration parameters for all subsequent image captures. Old images that you load, however, will still have the calibration parameters in effect when you collected the image.

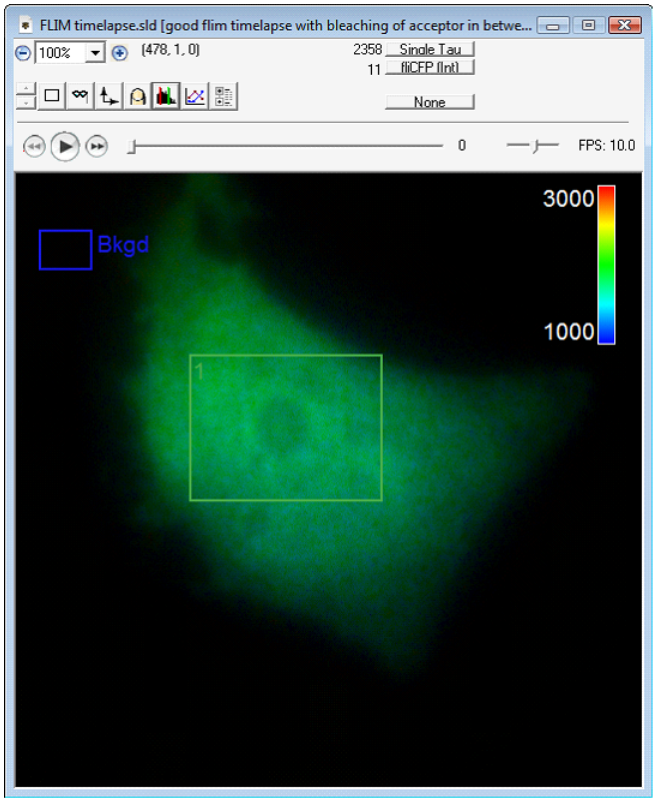
Acquiring and Viewing a Lifetime Image

Acquiring a lifetime image, once the system has been calibrated, is easy. Simply select the lifetime channel that corresponds to the excitation wavelength that you want when you are setting up a new experiment in ***Capture***. You can collect several lifetime channels or you can mix lifetime and non-lifetime channels.

Creating a Single-τ Channel

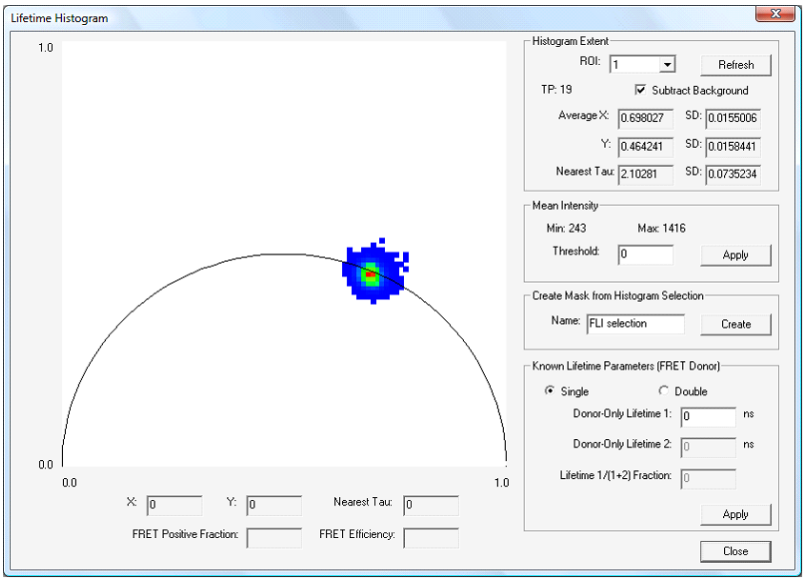
Acquiring a FLIM image produces three component channels, (**X**), (**Y**), and (**Int**). While the x and y component values are related to lifetime, and can be useful to generate simple lifetime-based contrast, a proper scaled display requires that you create a fourth channel that maps to lifetime directly. You can do this by selecting ***Modules tab > FLIM > Create Single Tau FLI Channel***.

If you use the pseudocolor display mode for a view, and set the hue channel to the generated **Single Tau channel**, and the intensity channel to the (**Int**) component channel, and set the renormalization range of the **Single Tau channel** to a reasonable range of ns (x 1000), such as a ***Low*** of **1000** and a ***High*** of **3000**, then the display will map color to nearest single # with a range determined by the renormalization range. In the case below, we see a pseudocolored cell displayed in a range where blue is 1 ns (1000 ps) and red is 3 ns (3000 ps):



The Lifetime Histogram

The lifetime histogram displays the lifetime for a selected region using the polar plot representation:



In order to bring up the lifetime histogram, create at least one region in the image. If you believe there is a significant background component, you should also create a background region. Then, select ***Modules tab > FLIM > Lifetime Histogram***.

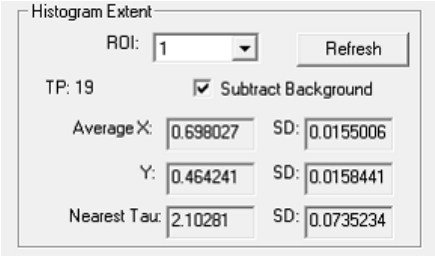
The polar plot itself gives a reading of the number of pixels in the selected region that have specific x and y values. The redder the display, the higher the number of pixels that have that corresponding x, y value.

Lifetimes with a single exponential fall on the semicircle. The lower right position at (1.0, 0.0) represents zero lifetime, and the lower left position at (0.0, 0.0) represents infinite lifetime.

If you see a single, circular, relatively compact cluster, as in above, you are looking at a region of spatially homogenous lifetimes with good signal to noise. If you see a multi-modal cluster or multiple clusters, you are looking at a region with a spatially inhomogeneous mixture of lifetimes. If the cluster is spread out, or potentially filling much of the plot, you probably have insufficient signal to make a good determination: one of the nice properties of the polar plot is that the compactness of the cluster correlates with the degree of error.

Histogram Extent and Background Correction

The upper right corner of the dialog allows you to select which region to plot as well as whether it is performing background correction. It also gives useful statistics about the lifetime within the selected region.



In order to change to view a different ROI, simply select a different region using the ***ROI*** dropdown.

If you move a region on the corresponding view, or change which timepoint that view is displaying, then you will need to click the ***Refresh*** button to update the display.

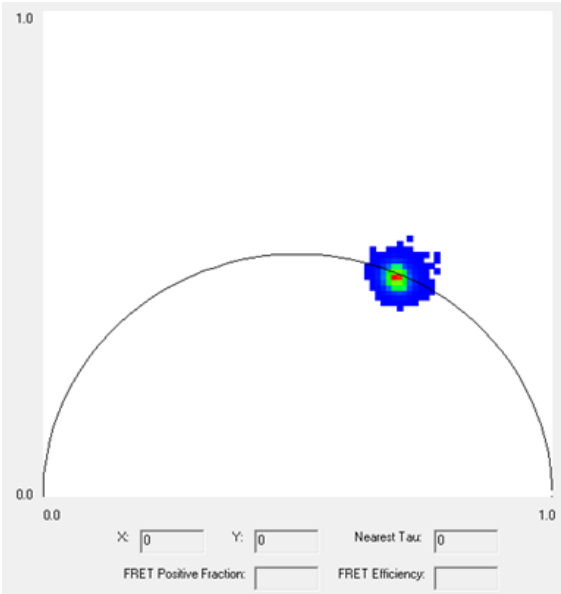
If you click on ***Subtract Background***, the plot will compute the lifetime of the background region and shift the plot accordingly. Note that background here is strictly defined to be regions that have fluorescent signal but are not the label of interest. So, for instance, if you are imaging a GFP-RFP FRET construct that is expressed in either only a fraction of cells or a spatially-resolved compartment within each cell, against a background autofluorescence, you would want to select a background region that contained only the autofluorescence.

The plot displays the average and standard deviation for the x and y values as well as the nearest tau, which is the point on the semicircle closest to the weighted center of the region's cluster in the plot.

Calculating FRET using Lifetime

In order to calculate FRET using lifetime data, you must either know the lifetime of the donor in the absence of any acceptor, or measure it in a control experiment. Probably the best way to do the experiment is to take a sample with the same FRETting constructs as your intended experiment and bleach out the acceptor.

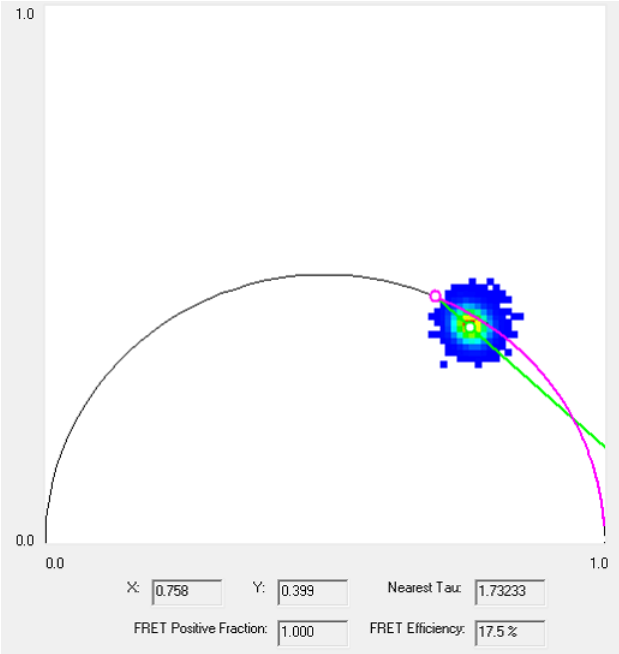
A quick visual aid for determining FRET for a region is available in the lifetime histogram.



For instance, in the histogram example above, we are looking at the lifetime of a region of a sample after progressive bleaching of the acceptor for 19 timepoints. If we assume that we have fully bleached the acceptor, the donor-only lifetime is 2.1 ns. Since it lies so close to the semicircle, we will assume it is a single exponential.

If you select ***Single*** in the ***Known Lifetime Parameters (FRET Donor)*** section of the histogram dialog box, and enter 2.1 as the ***Donor-Only Lifetime 1***, then press ***Apply***, the histogram will draw a magenta circle at the 2.1 ns position along the semicircle and then re-color the portion of the semicircle that represents the 0% FRET to 100% FRET "trajectory" as magenta as well. Any (potentially) FRETting sample with the same donor will lie either somewhere along this magenta portion or within the chord that goes from the 2.1 ns position to the (1.0, 0.0) endpoint.

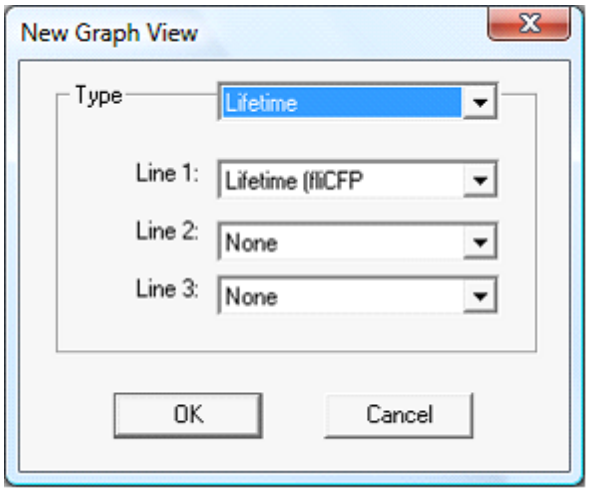
If we double-click on the cluster in an image—in this case it is the first (pre-bleach) timepoint of the same timeseries—then the histogram will draw a straight line from the 2.1 ns position, through where you double-clicked, and on through the semicircle.



Under the plot, the dialog box reports the nearest single-exponential # of where you clicked as well as the average FRET efficiency (in this case 17.5%) of the region.

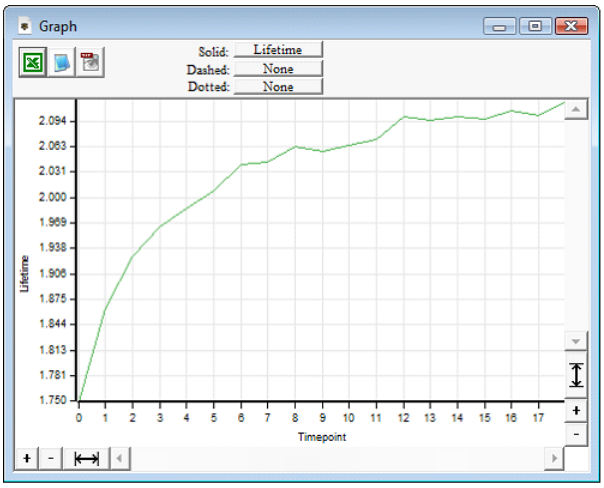
Graphing Lifetime

Graphing lifetime in timelapse images is straightforward. Create regions (and optionally a background) and select ***Graph***:



Select ***Lifetime*** for the graph ***Type***, and then choose which lifetime channel(s) you wish to display.

The resulting graph will show lifetime versus timepoint:



Multiphoton Scanning Module

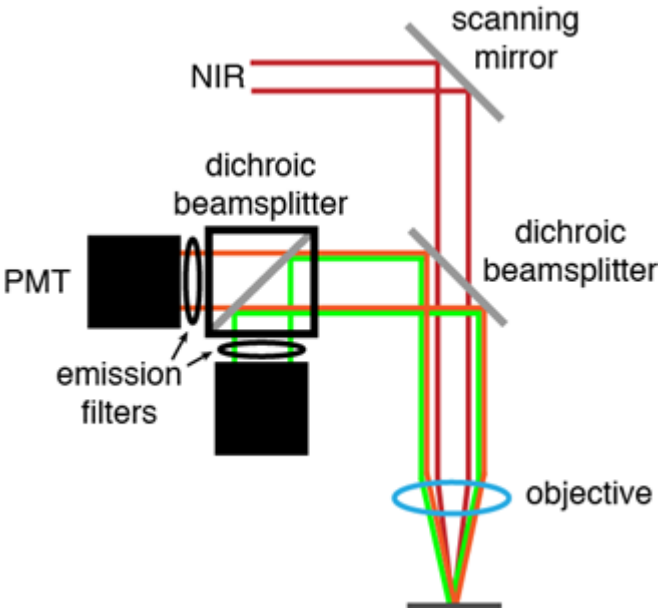
One of the principal design goals of the SlideBook scanning interface is that it be a natural extension of SlideBook and take full advantage of the many tools that comprise SlideBook and have been developed over the past sixteen years.

As many of 3i's Vivo™ Multiphoton and Marianas™ Multiphoton systems combine both scanning and camera-based acquisition, it is important that you can move easily between the two modes. Therefore, you will find that many aspects of the control and acquisition user interface, such as the focus window and capture dialog, will behave very similarly in both modes. In fact, some systems are customized to allow for simultaneous camera and scanning operation.

Principles of Scanning and Scanning Controls, are vital to fully understand the differences between camera and scanning capture and the additional settings that need to be adjusted for scanning.

Principles of Scanning

A scanning system offers tremendous flexibility in terms of how images are captured. Unlike a camera-based system, you are not tied to a particular resolution or image extent.



There are five parameters that are all related, and understanding their interaction will help you choose the best values for a particular experiment:

- scan extent
- resolution
- microns per pixel
- pixel dwell time
- frame time

Scan extent is the physical extent, in microns, of the specimen plane that you wish to scan. Depending on its optical and software configuration, a Vivo Multiphoton system equipped with a 20x/1.0 W objective can have an extent (up to 500microns / in excess of 750 microns in both axes.

Resolution is the number of pixels along the x- and y-axis that the scan will be divided into. Typical values may be 256 x 256, 512 x 512, 1024 x 1024, but they do not need to be restricted to powers of two or have the same number of pixels along each axis.

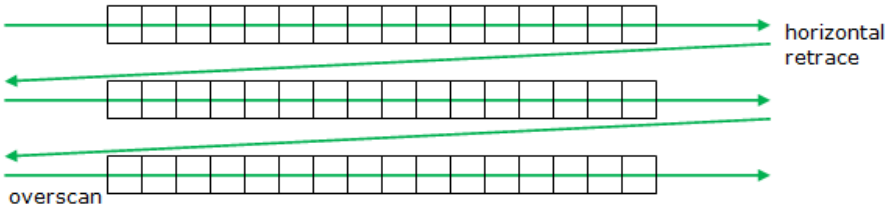
Microns per pixel is the spacing from one pixel to the next in the specimen plane. The physical nature of light sets a practical limit on how small this can be set and for you to expect to resolve additional detail in the sample.

Pixel dwell time is the total time (expressed in microseconds) that the beam effectively "hovers" over a pixel in order to record emitted photons before proceeding to the next pixel. Of course the galvanometers are in constant motion when scanning an image, but the concept remains valid.

Frame time is the amount of time it takes to collect an entire frame.

Scan Pattern

Because the scan is being performed by mirrors that must move in a continuous motion, there are two additional factors to consider: *retrace* and *overscan*.



In unidirectional scanning, the beam moves in a horizontal line and data are collected for the corresponding row in the resulting image. Then the beam is returned to the same horizontal position it started at before, with an adjustment in the vertical position, and data are collected for the next row. No data are collected during this horizontal repositioning, which is called a *horizontal retrace*.

In bidirectional scanning, data are collected as the beam is moved in both directions.

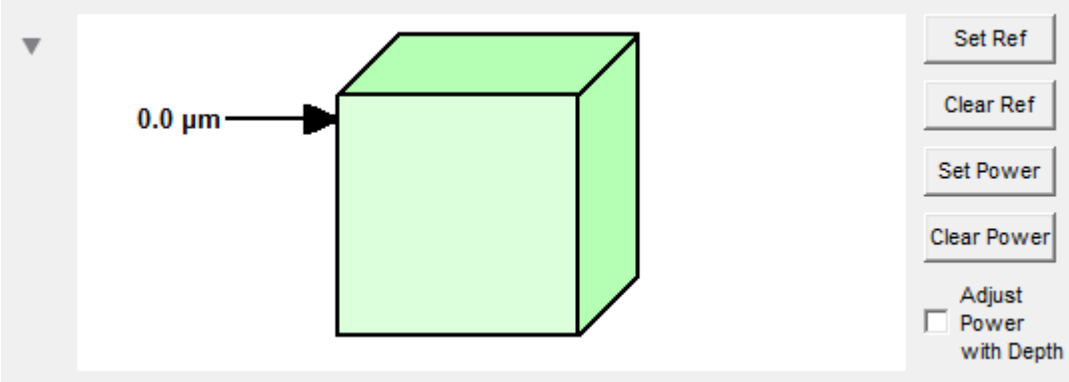
In either type of scanning, it is important that the mirrors are moving at a constant angular velocity while data are being collected. As mirrors have mass, it is therefore necessary to decelerate the mirrors at the end of an image's row and then accelerate them in the opposite direction. The path that the beam takes during this reversal is called overscan, and no data are recorded during it. In fact, in a Vivo™ Multiphoton system, the beam is actually turned off during the overscan motion in order to reduce the amount of excitation light that the sample sees that does not contribute to the image signal.

Adjusting Power for Depth

When imaging using your multiphoton system, you may notice intensity levels dropping off as you image deeper into a sample. This is due to tissue absorption and scattering. SlideBook can compensate for this by increasing the laser power as a function of z depth. Follow the instructions below to calibrate laser power for z depth.

1. Open the Scanning dialog, bring the specimen into focus and adjust scanning parameters as desired.

2. Expand the section below ***Scan Field***, the following portion of dialog will appear.

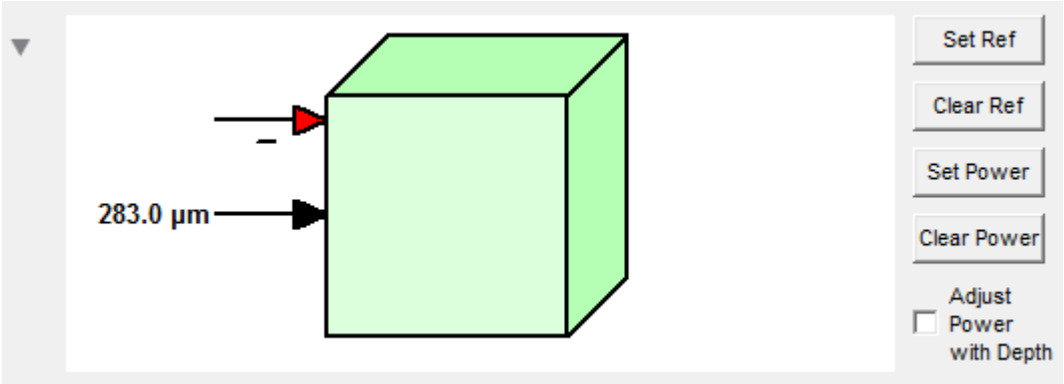


3. Adjust the focus of the microscope to the top of the sample (side closest to the objective lens). If you are using a piezo z stage, proceed to the next step. If you are using an analog (microscope stand) z control, Click ***Set Ref***. A red arrow will appear next to the green cube and the stage value will be displayed.

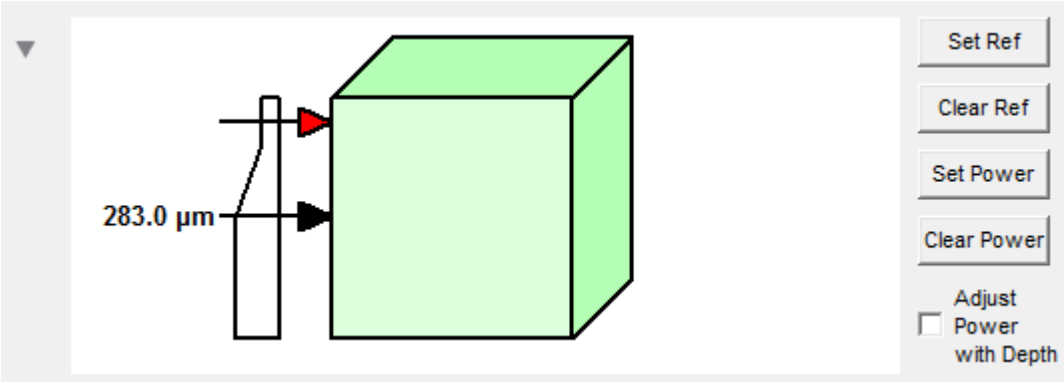
> **NOTE:** ***Set Ref*** is used to set the range of the green diagram cube for visualization purposes when using the microscope's internal z drive. The cube will represent 1000um in this case. When using a piezo z device, the cube represents the full range of travel for the piezo drive (usually 400um).

4. Move near the top of the imaging range, You will notice a black arrow moving away from the red arrow. Adjust the laser power to produce the desired results. You may wish to ***Snap*** an image from the Focus Controls or refer to the ***Max Signal*** level displayed for the PMT. Once you are satisfied with the image quality, click on ***Set Power***.

5. Continue moving deeper into the sample. You will notice a dash where you set the previous laser power and a red arrow if a reference position was set.



6. Once you are near the maximum desired z depth, adjust the laser power as described in step 4 and click ***Set Power***. A power ramping wedge will be drawn to indicate the first power setting ramped up to the current power setting. Notice the increase starts below the red reference arrow, where the dash was in step 5.



7. Check the box to ***Adjust Power with Depth***. Focus through the imaging range to ensure intensity remains consistent. If you are satisfied with the results continue on to set up image acquisition. If you are not satisfied, click ***Clear Power*** and repeat the laser power setting calibration using the steps above.

Line Scanning

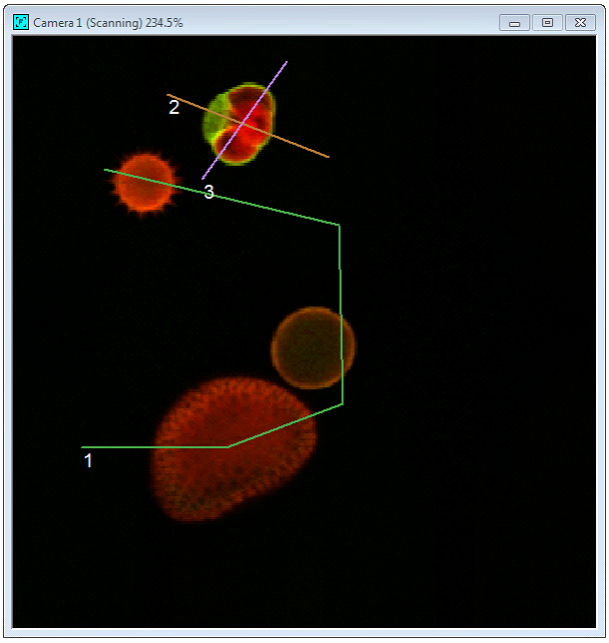
It is possible to scan along a single line back and forth very quickly to capture dynamics up to the several kHz range.

1. Using the Scanning dialog and Focus Window, locate the specimen and focus on it using standard 2 Photon functions find the region of interest in the live window.

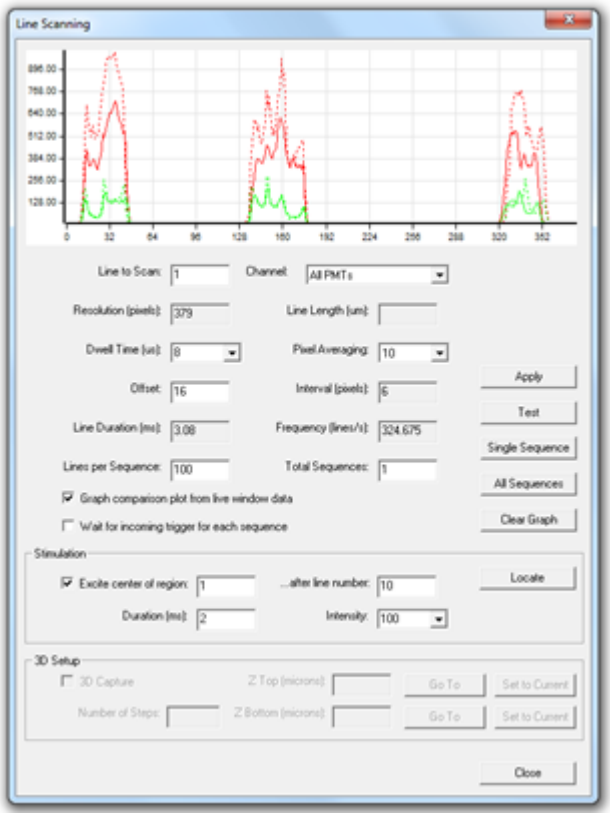
2. Draw a line using line region tool

SlideBook uses line annotations in the live focus window to define where you want to line scan. You can create a line by selecting the line annotation tool from the Regions group of the Home tab.

Click to define the end points of the line across the feature in the sample where you want to perform line scanning. If you want to draw a line that has multiple segments, just hold the **Ctrl** key down and click for each "vertex" except for the very last one. Please refer to Region Tools for more information on using these tools.



When you have created a line (or lines) that you want to scan, you can open up the ***Line Scanning*** dialog in the Home tab, Acquire group, ***Multiphoton*** menu > ***Line Scanning***.

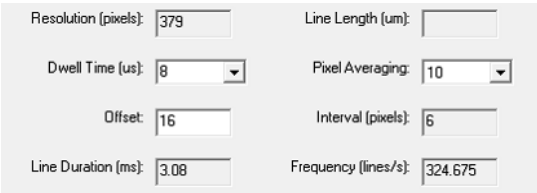


The ***Line to Scan*** edit field specifies which of the numbered line regions in the live focus window will be scanned.

Dwell Time (µs) determines the pixel dwell time just as in the Scanning dialog box.

Once you click the ***Apply*** button, SlideBook will determine how fast it can perform line scanning given the length of the line and the dwell time specified. Because of mechanical limitations on the speed and acceleration of the galvanometer scanners, you may be asked to extend the dwell time to effectively slow down the line scanning.

For instance, after selecting line 1 to scan and setting the ***Dwell Time (µs)*** to **8.0**, and clicking ***Apply***, the ***Resolution (pixels)***, ***Interval***, ***Pixel Averaging***, ***Line Duration (ms)***, and ***Frequency (lines/s)*** fields are filled in.



Resolution (pixels) is the length of the line in pixels and represents how many pixels will actually be recorded from. **NOTE:** The number of pixels and the microns per pixel of the line is determined by the current resolution and pixel size of the region in the live focus window. For example, if the live window focus represents a 256 × 256 resolution with 0.5 micron pixels, and you draw a horizontal line that spans half the window, it will have a resolution of 128 pixels that will each be 0.5 microns in size.

Pixel Averaging is currently set to find the optimal number of samples to take per pixel without exceeding the limits of the acquisition hardware.

Offset accommodates the fact that there is a finite delay between when the galvanometer scanners are sent to a position in the field and when they actually get there. By default, it is set to the ***Default Scanning Offset*** in the ***Vivo 2p Settings*** section of the hardware properties, adjusted for the currently specified dwell time.

Interval (pixels) is the number of additional pixels that must be added to each line in order to give time for the galvanometer scanners to decelerate, reverse direction, and accelerate so that the recorded line itself represents a linear scan.

Line Duration (ms) is the amount of time it takes to record a single line (including the interval mentioned above).

Frequency (lines/s) is the reciprocal of the line duration.
NOTE: Currently line scanning is unidirectional, which means that after scanning a line in one direction, the galvanometer scanners are returned to their original position to scan in the same direction again. A future version of SlideBook will support bidirectional scanning which will increase the maximum frequency of line scanning, particularly for smaller scans.

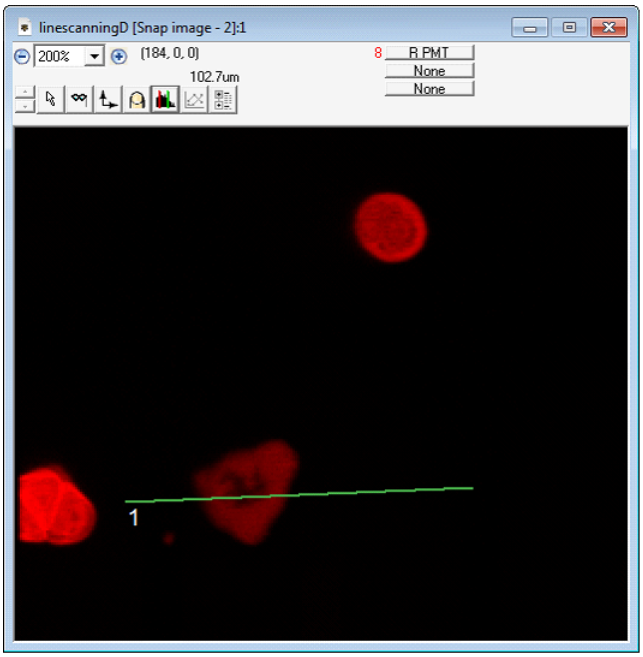
Lines per Sequence specifies the number of lines that are scanned at once. SlideBook organizes a sequence of line scans as a 2D image plane, where the x axis is the recording along a single line scan and the y axis is which line scan in the sequence it represents.

Clicking ***Single Sequence*** will initiate scanning for one sequence and add the resulting 2D image to the current slide. If you have ***Wait for incoming trigger for each sequence*** checked, SlideBook will wait for the configured external device to provide a pulse before beginning the sequence. The graph at the top of the Line Scanning dialog box will update to represent the line recording averaged across all lines in the sequence.

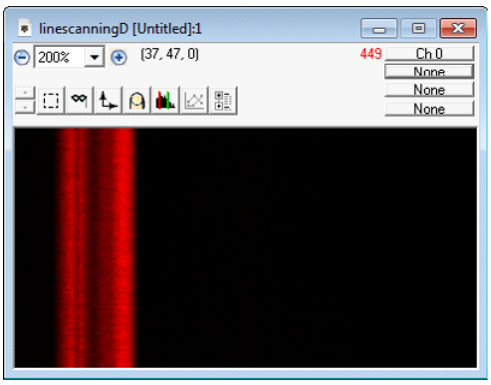
Total Sequences specifies the number of sequences to be run in a capture. Each sequence will be added as a new plane in a 3D image, where the x axis is the recording along a single line scan, the y axis is which line scan in the sequence, and the z axis is which sequence in the entire capture.

Clicking ***All Sequences*** will initiate scanning for as many sequences as you specified with ***Total Sequences***, and if ***Wait for incoming trigger*** for each sequence is checked, then the external device will need to provide a pulse to initiate each sequence.

Below is an example of a selected line in the live focus window:



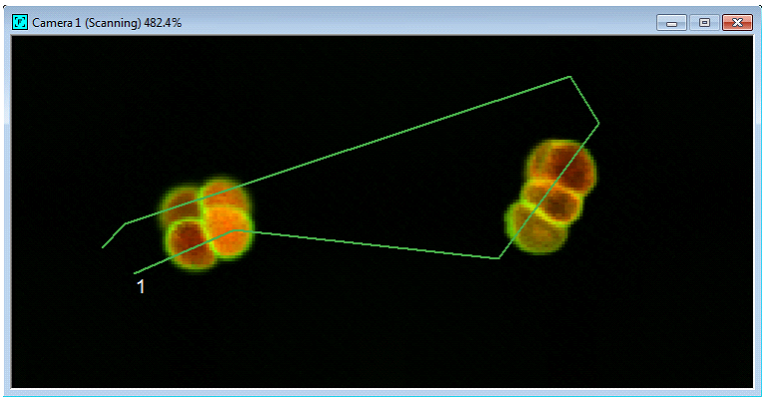
And the resulting image produced by a single line scan sequence:



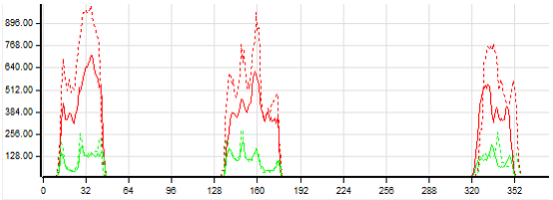
The line was 144 pixels in length, so the resulting line scan image is 144 columns wide and 100 rows high (where each row represents a single scan in the sequence of 100 scans).

If you select ***Graph comparison plot from live window data***, in addition to the averaged plot lines for the two PMT channels, SlideBook will draw additional (dotted) lines that represent the intensity values along the line in the live window image. This serves as a confirmation that the path taken during the line scanning corresponds closely to the line specified.

For instance, if the path specified is as follows:



and the user runs a single sequence, the graph will be updated with the actual (average) line scan intensity as well as the intensity along the line annotation in this image.

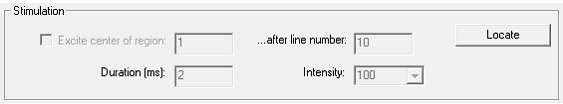


> **NOTE:** The direction of travel of the line scan is always from the end of the line annotation with the number to the end without. In this example, the scan is in a counterclockwise direction.

To clear the plot, simply click on the ***Clear Graph*** button.

"In-line" Photostimulation

For systems equipped to perform photostimulation using the same scanhead (but a different laser line) you can specify such an event using the ***Stimulation*** settings:



Use ***Excite center of region*** to turn on this mode and specify which region you wish to stimulate. The point that is stimulated will be the center of the region you specify, or if you select a line, it will be the midpoint of that line.

Once after line number of line scans have been performed, the galvanometers will move the beam to the specified position and the photostimulation laser will be turned on for exactly ***Duration (ms)***. Laser intensity can be set using the ***Intensity*** dropdown list. Afterward, the galvanometers will return the beam to the line being scanned and resume scanning.

> **NOTE:** Currently intensity dictates the percentage of the maximum modulating voltage applied to the laser diode or the AOTF. It is not necessarily linear.

Determining Actual Path Taken

If parameters are chosen such that the line scanning is extremely fast (e.g., > 2 kHz) the path taken may be either slightly shortened from the line drawn or, in the case of a multi-segment line, the path taken may be slightly curved with respect to the line segments drawn.

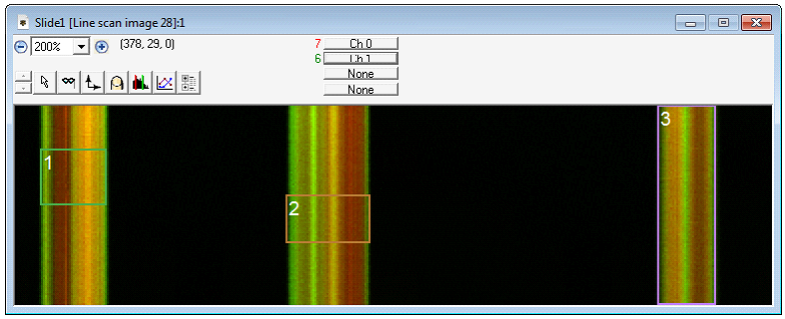
VIVO™ Multiphoton systems are equipped to record the exact path taken, and you can display this on a snapped (2D) image of the field in which the line was drawn.

Either using the ***Snap*** button in the focus controls, or by doing image capture, acquire an image of the field in which the line was specified and select that image in the corresponding slide view. Then, click ***Test***. SlideBook will run a test scan and create a new mask in the selected image that displays the actual path taken.

Graphing Line Scans

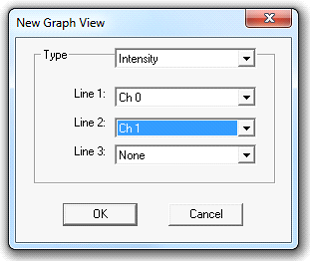
In order to graph a line scanning sequence over time, open the line scan image of interest and create rectangular regions just as you would in a normal image (see also Region Tools). In a line scan image, SlideBook will automatically assume you want to see the line scan across all timepoints, regardless of where you place the region vertically. However, the portion of each line scan that it averages to compute each position in the plot is determined by the horizontal extent of the region.

So, for instance, in order to graph three different sections of a line scan image…

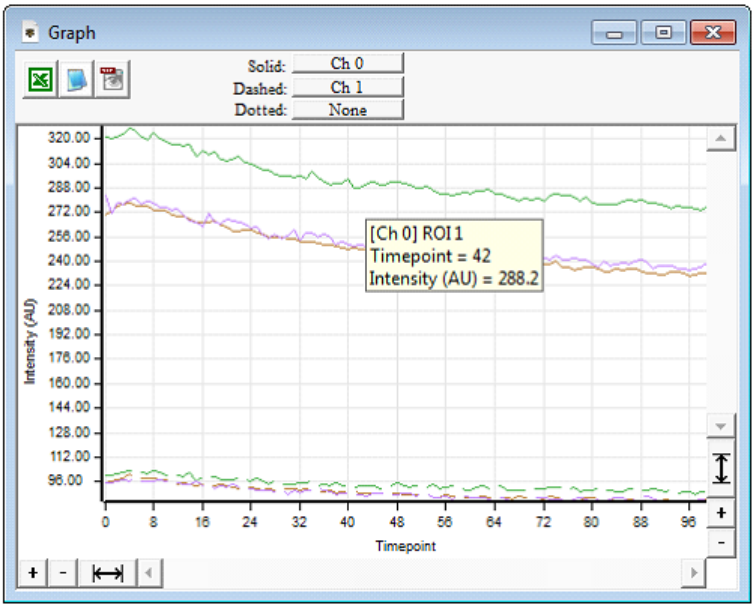


You create three different regions and then select ***Graph*** either in the Evaluate group of the Process tab or by the ***Graph*** icon in the view window.

This brings up the following dialog box, where you can select which channels you wish to graph as well as what you want on the vertical axis (e.g., intensity, ΔF / F, etc.):



The resulting graph view will show the first channel intensities as a solid line and the second channel intensities as a dashed line. You can easily change which channels are displayed by selecting the corresponding Solid, Dashed, and Dotted dropdown lists at the top of the new view.



Each region’s plot line is the same color as the corresponding region in the original view.

Placing the mouse cursor over a plot line will pop up channel, region, timepoint, and intensity information at that position.

Note that the horizontal axis of the plot is the same (and covers all timepoints) for all three regions, despite the fact that their vertical placement (and size) differs.

Additional buttons within the graph view allow you to zoom in on particular sections.

Lattice LightSheet Module

Background

Live cell imaging allows for the understanding of spatially complex, rapidly evolving cell physiology that cannot be accomplished by structural imaging alone. However, live cell imaging requires tradeoffs of spatial resolution, temporal resolution, and phototoxicity. This is especially true when imaging in three dimensions. The Lattice LightSheet (LLS) microscope, developed by Dr. Eric Betzig of the HHMI Janelia Research Campus, allows scientists to overcome these limitations. Ultra thin light sheets (~400nm) derived from two dimensional (2D) optical lattices are scanned plane by plane through the specimen to generate a 3D image. The fluorescence signal emitted from the in focus plane is detected simultaneously over the entire field of view, thus increasing imaging speeds over conventional confocal scanning techniques. The thinness of the sheet generates diffraction limited and beyond axial resolution with excellent signal to noise ratio and negligible photobleaching.

Optical Path

Lasers are individually expanded in the laser launch to 2.5mm, collimated and aligned to be co-linear. All lines pass through an Acousto-Optic Tunable Filter (AOTF). Frequency modulation of the AOTF regulates the degree of higher order light that is transmitted, thus regulating the laser power input into the system. Once in the Lattice LightSheet optical path, a set of cylindrical lenses expands the 2.5mm input beam in X to 25mm to uniformly illuminate a stripe on the Spatial Light Modulator (SLM). The SLM is programmed to display binary images of user generated multi-Bessel patterns generating an optical lattice of Bessel beams. The Bessel beam is projected onto an annular mask, which filters the zeroth order, removes artifacts and lengthens the sheet. The mask is serially conjugate to Z and X galvo mirrors as well as the rear pupil of the excitation objective, allowing the light sheet to be translated in y and z and rapidly oscillated in x for the dithered mode of operation. The beam is focused through the illumination objective to create a pattern of the Bessel beams at the sample plane that is conjugate to the projection off of the SLM. This pattern is dithered by the X galvo to form the sheet of illumination that is observed by the sCMOS camera, through the detection objective. The 25X detection objective, in conjunction with the 500mm tube lens, gives a 62.5X magnification at the camera.

Starting the System

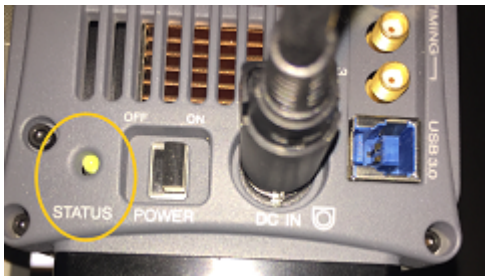
This chapter covers turning on the hardware and software to begin using the 3i Lattice LightSheet system. The system should be powered up and allowed to stabilize for 1 hour before alignment or use. Alternatively the system can be left on and the MPB lasers, discussed below, can be turned off. By leaving the system on, the launch and in particular the AOTF stays powered and thus reduced startup thermal instability.

Powering on Hardware

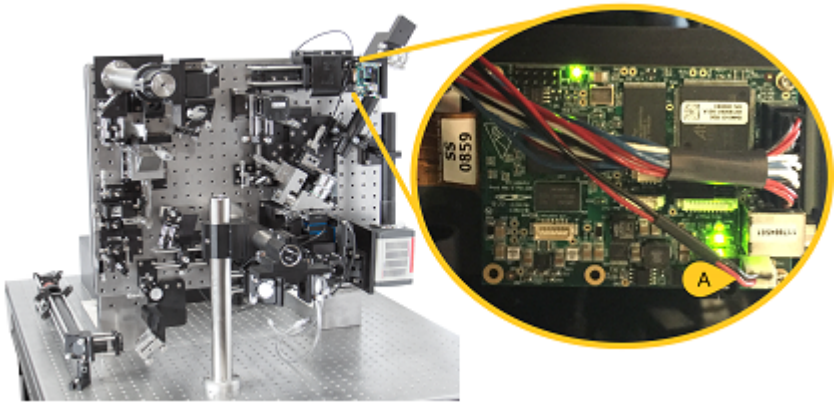
For proper system operation, it is important to adhere to the following procedure when powering on your system.

1. Turn on the main system power switch.

2. Confirm power is on for Orca Flash 4.0, light will be illuminated on bottom of camera. Confirm the FPGA and galvo chassis are switched on by the red LED indicator lights.



3. Confirm SLM has power. There will be green flashing lights on the SLM control board. If these green LEDs are not illuminated, unplug the power (A) then plug it back in. This should not be a frequent issue and if it becomes so, please contact support@intelligent-imaging.com.



4. Make sure the Laser Safety Shutter key (A) is turned to the ON position and that the 642nm (B) and 560nm (C) laser keys are turned to the LASER ON position.

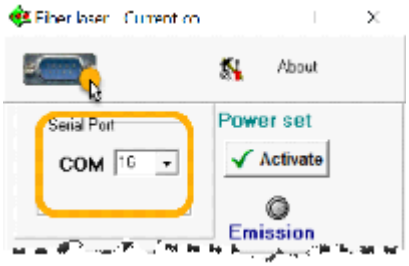
A
B
C

Starting the Software

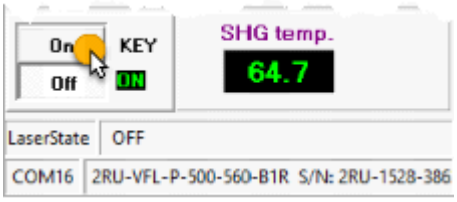
Multiple software programs need to be initialized and set up to get the system running.

1. Open a GUI VFL program window for each MBP laser the system contains (usually 560nm and/or 642nm)

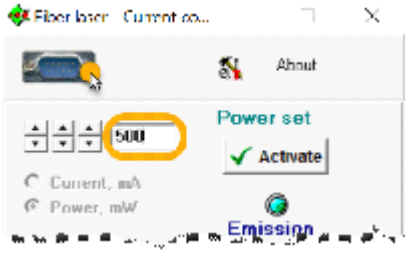
2. Click the serial port button (looks like the plug itself) to access ***Serial Port*** selection. Ensure correct ***COM*** port is displayed for each laser. This can be determined in Device Manager/Ports (Com & LPT). The MPB lasers will be designated MPB Serial Device in Windows 7 and USB Serial Device in Windows 10.



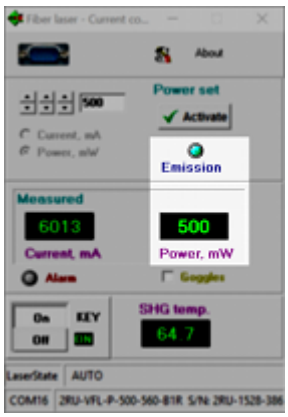
3. Turn each laser key off and on again. Click ***On*** button in software for each laser.



4. Click the serial port button to access the laser power setting. Set laser powers to 500mW, click ***Activate***.



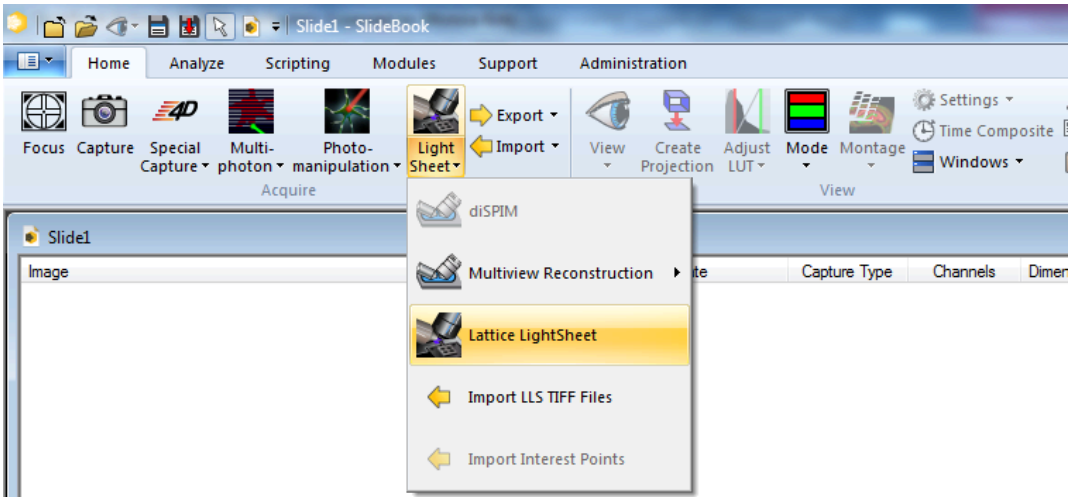
5. Lasers will take a moment to warm up. You will see the ***Emission*** indicator turn on and note the ***Power*** displayed is in range when lasers are ready.



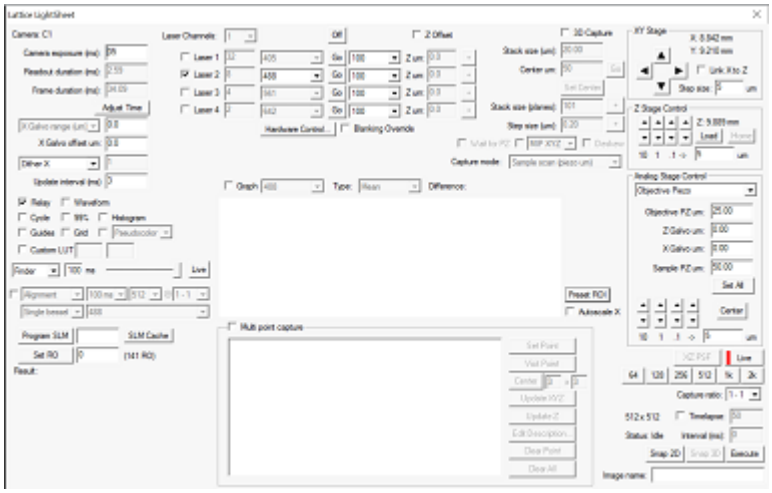
NOTE: The MPB lasers must be tuned regularly. When the laser is at 500mW output, observe the ***Measured*** output. If this value is over 8000mA, the laser needs to be tuned. To do this, see Tuning Lasers.

6. Ensure the objective assembly is in the raised position and no sample holder is mounted. Open SlideBook.

7. Open the ***Lattice LightSheet*** module.



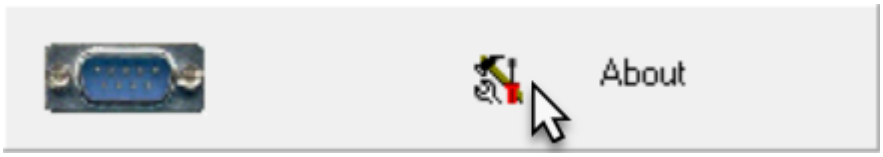
8. The following Lattice LightSheet user interface will appear.


Lattice Light Sheet
Camera: C1
Camera exposure (ms)
Readout duration (ms)
Frame duration (ms)
Adjust Time
X Galvo range (um)
X Galvo offset um
Dither X
Update interval (ms)
Relay
Waveform
Cycle
WFL
Histogram
Guides
Grid
Pseudocolor
Custom LUT
Finder
100 ms
Live
Alignment
100 ms
512
1 - 1
Single kessel
Program SLM
SLM Cache
Set RO
(141 RO)
Result:
Laser Channels:
Off
Z Offset
Laser 1
Laser 2
Laser 3
Laser 4
405
488
561
642
Go
100
Z um
Hardware Control
Blanking Override
3D Capture
Stack size (um)
Center um
Set Center
Stack size (planes)
Step size (um)
Wait for PZ
RIP XYZ
Deskew
Capture mode
Sample scan (piezo um)
XY Stage
X: 5.542 mm
Y: 9.210 mm
Step size
um
Link X to Z
Z Stage Control
Z: 9.889 mm
Load
Home
Graph
Type:
Mean
Difference:
Preset ROI
Autoscale X
Multi point capture
Set Point
Visit Point
Center
Update XYZ
Update Z
Edit Description...
Delete Point
Delete All
Analog Stage Control
Objective Piezo
Objective PZ um
Z Galvo um
X Galvo um
Sample PZ um
Set All
Center
XZ PSF
Live
64
128
256
512
1k
2k
Capture ratio:
1 - 1
512 x 512
Timelapse
Status: Idle
Interval (ms)
Snap 2D
Snap 3D
Execute
Image name:

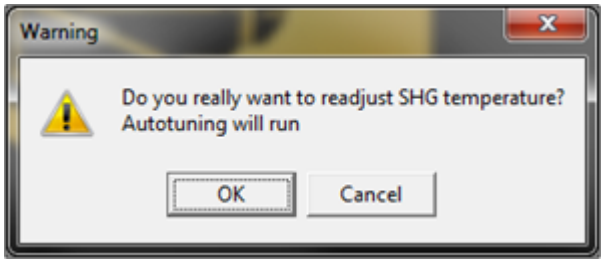
Tuning Lasers

The MPB lasers must be tuned regularly. When the laser is at 500mW output observe the ***Measured*** output. If this value is over 8000mA, the laser needs to be tuned.

1. Click the settings/tools icon.



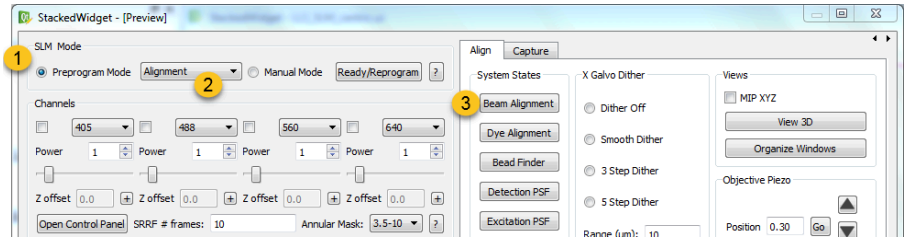
2. A warning message will open. Click ***OK***.



3. The tuning process will run in the background and close when complete.

LLS Initial Beam Alignment

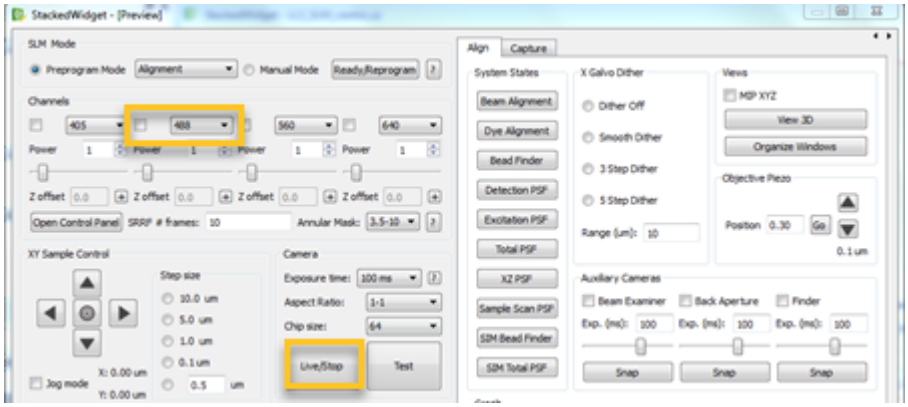
1. In the Lattice LightSheet dialog, select ***Preprogram Mode*** (1) then ***Alignment*** (2) from the dropdown menu. In the Align tab, Select ***Beam Alignment*** (3)from System States.



NOTE: The laser dropdown menu controls both the laser and loaded Bessel pattern. For 488, there are two options: ***488*** and ***488SB***. Select 488 to load the multi Bessel pattern (as opposed to the single Bessel (SB) pattern). Loading Bessel Patterns outlines how to load new patterns into SlideBook.

If the laser checkboxes are grayed out, confirm that the ***Alignment*** dropdown checkbox is unchecked.

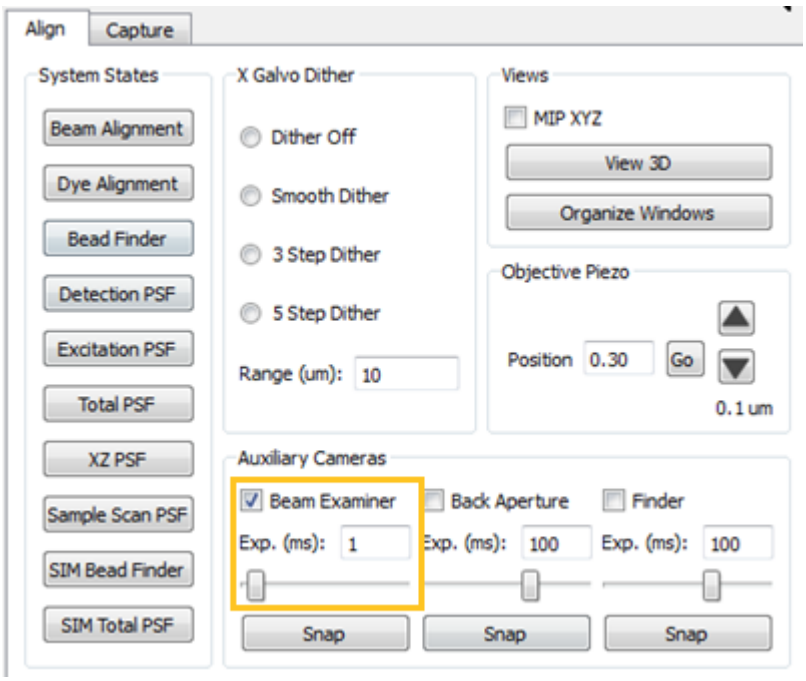
2. Check the box to select the desired channel. Click ***Live***.

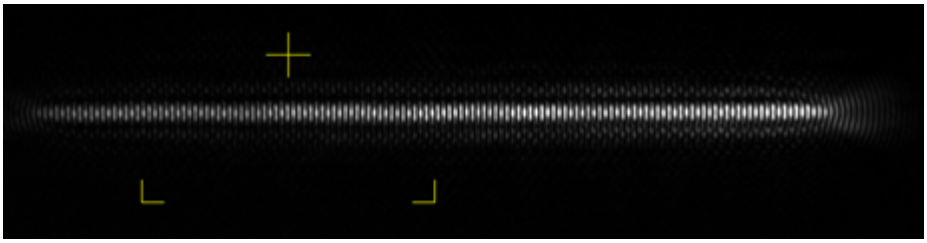


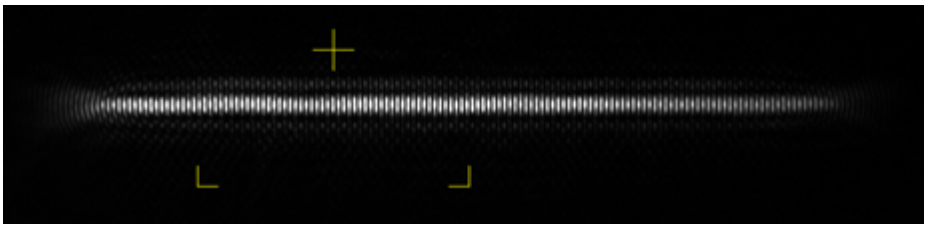
3. You should observe the desired laser entering the system. If you do not, toggle the interlock key. When the capture initiates, the Live Camera window will open. It may take a moment for the live window to open while the SLM is programmed. (not sure if this will be relevant - You may monitor the SLM progress by viewing the status messages in the lower left corner of the Lattice LightSheet dialog. If the camera window does not fully open, click *Stop* then click *Live* again.)

Once system is started and acquiring, wait at least one hour before starting the beam alignment outlined below.

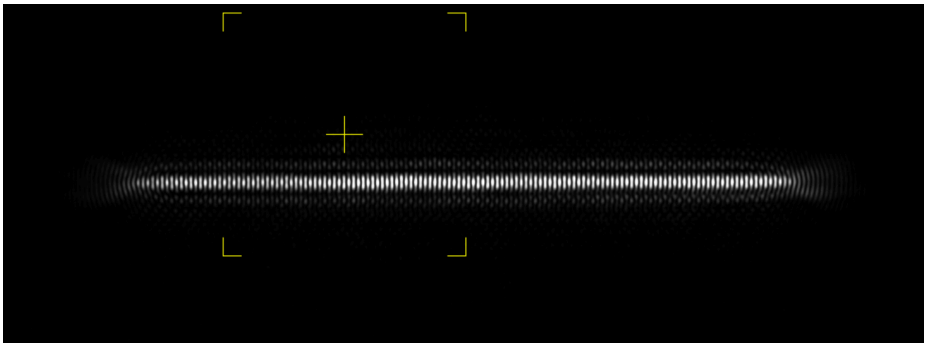
4. In the Align tab, check the box next to ***Beam*** and use the slider or text field to reduce the exposure time to 1ms. (can this happen as part of the system state?) A new window will open and you will be able to view the multi Bessel beam.







NOTE: When you first start up the system, it is imperative that you allow the system to warm up to a baseline temperature. This can be monitored using the multi Bessel beam profile in the ***Beam*** examining camera. During this initial warm up the pattern will be non-uniform horizontally, as seen in the examples above; it is critical that any alignment is not initiated until the beam is close to uniform as shown in the following image. The approximate time needed to wait is **at least one hour** for most systems.



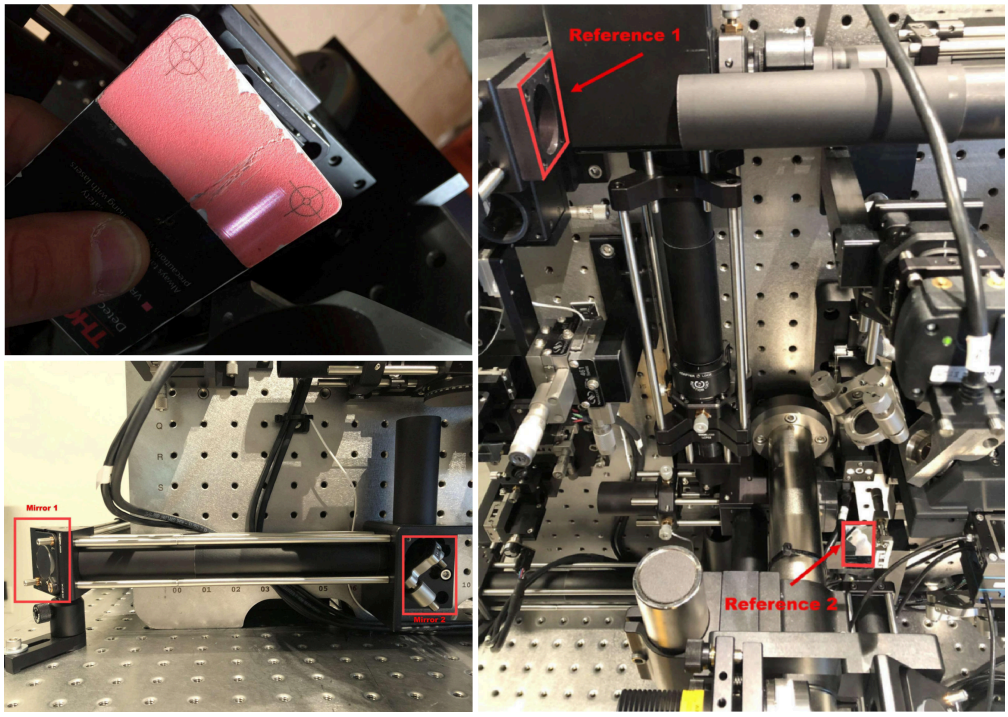
5. If the multi Bessel beam profile is uniform, proceed to Daily Dye Alignment. If, after 1 hour, you still observe non-uniform illumination, you will need to adjust the input of the laser into the system. Continue reading Adjusting Laser Input to the System.

TUTORIAL: LLS Adjusting Laser Input to the System

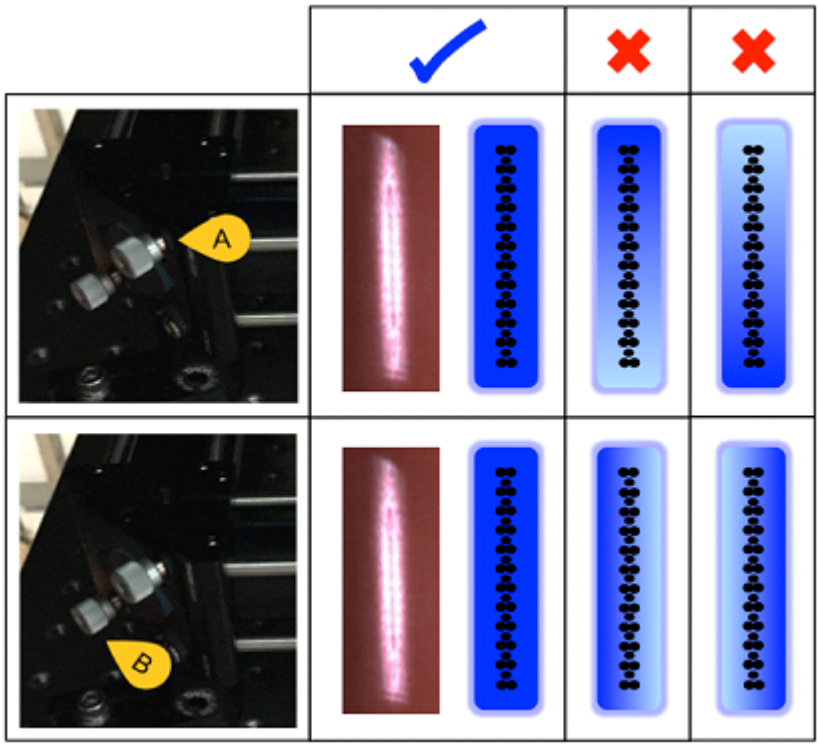
This is a common alignment procedure that will need to be performed periodically during the alignment and usage of the system
To reduce temperature related alignment drift, keep the enclosure and laser lid closed as much as possible
The goal of this procedure is to align the input excitation beam into the system, using the SLM and the annular mask as reference points.

1. The first step in adjusting the laser input is to verify illumination on the SLM. To do so, hold a card in the light path just beyond the SLM towards the Fourier transform lens. The adjustments you make will be using the knobs on the first alignment mirror:
    a. Program the SLM for a 488 MB 0.55 0.493 pattern
    b. Set the X dither value to 0
    c. Select Live
    d. Use the Beam Examining camera to view the pattern

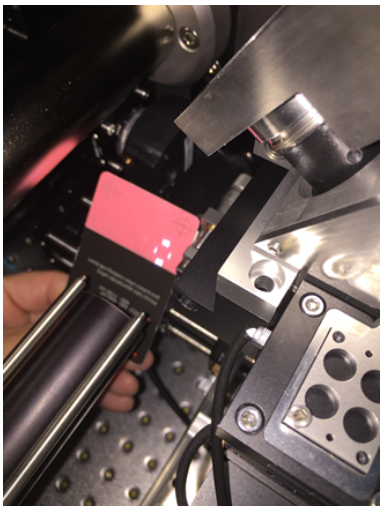
2. Align the beam to the SLM
    a. Hold a Thorlabs detector card in the lightpath just after the SLM (**Reference 1**)
    b. Use **Mirror 1** on the input path to center the beam to the pattern horizontally and center the illumination vertically



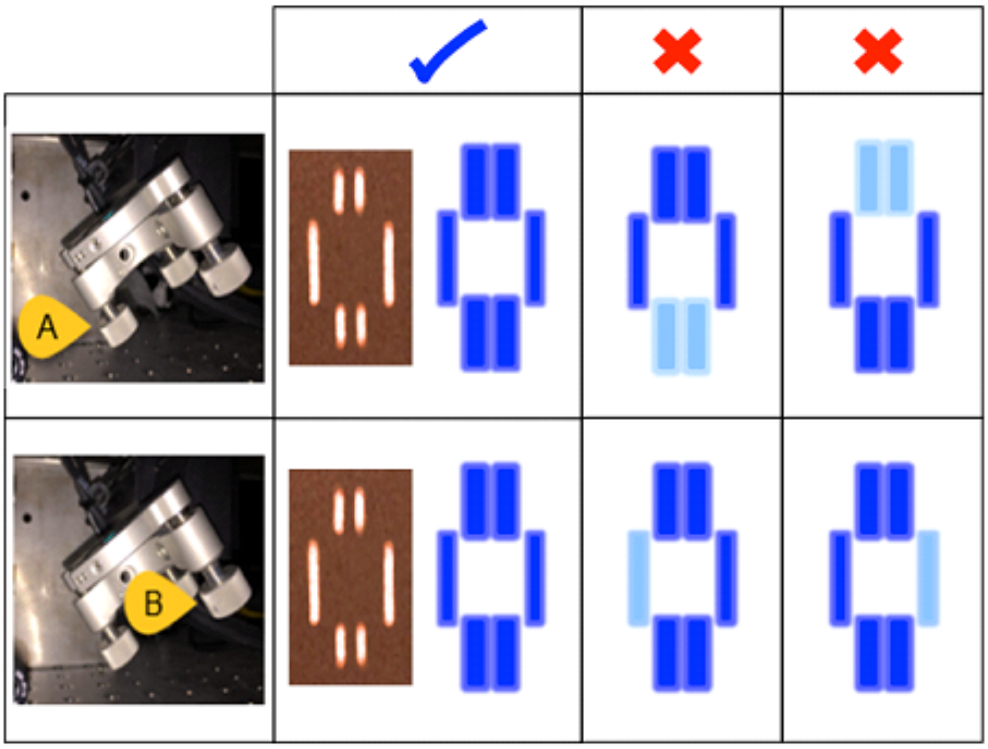
c. Use knob **A** (upper right) to adjust evenness of vertical illumination. Use knob **B** (lower left) to adjust evenness of horizontal illumination.

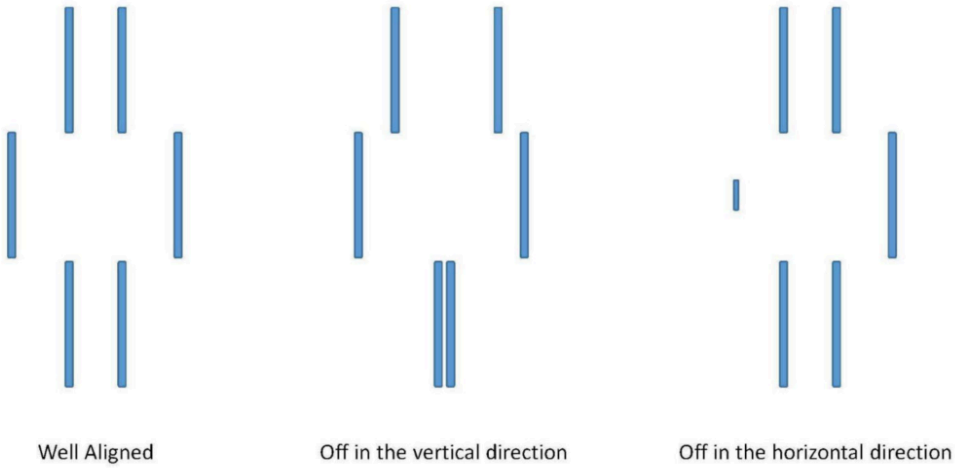


3. Align the beam through the annular mask:
    a. Hold the detector card immediately before the back aperture prism mirror (**Reference 2**) to view the pattern
    b. Use **Mirror 2** on the input path to adjust the alignment until the pattern is symmetrical in the vertical and horizontal pattern lines as shown below



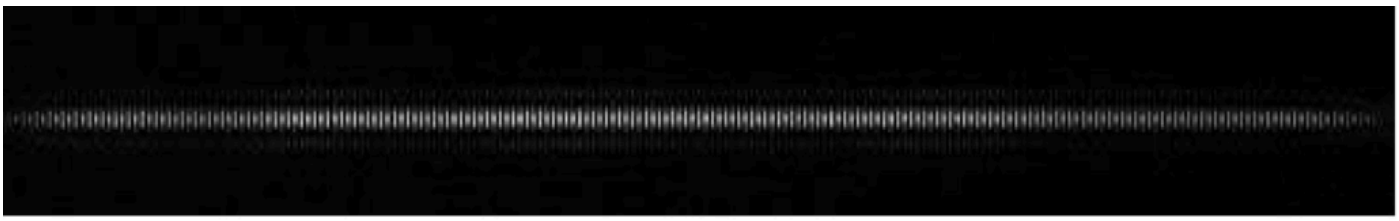
    c. The lower knob (**A**) adjusts vertical intensity while the front upper knob (**B**) adjusts horizontal intensity.

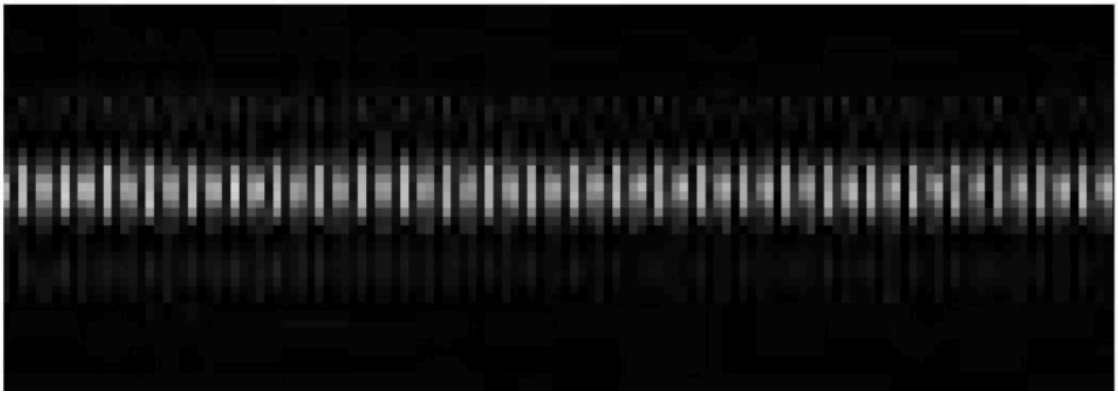




4. You will go back and forth between pre-annular mask (step 2) and post-annular mask (step 3) to fine tune and perfect the laser input. Remove both cards and verify the alignment with the Beam Examining camera:

   a. Intensity is centered and evenly distributed across the pattern
   b. The bessels are clearly discernible in the pattern and there are no aberrations across the pattern





5. If you do more than 10 iterations and the two alignments are still countering each other (you cannot achieve even illumination at both locations at the same time), please contact support@intelligent-imaging.com before attempting any additional corrections.

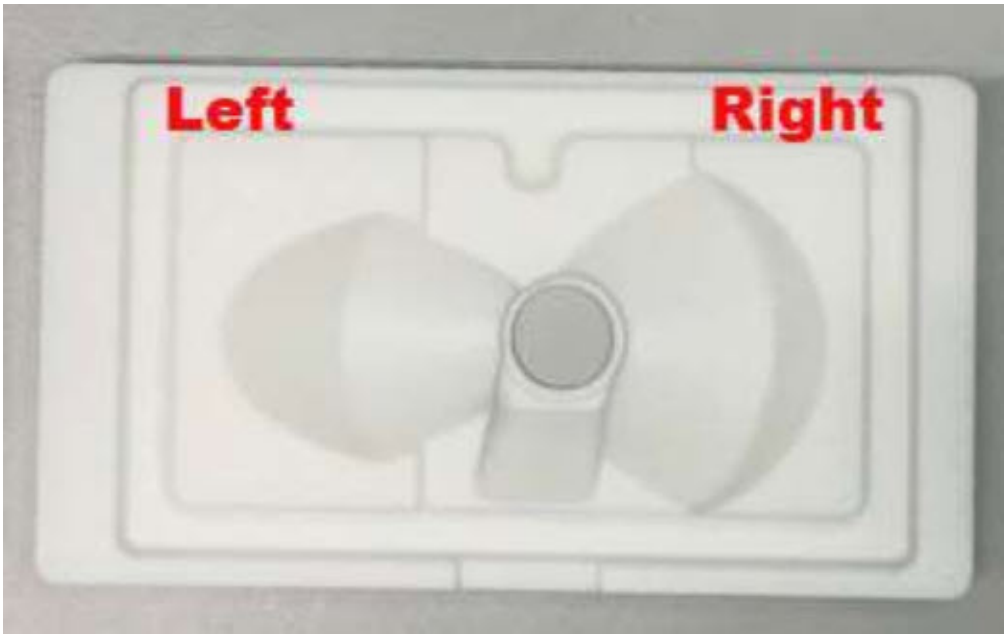
6. Once even illumination, symmetry, and clarity are achieved, proceed to Daily Dye Alignment.

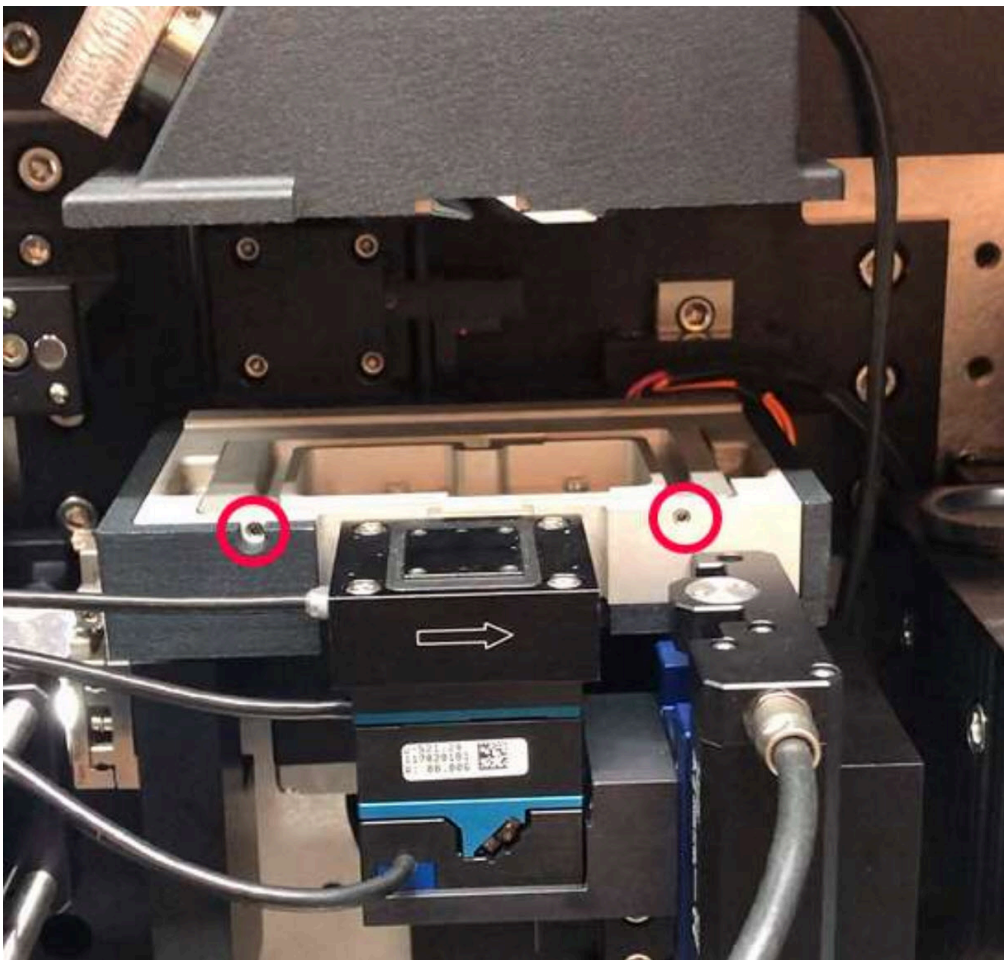
TUTORIAL: LLS Daily Dye Alignment

1. Power on the Objective Piezo E-709 if not running yet.

2. Install the 3D printed bath insert and secure the set screws

   NOTE: Ensure the insert is installed with the larger cavity to the right. Installation with incorrect orientation can lead to damage to the Nikon objective.





3. Mix 20µl of 100nM Fluorescein (in deionized water) with 3 ml deionized water and add into the bath insert.

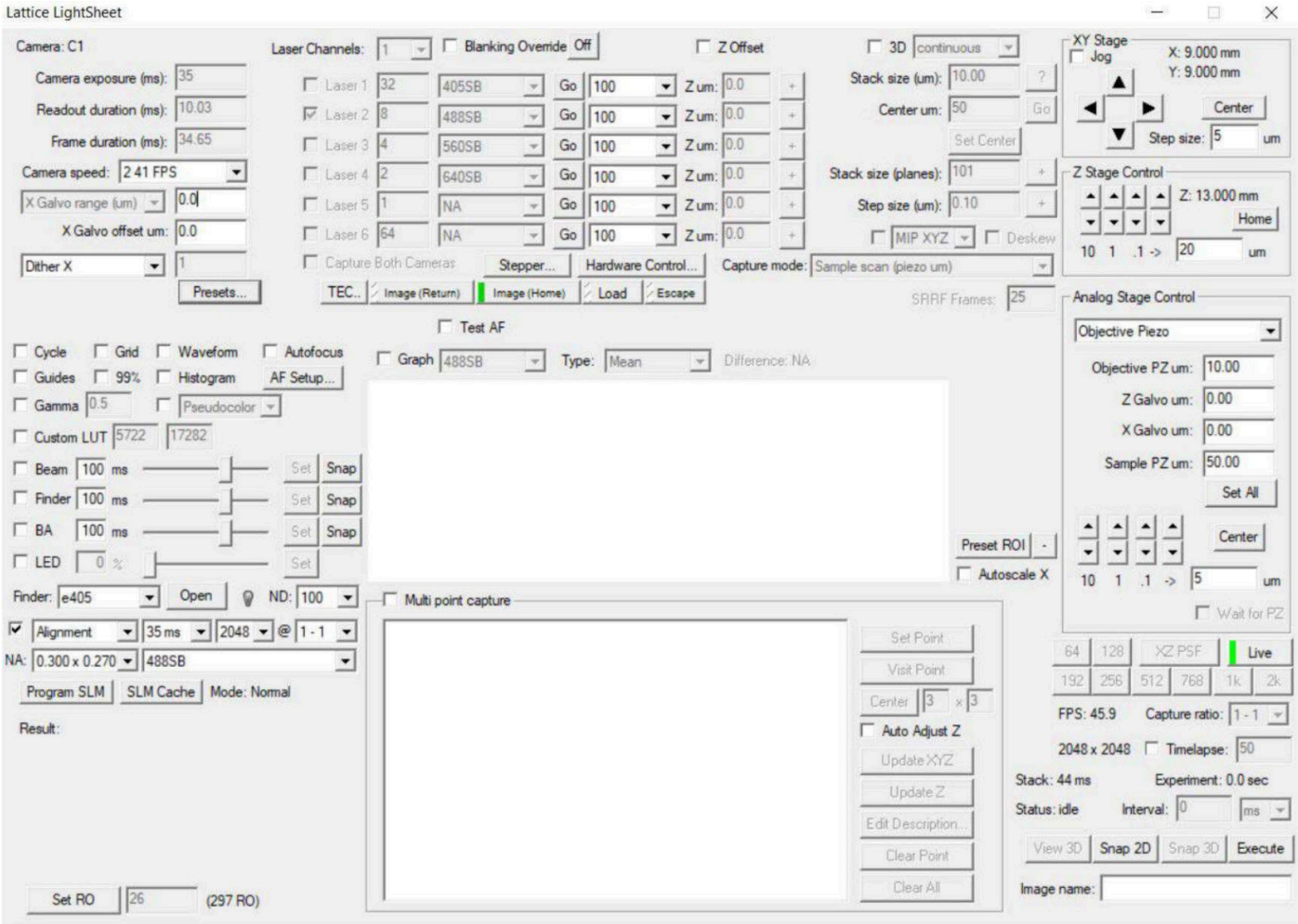
4. Bring the Bath assembly to the Image position and move the special optics objective stage to its neutral position.

5. Allow the media and dye to come to temperature, about 5-15 minutes. This time can be reduced by having the media at imaging temperature in a water bath or incubator.

6. In Administration>Filters change the camera assignment of 488SB to camera 1

7. Start a full chip live capture with the parameters below.

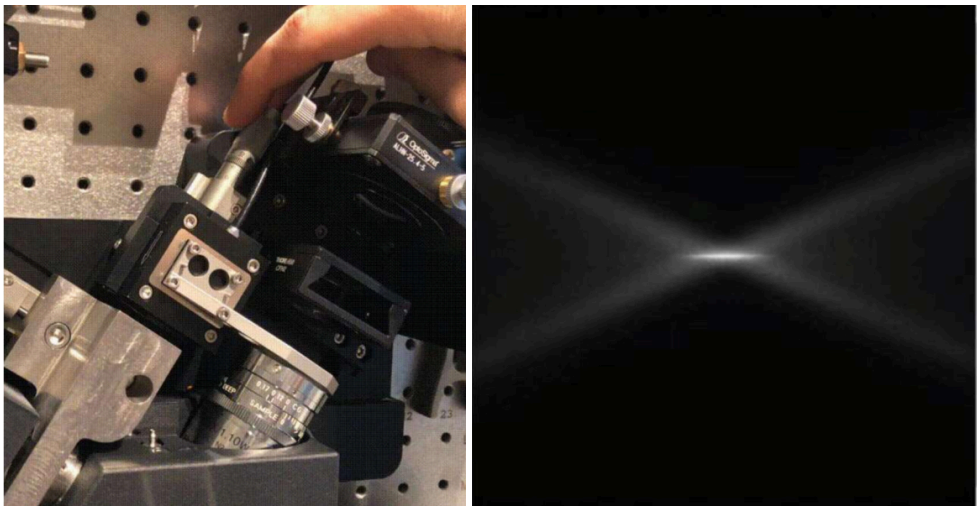
NOTE: If you have a Sapphire laser set the emission to 300mW in Sapphire Connection



8. Bring the beam into focus using the Nikon objective stage micrometer

NOTE:

- Be careful not to drive the Nikon objective into the special optics objective
- It is possible that the image viewed on the screen will not update quickly in response to your adjustments so you may need to make small adjustments and wait
- Verify the refresh rate by shining a flashlight on camera 1

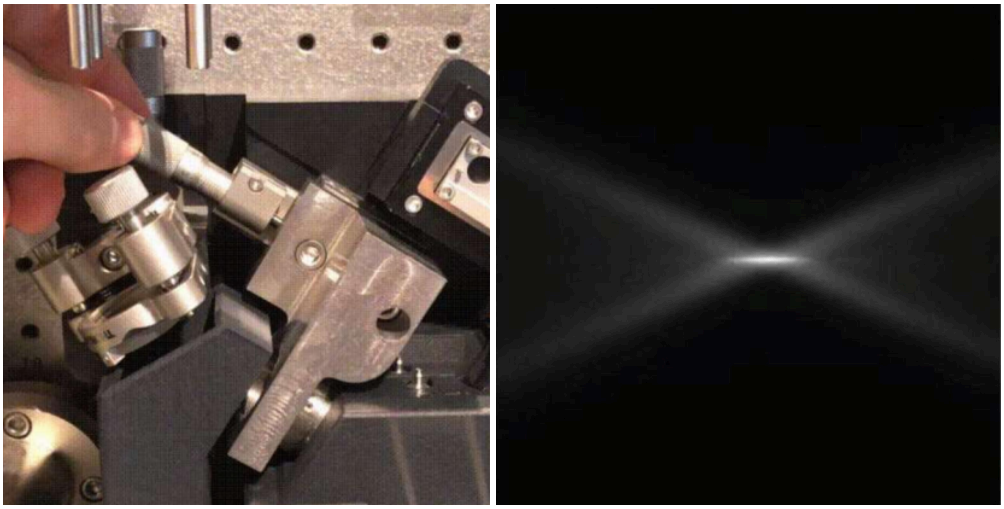


9. Once the beam is visible on the camera roughly center the beam to the camera

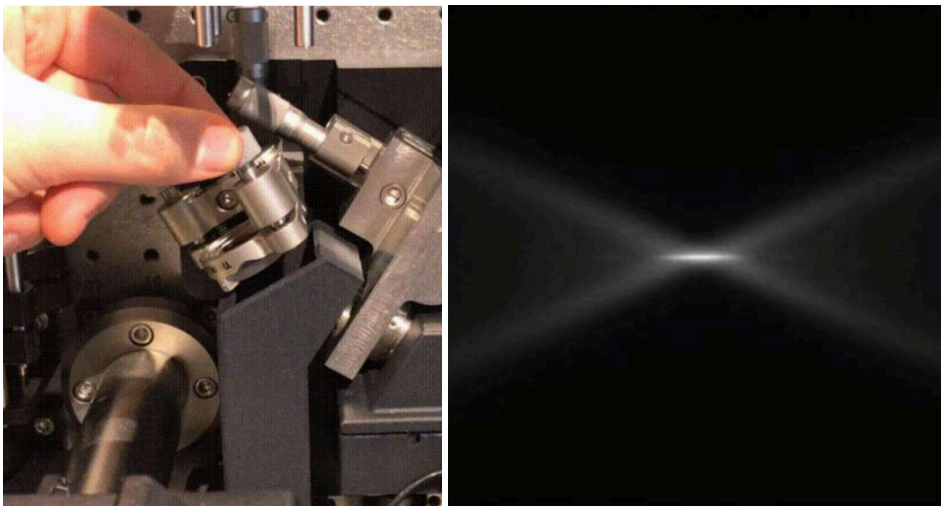
NOTE: If the vertical starting position of the beam is more than 512 pixels from the center it may be necessary to shim one of the objectives

10. Observe a single Bessel beam in the dye solution. This is the condensed interference pattern of the single Bessel observed from the perspective of the imaging 25X objective. At this point, regardless of focus and centration, the input beams should display even illumination. If you notice some intensity differences between the input beams, it means that there may be a bubble in the media or the media is not filled to the appropriate level. Check the sample chamber, fix any issues, and return to this step. If you still see illumination asymmetry, contact 3i Support.

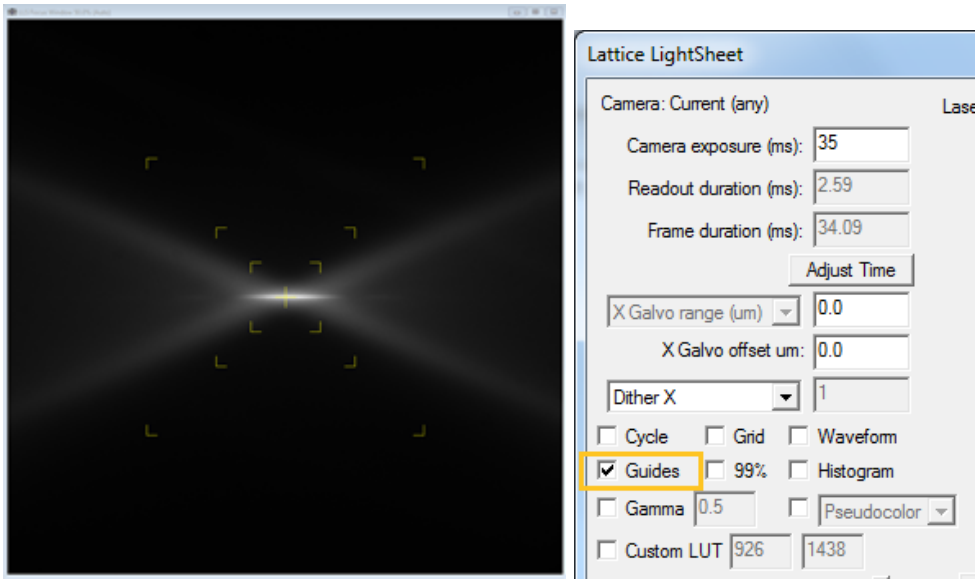
11. Translate the beam across the field in the X direction using the illumination objective micrometer



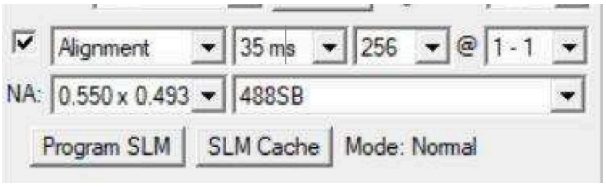
12. Translate the beam across the field in the Y direction using the adjustment on the flipper mirror closer to the user



13. To assist in the initial centering of the beam, check the ***Guides*** checkbox to produce a centering guide in the live camera window.

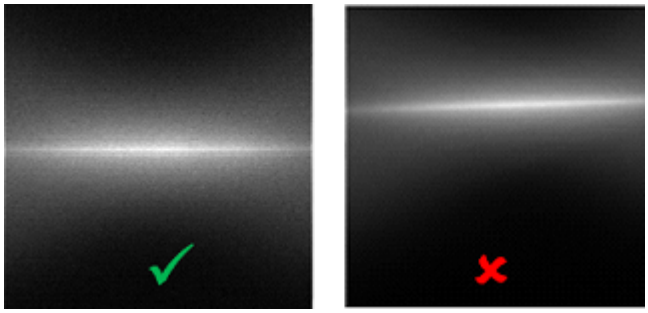


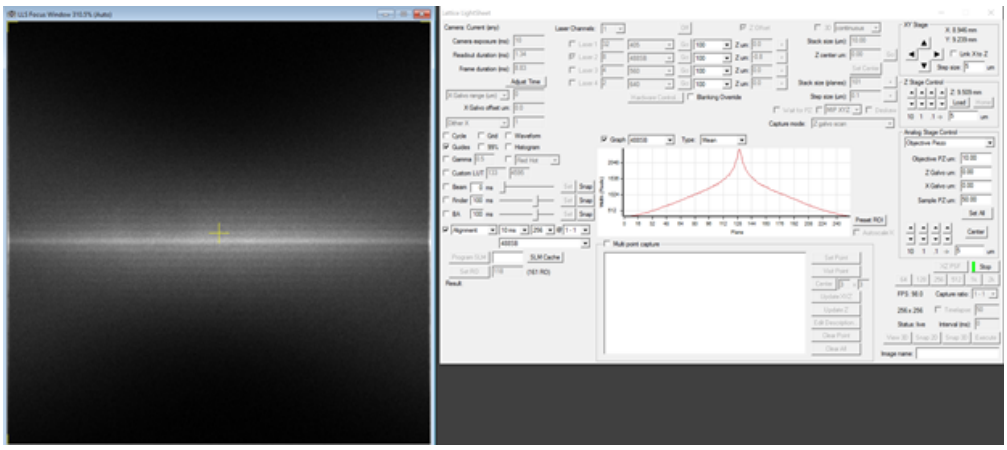
14. Update the capture settings as below



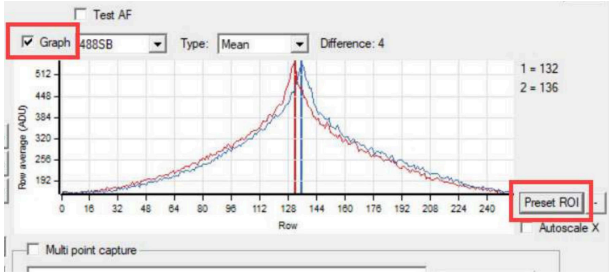
15. Click ***Live***. You are now zoomed in to the center of the pattern we previously examined

NOTE: When you zoom in to 256 x 256, you will be looking at the very center of the Bessel pattern. Ideally, the line displayed should appear vertically centered and horizontally level as shown on the left in the following figure. Often, once you zoom into 256x256, refinement is required as shown on the right in the following figure. Either way, we will use regions to perfect this alignment in the next steps.

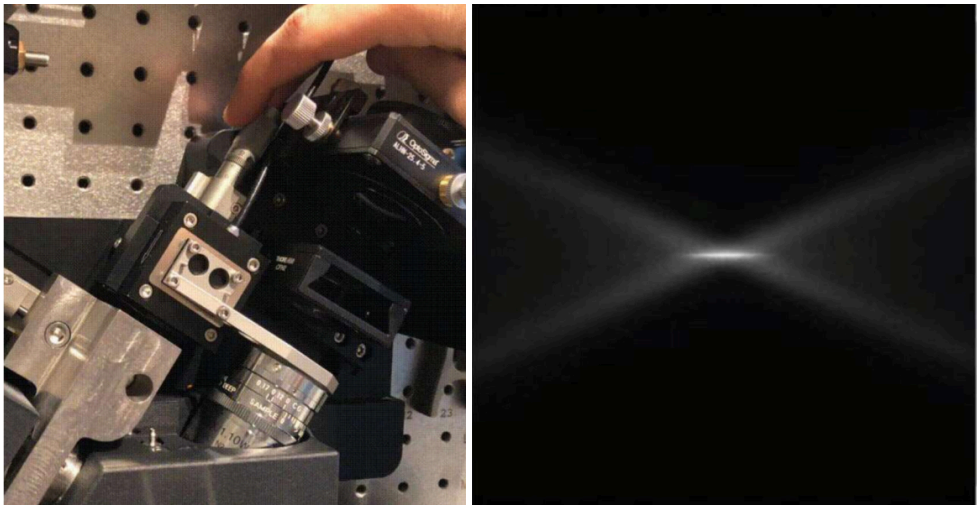




16. Check the ***Graph*** box and select ***Preset ROI*** in the main Lattice window

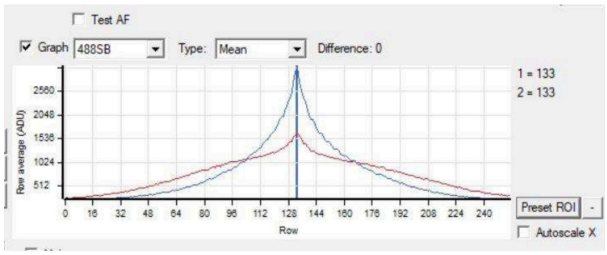


17. Check focus on the single bessel by adjusting the Nikon objective stage micrometer



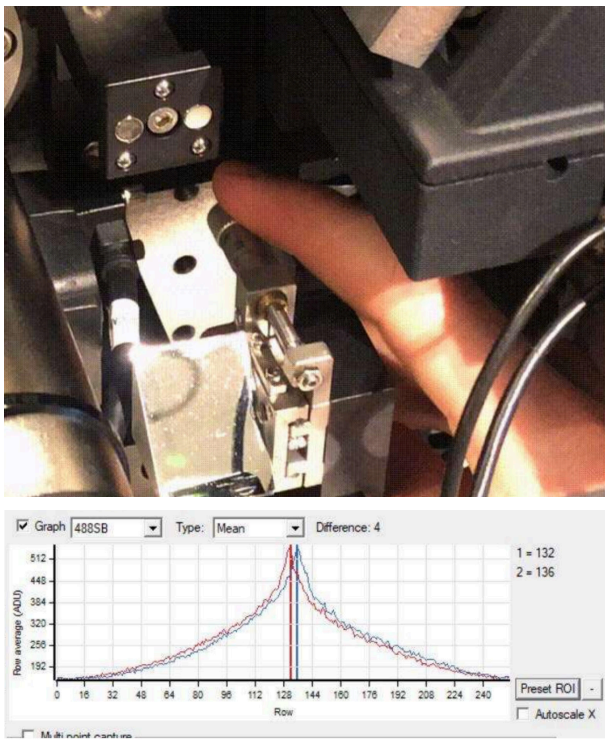
18. Now, we will use the row profiles of the two drawn regions to refine the beam alignment. In this alignment, you will adjust the beam in three ways:

a. Horizontal centration (translation in X) – red and blue peaks are equal height: Centration in the X axis is represented by the intensity distribution of both regions overlapping



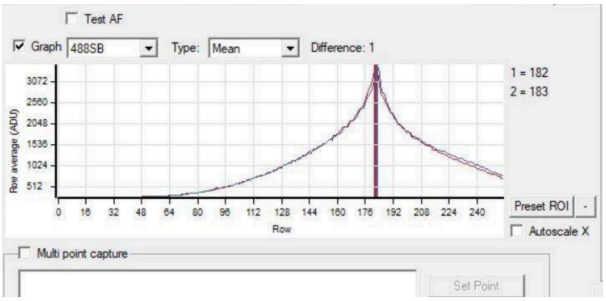
Single Besel in Dye off in the X axis

b. Horizontal tilt – red and blue peaks overlap at peak row: Adjust the rotation of the single bessel so that it is precisely horizontal by translating the back aperture prism mirror. The rotation of the beam is represented by the horizontal overlap of the intensity peaks and is quantified by the Difference value. The beam is rotated properly when the difference equals 0



Single Besel in Dye off rotationally

c. Vertical centration (translation in Y) – both peaks are in center of row profile: Centration in the Y axis is represented by the 1&2 values on the right acceptable centration is between 123-132

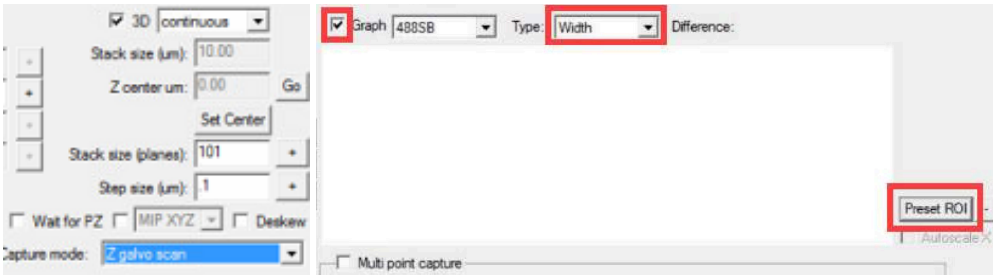


Single Besel in Dye off in the Y axis

The process is iterative; you will go back and forth between (a) and (b) bring into center with (c), then fine tune as needed.

19. Click ***Stop*** to stop the acquisition.

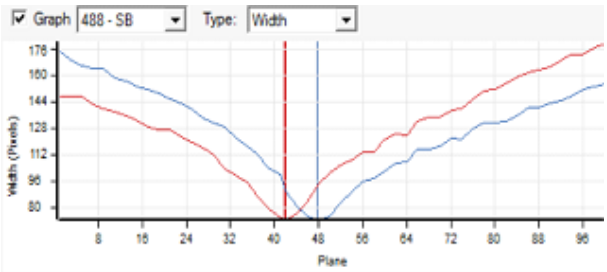
20. Check the box for ***3D Capture***. Select ***Continuous*** from the dropdown menu. Use the ***Capture mode*** drop down menu to select ***Z Galvo Scan***.

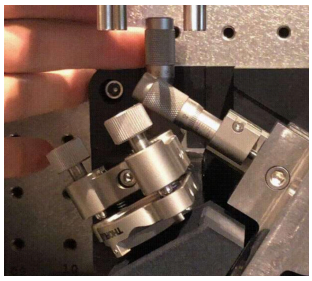


21. Set the following capture parameters:
    X Galvo range: 0.0
    X Galvo Offset: 0.0

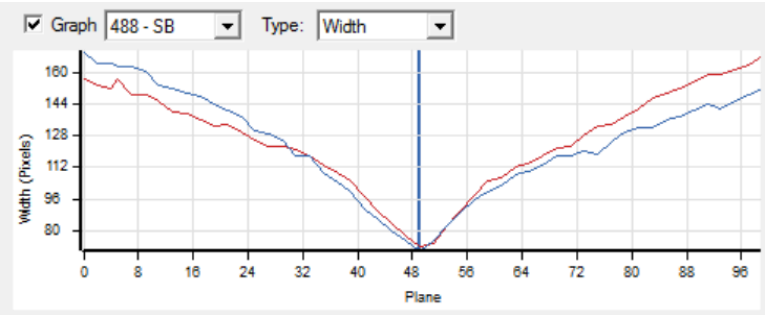
Stack Size (planes): 101
Step size (um): 0.2
Enable: ***Graph***
Type: Width
Enable: ***Preset ROI***

22. Click ***Live***. To create a stack profile, check the box next to ***Graph*** and choose ***Width*** from the ***Type*** dropdown menu. The goal is to have the peak for the red and blue line overlap at the same plane. This tells us that the beam is parallel to the image plane. If the beam is coming in at an angle relative to the image plane, the red and blue lines will be separated and at different width values for planes 0 and 100. If the peaks do not overlap as illustrated in the following figure, you will need to adjust the flip mirror micrometer until you see the red and blue lines overlap.





23. Once the peaks overlap, you are ready to begin the daily PSFs. Click ***Stop*** to stop the cameras.



Preparing for PSFs

Once the beam alignment and dye alignment are optimized, you can begin taking the alignment verification PSFs. To do so, you will need to clean the system from the dye alignment and load your bead sample. Fill the lower sample chamber with 8ml of the media you will be using with your sample that day and load your bead sample. If you switch imaging media during a session, you should repeat the daily PSFs to correct any spherical aberration in the LLS.

Cleaning after Dye Alignment

1. Slowly and carefully, raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

2. Wash the lower sample chamber 4-5 times with deionized water to remove as much of the dye as possible.

3. Wipe the tips of the two microscope objectives (lens and portion of body immersed in liquid) with lens cleaning tissues and liquid lens cleaner.

Bead Sample Preparation

The PSF sample is fluorescent beads (preferably 100nm green beads) sparsely attached to a 5mm diameter cover slip. A well-prepared sample should last at least a week. Please refer to Supplementary Materials for information on cover glass preparation and bead coverslip preparation.

NOTE: If you will be performing multichannel acquisition later in the day, you should have bead coverslips prepared for all wavelengths so you can perform Z Galvo Offsets for Additional Wavelengths.

Loading a Sample

1. Slowly and carefully raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

2. Add media if necessary. The sample chamber holds a maximum volume of 8ml.

> **NOTE:** You must be extremely careful when adding water as stages and piezos are very sensitive to water exposure. Please cover the stages with the provided stage cover.

3. Load the sample holder containing the sample coverslip with the coverslip up. See also Loading Coverslip to Holder.

4. Secure using set bolts.

5. Grasp the objective assembly handle to stabilize it. Remove the block that was holding the assembly up in the load position. Slowly lower the objective assembly using the handle.

> **NOTE:** There are hard limits in the software that will prevent positioning that would damage the objective but caution should still be exercised.

Removing a Sample

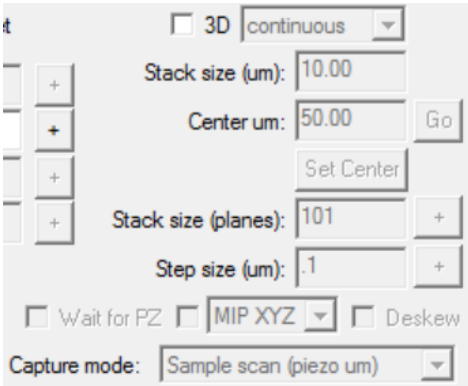
1. Slowly and carefully raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

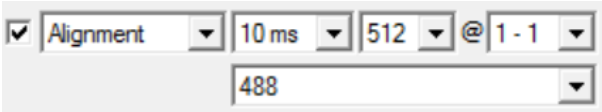
2. Remove the set bolts.

3. Remove the sample holder. If it is the end of use for the day, see also Clean Up and Shut Down.

Finding a Bead

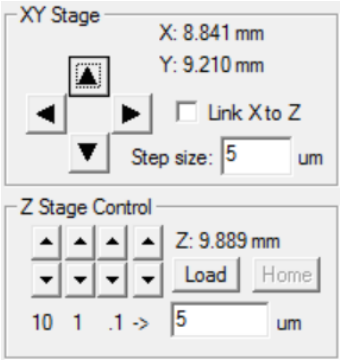
1. Under ***Capture mode*** choose ***Sample Scan***. Uncheck the box for ***3D***. Check the box for ***Alignment***. In ***Laser Channels*** choose the appropriate wavelength for the beads that you will be imaging. For PSF and biology acquisition, use the multi-Bessel for each wavelength and you initially use a non-3D capture to find the bead. In ***Alignment***, change the parameters to ***10ms*** exposure, ***2048*** field, and ***488***.



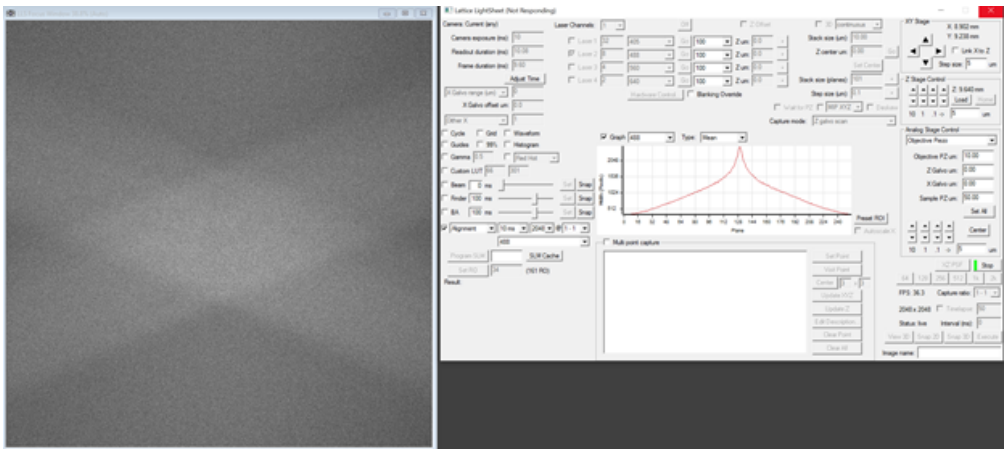


2. Click ***Live*** to begin live camera streaming.

To find beads, use the ***XY Stage*** and ***Z Stage Control*** interfaces in the top right corner of the Lattice LightSheet dialog to move the sample holder to bring beads into the field of view. The values that are referenced are the last position of the X, Y and Z stages. Do not click ***Link X to Z***. Move the XY stages by clicking the arrows that designate the direction in which you would like the bead to move. The ***Step size*** field is used to set a value for the movement made each time you click an arrow button. The default value is 5µm and this is a good starting value for the initial location of beads. For the Z stage control there are pre set values of 10, 1 and 0.1um as well as a fourth user-defined value all linked to up and down arrows.



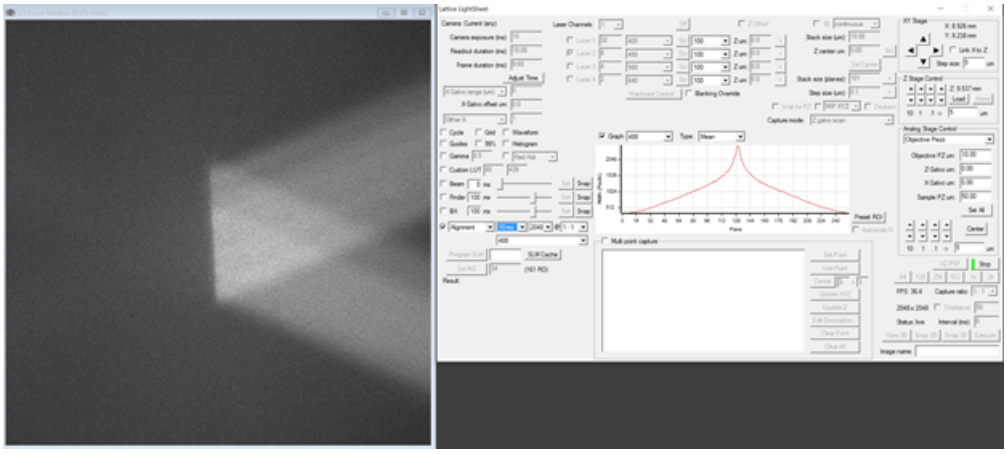
Use the arrow buttons to focus through the sample to get your bearings. In the full 2K FOV using 488nm, you may see a faint multi Bessel pattern due to a slight amount of residual dye. Ironically, for new users this is a useful tool for finding the coverslip as will be covered below. As you become more and more proficient and comfortable locating samples, we recommend that you wash the sample chamber to try and remove as much residual dye as is possible.



Using the Z stage controls, move the stage up. As the stage is physically moving up to move the sample holder closer to the objective, the value for the Z stage position will become more negative.

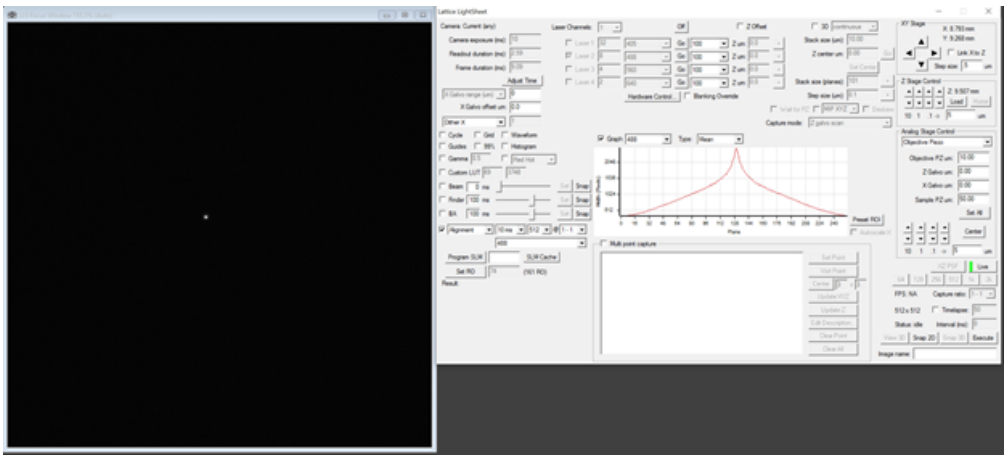
NOTE: We recommend that when changing samples or beads that you lower the Z stage. This will allow you to place a new sample on the system and lower the objective assembly without concern of lowering the objective onto the sample holder.

1. As you raise the sample holder, you will observe the sample come into the field of view from the left. This will appear as a line through the Bessel with the FOV to the left of the Bessel being much darker than the FOV to the right of the Bessel. This line that demarcates these halves of the overall FOV is the point where the Bessel is making contact with the coverslip.

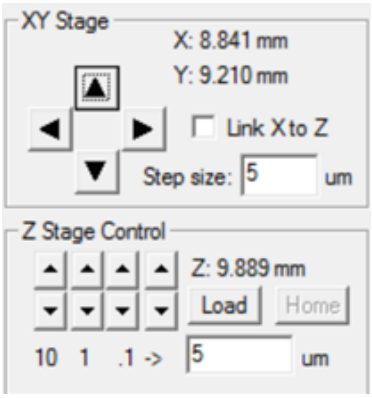


2. Click ***Stop*** to stop the acquisition. In alignment, change the field size to ***512*** then click ***Live*** to resume live capture.

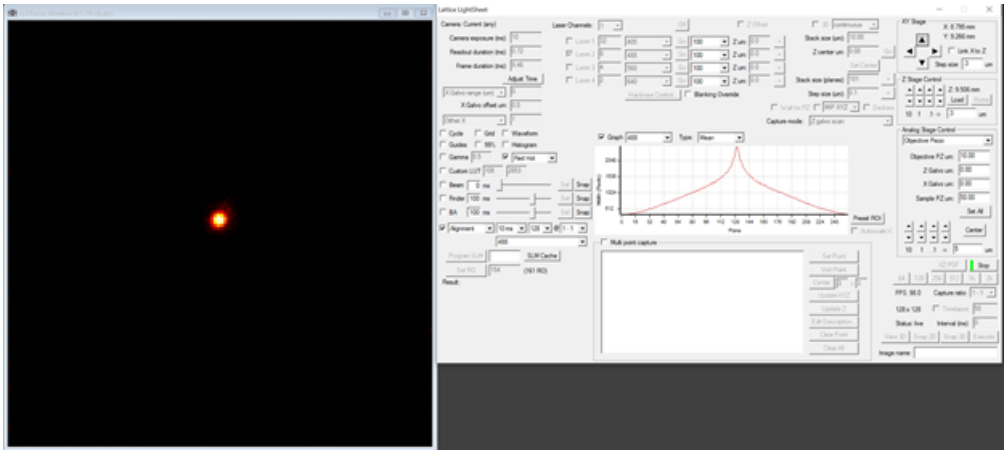
The LLS has an effective magnification of 62.5X so 1 pixel is 0.104um giving a FOV of 53.2um at 512 x 512) Use the XY stage control (we recommend that you initially use 5um step size) to move the sample to locate and center a bead. You will need to locate a single bead with no other beads within ¼ of the field of view. As you move the bead in X, you will need to re-center the beam using the Z stage. This iterative process is required because of the geometry of the illumination and detection relative to the sample. As you move the sample (in this case beads) in X, the sample will move out of the Bessel beam and you will need to raise or lower the coverslip to return the sample to the Bessel beam.



3. Decrease X, Y, and Z step size to 0.2.



4. Center the bead. You can use the ***Guides*** to assist in centering the bead.



5. After you center the bead at 512 x 512, click ***Stop***. Reduce the field to ***128***. Click ***Live***. Adjust Y using the up and down buttons so that the bead is vertically centered. Iteratively adjust X and Z to get the bead focused and centered horizontally. You may notice another bead contributing to the field. If this happens, increase the X step size to 2.2 and continue moving in the + direction looking for another, more isolated bead. When you find the next bead, adjust Y, and then iteratively X and Z.

6. Once you have a nice bead centered and in focus, click ***Stop***.

Daily PSFs

It is important to find an isolated bead per the previous instructions. Any contributing emission from other beads in the field will be problematic as will excessive background due to excessive residual dye from the dye alignment.

Throughout the PSF process (and when imaging samples) you should intermittently evaluate the beam-examining camera to ensure the multi Bessel beam has not drifted. Failure to do so could cause subsequent unnecessary adjustments that could further degrade system performance.

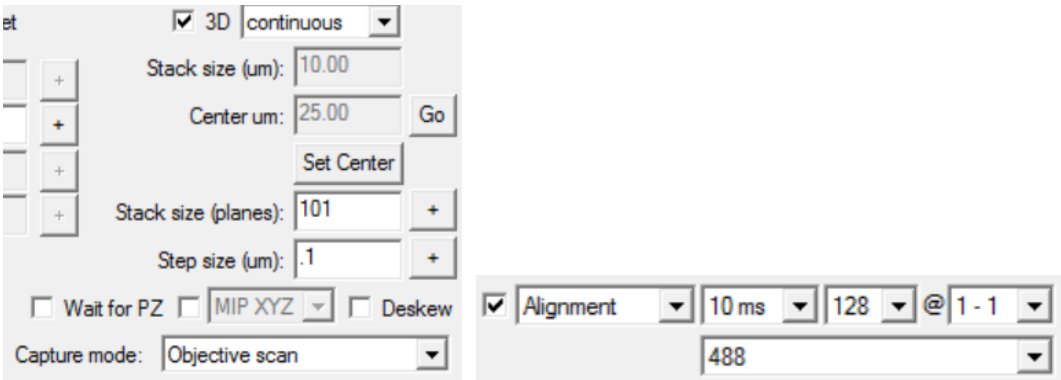
If you notice a bead bleaching during the PSF process, move to a fresh bead per the Finding a Bead instructions.

Detection PSF

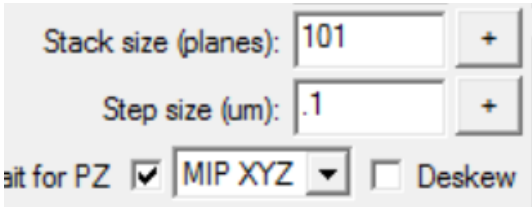
The Detection PSF (also called Emission PSF) describes the performance of the detection path independent of the light sheet excitation path. It uses a fixed sheet focused on the bead and moves the detection objective with the Z piezo.

Purpose: Eliminate spherical aberration, check for coma and astigmatism.

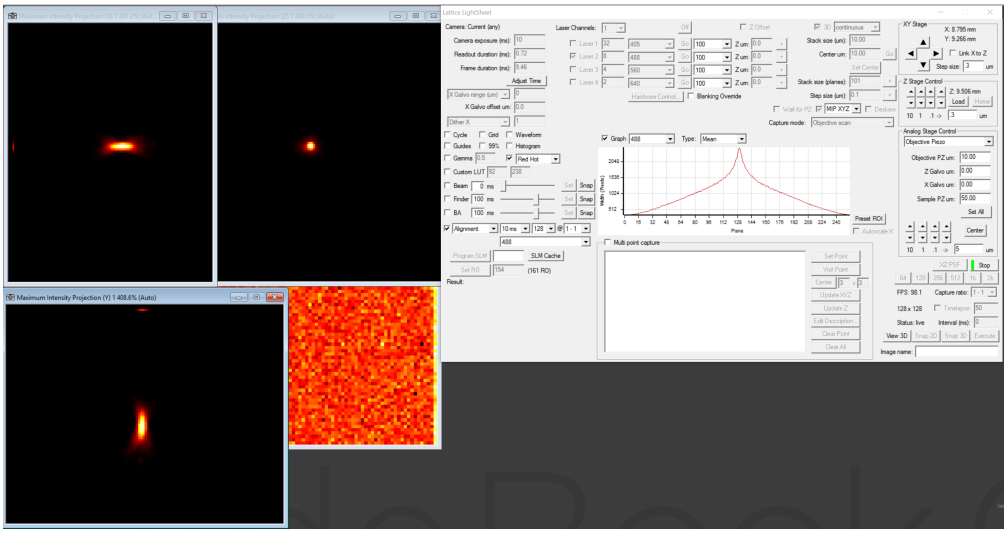
1. Check the box for ***3D***, choose ***continuous***. Select ***Objective scan*** as the scan type. You will be using the alignment parameters previously set of ***10ms*** exposure, ***128*** field size, and ***488*** channel with 100% laser power.



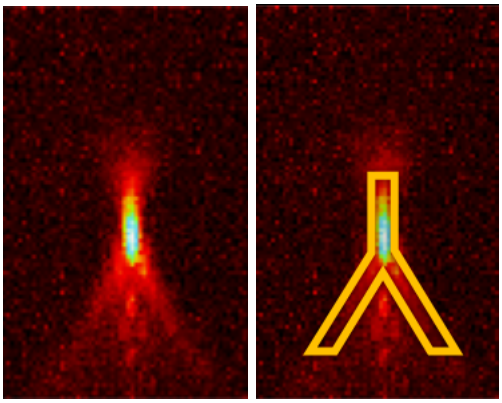
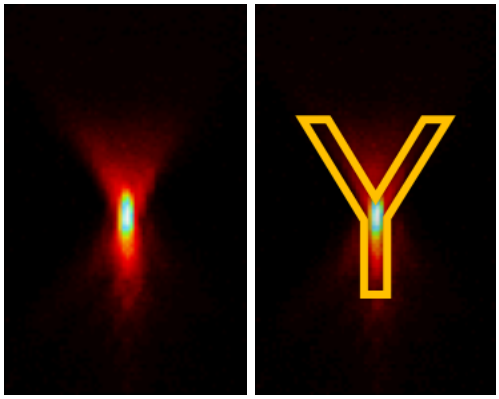
2. Set ***Stack size (planes)*** to **101** and ***Step size (um)*** to **0.1**. Check the box for ***MIP XYZ***.



3. Using a centered and focused bead, click ***Live***. By clicking ***MIP XYZ***, will display XY, XZ and YZ Max Projections of the emission PSF in separate windows. You will need to resize the MIP windows to visualize all in the workspace. You may wish to turn on ***Red Hot*** as a lookup table near the Guides checkbox.



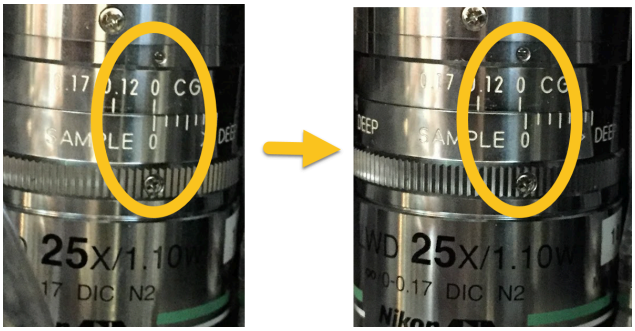
4. The stack projections should be symmetrical in both horizontal and vertical axes. First, look for and eliminate spherical aberration. Spherical aberration is evident when the spread of light above and beneath the bead are not symmetrical (creates a "Y' shape instead of an "X" shape) and can occur when switching to a media of a different refractive index.

	
Occurs when switching to media of a higher refractive index. Compensate by turning correction collar counter-clockwise and objective micrometer clockwise.	Occurs when switching to media of a lower refractive index. Compensate by turning correction collar clockwise and objective micrometer counterclockwise.

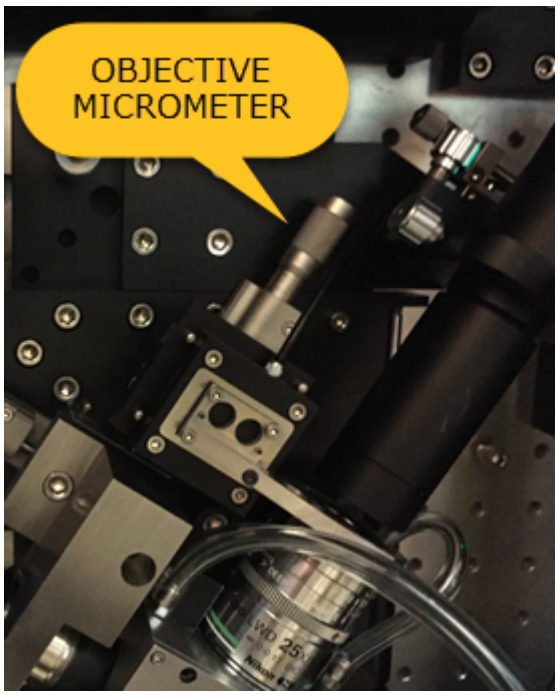
NOTE: The XZ and YX projections should exhibit similar spherical aberration. If you notice the "Y" flare of spherical aberration is flipped between the XY and YZ projections, this is a form of astigmatism and you should contact 3i Support for assistance in resolving the alignment issue. Remember to evaluate the pattern in the ***Beam*** camera window to confirm the multi Bessel beam is evenly illuminated and crisply focused.

To correct for spherical aberration, you will move the correction collar on the detection objective a discreet amount in the direction indicated by the preceding chart.

An example counterclockwise correction was made in the following figure; note it was about 1/3 of a tick mark distance.



5. Each move on the correction collar requires a counter move on the objective micrometer. Turn the objective correction collar the direction required (about 1/3 of a tick mark). Adjust the objective micrometer the opposite direction in the live feed to bring the bead back into focus.

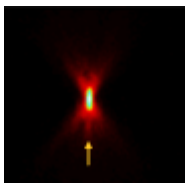


6. Evaluate for spherical aberration and repeat corrections as necessary. If you cannot resolve the spherical aberration using this method and have confirmed the multi-Bessel is evenly illuminated and crisply focused in the Beam camera window, contact support@intelligent-imaging.com for additional assistance.

7. Once you have a PSF that shows little to no spherical aberration, evaluate the PSF for coma or astigmatism. These aberrations may manifest as any of the following:

- Flipping of the spherical aberration "Y" between the XZ and YZ projections
- Rotation of the "X" shape either clockwise or counterclockwise in either XZ or YZ projection
- Lateral asymmetry in the X shape in either XY or YZ projection

A good detection PSF is shown in the following figure. There is a small interference pattern beneath the bead caused by the coverslip. This is acceptable.



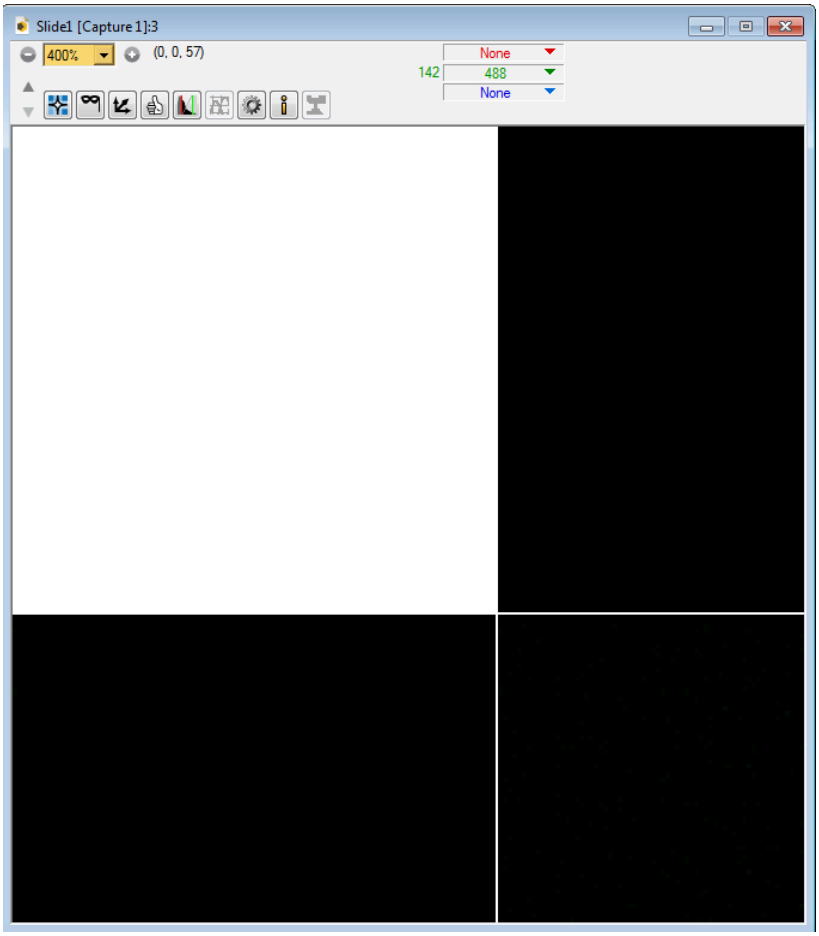
If you notice astigmatism or coma please contact 3i Support.

Warning: Do not attempt tube lens adjustments without approval from 3i Support.

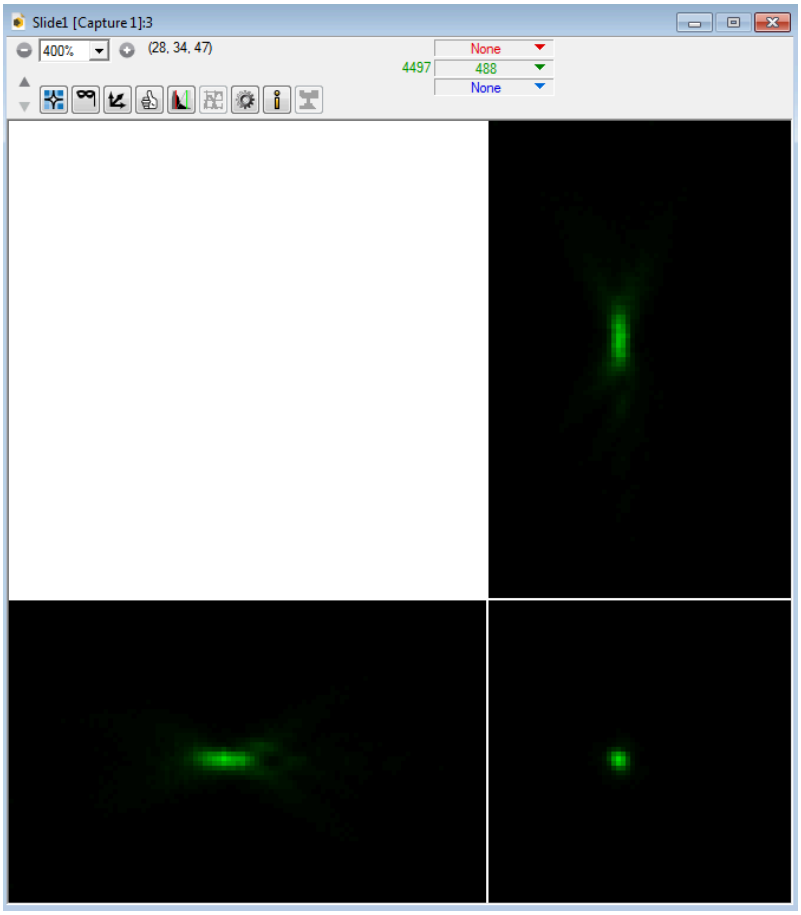
8. To best evaluate the detection PSF, capture the PSF by clicking ***Stop*** to stop live streaming and ***Execute*** in the lower right corner of the Lattice LightSheet dialog. This will acquire a single z stack, write it to the **.sld**, and open the image in a view window.

9. With the image window active, type **Ctrl + T** to open a Three View. Different view types can also be accessed from the ***View*** menu in the Home tab.

10. The Three View will default to 800%. Use the ***Zoom*** dropdown menu to change to 400%.



11. Use the ***Pointer tool*** to select a point in the XY, XZ, or YZ area. The other areas will update to focus around the selected voxel. Alternatively, you may click and drag through a selected area to create a flythrough in other dimensions. Clicking near the center of any panel should bring the bead into view.



12. Click the ***Renormalize*** icon to open the Renormalization dialog. Adjust gamma to better visualize the PSF.

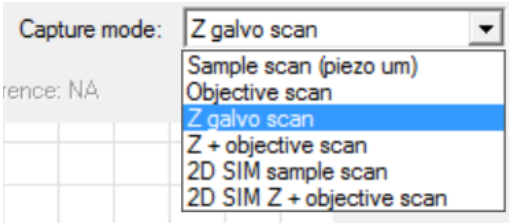
13. You may wish to save the Detection PSF for your records.

Excitation PSF

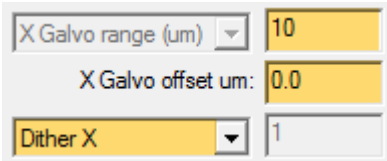
The Excitation PSF (also called Illumination PSF) demonstrates the alignment of the excitation path. In this PSF, we will sweep the dithered beam over the bead and keep the z piezo stationary.

Purpose: Confirm the sheet is optimized, including axial thickness.

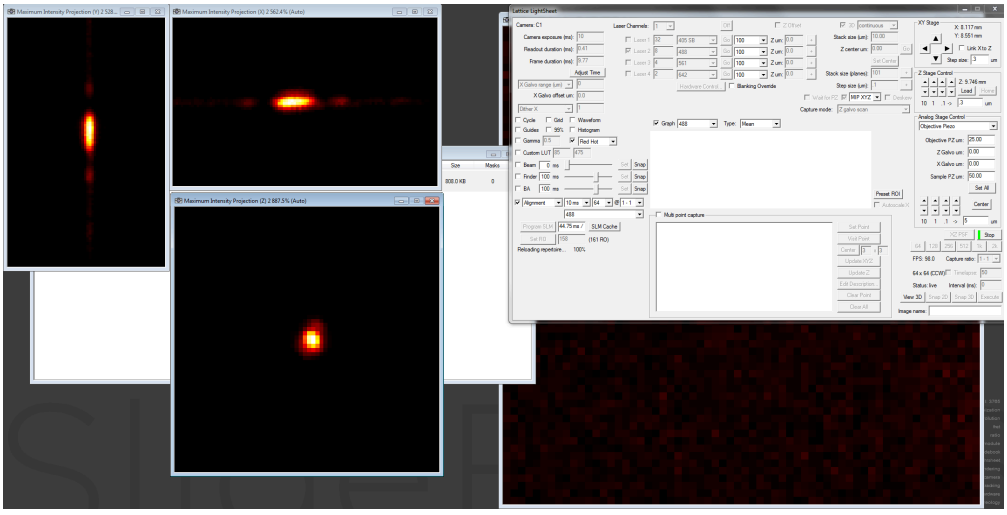
1. Change the Capture mode to Z galvo scan. Use all of the other settings configured for the Detection PSF.



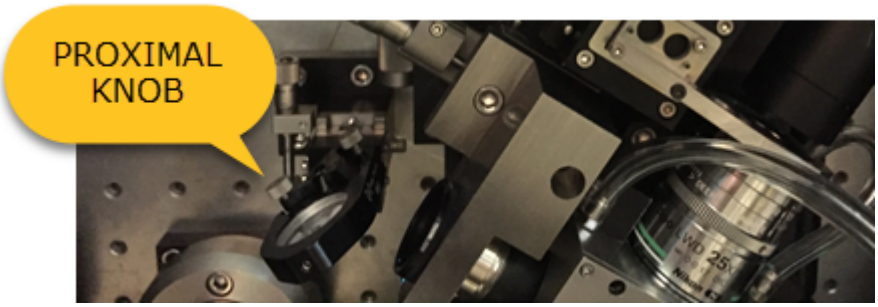
2. Set the ***X Galvo range*** to **10** to begin dithering the lightsheet.

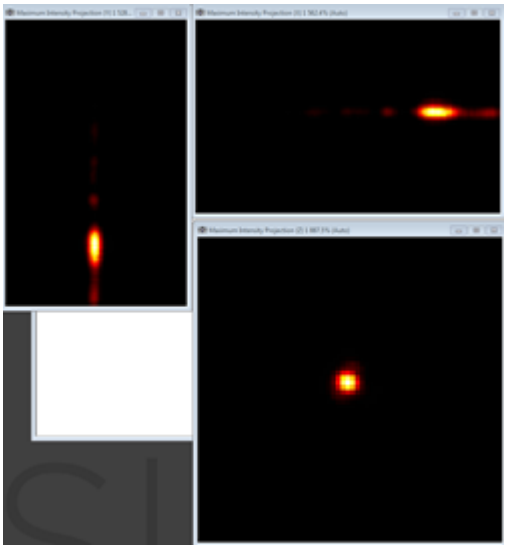
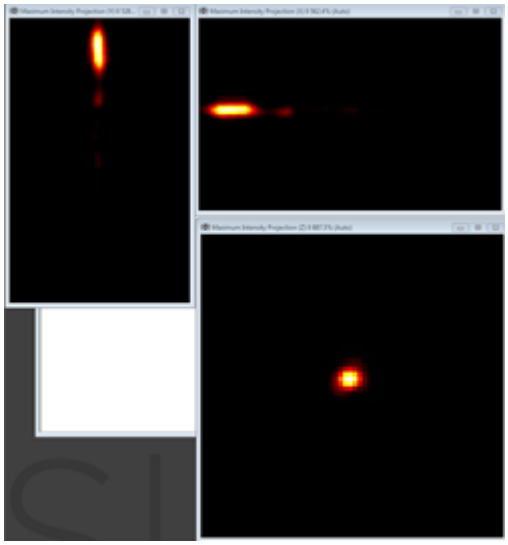


3. Using the bead that you centered and focused click ***Live***. By clicking ***MIP XYZ***, SlideBook will display XY, XZ and YZ maximum intensity projections of the Excitation PSF in separate windows. You will need to resize the MIP windows to visualize all in the capture window



4. If the PSF is not vertically centered in the YZ view (upper left displayed above), first check the Beam camera window to ensure the multi Bessel pattern is evenly illuminated and well-focused. If that looks good, you will need to adjust the proximal knob on the flip mirror.



PSF is Vertically Low	PSF is Vertically High
	
Turn proximal knob clockwise	Turn proximal knob counterclockwise

NOTE: This adjustment using the proximal knob on the flip mirror should only be performed at one wavelength during initial dye alignment. Other wavelengths will be compensated for by using Z galvo offsets discussed later in this manual.

5. Once you have the PSF vertically centered, click ***Stop*** to stop the acquisition.

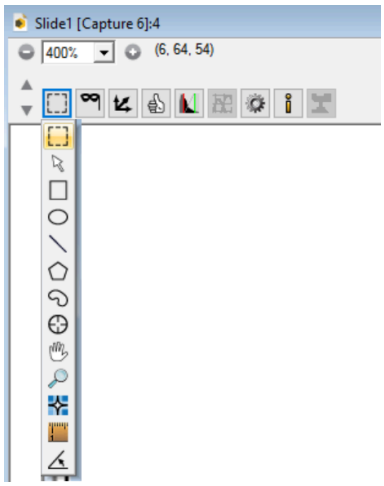
6. To best evaluate the Excitation PSF, capture the PSF by clicking ***Execute*** in the lower right corner of the Lattice LightSheet dialog. This will acquire a single z stack, write it to the **.sld**, and open the image in a view window.

7. With the image window active, type **Ctrl + T** to open a Three View. Different view types can also be accessed from the ***View*** menu in the Home tab.

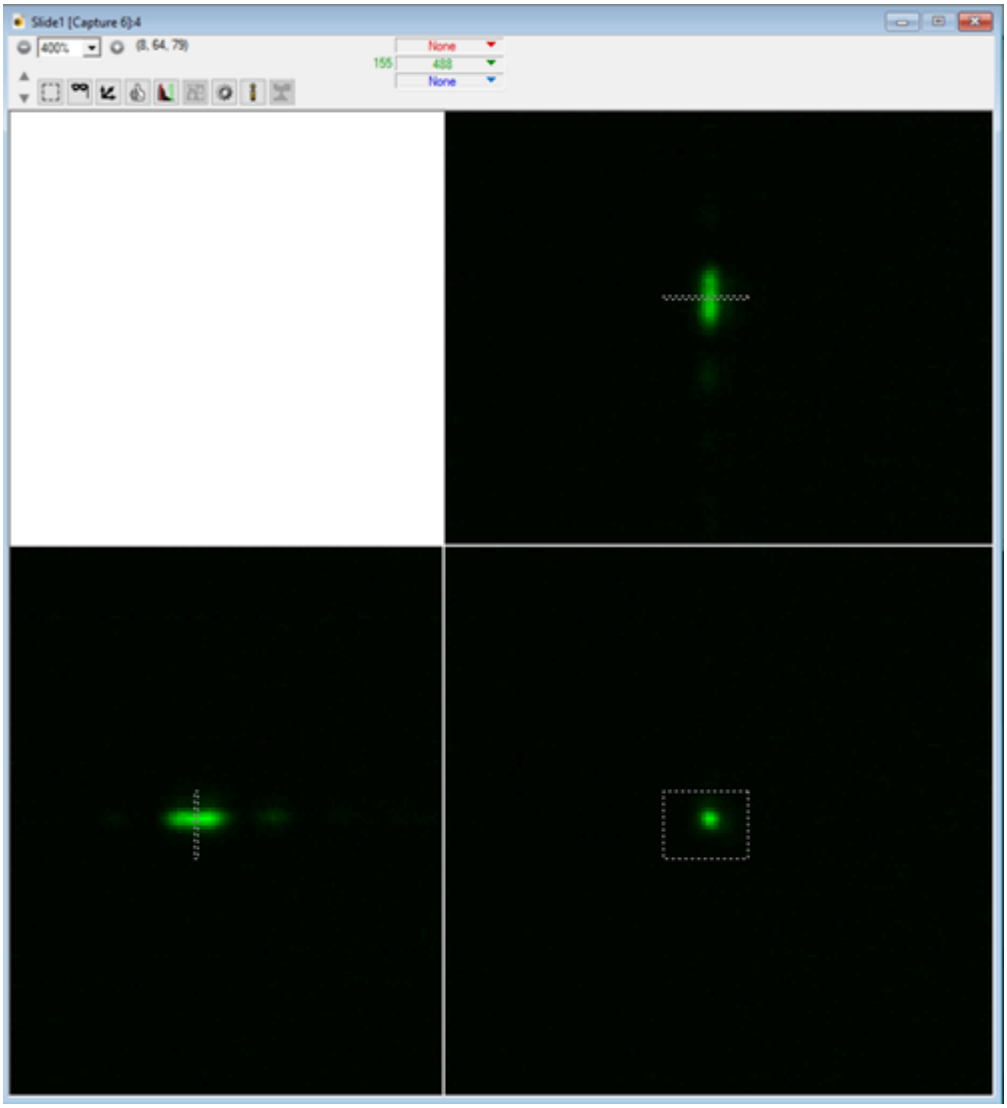
8. The Three View will default to 800%. Use the ***Zoom*** dropdown menu to change to 400%.

9. Use the ***Pointer tool*** to select a point in the XY, XZ, or YZ area. The other areas will update to focus around the selected voxel. Clicking near the center of any panel should bring the bead into view.

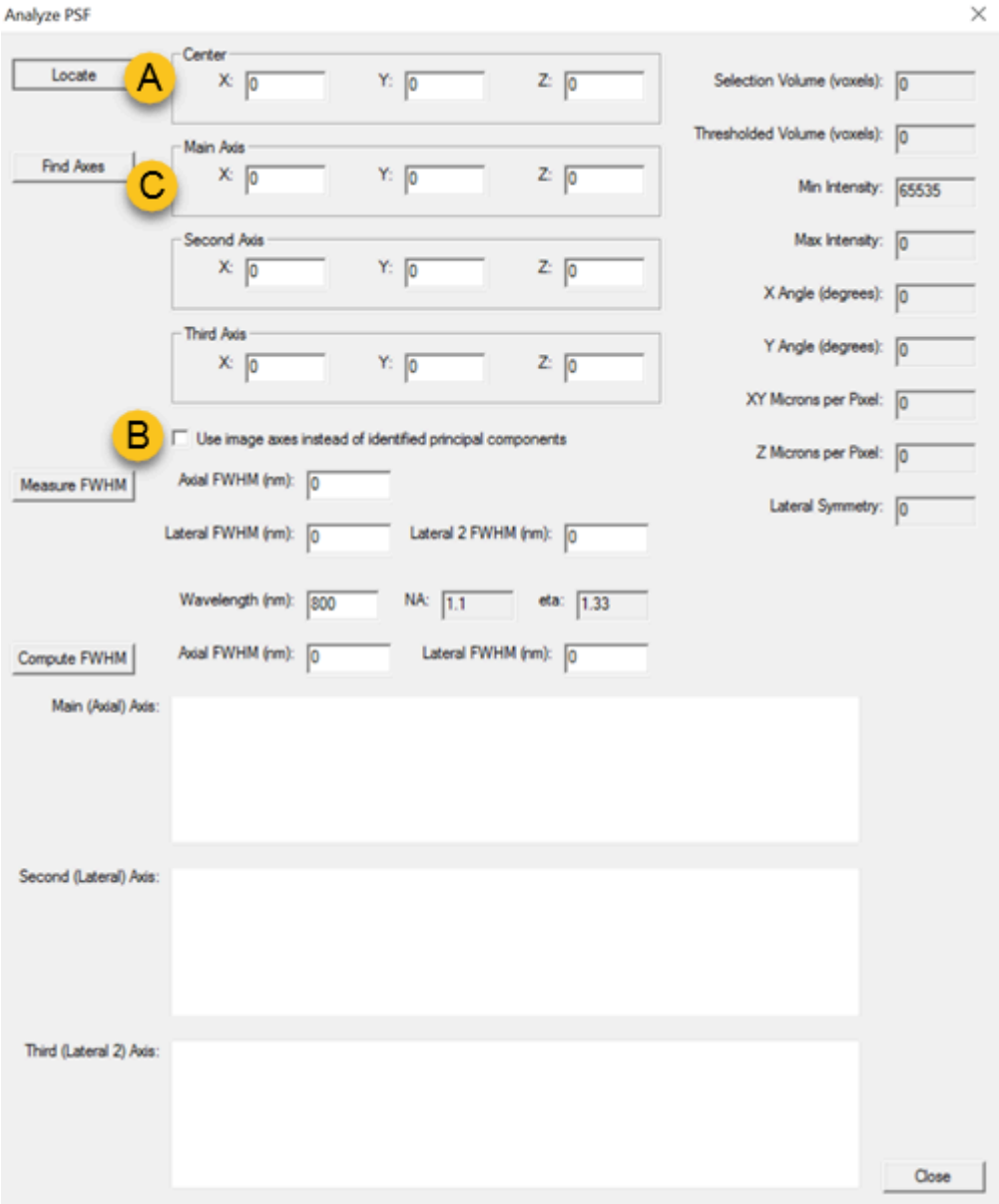
10. Select the Marquee tool from the tool menu in the Three View window.



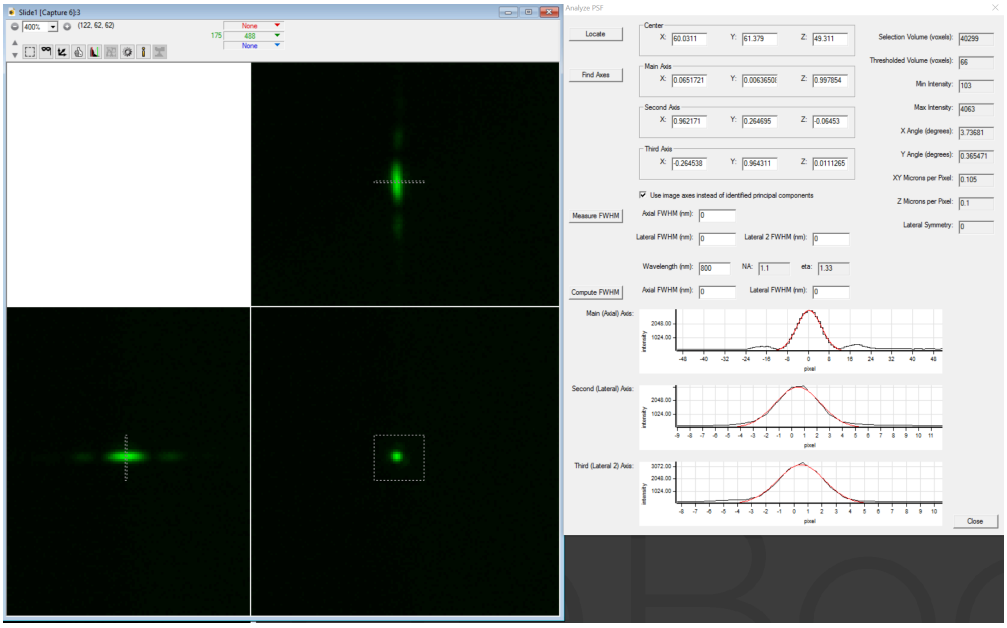
11. Draw a rectangle around the XY view of the bead.



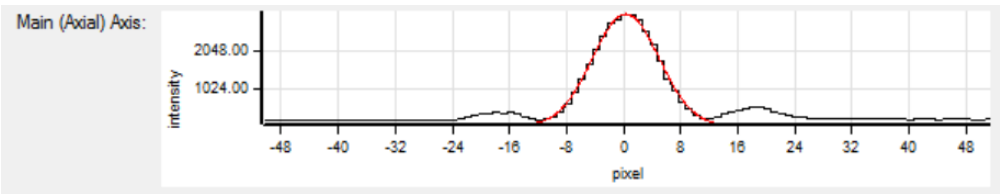
12. In the main SlideBook ribbon toolbar, go to the Analyze tab, Evaluate group, ***Extract Measurements > Analyze PSF***. The Analyze PSF dialog will open.



13. Click ***Locate*** (A). Check the box to ***Use image axes instead of identified principal components*** (B) . Click ***Find Axes*** (C).



14. You should observe the following pattern in ***Main (Axial) Axis***. This is a profile of the excitation PSF.



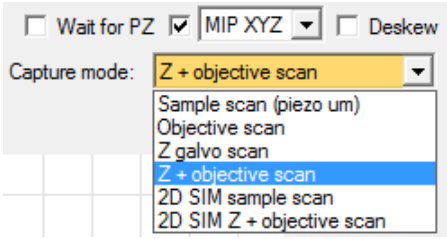
15. If desired, save the PSF for your records.

Total PSF

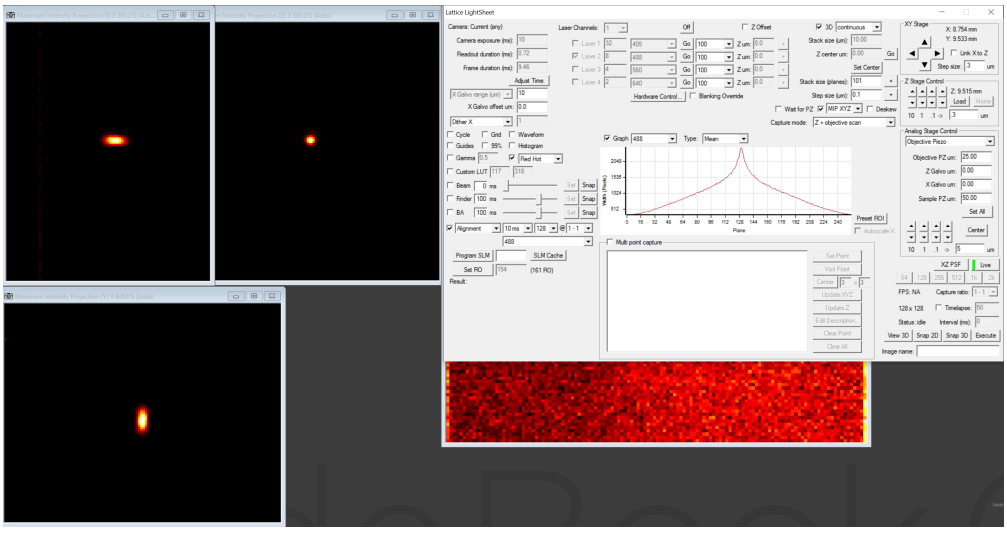
The Total PSF incorporates both excitation and emission paths to demonstrate the optical performance of the light sheet scope. This PSF uses dither of the x galvo, sweeps the beam with the z galvo, and coordinately moves the objective piezo.

Purpose: Demonstrate axial performance of the system and set Z Galvo Offset for the imaging session.

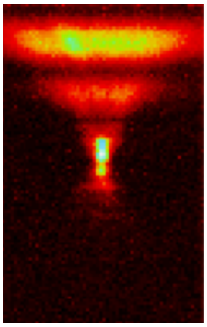
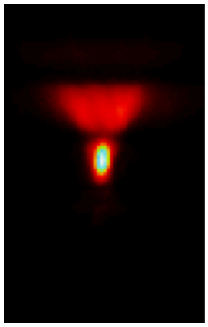
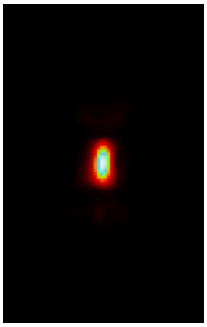
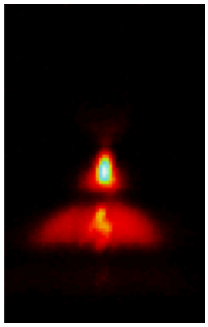
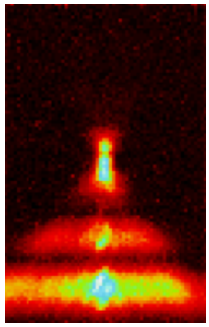
1. Select ***Z + Objective scan*** as the scan type. You will use all; of the previous settings used for the Excitation PSF.



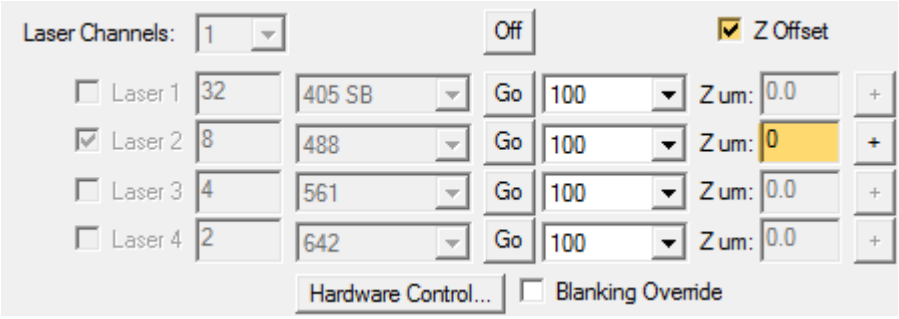
2. Using the bead that you centered and focused, click ***Live***. By clicking ***MIP XYZ***, SlideBook will display XY, XZ and YZ maximum intensity projections of the Total PSF in separate windows. You will need to resize the MIP windows to visualize all in the capture space.



3. You are looking for a nice tight oval bead in the YZ projection panel. If you see any flare above or beneath the bead, the ***Z Galvo Offset*** needs to be adjusted. Refer to the following chart for guidelines on adjusting this value.

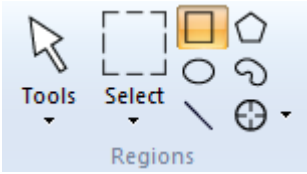
 		 
Increase Z Galvo Offset value	Z Galvo Offset is correct	Decrease Z Galvo Offset value

4. If you need to adjust z galvo offset, click ***Stop***. Check the box for ***Z Offset***. Enter a test value for ***Z um***. Click ***Live*** to recapture the PSF. Repeat until you achieve the correct offset value. Once the correct value is determined, ***Stop*** the capture and click **+** next to the offset to save the Z offset value for the imaging session.

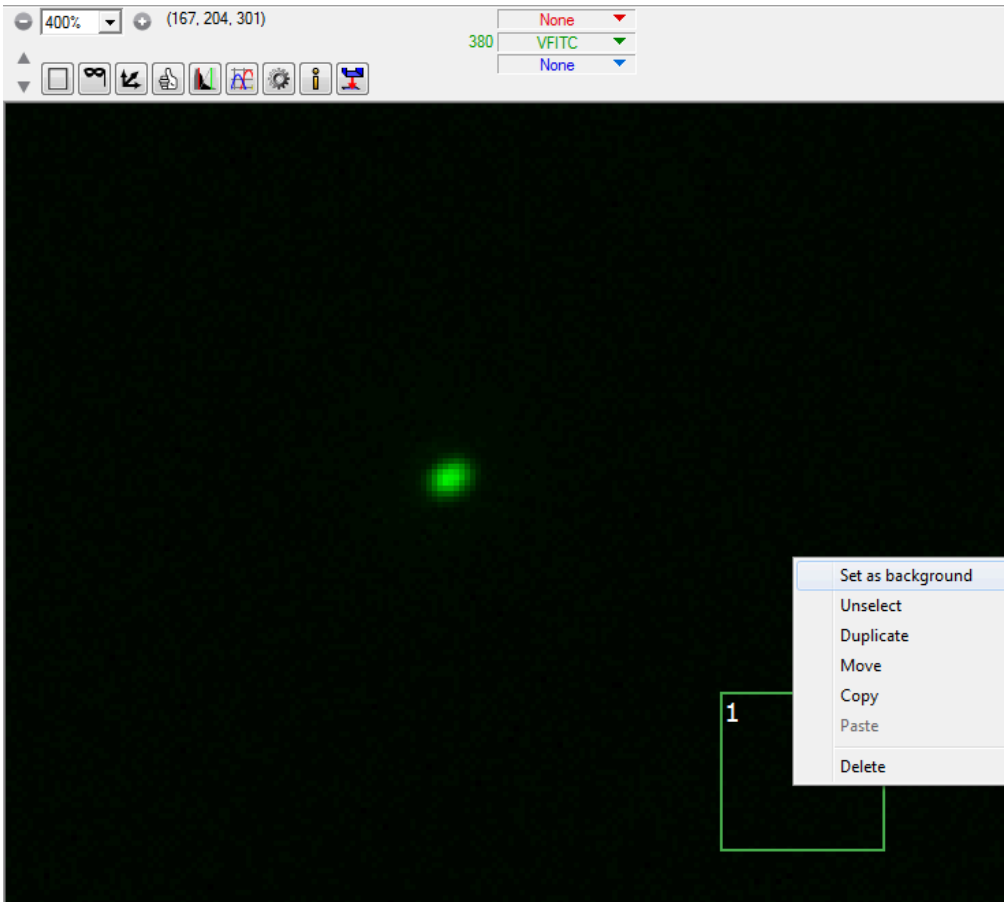


5. To best evaluate the Total PSF, capture the PSF by clicking ***Execute*** in the lower right corner of the Lattice LightSheet dialog. This will acquire a single z stack, write it to the **.sld**, and open the image in a view window.

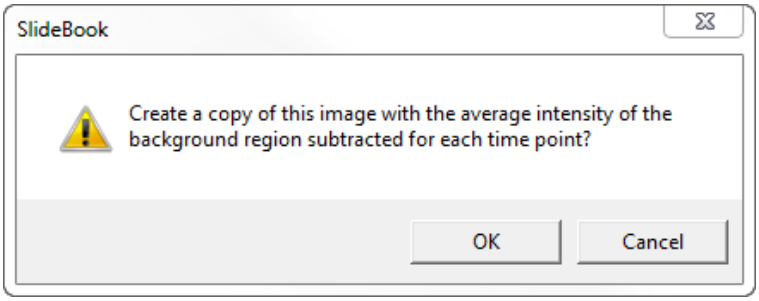
6. Before evaluating the Total PSF, you will need to perform a background correction. In the main SlideBook ribbon toolbar, go to the Home tab, Regions group and select the rectangle region drawing tool.



7. Click and drag to draw a rectangular region in a corner of the image view window. Click to select the region (the number "1" will be white when the region is selected). Right click and select ***Set as Background***.

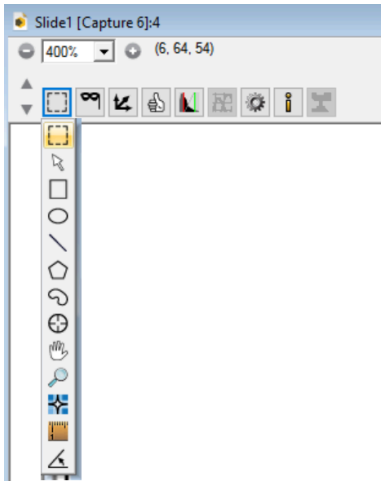


8. Go to the Analyze tab, Process Image group, ***Math menu > Subtract Region Background***. A window will open asking if you would like to create a copy of the image with the average intensity of the background region subtracted. click ***OK***.

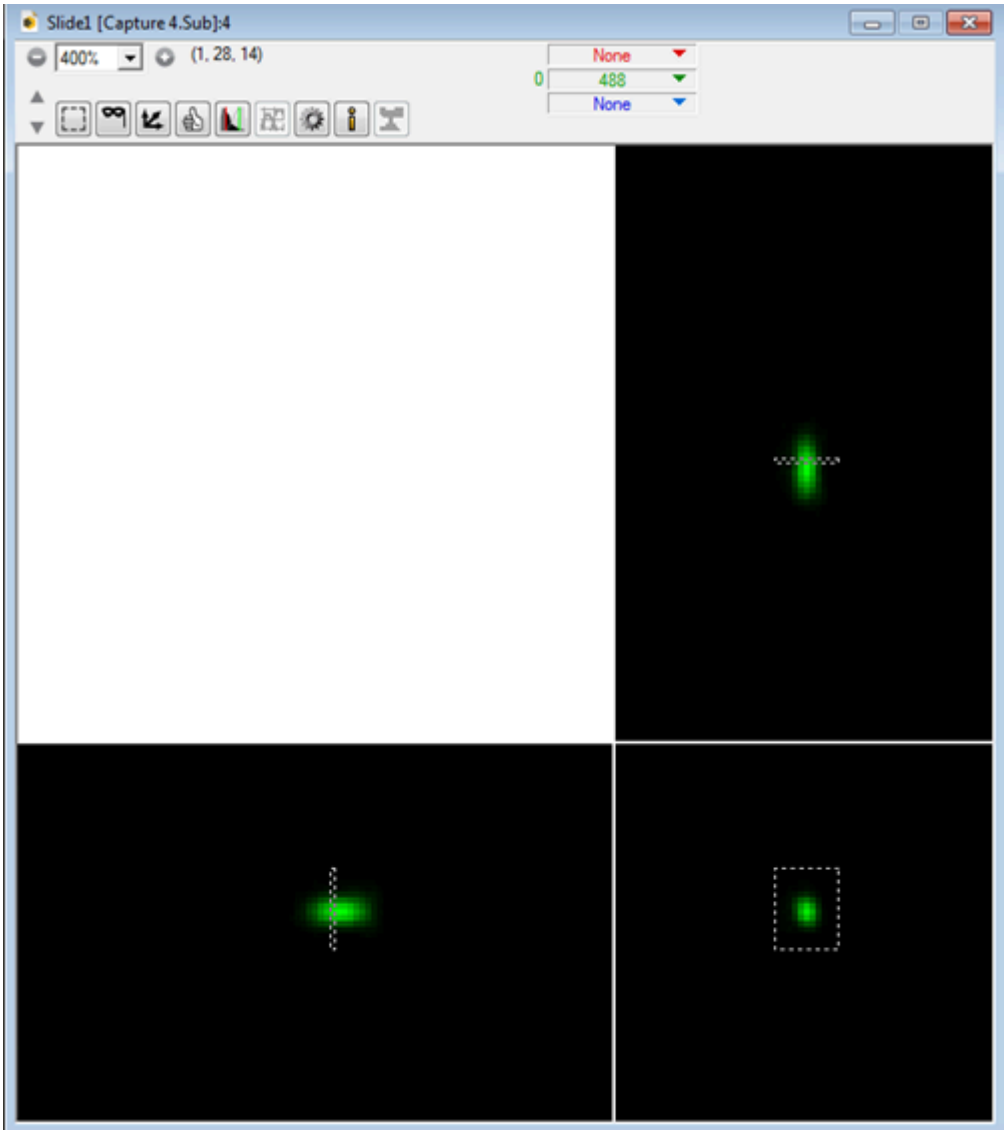


9. Locate the new image in the **.sld**, the image name will be appended with **.Sub**. Click on the image name then type **Ctrl + T** to open a Three View. All views will appear blank upon initial opening, click near the center of any panel to bring the bead into view.

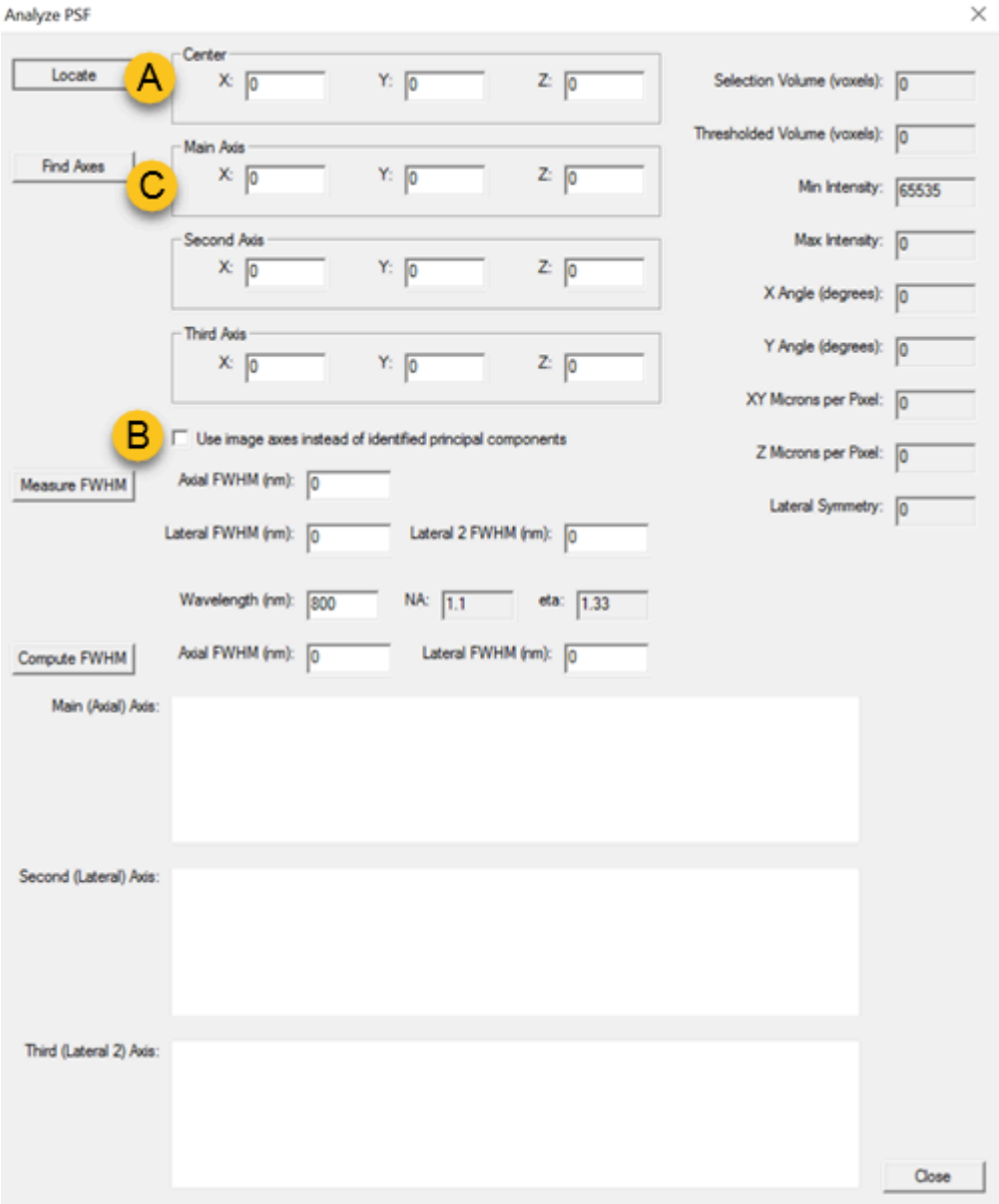
10. Select the Marquee tool from the tool menu in the Three View window.



11. Draw a rectangle around the bead in the XY panel.

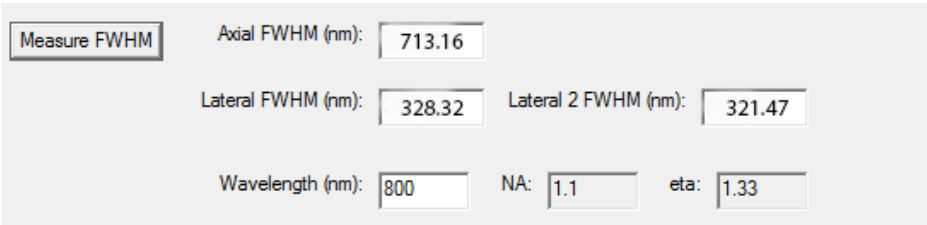


12. In the main SlideBook ribbon toolbar, go to the Analyze tab, Evaluate group, ***Extract Measurements > Analyze PSF***. The Analyze PSF dialog will open.



13. Click ***Locate*** (A). Check the box to ***Use image axes instead of identified principal components*** (B) . Click ***Find Axes*** (C).

14. Click ***Measure FWHM***. You need to achieve an XYZ resolution of greater than 375 x 375x 775nm for 488nm beads. If you do not, it may be necessary to evaluate the detection and excitation PSF as any spherical aberration in the detection PSF and any non-centration in the excitation PSF will cause decreased resolution. In addition, you may need to evaluate the Z galvo offset and adjust accordingly.



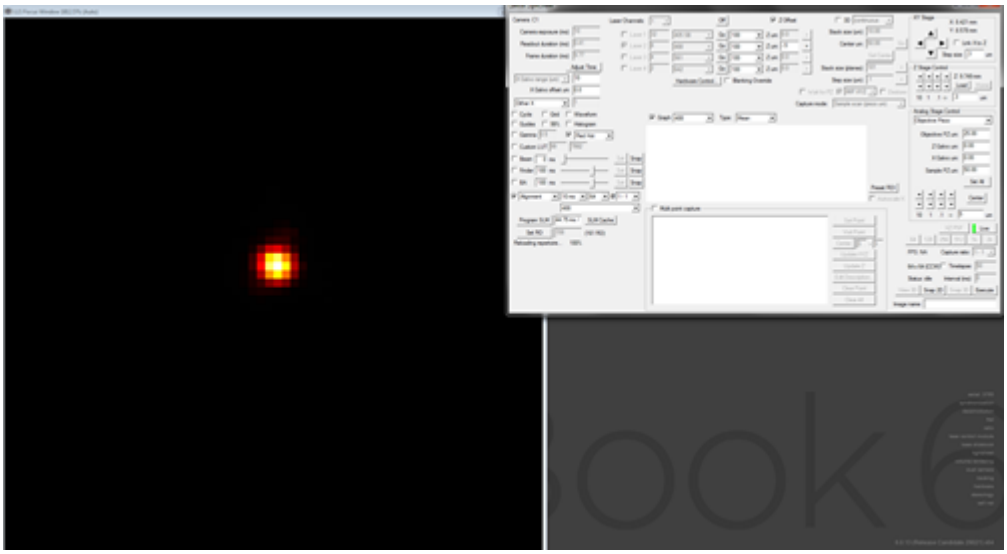
15. Save the PSF for your records if desired.

XZ PSF

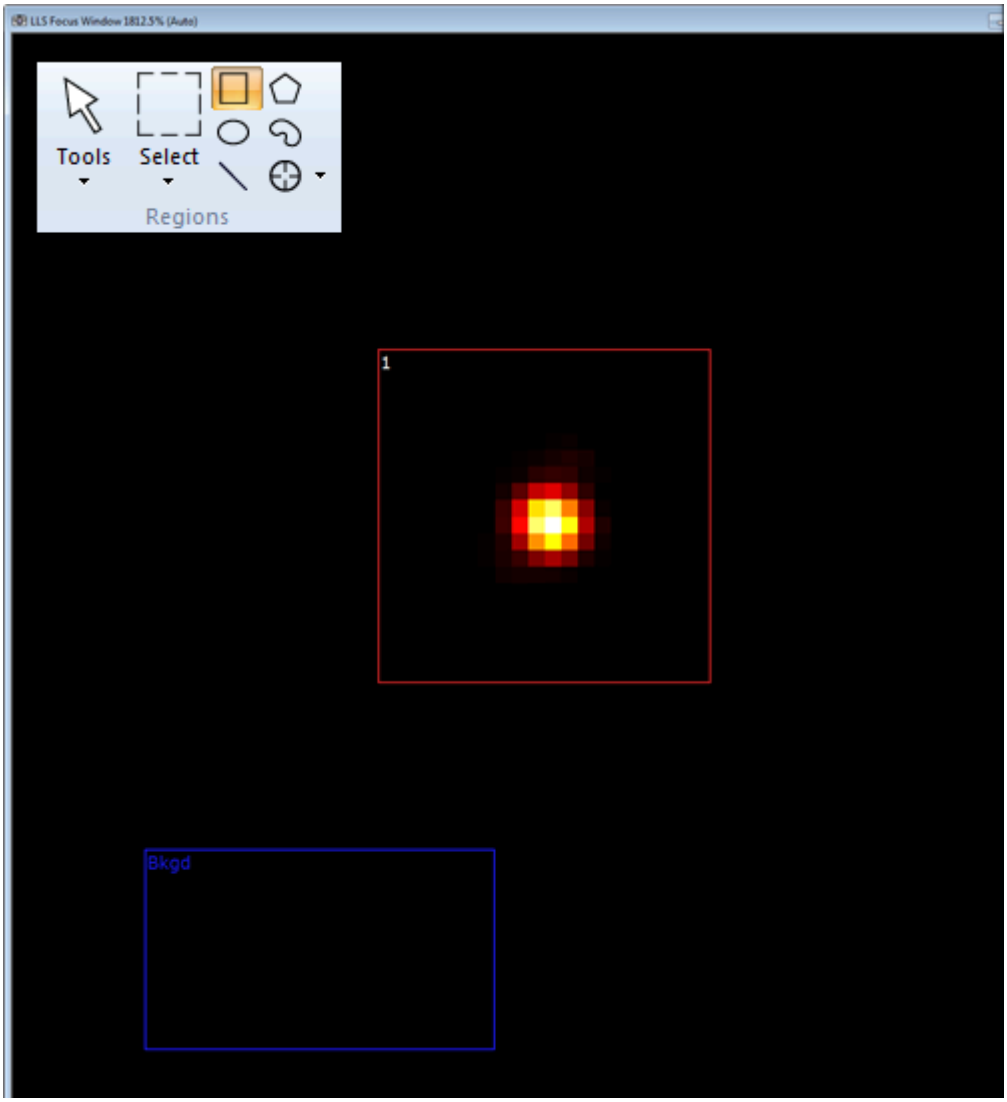
The XZ PSF maps out the shape of the lattice light sheet. This PSF rasters the beam with the X galvo for dither and uses the Z galvo for sweep. It rasters over a stationary bead and takes a maximum intensity projection at each acquisition. We previously used the beam-examining camera to examine the incoming beam but now we will evaluate the beam in the sample plane using intensity of the bead as a detector.

Purpose: To further evaluate the multi Bessel beam pattern.

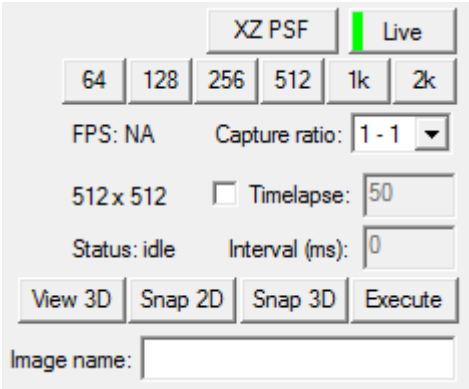
1. Uncheck the box for ***3D***, click ***Live***. Center and focus a bead.



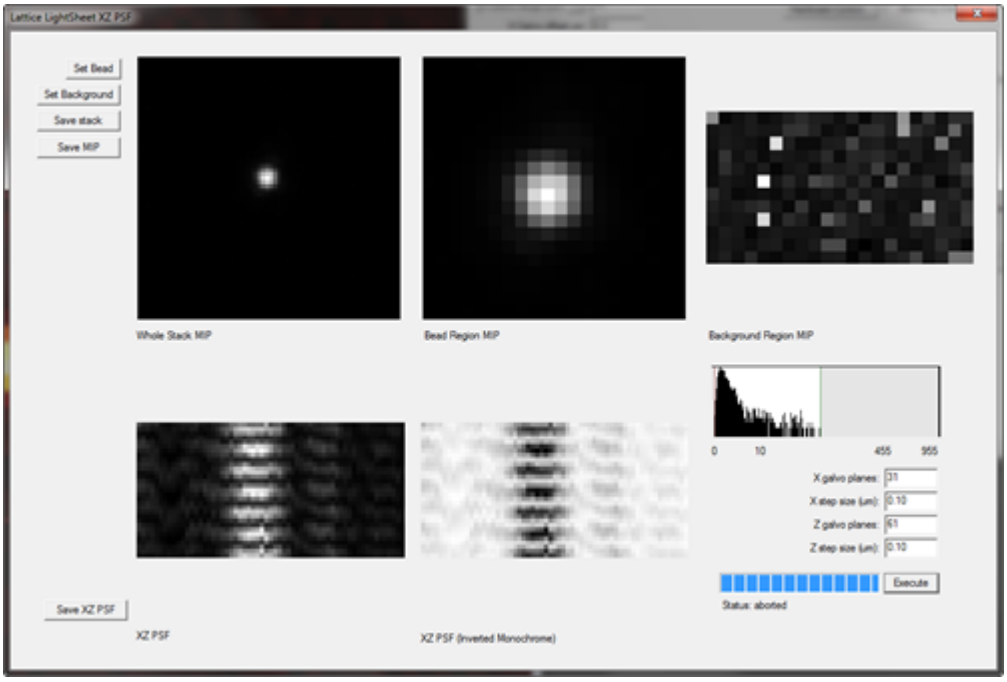
2. In the main SlideBook ribbon toolbar, go to the Home tab, Regions group and select the rectangle region drawing tool. Draw a region away from the bead, right-click and Set as Background. Draw a region around the bead.



3. Click ***Stop*** to stop acquisition. Check the box for ***3D***, click ***XZ PSF*** (by the Live button). The XZ PSF is acquired using preset acquisition parameters and thus it does not matter on what type of capture mode you use.



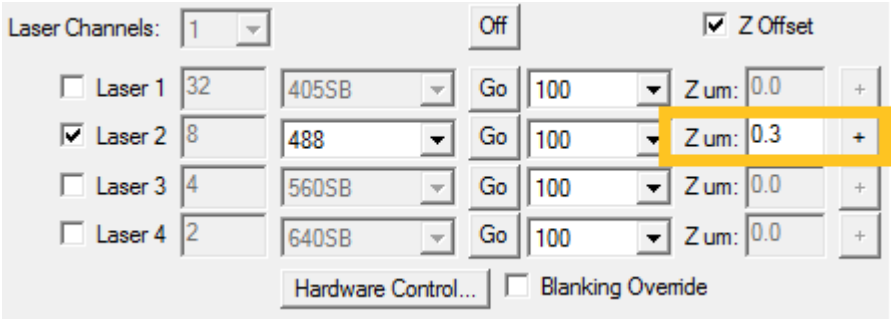
4. A large Lattice LightSheet XZ PSF dialog will open. Click ***Execute*** in the lower right corner of the dialog. Once the acquisition is complete the following XZ images will appear. You should observe a symmetrical and perfectly vertical XZ PSF.


Lattice LightSheet XZ PSF
Set Bead
Set Background
Save stack
Save MIP
Whole Stack MIP
Bead Region MIP
Background Region MIP
0
10
455
955
X galvo planes:
31
X step size (um):
0.10
Z galvo planes:
61
Z step size (um):
0.10
Execute
Status: aborted
Save XZ PSF
XZ PSF
XZ PSF (Inverted Monochrome)

Z Galvo Offsets for Additional Wavelengths

After you capture the four different daily PSFs (Detection, Excitation, Total, and XZ) and adjust the alignment, you should be confident that the system is well aligned for the 488nm wavelength. It is important to note that the Z Galvo offset will vary slightly for each wavelength. Before you proceed with imaging biological samples, you should repeat the Total PSF for each wavelength you anticipate using for the session.

1. Reload a bead coverslip with beads of desired fluorophore. Refer to Finding a Bead and Total PSF to determine ***Z Galvo Offset*** value (step # 5 of Total PSF). Once you have determined the appropriate Z galvo offset, be sure to click + to save the value for use.



NOTE: Once you click +, the value will change to **0.0**. This is because you have essentially created a new zero offset position.

2. At Step 15 of the Total PSF, refer to the following chart for the target values for XYZ resolutions.

Wavelength	X	Y	Z
488nm	375nm	375nm	775nm
560nm	435nm	435nm	888nm
642nm	525nm	525nm	1100nm

3. You are now set up to perform multi-channel imaging.

Total PSFs using Sample Scan

We previously discussed the Total PSF captured using Z Galvo/Z Piezo capture (also called Objective Scan mode), which generates a deskewed PSF. While this is critical for daily evaluation of the alignment, when imaging biology, the predominant mode of capture is Sample Scan acquisition, which generates skewed data sets. For this reason, you will need to capture a matched Total PSF using Sample Scan capture mode. This PSF will then be available for use in downstream workflow, including deskewing and deconvolution.

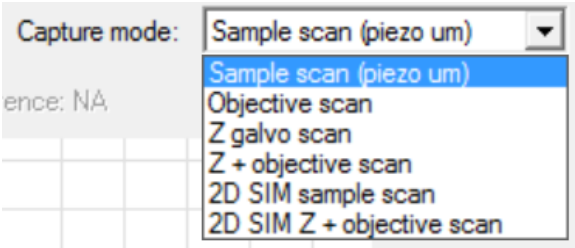
The following video covers and explains the math involved during Objective Scan and Sample Scan acquisition types and explains deskewing.

https://youtu.be/Icsj1NrIPlo

Purpose: To collect a PSF for use in deconvolution.

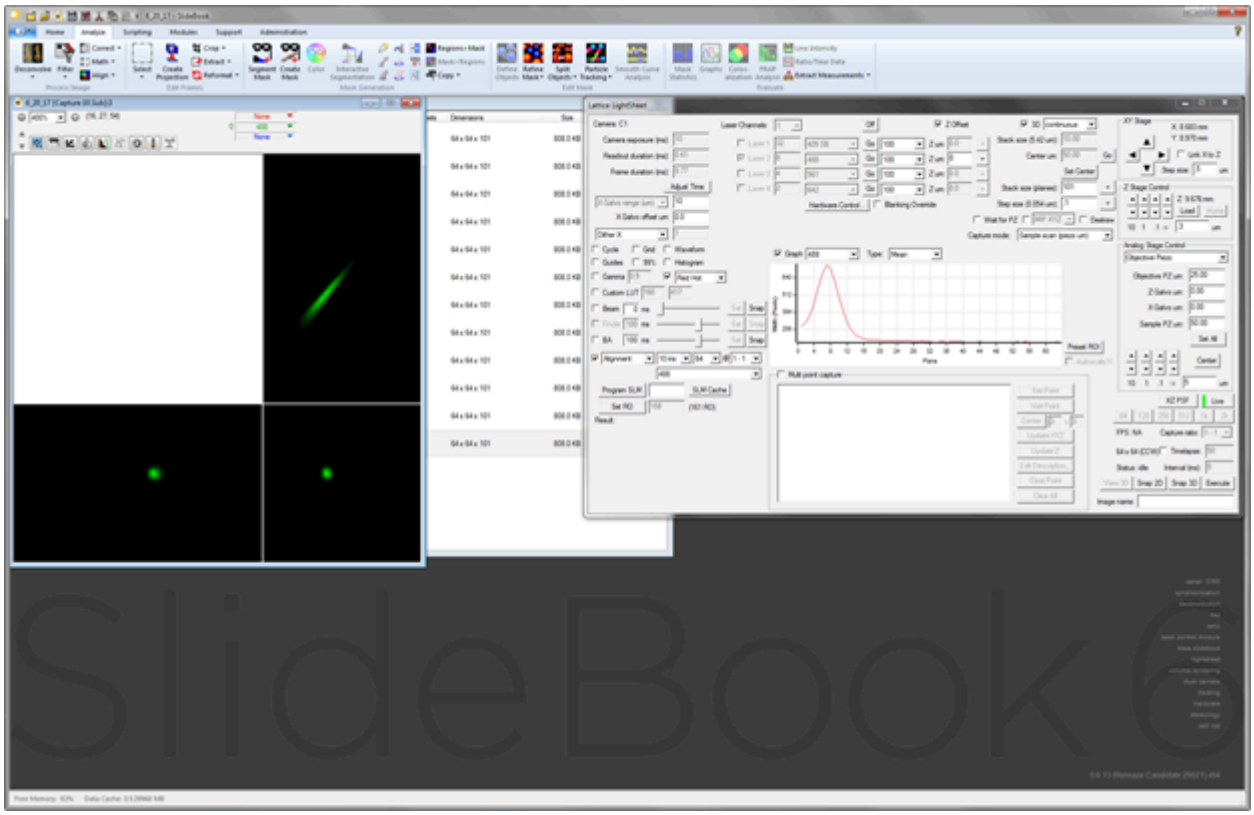
1. Bring a single bead into focus in the center of the field. If you can not locate a bead, return to ***Finding a Bead***.

2. Change Capture mode to ***Sample Scan***. Use previous PSF capture settings.



3. Click ***Execute***. The PSF will be acquired. Locate the new image in the **.sld**. Click on the image name then type **Ctrl + T** to open a Three View. All views will appear blank upon initial opening, click near the center of any panel to bring the bead into view.

4. Look at the XZ Projection within the Stack Projections tab and observe that the PSF is skewed. This is because the acquisition was taken in Sample Scan mode and will need to be deskewed. For more information on what this means, please refer to our short instructional video at https://youtu.be/Icsj1NrIPlo.



5. Save the stack for use in deconvolution later by clicking the save icon in the center. You may wish to include wavelength information in the filename.

6. Daily alignment and PSF verification is now complete. You can proceed to imaging biology. During biological imaging, periodically check in on the uEye software to examine the beam and be sure to maintain an adequate level of media in the chamber sample. Remember to repeat Daily Dye Alignment and Daily PSFs should you change imaging media.

LLS SIM Acquisition Guide

The Lattice LightSheet Microscope is capable of two modes of acquisition. The first and most commonly used is the dithered mode, where the light sheet is rapidly dithered along the X-axis and only one image is recorded per Z plane. The second is the structured illumination microscopy mode (SIM). During SIM acquisition, the lightsheet is moved in N discrete phase steps along the X-axis. Five raw images are collected that are reconstructed using a user generated optical transfer function (OTF) to produce a reconstructed image that is beyond the diffraction limit of the detection objective and the generated Lattice pattern, in the patterned direction. The obvious advantage to SIM acquisition is the increase in axial resolution from approximately 400nm to an achievable resolution of 280nm. The disadvantages of SIM for live cell microscopy are the decrease in temporal resolution due to the longer acquisition times needed to collect the 5 phase images for each Z plane and the increase in photobleaching and phototoxicity for the same reason. SIM acquisition should only be used when the need for temporal resolution is not as stringent and the sample lends itself to longer acquisitions, i.e: higher expression levels of the fluor that is to be detected.

Preparing for SIM PSFs

Once the beam alignment and dye alignment are optimized, you can begin taking the alignment verification PSFs. To do so, you will need to clean the system from the dye alignment and load your bead sample. Fill the lower sample chamber with 8ml of the media you will be using with your sample that day and load your bead sample. If you switch imaging media during a session, you should repeat the daily PSFs to correct any spherical aberration in the LLS.

Cleaning after Dye Alignment

1. Slowly and carefully, raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

2. Wash the lower sample chamber 4-5 times with deionized water to remove as much of the dye as possible.

3. Wipe the tips of the two microscope objectives (lens and portion of body immersed in liquid) with lens cleaning tissues and liquid lens cleaner.

Bead Sample Preparation

The PSF sample is fluorescent beads (preferably 0.1 488µm beads) sparsely attached to a 5mm diameter cover slip. A well-prepared sample should last at least a week. Please refer to Supplementary Materials for information on cover glass preparation and bead coverslip preparation.

NOTE: If you will be performing multichannel acquisition later in the day, you should have bead coverslips prepared for all wavelengths so you can perform Z Galvo Offsets for Additional Wavelengths.

Loading a Sample

1. Slowly and carefully raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

2. Add media if necessary. The sample chamber holds a maximum volume of 8ml.

> **NOTE:** You must be extremely careful when adding water as stages and piezos are very sensitive to water exposure. Please cover the stages with the provided stage cover.

3. Load the sample holder containing the sample coverslip with the coverslip up. See also Loading Coverslip to Holder.

4. Secure using set bolts.

5. Grasp the objective assembly handle to stabilize it. Remove the block that was holding the assembly up in the load position. Slowly lower the objective assembly using the handle.

> **NOTE:** There are hard limits in the software that will prevent positioning that would damage the objective but caution should still be exercised.

Removing a Sample

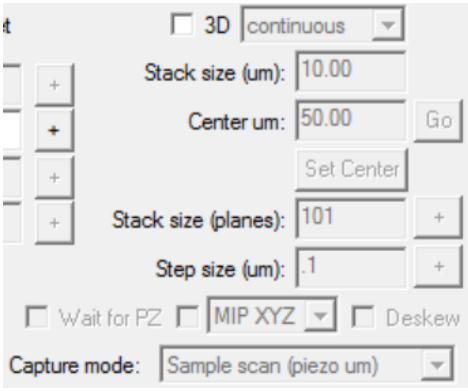
1. Slowly and carefully raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

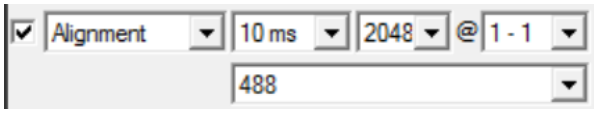
2. Remove the set bolts.

3. Remove the sample holder. If it is the end of use for the day, see also Clean Up and Shut Down.

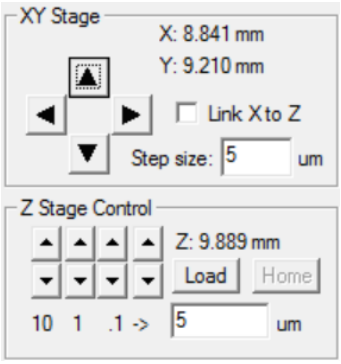
Finding a Bead for SIM

1. For PSF and biology acquisition, use the multi-Bessel for each wavelength and a non-3D (uncheck ***3D*** box) capture to find the bead. In ***Alignment***, change the parameters to ***10ms*** exposure, ***2048***, and appropriate wavelength.

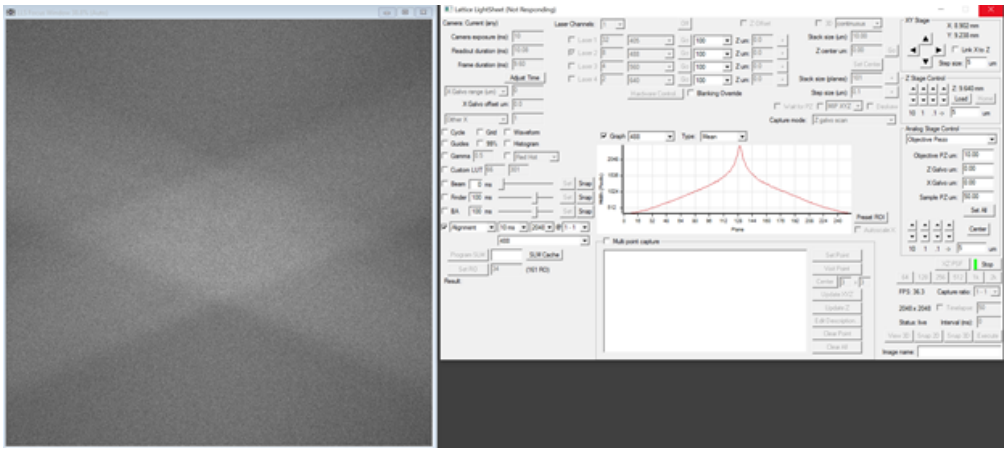




2. To find beads, use the ***XY Stage*** and ***Z Stage Control*** interfaces in the top right corner of the Lattice LightSheet dialog to move the sample holder to bring beads into the field of view. The values that are referenced are the last position of the X, Y and Z stages. Do not click ***Link X to Z***. Move the XY stages by clicking the arrows that designate the direction in which you would like the bead to move. The ***Step size*** field is used to set a value for the movement made each time you click an arrow button. The default value is 5µm and this is a good starting value for the initial location of beads. For the Z stage control there are pre set values of 10, 1 and 0.1µm as well as a fourth user-defined value all linked to up and down arrows. The default value is 5µm.



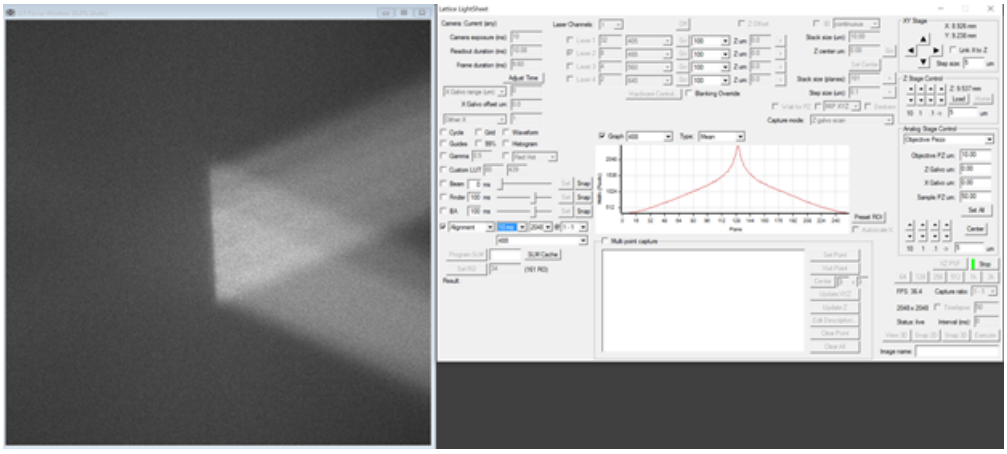
Use the arrow buttons to focus through the sample to get your bearings. In the full 2K FOV using 488nm, you may see a faint multi Bessel pattern due to a slight amount of residual dye. Ironically, for new users this is a useful tool for finding the coverslip as will be covered below. As you become more and more proficient and comfortable locating samples, we recommend that you wash the sample chamber to try and remove as much residual dye as is possible.



Using the Z stage controls, move the stage up. As the stage is physically moving up to move the sample holder closer to the objective, the value for the Z stage position will become more negative.

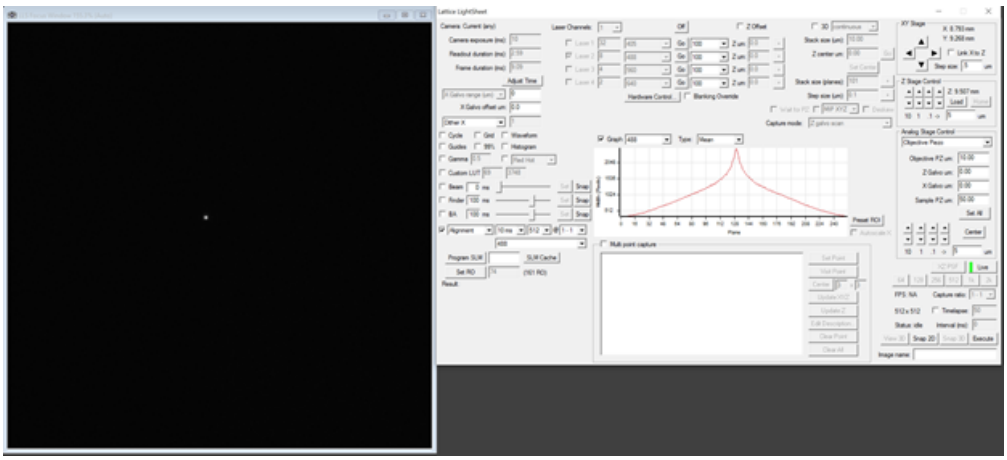
NOTE: We recommend that when changing samples or beads that you lower the Z stage. This will allow you to place a new sample on the system and lower the objective assembly without concern of lowering the objective onto the sample holder.

3. As you raise the sample holder, you will observe the sample come into the field of view from the left. This will appear as a line through the Bessel with the FOV to the left of the Bessel being much darker than the FOV to the right of the Bessel. This line that demarcates these halves of the overall FOV is the point where the Bessel is making contact with the coverslip.

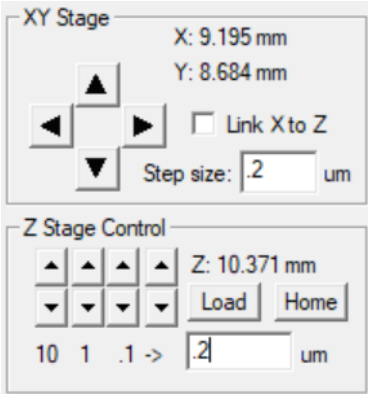


4. Click ***Stop*** to stop the acquisition. In alignment, change the field size to ***512*** then click ***Live*** to resume live capture. Any time you change acquisition parameters (with the exception of laser power), the acquisition will need to be stopped and restarted.

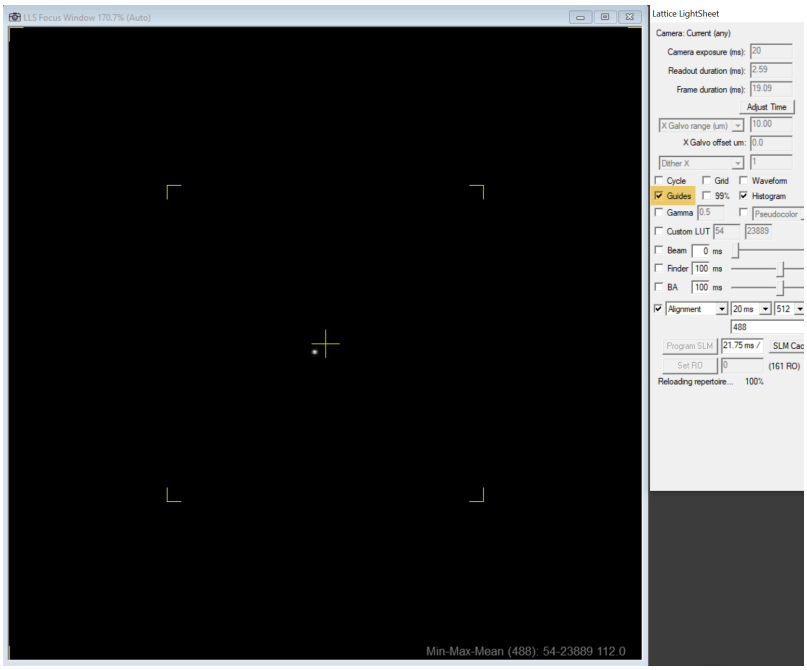
NOTE: The LLS has an effective magnification of 62.5X so 1 pixel is 0.104µm giving a FOV of 53.2µm at 512 x 512) Use the XY stage control (we recommend that you initially use 5µm step size) to move the sample to locate and center a bead. You will need to locate a single bead with no other beads within ¼ of the field of view. As you move the bead in X, you will need to re-center the beam using the Z stage. This iterative process is required because of the geometry of the illumination and detection relative to the sample. As you move the sample (in this case beads) in X, the sample will move out of the Bessel beam and you will need to raise or lower the coverslip to return the sample to the Bessel beam.



5. Decrease X, Y, and Z step size to 0.2 µm.



6. Center the bead. You can use the ***Guides*** to assist in centering the bead.



7. After you center the bead at 512 x 512, click ***Stop***. Reduce the field to ***128***. Click ***Live***. Adjust Y using the up and down buttons so that the bead is vertically centered. Iteratively adjust X and Z to get the bead focused and centered horizontally. You may notice another bead contributing to the field. If this happens, increase the X step size to 2.2 and continue moving in the + direction looking for another, more isolated bead. When you find the next bead, adjust Y, and then iteratively X and Z.

8. Once you have a nice bead centered and in focus, click ***Stop***.

SIM PSF and OTF Generation

It is important to find an isolated bead per the previous instructions. Any contributing emission from other beads in the field will be problematic as will excessive background due to excessive residual dye from the dye alignment.

Throughout the PSF process (and when imaging samples) you should intermittently evaluate the beam-examining camera to ensure the multi Bessel beam has not drifted. Failure to do so could cause subsequent unnecessary adjustments that could further degrade system performance.

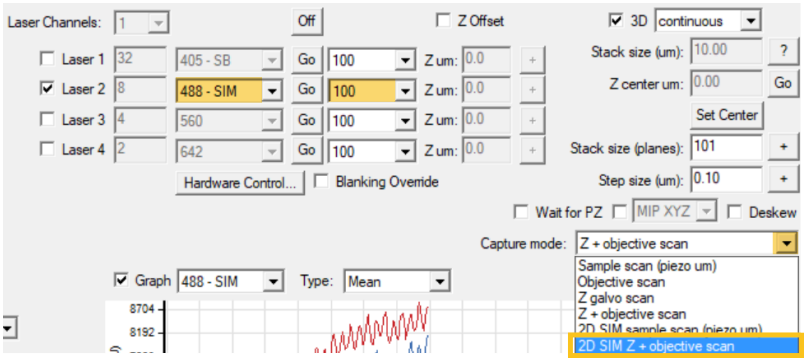
If you notice a bead bleaching during the PSF process, move to a fresh bead per the Finding a Bead instructions.

Collect SIM Total PSF

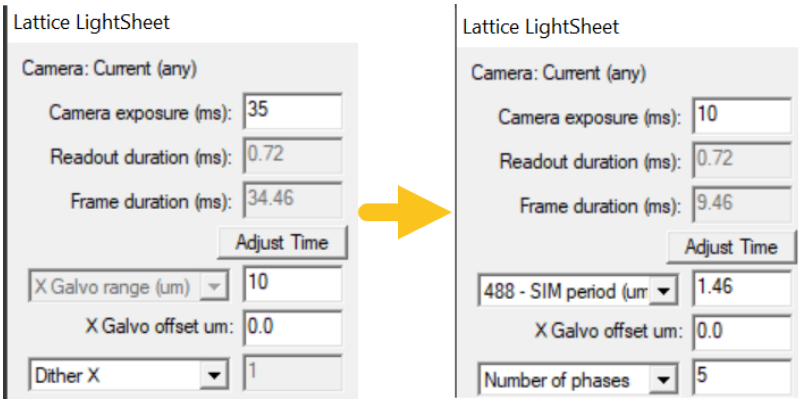
The Total SIM PSF incorporates both excitation and emission paths to demonstrate the optical performance of the light sheet scope. During SIM acquisition the Z galvo moves the lattice in 5 discrete phase steps, sweeps the beam with the z galvo, and coordinately moves the objective piezo.

Purpose: Demonstrate axial performance of the system and set Z Galvo Offset for the imaging session. At this point you should have already performed the dye alignment and the standard daily acquisition of the Detection/Emission, Excitation and Total PSFs. The acquisition of a Total SIM PSF is required to generate the proper Optical Transfer Function (OTF), necessary for the proper reconstruction of generated SIM data.

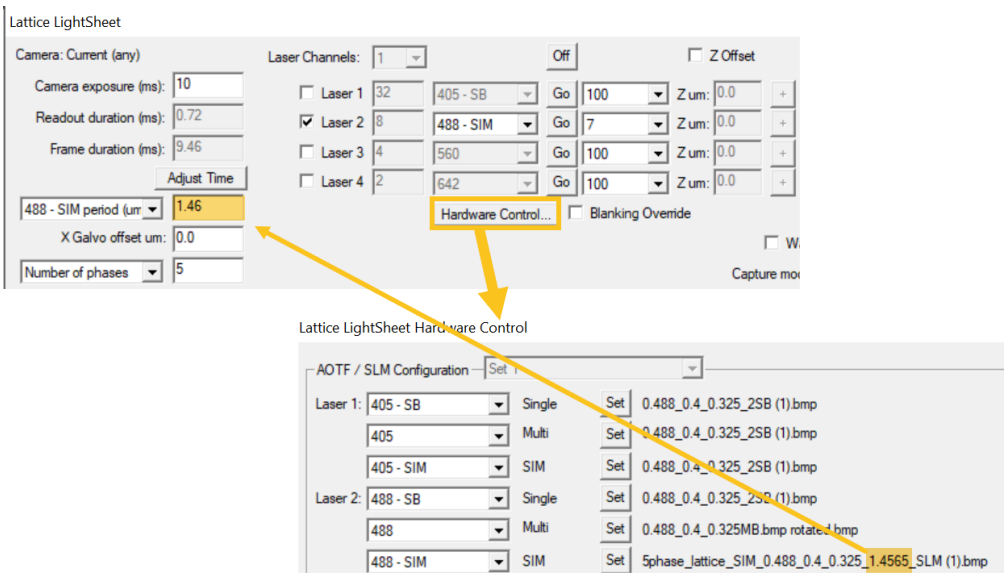
1. Select ***SIM Z + Objective Scan*** as the scan type. In the Laser channels group, select ***488 - SIM*** to use the 488 nm laser with multi-Bessel SIM pattern loaded. Use ***100***% power.



2. When you set the acquisition to 2D SIM Z + objective scan, the SIM pattern and phase options replace the dither options. This is critical, as you will need to enter the proper SIM period for the generated SIM pattern. These patterns will have initially been generated by 3i and placed into a **Patterns** folder on the desktop.

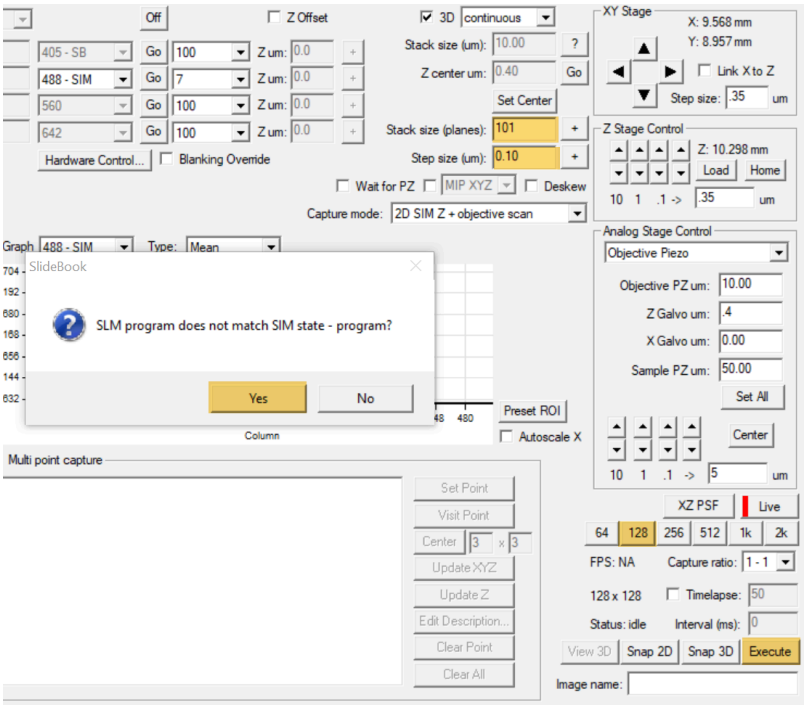


3. Click ***Hardware Control*** to open the Lattice LightSheet Hardware Control dialog. The period will be designated at the end of the pattern name. Enter this value in the text edit field next to the SIM pattern selection dropdown menu.



4. Confirm imaging parameters are set to 101 z steps at 0.1 µm step size and a 128x128 field. With the bead centered and focused, click ***Execute***. A dialog will open asking if you would like to program the SLM, click ***Yes***.

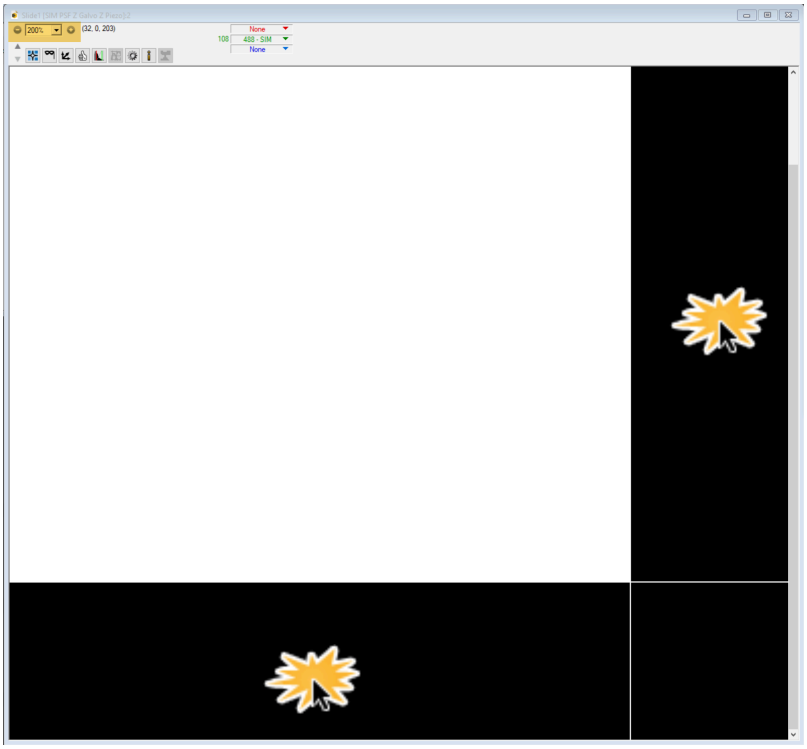
NOTE: It is critical to reprogram the SLM every time you change an imaging parameter with SIM acquisition.



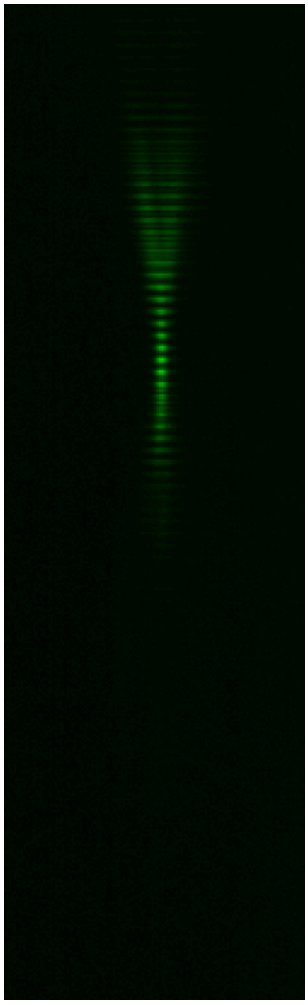
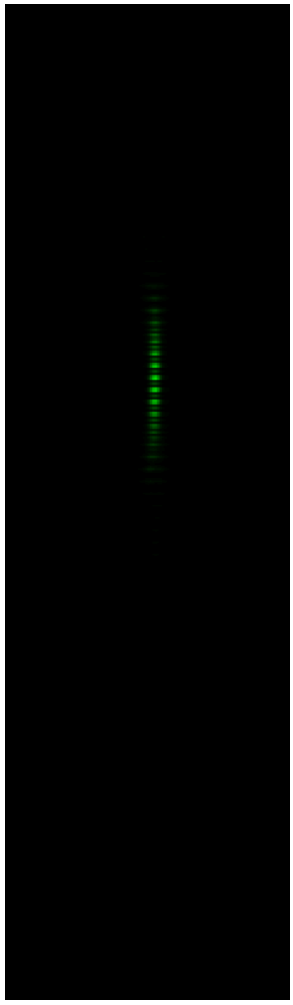
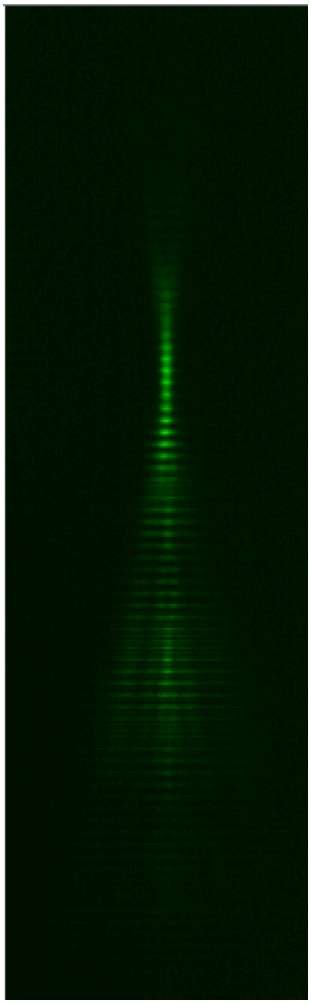
Evaluate Z Galvo Offset in SIM Total PSF

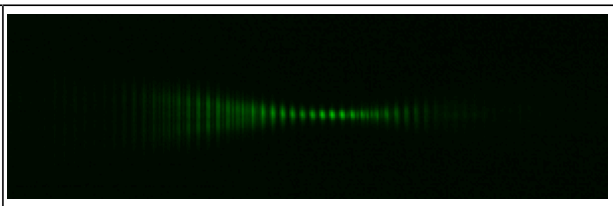
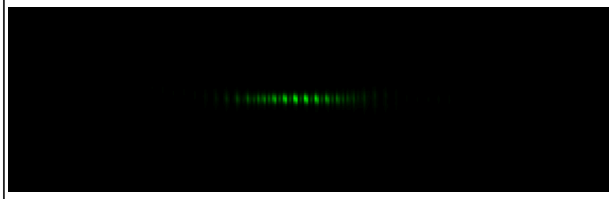
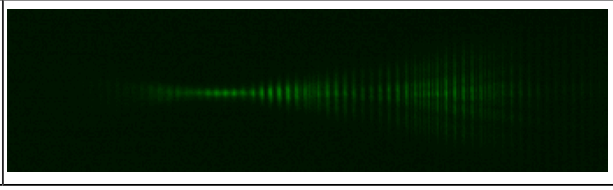
After collecting a SIM Total PSF, you will want to confirm beam offset and focus by evaluating the Z Galvo Offset.

1. After the SIM Total PSF has been acquired, select the image on the .sld then type **Ctrl + T** to open a Three View window. The Three View window will need to be resized as 800% is the default size. Reduce the size to 200% using the zoom buttons or drop down menu. Click in the center of the XZ and YZ panes to bring the SPF into the center of the field.

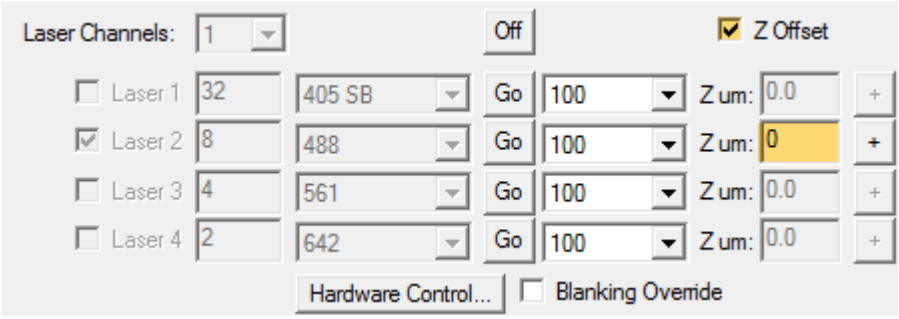


2. The shape of the PSF will determine if you need to change the Z Galvo offset. The PSF should be symmetrical along the horizontal and vertical axes and taper evenly from the center of the PSF.

		
Increase Z Galvo Offset value	Z Galvo Offset is correct	Decrease Z Galvo Offset value

Increase Z Galvo Offset value	
Z Galvo Offset is correct	
Decrease Z Galvo Offset value	

3. If the Z Galvo Offset is correct, proceed to Generate SIM OTF. If you need to adjust the Z Galvo Offset, return to the Lattice LightSheet dialog. Check the box for ***Z Offset***, enter a test value for ***Z um***.



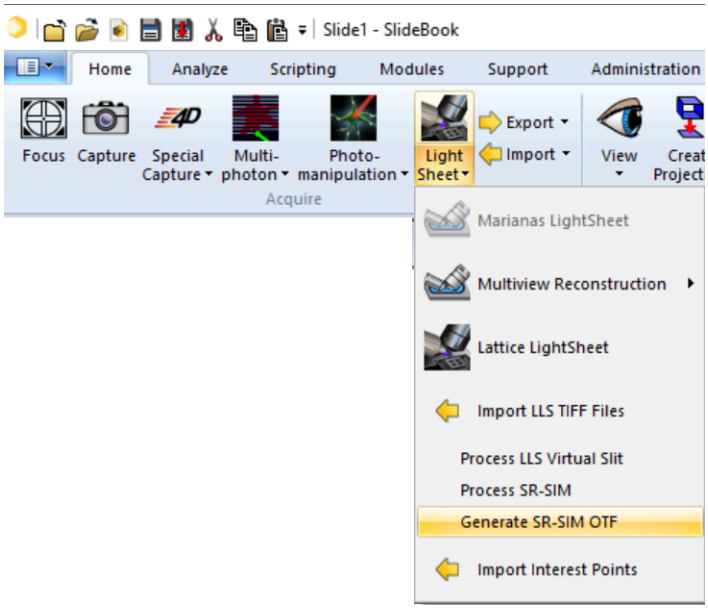
4. Click ***Execute*** to recapture the PSF and evaluate as described earlier. Repeat until you achieve the correct offset value.

5. Once the correct value is determined, return to the Lattice LightSheet dialog and click the **+** button next to the offset to save the Z offset value for the imaging session.

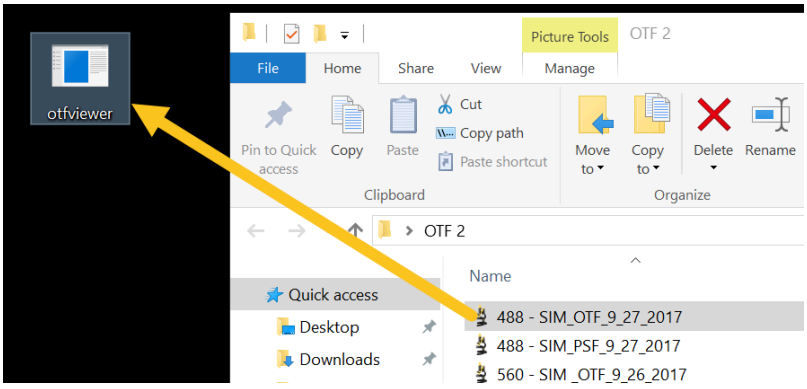
Generate and View SIM OTF

After you have collected the SIM Total PSF and perfected the Z Galvo Offset, you can proceed with generating the SIM Optical Transfer Function (OTF).

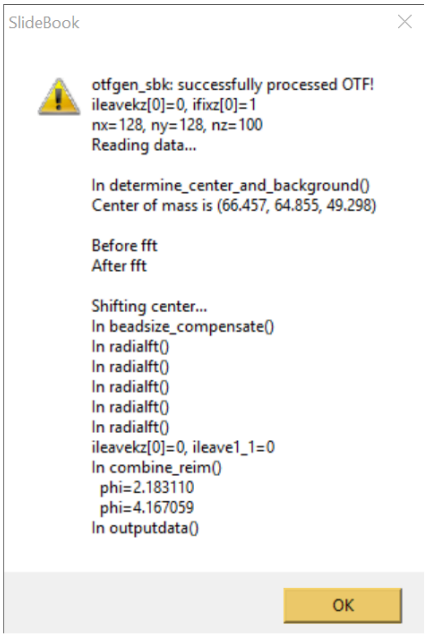
1. In the Home tab of SlideBook, go to ***LightSheet > Generate SR-SIM OTF***.

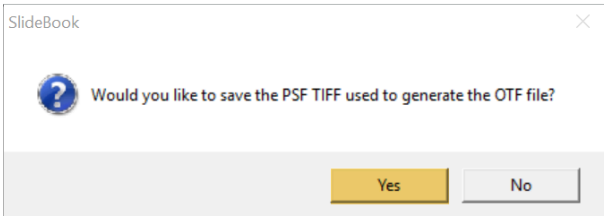


2. You will be prompted to choose a destination folder. Select ***Desktop*** as the destination then ***Make New Folder***. Name the folder OTF, select it as the destination, and click ***OK***.

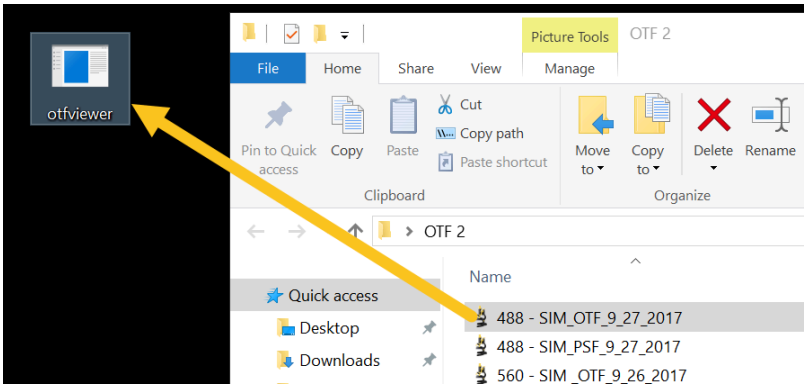


3. A dialog status box will open as the OTF is generated click ***OK***. When prompted to save the PSF TIFF used to generate the OTF, select ***Yes***.

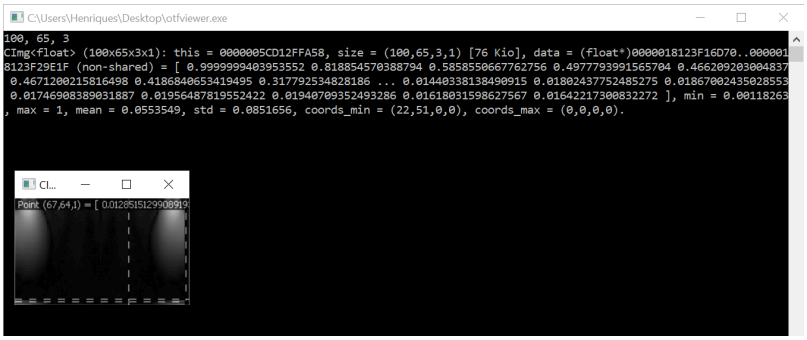




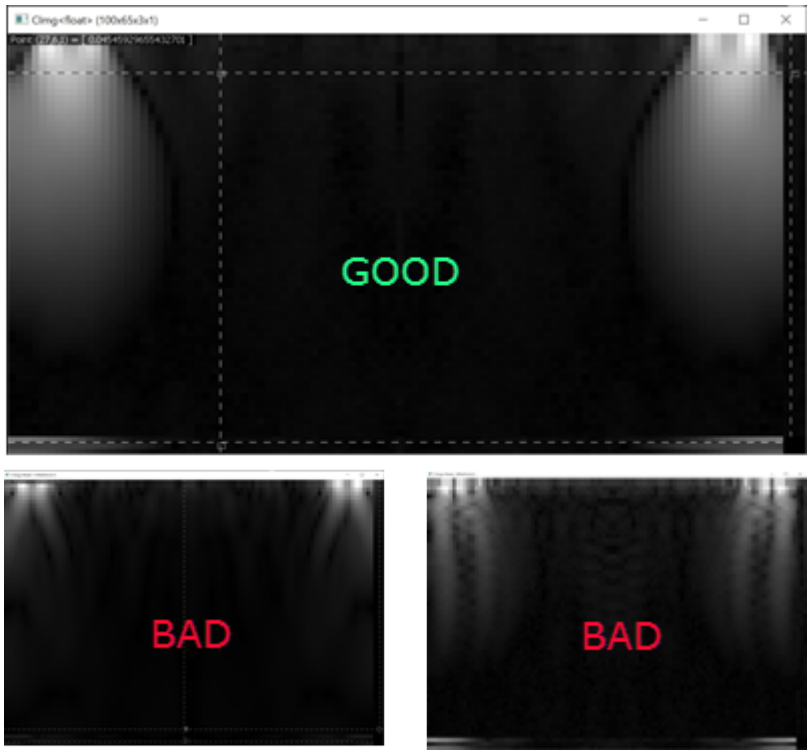
4. Minimize SlideBook to access the desktop. Open the ***OTF*** folder. Click on the newly generated SIM OTF and drag it into the ***OTF Viewer*** application icon.



5. The OTF Viewer application will open and a small image of the OTF will open. Click and drag the corner to enlarge the OTF Image.



6. Visually examine the SIM OTF. There should be two clear lobes of frequency information that are uniform in size and intensity. There should be no vertical or horizontal striping.



7. If the OTF passes inspection, proceed to Evaluating the SIM OTF. If you notice non-uniformity or striping, check alignment and z galvo offset.

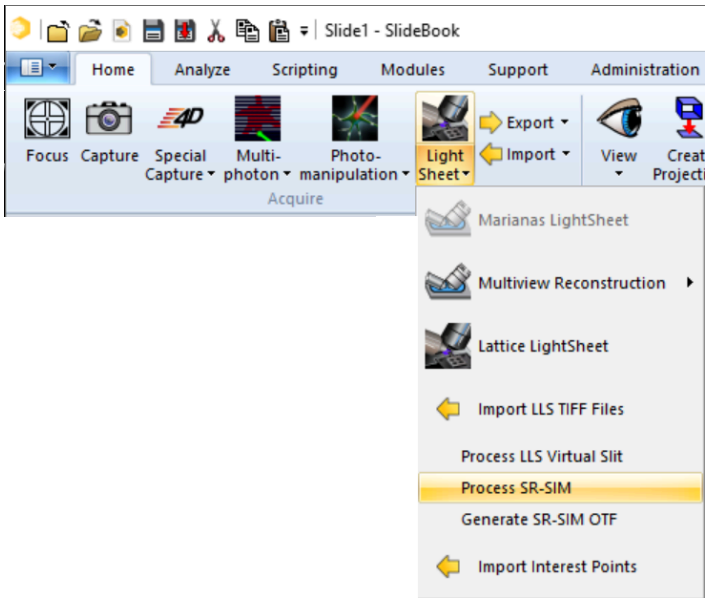
Evaluate SIM OTF

The best way to evaluate a SIM OTF is to test it on a SIM Total PSF. To proceed with this step, you should have already collected the initial SIM Total PSF, adjusted the Z Galvo Offset, and generated a SIM OTF.

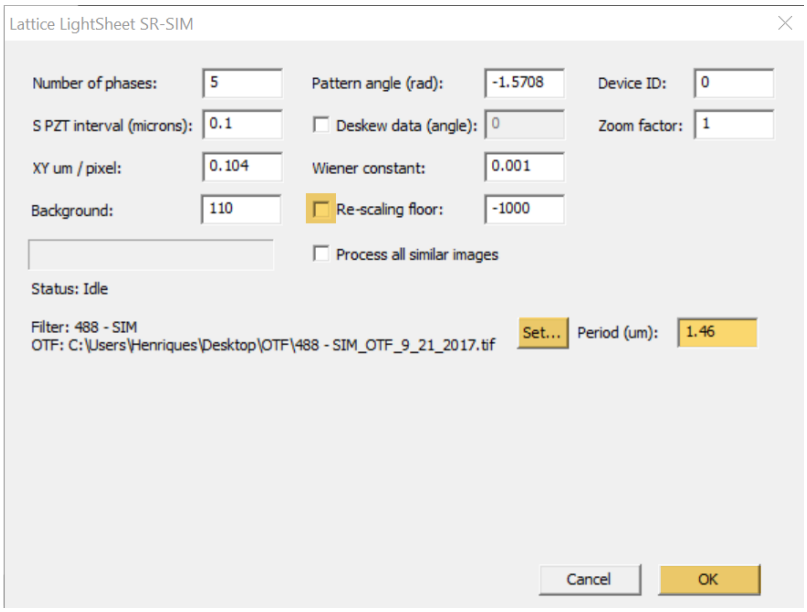
1. Capture a fresh SIM Total PSF. Ideally, you can use the same bead, click ***Live*** to fine tune the focus, then ***Execute*** to acquire the SIM Total PSF. Refer to the tutorial on collecting a SIM Total PSF or Finding a Bead for SIM for additional assistance.

> **NOTE:** When changing any parameters, including switching to ***Live*** capture, you will need to reprogram the SLM. This can be done by clicking ***Program SLM*** after making any changes. Otherwise, you will be prompted by a dialog asking to reprogram, always select ***Yes***.

2. With the new SIM Total PSF open and selected in a view window, go to ***LightSheet > Process SR-SIM***.



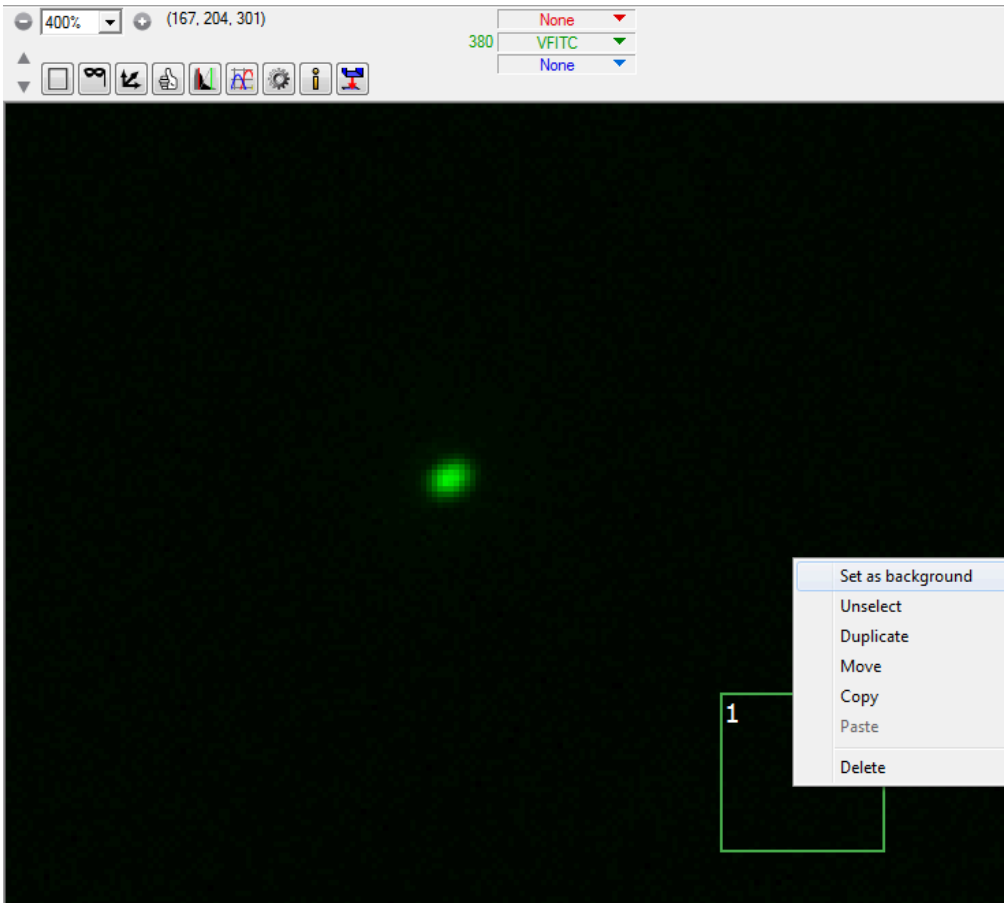
3. The Lattice LightSheet Process SR-SIM dialog will open. Uncheck the selection for ***Re-scaling floor*** (the default status is checked but we recommend unchecking this option for the initial attempt). Click the ***Set*** button to select the OTF for the reconstruction. Navigate to the desktop OTF directory, select the desired OTF then click ***Open***. Type in the SIM ***Period*** value used for acquisition of the PSF (see step 3 in Collect SIM Total PSF). Click ***OK*** to process the PSF.



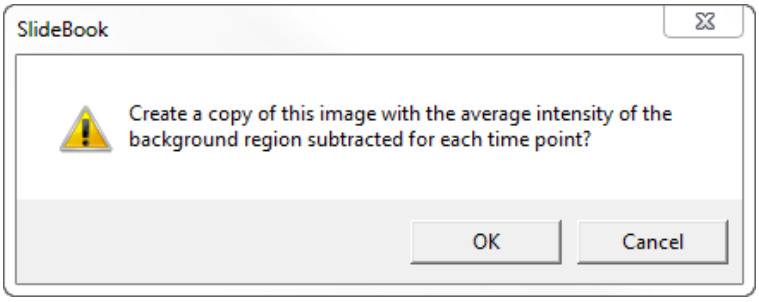
4. Once the SIM data is reconstructed, the processed image will be written to the .sld directly below the original SIM capture. It will be designated LLSIM: Capture followed by the name of the original image and the imaging parameters. Double click on the image to open.

5. Before you analyze the XYZ resolution, you will need to perform a background correction. In the main SlideBook ribbon toolbar, go to the Home tab, Regions group and select the rectangle region drawing tool.

6. Click and drag to draw a rectangular region in a corner of the image view window. Click to select the region (the number "1" will be white when the region is selected). Right click and select ***Set as Background***.

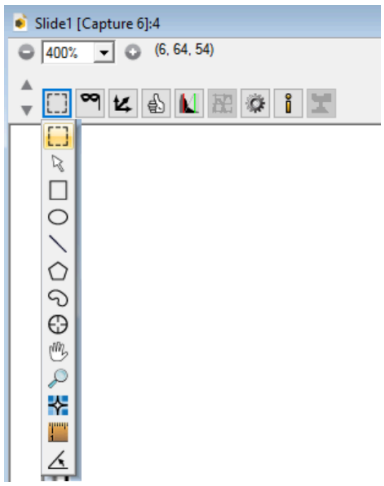


7. Go to the Analyze tab, Process Image group, ***Math menu > Subtract Region Background***. A window will open asking if you would like to create a copy of the image with the average intensity of the background region subtracted. click ***OK***.

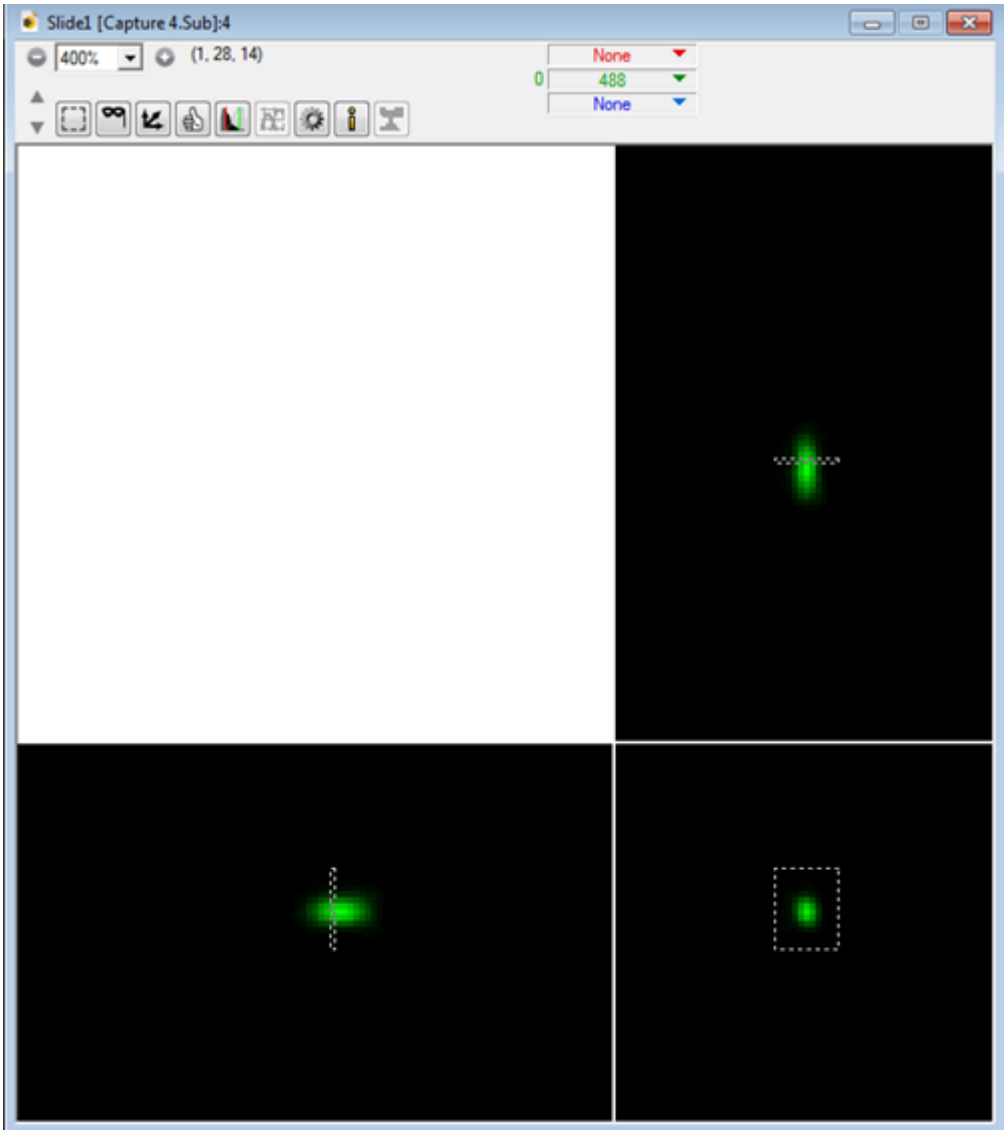


8. Locate the new image in the **.sld**, the image name will be appended with **.Sub**. Click on the image name then type **Ctrl + T** to open a Three View. Resize the window to ***400%***. All views will appear blank upon initial opening, click near the center of any panel to bring the bead into view.

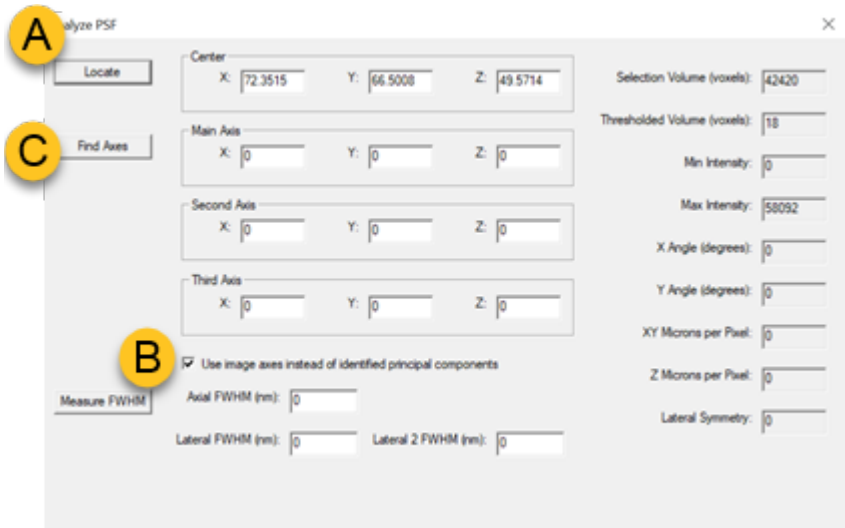
9. Select the Marquee tool from the tool menu in the Three View window.



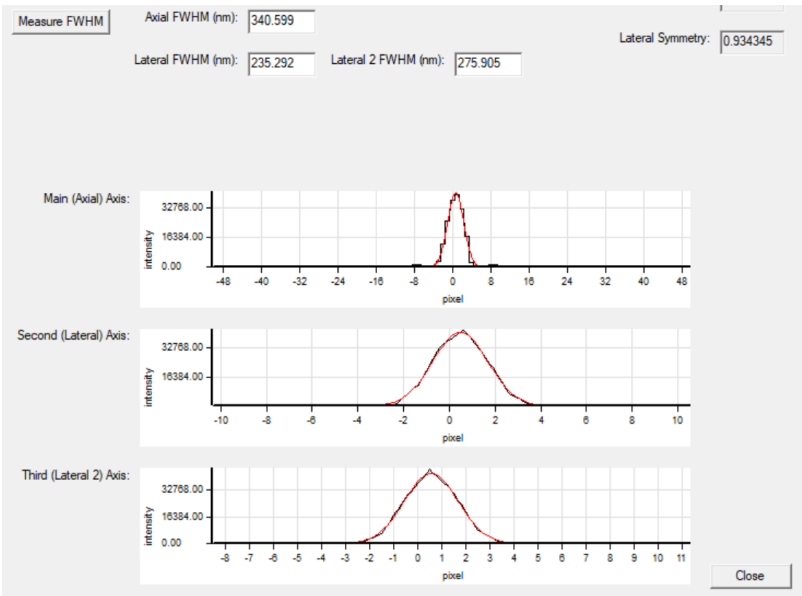
10. Draw a rectangle around the bead in the XY panel.



11. In the main SlideBook ribbon toolbar, go to the Analyze tab, Evaluate group, ***Extract Measurements > Analyze PSF***. The Analyze PSF dialog will open. Click ***Locate*** (A). Check the box to ***Use image axes instead of identified principal components*** (B) . Click ***Find Axes*** (C).



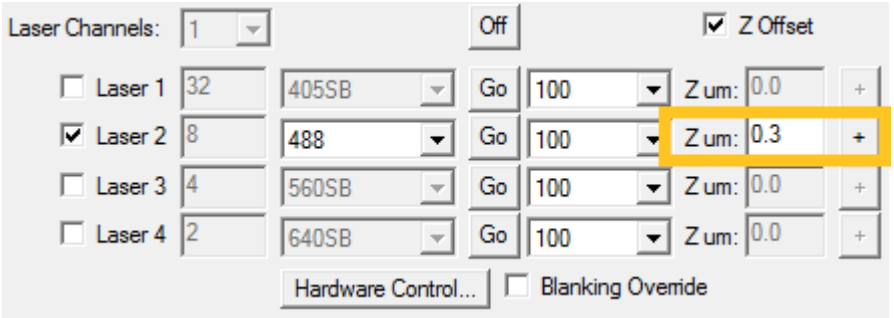
12. The graphs will be populated with axis information. Click ***Measure FWHM***. The goal is to achieve 300 or less for lateral FWHM and 425 or less for axial FWHM.



SIM Z Galvo Offsets for Additional Wavelengths

After you capture and reconstruct the SIM Total PSF you should be confident that the system is well aligned for the 488nm wavelength. It is important to note that the Z Galvo offset will vary slightly for each wavelength. Before you proceed with imaging biological samples, you should repeat the above steps for OTF generation and SIM PSF reconstruction for each wavelength you anticipate using for the session.

Reload a bead coverslip with beads of desired fluorophore. Refer to Finding a Bead, Collect SIM Total PSF, and Evaluate Z Galvo Offset to determine Z Galvo Offset value for the additional wavelengths.



As you set each z galvo offset, be sure to Generate the SIM OTF for that wavelength and Evaluate the SIM OTF to confirm performance. You are now ready to perform multi-channel imaging.

Imaging Biology with SIM LLS

The first step is to load the biology. Cells must be grown or attached to the 5mm coverslips. Please note to add a drop of warm imaging media to the underside of the mounted coverslip to remove and prevent any air bubbles from forming when loading sample. Load the sample holder with the mounted coverslip into the 8ml sample bath.

Slowly and carefully raise the objective assembly. Insert the block to hold the objective assembly in the raised position.

Add media if necessary. The sample chamber holds a maximum volume of 8ml.

> **NOTE:** You must be extremely careful when adding water as stages and piezos are very sensitive to water exposure. Please cover the stages with the provided stage cover.

Load the sample holder containing the sample coverslip with the coverslip up. See also Loading Coverslip to Holder.

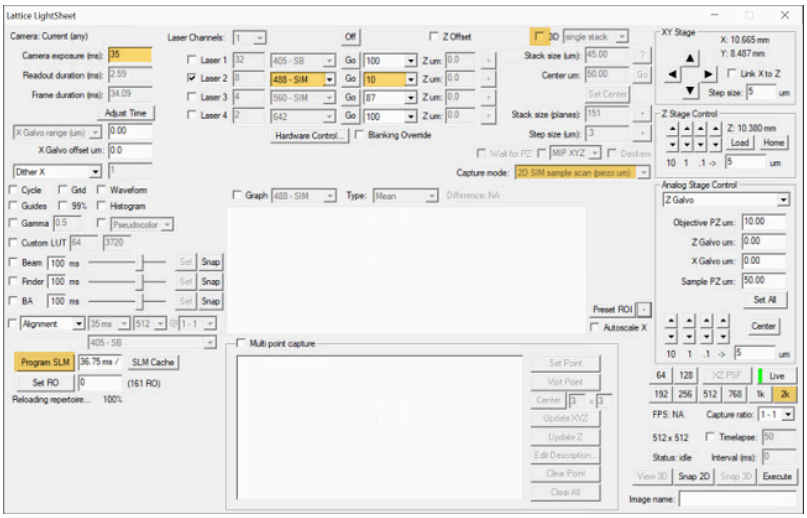
Secure using set bolts.

Grasp the objective assembly handle to stabilize it. Remove the block that was holding the assembly up in the load position. Slowly lower the objective assembly using the handle.

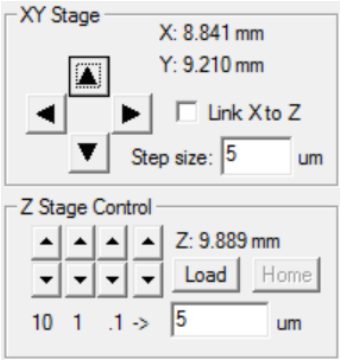
> **NOTE:** When loading any new sample, lower the sample holder to the lower limit. There are hard limits in the software that will prevent positioning that would damage the objective but caution should still be exercised.

1. For biology acquisition, we will begin in 2D SIM mode. Select the appropriate channel with the SIM pattern. Lower the laser power to a low value such as ***10***. Set the Capture mode to ***2D SIM sample scan***. Set the ***Camera Exposure*** to 35ms and click the button to set the FOV to 2K. After all of these adjustments, select ***Program SLM*** to use the settings on the SLM.

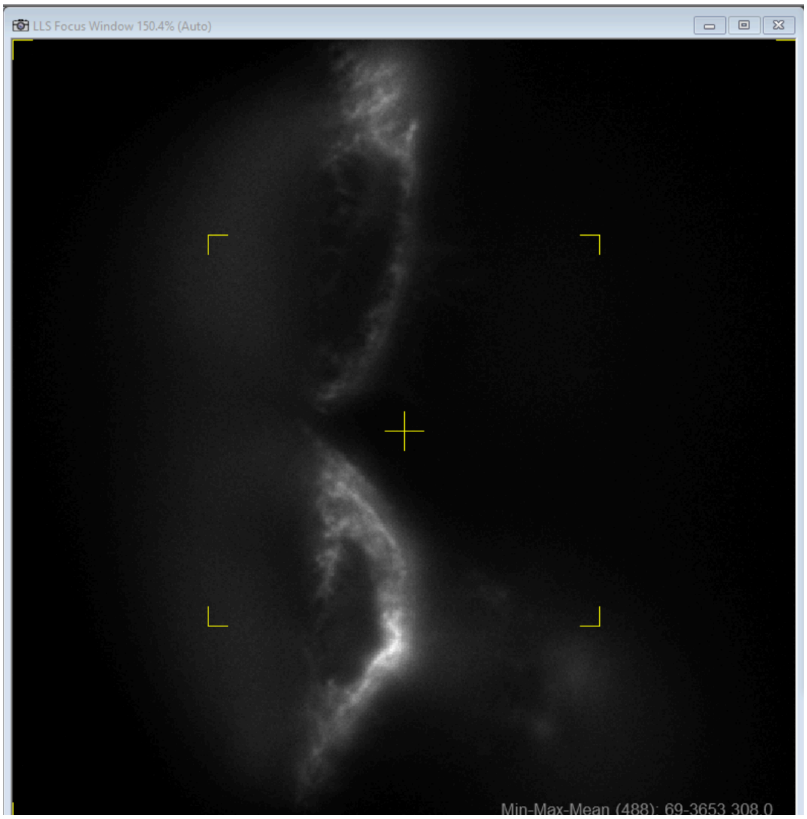
> **NOTE:** The SLM will need to be programmed when any changes to imaging parameters are made. You can change laser power without reprogramming the SLM. Any time a dialog opens asking to reprogram the SLM, select ***Yes***.



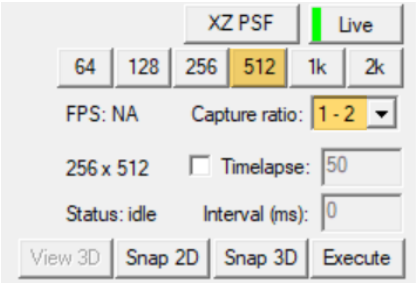
2. To find cells, use the ***XY Stage*** and ***Z Stage Control*** interfaces in the top right corner of the Lattice LightSheet dialog to move the sample holder to bring cells into the field of view. The values that are referenced are the last position of the X, Y and Z stages. Do not click ***Link X to Z***. Move the XY stages by clicking the arrows that designate the direction in which you would like the cell to move. The ***Step size*** field is used to set a value for the movement made each time you click an arrow button. We recommend a value of 10µm afor the initial location of cells. For the Z stage control there are pre-set values of 10, 1 and 0.1µm as well as a fourth user-defined value all linked to up and down arrows. . As you should have lowered the sample holder prior to changing the sample, the first step is to raise the sample using the up arrows for the ***Z Stage Control***.



3. Raise stage until the coverslip comes into view. You will now need to move the sample in X and Y to find cells and also use the Z stage control to keep the coverslip in the center of the field of view. Select ***Stop*** to stop the live stream when you are satisfied with the sample orientation.



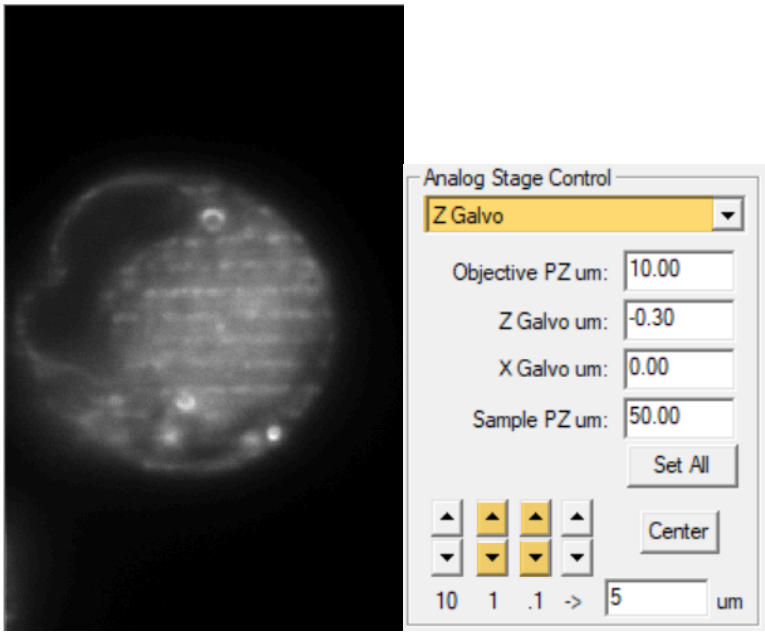
4. The recommended image capture field for most live cells is 512 x 256. To set this image size, select the ***512*** FOV button. Select ***1-2*** from the ***Capture ratio*** drop down menu.



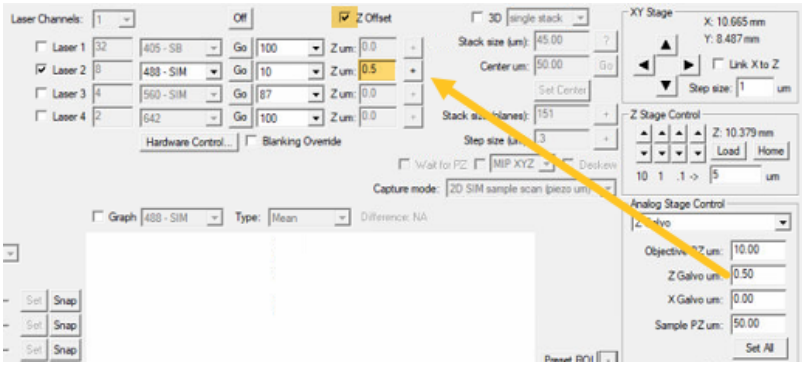
5. Select ***Program SLM***. Once the reprogramming reaches 100%, click ***Live*** to resume live streaming.

6. Next you will refine the focus by adjusting the Z galvo offset. Even though we previously adjusted Z galvo offsets during PSFs, it is important to adjust these baseline values to the specific sample. In the Analog Stage Control group, select ***Z Galvo*** from the dropdown menu. Use the up and down arrows for 1 and 0.1 µm steps to find a crisp focal plane where you can see the illumination pattern on the sample.

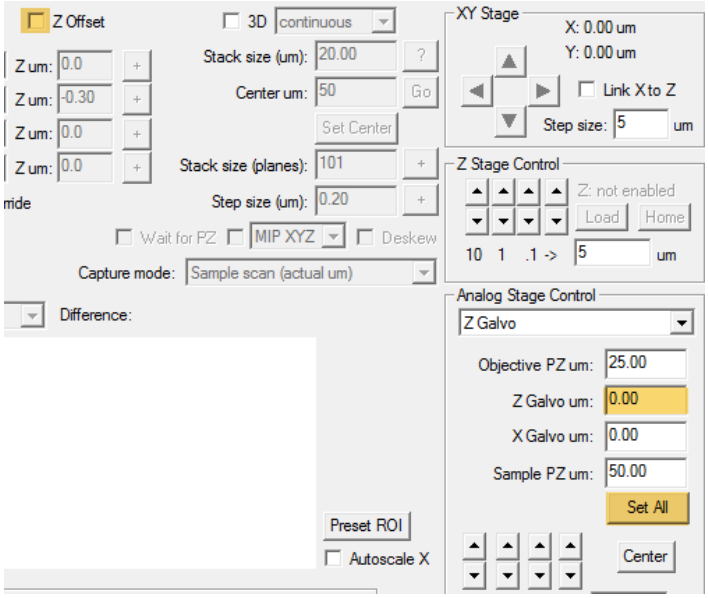
> **NOTE:** You may wish to adjust laser power or exposure time at this time. To do so, you may find it useful to view Min - Max-Mean intensity values in the live window by checking the ***Histogram*** box in the center of the left side of the Lattice LightSheet dialog.



7. In the Laser Channels group, check the box for ***Z Offset***. Enter the value determined in the ***Z Galvo*** field into the ***Z µm*** field for the active laser.

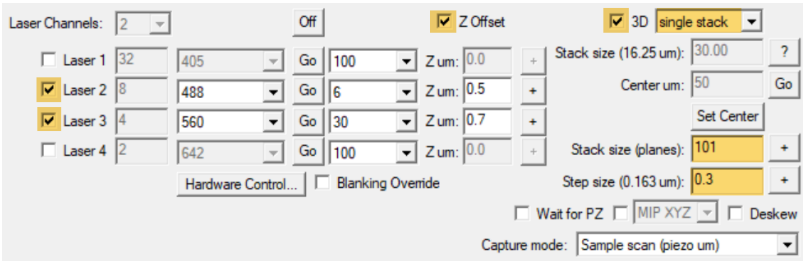


8. Uncheck the box for ***Z offset***. Enter **0.00** in the ***Z Galvo*** field and click ***Set All***. This will allow you to move on to setting up Z acquisition parameters, click ***Stop*** to stop the Live stream.



9. If you will be imaging additional channels, uncheck the current laser and check the box for the channel you would like to set up. Adjust laser power to a low value around ***10***. Repeat steps 5-8 to reprogram the SLM, adjust laser power, and set the Z galvo offset.

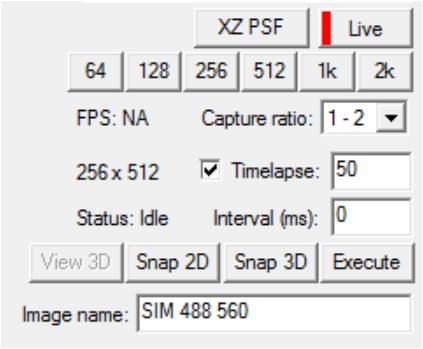
10. Once all channels are set up, check the box for ***Z offset*** (to use the Z galvo offsets you just determined). Check the boxes for each channel you will be imaging. Check the box for ***3D*** and choose ***single stack*** from the dropdown menu. Set ***Step size*** to **0.30** µm (this will yield 0.163 µm step size in the deskewed data and is close to Nyquist sampling). Set the ***Stack size*** to **101** planes which will image 16.25 µm total z stack size.



11. Click ***Program SLM*** to reprogram the SLM then ***Live***. This will initiate a single z stack collection in the live window (it will not be a saved image file). Watch the live camera window to confirm the entirety of the cell was acquired. If the range was insufficient, adjust the stack size or re-orient the sample using the XY Stage controls as necessary.

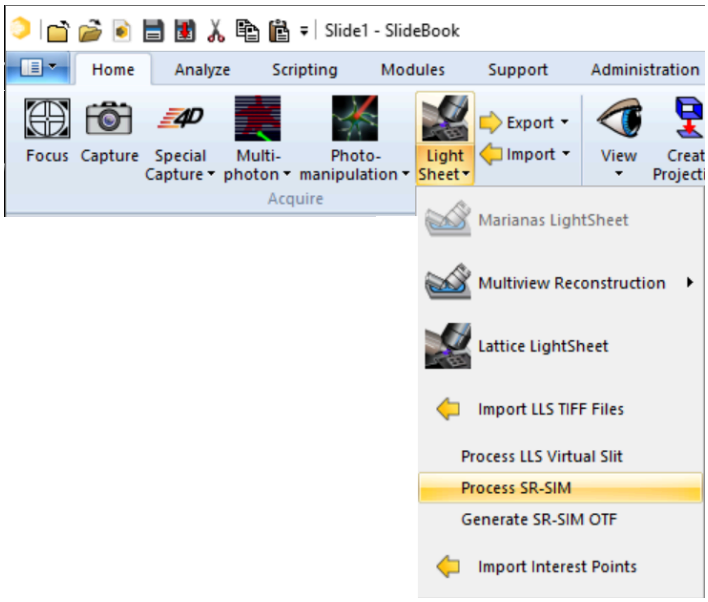
12. Once the 3D parameters are set as desired, you are ready to set up the capture parameters. To image a single 3D stack, clicks ***Snap 3D***. To image a 3D time series, check the box for ***Timelapse*** and enter a value for the number of time point you would like to collect. If desired, enter an ***Interval*** for the amount of time between time points. Enter an ***Image Name***. Click ***Execute*** to begin acquisition. If you are prompted to reprogram the SLM, always say ***Yes***.

> **NOTE:** The interval of **0** will collect as fast as possible. Any interval less than the time it takes to acquire a single stack is the equivalent to an interval of **0**.

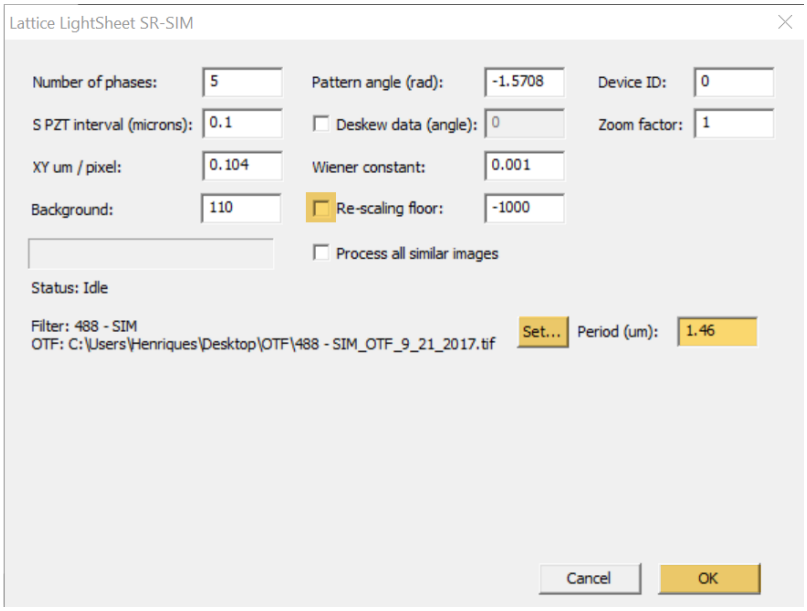

XZ PSF
Live
64
128
256
512
1k
2k
FPS: NA
Capture ratio:
1 - 2
256 x 512
Timelapse:
50
Status: Idle
Interval (ms):
0
View 3D
Snap 2D
Snap 3D
Execute
Image name:
SIM 488 560

Processing Biological SIM Data

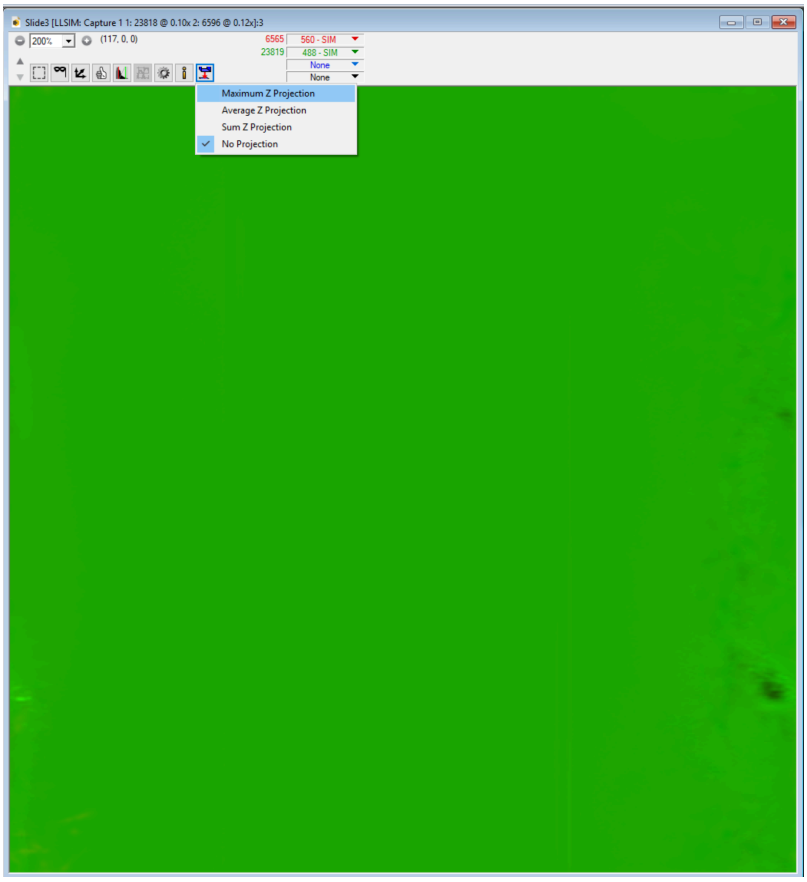
1. With the new biological SIM data open and selected in a view window, go to ***LightSheet > Process SR-SIM***.



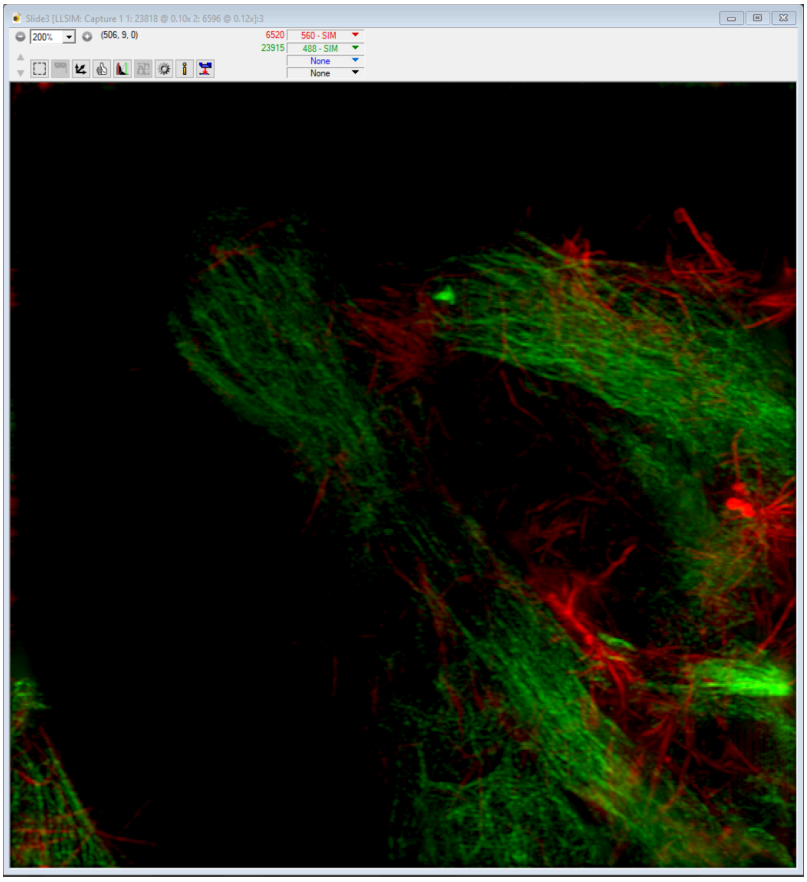
2. The Lattice LightSheet Process SR-SIM dialog will open. Uncheck the selection for ***Re-scaling floor*** (the default status is checked but we recommend unchecking this option until you are very comfortable with SIM analysis). Click the ***Set*** button to select the OTF for the reconstruction. Navigate to the desktop OTF directory, select the desired OTF then click ***Open*** (see also Generate and View SIM OTF). Type in the SIM ***Period*** value used for acquisition (see step 3 in Collect SIM Total PSF). Click ***OK*** to process the data.



3. Once the SIM data is reconstructed, the processed image will be written to the .sld directly below the original SIM capture. It will be designated **LLSIM:** followed by the name of the original image and the imaging parameters. Double click on the image to open. Go to the ***Projection icon > Maximum Z Projection***.



4. Click on the ***Renormalize icon*** to open the Renormalize dialog. Adjust the display for each channel of data the data as desired.



5. Should you wish to perform additional rendering or analysis, you may find it useful to review the View menu, Annotations Group, and Mask Generation Group help sections for additional information.

Supplementary Materials

Supplementary materials include the following:

5mm Cover Glass Preparation

Due to the sensitive nature of the Lattice LightSheet system, we recommend the highest quality cover glass available. Our current recommendation is https://www.warneronline.com/product_info.cfm?id=11

Coverslip Cleaning Protocol:

1. Soak coverslips in 0.1M HCl for 1 hour.

2. Rinse multiple times with DI (deionized) water.

3. Soak coverslips in 0.1M NaOH for 1 hour.

4. Rinse multiple times with DI (deionized) water.

5. Rinse multiple times with 70% EtOH.

6. Store in 70% EtOH in a 15ml conical tube until you are ready to use.

Bead Coverslip Preparation

Prepare cover glass as described previously. We recommend the following beads:

405nm: https://www.thermofisher.com/order/catalog/product/F8805?ICID=search-f8805

488nm: https://www.thermofisher.com/order/catalog/product/F8848?ICID=search-f8848

561nm: https://www.thermofisher.com/order/catalog/product/F8810?ICID=search-f8810

642nm: https://www.thermofisher.com/order/catalog/product/F8807?ICID=search-f8807

Bead Dilution Protocol:

1. Set up 6 tubes (1.5 ml Eppendorf tubes) with 1 ml DI (deionized) water and 1 tube with 1ml of poly-L-lysine solution.

2. Add 1 µl of beads into the tube with poly-L-lysine solution.

3. Bath Sonicate for 15 minutes.

4. Add 100µl of this bead/ poly-L-lysine solution to the first tube of DI water and pipette well.

5. Take 100µl from this tube and add to the second tube of DI water and repeat this step to last tube of DI water.

6. Keep all tubes.

7. Start by using the last tube of beads, most dilute, and add enough to cover an entire coverslip and let dry. You may need to use other dilutions to get the density of beads you desire for any specific application.

Loading Coverslip to Holder

It is important to use the recommended coverslips that have been prepared according to the 5mm Cover Glass Preparation instructions.

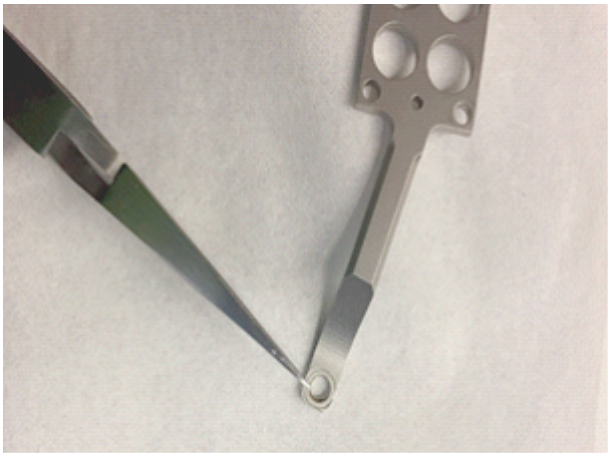
Equipment Required:

Grease
https://www.amazon.com/Dow-Corning-Vacuum-Lubricant-5-3oz/dp/B001UHMNW0

Forceps
https://www.fishersci.com/shop/products/fisherbrand-straight-veryfine-point-reverse-actionforceps/16100126#?keyword=reverse+action+forceps

Procedure:

1. Using forceps, place a small amount of vacuum grease on the sample holder along the rim where the 5mm coverslip is to be placed. This grease is biologically inert.



2. After you add the grease, carefully place the coverslip onto the holder sample side up. The coverslip should be seated in the depression shown in the preceding image.

3. Press down on the coverslip gently using a pipette tip.

4. Turn the sample holder over (so the coverslip is on the bottom) and carefully pipette in about 25µl imaging media into the space directly beneath the coverslip. If you do not do this, there is a chance that an air bubble will get in this space and disrupt your imaging.

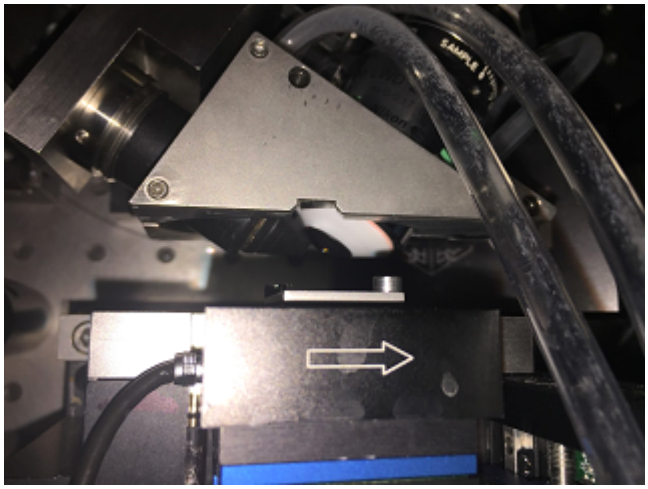
5. Turn the holder back to coverslip up and place into the lower sample chamber. See also Bead Coverslip Preparation.

6. The PSF sample is fluorescent beads (preferably 100nm green beads) sparsely attached to a 5mm diameter cover slip. A well prepared sample should last at least a week. Please refer to Supplementary Materials for information on cover glass preparation and bead coverslip preparation.

> **NOTE:** If you will be performing multichannel acquisition later in the day, you should have bead coverslips prepared for all wavelengths so you can perform Z Galvo Offsets for Additional Wavelengths.

Clean Up and Shut Down

Once you are done imaging for the day, you will need to rinse out the lower sample chamber that holds the imaging media. Rinse and drain the lower sample chamber multiple times with DI (deionized) water. In addition, you will need to use a squirt bottle or transfer pipette to rinse the objectives. A transfer pipette works well as you will need to squirt DI water onto the objectives to rinse off any imaging media.



You will need to be very careful during this process to not splash or drip water onto the stages.

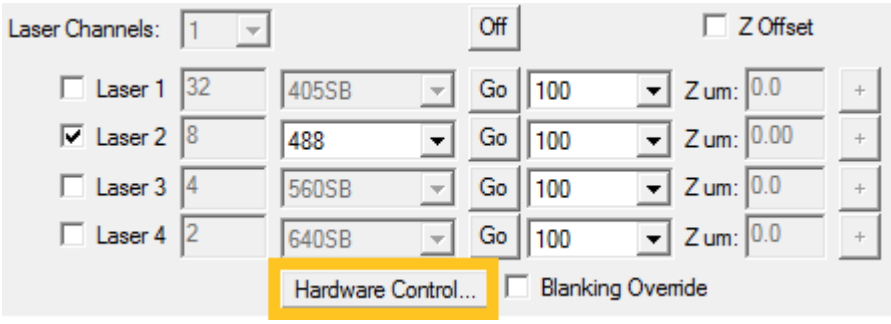
Once the lower sample chamber and objectives are rinsed, leave the objective assembly raised when not in use to allow everything to dry.

Shut down the LatticeScope, GUI VFL and µEye Cockpit software and turn off the hardware.

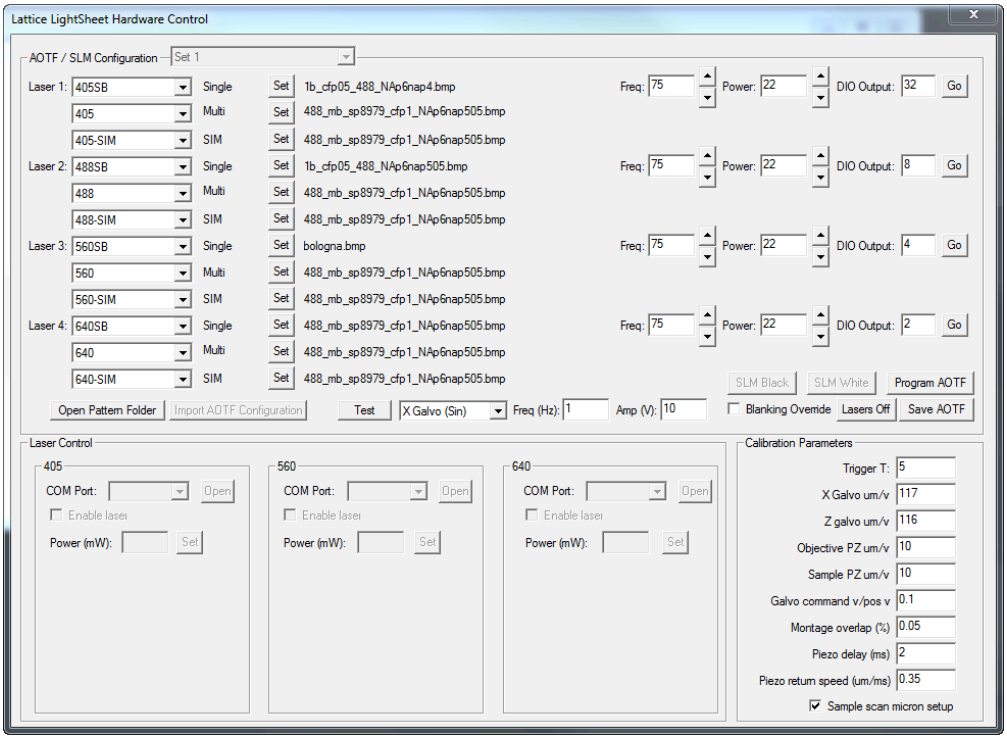
Loading Bessel Patterns

SlideBook comes with pre-loaded single and multi Bessel patterns. Users may load their own patterns by following these instructions.

1. In The Lattice LightSheet dialog, click Hardware Control (located below the laser channel selections.



2. The Lattice LightSheet Hardware Control dialog will open. Click ***Set*** for any of the laser channels for which you want choose an alternative pattern. A file browser will open. Select the desired bitmap file and click ***Open***.



3. You will be prompted to copy the file to the Pattern Folder. Say ***Yes***.

4. Repeat for any additional channels. Click the X in the top right corner to close the dialog when finished.

Marianas LightSheet Module

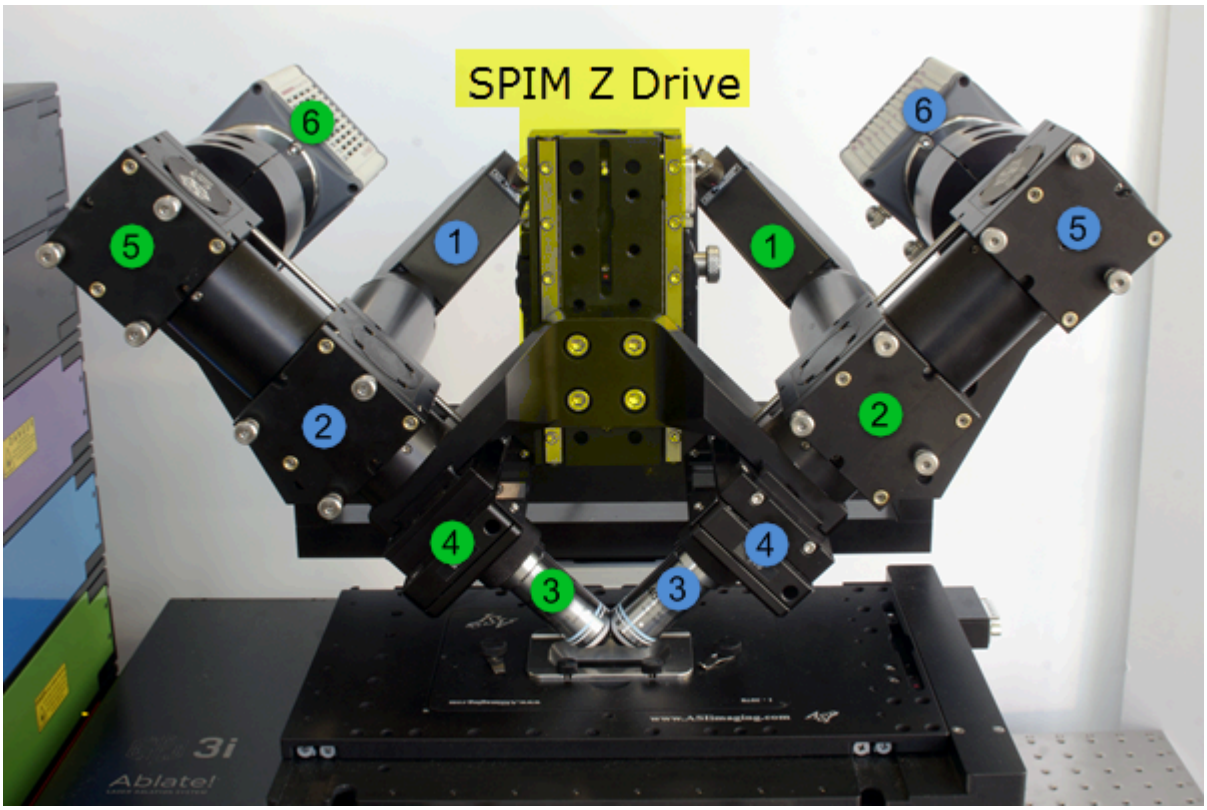
The Marianas LightSheet (MLS) is our turnkey dual inverted selective plane illumination (diSPIM) imaging solution. The scanned-sheet method's low phototoxicity, three-dimensional imaging makes long time lapses at high spatial and temporal resolution possible. The open geometry of the sample chamber allows a wide variety of sample preparations without the need for refractive index-matched capillary tubes or special slides.

MLS uses a pair of identical optical axes, oriented perpendicular to one another, to alternatively excite and detect fluorophores. The overlapping datasets are then fused and deconvolved into a single, three-dimensional dataset with isotropic X, Y, and Z resolution from only two views. This arrangement allows for faster data collection, better axial (Z) resolution, and lower phototoxicity compared to other multi-view techniques.

The paired, matching dipping objectives can have an NA of up to 0.8, and the working distance to enable access to many different sample preparations. Each objective is used for both excitation and detection, and images are collected on identical high-speed, high-resolution sCMOS cameras. A third objective, positioned on the turret beneath the specimen chamber, can be used to locate the sample. This objective can also be used for photomanipulation when an Ablate!, Phasor or Vector photomanipulation system is mounted via the microscope's side or Epi-port.

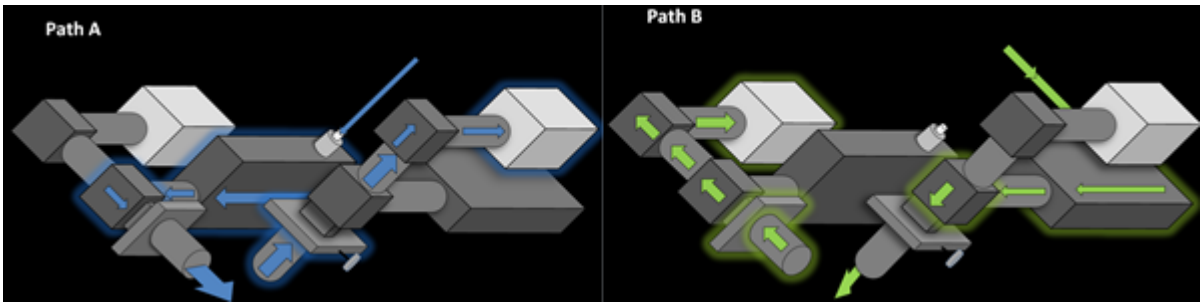
MLS System Hardware

The system consists of two symmetric optical paths (except in the case of Single View SPIM systems) for light sheet imaging. Two objectives are placed at right angles above a sample mounted horizontally. A light sheet is projected from one objective and imaged using the other objective. A stack of images is collected by moving the light sheet through the sample. The role of the two objectives can be exchanged to collect another stack from a perpendicular direction, and then the two datasets can be computationally merged to yield a 3D dataset with isotropic resolution. The diSPIM assembly is mounted on a linear stage, ***SPIM Z Drive***, which raises and lowers the objectives from the sample chamber.



(1) Scanner; (2) Dichroic Mirror; (3) Imaging Objective; (4) Piezo; (5) Camera (Upper) Mirror; (6) Camera
Blue depicts Path A components Green depicts Path B components

The MLS system comes as the dual light path module mounted on any inverted Zeiss, Nikon, Olympus, or Leica microscope frame. For each imaging channel, two filters will need to be configured, one for Path A and one for Path B as depicted below. It is also important to note that both objectives are used in each path, one for illumination and one for imaging and this switches depending on the path.



The ASI hardware of the MLS can be controlled in SlideBook or by the accompanying joypod. Review the following diagram to become familiar with the joypod functions. The left wheel (1) controls the SPIM Z Drive by default, can be set in the software. The right wheel (2) controls the Piezo or Slice Position and can be set in software. The joystick controls the XY stage. The button on the joystick (3) toggles the XY stage movement between fast and slow. **@** saves the current XY stage and Z-Drive position, ***HOME*** will drive XY stage and SPIM Z Drive to their zero position. ***NOTE:*** Make sure all Objectives are removed or retracted

before pressing this button. ***HALT/ZERO*** stops all ASI hardware (similar to the ***STOP*** button in the MLS window) and reset the position of all ASI hardware to zero. ***NOTE***: with the SPIM Z Drive zeroed in engaged position DO NOT hit the Home button. If this button was pressed to halt the engagement process withdraw the SPIM Z Drive completely by clicking ***Load*** and zero the system again.

MLS Alignment and Calibration

MLS system alignment is an integral part of system use. Alignment and calibration should be checked and refined any time you change sample type/thickness, sample buffer, room temperature, or any other factor that could impact optical properties of the setup. Optimal image quality can only be achieved when alignment and calibration are complete.

This section of our help contains several tutorials. No tutorial is a standalone document; the "steps" may need to be repeated several times in an alternating fashion until alignment is complete. It is recommended to first attain a close approximation for each alignment, proceed with the rest of the steps, and then cycle through the steps again. It is easiest to perform the fine alignment in a dye solution like fluorescein (beam alignment requires a high signal to noise ratio). The final steps can also be performed with a 2D sample of fluorescent beads.

Broadly speaking, the tutorials cover bringing the specimen into focus, focusing the objective bushings, eliminating focus and rotational tilt, and calibrating the piezo/slice relationship.

Equipment needed for alignment and calibration:

- dye solution (ex. Fluorescein) and/or a dense solution of beads/ fluorescent particles
- Allen wrench (3/32" size) - applies to systems installed before November 2017 only

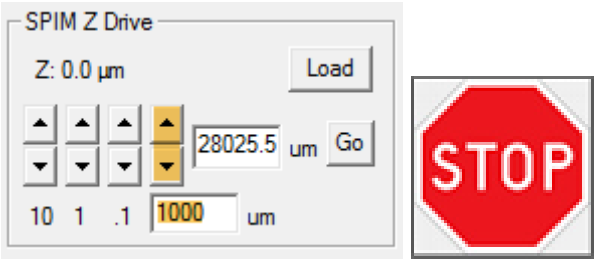
TUTORIAL: Using the SPIM Drive to Introduce Objectives into the Sample Chamber

Lowering the objectives into the sample chamber is a delicate task; it is essential that the sample chamber is centered below the objectives.

1. Open the ***Marianas LightSheet*** controls within SlideBook, go to the Home tab, Acquire group > ***LightSheet*** > ***Marianas LightSheet.***

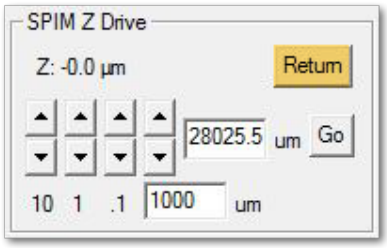
2. When using the system for SPIM, you will need to raise and lower the objectives using the ***SPIM Z Drive*** in SlideBook. To do so, enter a value (such as **1000** or **100**) when closer to the coverslip in the custom Z step size field and use the up and down arrows to control the drive. The large ***STOP*** button to the right of the SPIM Z Drive group can be used as an emergency brake for the SPIM Z Drive at any time; the ***HALT*** button on the joystick also works as an emergency brake but zeroes all hardware components.

> **NOTE:** When the system was withdrawn with Load after the last session it is fine to engage the liquid with a value of ***30000 um*** then click the ***arrow down button*** <u>while hovering with the mouse over the ***STOP***</u>. Hit the ***STOP*** button the moment the objectives dip into solution.



3. You will need to raise the objectives using ***SPIM Z Drive*** to load a sample. Use the ***Load*** button to withdraw the objectives completely.

> **NOTE:** The ***Load*** button will become the ***Return*** button when the drive has reached its maximum height.

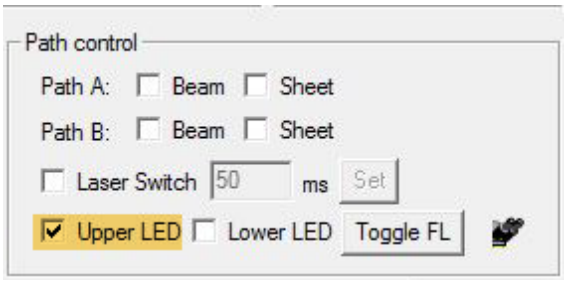


4. Once the sample is in place, you will want to move the SPIM Z Drive so the front lens of the objectives are fully submerged, this configuration will typically put you in position just above the coverslip.

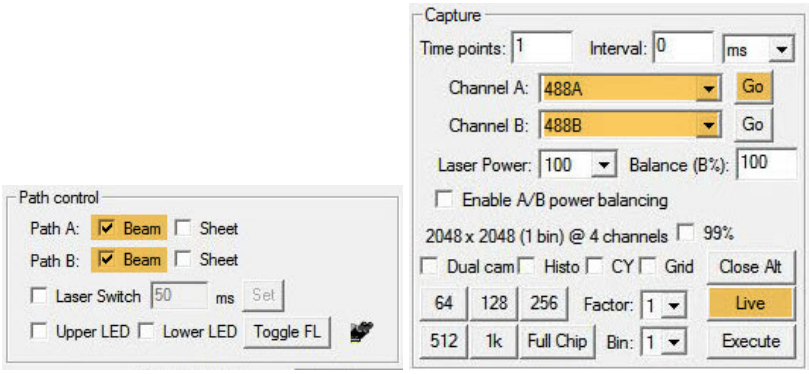
> **NOTE:** If you use the ***Return*** button, the drive will lower to the previous sample position. Make sure your coverslip or sample is not higher.

5. Incrementally step down SPIM Z Drive by entering a value into the ***SPIM Z Drive*** group as described in step #2.

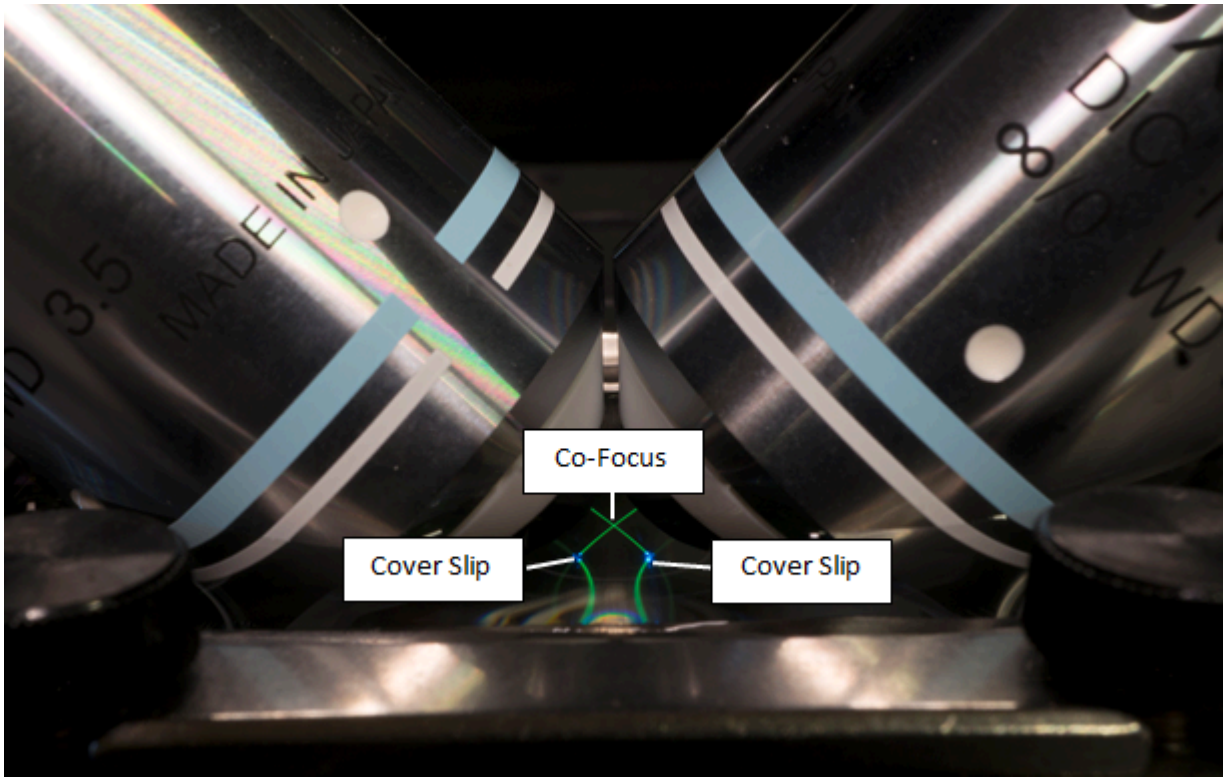
> ***NOTE:*** Turning on the ***Upper LED*** in Path control may add helpful additional illumination



6. In the Path Control group, select ***Beam*** for both paths. In the Capture group, select a filter for ***Channel A*** and ***Channel B*** respectively. Click ***Go*** for either of the channels then ***Live*** to activate lasers.



7. The next tutorials will cover co-focusing and aligning the objectives. For imaging samples, you will want both laser paths to be present in the middle of the field of view through the oculars. For alignment, you will want both laser paths to be present on the edges of the field of view through the oculars as shown below. Also see the Quick Alignment Guide.



See further:

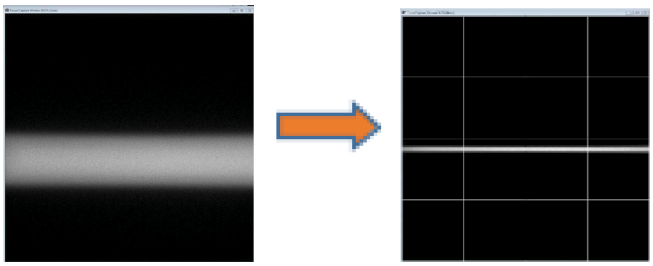
[TUTORIAL: Co-focusing the SPIM Objectives]
[TUTORIAL: Adjusting the Tilt of the Pencil Beam]
[TUTORIAL: Co-aligning the Beams]
[TUTORIAL: Calibrating the Piezo and Slice Position]
[TUTORIAL: Setting Sheet Width and Offset]
[TUTORIAL: Alignment for Equalizing Beam Power]

TUTORIAL: MLS Daily Quick Alignment Guide - Dual Sided

• Start with 488***A*** chosen for channel ***A*** and 488***B*** chosen for channel ***B***. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** & ***Live***, best with ***Dual cam***: (A) red/cyan, (B) green/yellow
• After objective swap check with white paper underneath objectives that beams are projected roughly underneath the opposite objective
• Check that the sample chamber is centred below the objectives
• Use ***Upper LED*** to better visualize
• Use the SPIM Z-drive, drive into dye solution

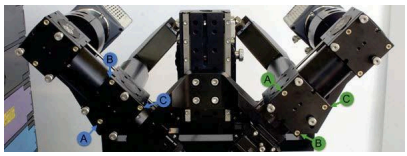
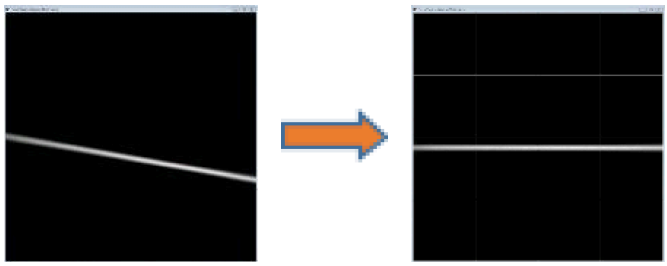
Detailed steps:
TUTORIAL: Co-focusing the SPIM Objectives
TUTORIAL: Adjusting the Tilt of the Pencil Beam
TUTORIAL: Co-aligning the Beams

1. Check Beams & Focus
   a. When the beams are not focused at all or focus is far out (like in picture), focus each side's beam by rotating the black bushings (best with ***Dual cam***) – (***A***) red/cyan = right objective; (***B***) green/yellow = left objective (clockwise rotation brings objectives apart)



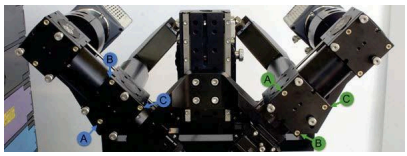
   b. Make sure you are on pencil beam (closed iris) for the following alignment steps: Close scanner iris mostly (lever towards right when looking from front of the scope)

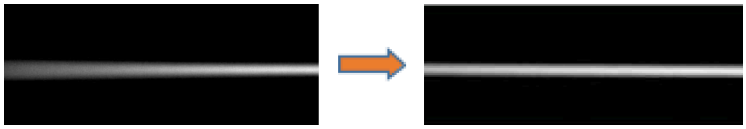
2. Adjustment of Rotational Tilt
   a. Untick ***Dual cam*** to observe beams separately, use ***Grid***
   b. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** for 488***A***, Adjust dichroic mirror (left cube) with ***B*** & ***C*** screws to bring beam parallel to the grid
   c. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** for 488***B***, Adjust dichroic mirror (right cube) with ***B*** & ***C*** screws to bring beam parallel to the grid

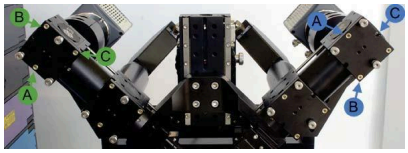
3. Adjustment of Focus Tilt
   a. Observe beams separately
   b. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** for 488***A***, Make sure right joy-pod wheel is set to ***Slice A***

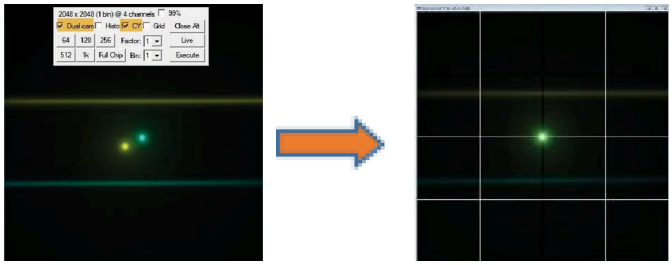
c. Check that beam goes out of focus evenly when changing the slice position with the right wheel
d. To adjust use dichroic mirror (left cube) with the ***A*** screw to bring beam rolling parallel in and out focus
e. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** for 488***B***, Make sure right joy-pod wheel is set to ***Slice B***
f. Check that beam goes out of focus evenly when changing the slice position with the right wheel
g. To adjust use dichroic mirror (right cube) with the ***A*** screw to bring beam rolling parallel in and out focus



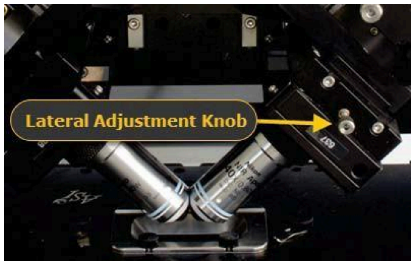


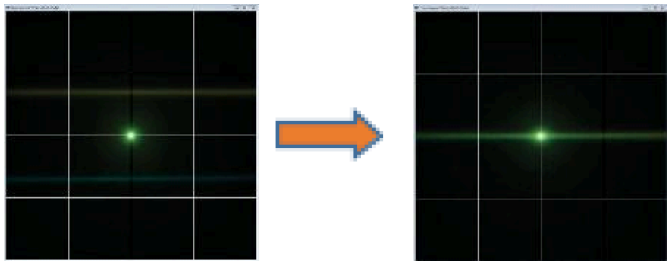
4. Adjustment of Camera Centration
a. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** & ***Live***, best with ***Dual cam***, tick ***Laser Switch*** and ***Grid***.
b. To adjust the spots to the centre use camera mirror ***B*** (upper left cube) with the ***A, B, C*** screws and camera mirror ***A*** (upper right cube) with the ***A, B, C*** screws





5. Final Adjustments
a. Tick beam for ***Path A*** & ***B***, hit ***Neutral all*** and then ***Go*** & ***Live***, ***Dual cam***, ***Histo*** and ***Grid***, untick ***Laser Switch***
b. Adjust both irises to the desired beam shape meanwhile checking the histogram (bottom of live window) for similar intensity
c. Set the calibration for either beam with desired piezo range (untick ***Dual cam*** and hit ***Go*** for desired path)
d. Tick beam for ***Path A*** & ***B***, (Do Not hit ***Neutral all*** anymore when beams are calibrated) and then ***Go*** & ***Live***, ***Dual cam*** and ***Grid***
e. Use the **lateral adjustment screw** to move both beams to overlap at the FOV centre (here shown with ***Laser Switch*** on)



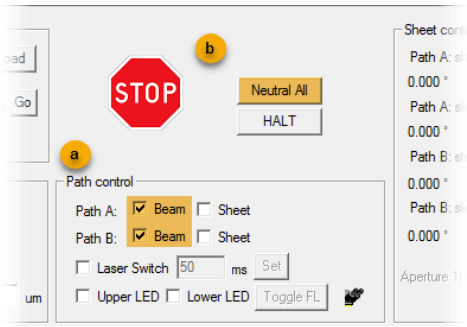


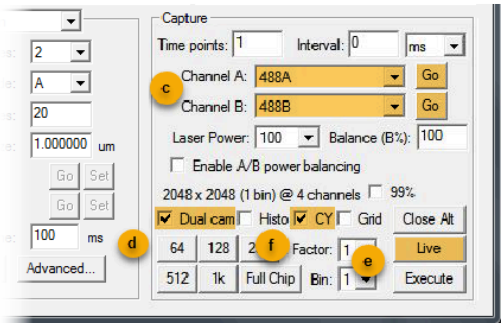
TUTORIAL: Co-focusing the SPIM Objectives

1. Open the ***Marianas LightSheet*** controls within SlideBook (Home tab, Acquire group > ***LightSheet*** > ***Marianas LightSheet***).

2. Refer to [TUTORIAL: Using the SPIM Drive to Introduce SPIM Objectives into the Sample Chamber](#).

3. To prepare for co-focusing the SPIM objectives, perform the following steps:

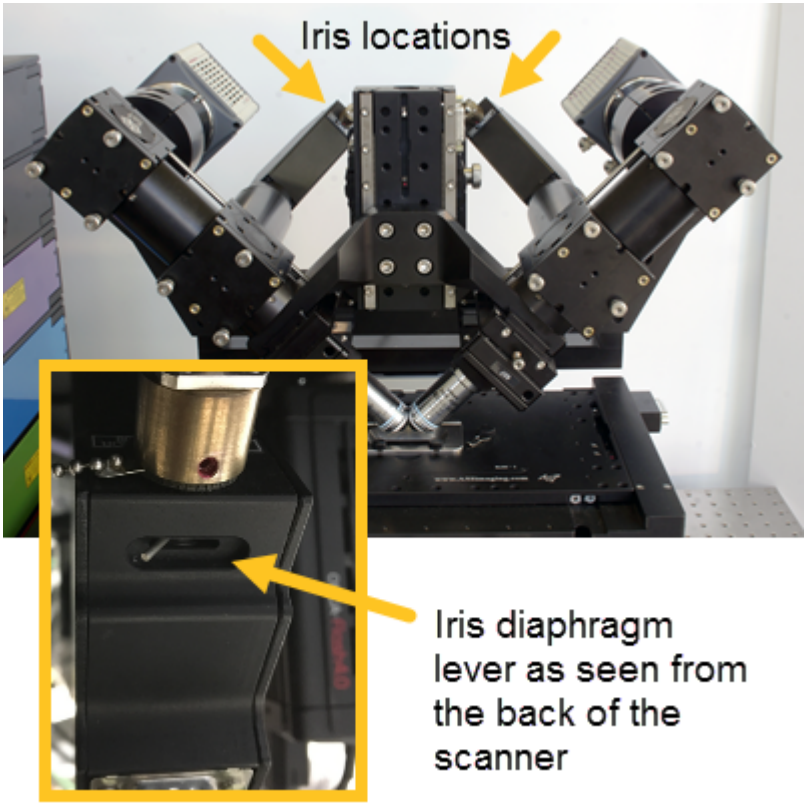




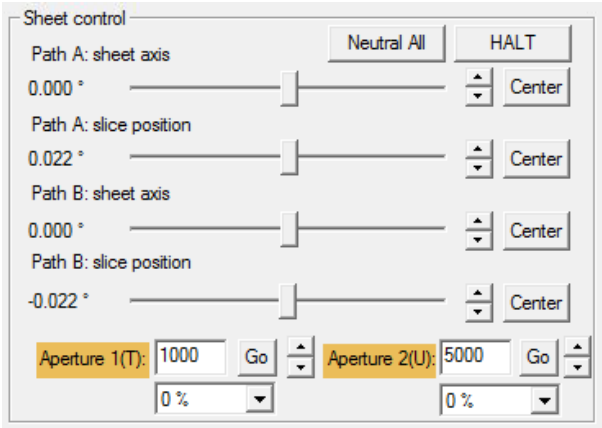
a. In Path control, select ***Beam*** for both paths.
b. In Sheet control, click ***Neutral All*** to return the slice position to its neutral position.
c. In Capture, select the same filter configuration for ***Channel A*** and ***Channel B***. Be sure to use opposite path designations. Click ***Go*** for each.
d. Check the box ***Dual Cam*** to see both beams.
e. Click ***Live*** to start a live stream from the cameras.
f. (Optional) When in dual camera mode, red represents Path A and green represents Path B. You can check the box ***CY*** to view Path A in yellow and Path B in cyan.

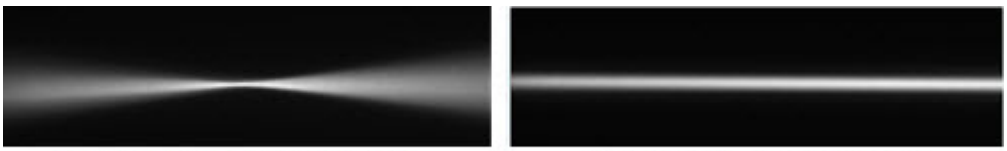
4. Use the iris on the back of each scanner to adjust each beam to a pencil beam. The iris is fully closed when the small lever is to the right as seen from the front of the microscope (pictured from the back of the microscope below).
5. When your system is equipped with motorized irises, go to the Sheet control group, type a small value (can be -70000) and click ***Go*** or set 0% using the drop down menu for each iris ***Aperature T*** and ***U*** to achieve a pencil beam.

Manual Iris



Motorized Iris





Open iris = Gaussian beam **Closed iris = pencil beam**

6. Select ***Channel A > Go***, adjust the imaging objective's focus on the beam using the corresponding ***Objective Threaded Bushing (A)*** to focus pencil beam. Select ***Channel B > Go***, adjust the beam's focus using the corresponding ***Objective Threaded Bushing (B)*** to focus pencil beam. Alternate between adjusting the two Objective Threaded Bushings; you will need to click ***Go*** for the corresponding channel each time. Either move half way to the focus at a time before alternating sides to achieve convergence (even if there is a bit of play in the Objective Threaded Bushings) or adjust while in the ***Dual Cam*** mode.

> **NOTE:** If you have the feeling one objective is much further out than the other, turn both in by rotating the bushings clockwise and start the focusing anew, making sure you turn both bushings out about the same way each time.

7. If the pencil beam is effectively focused, you may see an interference pattern present around the beam and can proceed to TUTORIAL: Co-aligning the Beams.

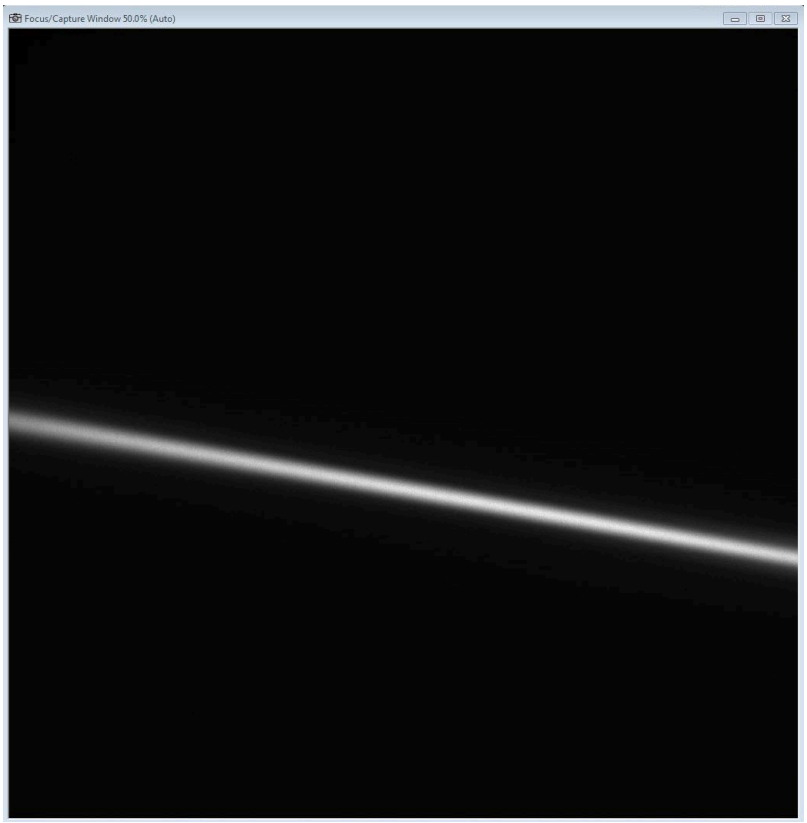
8. After the pencil beams for both channels are focused, you may notice some tilt (either rotational or focus). Please see TUTORIAL: Adjusting the Tilt of the Pencil Beam to correct for horizontal rotation (YZ tilt) or focus inconsistency (XY tilt).

TUTORIAL: Adjusting the Tilt of the Pencil Beam

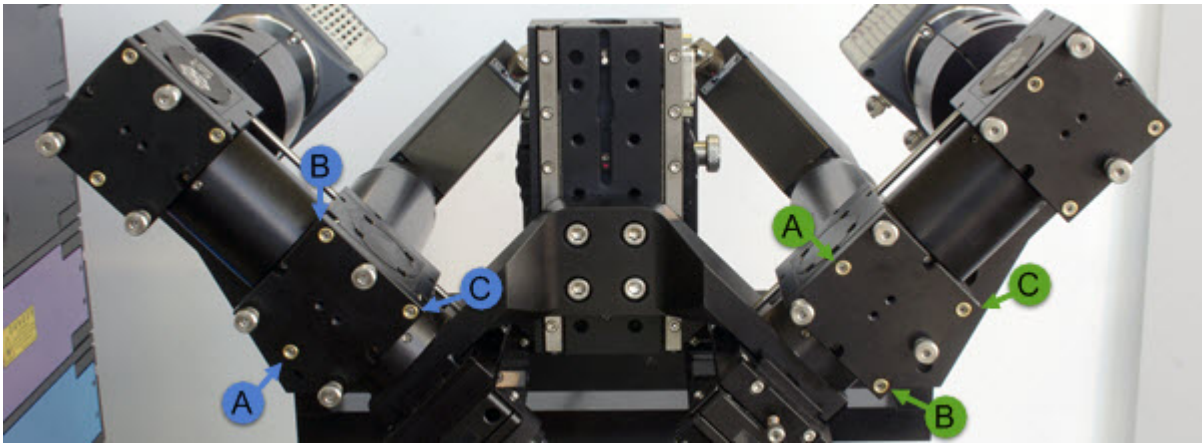
Two major checks are required when starting the system: (1.) Check of the rotation of the beam/ sheet and most importantly (2.) Check the yawn of the beam/ sheet is respect to the focus plane.

1. **YZ Tilt (rotational)**

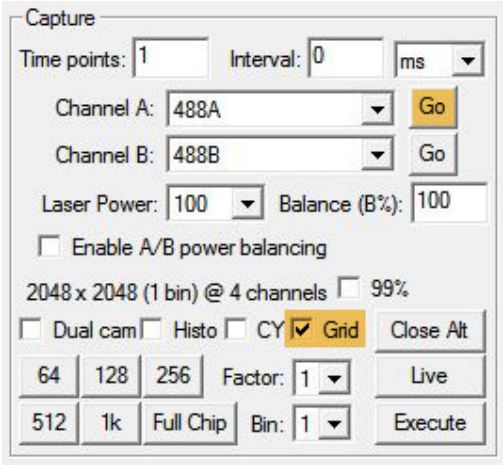
The YZ Tilt is apparent when viewing the beam through the camera; if the YZ tilt needs to be adjusted the beam is slightly rotated in the YZ dimension.



To correct the YZ Tilt, adjust the dichroic mirror using both screws B and C on the filter cube of the desired path.

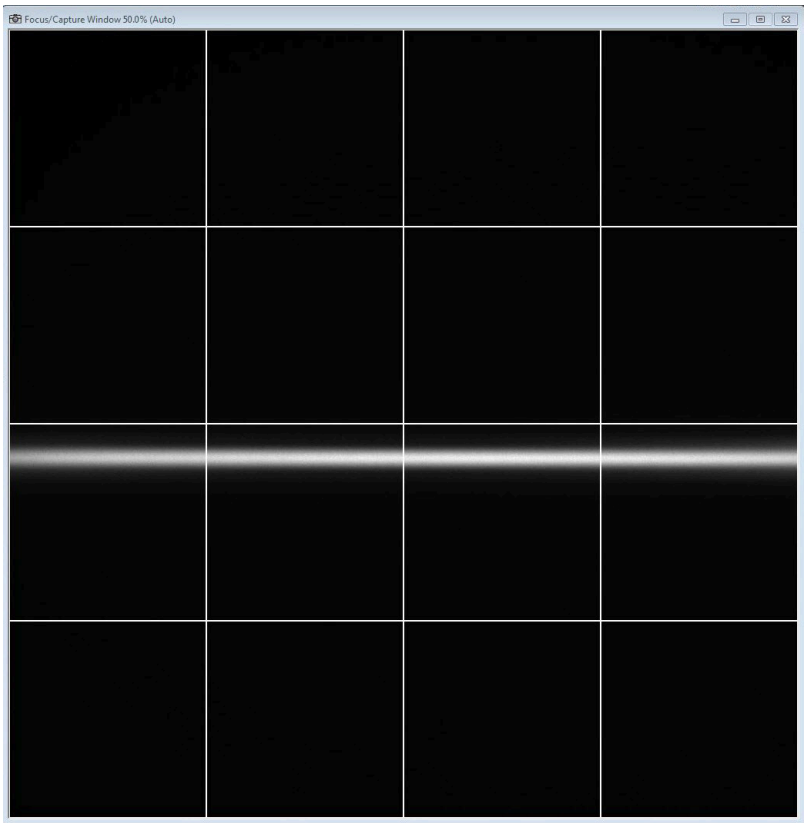


1. Turn on gridlines in the live view by checking the ***Grid*** box, click ***Go*** for ***Channel A*** to activate **Path A**.



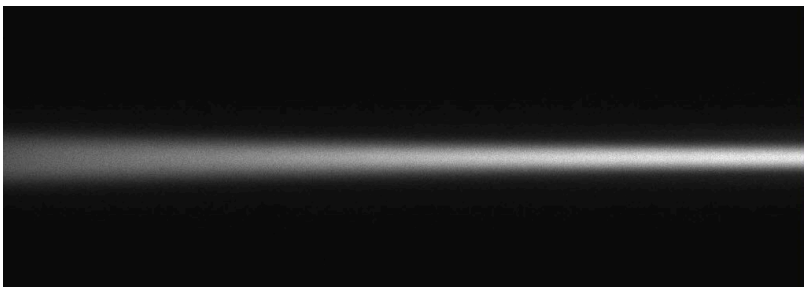
2. Adjust both **Screw B** and **Screw C** of the **Path A** dichroic mirror until the beam is horizontal and parallel to the grid lines.

3. Repeat for **Path B** (by clicking ***Go*** for ***Channel B*** and adjusting both **Screw B** and **Screw C** of the **Path B** filter cube) until both beams are in focus and parallel with the horizontal gridlines across the field of view. Do not be concerned if beams are not in center of field of view (the beam just needs to be uniform at this point).

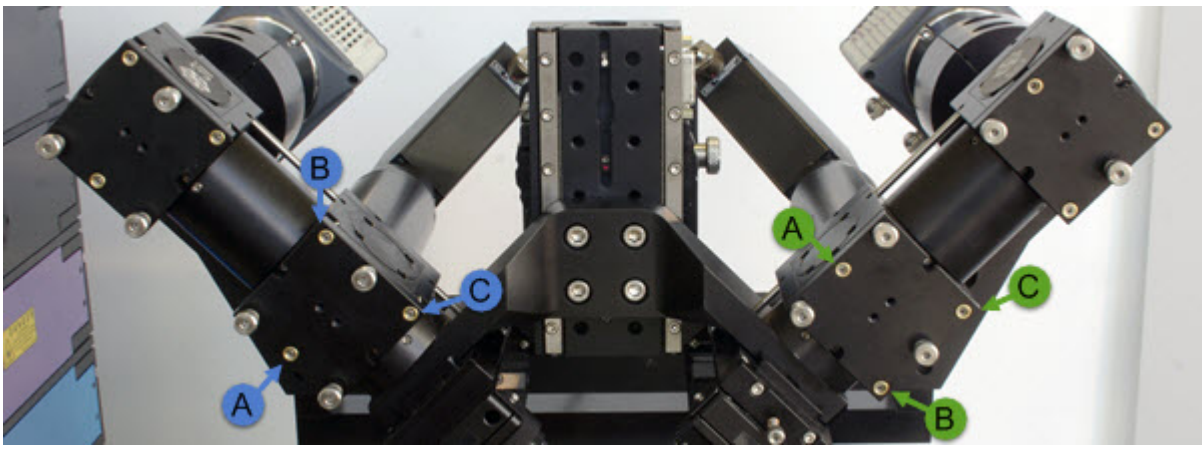


2. XY Tilt (focus)

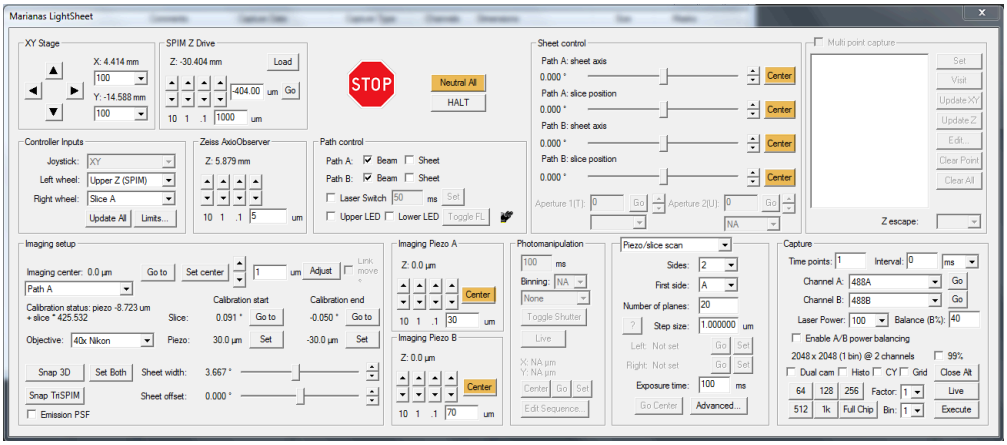
The XY Tilt is apparent when viewing the beam with the camera; if the XY tilt needs to be adjusted, the beam will appear as if it is coming in an angle toward (and away from) the camera instead of going parallel to the image plane.



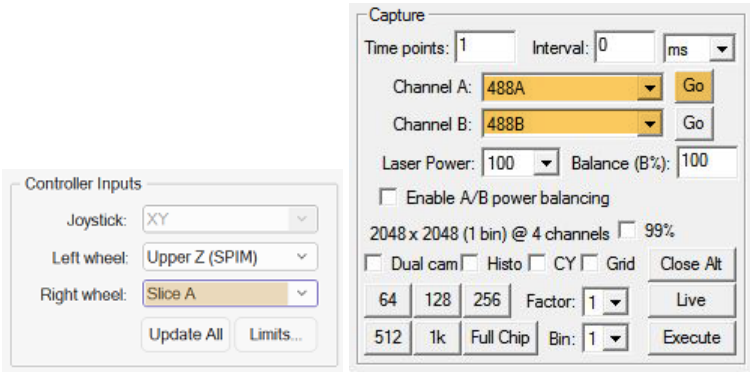
To correct XY Tilt for Channel A, you will adjust the dichroic mirror using **Screw A only** of the **Path A** filter cube. To correct XY Tilt for Channel B, you will adjust the dichroic mirror using **Screw A only** of the **Path B** filter cube.



1. Make sure sheets and slices are centered by clicking ***Neutral All*** and ***Center*** for all sheet and slice positions.

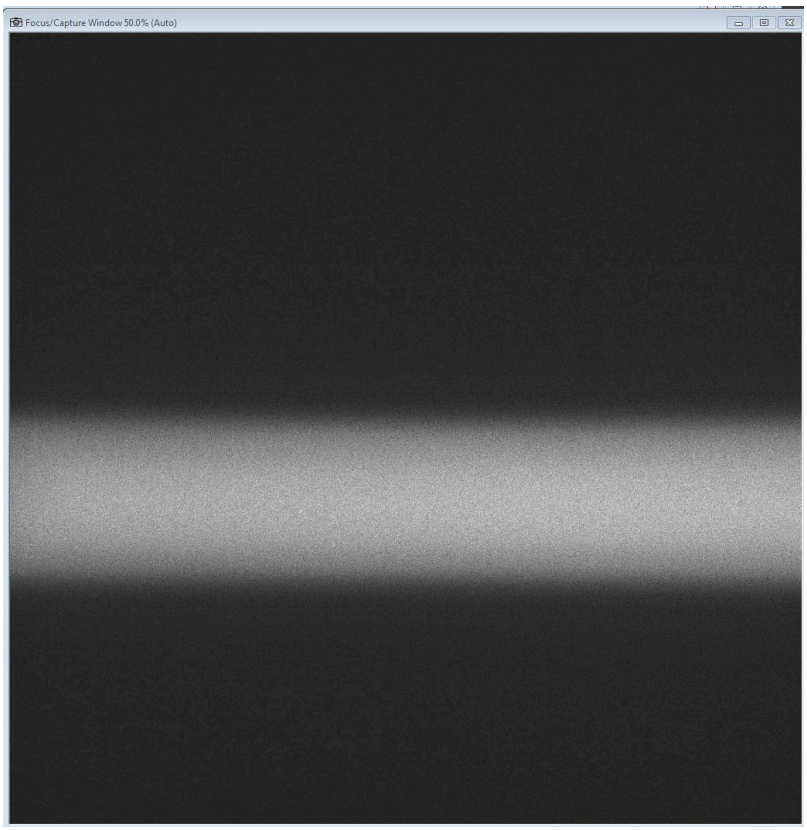


2. In the Controller Input group of Marianas LightSheet controls, make sure the ***Right wheel*** is showing to ***Slice A*** for Path A using the drop-down selection. Confirm that Channel A and Channel B (in the Capture group) are set to the same filter for their respective paths (e.g. Channel A: 488A, Channel B: 488B). Click ***Go*** for ***Channel A*** to activate **Path A**.



3. De-focus the beam using right wheel on the joystick.

4. Adjust **Screw A** of **Path A only** or **Screws B & C together** on the filter cube manually with an Allen wrench until beam is uniform across the field of view.



5. Select ***Neutral All*** to restore the slice position to its neutral position.

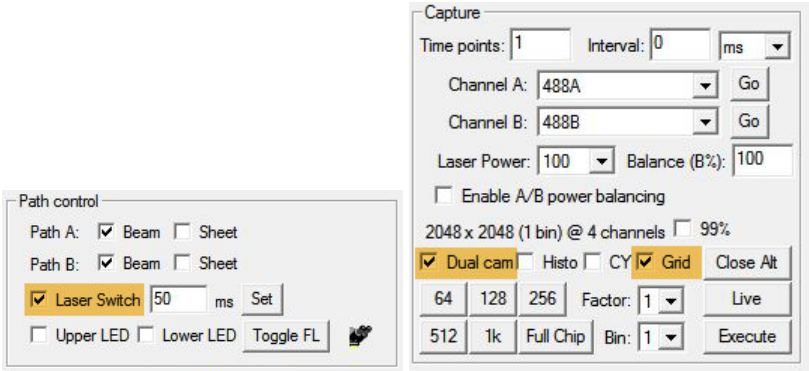
6. Refocus both pencil beams if necessary using the objective threaded bushings (as described in TUTORIAL: Co-focusing the SPIM Objectives) until pencil beam is uniform across the field of view.

7. Repeat steps 1-5 for Path B (making sure the ***Right wheel*** is set to ***Slice B***, clicking ***Go*** for ***Channel B***, and using **Screw A only** of **Path B** filter cube) until both beams are in focus across the field of view. Do not be concerned if beams are not in center of field of view (the beam just needs to be uniform at this point).

TUTORIAL: Co-aligning the Beams

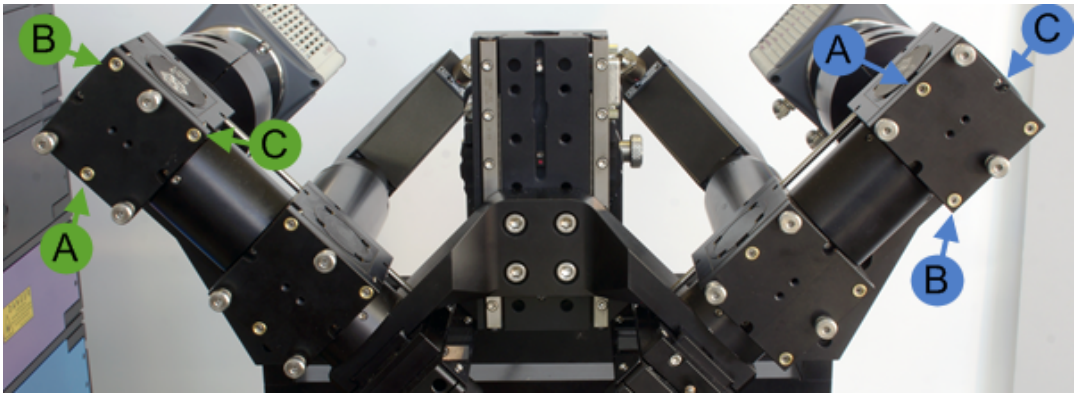
Prepare a sample for the MLS alignment, use the imaging media you will be imaging your samples in (water or PBS are acceptable but not ideal). To better visualize the beams, add a small amount of fluorescein or phenol red. For this tutorial, you will need to show beams and spots from both paths. To do so, check 3 boxes in the Marianas LightSheet dialog:

- ***Dual Cam***
- ***Laser Switch***
- ***Grid***



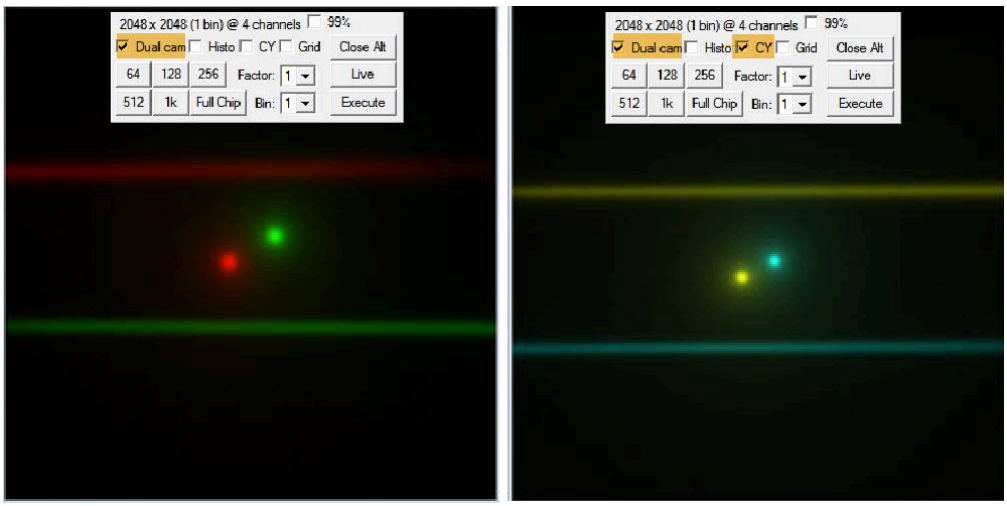
Throughout the tutorial, you will be adjusting the Camera (Upper) Mirror for each path. You will be using an Allen wrench in or just the Fine Adjustment Screws A (left-right), B, and C (diagonally up-down) of **Path A** and **Path B** as depicted below.

- Use ***Screw A*** on the Camera (Upper) Mirrors to move the beam and spot left and right for each path.
- Use ***Screws B*** and ***Screw C*** on the Camera (Upper) Mirrors to move the beam and spot vertically with a slight horizontal bias for each path



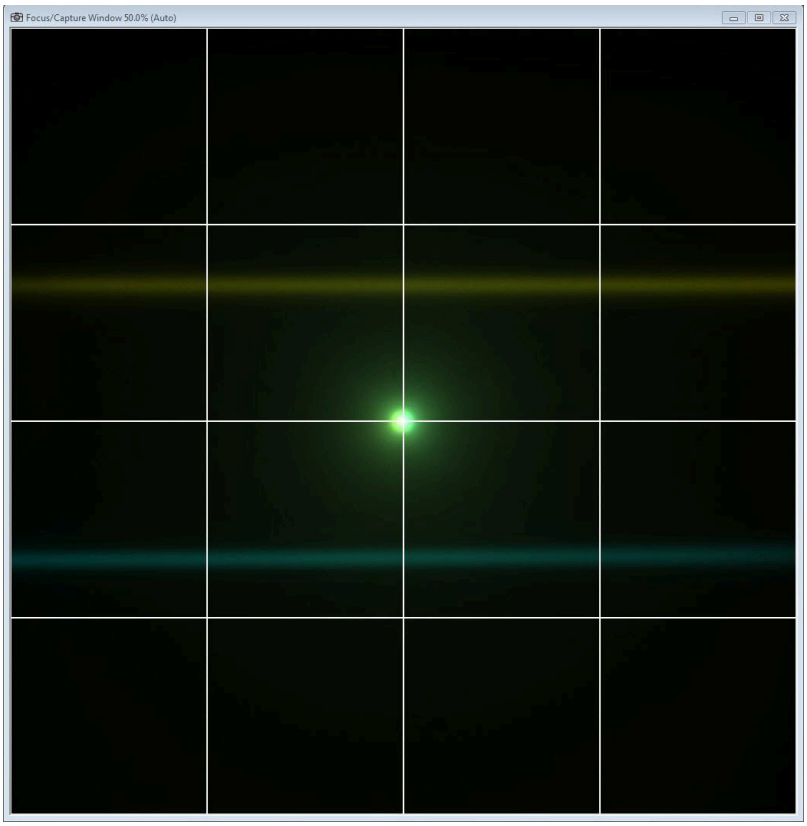
In the live window, **Camera 1** (**Path A**) will be pseudocolored red while **Camera 2** (**Path B**) will be pseudocolored green.

NOTE: For our color blind users, activate ***CY*** checkbox (under the Grid checkbox) to view beams and spots as Cyan (**Path A**) and Yellow (**Path B**)

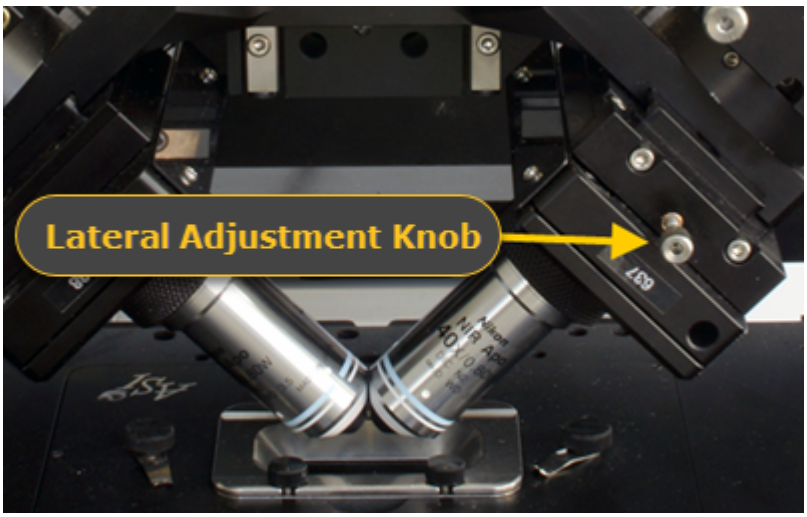


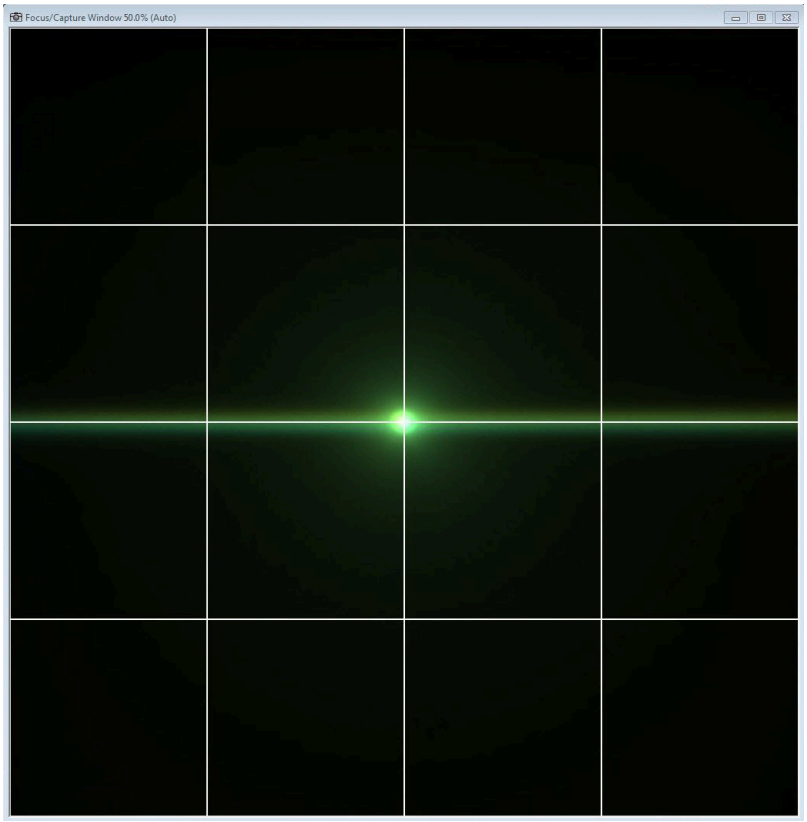
1. Use a combination of screws A, or B and C to move both the green and red spots to the center of the grid. Do not worry about the vertical location of the beams at this point.

NOTE: The image below shows the ***CY*** pseudocoloring for color blind users.



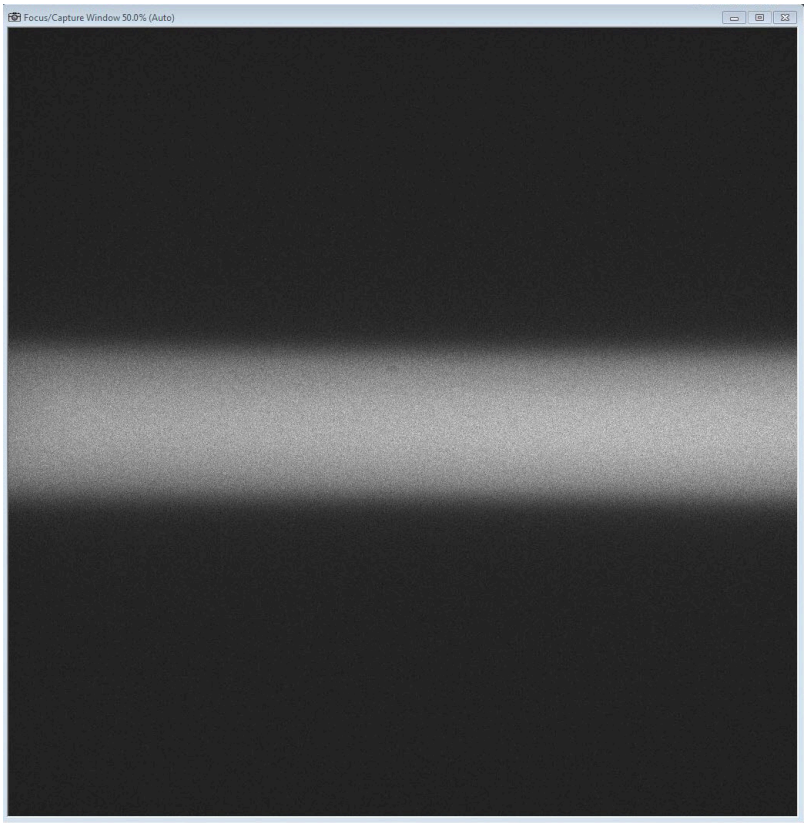
2. Use the Lateral Adjustment Knob (located on the front of Piezo B) to overlay all beams and spots at the center of the grid.





3. Unselect ***Dual Cam*** and ***Laser Switch***. The pencil beams for both path A and B should be uniform and centered.

4. To test uniformity, you can use the ***Right wheel*** to adjust the slice position to see the beams go out of focus uniformly. To do so, click ***Go*** for ***Channel A***, this should set ***Slice A*** to the Right wheel. Rotate the wheel and check the beam goes out of focus evenly in both directions. Click ***Neutral All*** to bring the slice position back to center. Repeat the same for beam B by setting ***Slice B*** to the Right wheel by clicking ***Go*** for ***Channel B***.

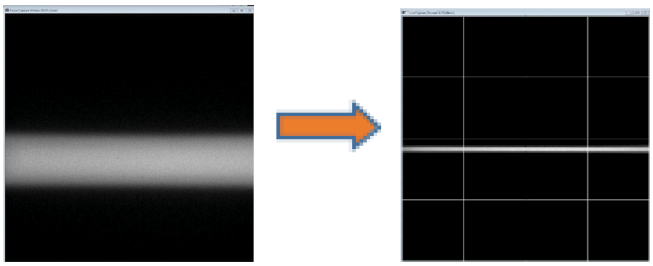

Focus/Capture Window 50.0% (Auto)

TUTORIAL: MLS Daily Quick Alignment Guide - Single Sided

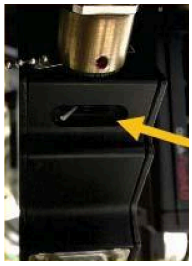
- Start with 488***A*** chosen for channel ***A***. Tick beam for ***Path A***, hit ***Neutral all*** and then ***Go*** for 488***A*** & ***Live***
- After objective swap check with white paper underneath objectives that beams are projected roughly underneath the opposite objective
- Check that the sample chamber is centred below the objectives
- Use ***Upper LED*** to better visualize
- Use the SPIM Z-drive, drive into dye solution

1. Check Beam & Focus

   a. When the beam is not focused at all or focus is far out (like in picture), focus by rotating the objective ***A*** (right) black bushing, sometimes it can be advised to rotate both bushings at the same time to keep them evenly close to the surface (clockwise rotation brings objectives apart)



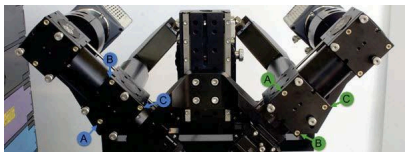
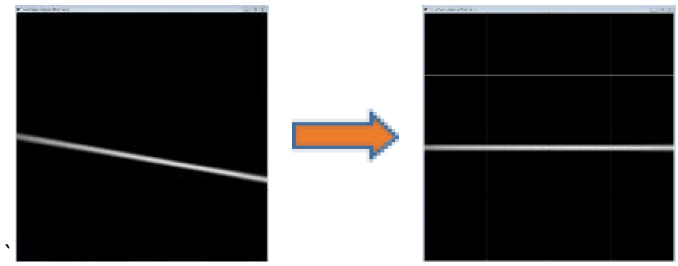
   b. Make sure you are on pencil beam (closed iris) for the following alignment steps: Close scanner iris mostly (lever towards right when looking from front of the scope)



2. Adjustment of Rotational Tilt

   a. Untick ***Dual cam*** to observe beams separately, use ***Grid***

   b. Tick beam for ***Path A***, hit ***Neutral all*** and then ***Go*** for 488***A*** & ***Live***, Adjust dichroic mirror (left cube) with ***B*** & ***C*** screws to bring beam parallel to the grid

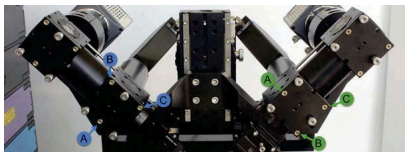
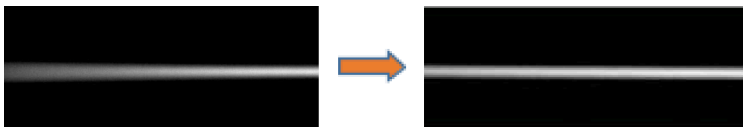
3. Adjustment of Focus Tilt

   a. Observe beams separately

   b. Tick beam for ***Path A***, hit ***Neutral all*** and then ***Go*** for 488***A*** & ***Live***, Make sure right joy-pod wheel is set to ***Slice A***

   c. Check that beam goes out of focus evenly when changing the slice position with the right wheel

   d. To adjust use dichroic mirror (left cube) with the ***A*** screw to bring beam rolling parallel in and out focus

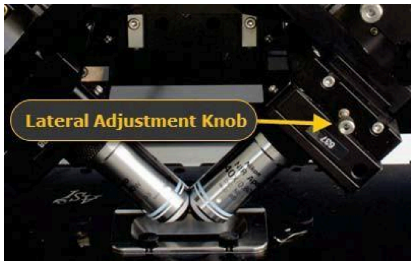
4. Adjustment of Camera Centration

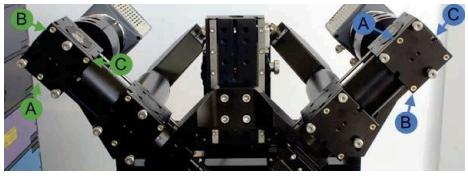
a. Tick beam for ***Path A***, hit ***Neutral all*** and then ***Go*** & ***Live***, and ***Grid***

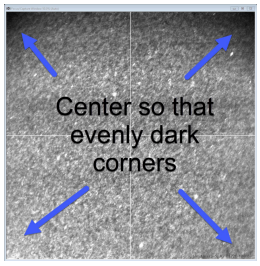
b. Use the **lateral adjustment screw** to move both beams to overlap at the FOV centre

c. Tick sheet for ***Path A*** and untick ***Grid.*** Open the sheet width to fill the full chip if required, tick ***99%*** and select ***2x2*** or ***4x4 binning*** to see the vignette

d. Adjust the sheet intensity evenly within the FOV using camera mirror ***A*** (upper right cube) with the ***A, B, C*** screws







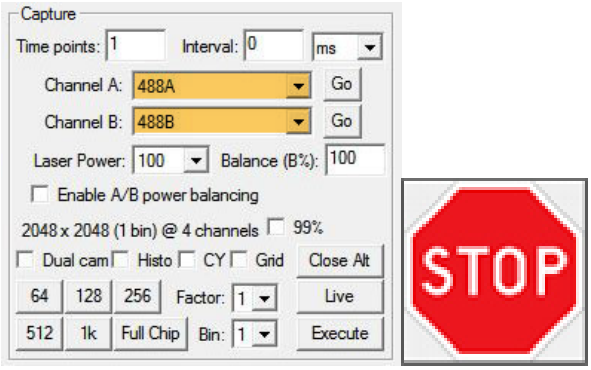
5. Final Adjustments

a. Set the calibration for either beam with desired piezo range (untick ***Dual cam*** and hit ***Go*** for desired path)

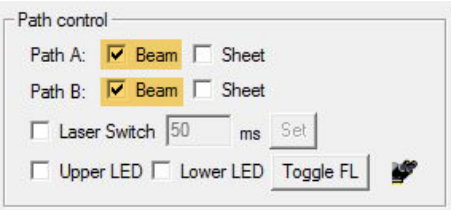
TUTORIAL: Calibrating the Piezo and Slice Position

Before you begin this final step of alignment and calibration, it is essential that pencil beams for both Path A and Path B are both uniform and centered as described in the earlier tutorials. To get ready for this tutorial, you will need to click ***Neutral All***, and have the appropriate filter selected for Channel A and Channel B.

> **NOTE:** It is recommended to use the imaging media you will use for your sample for this calibration or, at the very least, a solution with the same refractive index.

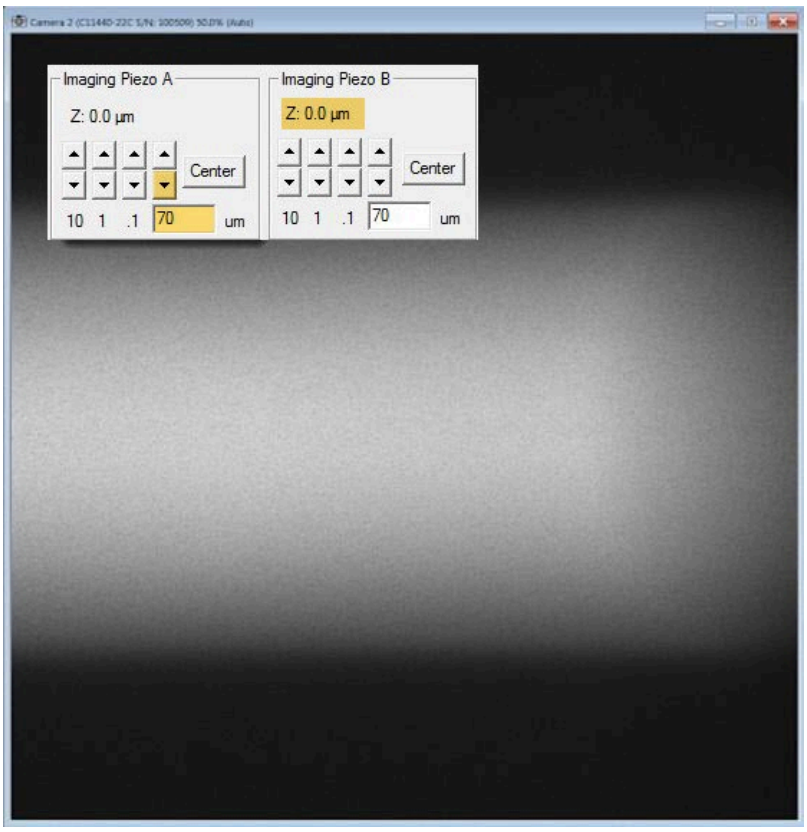


1. Check the box for ***Beam*** for Path A and Path B.

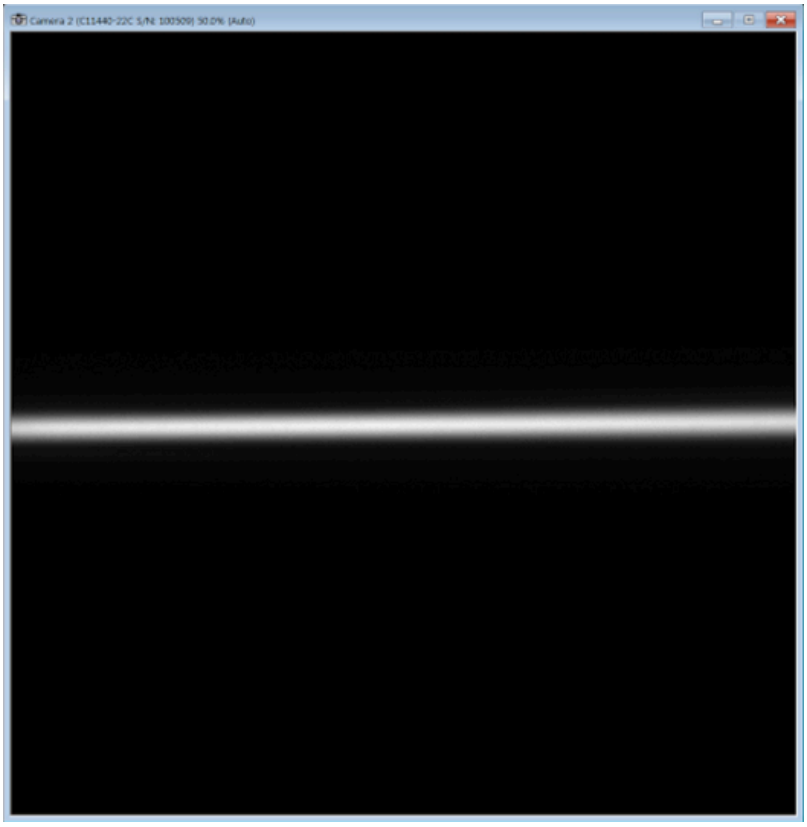


2. For ***Channel A***, click ***Go***. In the ***Imaging Piezo A*** control, enter **70** and click the down arrow to drop Piezo A down 70μm (for a 150μm travel piezo), the beam will go out of focus.

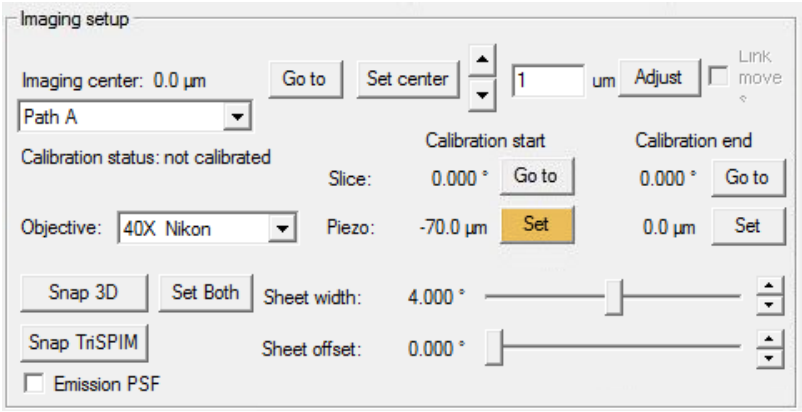
> **NOTE:** A Z value is displayed above the arrows, this will indicate the current position of the piezo. For a 150 μm Piezo it will range between -75 to 75 max. Always make sure the opposite piezo is at center position when starting the calibration.



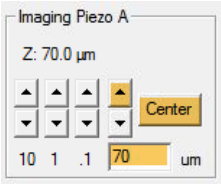
3. Adjust the right wheel on the joystick (confirm Controller Inputs, ***Right wheel*** = ***Slice A***) until the beam is in focus and uniform.



4. In Imaging Setup, click ***Set*** to select the Calibration start for the piezo.

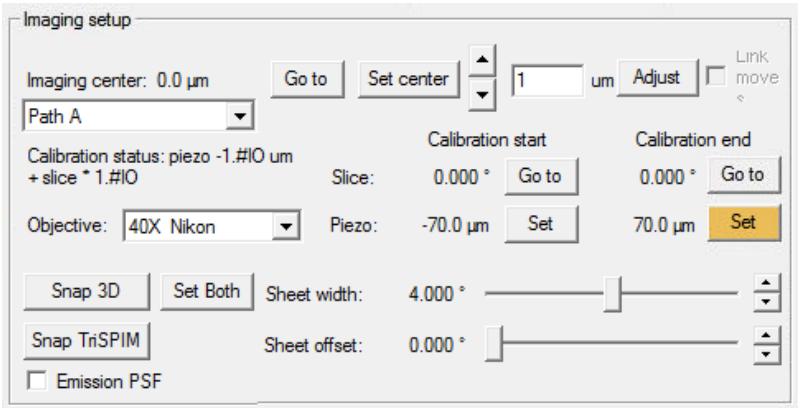


5. In the Imaging Piezo A control, click ***Center*** to bring the piezo back to 0.0um then click the up arrow to raise it 70um.



6. Adjust the right wheel on the joystick until the beam is in focus and uniform.

7. In Imaging Setup, click ***Set*** to select the Calibration end for the piezo.



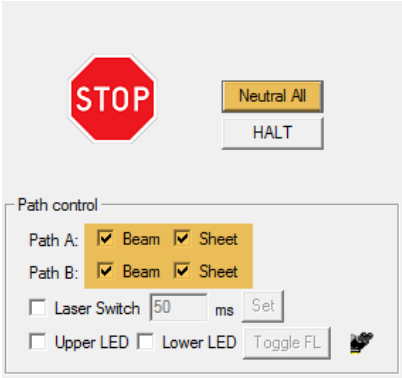
8. Return Piezo A to center by selecting ***Center*** (as in Step #4) and select Adjust (the beam should become focused again) to check the calibration.

Repeat steps #1-#8 for Path B and Piezo B.

TUTORIAL: Setting Sheet Width and Offset

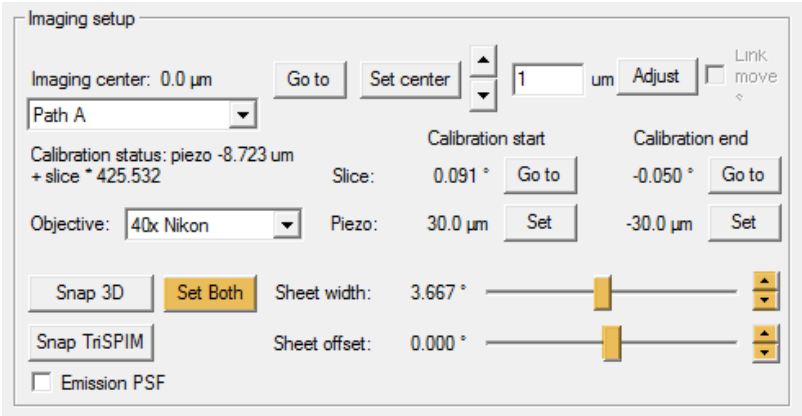
Once the Beams are aligned and calibrated return to the neutral position and continue to set the sheet width and offset.

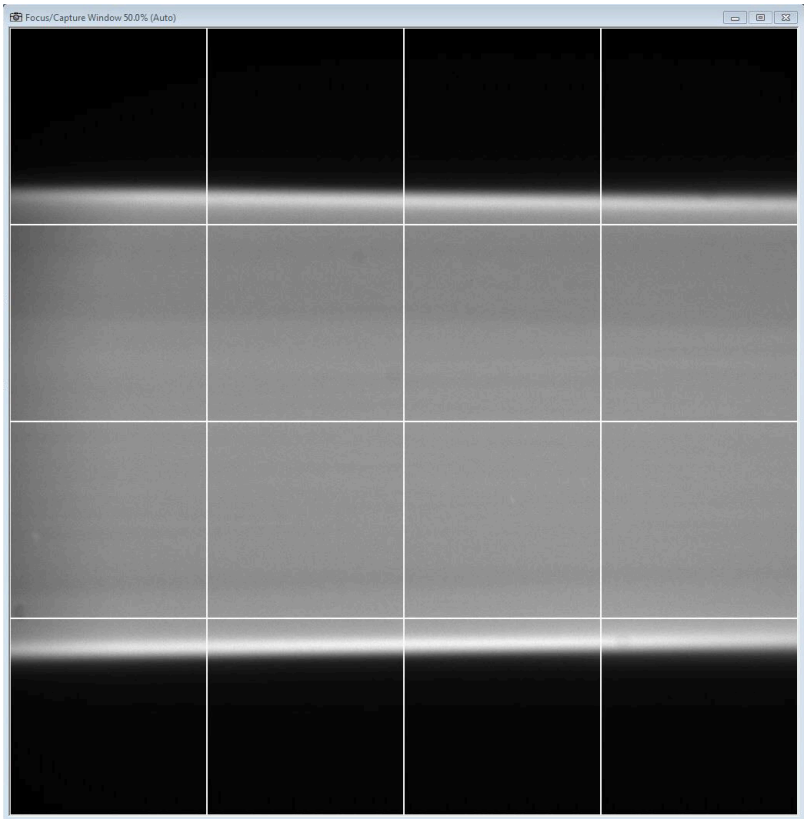
1. In Path Control, check the boxes for ***Beam*** and ***Sheet*** for Path A and Path B. Click ***Neutral All***.



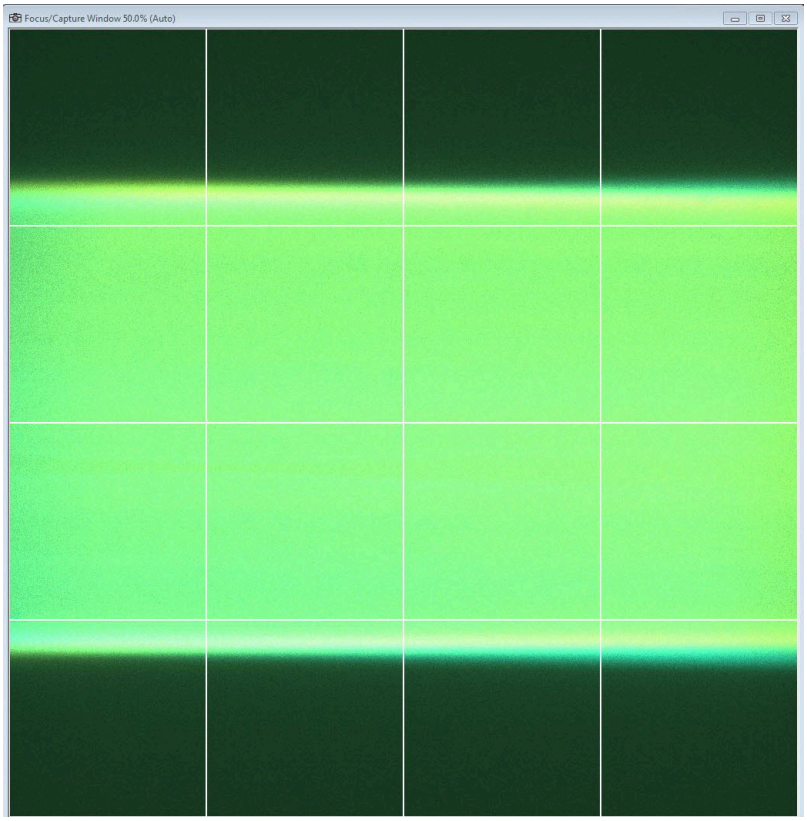
2. Check the box to turn on the ***Grid*** for easier alignment. Adjust the ***Sheet width*** (displayed as the vertical height of the sheet) to just outside the field of view. In the following example, the field of view is the center four squares of the grid. If the sheet is not perfectly centered, adjust the ***Sheet offset*** using the slider or arrows. Once the sheet width is correct and centered in the field of view, click ***Set Both*** to apply the width to the other sheet.

> **NOTE:** The arrows to the right of the sliders can be used to adjust sheet width and offset. The step size for a single arrow button click is 0.001 units. Hold CTRL while clicking the arrows to use a step size of 0.01 units.

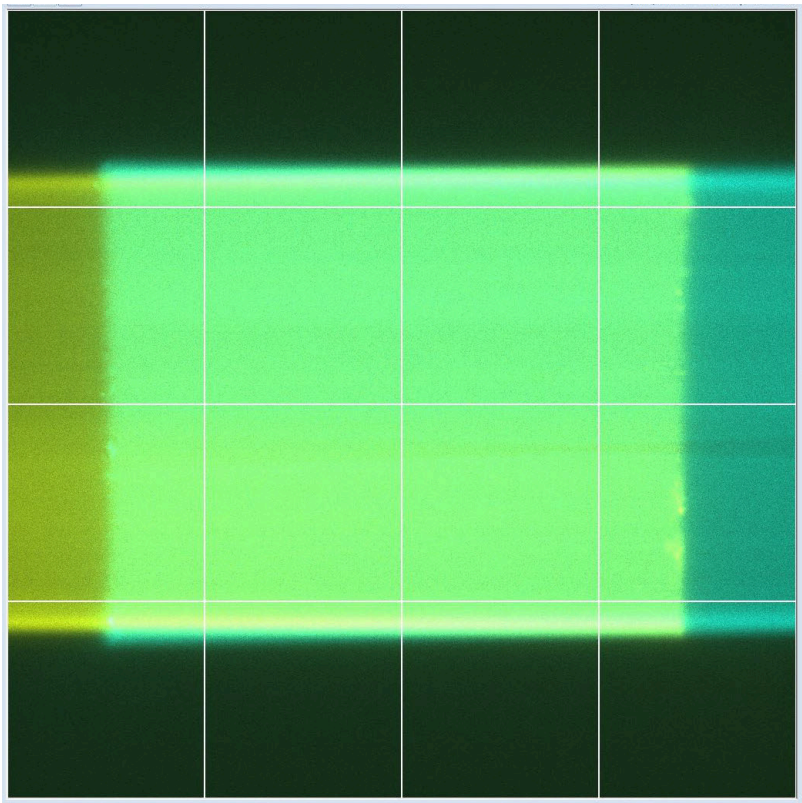




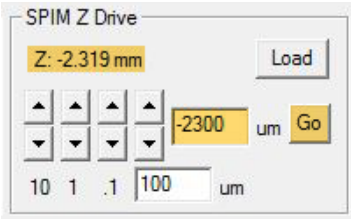
3. Check the box for ***Dual Cam*** mode to check the sheets are overlaid. When you are in ***Dual Cam*** mode, the sheet width and offset will only adjust for the active channel, the channel for which you last clicked ***Go***. Use the Go buttons to toggle between Channel A and Channel B while adjusting the offset until the sheets are well aligned as shown below.



4. At this point, you should find a good Z location for loading and unloading samples. Using the SPIM Z Drive group arrows, slowly lower the Z drive until the sheets start to hit the coverslip as shown in the following image.



5. Move back up 200-300 μm and write down/remember the Z position. You can later return to this position by typing the value in the text field and clicking Go once your sample is loaded.

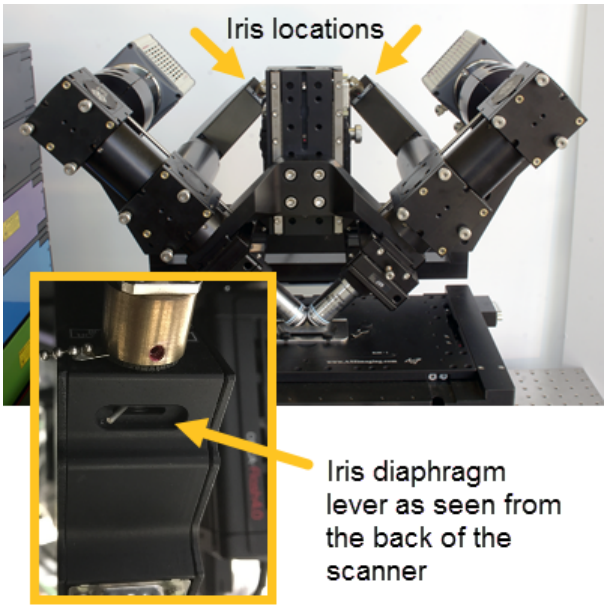


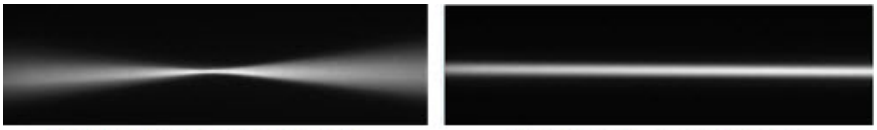
TUTORIAL: Alignment for Equalizing Beam Power

Normalizing laser power between Path A and Path B will yield the best reconstruction and analysis results. This power balancing has a hardware component and a software component. Read below for instructions on the hardware component.

1. Align the beams (in pencil beam mode) according to MLS alignment protocol: MLS Alignment and Calibration (all steps).

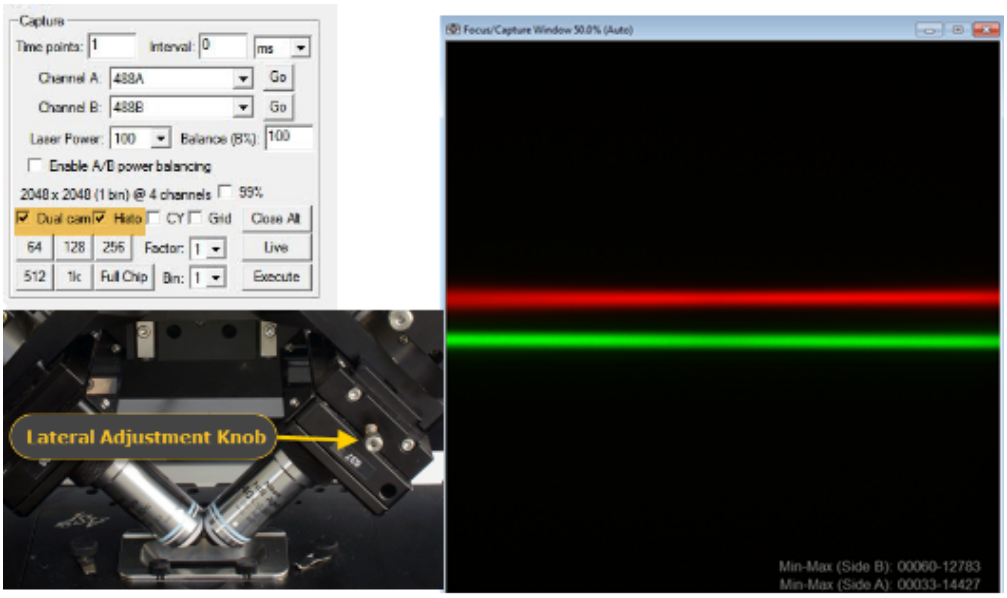
2. Adjust the scanner irises in beam mode so that the beams/ beam waists have the desired thickness.



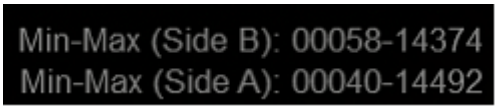


Open iris = Gaussian beam **Closed iris = pencil beam**

3. In the MLS window check ***Dual Cam*** and ***Histo*** to see both channels and their maximum and minimum intensities the same time. Bring the beams slightly apart using the Lateral Adjustment Knob as shown in the following image.



4. Carefully adjust the irises so that each beam shows a similar shape as well as a similar intensity is reported in the lower right of the live window.



NOTE: It is best to refine this in sheet mode and using your sample, if necessary you can also use Software Integrated Power Balancing.

5. Bring the beams back to overlap in the center and continue with the usual set-up of the experiment.

MLS Operation

Once the beams are aligned, a sensible sheet width is set, and piezo and slice distances are calibrated, you can start imaging your samples. This section of help pages will walk you through MLS operation from locating your sample to refining the calibrations through to image acquisition.

Locating and Positioning the Sample

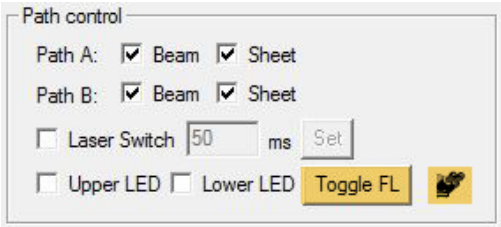
Locating the Sample

1. Use the ***Load*** button to fully withdraw the SPIM Z Drive.

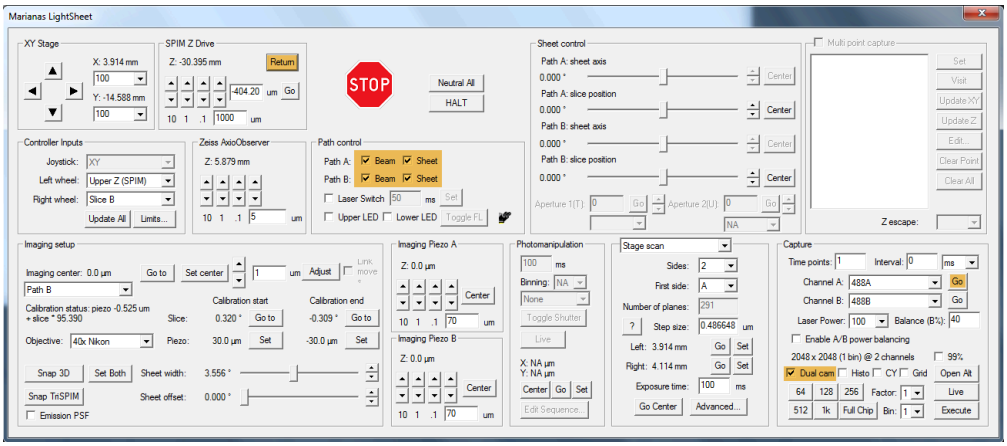
2. Load the sample, it should be prepared and placed on a 24 x 50 mm slide in its chamber (ideally within a centrally located circle of ~5mm diameter) or 10 mL petri dish .

3. Click on the ***binocular*** icon to direct light to the oculars and if you have activated an LED channel in the Focus window or on the LED touch pad click the ***Toggle FL*** button to turn the fluorescence on and off. Observe with one of the lower objectives. Focus with the microscope drive and position an object of interest by carefully moving the xy stage using the joystick.

> **NOTE:** The button on top of the joystick handle changes the XY travels speed from fast to slow. Be sure to use the slow drive when the upper objectives are immersed to prevent objective crashing and damage, especially when using the stainless steel imaging chamber.



4. Once the sample is located in the center of the field of view through the oculars, check the boxes to select ***Beam*** and ***Sheet*** for both paths and click ***Go*** and ***Live***. You might want to use ***Dual Cam*** mode while driving the Z Drive down to the sample using the ***Return*** button.



Approaching the Sample

1. Check the boxes for ***Beam*** and/or ***Sheet*** and turn down ***Laser Power*** to about 5%.

2. Select a filter for ***Channel A*** and ***Channel B*** and click ***Go*** for one path.

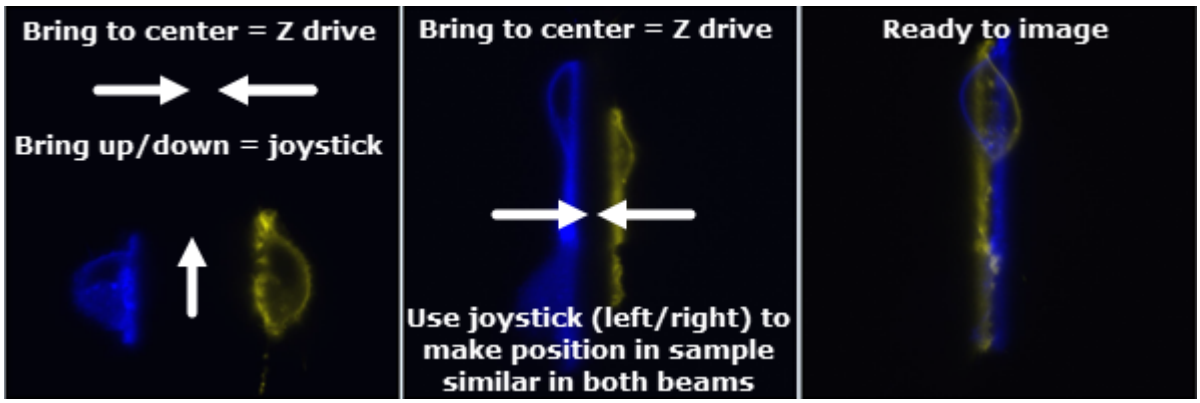
3. Lower objective without crashing into the sample, as outlined in [TUTORIAL: Using the SPIM Drive to Introduce Objectives into the Sample Chamber](#).

> **IMPORTANT INFORMATION TO NOTE:**
>
> • The beams are quite powerful when set to 100%, they will photobleach your sample (adjust sheet/beam power as necessary for your sample).
>
> • Move the Z/SPIM drive to a "safe" position (this is a position we determined at the end of [(TUTORIAL: Setting Sheet Width and Offset](#)). From this safe position, move in small increments toward your sample, being careful.

• Select both ***Path A*** and ***Path B Sheets***, and activate ***Dual Cam*** function as the objectives are lowered to visualize the co-focus point in relation to the sample and visualize Path A and Path B on the screen.

• Once the sample enters the field of view of the cameras, continue to move the SPIM drive towards the sample using the Left wheel of the joystick until Path A and Path B are overlapping in the center.

• Once the sheets are overlapping (both hitting the sample at the center), move slightly left and right and up and down with the joystick to bring the sample of interest in the center and similarly hit by the sheets.



• To image smaller objects, consider cropping the camera field of view to an appropriate size by clicking ***1k*** through to ***64 x 64*** pixels or narrow the field of view using the ***Factor*** drop down. This will save considerable space in data capture. And there is also an option (drop down ***Bin***) for binning if you are OK sacrificing resolution for signal.

• Adjust the piezo range to your field of view or vice versa before capture.

• When imaging a small volume (e.g. cells on a coverslip), open the scanner iris slightly in order to narrow the beam waist. Adjust the beam width while in ***Dual Cam*** mode in solution (Z drive is slightly above samples).

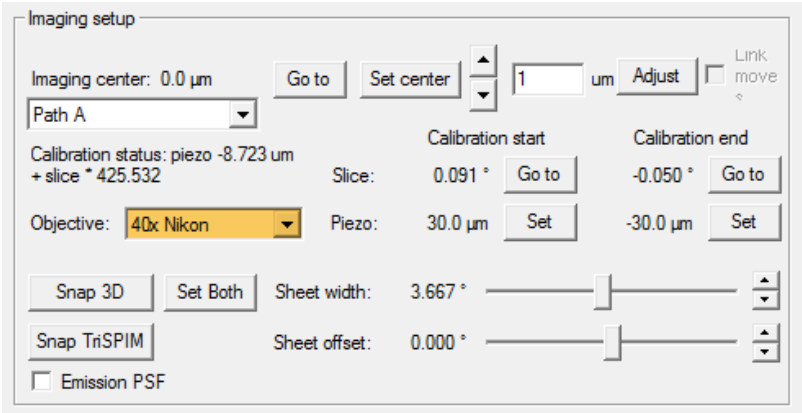
Before Capture

Before you set up an experiment to capture, do the following:

1. Save the **.sld** file.

2. Ensure sheets are completely overlapped and your sample of interest centered to maximize the acquisition area for alignment and post-image processing.

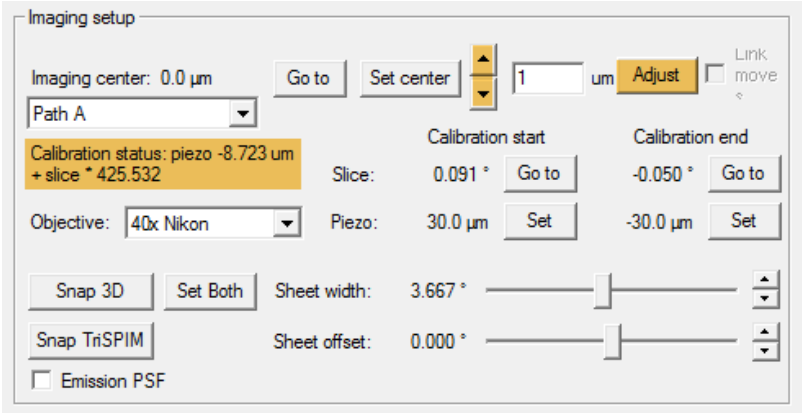
3. Select the correct objective.



4. Consider the objective and camera crop you are using for choosing the appropriate number of planes and step size (Hamamatsu 6.5 µm/px with 40X gives you 160 µm with 1k = use full range of piezos, 83 µm with 512x512 = use about 80 µm of the piezo range).

5. Consider the camera crop and adjust the sheet width accordingly (just slightly over the field of view).

6. Test calibration in sheet mode by moving Z in the Imaging Setup group. The sample should stay in focus when the Z coordinate is changed.



7. Make sure that slice position is adjusted for piezo positions using the previously determined calibrations. To display the calibrated values, select ***Adjust*** in MLS Imaging Setup window. Repeat for Path B.

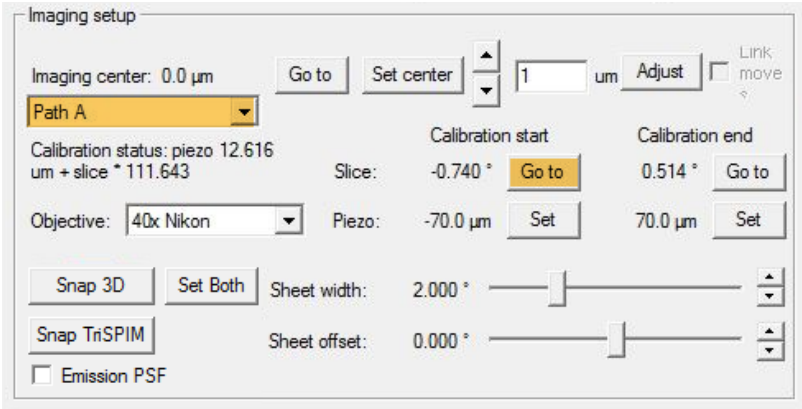
> **NOTE:** If refining the calibration is necessary, see Refine an Existing Calibration.

8. Check that the ***Imaging center*** is set to **0.0** µm for both sides.

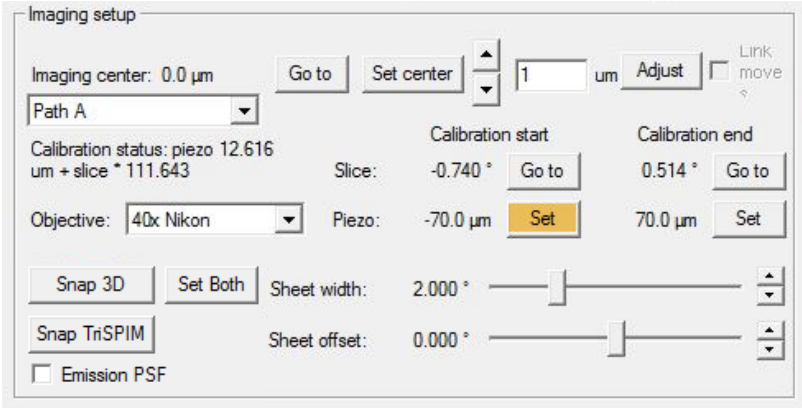
Refine an Existing Calibration

If an existing calibration has been set and the pencil beams are uniform and centered, you can fine tune the calibration using following instructions.

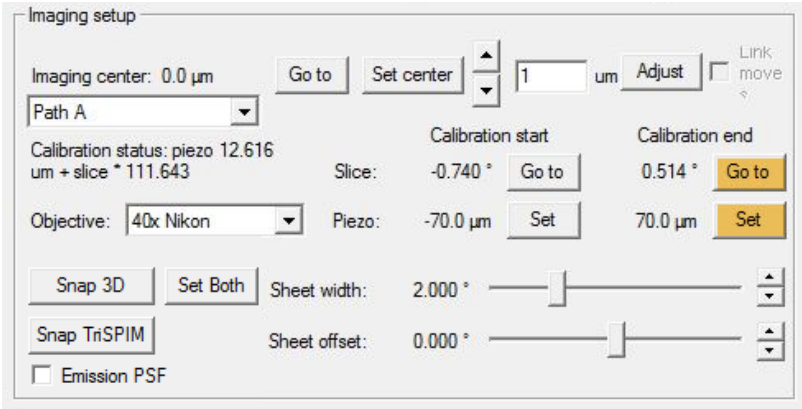
1. Click ***Go*** for ***Channel A***. In Imaging setup, select ***Path A*** and click ***Go to*** for Slice, Calibration start.



2. Use the right wheel on the joystick to refine the focus of the beam. Click ***Set*** when satisfied with the focus and uniformity.



3. Adjust the ***Calibration end*** Slice by clicking ***Go to***. Refine the focus using the right wheel, click ***Set***.



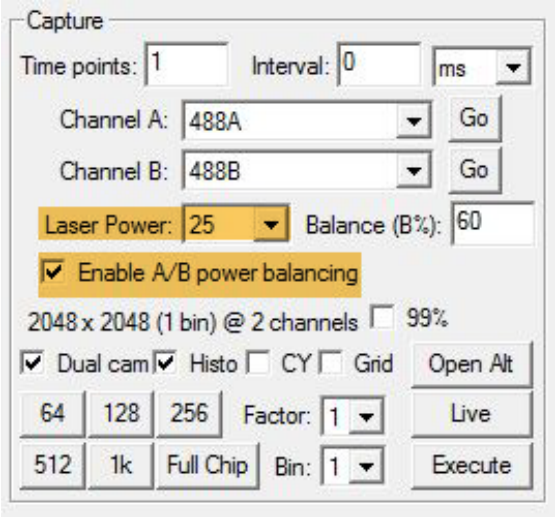
4. Repeat steps #1-3 for Path B.

Software Integrated Power Balancing

Normalizing laser power between Path A and Path B will yield the best reconstruction and analysis results. This power balancing has a hardware component and a software component. Read below for instructions on the software component.

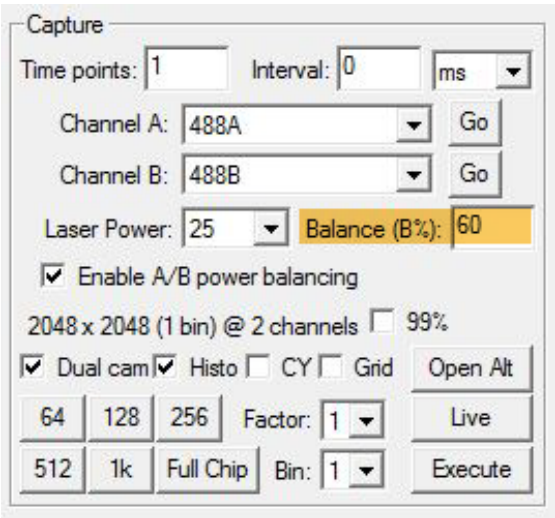
1. Take a test capture with the desired wavelengths and imaging mode. Open the image and check the histograms (***Renormalize*** icon) for each channel (wavelength and side).

2. If you need to adjust the laser power per side, set the desired ***Laser Power*** and check ***Enable A/B power Balancing***. The set laser power will then apply to side A of each wavelength.

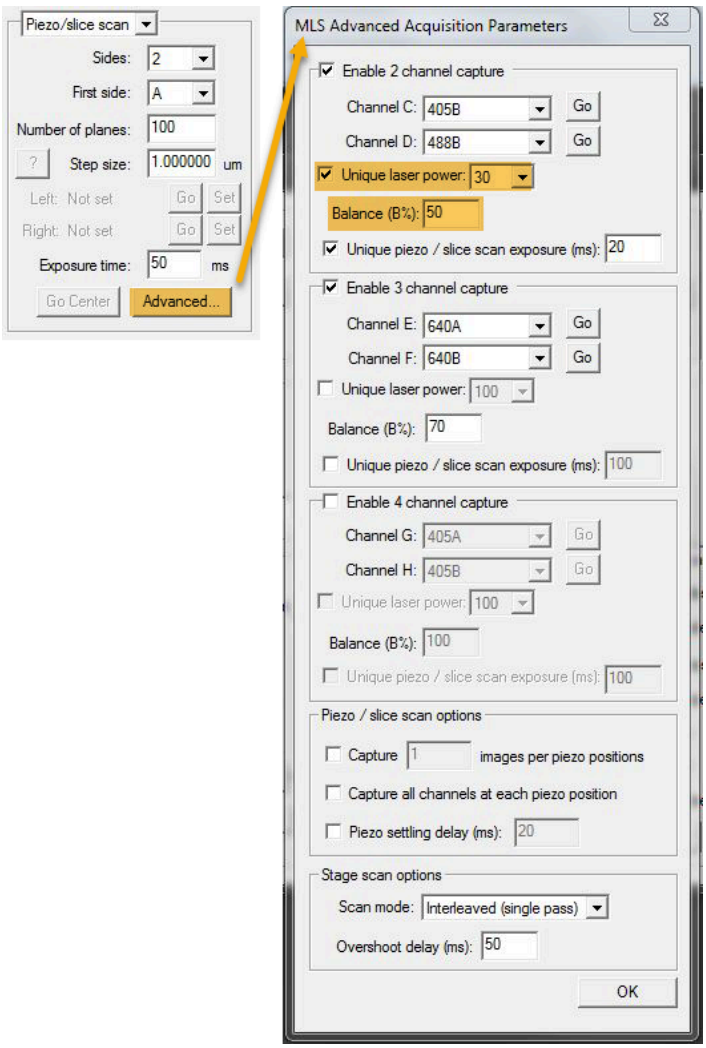


3. To alter the laser power for side B of each wavelength, enter a value for ***Balance (B%)***. With B%=100 side B will have the same laser power as A (set in the general Laser Power box).

> **NOTE:** The power of side B is considered a % of side A. When the Laser Power (for side A) is set to 25% and B should be 12.5% enter B%=50, when A is set to 25% and B should be 50% enter B%=200. Leaving B%=100 will result in A and B at 25% laser power.



4. This adjustment is available for every other wavelength individually if required. Use the ***MLS Advanced Acquisition Parameters*** dialog as shown in the following figure.


Piezo/slice scan
Sides: 2
First side: A
Number of planes: 100
?
Step size: 1.000000 um
Left: Not set
Go
Set
Right: Not set
Go
Set
Exposure time: 50 ms
Go Center
Advanced...
MLS Advanced Acquisition Parameters
Enable 2 channel capture
Channel C: 405B
Go
Channel D: 488B
Go
Unique laser power: 30
Balance (B%): 50
Unique piezo / slice scan exposure (ms): 20
Enable 3 channel capture
Channel E: 640A
Go
Channel F: 640B
Go
Unique laser power: 100
Balance (B%): 70
Unique piezo / slice scan exposure (ms): 100
Enable 4 channel capture
Channel G: 405A
Go
Channel H: 405B
Go
Unique laser power: 100
Balance (B%): 100
Unique piezo / slice scan exposure (ms): 100
Piezo / slice scan options
Capture 1 images per piezo positions
Capture all channels at each piezo position
Piezo settling delay (ms): 20
Stage scan options
Scan mode: Interleaved (single pass)
Overshoot delay (ms): 50
OK

Data Acquisition: Piezo/ Slice Scan

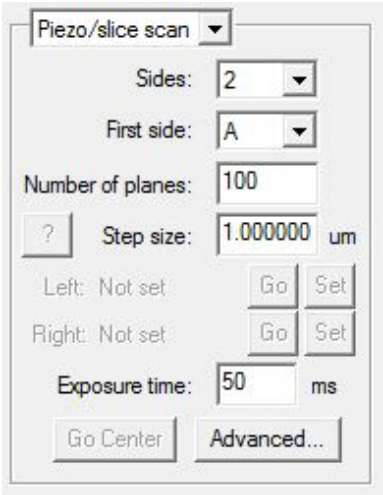
1. Have ***Beam*** and ***Sheet*** checked for both Paths.

2. In the Capture group:

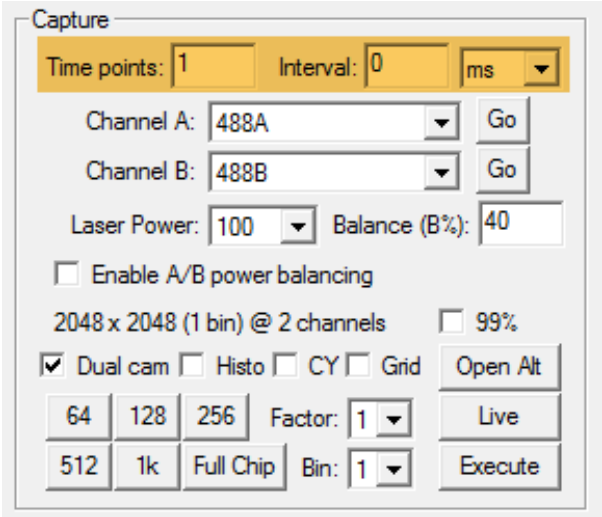
    a. Select the (first) filter for each channel A and B respectively.
    b. Lower the sheet power (5 % recommended).
    c. Check ***Dual Cam*** and ***Live*** to view your sample.
    d. Make sure the imaging FOV (camera cropping) of choice is selected.
    NOTE: Click ***Stop*** again to not bleach your sample

3. In the Capture Setup and Capture groups do the following:

    a. Select ***Piezo/slice scan*** from the drop down menu
    b. ***Sides*** – select 2 for Dual View SPIM, 1 for Single View SPIM (the second side choice will then be greyed out)
    c. ***First Side*** - can be either A or B (only significant for Single View SPIM)
    d. Set the ***Number of planes*** and ***Step size*** (consider the camera crop)
        NOTE: These values combined cannot exceed the travel of the piezo.
        For example: 10X objective + 150 um Piezo
        Step Size = 1μm, Number of planes = 150
        or
        Step size = 2 μm, Number of Planes =75
    e. Set ***Exposure time*** - For USB3 connected cameras recommended minimum exposure is 5 ms at 512 px or smaller chip size, above 15 ms at 1k px, above 36 ms at full chip
        NOTE: Check for the appropriate exposure time in LIVE mode.

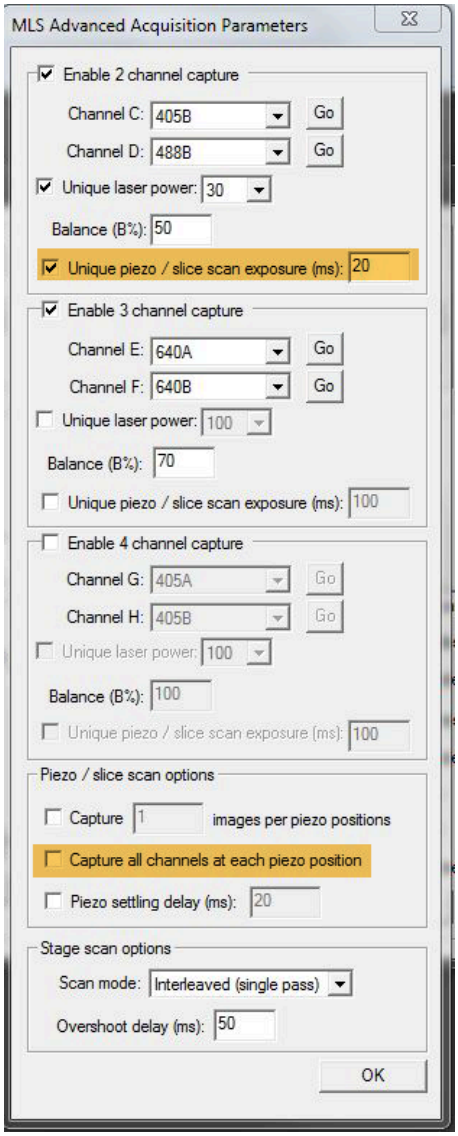


4. For timelapse acquisition choose the number of ***Time points*** and ***Interval*** at the top of the Capture group.



5. Click ***Advanced*** to open the dialog to add channels.
    a. Check 2, 3, or 4, channel capture and select the filter pairs for additional channels.
    b. Check ***Unique Laser Power*** to enter an individual laser power value for each channel.
    c. Enter a laser power for side B in ***Balance B (%)*** when power balancing is enabled.
    d. Check ***Unique piezo /slice scan exposure (ms)*** to enter an individual exposure time.
    e. For colocalization assays and Single View SPIM, check ***Capture all channels at each piezo position*** at the bottom of the advanced window.
    f. Click ***OK*** to close the Advanced Capture mode window.

    Note: When capturing all channels at each piezo position it will disable the possibility to assign different exposure times to different channels for capture timing reasons.



6. Click ***Execute*** in the Capture group to start the capture.

Data Acquisition: Stage Scan

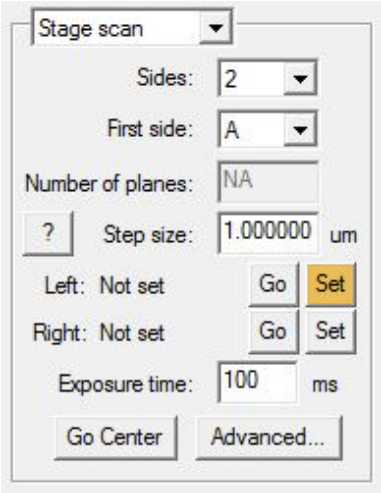
1. Have ***Beam*** and ***Sheet*** checked for both Paths.

2. In the Capture group:

    a. Select the (first) filter for each channel A and B respectively.
    b. Lower the sheet power (5 % recommended).
    c. Check ***Dual Cam*** and ***Live*** to view your sample.
    d. Make sure the imaging FOV (camera cropping) of choice is selected.
    NOTE: Click ***Stop*** again to not bleach your sample

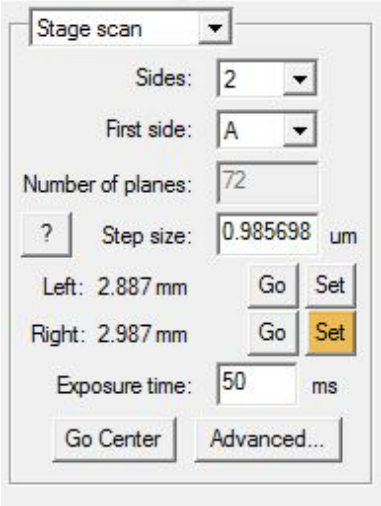
3. In the Capture Setup and Capture groups do the following:

    a. Select ***Stage scan*** from the drop down menu
    b. ***Sides*** – select 2 for Dual View SPIM, 1 for Single View SPIM (the second side choice will then be greyed out)
    c. ***First Side*** - can be either A or B (only significant for Single View SPIM)
    d. Set the ***Step size***.
    e. Use the Joystick or the arrows in the XY Stage Group to move the sample in X. Set the stage scan extents by clicking ***Set***.



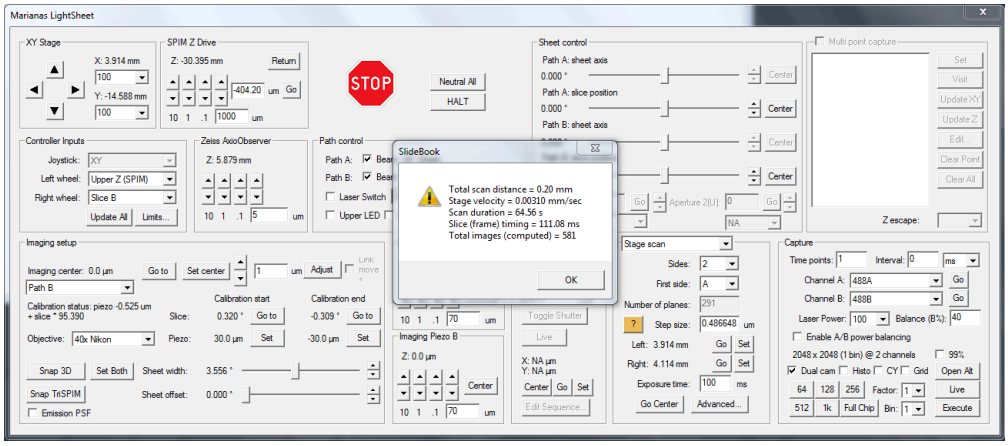
    f. Repeat for the opposite end of the sample. The ***Number of planes*** will be calculated automatically from the set ***Step size***.
    g. Once the extents are set you can visit the start and end using the ***Go*** buttons, ***Go Center*** will move the stage to the center position of the stage travel range.

    NOTE: The calculated step size is also determined by the stage specifications and might vary slightly from the typed value. The number of planes is calculated from the travel distance and the step size as follows: Travel Distance/(Step Size/sin(45))

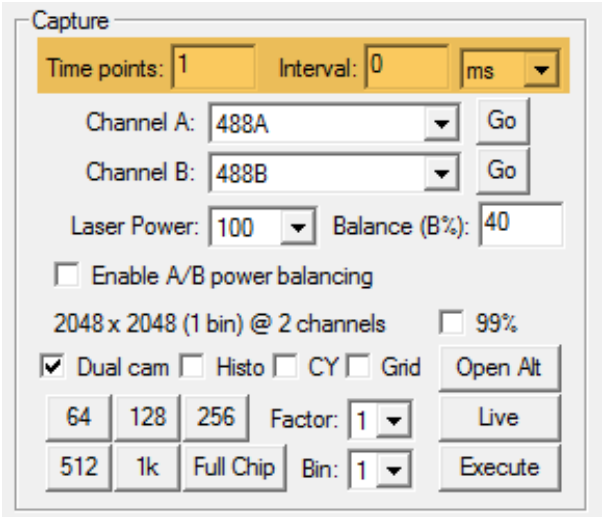


h. Set ***Exposure time*** - For USB3 connected cameras recommended minimum exposure is above 50 ms for full chip, 30 ms for 1k.

NOTE: Check for the appropriate exposure time in LIVE mode
NOTE: Once the capture parameters are set you can check the Total scan distance, Stage velocity, Scan duration, Slice (frame) timing and number of Total images when you click the "?" button next to the stage travel extents.



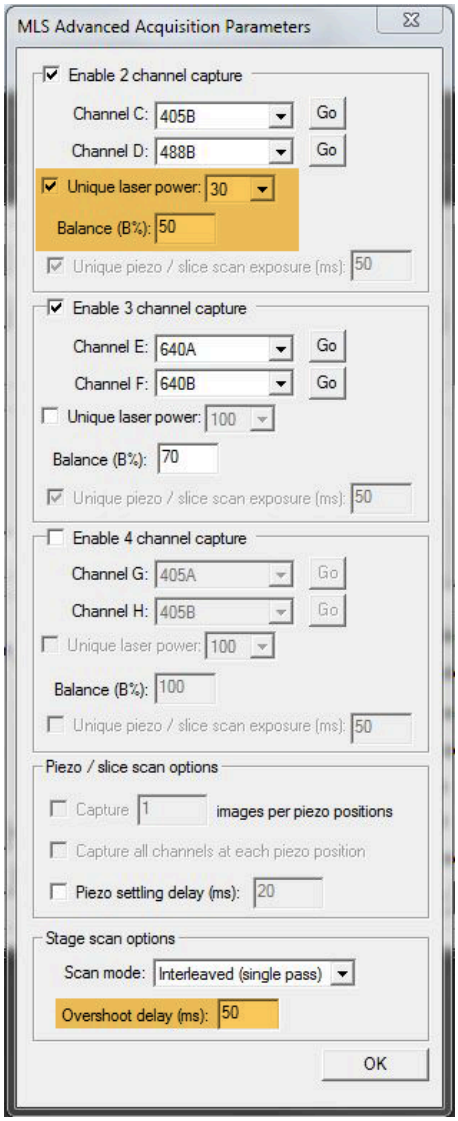
4. For timelapse acquisition choose the number of ***Time points*** and ***Interval*** at the top of the capture group.



5. Click ***Advanced*** to open the dialog to add channels.
   a. Check 2, 3, or 4, channel capture and select the filter pairs for additional channels.

b. Check ***Unique Laser Power*** to enter an individual power value.
c. Enter a laser power for side B in ***Balance B (%)*** when power balancing is enabled.
d. In the drop down you have the choice of three scan modes: ***Interleaved (Single Pass)*** will capture all channels of all sides in one run. ***Per-Side (Bidirectional)*** will capture all channels of the first side in one run and all channels of the second side on return. ***Per-Side (Unidirectional)*** will capture all channels of the first side in one run, return the stage and capture all channels of the second side in another run.

e. Enter an appropriate value for the ***Overshoot delay***. The required delay (in ms) is calculated as follows: Stage Backlash in um (for default use 7 um)/ stage speed in mm/s (for info click the "?" button next to the stage travel extents)



f. Click ***OK*** to close the Advanced Capture mode window.

NOTE: For stage scan captures set the calibration over a narrow range (e.g. 10 um), especially when you are working with more opened scanner irises to create a narrower sheet.

6. Click ***Execute*** to start the capture.

Data Acquisition: Combined SPIM Capture and Photomanipulation Experiments

Before you begin this process, you must first set the appropriate MLS capture parameters as described in Data Acquisition: Piezo/Slice Scan and Data Acquisition: Stage Scan. Make sure the center of your SPIM image is the field of view of the finder camera.

1. For Piezo/Slice Scan the stage position for photomanipulation will be similar to the SPIM image position.

2. For a stage scan, set the bounds of the scan
   a. Enable the view through the finder camera: In the Photomanipulation group of the MLS window adjust exposure time and binning, set channel in the drop down menu, click ***Toggle Shutter*** and ***Live***. Note: Make sure the SPIM lasers are off or an appropriate filter is selected in the turret to avoid over exposing the finder camera with the lasers above.
   b. Focus on the sample from below using the microscope focus or the Lower Z Stage group of the MLS window.
   c. Either click ***Center*** or move the stage along X between the stage scan bounds to the field of view of interest.
   d. Click ***Set*** to select these coordinates for the photomanipulation experiment.

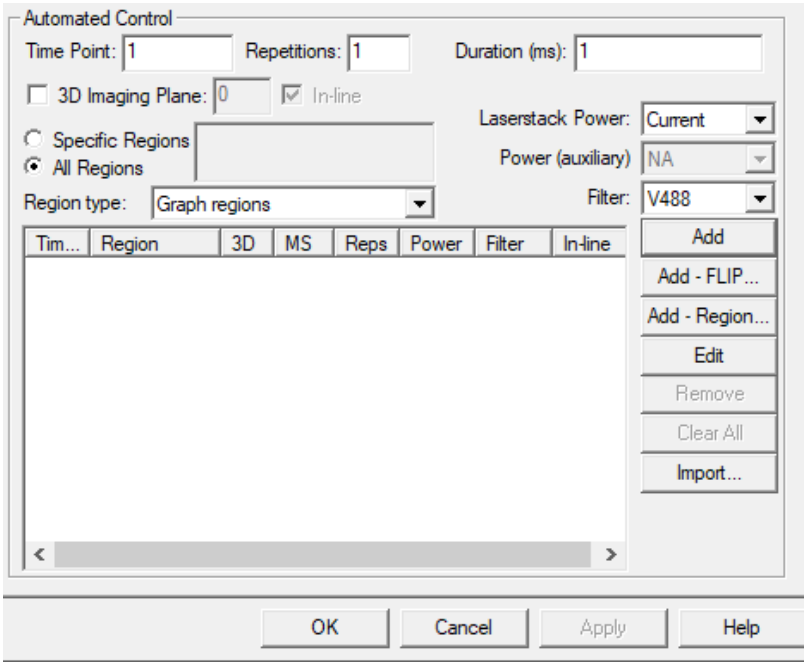
3. Use the region tools to create a region or multiple regions in the image. These regions are where the photomanipulation will occur.

4. Click ***Edit Sequence...*** to open the Photomanipulation settings.

6. Begin creating events by entering the ***Time Point***, ***Repetitions***, ***Duration***, ***Laser Power*** and either ***Specific regions*** or ***All regions*** to be affected by the event.

5. Select the desired ***Photomanipulation filter*** from the drop down menu. This filter is usually named by the schema V*wavelength*. For example, the Vector FRAP filter selection for 488 would be called **V488**.

NOTE: Always set a specific laser power - leaving it at 'Current' will not initiate the FRAP lasers during MLS capture



7. Click ***Add*** to save the first event.

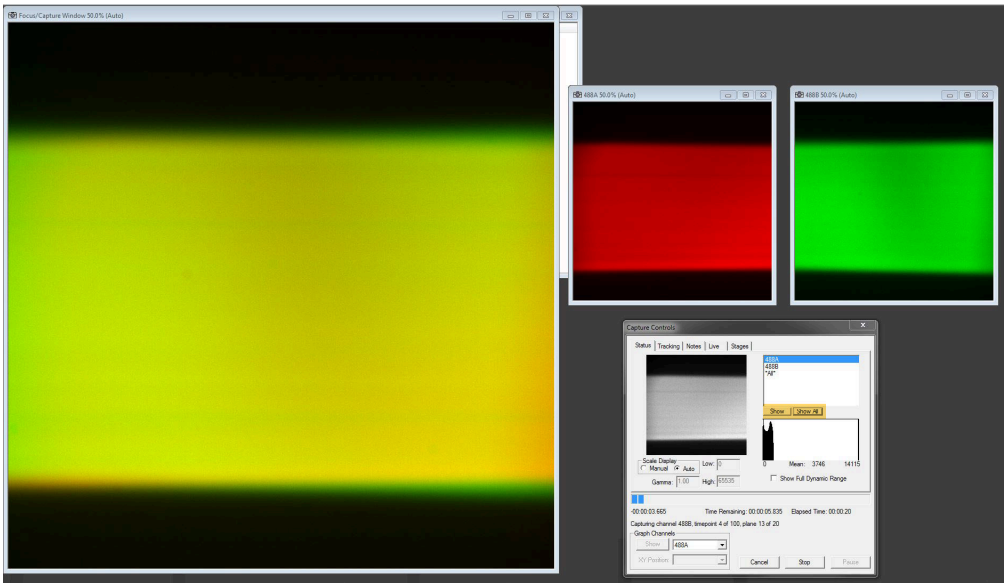
8. Create additional events as desired by repeating steps 6-7. When all events are entered, click ***OK***. This will close the ***Capture Parameters*** dialog and take you back to the MLS controls window.

9. Set a timelapse by entering a value above 3 in ***Timepoints*** and set the desired ***Interval*** in the Capture group of the MLS window. ***NOTE:*** The photomanipulation will initiate after the SPIM capture of specified timepoint.

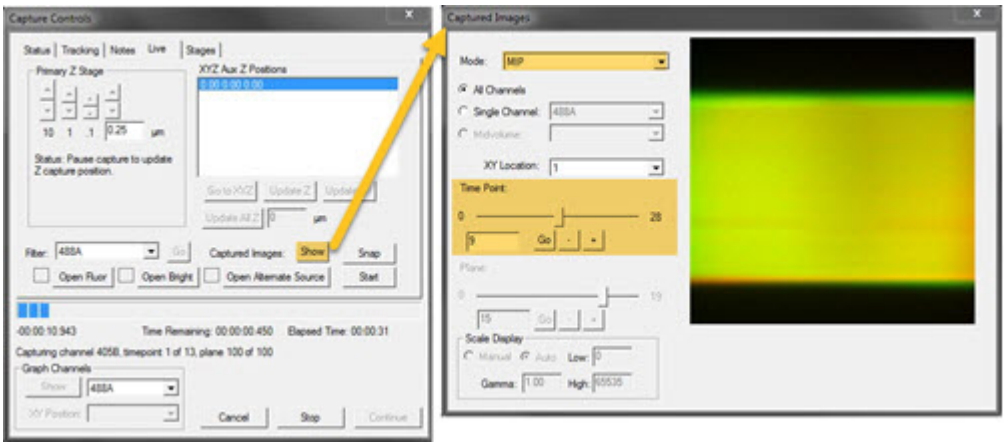
10. Click ***Execute*** to begin acquisition.

During MLS Capture

During image capture, a live monochrome window shows individual planes as they are acquired. You may open separate windows for each channel by selecting a channel in the Status tab of Capture Controls and clicking ***Show*** or ***Show All*** to open a window for all available channels.



For timelapse data acquisition, go to the ***Live*** tab of Capture Controls and click ***Show*** Capture Images. This will open the Captured Images dialog that allows you to view a MIP (maximum intensity projection) image and previous timepoints before capture completes.



MLS Post-Acquisition Processing

When using Dual View SPIM mode of MLS, the two paths will need to be fused using reconstruction algorithms. In addition to this, any data collected using stage scan mode will need to be deskewed first to put it back into a geometrically correct location. General information about deskewing can be found in this video about our Lattice LightSheet system. The concept is the same but the acquisition angle is 45 degrees for Marianas LightSheet.

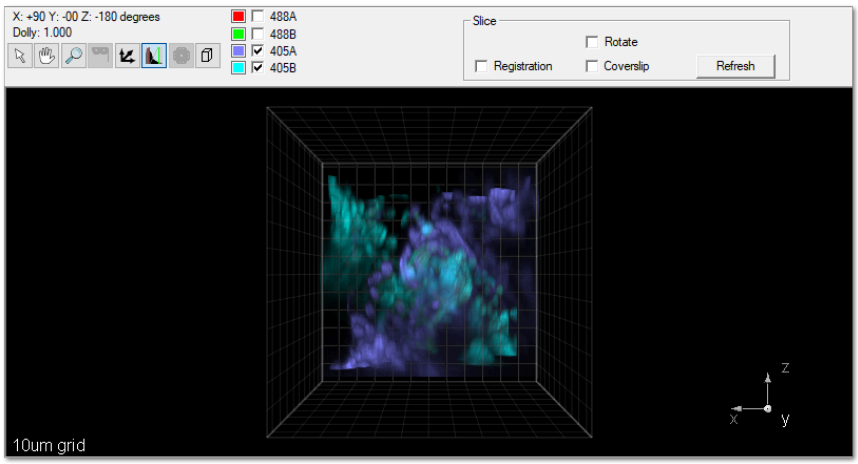
TUTORIAL: 3D/ 4D Volume Preview and Channel Transforms

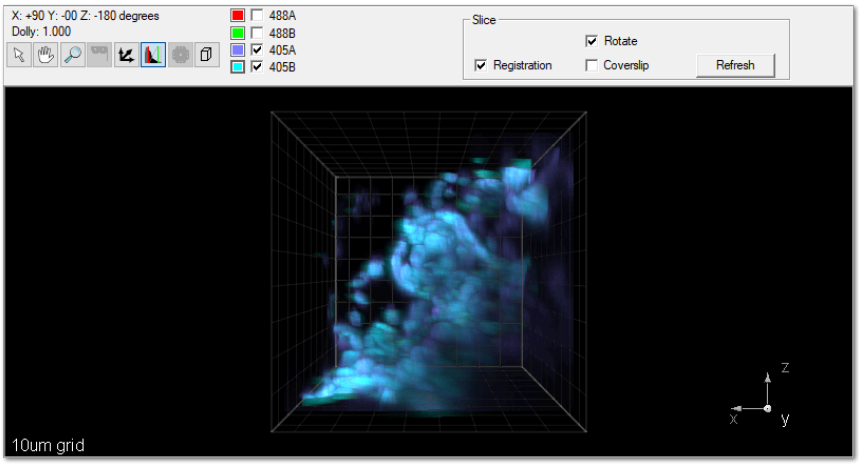
This tutorial will show how to create a 2D/3D-Transform for previewing your data or preparing them for an export as TIF file.

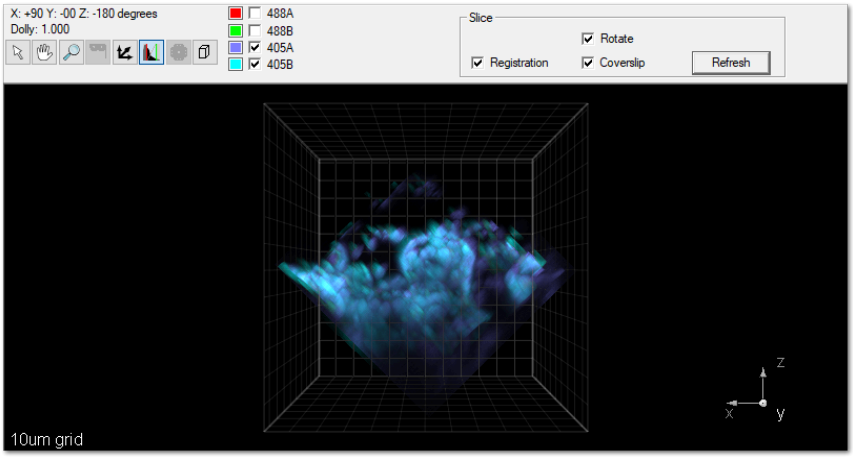
MLS Data in the 3D/ 4D Volume Viewer

1. Save the **.sld/ .sldy** before reviewing your captures with either the 3D/ 4D Volume Viewer or the Preview tool.

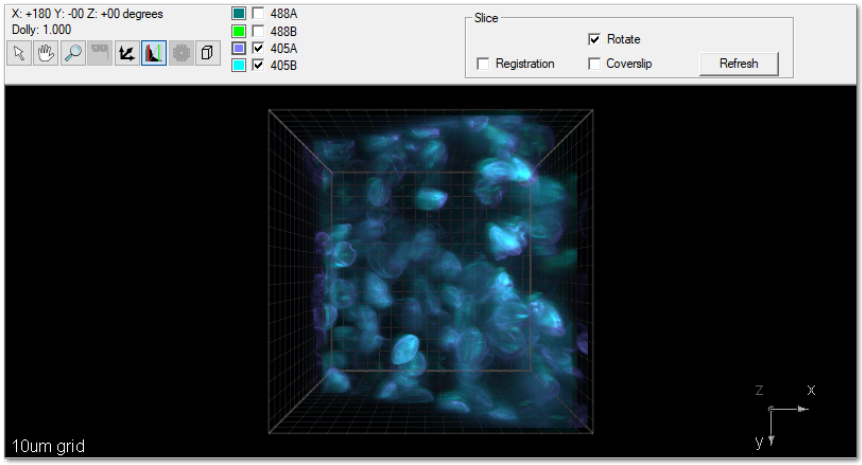
2. Select (highlight) the captured piezo/slice or stage scan. In the Home tab of SlideBook, select ***View > 3D/4D Volume View***. The 3D/ 4D Volume Viewer will open a rendering of your capture in order to decide whether to process it further.
3. In order to check your data and the transform you will have the selection to view your data with the following options:
    - ***Deskew*** - For Stage scan data this will be ticked by default.
    - ***Rotate*** - This rotates the second side of a Dual View SPIM capture roughly above the other side (90° rotation).
    - ***Coverslip Correct*** - This rotates the data another 45° so that the sample can be viewed like a conventional Z-Stack.

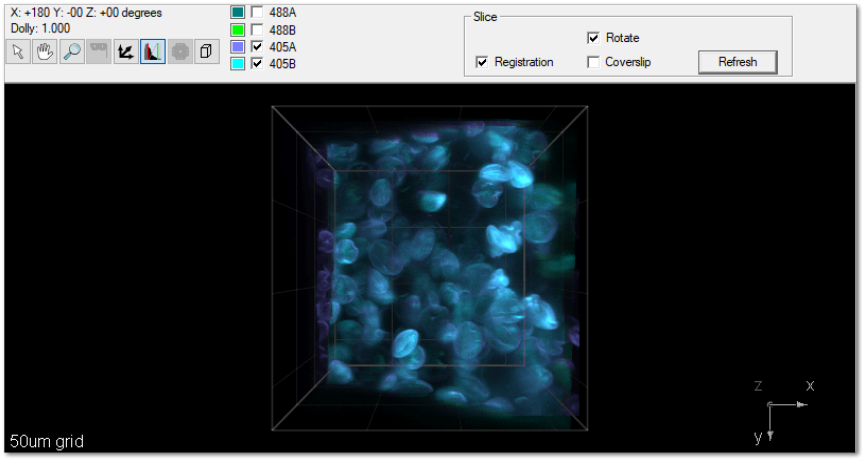






• ***Registration*** - This applies either a previous transform saved in SlideBook or it's on transform if present.



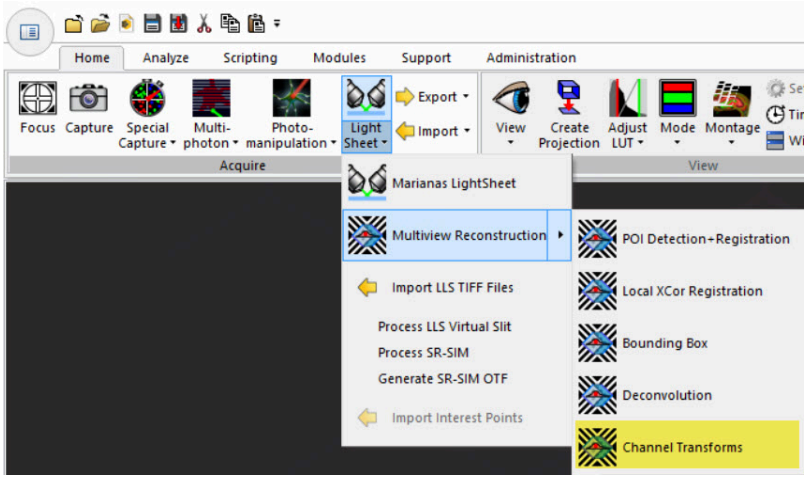


• The **Bounding Box** allows for orthogonal slicing of a 3D or 4D volume. This is particularly useful to highlight a certain area in a volume or select the Data or PSF Bounding Box for the steps below.
See also:
[3D/ 4D Volume View]

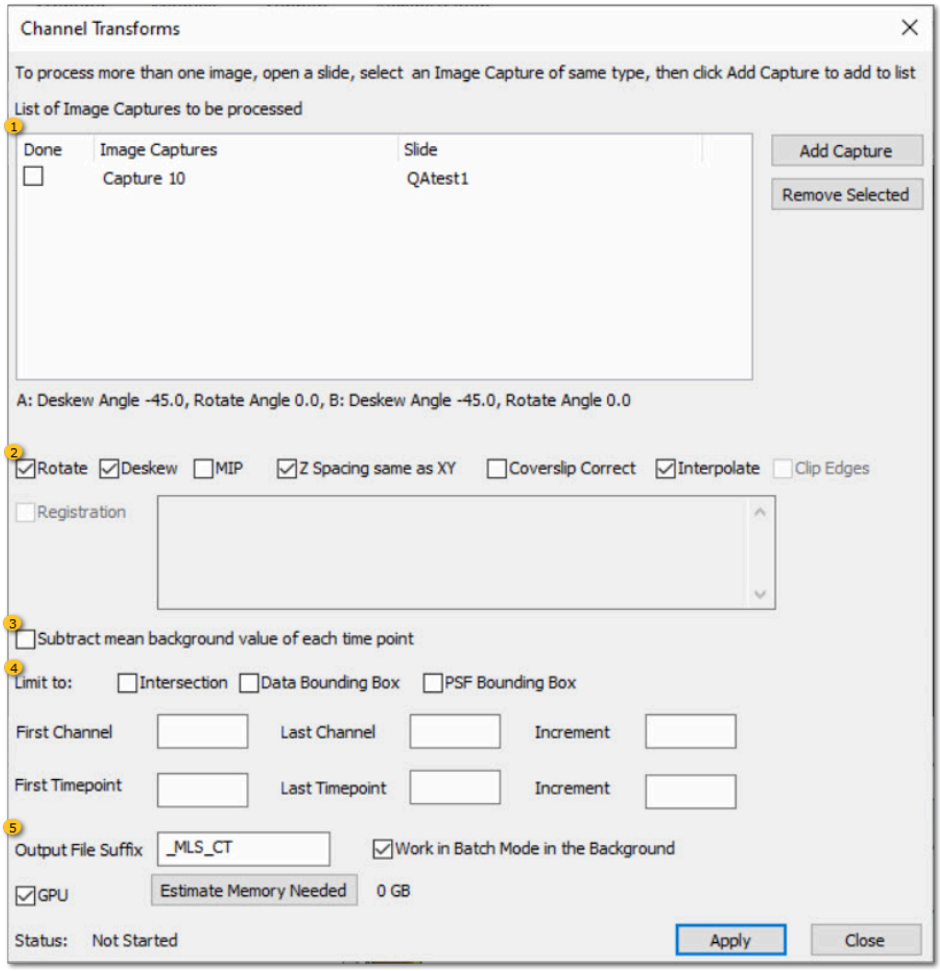
For Single View SPIM data or should you wish to generate an image/ series that is transformed and/or de-skewed, continue on with this tutorial. If you have a Dual View SPIM capture and wish to run combined deconvolution, continue to with the [Multiview Reconstruction] and joint deconvolution in SlideBook.

Channel Transforms of MLS Data

1. Select (highlight) the time lapse or single 3D image of the captured slice or stage scan, go to ***LightSheet >*** ***Multiview Reconstruction > Channel Transforms***.



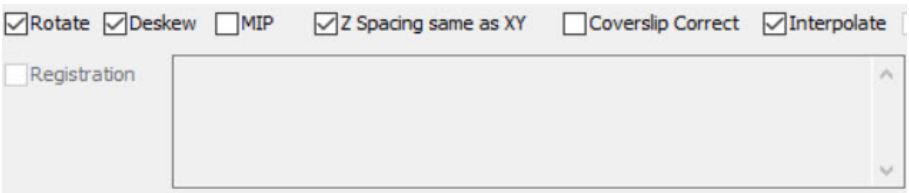
2. The ***Channel Transforms*** dialogue will open.
3. Select the captures to process, transform or projection options, data cropping and output options in the ***Channel Transforms*** window as detailed below.
4. Hit ***Apply*** and ***Save*** the slide after the process.



1 List of Image Captures to be Processed

Channel Transforms calculations can be done in batch. If you want to process several captures of the same type at once highlight them in their respective Slide file and ***Add Capture***. To remove an image from the list, highlight the capture in the batch list and ***Remove Selected***. The list shows the name of each selected capture in the ***Image Captures*** and the name or their original Slide file in the ***Slide*** . The tick box in the ***Done*** column indicates if the capture has already been processed an updates during the batch process.

2 Transform Options



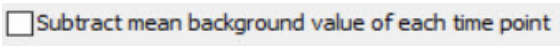
The window will open with the tick boxes already ticked for each capture format.

• ***Rotate*** - This rotates the second side of a Dual View SPIM capture roughly above the other side (90° rotation) - will be ticked for piezo/slice and stage scan types.

• ***Deskew*** - This will align planes to generate a geometrically correct image - will be ticked for stage scan captures only.
• ***MIP -*** This will create a maximum intensity projection of all 3 views (XY, XZ & YZ).
• ***Z spacing same as XY*** - This will create isometric voxels. **NOTE:** this is only required if you want to use the data in Fiji Multiview reconstruction from the created file. For e.g. only de-skewing SPIM files un-check this option to save data storage space.

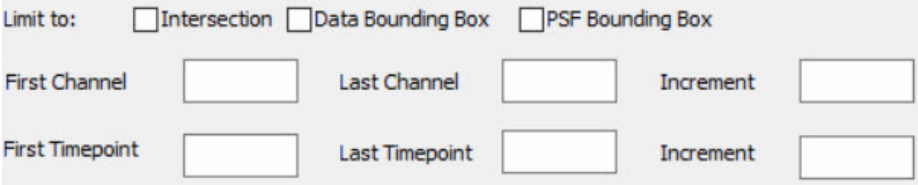
• ***Coverslip Correct:*** - This will re-slice the data so that Z-planes appear parallel to the coverslip and not in the 45° orientation.
• ***Interpolate -*** (Re-)Construction of the image information to the new voxel arrangement - gives a high quality image.
• ***Registration*** - If you want to use the saved registration (and not only a rigid rotation), check ***Registration***.

3 Subtract Background



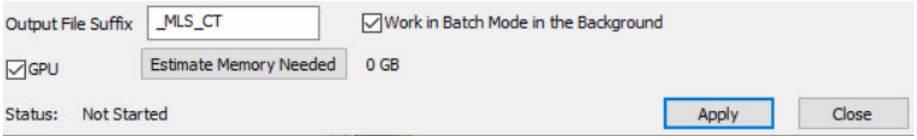
Subtract background automatically calculates the mean background value of the data and subtracts it from each time point in the output file.

4 Limit Data



• ***Intersection*** - This will create the preview of the volume where both volumes of each side are overlapping.
• ***Data Bounding Box*** - This will create an image of only the Data volume you assigned with the ***Bounding Box*** tool (Multiview Reconstruction).
• ***PSF Bounding Box*** - This will create an image of ony the PSF volume you assigned with the ***Bounding Box*** tool. It is a great tool to create a PSF file for the 3i joint deconvolution (***Deconvolution***) of Dual View SPIM data.
• To crop channels (e.g. create a single view from dual view capture), enter the first and last channel to use and enter the increment. **NOTE:** Use the channel numbers, not the names. E.g. ***First Channel:*** 1, ***Last Channel:*** 5, ***Increment:*** 2, this creates a 3 channel single view capture containing the odd-numbered captures from the original dataset.
• To crop time points enter the first and last time point to use and enter the increment. **NOTE:** SlideBook uses "0" for the first timepoint. E.g. ***First Timepoint:*** 0, ***Last Channel:*** 99, ***Increment:*** 2, this creates a timelapse of 50 time points skipping every second original time point.

5 Output Control



• SlideBook will create a new capture in a new **.sld/ .sldy** file with the suffix **_MLS_CT**. You can change this under ***Output File Suffix***. To save the output file in the same **.sld/ .sldy** file, delete the suffix.
• ***Work in Batch Mode in the Background*** enables the montage to work in the background when a batch list is present.
• If you have a CUDA-capable graphics card installed, check ***GPU*** for a faster result.
• ***Estimate Memory Needed*** will give the estimated memory size required for the highlighted capture.
• Status will indicate the progress of the batch process with Not Started, Processing (which image) and Finished.
• ***Apply*** will start the porcess.
• ***Close*** will close the Channel Transforms window and erase the batch list.

Select (highlight) the created 3D transform image. In the Home tab of SlideBook, select ***View > 3D/ 4D Volume View*** or ***4D Volume View***. The selected Volume viewer will open the rendering of your capture. For Single View SPIM captures you

can proceed directly to the SlideBook ***Analyze >*** ***Deconvolution*** with either the raw or deskewed file if desired. For Dual SPIM captures, especially a MIP with the Registration applied will give you a rough idea as to whether it is worth it to process the data using the ***MultiView Reconstruction*** (Multiview Reconstruction).

NOTE: some options how to process your data are listed below:

Single View SPIM data:

- For view and export only (no quantitative analysis) - Rotate) true; Deskew) true if stage scan; Z spacing same as XY) false; Coverslip Correct) true; Interpolate) true
- Prepare for SlideBook Constrained Iterative Deconvolution or export for analysis
  (1) Use raw data and raw beads for PSF (deskew a stage scans afterwards via Create Preview)
  (2) Rotate) false, Deskew) true if stage scan, Z spacing same as XY) false, Coverslip Correct) false, Interpolate) true. Apply the same for the beads file you are going to use as PSF (coverslip correct afterwards)

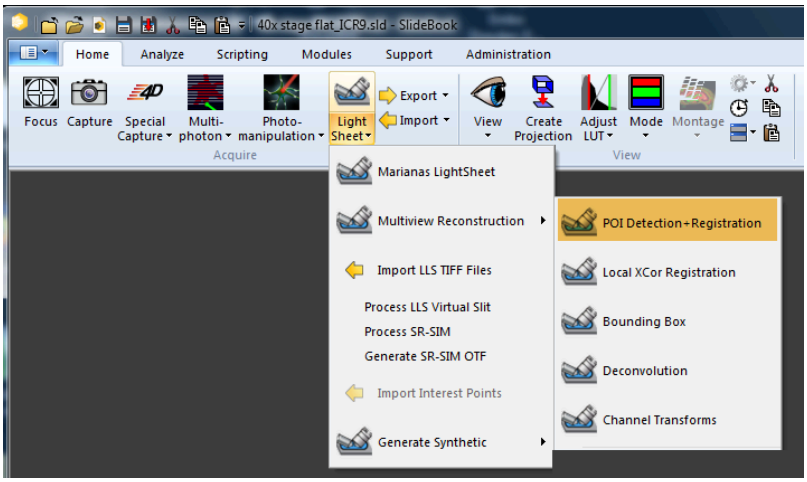
Dual View SPIM data:

- For transform preview and export only (no quantitative analysis) - Rotate) true, Deskew) true if stage scan, Z spacing same as XY) false, Coverslip Correct) true, Interpolate) true, Default Transform) true.
- For MVR in Fiji only
  (1) Piezo Slice scan: Best transfer the capture into its own `.sld` file, save and open in Fiji via the Multiview Reconstruction Plugin (A=0,l B=90)
  (2) Stage Scan: Rotate) false, Deskew) true if stage scan, Z spacing same as XY) true, Coverslip Correct) false, Interpolate) true, Default Transform) false. save and open the DR file in Fiji via the Multiview Reconstruction Plugin (A=0 B =90).
- For MVR in SlideBook - Highlight file, select In the Home tab of SlideBook ***LightSheet >*** ***Multiview Reconstruction*** (Multiview Reconstruction).

TUTORIAL: Multiview Reconstruction

This tutorial will walk you through aligning a Dual View SPIM image, defining a bounding box, fusing, and deconvolving the image.

1. Save the **.sld** before beginning.
2. For registration two algorithms are available: Point of Interest Detection and Registration (POID + Reg) or Local Cross Correlation (LXCOR). Start with a method of your choice.
3. For the Registration from Points of Interest (see 4. for alternative better suited for biological samples), select (highlight) the capture you would like to work with. In the Home tab of SlideBook, select ***LightSheet > Multiview Reconstruction > POI Detection + Registration***. The 3i POID + Reg dialog and the ***3D/ 4D Volume Viewer*** will open.



a. **Input:**

i. Choose the ***Channels*** for which to detect the interest points and run the automated registration in the drop down.

ii. Choose the ***Time Point*** for which to detected the interest points and run the automated registration using the arrows.

iii. Select ***Downsample*** the data to gain speed. A downsampling value of 3 would keep only every third value. The volume size is 27 times smaller.

iv. Limit To:

i. ***Intersect***: This is the data volume common to path A and Path B that has been (deskewed) rotated.

ii. ***Data Bounding Box***: You can draw a Data Bounding Box in the Bounding Box 3D/ 4D Volume Viewer beforehand.

b. **Background:**

i. ***Remove Background***: If the background is not 0, it should be removed so that the Difference of Gaussian filter does not create any artifact at the edges of the volume. If big enough, these artifacts can be picked as POI and ruin the calculation.

ii. ***Ch1***: The first channel (e.g. 488A) background value

iii. ***Ch2***: The second channel (e.g. 488B) background value

iv. ***Compute Background***: Choosing this option will compute the background, but it may be slow. Alternatively, you can choose and enter background values based on the channel histograms.

c. **Interest Point Detection:**

i. Sigma defines number and density of detected interest points and can be considered as intensity spot diameter. You can let the program range through a value of sigmas and determine automatically which gives you the smallest error.

ii. Select a ***Start Sigma***: the first sigma to use. Must be greater or equal to 0.5. - and an ***End Sigma***: the final Sigma value. Should be larger than Start Sigma. Only relevant if Number of Sigmas is greater than 1.

iii. Select a ***Number of Sigmas*** to decide for how many Sigmas in the set range the algorithm detects interest points.

iv. ***Output DOG Filtered Images***: Check this if you would like to see the result of the Difference of Gaussian filter. The larger the sigma selected above, the larger the "blobs" become.

NOTE: Testing about 1-3 Sigmas from a Start Sigma: 1 to End Sigma: 4 is a good starting point; this may need to be modified to better fit your data. The Program will automatically select from the best combination of interest points and registration accuracy.

d. **Registration:**

i. To start the algorithm with an ***Initial Shift*** click ***Compute Shift*** and the program will run a rough estimation.

ii. Set the ***Initial Max Distance (um)*** to an initial search radius.

iii. ***Set the Final Max Distance (um)*** to the distance between interest points of each side you want to achieve.

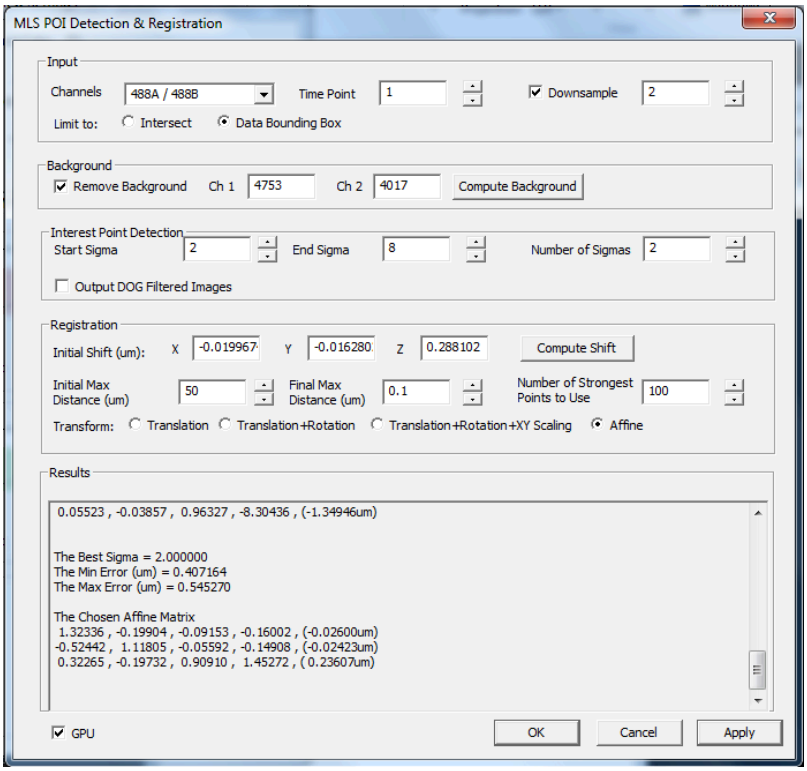
iv. Set the ***Number of Strongest Points to Use*** from the detected interest points.

v. ***Transform***: Check the box of the preferred transform. The default is ***Affine***, which applies a Translation, Rotation, Scaling and Shear.

e. Check the box to ***GPU*** to use CUDA accelerated processing.

f. Click ***Apply*** to start the process. It might take a while depending on file size and how many different parameters it is checking.

g. The ***Results*** panel will present the interest points found, the transform and the registration accuracy (min max distance) for each individual Sigma as well as the best transform from this range.



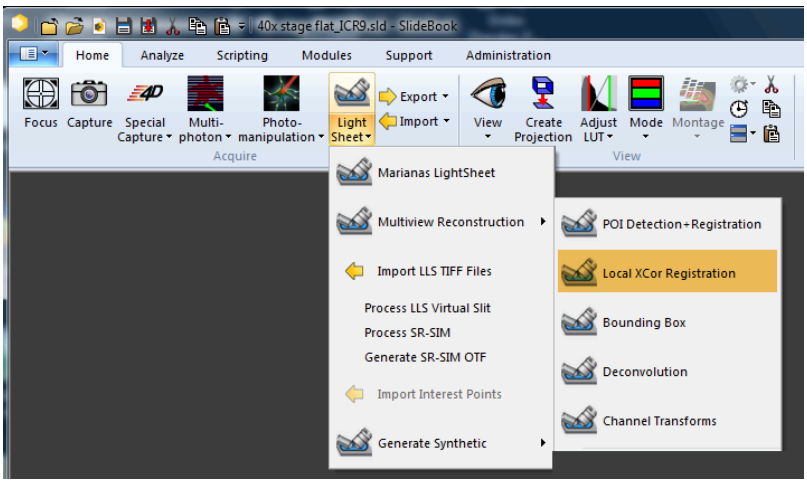
h. The ***3D/ 4D Volume Viewer*** will update and show the 'best' transform when the check box ***Registration*** is ticked.

i. If you are not happy with the transform select a new set of parameters and ***Apply*** again.

j. If you want to finish the process without saving the transform, click ***Cancel***. This will close the Registration dialogue and not update the Default Transform.

k. Once you are happy with the transform click ***OK***. This saves the registration with the capture metadata once you save the entire **.sld** file.

4. <u>Alternatively</u> to the POI Detection + Registration you can use 3i's Local Cross Correlation Registration: Select (highlight) the capture you would like to work with. In the Home tab of SlideBook, select ***LightSheet > Multiview Reconstruction > Local XCor Registration***. The 3i Local XCor Registration dialog and the ***3D/ 4D Volume Viewer*** will open.



a. **Input:**

i. Choose the ***Channels*** for which to detect the transform

ii. Choose the ***Time Point*** for which to detected the transform.

iii. Select ***Downsample*** the data to gain speed. A downsampling value of 3 would keep only every third value. The volume size is 27 times smaller.

iv. Limit To:

i. ***Intersect***: This is the data volume common to path A and Path B that has been (deskewed) rotated.

ii. ***Data Bounding Box***: You can draw a Data Bounding Box in the Bounding Box 3D/ 4D Volume Viewer beforehand.

b. **Background:**

i. ***Remove Background***: If the background is not 0, it should be removed, since a better contrast improves the results.

ii. ***Ch1***: The first channel (e.g. 488A) background value

iii. ***Ch2***: The second channel (e.g. 488B) background value

iv. ***Compute Background***: Choosing this option will compute the background, but it may be slow. Alternatively, you can choose and enter background values based on the channel histograms.

c. **Local Cross Correlation**

i. ***Subvolume Side Size (um)***:The process subdivides the volume in a number of smaller cube volumes. Specify the size in microns of the cube side.

NOTE: This volume needs to be small enough to offer a true localized cross-correlation, and yet large enough to include the maximum expected shifts.

ii. ***Percentage Overlap***: Set the percentage overlap between a subvolume and its adjacent one.

d. **Registration**

i. To start the algorithm with an ***Initial Shift*** click ***Compute Shift*** and the program will run a rough estimation.

ii. ***Number of Iterations***: Set the number of iterations to perform. At every iteration the current registration transform is applied to the data before the correlation process starts.

iii. Set the ***Quality Threshold***: First the maximum correlation in all subvolumes is detected. Then only subvolumes with correlation quality larger than the maximum correlation times the threshold are used.

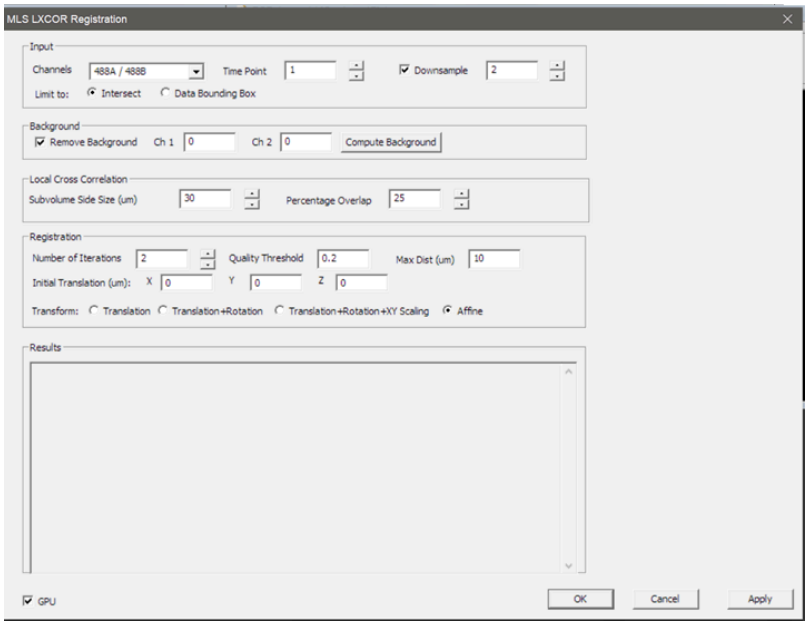
iv. When looking for the correlation maxima, restrict the search for shifts less than the maximum distance in ***Max Dist (um)***.

v. ***Transform***: Check the box of the preferred transform. The default is ***Affine***, which applies a Translation, Rotation, Scaling and Shear.

e. Check the box to ***GPU*** to use CUDA accelerated processing.

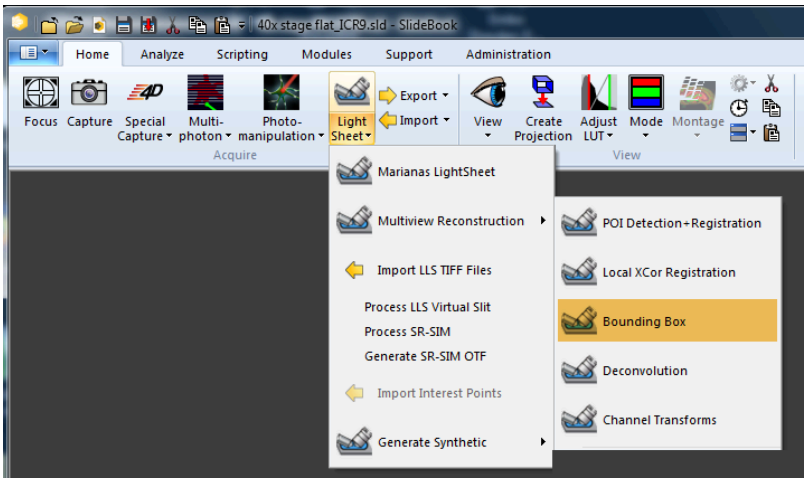
f. Click ***Apply*** to start the process. It might take a while depending on file size and how many different parameters it is checking.

g. The ***Results*** panel will present the transform.



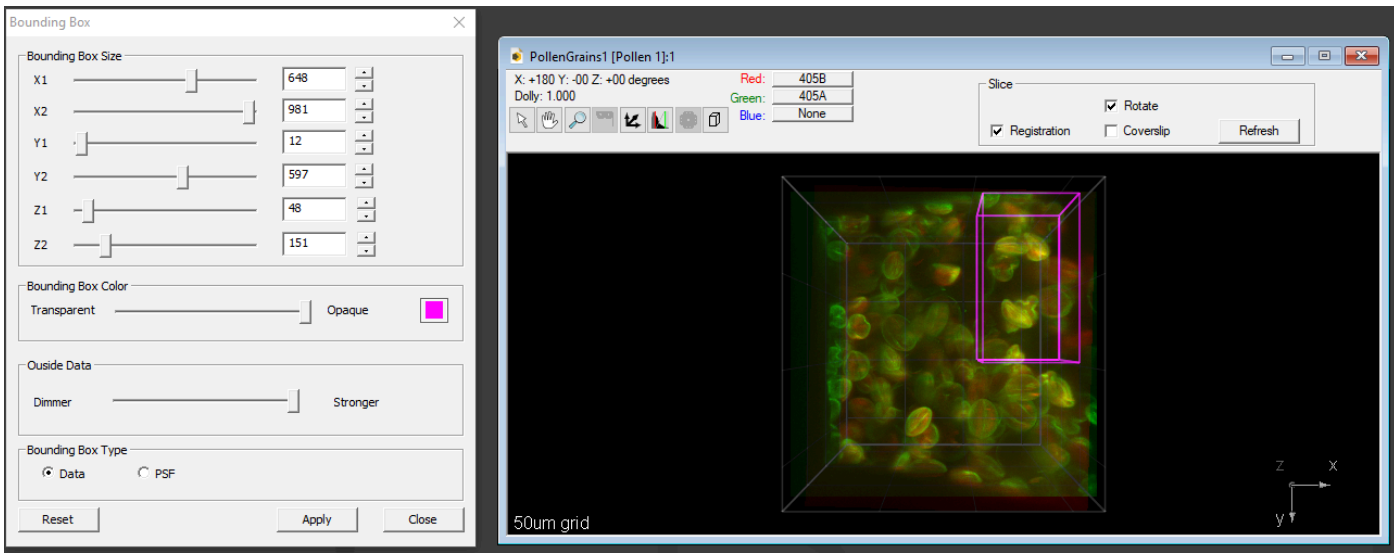
h. The ***3D/ 4D Volume Viewer*** will update and show the 'best' transform when the check box ***Registration*** is ticked.
i. If you are not happy with the transform select a new set of parameters and ***Apply*** again.
j. If you want to finish the process without saving the transform, click ***Cancel***. This will close the Registration dialogue and not update the Default Transform.
k. Once you are happy with the transform click ***OK***. This saves the registration with the capture metadata once you save the entire **.sld** file.

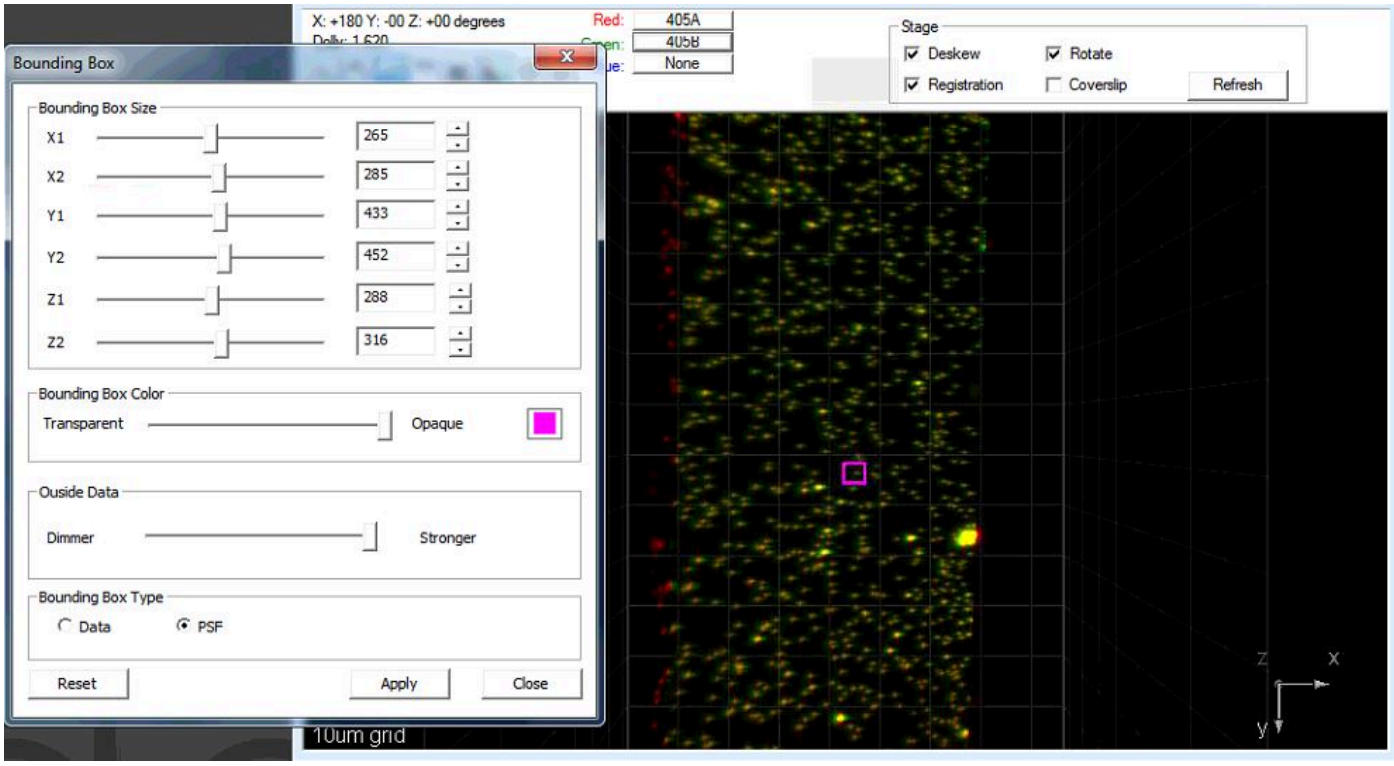
5. To define a bounding box around the volume you want to deconvolve, select ***LightSheet > Multiview Reconstruction > Bounding Box***. The default bounding box will be the intersection of the two volumes. Once a bounding box is applied and saved with the file it will show the last defined bounding box upon opening.



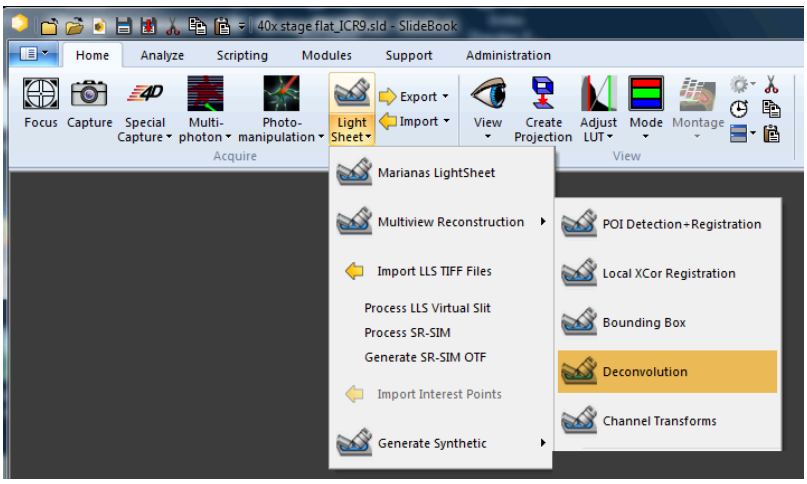
a. Click ***Reset*** if you are not happy with the previously set bounding box
b. Set the width, height and depth with the sliders.
c. Select the Bounding Box Type:
    i. ***Data***, to deconvolve the data within the set volume.
    ii. ***PSF***, to use the selection from beads as PSFs in the 3i Deconvolution. Select a single pair (in A and B) of beads in the capture.
d. Click ***Apply*** to set the bounding box and ***Close*** the dialogue.

NOTE: It is recommended to save the **.sld** file so the bounding box is saved with the capture for further processing.





6. Finally, highlight your capture of interest and select ***LightSheet > Multiview Reconstruction > Deconvolution***.



a. ***Input***:

i. ***Channel Selection***: Select the channels you want to deconvolve.

ii. ***Time Points***: Select/type in a single time point or tick ***All*** if you want to deconvolve all time points of a time lapse.

iii. ***Bounding Box*** and ***Registration*** will show you the defined values for this capture.

b. ***Background:*** Enter a value or choose ***Compute Background*** to subtract the background noise before deconvolution. For some captures this can greatly improve the deconvolution result.

c. ***PSF***: You can use beads in the captured file or a bead image captured with the same magnification as PSF. The beads capture needs to be registered beforehand using the ***3i POI Detection + Registration*** or ***Local XCor Registration*** and a single bead pair needs to be selected as PSF using the Bounding Box tool (tick PSF in this case).

i. Open a **.sld** file with a beads capture, with assigned registration and PSF bounding box or use the same file as you want to deconvolve with an applied PSF bounding box around a beads pare within.

ii. Highlight the file with the PSF bounding box and hit ***Load from Selected Image***.

iii. You can choose a channel in ***Channel Selection***, e.g. if you imaged beads in several colours. It is not necessary to use a PSF from beads imaged at the same wavelength as your capture to deconvolve.

iv. ***Bounding Box*** and ***Registration*** will show you the defined values for the PSF capture.

v. ***Background:*** Tick the check box and enter a value or choose ***Compute Background*** to subtract the background noise of the PSF before deconvolution. This is recommended when splitting the data to fir in RAM or CUDA FFT (see below).

d. ***Deconvolution Parameters***:

i. Check the box to ***GPU*** to use CUDA accelerated processing.

ii. Set the number of ***Iterations***. The default of 10 usually gives a good result. Increasing to 15 or 20 may provide further improvements. For a quick preview, choose 1 or 2 iterations.

iii. Normalize Each Channel will normalize the intensity per channel over all time points if applicable.

iv. ***Tikhonov Regularization*** can be applied for the Sequential algorithm if wanted.

v. ***Algorithm***: You can decide between the ***Multiplicative*** (similar to Fiji Big Data Stitcher) or ***Sequential*** (similar to conventional algorithm of the Fiji Multiview Reconstruction plugin).

e. ***Split Data to Fit in*** RAM or CUDA FFT: Use this when the ***Memory Needed (GB)*** indicated at the bottom of the window is larger than the RAM of the PC.

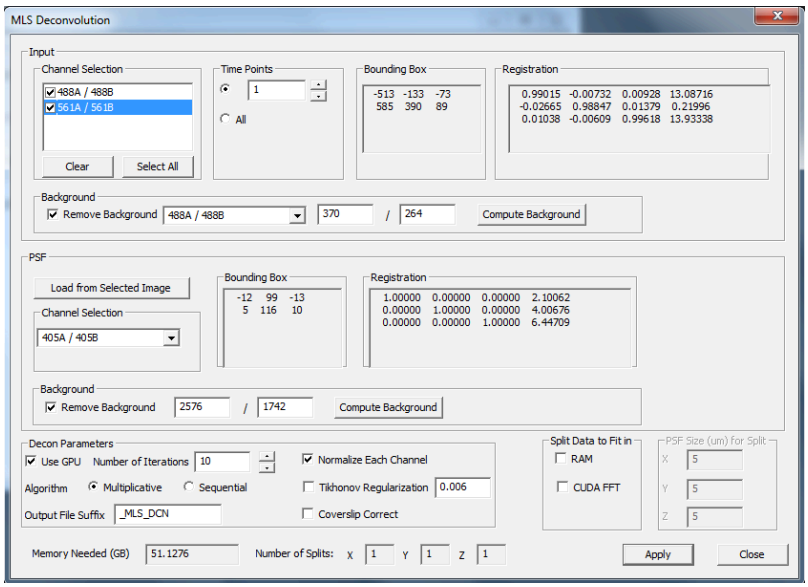
i. Check the box(es) for RAM only or RAM and CUDA FFT to use the CUDA for deconvolution.

ii. Enter a PSF size for the coordinates you want to split the image.

iii. Enable the background subtraction for the PSF.

iv. ***NOTE:*** Setting the PSF thickness correctly is required to properly re-montage the slabs after deconvolution. For captures with the 40x objectives PSF sizes of 0.1-0.5 um, for captures with the 10x objectives values about 5 um are recommended

f. The ***Memory Needed (GB)*** and the ***Number of Splits*** when splitting the data to fit into RAM and CUDA FFT is given at the bottom of the window.



7. Click ***Apply***. The deconvolution and/or fusion process can be quite intensive and may take a while depending on the computer. When the process is complete, a new image will appear in a new **.sld** file with the suffix **_MLS_DCN**. You can change this under ***Output File Suffix***. If you would like the output file to appear in the same **.sld** file, delete the suffix. ***Save*** the slide.

MLS Troubleshooting

For additional assistance, please contact 3i Support.

• If you are having trouble aligning the beams, turn everything off and reset the filter cubes and mirror cubes to zero (1mm gap between filter/ mirror face plates and the cubes on all sides), turn the objective bushings in (clockwise) and start the alignment procedure from scratch.

• If you do not see a live display during acquisition or it updates too slow: This happens, and depends on the frame rate.

• Beams/ Sheet appear out of focus:

a. Check with ***Neutral All*** and then ***Adjust*** after hitting ***Go*** for each path. If no change, repeat calibration.

b. Re-align and re-calibrate when buffers are switched.

• If your sample is not strongly attached to the coverslip/ dish surface and moves during calibration:Move joy stick off of air table during operation.

• The 3D/ 4D Volume Viewer does not rotate data correctly:

a. Make sure the comment of the captures contain either **piezo/ slice** or **stage** scan. To do so, select the image in the **.sld** and press ***Ctrl + I*** then review the Comments field.

b. Make sure the A is the first captured side. If it is B go to ***Analyze -> Reformate -> Rotate -> Flip Horizontal***

Stereology Module

SlideBook's Stereology module employs unbiased stereological techniques for accurate estimation of total number, volume, surface area, and length of objects in a biological structure. Stereology systematically samples 3D volumes from a series of tissue sections in a random fashion. The module integrates support for a number of stereological tools within the standard image capture framework. Montaging can be used to outline the boundaries of a structure in a tissue section and then counts performed at high magnification using standard capture tools. Support for both transmitted light and fluorescence microscopy is included, and an offline mode the permits acquisition and counting to be performed separately.

3i would like to thank Glen MacDonald of the NIDCD Research Core, V.M. Bloedel Hearing Research Center, Otolaryngology-HNS, University of Washington for his contributions on our Stereology help pages.

Summary of Operation

SlideBook begins with a montage collected at low magnification to map the extent of the structure containing the objects to be counted. A mask is created to identify that portion of the image over which SlideBook will place counting frames. Sampling sites within this sampling area will be randomly generated. A z-series is collected at each sampling site with sufficient magnification to accurately identify the objects and count them. Finally, each of the 3D stacks are opened in SlideBook, displaying a counting frame, and objects located within the counting frame are counted. The total number of objects within the structure is obtained by scaling the summed counts by the fraction of the structure's volume represented by the counting volumes. All steps are carried out in accordance with the principles of stereology.

Optical Disector Principle

Stereology allows estimation of objects within a structure by systematically sampling sub-regions within the structure. The total estimate of number, length, volume, etc of the objects is obtained by summing the estimates in each sub-region and scaling them to the total volume of the structure. Objects are counted in the disector method of stereology by comparing a "lookup" section to a subsequent "reference" section. Objects are only counted the first time they appear in the reference section. This method avoids double counting and bias from size, shape or tissue processing. The optical disector uses comparisons between adjacent focal planes in an optical volume. An initial focus plane is a "lookup" plane and the subsequent plane is a "reference" plane. Any object that appears in the lookup plane is not counted. New objects are counted as they come into focus in any subsequent reference plane. An object counted in a reference plane becomes the lookup plane for the subsequent optical planes through the volume.

Specimen Tilt Compensation

A slide specimen rarely sits perfectly parallel to the focal plane of the objective lens. This results in the specimen changing focus as you move across the slide. SlideBook 6 has the ability to calculate the amount of slide tilt in a montage in order to adjust the focus limits for 2D or 3D montages. Note, this may not correct for specimens that are not planar, such as wrinkled sections or mounted organs.

Collecting a Stereology Montage

Enable z-Tilt Compensation for the Montage

1. Open the ***Capture*** window from the Home tab.

2. Click ***Advanced*** in the upper right of the window.

3. Go to the ***Focus*** tab, check the box to ***Enable 3 point Z-interpolation for montage capture***.
   NOTE: This option will be greyed out if you have set any points in the XY tab of Focus Controls. Delete any existing points and try again.

4. Click ***OK***.

Define the Montage Area

1. Open the ***Focus*** window from the Home tab.

2. Select a low magnification objective for making the montage.

3. Scan the specimen to identify the extent of the region to montage.

4. Open the ***XY tab*** in the Focus window.

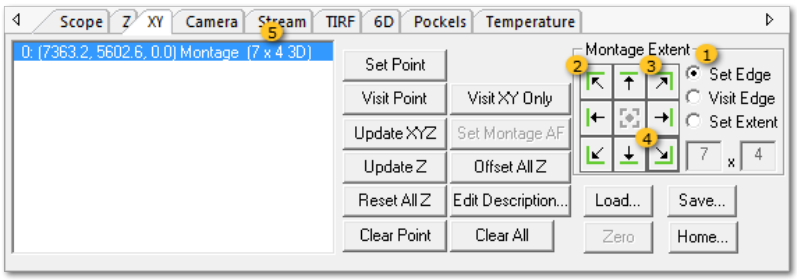
5. Make sure that ***Set Edge*** option is checked in the ***Montage Extent*** group (1 below).

6. Move to the first corner of the area, focus to an identifiable focus position, such as the bottom or top of the specimen

7. Click the montage button corresponding to the position of the current field (2 below)

8. Move to each of two other subsequent corners of the area and click on their corresponding buttons (3 & 4 below).

9. The single line for the montage region will display the number for frames for the montage as well as ***3D*** when tilt compensation has been configured and *z* plane was changed in setting the montage extents (5 below)



Tips

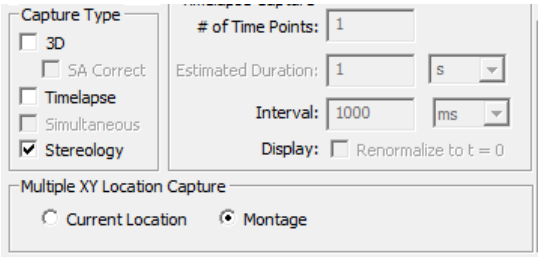
• Each corner must be set to the same focal level in the specimen in order to correctly calculate the degree of tilt for the specimen.
• The corners must be set in sequence, such as UL-UR-LR, or LL-UL-UR, etc.
• 2x2 is the smallest possible montage. 1 x 1, N x 1 or 1 x N will cause problems.
• If you make a mistake, delete the montage with ***Clear Point*** and start over.
• It may be easier to focus if you enlarge the focus window image by clicking on the ***Zoom***: **+** button, far left, middle of focus. Return to 50% with ***Zoom***: **-**.

Capture Stereology Montage

1. Open the ***Capture*** window from the Home tab.

2. Select the channel(s) to be included, set the exposure time for each channel (for more information on this, refer to the Adjust Exposure group).

3. Select ***Stereology*** for the Capture Type and select ***Montage*** from Multiple XY Location Capture.



4. Name the montage if desired, click on ***Start*** to capture the montage.

5. Two images will appear in the Slide file, a stack of image tiles collected for the montage and a low-resolution merged working montage titled **Stereology Montage**.

Move on to define the area for analysis...

Optional: Refine "Plane" of Montage

Because the initial plane of imaging was defined using a low NA objective, you may wish to further refine the z positions for the montage extents before creating the point list of counting stacks. To do so, follow the procedure below. It is important to note that if there are wrinkles or other non-uniform aspects in the slice, you will need to update the z position for each stack individually.

After collecting the low magnification montage image...

1. Open the ***Focus*** window from the Home tab.

2. Click on the ***Scope*** tab and select the objective to be used for analysis.

3. Go to the ***XY*** tab. Select ***Visit Edge*** then click on a corner to visit in the montage.

4. Adjust the z plane using the z control buttons. Select ***Set Edge*** and click on the same corner from step 3 to update the z position.

5. Repeat steps 3-4 for 3 corners.

Continue to define the imaging area from the low magnification montage...

Define the Area

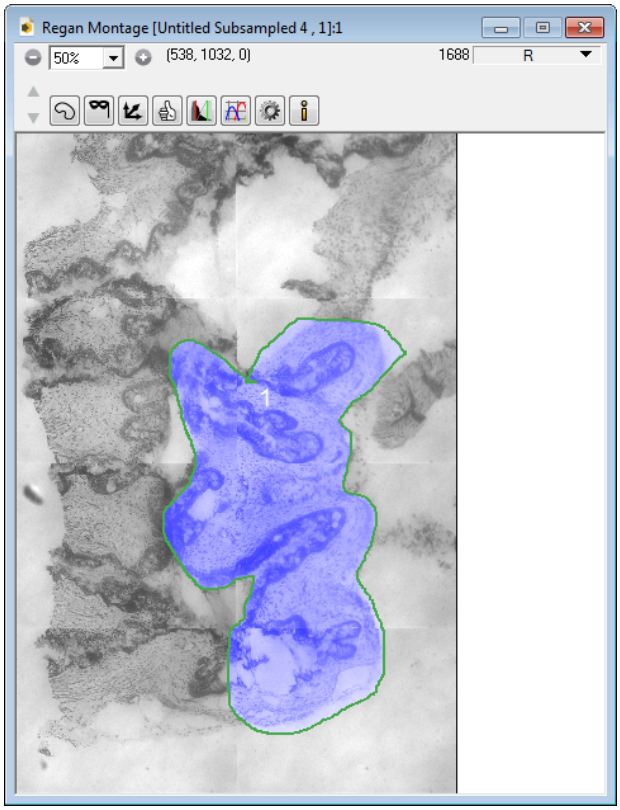
Define the Area for Analysis

1. Open the low-resolution montage image titled **Stereology Montage**

2. Select the ***Freehand*** region tool from leftmost menu in the view window (see also Region Tools).

3. Outline the area you would like to analyze.

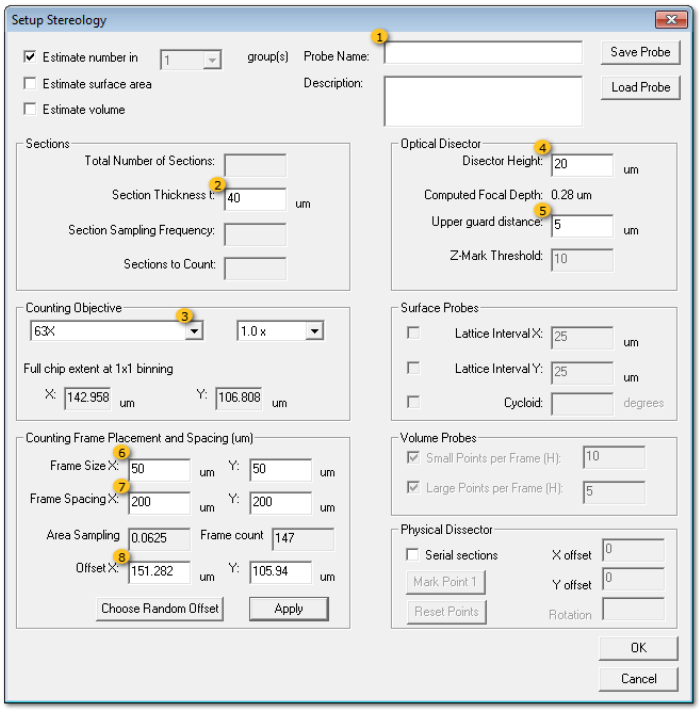
4. In the Mask Generation group of the Analyze tab, click on ***Region > Mask*** to covert the region into a mask object, name the mask if desired.



Move on to setup counting...

Setup Counting

1. Open the ***Setup*** dialog from the Modules Tab, ***Stereology*** menu. For detailed information on the terms used below, click on the linked numbers or refer to the Setup Stereology window page.



2. Enter the thickness of the section (1), as it is on the slide.

3. Select the higher magnification ***Counting Objective*** lens to be used for counting (2), X and Y dimensions of the full camera frame will be displayed below.

4. Optical Disector group, set ***Disector Height*** (3) and ***Upper guard distance*** (4) (also sets lower guard distance to same).

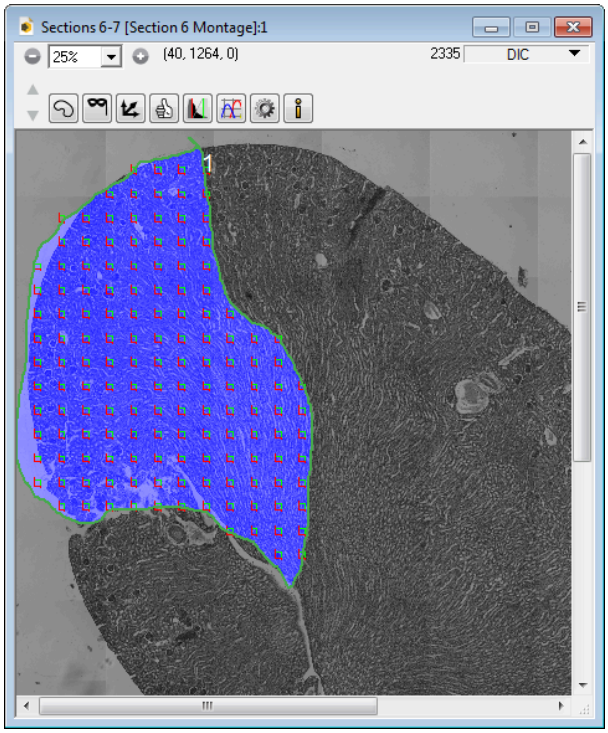
5. Counting Frame Placement and Spacing group, set ***Frame Size*** (5), ***Frame Spacing*** (6), and ***Offset*** (7). Click on ***Apply*** after changing any of these factors to update the ***Area Sampling*** and ***Frame count*** fields.

6. ***Click Choose Random Offset*** for systematic random sampling. Click on ***Apply*** to set the offset and calculate the counting frames. Once ***Apply*** is clicked, a ***Frame Count*** will display how many frames can be collected based on the masked area and setup parameters defined. Area Sampling = Frame Size/(Spacing X Frame Count). For example: (50 µm X 50 µm)/ (200 µm X 200 µm X frame count).

7. If desired, type a ***Probe Name*** and ***Description*** to save settings for future use (8).

8. Click ***OK*** to close the Stereology window. A message box will open telling you how many counting frames were generated for the current section and what the section area is. Click ***OK*** to close the message box.

9. The counting areas will then be displayed on the masked area of the montage as shown below.



continue on to [Capture Stereology Volumes](#)...

Setup Stereology window

1. Open the ***Setup*** dialog from the Modules Tab, ***Stereology*** menu.

2. Enter the thickness of the section (1), as it is on the slide.

3. Select the higher magnification ***Counting Objective*** lens to be used for counting (2), X and Y dimensions of the full camera frame will be displayed below.

4. Optical Disector group, set ***Disector Height*** (3) and ***Upper guard distance*** (4) (also sets lower guard distance to same).

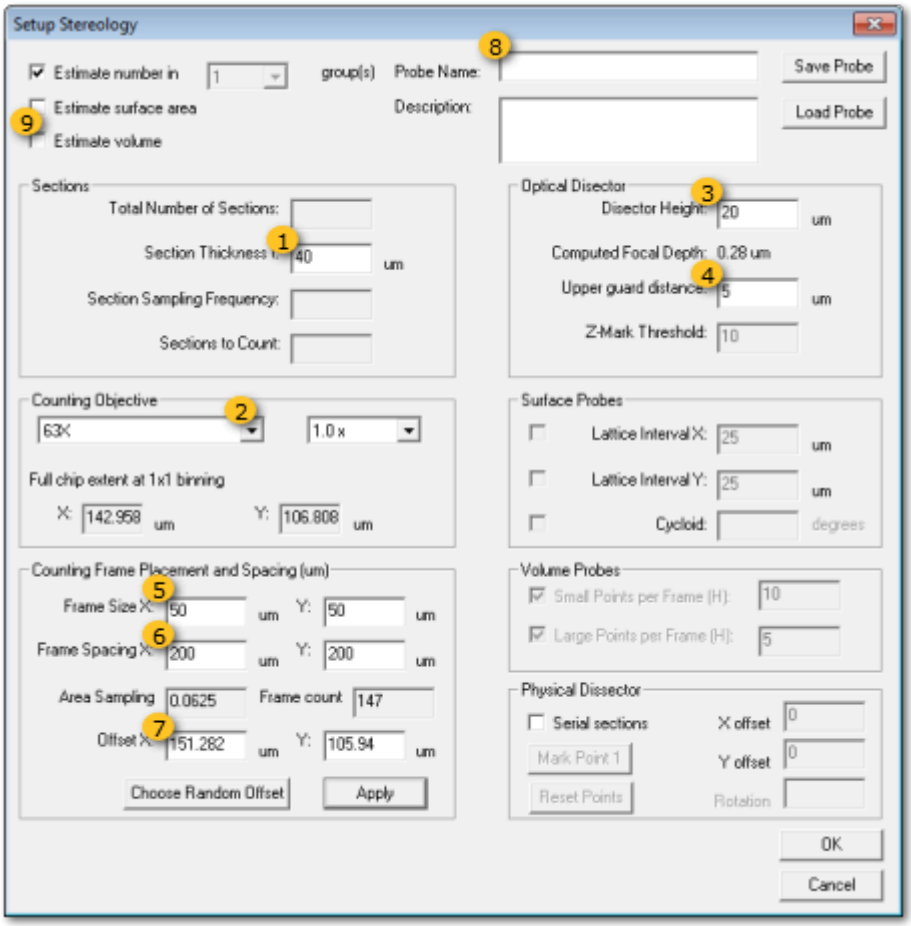
5. Counting Frame Placement and Spacing group, set ***Frame Size*** (5), ***Frame Spacing*** (6), and ***Offset*** (7). Click on ***Apply*** after changing any of these factors to update the ***Area Sampling*** and ***Frame count*** fields.

6. ***Click Choose Random Offset*** for systematic random sampling. Click on ***Apply*** to set the offset and calculate the counting frames. Once ***Apply*** is clicked, a ***Frame Count*** will display how many frames can be collected based on the masked area and setup parameters defined. Area Sampling = Frame Size/(Spacing X Frame Count). For example: (50 µm X 50 µm)/ (200 µm X 200 µm X frame count).

7. If desired, type a ***Probe Name*** and ***Description*** to save settings for future use (8).

8. Click ***OK*** to close the Stereology window. A message box will open telling you how many counting frames were generated for the current section and what the section area is. Click ***OK*** to close the message box.

9. The counting areas will then be displayed on the masked area of the montage.



1 Section Thickness

Set this to the average section thickness of the specimen. Remember, that most sections will shrink and thin to about ½ of their sectioned thickness when dried onto the slide. Shrinkage will also occur with water-compatible mounting media that harden, such as Fluoromount G and Prolong Gold. You may need to measure the final section thickness on the slide. Sections cut from epoxy and acrylic resins will not shrink when dried onto a slide.

2 Counting Objective

This is the objective that will be used to acquire images for analysis. Once selected, the size of the imaging area will be calculated.

3 Optical Disector Height

This field must be set to the minimum height that can be guaranteed across the entire section, minus an upper (and typically lower) guard region. For example, if the average thickness of the section is 40 µm and its minimum thickness is 30 µm, a 26 µm dissector allows a 2 µm guard region at the top and bottom of the minimum section thickness.

4 Upper Guard Distance

The guard distance excludes focal planes near the upper and lower section surfaces. Typical guard distances are 1/4 to 1/5 the axial thickness of the object being counted. In the case of counting 6 µm diameter nuclei, a guard distance of 1.2 µm to 1.5 µm would be sufficient, whereas counting nuclei based on the first appearance of a nucleolus may require 0.2 µm guard distance. If the estimated diameter of the counting objects is less than the z-step interval, then set the guard distance to the z-step value. Remember to set the z-step interval to 1/3 the thickness of the smallest object you need to resolve as a general rule.

The counting frame will only appear in focus planes within the disector height, not in the planes of the guard distance. The guard distance is used because tissue structures may be lost near section surfaces due to the physical forces of sectioning or washed out during labeling procedures. Failure to set the guard volumes in your counting volume may result in under-reporting the number of countable objects per volume.

5 Frame Size, X and Y

Specify the dimensions of the counting frame, in microns. These dimensions should fit within the extent of camera field collected with the high magnification objective used for counting. For example, the full frame is 1392 pixels by 1040 pixels, and the 100X objective has a pixel size of 0.06 µm, for field size of 83.52 µm X 62.4 µm. A counting frame of 50 µm X 50 µm leaves space for the exclusion boundaries for the 100X objective, or about 800 x 800 pixels.

6 Frame Spacing, X and Y

Specify how far to separate the upper left corner of each successive counting frame in the x and y axes.

7 Offset, X and Y

Specify the distance by which the counting frame grid is offset into the masked region. Unbiased stereology requires that every part of the masked region must have an equal chance of getting selected. Generally, you will click on ***Choose Random Offset*** to begin the grid of counting frames at a random location within the masked region determined by the ***Frame Spacing***, ***Frame Size***, and ***Offset***.

8 Probe Name and Description

You may name a probe then save it for use at another time. The description field can be used to write a brief description of what the probe is used for.

9 Estimate Surface Area or Volume

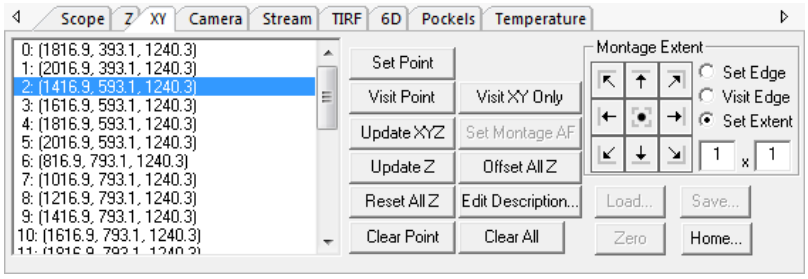
You may also set up the probe to estimate surface area or volume. Simply select the desired parameter and complete either the ***Surface Probes*** or ***Volume Probes*** group of parameters.

Capture Stereology Volumes

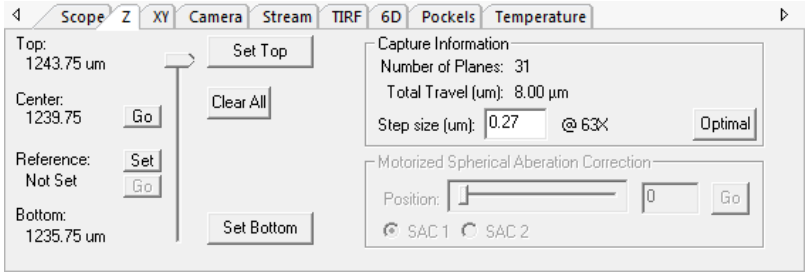
1. Open the ***Focus*** window from the Home tab.

2. Click on the ***Scope*** tab and select the objective to be used for analysis.

3. Go to the ***XY*** tab. Double click on a position in the XY list to move to a sample field.



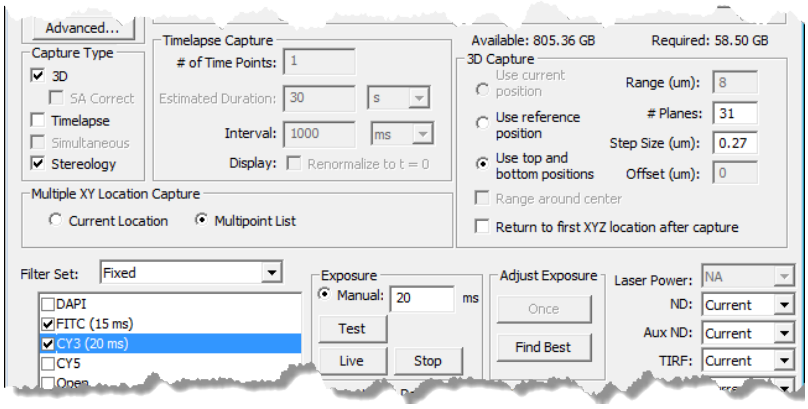
4. Select the ***Z*** tab and set the *z*-limits for a z-series (focus to top, click ***Set Top***, focus to bottom, click ***Set Bottom***). Drag the focus slider all the way to the top of the range as shown below.



5. Return to the ***XY*** tab, click on ***Update Z***. Repeat steps 3-5 for each point in the list. Alternatively, if you have a uniformly flat sample without wrinkles or other non-homogenous tilt, you can find top for the first point then click ***Offset All Z***.

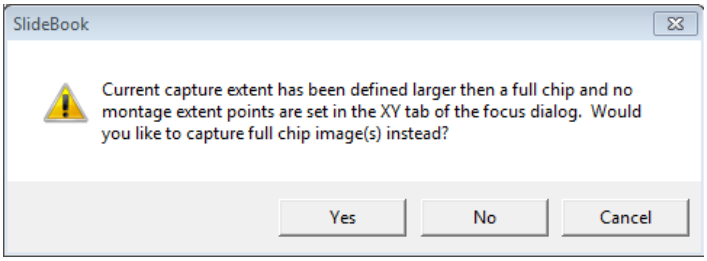
6. Open the ***Capture*** window from the Home tab. Under ***Capture Type***, select ***3D*** (leave ***Stereology*** selected as well), notice that ***Montage*** is now ***Multipoint Position***.

7. Set ***3D Capture*** to ***Use top and bottom positions***. Select channels for acquisition and find the best ***Exposure*** time (more on setting exposure times here).



8. Give a ***Name*** for the image series and click on ***Start***.

NOTE: If you see the following error message, click ***Yes***.



9. When capture is complete, save the **.sld**.

Continue on to [Counting in Stereology Volumes](#)...

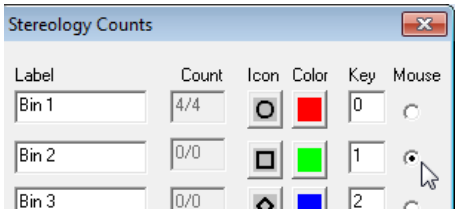
Counting in Stereology Volumes

1. A 3D stack has been collected for each counting frame.

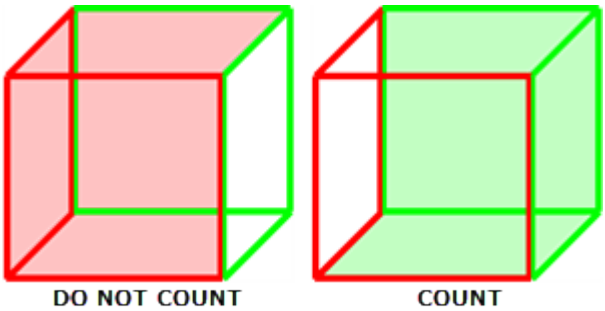
2. Open the first stereology volume, go to the Modules tab, ***Stereology*** menu > ***Count Offline***. A counting palette opens with a series of markers.

3. As you scroll through the z-stack of the image, note the appearance of the red and green counting frame. Go to the first plane displaying the counting frame.

4. Select a counting category by clicking on the radio button in the ***Mouse*** column of the palette.



5. The optical dissector principle states that you should only count objects that do not intersect the top plane of the counting frame stack or the left or bottom sides as indicated by the red boundary lines. Note the appearance of countable objects in the image, count nothing in focus in the first plane displaying the counting frame, this is the *lookup frame*. You will count any objects that intersect the green boundary lines and/or bottom plane even if they are not wholly contained in the counting volume unless the also intersect with a red boundary line.



6. From the first counting frame, the lookup frame, scroll to the next plane, the *reference plane*. Count objects that were not present in the lookup frame by clicking on the object.

7. Scroll to the next plane. The counted plane from step #6 becomes the lookup frame and the current plane is now the reference plane. You will notice markers on objects counted in previous planes will turn grey. Count objects that appear in the current reference plane but not in the previous lookup frame. Continue the through the stack with each reference frame becoming the lookup frame for the next image plane.

8. Switch between categories of objects to count using the different icons and colors available.

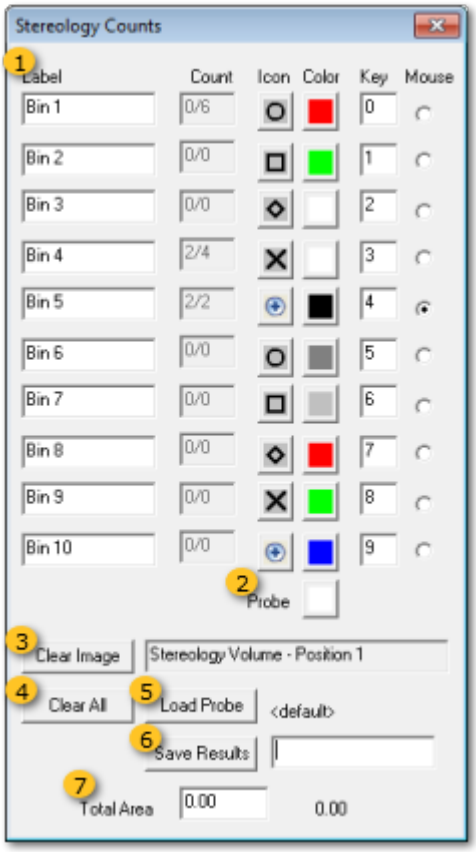
9. Open the next z-stack. Choose the ***Pixel Select*** tool from the leftmost menu in the image window to get the counting icon back. Proceed counting the stack as described above.

10. When finished, click ***Save Results*** to save the results as a delimited text file (.txt) compatible with Excel or other statistical packages.

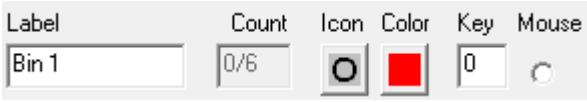
> **NOTE:** Do not enter any a filename into the box adjacent to ***Save Results***. No file will be saved. Leave this box blank and name the results file in the ***Save*** dialog.

Stereology Counts window

The Stereology Counts window is accessed in the Modules tab, ***Stereology*** menu > ***Count Offline***. For more information on using this dialog, refer to the Stereology Module section of help or, more specifically, Counting in Stereology Volumes.



1 Counting Rows



The counting dialog allows you to count 10 different categories of object. You can name or describe the object in the ***Label*** field. The ***Count*** field displays the number of objects counted in the current image/all images of the collected set. ***Icon*** and ***Color*** of the counting markers can be changed by clicking on the respective buttons to cycle through the options. When you scroll to a different *z* plane, markers will turn gray. Click on the radio button in the ***Mouse*** column of the desired counting row to select the active counting category.

The ***Key*** field is used if you would like to use the keyboard to count without marking the image. You can change the keys used by typing in the edit field.

Delete a marked count by centering the cursor over the unwanted marker and then click while holding down the **SHIFT key + left click the mouse**.

2 Probe

If you selected ***Estimate surface area*** or ***Estimate volume*** in the Stereology Setup dialog, this is the display color used for the probe selected.

3 Clear Image button

Clear Image

Pressing ***Clear Image*** will delete all counts made in the current image.

4 Clear All button

Clear All

Pressing ***Clear All*** will delete all counts made in all images.

5 Load Probe button

Load Probe

Press ***Load Probe*** to load a different saved probe (from Setup).

6 Save Results button

Save Results

When finished, click ***Save Results*** to save the results as a delimited text file (.txt) compatible with Excel or other statistical packages.

NOTE: Do not enter any a filename into the box adjacent to ***Save Results***. No file will be saved. Leave this box blank and name the results file in the ***Save*** dialog.

7 Total Area

Total Area 0.00 0.00

Reports total volume calculated in the Stereology Setup dialog.

SlideBook Reader

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- Graph regions of interest
- Export graph and graph data
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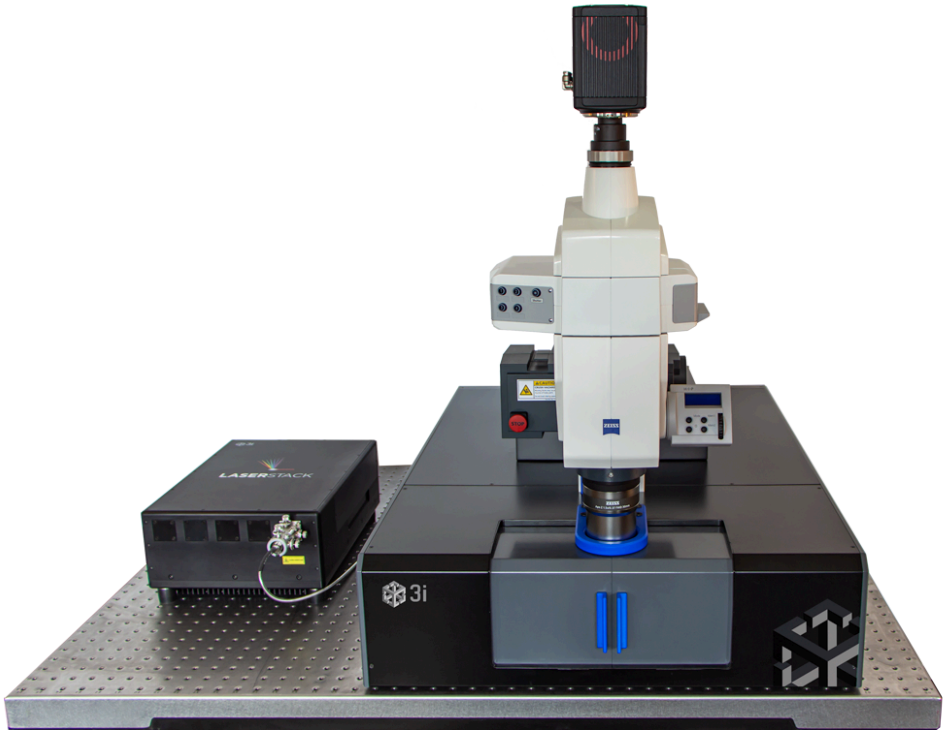
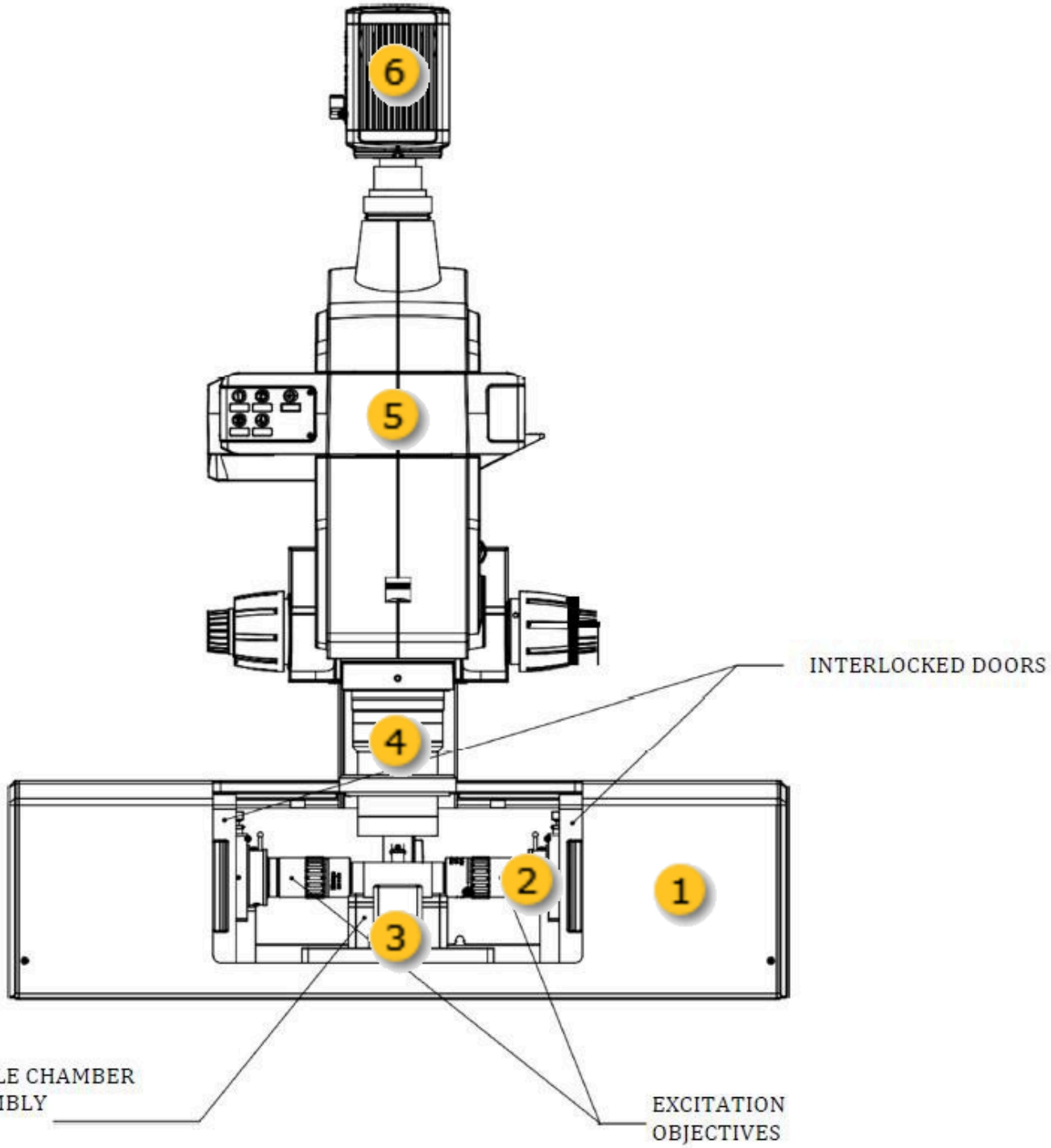
Cleared Tissue LightSheet Module

The Cleared Tissue LightSheet (CTLS) microscope is our turnkey system that leverages Light-Sheet Fluorescence Microscopy (LSFM) to interrogate large, fixed, optically transparent fluorescent specimens. The centerpiece of the CTLS microscope is a high resolution, high speed spatial light modulator (SLM). This SLM is used to shape a Gaussian beam to maximize its optical sectioning capability. In brief, the SLM is used to alter lightsheet thickness. It is also used to remotely focus the beam and translate it across the imaging field of view in a process called tiling (Q. Fu, B.L. Martin, D.Q. Matus and L. Gao, Nat. Commun., (2016) 7:11088). The result is clear: by using the tiling lightsheet method, CTLS provides near confocal-like resolution of fluorescent cells in tissues, organs and organisms that have been rendered transparent via refractive index matching chemistry.



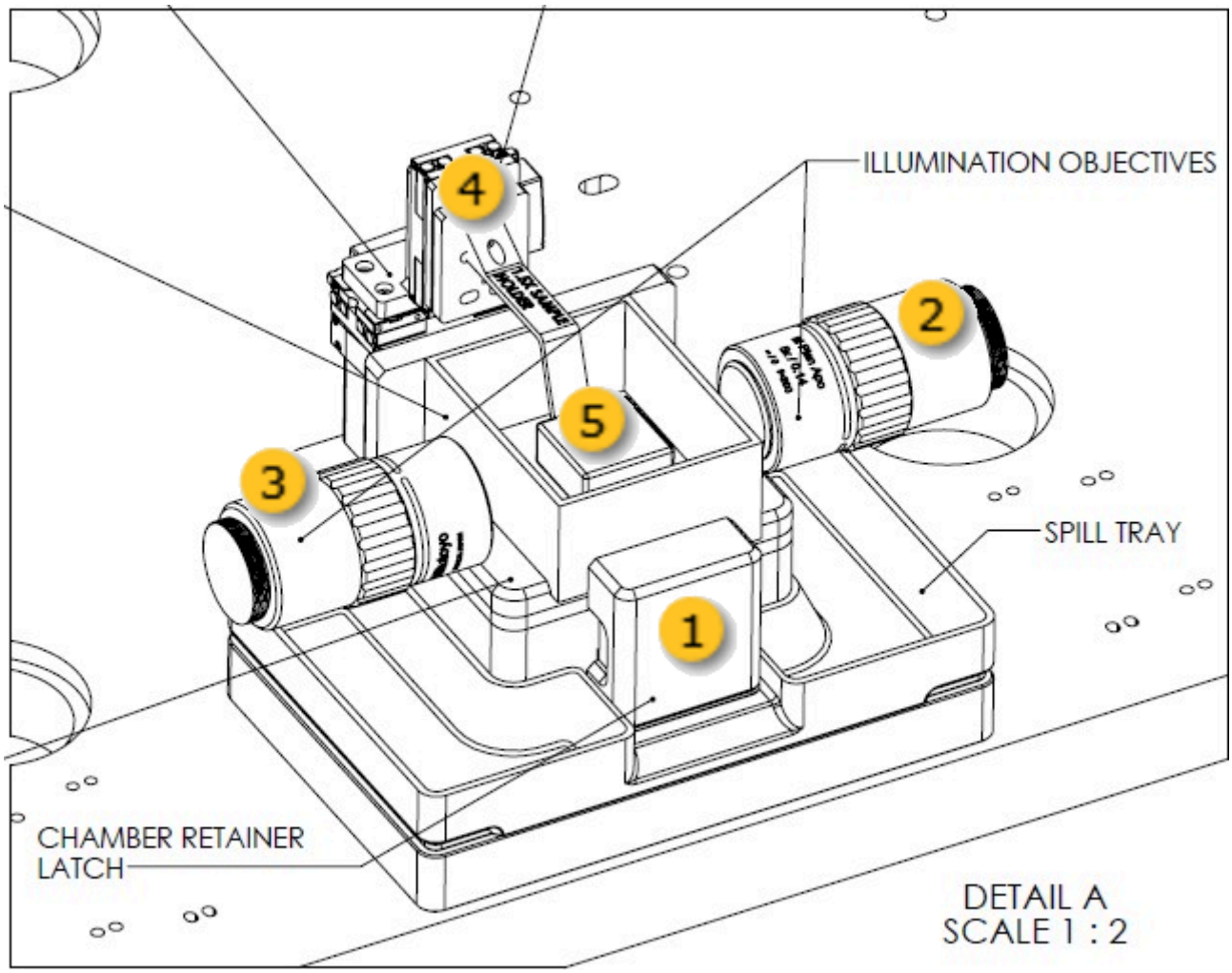
CTLS System Hardware

The CTLS microscope is composed of the scanner unit that houses the spatial light modulator and high speed galvanometer, a pair of excitation objectives, a thermo-coupled imaging chamber and high speed piezo XYZ stage, a macroscopic imaging objective, a fully motorized stereo-microscope, and a high resolution SCMOS camera.



(1) CTLS Scanner Unit; (2) Excitation Objectives (one left, one right); (3) Thermo-coupled Imaging Chamber; (4) Macroscopic Detection Objective; (5) Motorized Stereo Microscope; (6) High Resolution sCMOS Camera

A detail of the Imaging Chamber area is illustrated below, showing the thermo-coupled chamber, the excitation objectives to the right and left of the imaging chamber, the high speed piezo XYZ stage and finally, the specimen mount, which we refer to as "the spoon".



(1) Thermo-Coupled Chamber Unit; (2) Right-side Excitation Objective; (3) Left-side Excitation Objective; (4) High-Speed Piezo XYZ stage; (5) Spoon

There are several power supplies, all of which will be plugged into our System Master Power Control Module. The Master Power Module is housed in our equipment rack and will be the located in the upper-most bay. The LaserStack's Control Module will be found immediately beneath the Master Power Control Module. After the system is turned on you will have to switch the Laser Safety Key on the front panel of the LaserStack's controller to the "ON" position. As a rule of thumb, anytime that system is being powered up, move the Laser Safety Key to the OFF and then to the ON position. You should hear the laser safety shutter engage.

A control module for the galvanometer that also houses the power supply and electronics for the thermo-coupled chamber will be found in the third bay of the equipment rack. There is an LED display that shows the temperature of the chamber. We have determined that the temperature displayed by the LED maybe 1 to 2-degrees higher than the temperature of the imaging media. Thus, if you want to set the temperature of the index matching media to 25C, set the temperature of the thermo-coupled chamber to 26 or 27C. The temperature of the thermo-coupled chamber is seamlessly controlled by SlideBook.

Every motorized component of the CTLS microscope is controlled by SlideBook. For convenience, we've included a System Control Panel called SYCOP. Coarse and fine focusing may be adjusted using SYCOP. Use the joystick (1) to coarsely adjust the focus of the microscope. Take special care with the joystick so that you don't drive the microscope's objective into the imaging chamber. Fine focus may be adjusted by turning the dial (2) to the left of the joystick.

ZEISS
SYCOP 3
Home
Objective
PlanNeoFluar Z 1.0x
Shut Down
Favorite
Eyepiece
10x/23Br foc
Z-Position
17.444 mm
Status
Microscope
4/30/2020 9:15
Magnification
43.3x
Setup
Field of View
5.3 mm
User
Resolution
1.1 µm
Depth of Field
23 µm
Display
2
1

CTLS Beam Alignment

Beam alignment is a critical part of system use. Beam alignment is a very simple procedure and should be checked and refined any time you change the index matching media, the specimen or the imaging chamber.

This section contains 2 tutorials. As a first step, we recommend that you practice beam alignment with an imaging chamber filled with a 0.05% fluorescein or rhodamine solution (dilute your fluorescent dye in water).

CTLS has been designed specifically to image large, transparent organs several mms or cms wide. Even though the specimens are "transparent", the lightsheet is subject to some scatter and a loss of photon density as it propogates through the sample. This is Beer's law. CTLS uses a pair of excitation objectives to maximize the photon budget of the sheet over space. The tutorials that follow will review the use of the 3-axis objective focus mount used to interrogate, align and center the beams. The steps executed for one objective need to be repeated for the other. We provide a tutorial that reviews co-alignment of the beams to ensure co-planarity. Finally, we provide an advanced tutorial to show how to adjust the width of the lightsheet to maximize photon budget.

Note that many index matching solutions will autofluoresce, therefore, it is not necessary to add any dye to the index matching solution. This appears to be especially true for solvent-based index matching solutons. If the beams cannot be seen in your index matching media, you will need to align the beams in a chamber filled with your index matching media and 0.1% fluorescent dye. When you're ready to image, you'll need to remove the contents of the chamber, clean it with 70% ethanol, allow the chamber to completely dry, then refill it with your index matching media for imaging. You will have to make some fine adjustments to center the beam.

There are two chambers provided with the CTLS. Both are approximately 50mm wide. The system is designed so that the center point of the Gaussian beam will be focus in the middle of the chamber, so let's call that 25mm.

Suppose a user were to image a sample that was processed for Clarity. The index-matching solution for Clarity has an RI of 1.45. However, the next user will image a sample cleared with PEGASOS. The RI of the PEG index-matching solution is 1.54. The displacement of the center-point of the beam will be about 1.5m as calculated by

$$Fx = 0.5C_W * [(RI_X/RI_O) - 1]$$

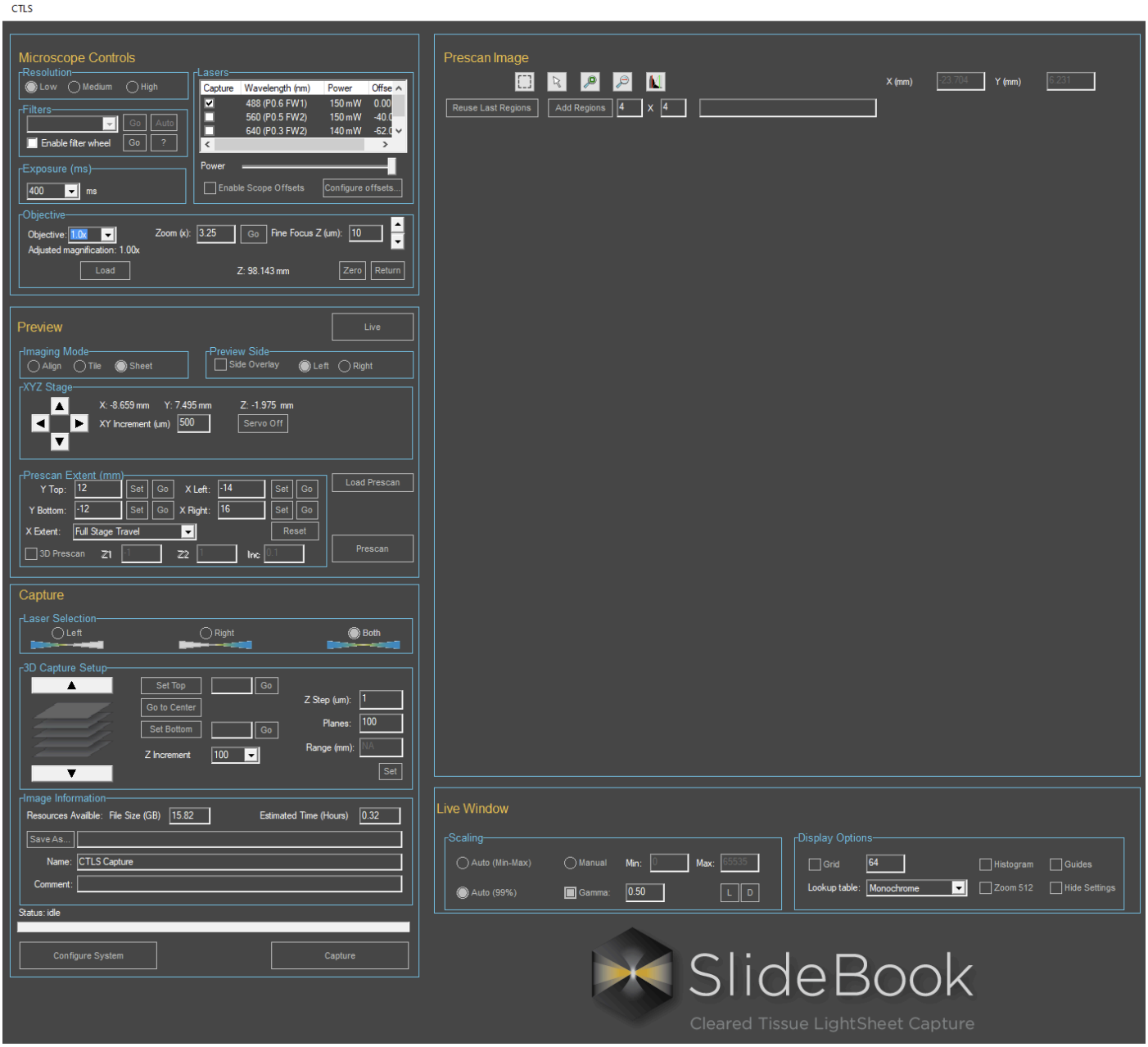
Where Fx = change in focus, C_W = chamber width and RI_X = new index-matching media and RI_O = old index matching.

This is very important: you cannot simply change index-matching media and expect to image with a beam that is optimized for optical sectioning. Fortunately, alignment and centration of the beam is easy to do in SlideBook, thanks to the 3-axis focus mount.

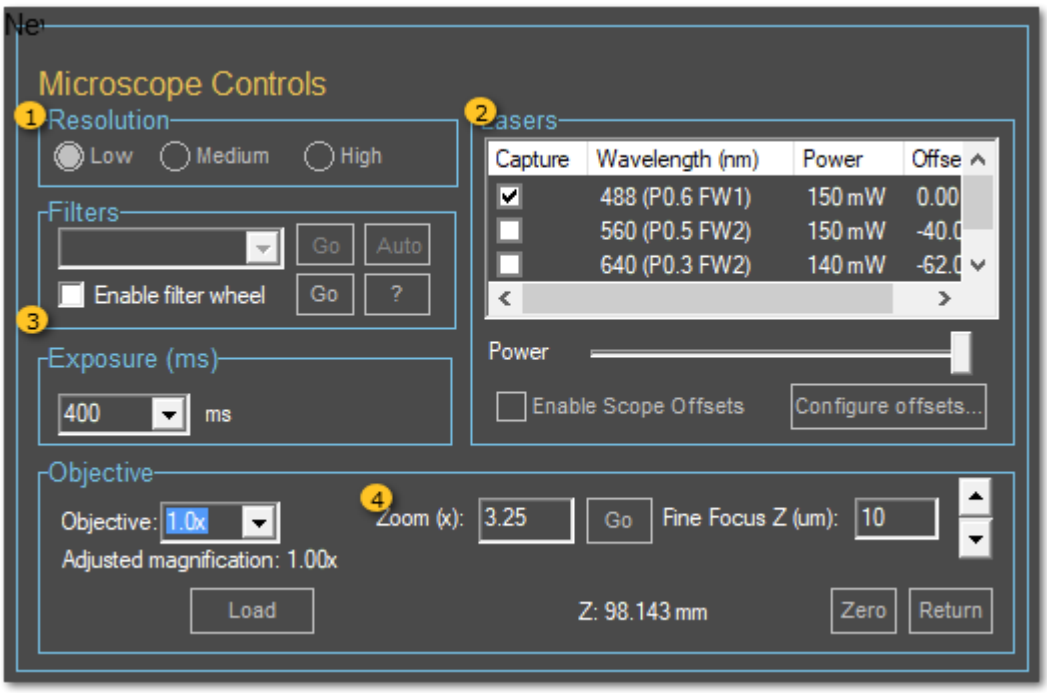
TUTORIAL: Aligning the CTLS Gaussian Beam

For the purposes of the tutorial, we recommend that you start with a 0.05% solution of a fluorescent dye such as fluorescein or rhodamine diluted in water. The chamber volume for the 1.0X objective is 130mls; the chamber volume for the 1.5X objective is approximately 70mls.

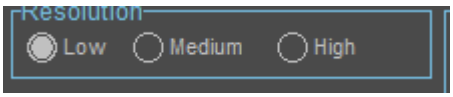
1. Open the ***CLTS Capture*** control interface: go to the Home tab, Acquire group > ***LightSheet*** > ***CTLS Capture.*** This interface will appear on the desktop.



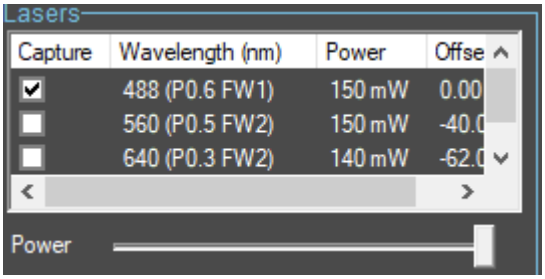
2. Navigate to the upper left portion of the interface to ***Microscope Controls***.



1 Set Resolution, e.g. LOW



2 Set Laser Wavelength



Hover the cursor over any laser and click the left mouse button to select it for imaging.

3 Set Camera Exposure

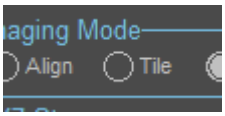
This is a pulldown menu. Click on the arrow key to the right of the variable box and select the camera's exposure time.

4 Set Zoom State

Navigate to the **Zoom (x)** variable window. Zoom (x) displays the current zoom factor. In case you want to view a low magnification overview, type 1x and click the **Go** button to set the zoom factor. This will help you find the beam or sample.

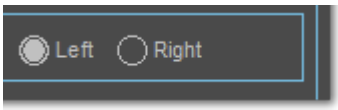
3. Once you've loaded these settings navigate to the ***Preview*** group.

1 Choose an Imaging Modality



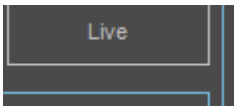
Select the ***Align (low)*** radio button.

2 Select an Excitation Objective



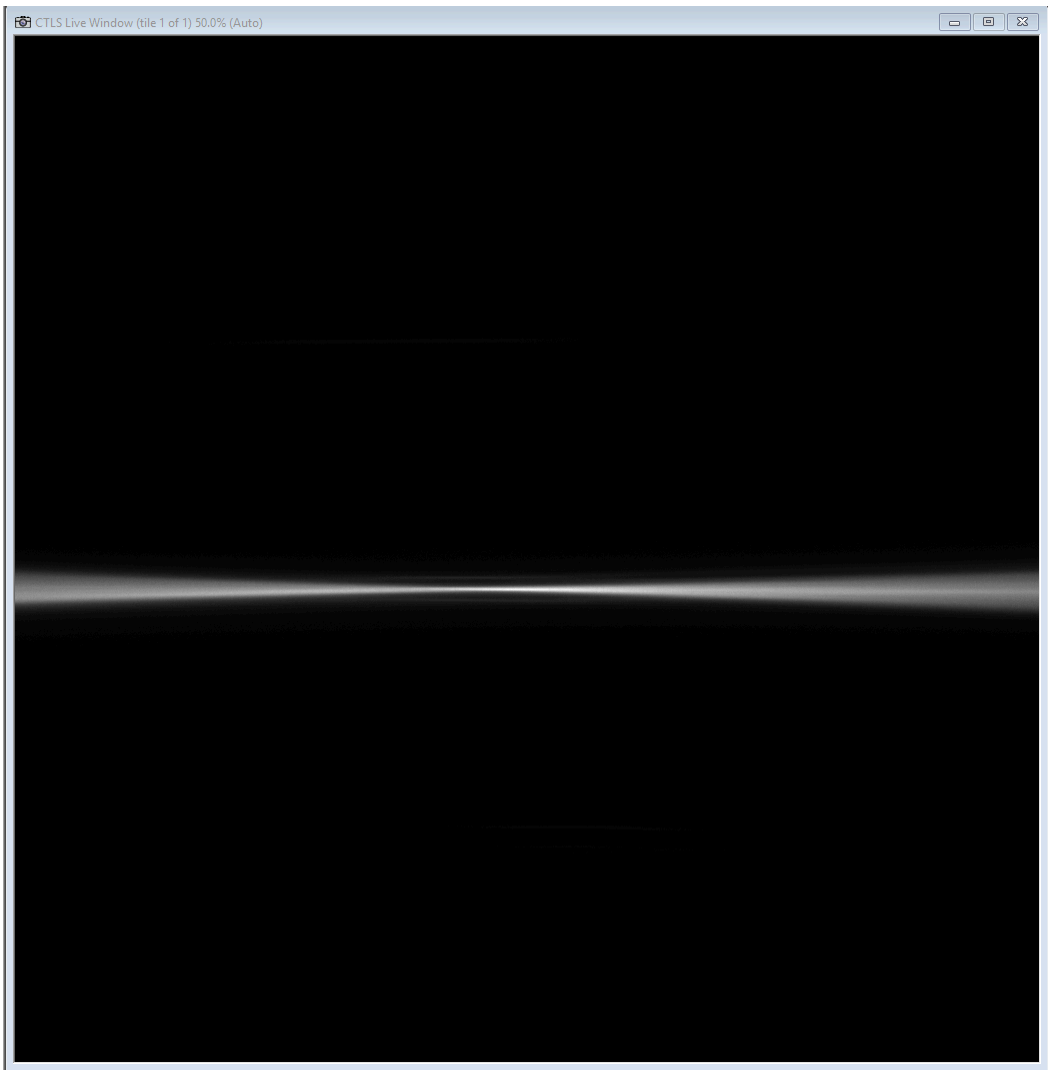
In the ***Preview Side*** group select an objective, either the one exciting the sample from the left side, or the right. You'll need to repeat these steps for both excitation objectives but the process will vary just a slightly. Whatever objective you choose here should be considered a "reference" objective. In this case, the "reference objective" will be on the ***Left***.

3 Look at the beam

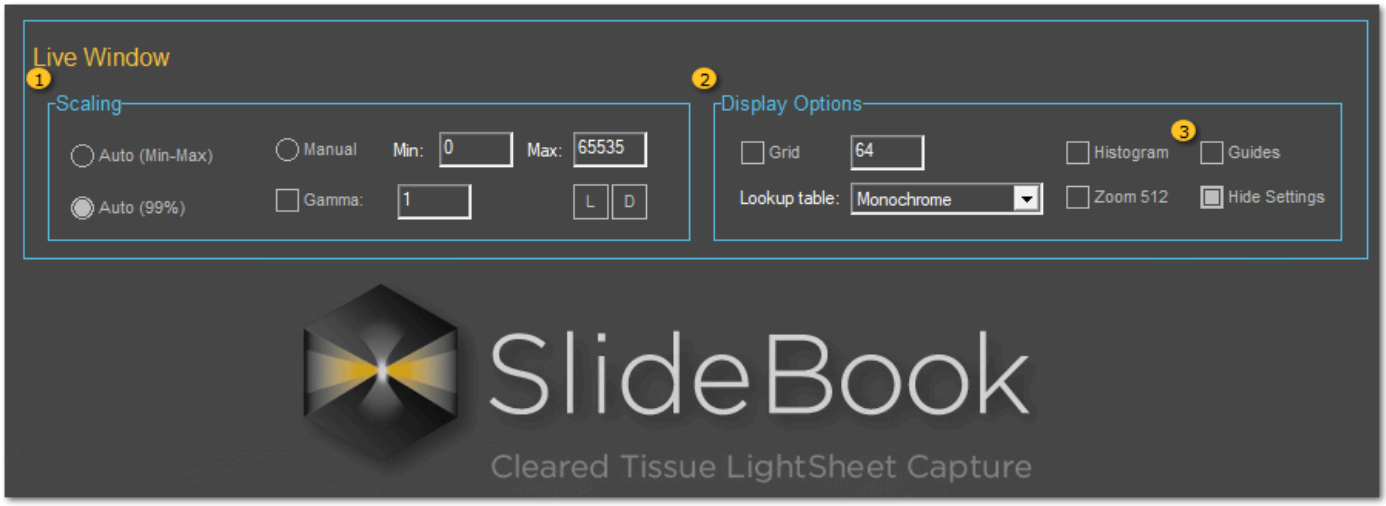


Click the ***LIVE*** button. A new live window will appear.

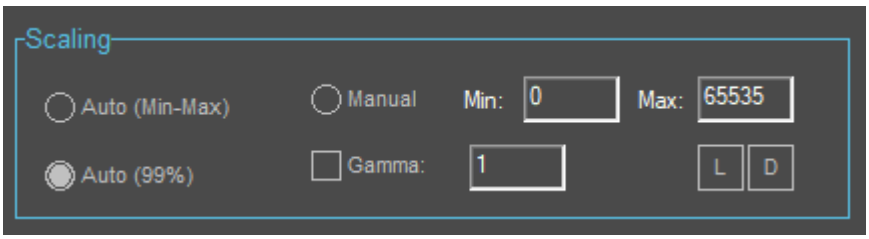
4. You will need to adjust the coarse and then the fine focus of the microscope. The coarse and fine focus adjustments of the microscope may be found on the SYKOP. You may also adjust the coarse and fine focus of the microscope in SlideBook. Moving the SYCOP's joystick forward will lower the head of the microscope for coarse focus. The wheel to the left of the joystick adjusts fine focus. The focused beam will look something like this:



5. Navigate to the ***Live Window*** group at the bottom center of the interface, just above the SlideBook banner. You'll find some important tools here to help you interrogate the beam.

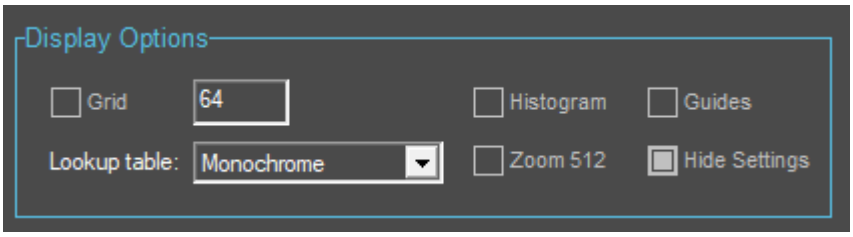


1 Seeing your image



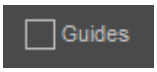
There are several tools in the ***Scaling*** group that can help you see the beam. Normally, the beams are pretty bright so the default setting is to scale at 99%.

2 Look Up Tables

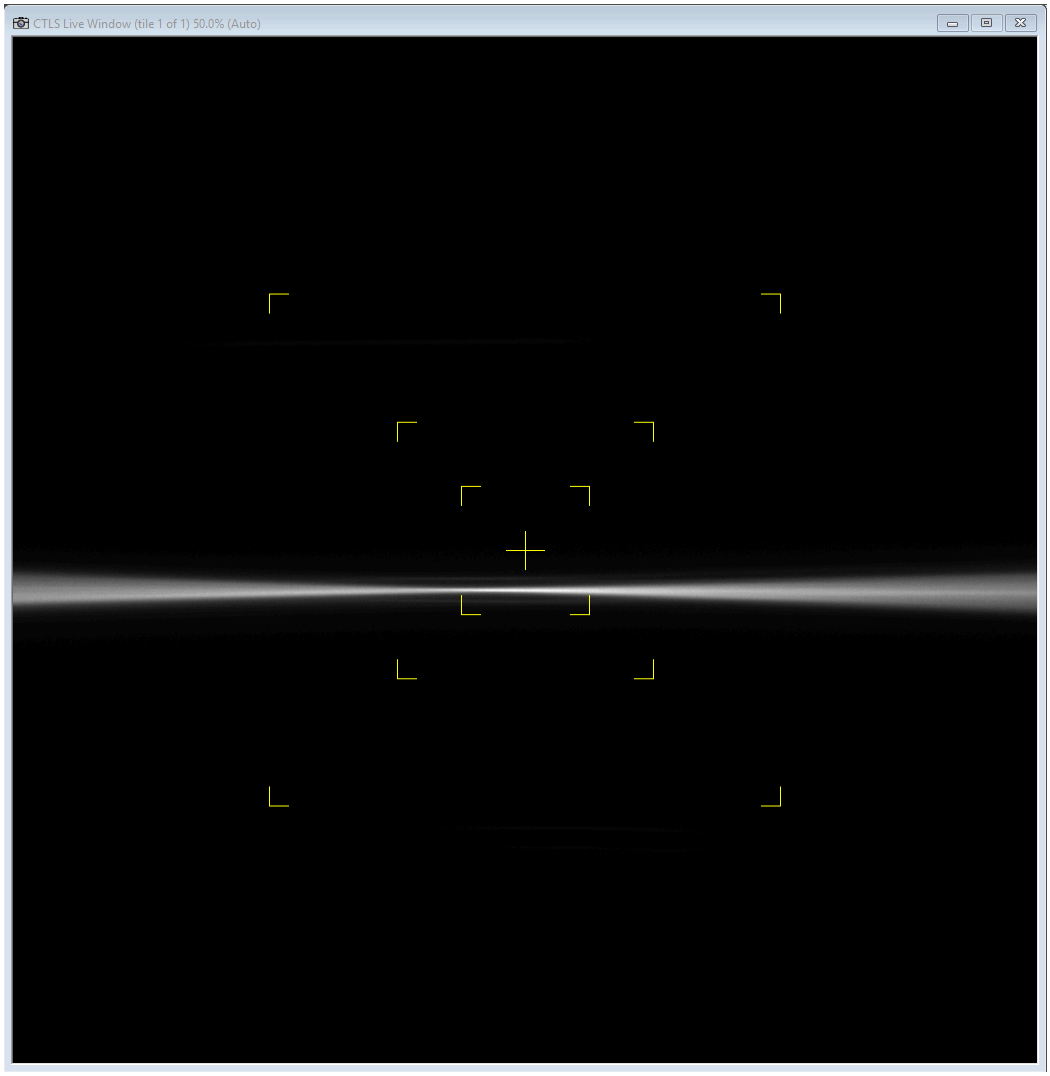


In the ***Display Options*** group you can choose to see the beams using one of three Look Up Tables (LUT). The default setting is ***Monochrome***. If you click the arrow on the right side of the variable box a pulldown menu will appear where you may select ***Pseudocolor*** or ***RedHot*** LUTs

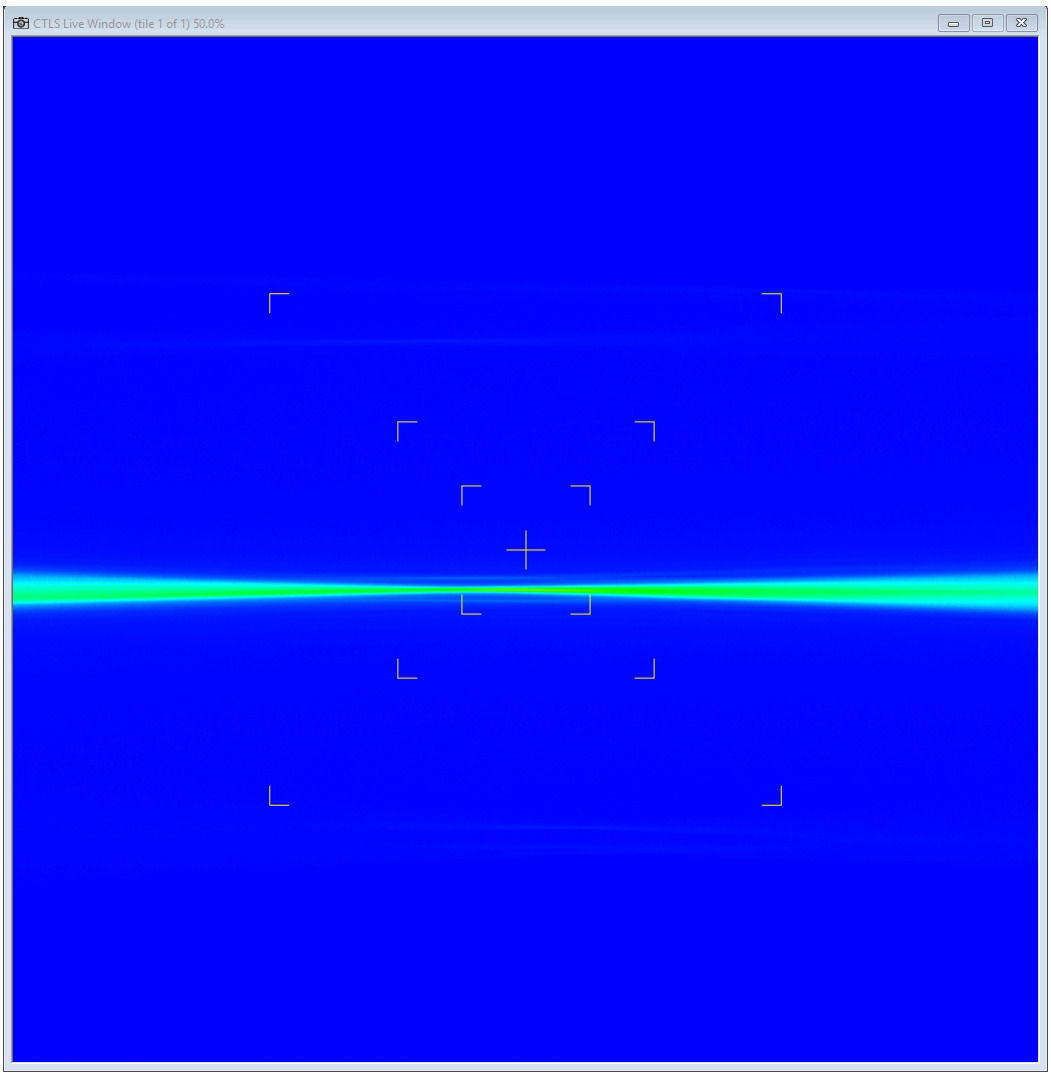
3 Set Guides



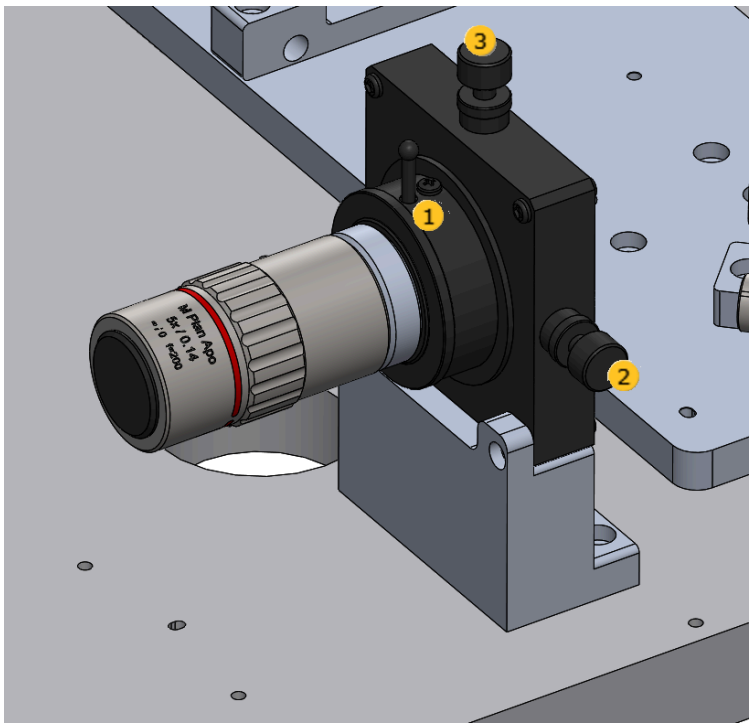
For this exercise you will definitely need to enable ***Guides***. This tool will overlay a crosshair in the live image window.



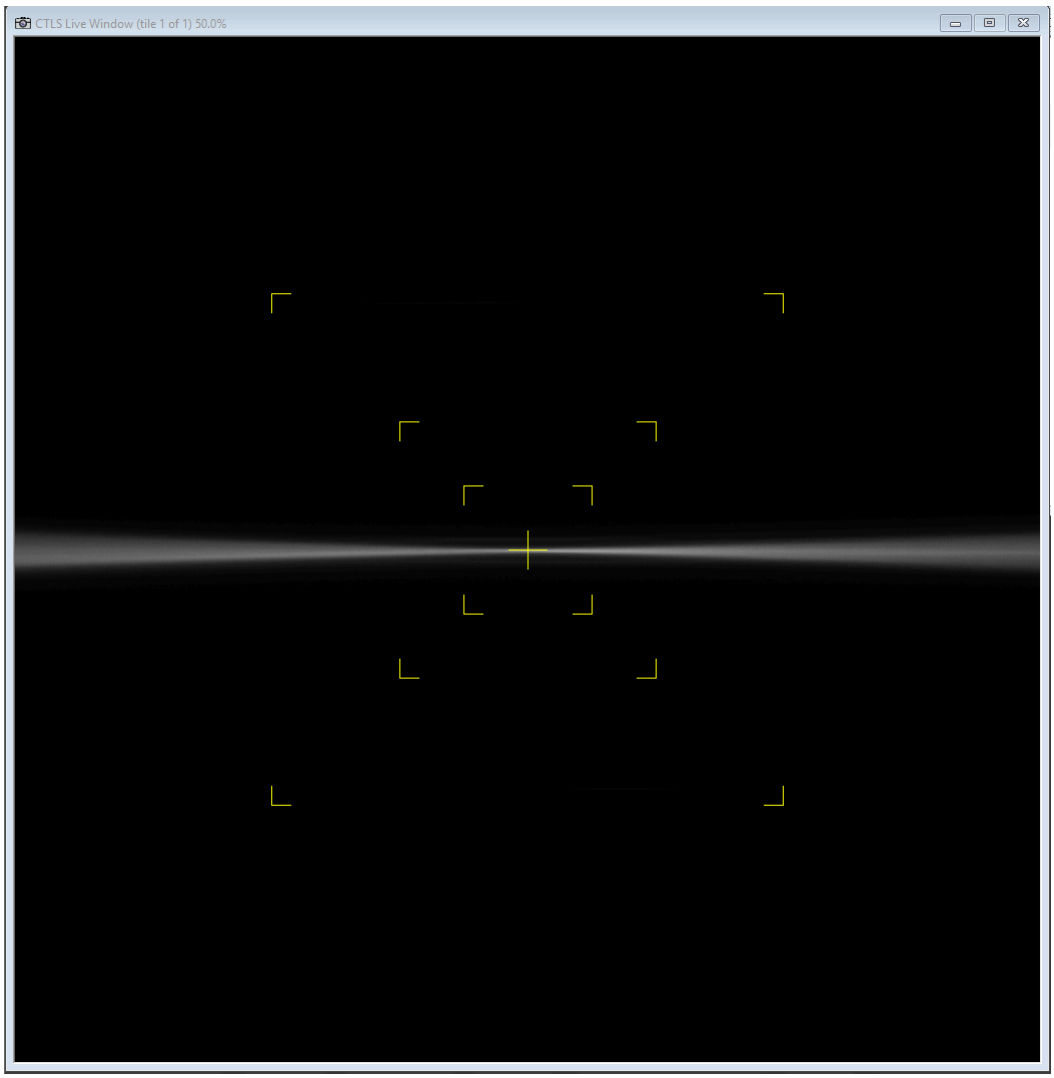
6. The cross hair marks the center of the field of view. The beam should overlap with the horizontal bar of the crosshair. In the example shown below we've applied the Pseudocolor LUT to demonstrate that this beam is not properly centered in the field of view. Note that the waist of the beam is located just to the left of the cross hair. We also want to place the beam so that it's length runs parallel to the horizontal bar of the cross hair.



The beam can be moved using the knobs on the 3-axis focus mount. This mount is used to translate the beam to the left or right using the lever marked "***1***" in the picture below and centration of the beam is achieved by moving the knob "***2***". The beam may also be focused using the knob "***3***". For the purposes of this exercise, critically focus the beam using the microscope and then make the final focal adjustments using knob "***3"*.**



7. After you've made the adjustments using the 3-axis focus mount the beam should look like this:



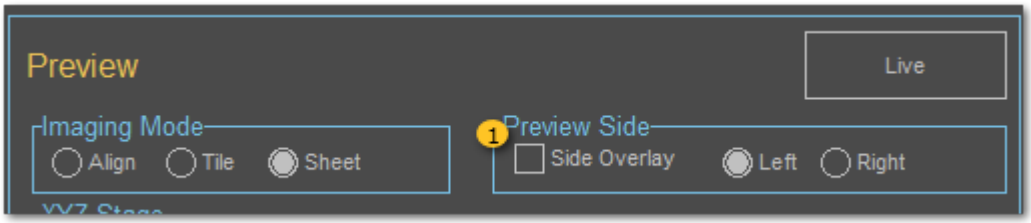
8. You will need to repeat the same procedure for the other objective. If you started with the left-side beam then go back to the ***Preview*** panel >***Preview Side*** and choose ***Right*** radio button.

NOTE: if the other beam is out of focus **DO NOT** adjust the focus of the microscope. **You must focus focus the beam using Knob "3" of the 3-axis focus mount.**

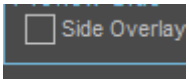
Adjust Knob 2 on the 3-axis focus mount and potentially Lever 1 so that the two beams overlap.

TUTORIAL: Ensuring Co-planarity

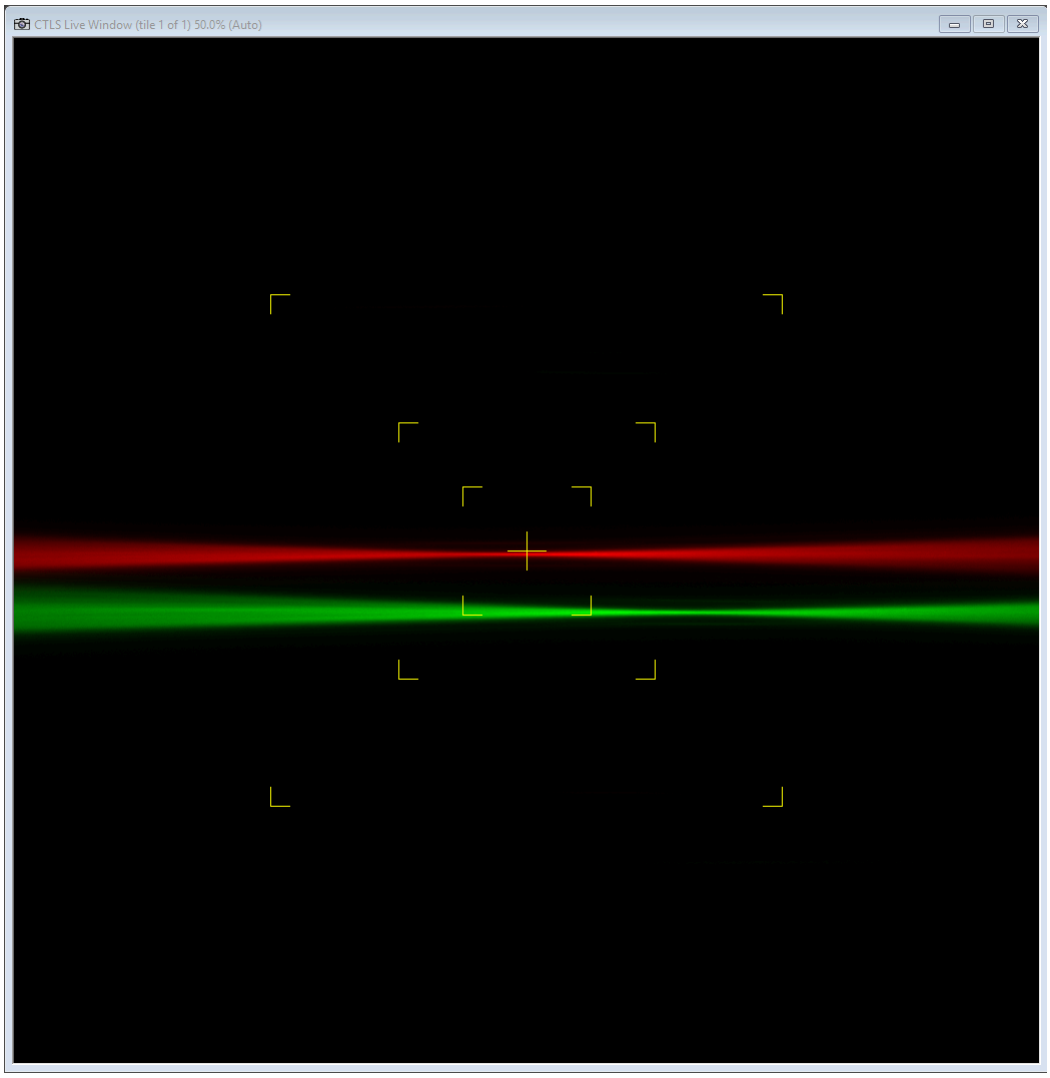
Now that you've focused the two beams, you want to make sure that they are centered and overlapping. First, stop the Live video. Navigate to the ***Preview*** panels. Navigate to the ***Preview Side*** group and enable the ***Side Overlay*** tool.



1 Visualizing the beams from the two excitation objectives

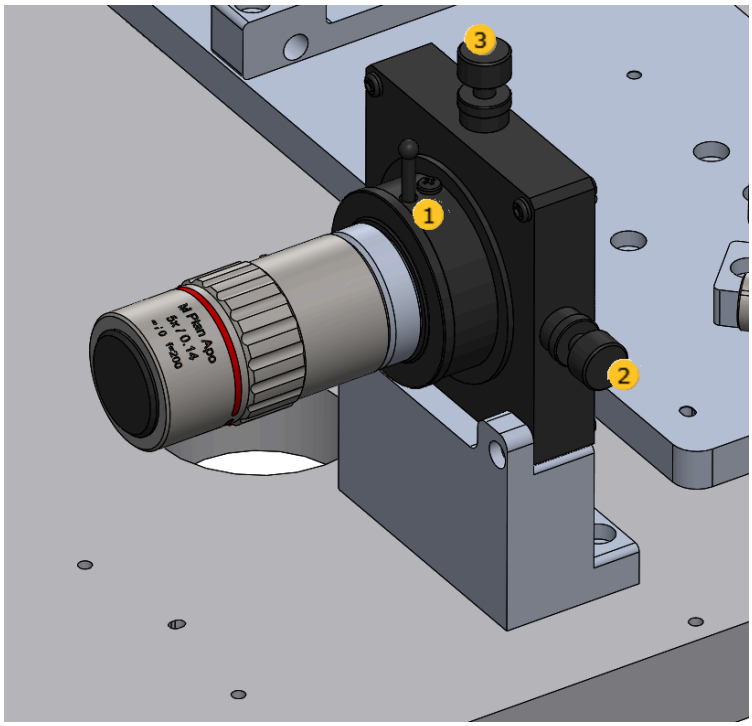


Select the Side Overlay tool. When this tool is enabled, the two beams will appear, pseudo colored with a Green and a Red lookup table. The Red beam represents the laser coming from the left-side excitation objective, the Green beam from the right-side.



1. Use ***Knob 2*** on the 3 axis focus mount to translate one of the beams up and down (on the camera). For the purposes of this exercise, let's maintain that the left-side laser is our reference. Therefore, adjust ***Knob 2*** to move the beam from the right

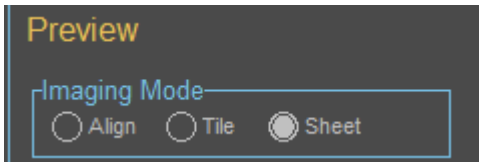
(Green beam illustrated above) excitation ***up*** so that it overlaps with the beam from the left excitation objective (Red beam illustrated above).



2. Next use **Lever 1** from the right-side focus mount to center the waist of the right-side excitation objective so that it overlaps with the waist of the beam from the excitation objective on the left.
3. **Knob 3** shifts the beam in and out of focus. It is important that you change **Knob 3** only on one side and never touch the other side. For example, left side remains in the fix position; put a tape around the knob so that no one touches it.

- Go live on the left side
- Focus using the z-drive of the microscope
- switch to the right side illumination
- use Knob3 to bring the beam in focus

This gets you very close to ensuring that the beams are co-planar. However, you'll have to repeat this process with your tissue. When you're interrogating your sample, you'll follow the exact same procedures. The notable exception is that the ***Imaging Mode*** will be set to ***Sheet***.

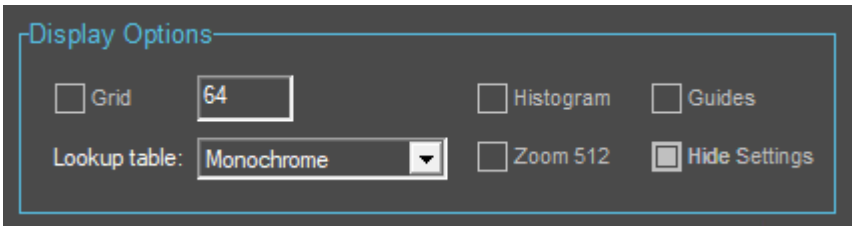


Be sure to image a field that is at the surface of the specimen. This ensures that both sides have an unobstructed access and illuminate the exact same features. Start with the left side illumination, focus on the specimen using the z-drive of the microscope. Then enable ***Side Overlay***. Now adjust Knob3 on the right side excitation objective mount until you see the same structures in both sides. Since the two sides are pseudo-colored Red and Green, you'll want to tune knob 3 until objects in the field of view are more "yellow" than either strongly red or strongly green.

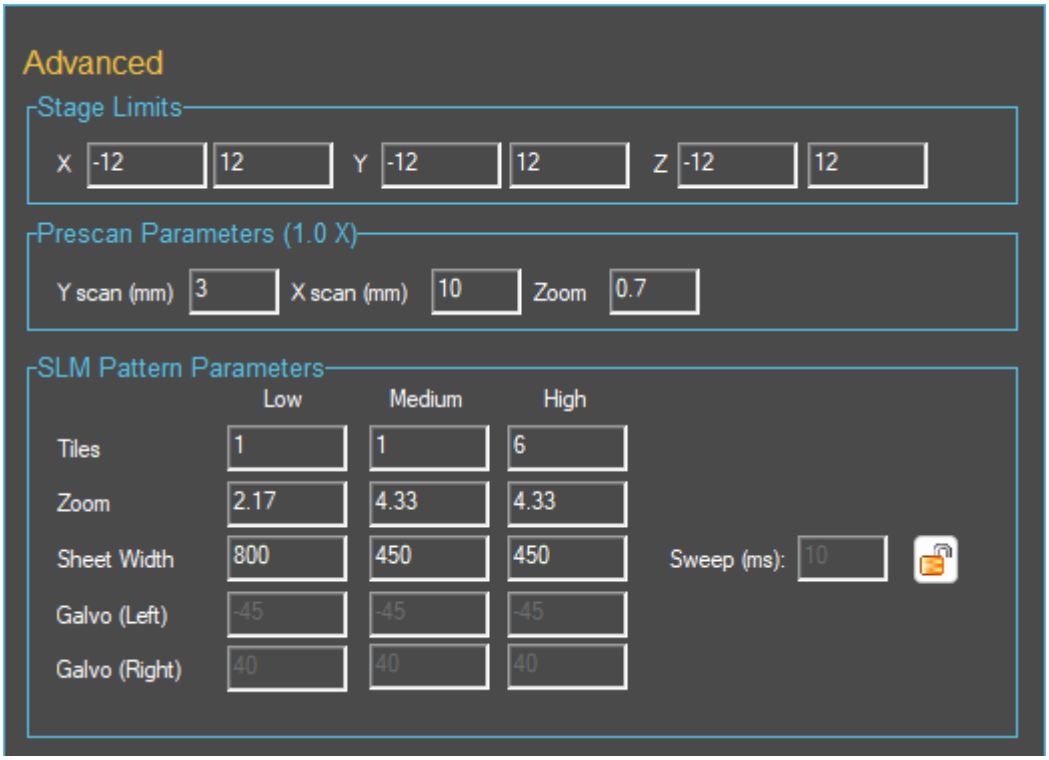
TUTORIAL: Setting Sheet Width (ADVANCED SETTING)

The width of the sheet is fixed during the installation of the system. The width of the sheet is set to ensure that the field of view can be visualized. Modifying the width of the sheet can buy back some photons, useful in situations where fluorescent emissions have been compromised by the clearing procedure. In this tutorial, begin with a sample-free chamber; we'll just image the index matching media. Should you have a sample in the chamber, that's okay, just move the sample to an area of the chamber so that you can image the obstructed beam in the media.

1. Navigate to the bottom right corner of the CTLS capture window to the **Display Options** group. Click the box ***Hide Settings***



This will reveal the ***Advanced*** settings panel. Navigate to the ***SLM Pattern Parameters*** group.



The ***SLM Pattern Parameters*** group contains all of the variables that are required to program the Spatial Light Modulator. The columns, ***Low, Medium*** and ***High*** define the resolution states that you've seen in the ***Microscope Controls*** group**.** ***Tiles*** defines the number of focus shifts of the lightsheet that the SLM will generate, so in the example shown here, the ***Low*** resolution state will create a single tile pattern that realizes one long beam while the ***High*** resolution state will create a 6-tile pattern, or 6 shorter beams that will be translated across the field of view.

The row of variables underneath ***Tiles*** defines the zoom factor of the microscope during image acquisition. In the example shown here, at *Low there is a 2.17X zoom factor, and in Medium* and *High* resolution, the zoom factor does not change. The zoom factor may be edited, but changing this variable will also change the lateral resolution of the system, and new SLM patterns need to be generated.

NOTE: The lateral resolution of the microscope is linked to the magnification of the imaging objective, the zoom factor of the Zeiss AxioZoom, and the pixel size on the detector. In this case, the imaging objective is 1.5X, the zoom factor is 4.33X, and the size of the camera pixel is 6.5um.

6.5um / (1.5X * 4.33X) = 1 um per pixel lateral resolution

The Sheet Width describes the scan range of the galvanometer in mV. The smaller the value, the less displacement, zero means a beam and 800 (the maximum scan range) will scan a light sheet of about 4mm in width.

2. Navigate to the ***Microscope Controls*** panel. Select the following:

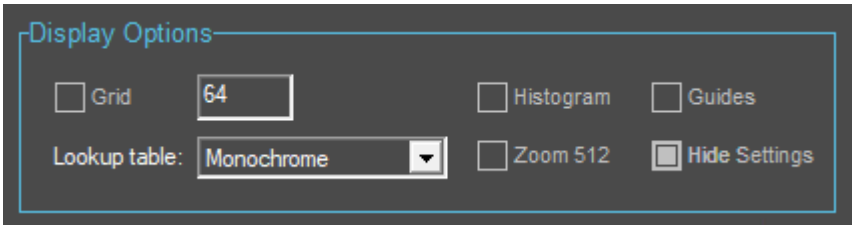
    a. The ***Low*** resolution radio button
    b. A laser and set its power
    c. An exposure time
    d. The ***Zoom (x)*** factor to a value of ***2***

3. In the ***Advanced*** Settings Panel, set the ***Beam width*** to 550. This should be a default setting.

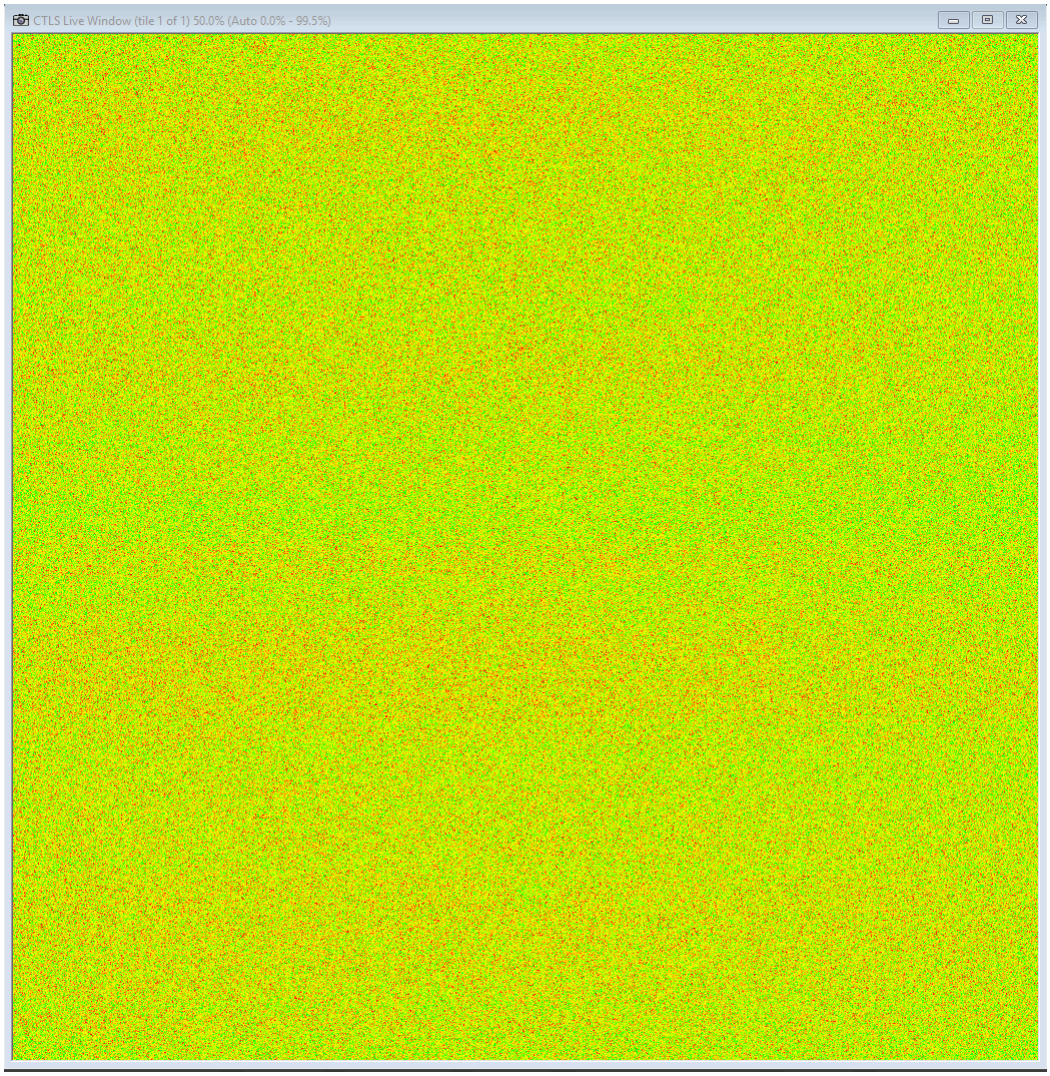
4. Navigate to the ***Preview*** panel. Select the following

    a. ***Imaging Mode*** = ***Sheet***
    b. Choose a ***Preview Side*** (SlideBook will default to the left-side)
    c. Click the ***LIVE*** button

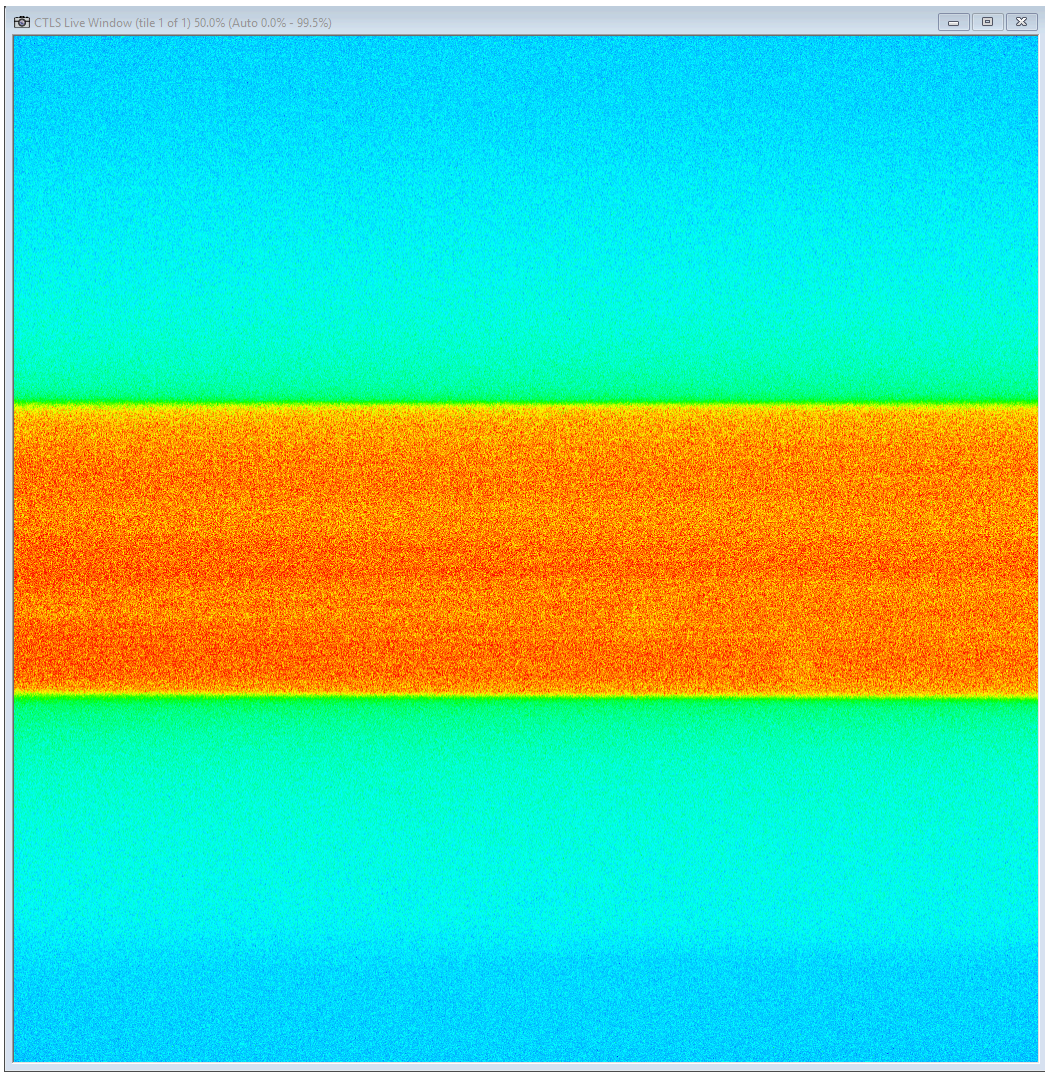
5. Navigate to the ***Display Options*** panel and click ***Lookup table.*** Select the ***Pseudocolor*** palette in the pulldown menu.



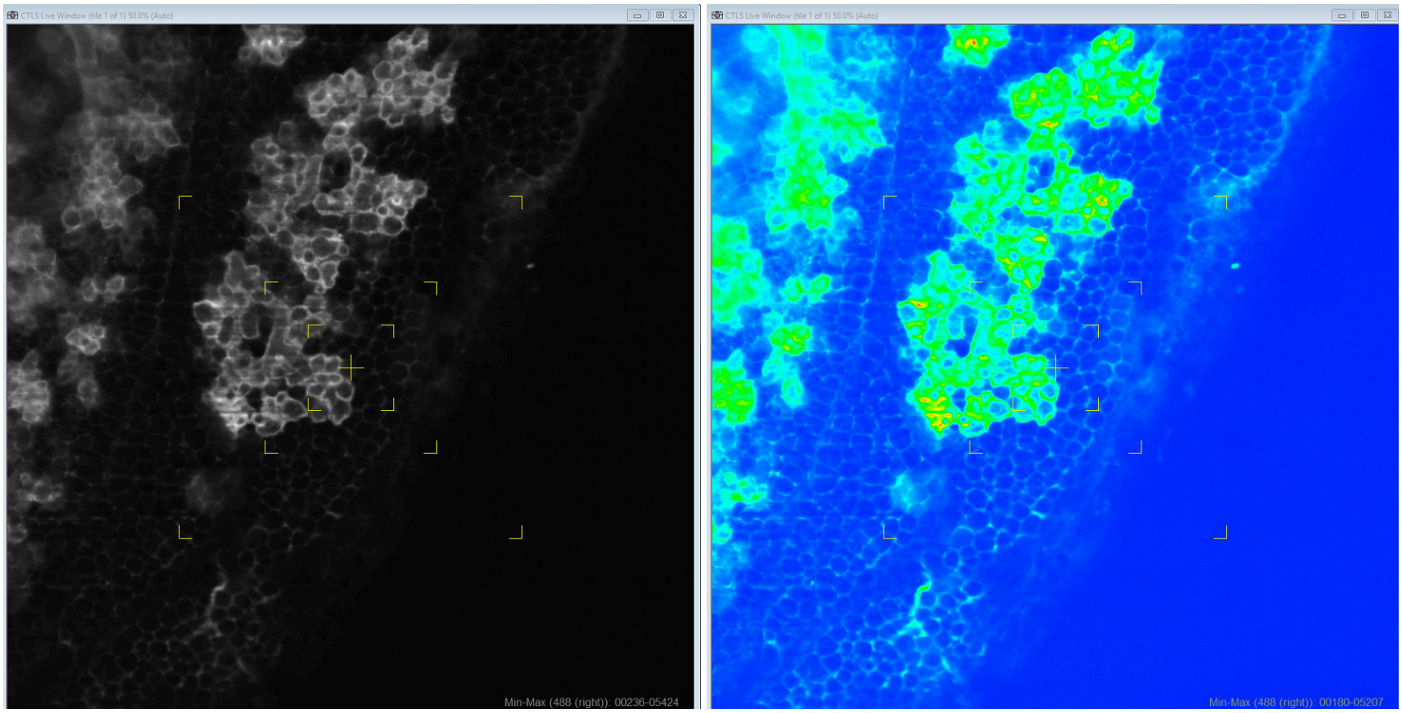
The image in the ***Live Window*** should look something like this:



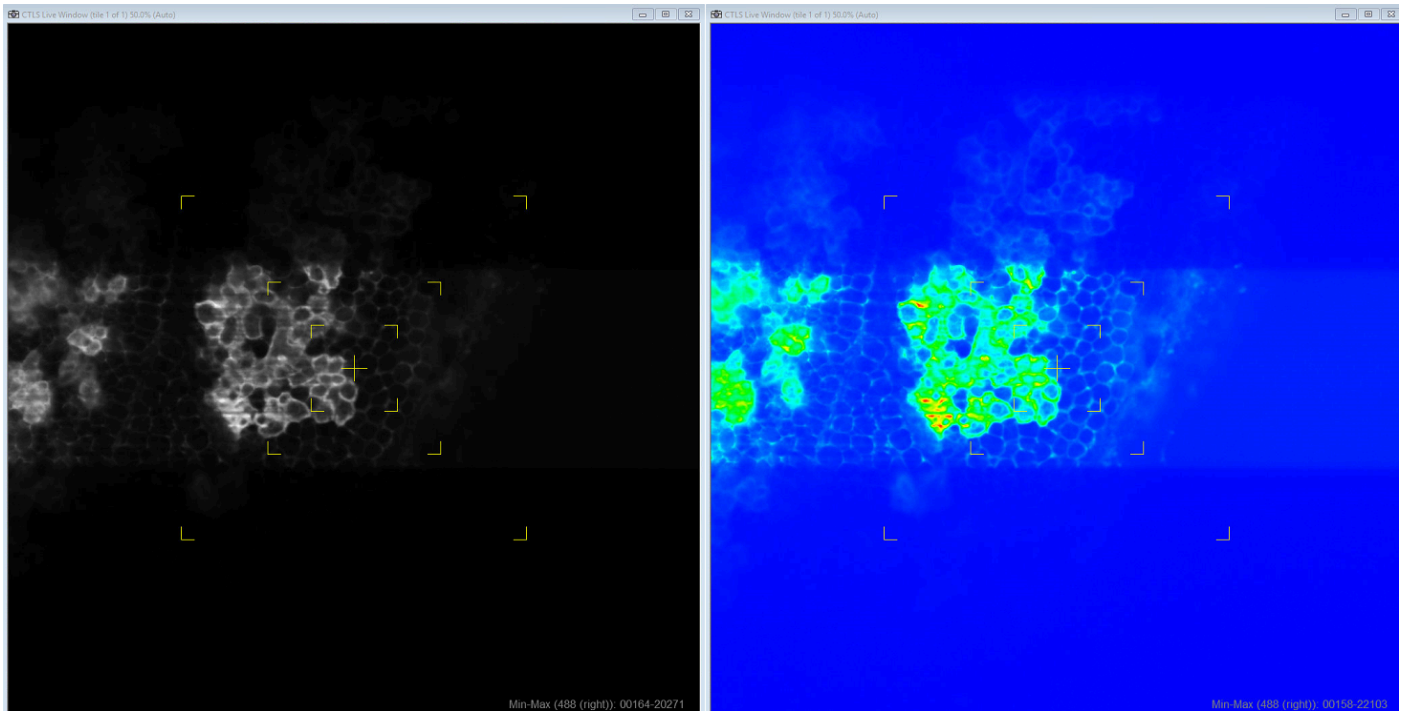
6. Navigate back to the ***SLM Pattern Parameters*** group and change the ***Beam Width*** value to 100. The image in the Live Window should now look something like this:



In this exercise you've demonstrated that you can easily adjust the width of the lightsheet. By adjusting the width of the lightsheet you can maximize your sheet's photon density. This could be a valuable tool if your fluorescent signal needs to be boosted and you are interested in only a small area. Let's look at the examples below.



This is a an image of a specimen visualized with the galvanometer's displacement set to 550mV, SlideBook's default setting. The image on the right is the same image with the Pseudocolor LUT applied. Note, in the bottom right corner of the image the min-max grey count. For this image, with the galvanometer displacement set to 550mV, **the max gray counts are 5500.**



Here is that exact same field captured with the galvanometer's displacement set to 100mV. Note that the maximum number of gray values **has increased nearly 4-fold to 20,000 counts.**

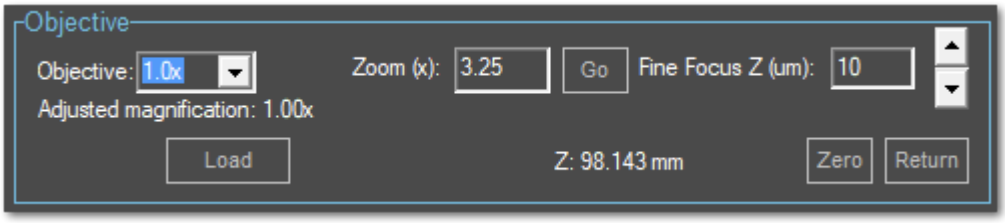
CTLS Operation

Once the beams are aligned, you may start imaging your samples. This section of help pages will walk you through CTLS operation from locating your sample to image acquisition.

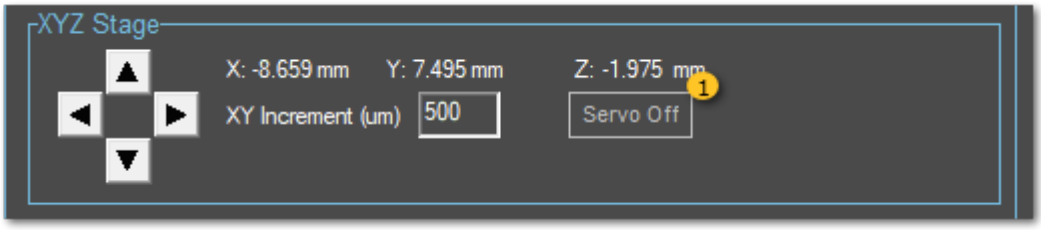
Loading and Positioning the Sample

Loading the Sample

1. Move the microscope up to gain access to the sample chamber. Use the **Sycop Joystick, HIP** or **Fine Focus** software control.



2. Turn Servo OFF



1 Servo Off

When you load the sample, it's important to turn off the Servo controlling the stages as the stages are very sensitive to pressure and vibration. Click the ***Servo Off*** button and then attach the sample to magnetized sample holder.

2. Once the specimen has been loaded into the chamber, Click the ***Servo On*** button.

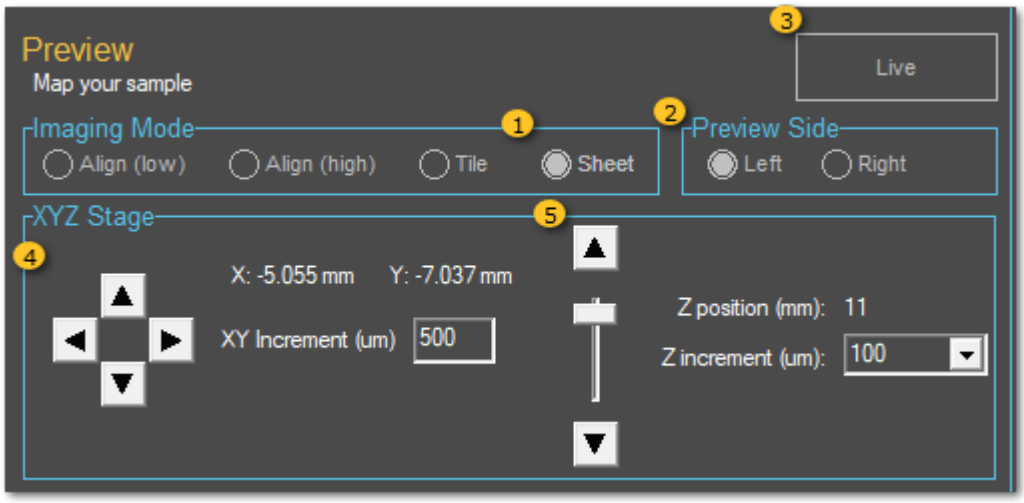
3. Return the objective into the imaging chamber.

Specimen Interrogation

1. Open the ***CLTS Capture*** control interface within SlideBook: go to the Home tab, Acquire group > ***LightSheet*** > ***CTLS Capture.***

2. Navigate to the ***Microscope Controls*** panel. Select the following:
    a. The ***Low*** resolution radio button
    b. A laser and set its power
    c. An exposure time
    d. The ***Zoom (x)*** fto actor to a value of ***2***

3. Navigate to the ***Preview*** panel



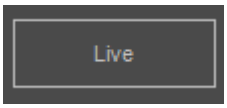
1 Select an Imaging Modality

Click the ***Sheet*** radio button. This is trigger the galvanometer to sweep the beam back and forth to create the light sheet

2 Select an Excitation Objective

SlideBook defaults to using the Left-side excitation objective. Depending on the properties of the sample, feel free to choose the Right-side objective.

3 Click LIVE

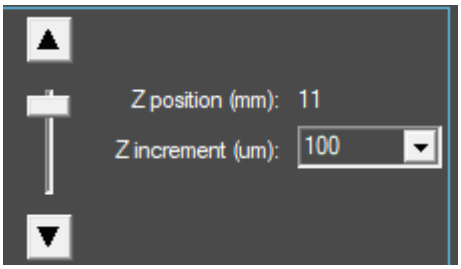


Click the ***LIVE*** button will launch a new Live window. Hopefully you'll see your specimen, and with a little luck, it may nearly be in focus.

4 XY Stage Control

Values, in microns, many be typed into the variable box next to the XY stage control arrows. Type in a variable to translate the specimen in X and Y.

5 Z Stage Control



The variable box next to the Z-slide bar is a menu. Click the down-arrow button to reveal the values in the menu. You may want to start my translating the stage up or down at millimeter increments (1000 microns).

Keep your eye on the *CTLS Live Window* and use SYKOP or the course and fine focus buttons in SlideBook to focus the sample. Begin focusing on a portion of the sample close to the left-excitation objective and then move to the right side of the specimen and focus here using the right-excitation objective by selectiing the radio buttons in the *Preview Side* group.

Prescan

In order to capture a 3D dataset a 2D map of the specimen needs to be created. This 2D map will be used to set regions to interrogate the specimen in Z. You can use this map to interrogate the entire specimen or choose smaller regions to visit.

1. Open the ***CLTS Capture*** control interface within SlideBook: go to the Home tab, Acquire group > ***LightSheet*** > ***CTLS Capture.*** This interface will appear on the desktop.

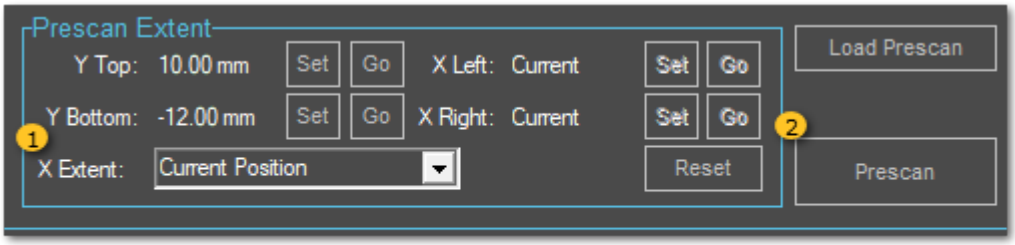
2. Navigate to the ***Microscope Controls*** panel. Select the following:

    a. The ***Low*** resolution radio button
    b. A laser and set its power
    c. An exposure time
    d. The ***Zoom (x)*** factor to a value of ***2***

3. Navigate to the ***Preview*** panel. Select the following

    a. ***Imaging Mode*** = ***Sheet***
    b. A ***Preview Side*** (SlideBook will default to the left-side)
    c. Click the ***LIVE*** button
    d. Use the XY stage controls to move the specimen so that you will view the center of the object
    e. Use the Z stage controls to navigate to what you believe to be the widest and longest portion of the specimen

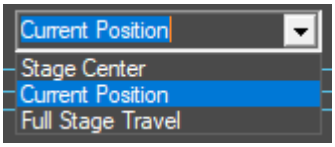
4. Navigate to the bottom of the ***Preview*** panel to find the ***Prescan Extent*** group



1 Setting Prescan dimensions in X

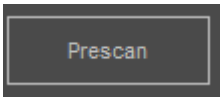
The variable window in X Extent is pulldown. Click on the arrow to reveal 3 options

    a. Stage Center
    b. Current Position
    c. Full Stage Travel

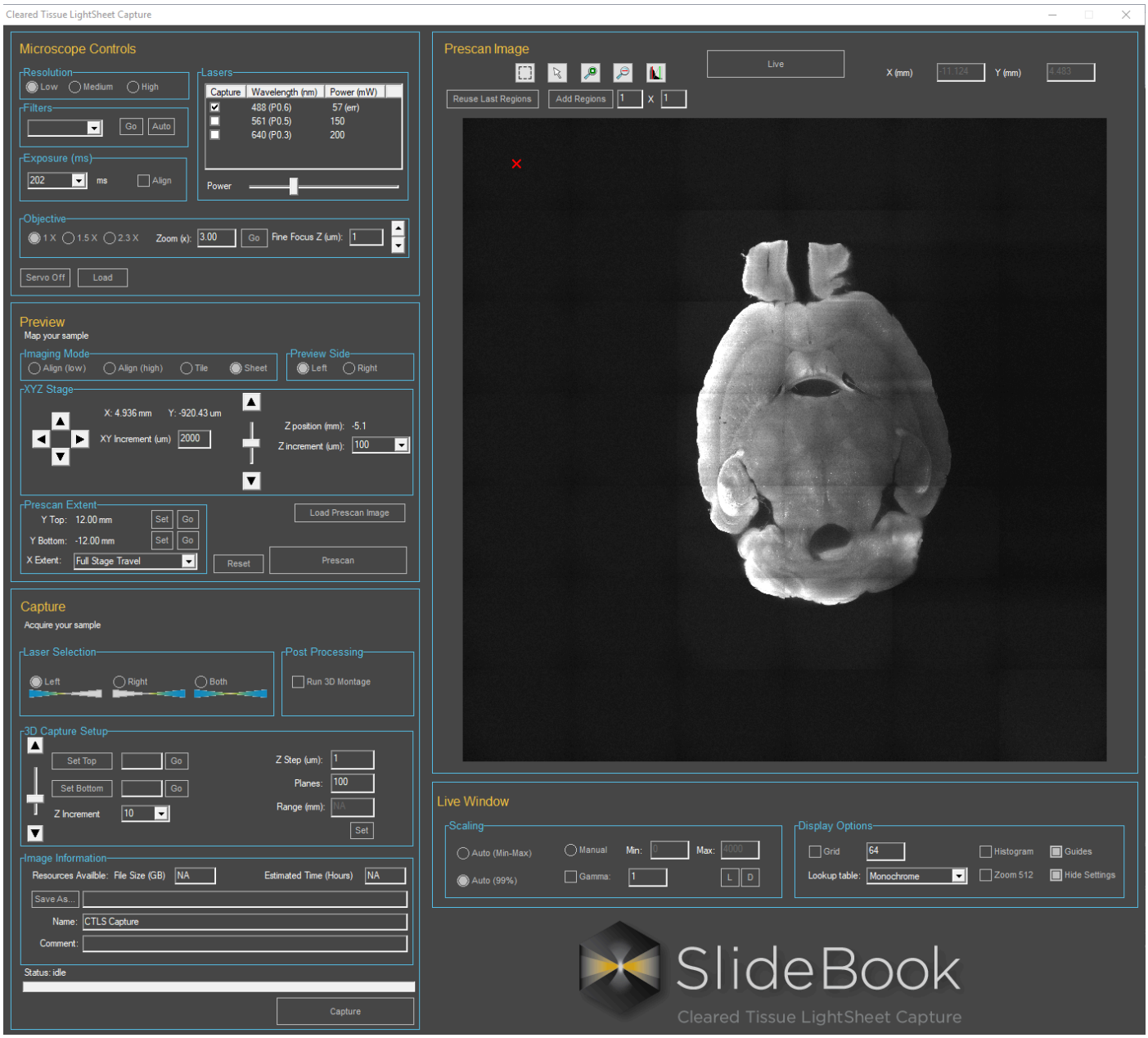


NOTE: Selecting ***Stage Center*** will translate the specimen to the center of the chamber then proceed to step the chamber through a single Y column. Selecting ***Current Position*** will translate the specimen through a single Y column based on the stage's present location. Selecting ***Full Stage Travel*** will translate the specimen across the entire chamber. A Prescan map may be generated quickly by choosing ***Stage Center*** or ***Current Position***. Selecting ***Full Stage Travel*** will take a few minutes but it will provide a full view of the specimen.

2 Create a 2D Map of Your Specimen

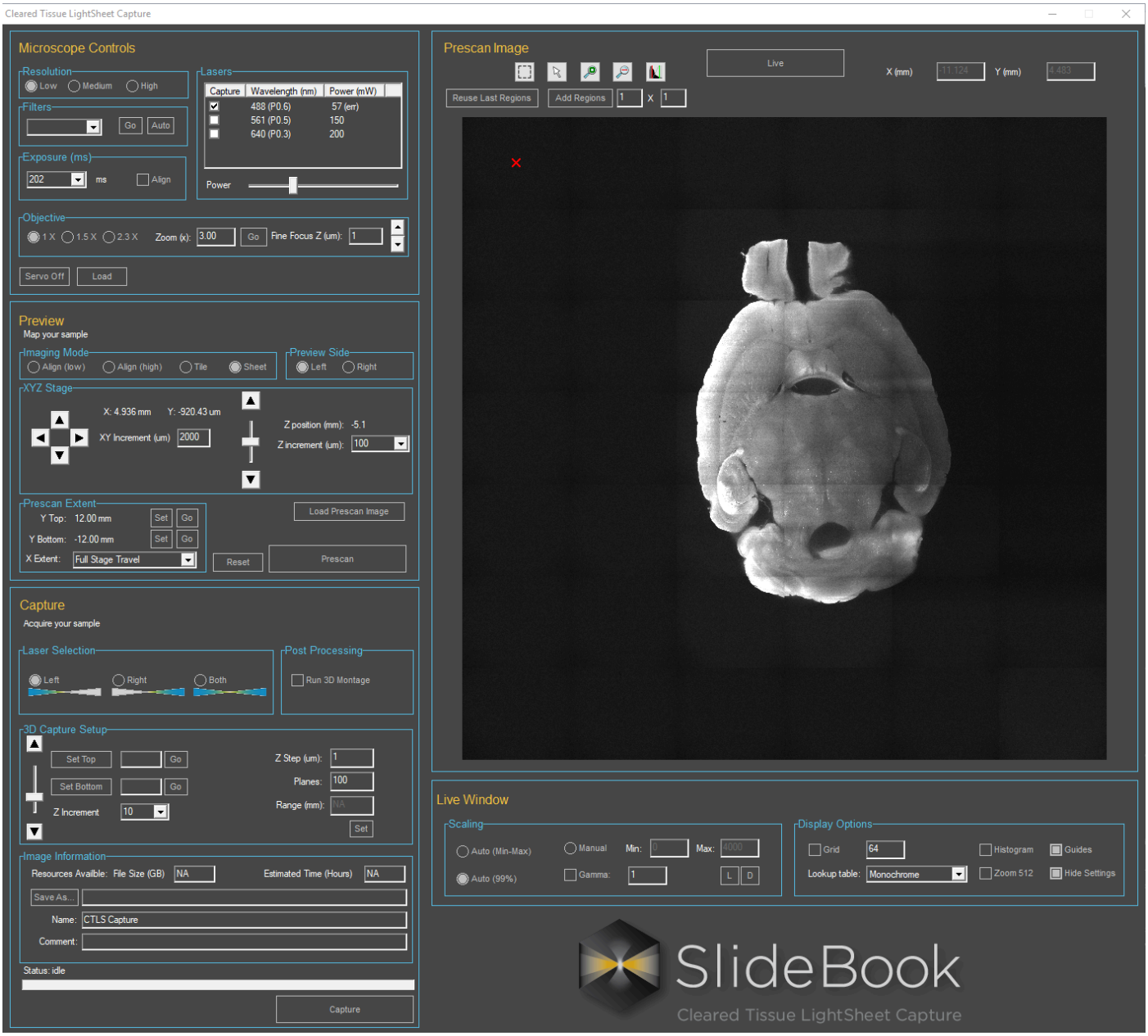


After you've selected an X Extent, click ***Prescan***. Based on the ***X Extent*** variable SlideBook will translate the stage through the chamber, capture a 2D image at each point and then stitch the individual images together to create a 2D map. An image of the map is presented in the ***Prescan Image*** window and a thumbnail of the map is saved in an open .sld.



TUTORIAL : Creating Regions of Interest in the Prescan Map

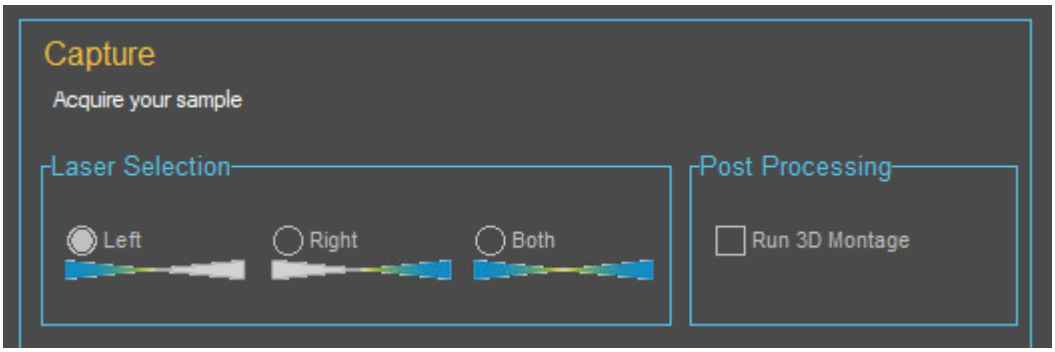
Once the 2D map has been created Regions of Interest need to be created. Regions of Interest (ROIs) are sites that SlideBook will visit and execute a Z-stack. In this tutorial we'll explore how to set regions for single-sided and dual-sided illumination, how to ensure that there is sufficient overlap between regions to maximize photon budget and reduce montage errors, and how to skip regions that do not contain any information to reduce the total time required to capture a 3D data set.



The tools that you will need to create ROIs are found in the upper left corner of the ***Prescan Image*** panel.

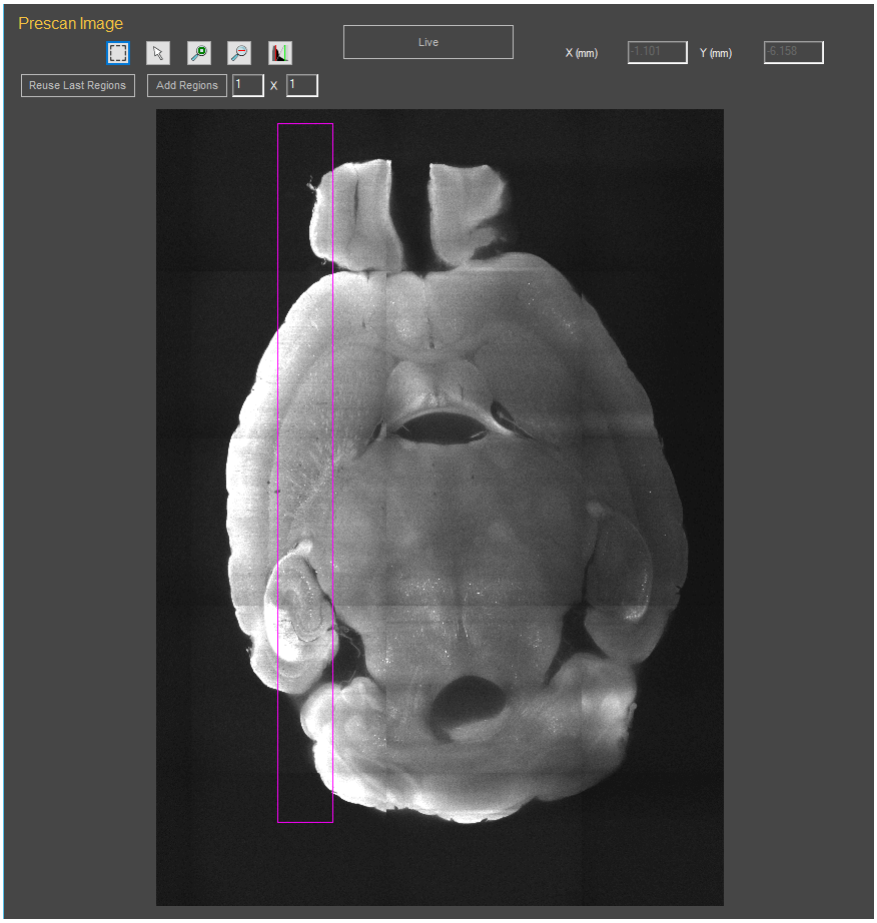
From left to right are the Marquee tool, the Selector tool, Zoom to Region tool, Reset Zoom tool and a Histogram tool that may be used to alter the LUT for the image in the ***Prescan Image*** Window.

1. Navigate to the ***Capture*** panel and in the ***Laser Selection*** group choose the ***Left*** excitation objective in the ***Laser Selection*** group.

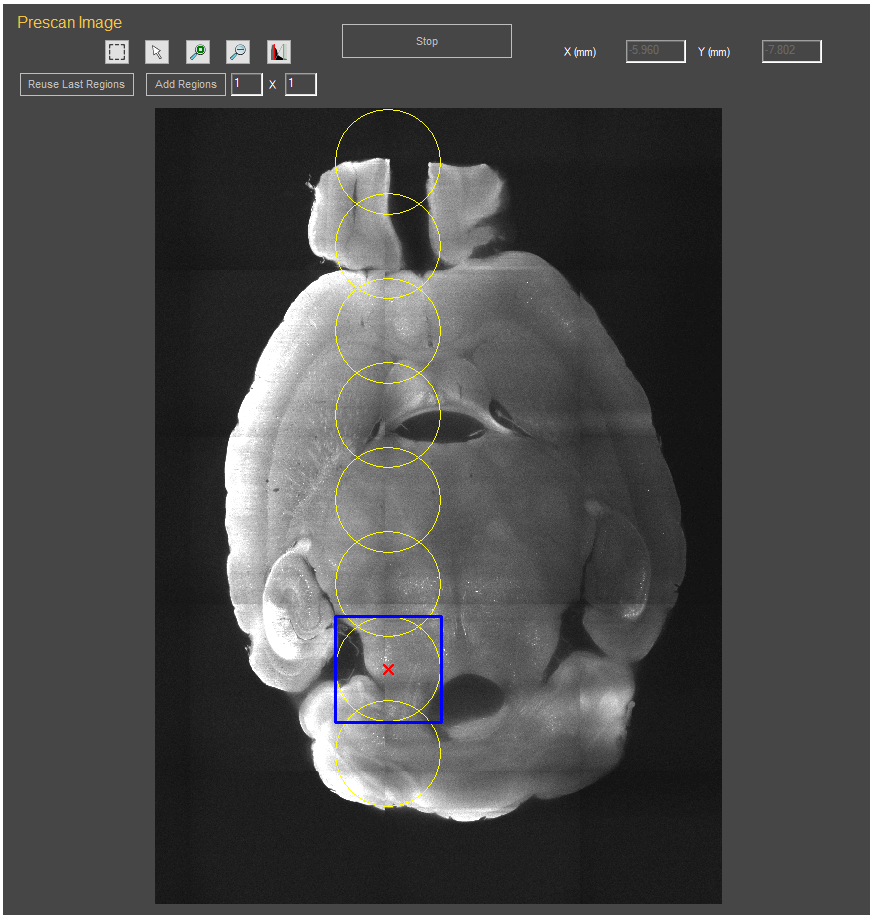


2. Choose the Marquee tool and then navigate your cursor to any portion of the 2D specimen map.

3. Click the left mouse button and drag the cursor over any region of the 2D specimen map. A magenta colored box will appear that marks an area of the 2D specimen map where regions will be created.

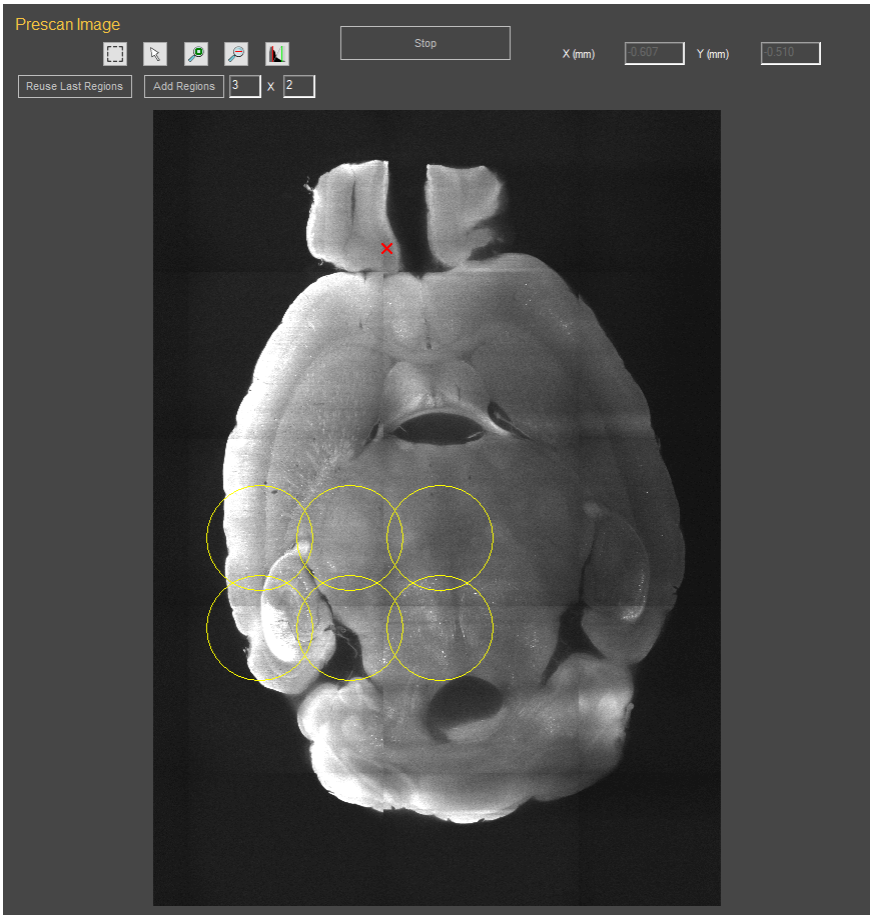


4. When you release the left mouse button regions will appear in the area that you previously described by the magenta box. In this example, the regions appear as yellow circles, representing regions that will be interrogated by the left excitation objective. If the circles are blue, they represent regions that will be imaged with the right excitation objective. Choose the Selector tool and navigate to a region. Click on that region and a blue bounding box will appear over that region and a red X will appear in the center of the yellow circle. This region will now appear in the ***Live Window*** at a zoom-factor that is defined in your capture settings.

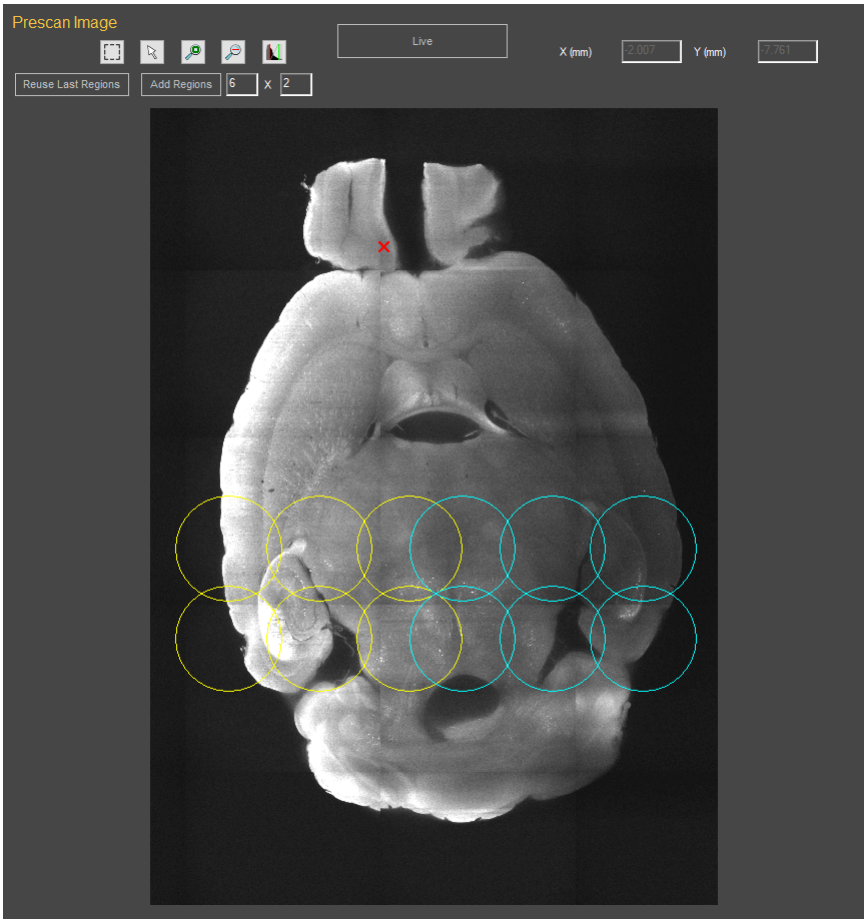


5. You may also choose to have SlideBook automatically add regions. Navigate to the upper left corner of the ***Prescan Image*** group. Immediately beneath the ***Prescan Image*** tools, navigate to ***Add Regions.***

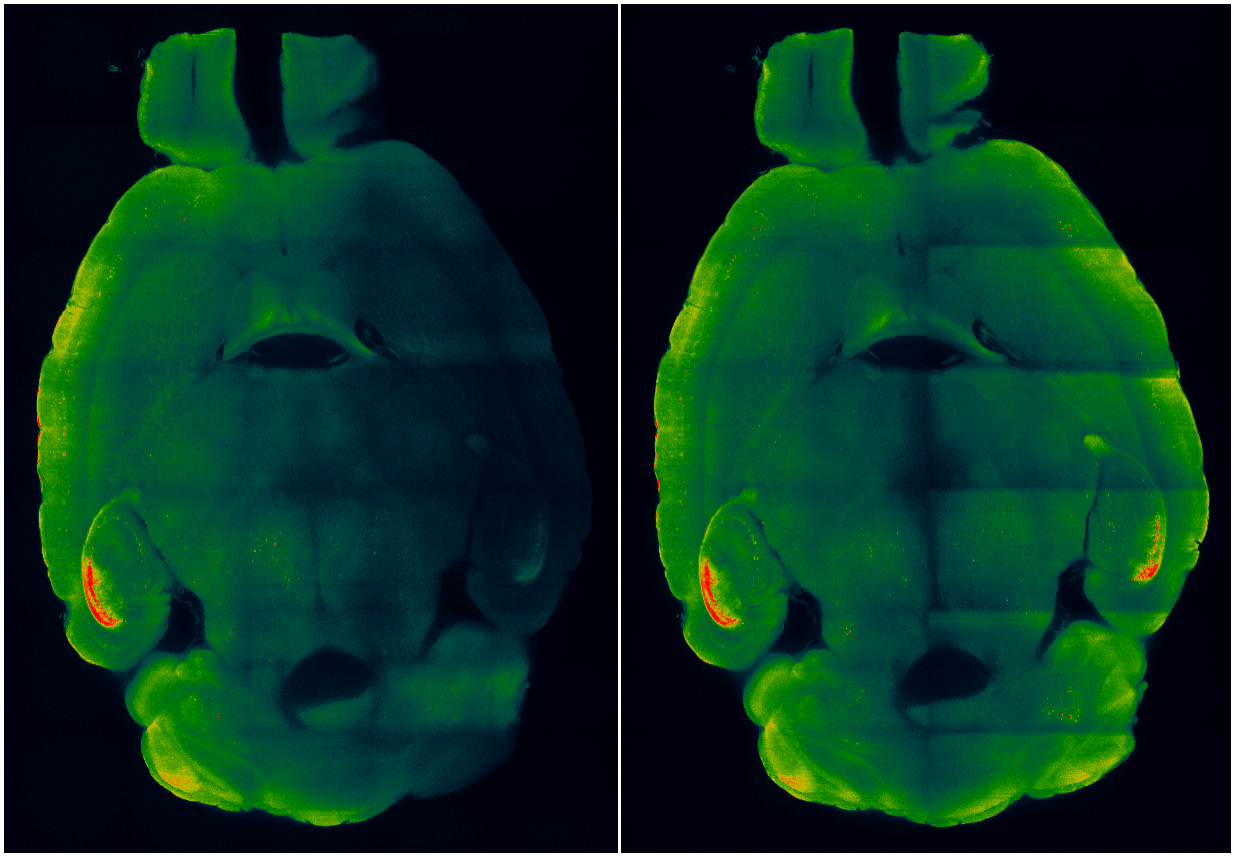
The variable boxes are editable. The first box represents the columns SlideBook will create, the second, the number of rows. Enter the value ***3*** in the first variable box, enter the value ***2*** in the second box. Click ***Add Regions***. A total of six regions will appear on the 2D Specimen Map. ***HINT***: a right-mouse button, click and drag can move those regions anywhere on the 2D Specimen Map.



6. Finally, navigate over to the ***Capture*** panel and in the ***Laser Selection*** group choose ***Both***. In the ***Add Regions*** variable boxes, insert the values ***6*** and ***2,*** to create 12 regions, 6 columns and 2 rows. Click ***Add Regions***.



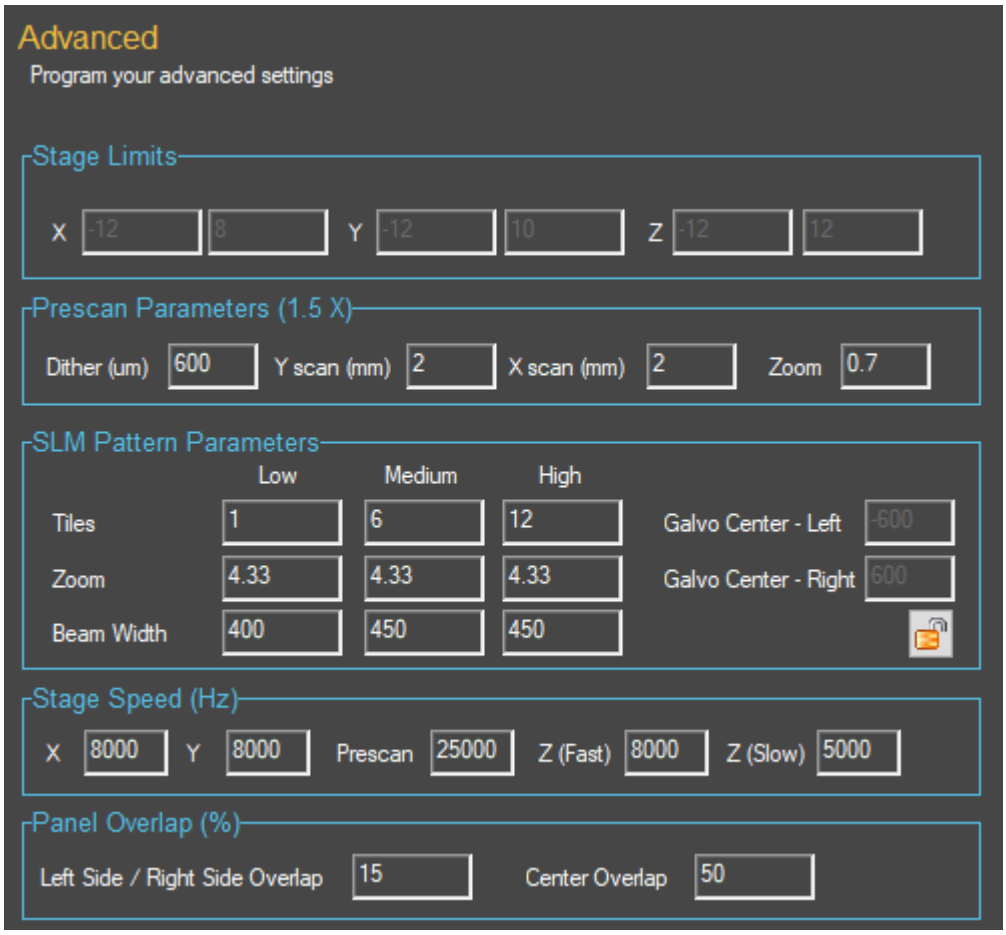
Note: Yellow circles represent images that will be interrogated by the left excitation objective, the blue circles by the right excitation objective. Depending on the size of your sample, you may need to image the specimen with both the left and the right excitation objectives. Per Beer's law, the photon budget of the light sheet will be reduced as the sheet propagates through space. This is demonstrated in the image set below which shows this brain, measuring 10mm in width, illuminated by just the left side laser (image on the left) and both lasers (image on the right).



TUTORIAL: Setting Region Overlap

The aim of the CTLS microscope is to create a 3D view of a large, cleared specimen. This is achieved by capturing several Z-stacks, region x region, as defined in the 2D specimen map and then stitching them together. In order to ensure that the regions are stitched together seamlessly, in this case, meaning without any obvious seams between each regional Z-stack, SlideBook will enforce some region x region overlap. If the overlap between each region is set to 0% you could reduce the total number of regions that need to be visited to visualize the sample. However, when the regions are stitched together, there may be a risk of montaging errors that will appear as seams, horizontal or vertical "shadows" in the image. In this tutorial, we'll learn how to set Region Overlap to maximize image capture duration or ensure the best quality 3D image rendering of the sample.

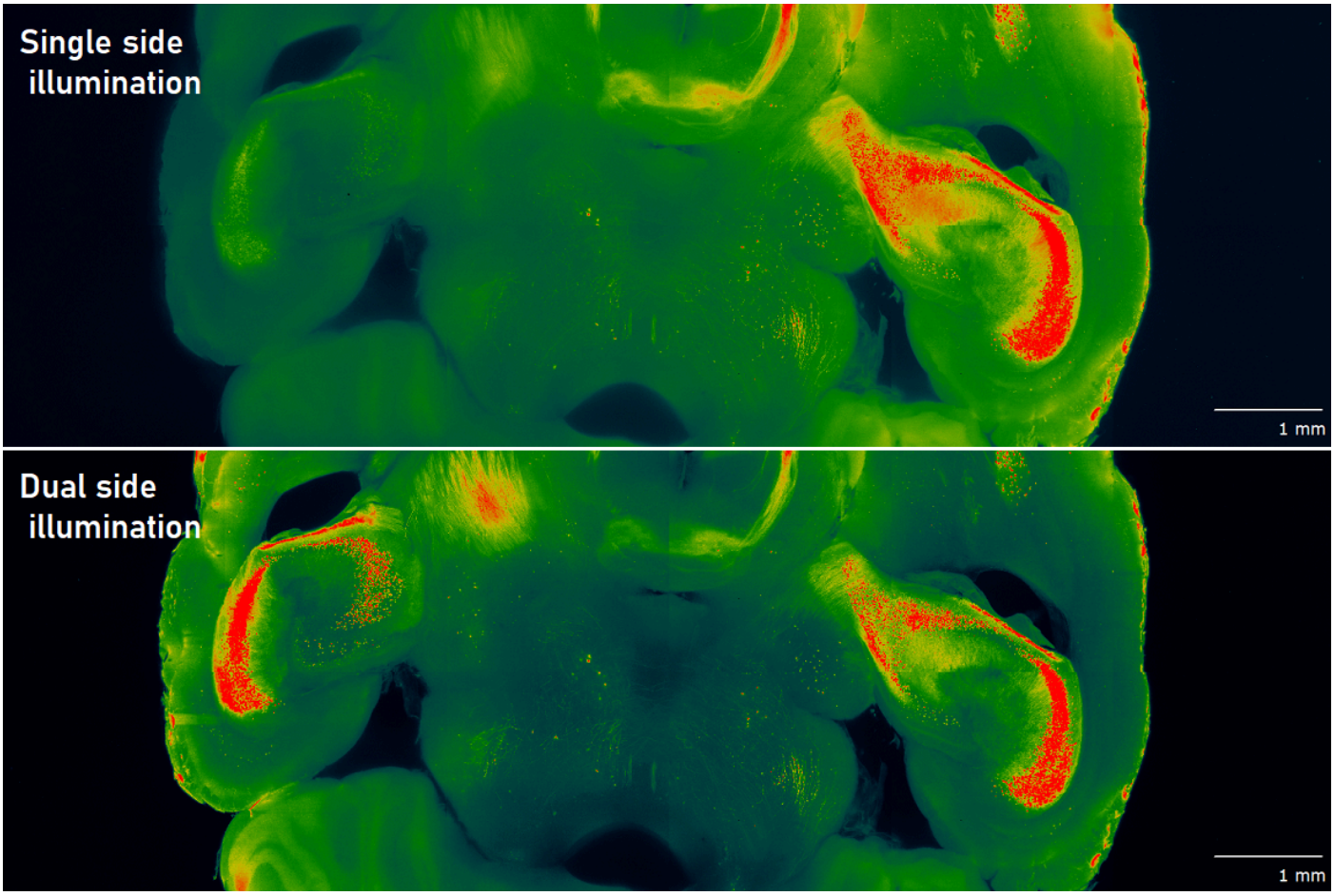
The region overlap tools are located in the ***Advanced*** Settings panel. Navigate to ***Panel Overlap (%)***



The ***Panel Overlap (%)*** group is composed of two editable variable boxes. ***Left Side / Right Side Overlap*** defines the percent overlap that SlideBook will impose on regions that will be interrogated by the excitation objectives to the left or right of the specimen. ***Center Overlap*** will define the percent overlap of the center-most regions that are set in the 2D Specimen map. The default values are 15% and 50% respectively.

Suppose that we have a totally of 6 regions plotted across the width of a specimen, let's call them R1-R6.

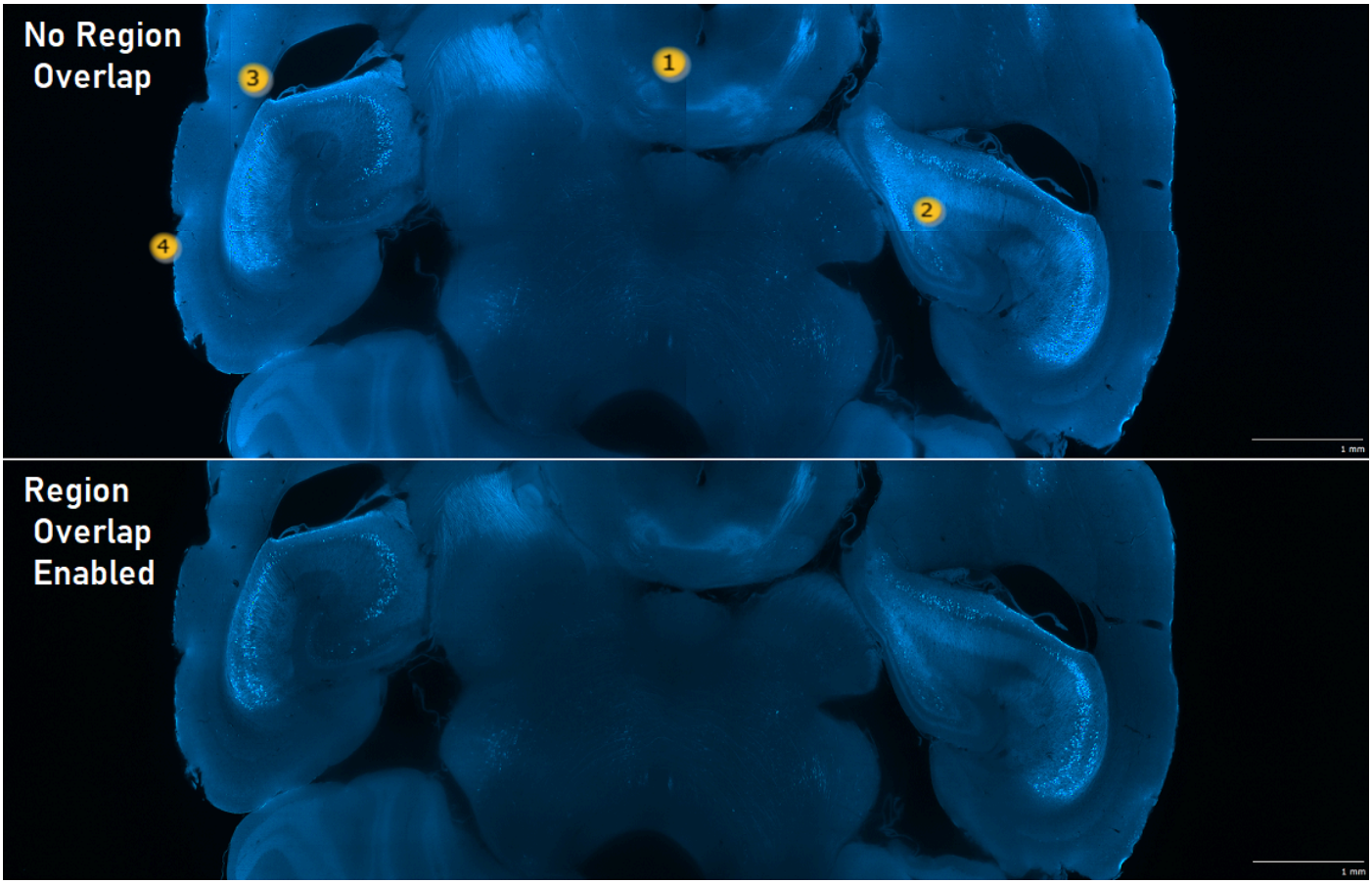
Each region will be approximately $2mm^2$. Thus in the example provided here, CTLS will be scanning across a 12 mm horizontal strip. Since the Gaussian beam will be subject to scatter the photon budget applied at region 1 will be quite different from that applied at region 6. If you use single-sided illumination to interrogate this strip, you'll end up with data set that is unevenly illuminated as illustrated below. In the example illustrated below, for single side illumination, the right illumination objective is used.



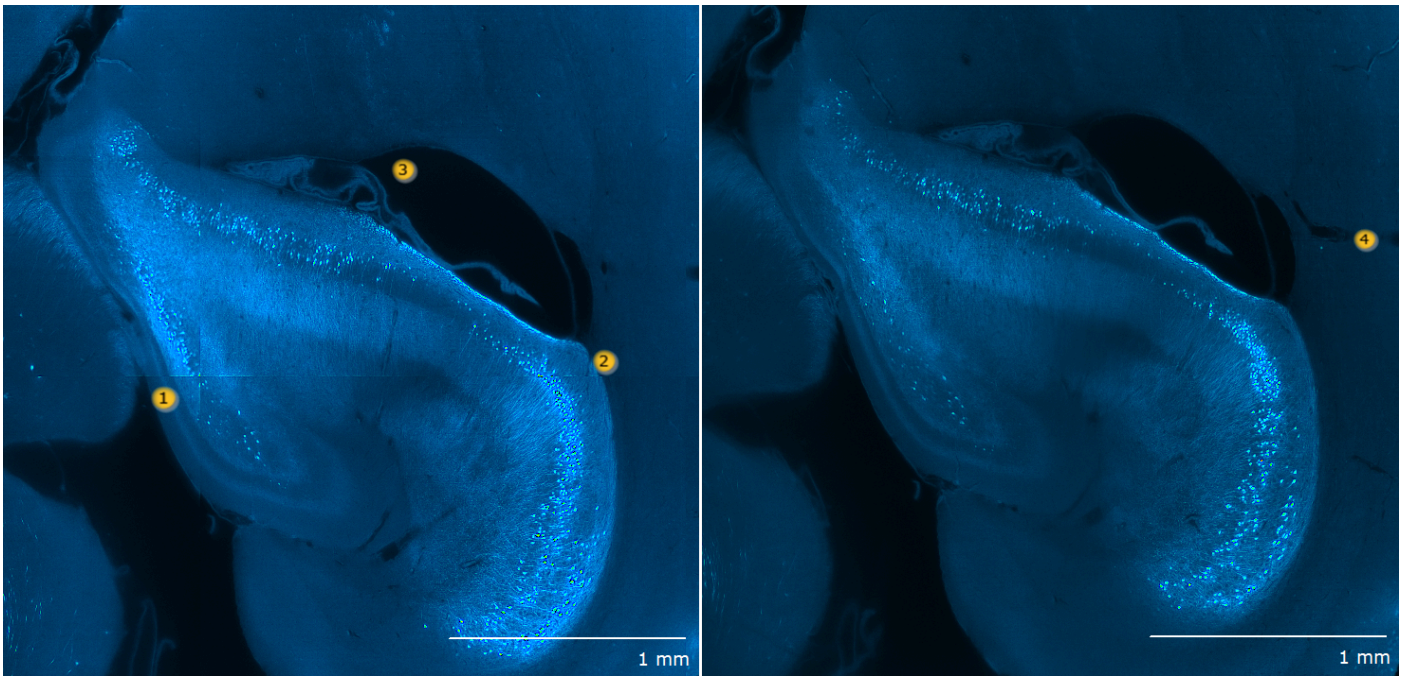
Since regions 1-3 will be interrogated from by the left excitation objective let's further define these as L1-L3. Where L1 is the region closest to the objective, the L3 the most distal. Thus R4-6 may be described as R1-3, the balance of the regions that will be interrogated by the excitation objective on the right. Similarly, we will describe region R1 as being closes to the excitation objective to the right side of the specimen, and R3 being furthest away.

By interrogating the sample with both excitation objectives, left and right, we realize a more uniform deliverable.

In the example illustrated below, regions have been acquired with 0% overlap. as it visits all of these regions. In this data a 2D Laplacian filter was administered to strongly visualize the seams. These are quite obvious at the midline (1) and in the hippocampus (2); a vertical seam can also be seen in the left hemisphere (3) as well as a horizontal seam (4). These seams can be neutralized by SlideBook be applying ***Panel Overlap*** values. In this case, we''ll use the default values of 15% overlap between regions L1 & L2 and R1 and R2; regions L3 and R3 will overlap 50%.



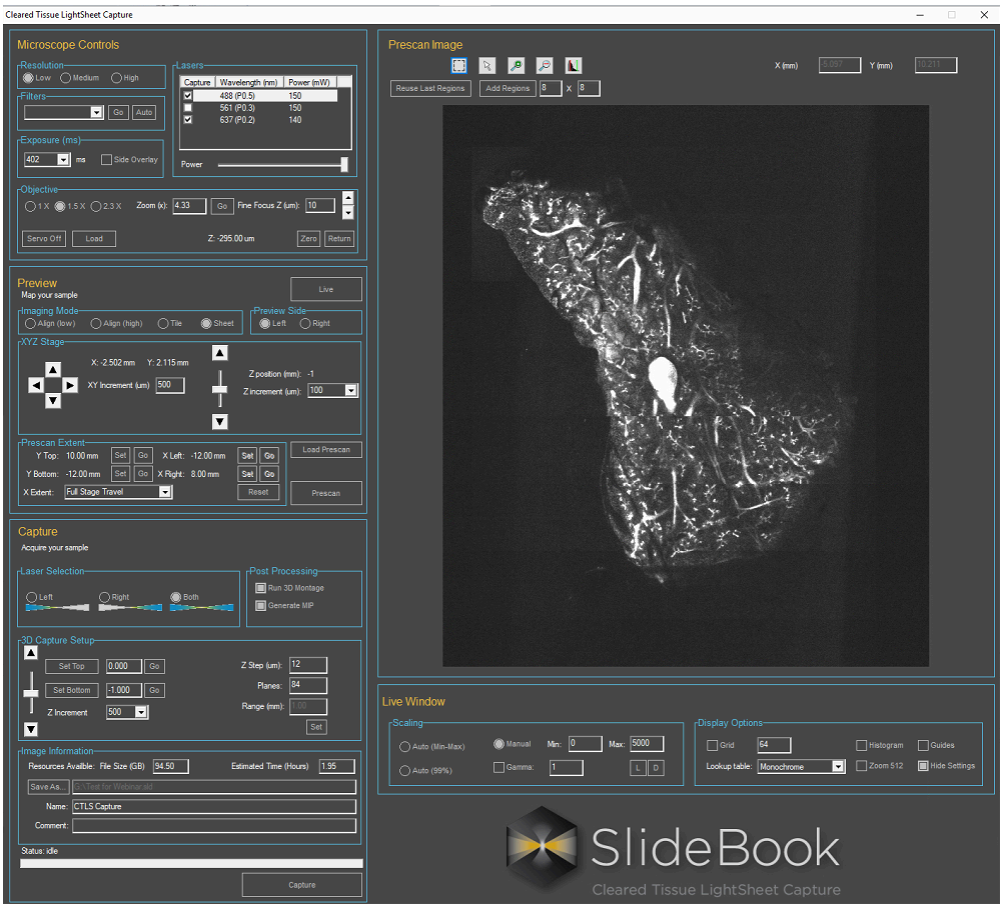
Here's a detail of these data, showing the hippocampus on the right side. In this illustration, the seams in the dentate gyrus of the hippocampus (1) are very easy to see, as well as a horizontal seam at the CA2 region. These are completely neutralized when ***Panel Overlap*** is administered during image capture. However, compare the morphology of the ventricle (3) and the blood vessel in the right cortical hemisphere (4). A keen understanding of the anatomy of your structure will be critical as you apply ***Panel Overlap***. You obviously don't want to see any seams in your 3D montage, but you also don't want to misrepresent the data by being over-aggressive in administering the region x region overlap (as we were here).



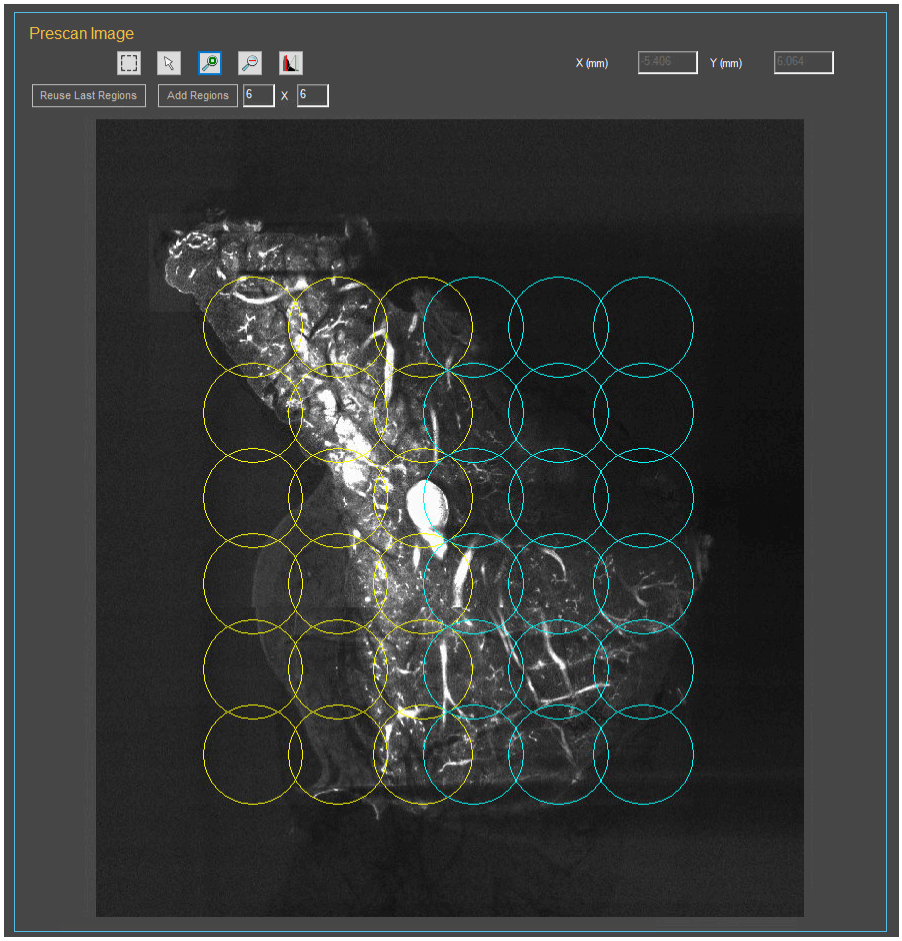
TUTORIAL: Omitting ROIs to Visit

The CTLS microscope has several features that are aimed towards maximizing efficiency. In this tutorial we will learn how to skip Regions of Interests, thus minimizing the time required for CTLS to provide you with a deliverable. Region Omission is a unique feature of the CTLS microscope and the data that are generated may only be viewed in SlideBook's 3D viewer. In order to complete this tutorial we will assume that you know how to create a 2D specimen man using ***Prescan*** and that you are comfortable creating Regions of Interest.

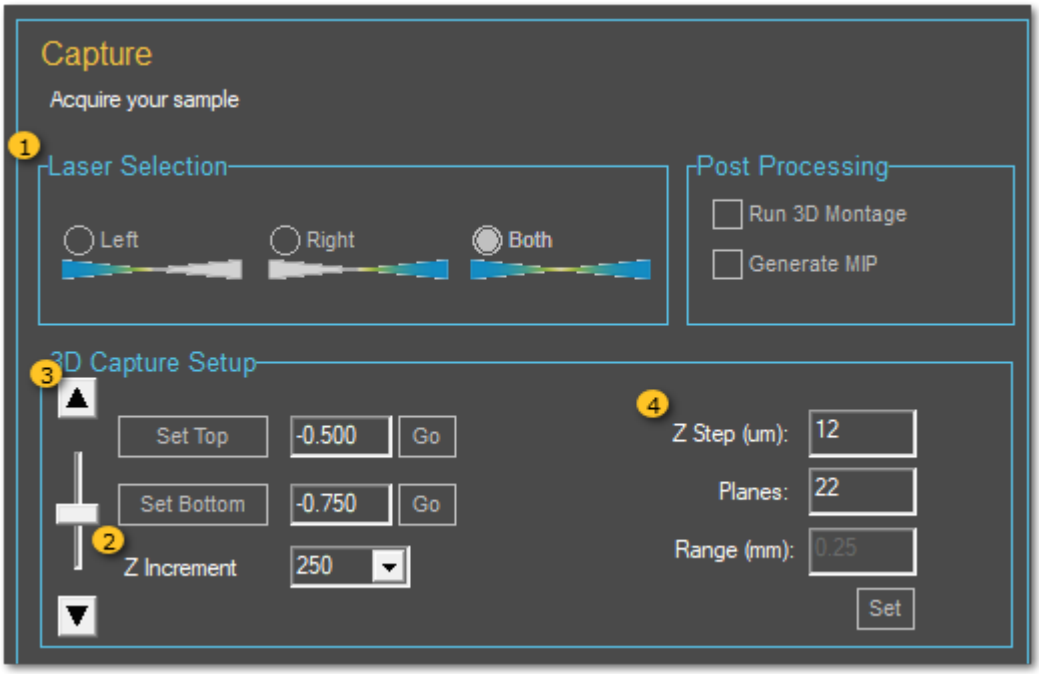
1. Setup a specimen in the imaging chamber and capture a 2D specimen map using ***Prescan.***



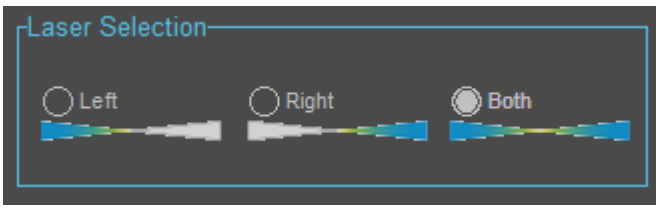
2. Using the ***Add Regions*** tool create 6 x 6 regions and then center those regions over the 2D Specimen map ***(press right-mouse button and drag)***



3. Navigate to the ***Capture*** panel



1 **Select Both Excitation Objectives**



In the ***Laser Selection*** group click on the ***Both*** radio button

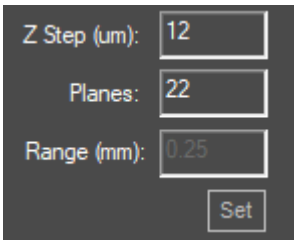
2 Set Z Volume

Navigate to the ***3D Capture Setup*** group. Find the ***Z Increment*** variable window. Click on the down arrow to reveal a menu of Z steps in microns. For the purposes of this exercise choose 250. This will set the stage to travel 250 microns.

3 Set the upper and lower limits of the Z stack

Navigate to the Z translation tool and use the up and down arrows to ***Set Top***, that is the upper limit of the Z-stack and then ***Set Bottom***, to define the lower limit of the Z-stack.

4 Set the Z internal



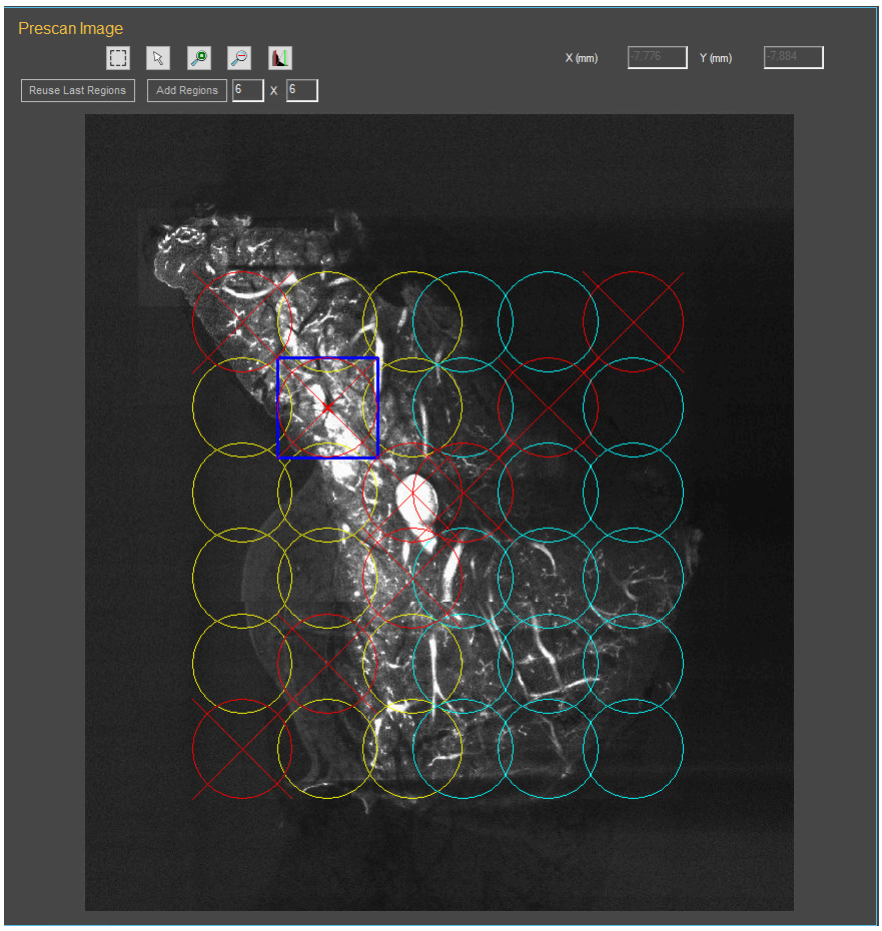
The variable box next to ***Z Step (um)*** is editable. For purposes of this exercise, let's set this value to 12. This means, that an image will be captured after Z-stage steps 12 microns. Thus, in a 250-micron volume, the stage will step 22 times. Click ***Set.***
4. Navigate to the very bottom of the GUI and just to the left of the SlideBook Banner you'll find the ***Capture*** button. Click it.

Be sure to give this file a unique name, something like "all 36 regions".

TIME HOW LONG IT TAKES TO CAPTURE YOUR DATA SET.

SlideBook will now travel to all 36 regions that have been defined, capturing a Z-stack in each of the regions. In the example as described above, it took approximately 4 minutes to capture Z-stacks in all 36 regions. The data will be saved in the open SlideBook file on your desktop.

5. Navigate back to the 2D Specimen map. Now, hover over a region, then press the SHIFT key on your keyboard and click the right mouse button. A red X will appear over that region. Repeat this for 9 regions.

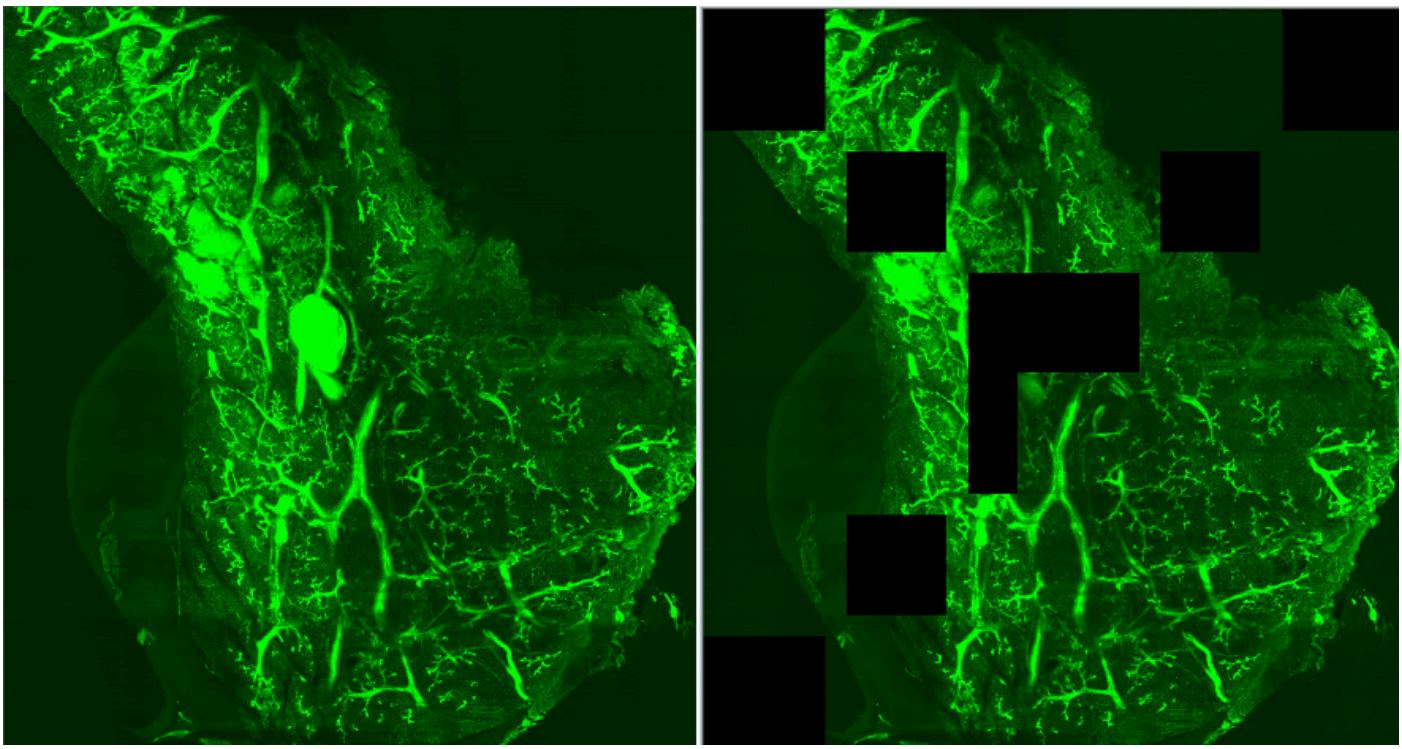


Navigate back to the ***Capture*** button. Click it. Don't forget to give this data set a unique file name.

TIME HOW LONG IT TAKES TO CAPTURE YOUR DATA SET.

Having removed 9 regions to visit, or 25% of the regions, SlideBook will be able to capture this dataset faster. In this particular case, the acquisition time was now reduced to 3 minutes from 4 minutes.

Let's take a quick look at these data. You'll learn how to visualize the data using SlideBook's ***3D Montage*** tool in another lesson.



The image on the left is a maximum projection of the data where all 6 regions were interrogated. The image on the right shows a maximum projection of the data where the 9 regions where omitted.

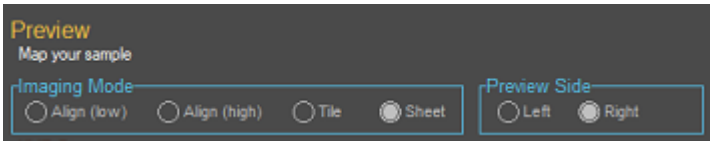
First, this is an extreme example to make the point about the functionality of the Omitting ROIs tool. Second, note that not all the regions are identically shaped. The shape of the omitted regions in the image on the right reflects the amount of region overlap that was defined in ***Advanced*** settings. Note that the shape of the regions in the three corners are identical whereas the three regions proximal to the 3 corners are a bit smaller. Those regions are smaller because (at least for the purposes of this exercise) there is 15% overlap with the adjacent regions above, below and to the left and right. The oddly shaped region in the center of the image is the result of three contiguous regions being skipped.

Use this tool selectively. The tool was designed to reduce the time required to capture a 3D dataset to evaluate. While these data are readily managed in SlideBook's 3DViewer, we do not know how other rendering packages manage these data.

Data Acquisition

Acquiring data is very easy. In this section, we will assume that you are familiar with beam alignment, the ***Prescan*** tool and that you can create regions of interest to visit.

1. In the ***Preview*** panel make sure that ***Sheet*** is selected in ***Imaging Mode.***



2. Select an objective to preview the specimen in the ***Preview Side*** group. SlideBook will default to creating a beam with the left-side excitation objective.

3. Click on the ***LIVE*** button. The ***CTLS Live Window*** will appear.

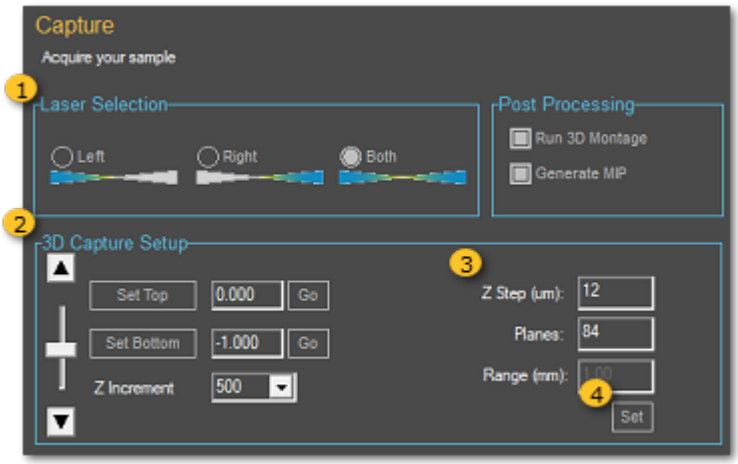
4. Choose any region that you've defined on the 2D Specimen map in the Interactive ***Prescan*** window.

NOTE: It might be useful to navigate to the ***Microscope Controls > Objective > Zoom(x)*** and set the Zoom factor to 1X or 2X.

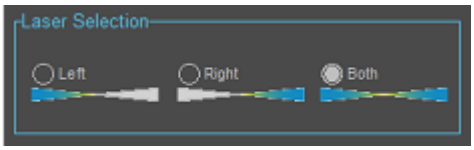
5. In the ***Preview*** panel navigate to the ***XYZ Stage*** group and use the up and down arrows on the Z-stage slider to discover the upper limit (top) and lower limit (bottom) of your sample.

NOTE: Visit several regions in the 2D Specimen map to confirm the upper and lower (top and bottom) limits of the specimen.

6. Once you're satisfied that you've discovered the upper and lower limits of the sample navigate to the ***Capture*** panel.



1 Select an imaging method



In the ***Laser Selection*** group choose your imaging method, single sided (***Left*** or ***Right*** excitation objective)or dual side (***Both***) if the specimen is wider than 4 mm.

2 Set the Top and Bottom limits of your specimen

Use the Z-stage slider to set the upper (***Top***) and lower (***Bottom***) limits of the specimen. The variable window, ***Z increment***, is a pulldown menu. Click on the arrow to reveal the menu and choose a Z-step from as little as 10 microns to 1000 microns.

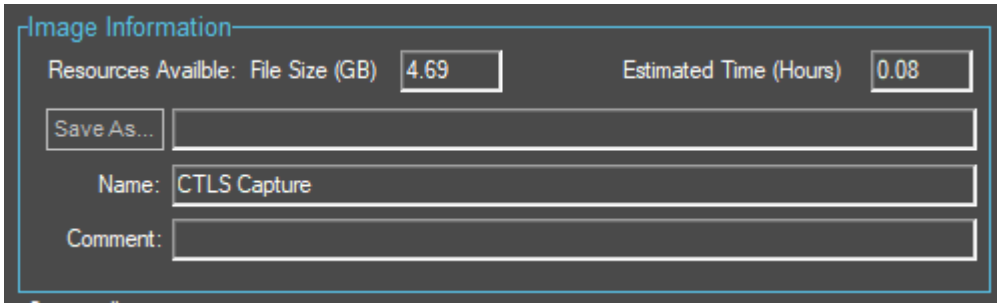
3 Choose a Z-interval

Z Step (um) is an editable variable window. Depending on the goal of your experiment, set the Z-interval here. If you are using the 1.5X objective, a Z-step of 3 microns will provide a near-nyquist sampling state. If you are using the 1.0X objective, a Z-step of 6 microns will provide near-nyquist sampling. If you want to acquire an image for publication, we recommend that you over sample.

4 Set the Acquisition Parameters

Click ***Set*** to save and load the capture parameters into SlideBook

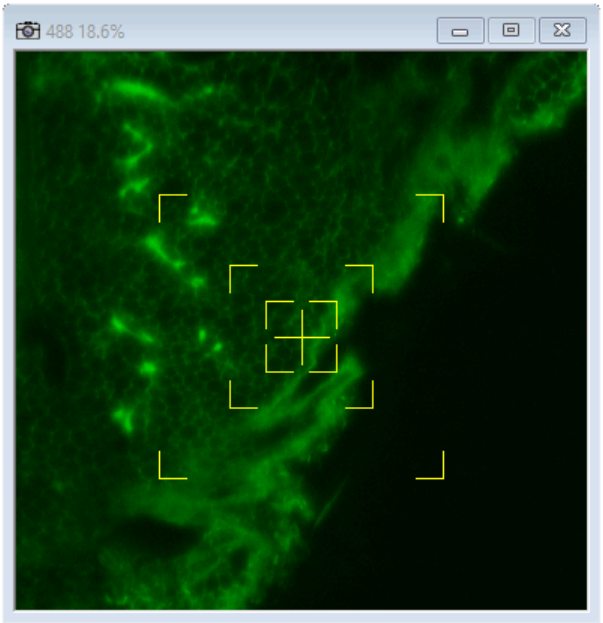
7. After you click the ***Set*** button the ***File Size (GB)*** and ***Estimated Time (Hours)*** will autopopulate in the ***Image Information*** panel. Take this time to provide a unique file name for your data in the ***Save As*** variable box. Be sure to place it in directory that will provide enough space to store the data. **DO NOT SAVE THE DATA ON THE DESKTOP.**

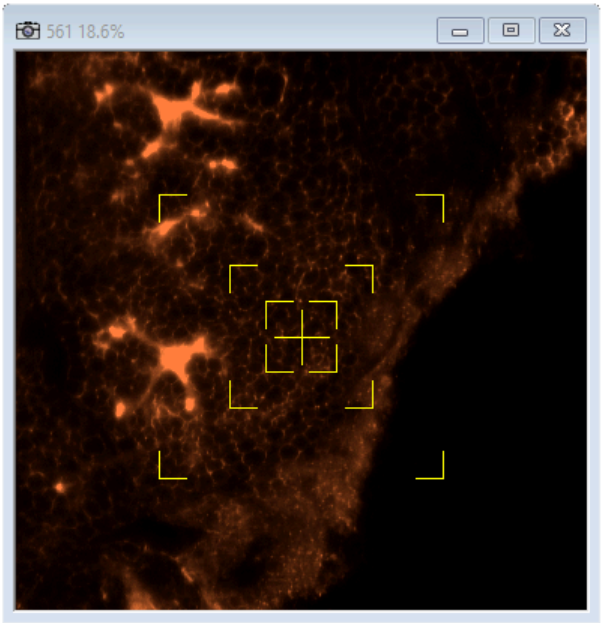


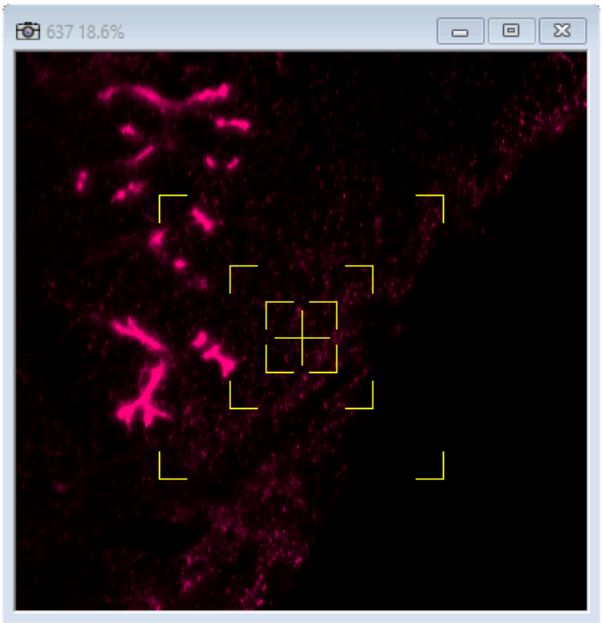
8. You're ready to go! Click the ***Capture*** button.

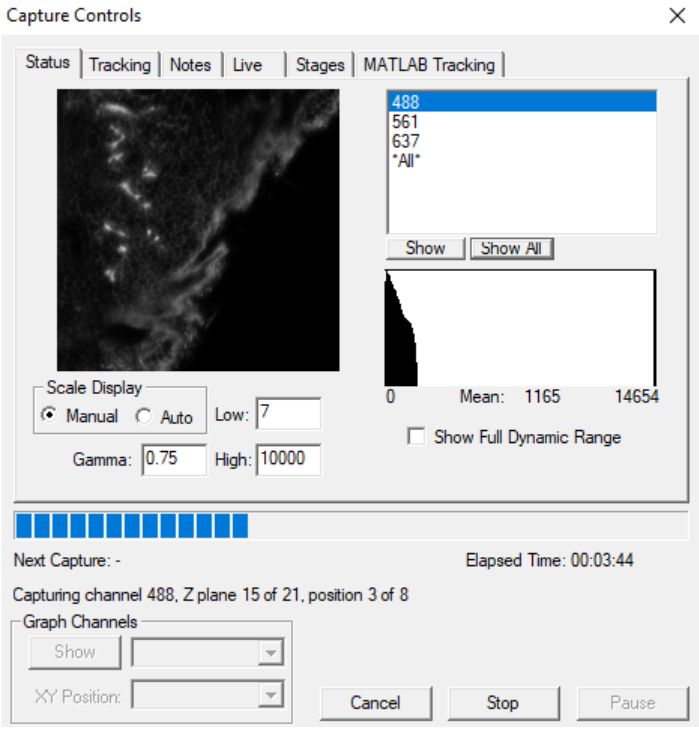
During CTLS Capture

During image capture, A live monochrome window shows individual planes as they are acquired. You may open separate windows for each channel by selecting a channel in the Status tab of Capture Controls and clicking ***Show*** or ***Show All*** to open a window for all available channels.









SlideBook is also capable of generating a 2D MIP of the dataset in real time. To enable this feature, navigate to the ***Capture*** panel > ***Post Processing*** and click the Box ***Generate MIP.***

As the data are being captured, SlideBook will populate on the fly a maximum image projection of each region. The aim of Real-Time MIP is to provide a window that shows the progress of image capture.

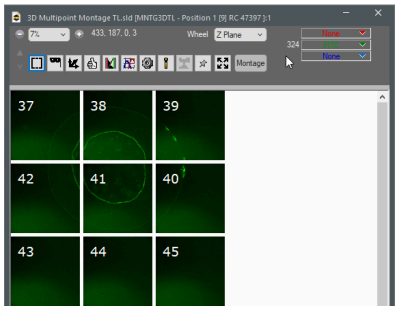
CTLS Post-Acquisition Processing

CTLS generates .sld files that are composed of individual regions, or fields of view. Each region is a Z-stack. In order to visualize the data as a 3D volume, these individual regions need to be montaged in 3D. However, the individual data, or the individual FOVs may also be viewed and interrogated by plane.

Montage Tile View
2D/ 3D Montage
3D/ 4D Volume View
Bounding Box
Movie Maker

To view the individual FOVs select/open the dataset in SlideBook. Navigate to the Quick Access Toolbar and choose **View > Montage Tile view**

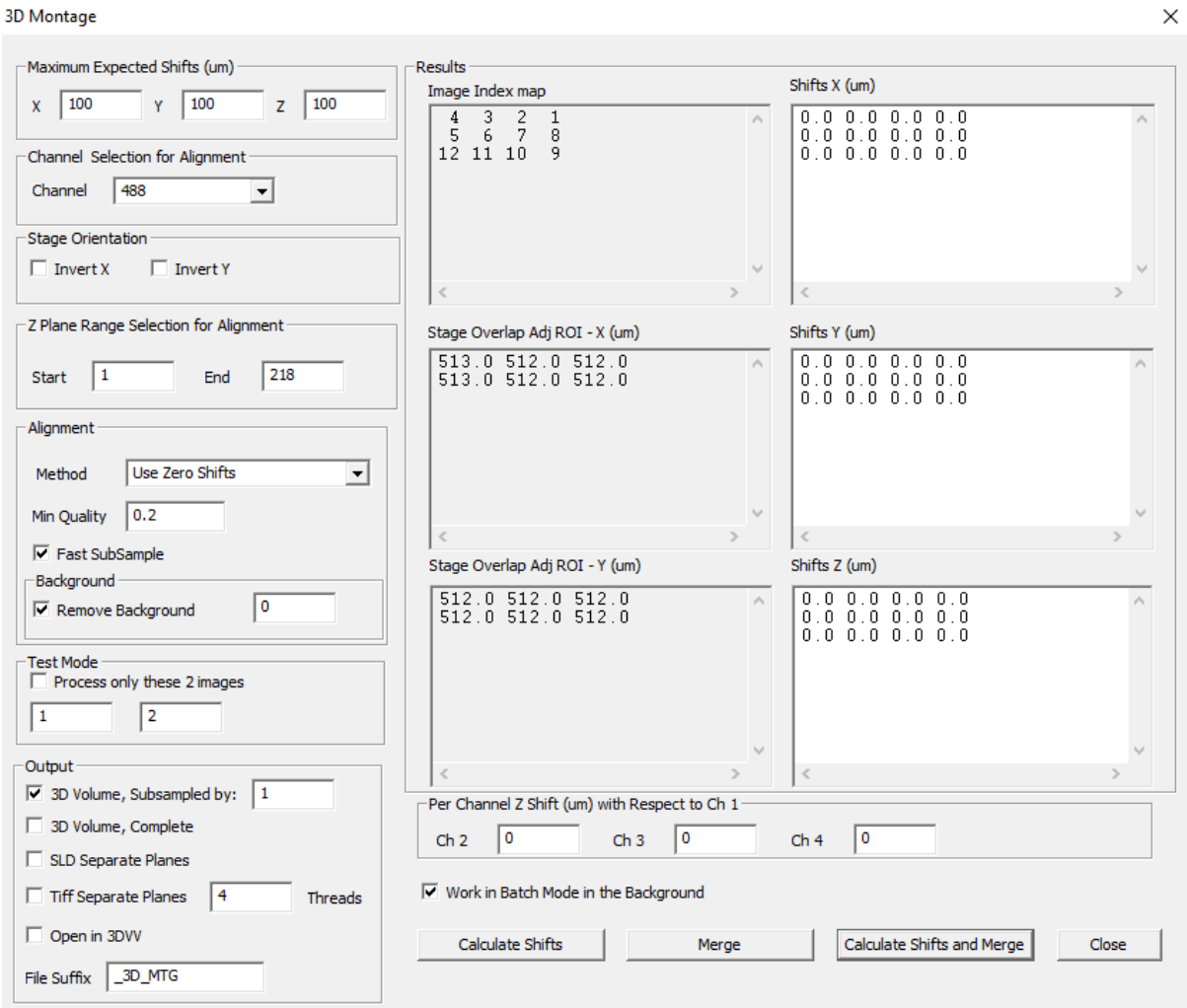
This tool will display all of the individual FOVs. You can browse individual planes by simultaneously pressing CTRL and G button. Add a value in the **Go to plane** variable window to browse that plane. When you enable ***Montage*** the image will sort the individual tiles according to the montage capture.



SlideBook can montage these data to create a 3D volume seamlessly and based on user inputs, quickly and with varying degrees of accuracy. The 3D montage may be saved as individual tiffs that may be imported into other 3rd party software applications for rendering or analysis. Data may also be montaged in 3D using a batch processor that will permit the user to have access to all the acquisition tools in the CTLS.

1. Highlight the dataset to be processed and then navigate to the Quick Access Toolbar and choose **Montage >2D/ 3D Montage**

2. Clicking Montage in The Timelapse Tile View and opening the 2D/3D Montage will launch this GUI



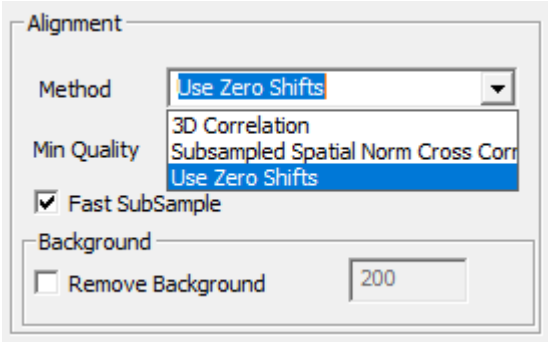
There are several tools here that may be leveraged to decrease the time required or optimize montaging. The values in **Maximum Expected Shifts** may be thought of as the search range that SlideBook will reference to build a 3D montage. The lower the value, the smaller the range, and the longer it will take to create the montage. Larger values will reduce the amount of time required to calculate a result. Values should NEVER exceed 2mm in X and Y. 100-microns is the default setting and returns very good results.

Channel Selection for Alignment identifies the channel that will be used as a reference for montaging. Choose a channel that has a plenty of structures for SlideBook to reference. The 488 channel is the default setting.

99.9% of the time the values in **Stage Orientation** will not need to be altered. These tools are here for support purposes should you discover a problem in executing the 3D montage.

Finally **Z Plane Range Selection** allows you to select the planes that SlideBook will use to build the 3D montage. It's best to identify about 100 - 200 planes **in the middle** of the sample for SlideBook to reference.

3. SlideBook can calculate a montage one of three methods, **3D Correlation**, **Subsampled Spatial Norm Cross Correlation**, or **Zero Shifts**



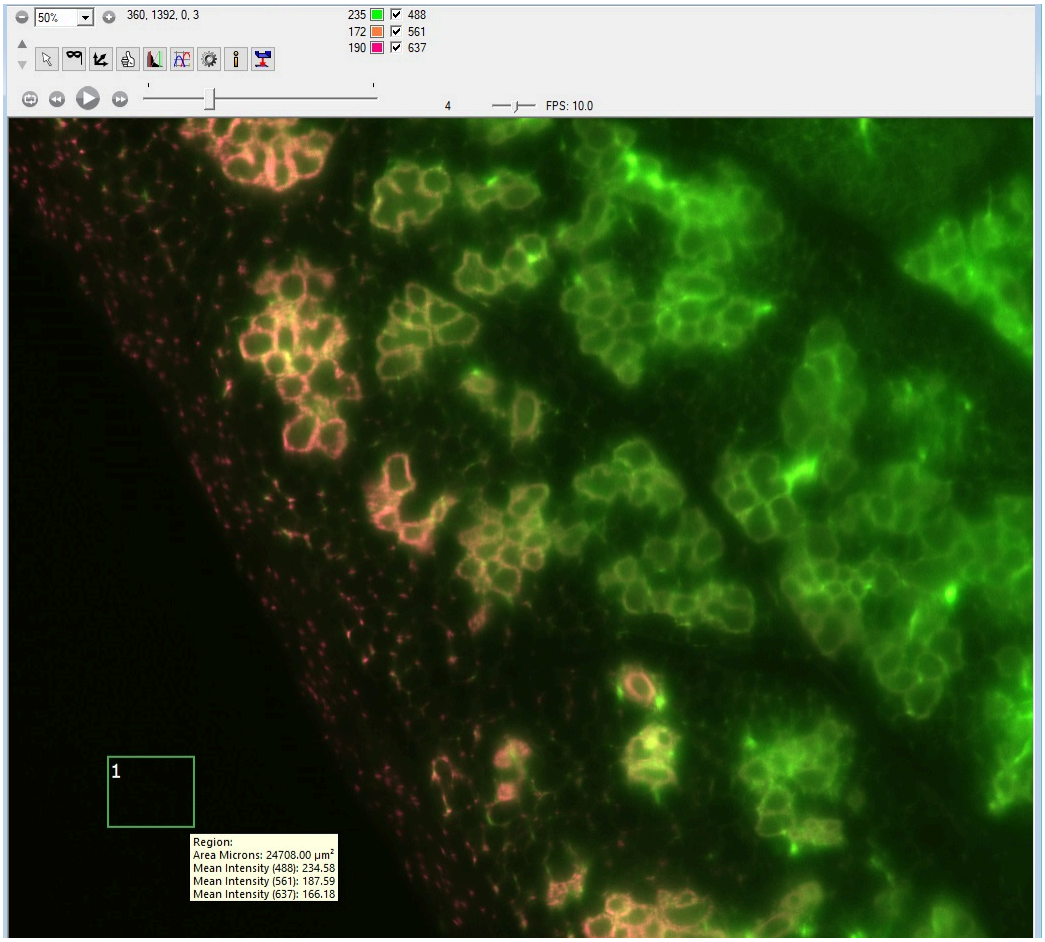
Briefly, when the **3D Correlation** algorithm is used SlideBook will calculate within an individual region and it's associated Z-planes a 3D FFT. This is a very time consuming algorithm to execute but results in a very accurate montage. The **Subsampled Spatial Correlation** algorithm will compress an individual region into a Maximum Image Projection (MIP) and then calculate the montage based on the intensity values of the MIP; also a very time consuming algorithm and based on the properties of your sample (such as a sample that is largely homogeneous in it's cell population) also very accurate. When **Zero Shifts** is chosen, SlideBook will make its best guess to montage the data in 3D using the Cartesian coordinates defined by the XY stage.

As a point of reference, **Zero Shifts** will create a 3D volume 3X faster than **Subsampled Spatial Correlation**. **3D Correlation** takes the longest, about 4-5X slower than **Zero Shifts**.

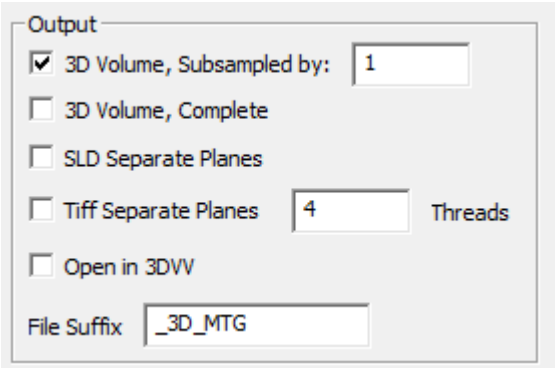
4. Depending on your sample, you may want to execute a background subtraction *a priori*. An *a priori* background subtraction will provide a nice, high contrast 3D volume and since you're already committing some time to produce a 3-volume, why not execute a background subtraction as well well.

In the Alignment group, click **Remove Background**. When you engage this tool, the variable window will become active and a background value may be set. To measure background navigate to the Tool menu and select one of the region tools. The Box usually works just fine.

Navigate your cursor over a portion of the image that does not contain any data ("background) and then click the right button and drag to create a region. Dwell the cursor over this region and a pop-up will appear that will show the area of the region as well as the mean intensity of the channels in this region. Use this mean intensity value to define the background value that you will subtract during the 3D Montage. Don't be too aggressive! ***A priori Background Subtraction*** doesn't happen "for free" since an additional algorithm is being executed. If it takes 10 minutes to build a 3D volume without **Remove Background** enabled, expect SlideBook to take twice as long to build the 3D volume with **Remove Background** enabled.



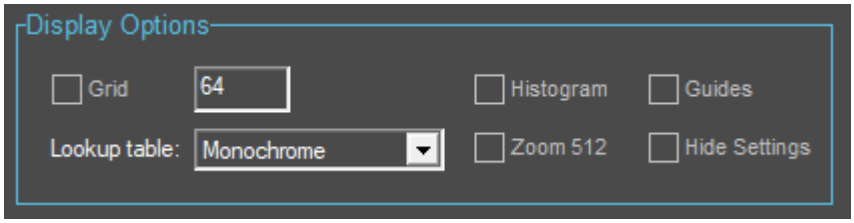
5. There are several ways to output the 3D volume in SlideBook



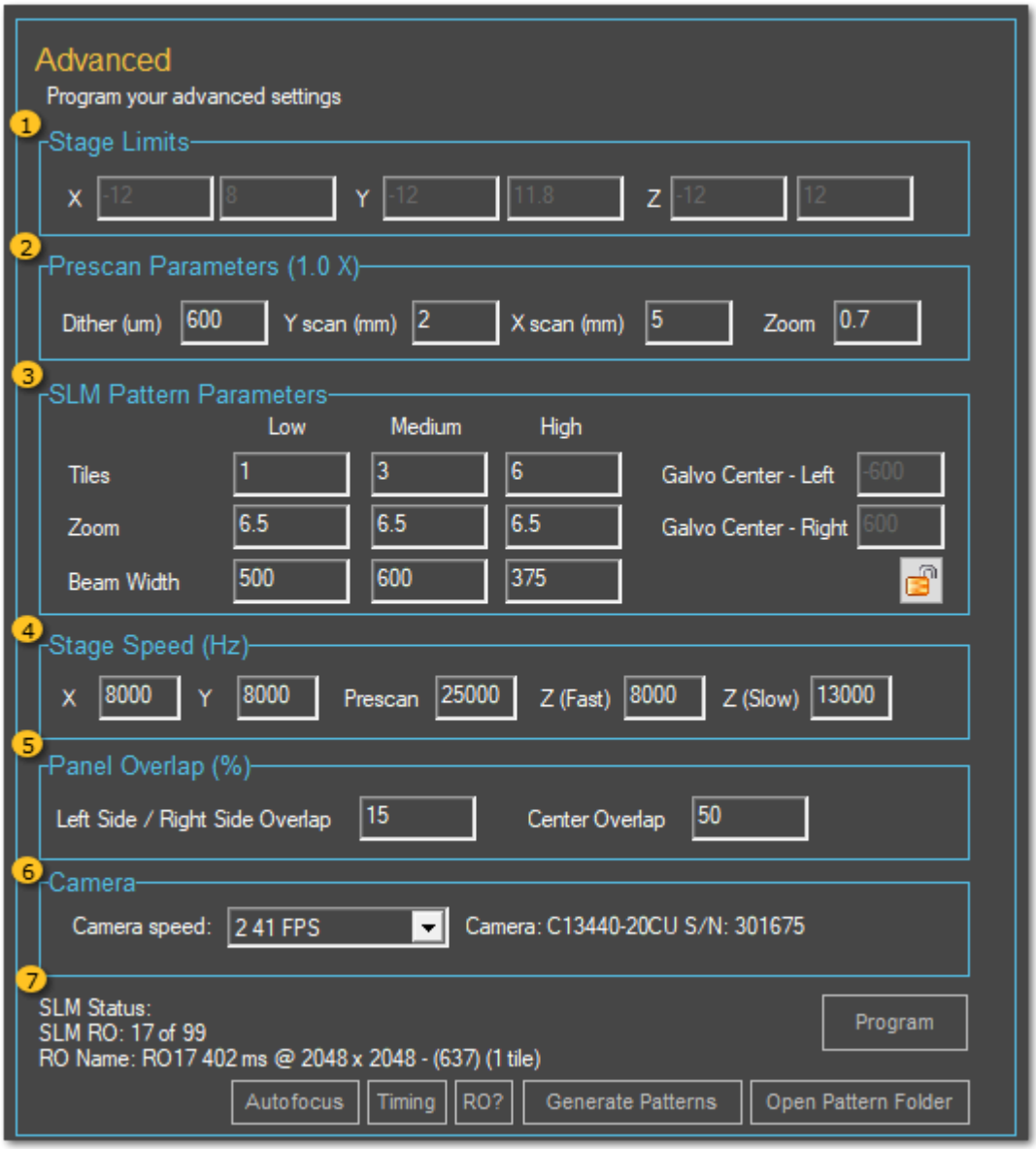
The quickest way to generate a 3D Volume is to subsample the data. SlideBook will automatically calculate the best subsampling needed to view the data in SlideBook's 3D viewer based on the amount of RAM present on your computer. A complete 3D volume may be executed, but based on the size of the data, it may not be visualized in SlideBook's 3D viewer based on the available RAM. In such cases. Individual Z planes may also be saved as .SLDs or as unique .tiff files that you may export to any number of 3D analysis/rendering 3rd party software.

CTLS Advanced Settings

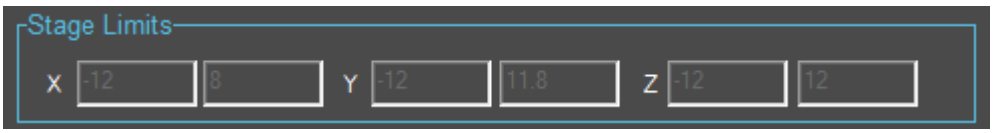
The ***Advanced Settings*** group is often hidden. It can be revealed by navigating to the **Display Options** group then enabling or disabling the box ***Hide Settings***



Nearly all of the variables in this group will be set upon installation of your systems. 3i will provide a detailed explanation of each of these settings at the time of installation. Here we will provide a brief overview of each setting and provide some background to variables that could be useful to manipulate. Generally speaking, try to avoid making significant changes to these settings.

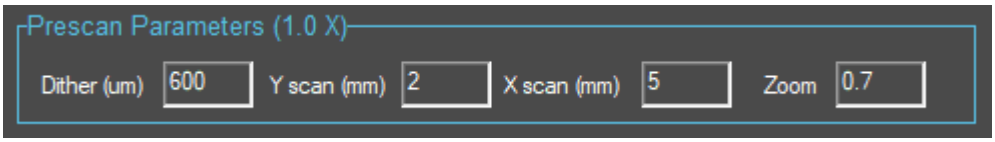


1 Setting the Stage Limits



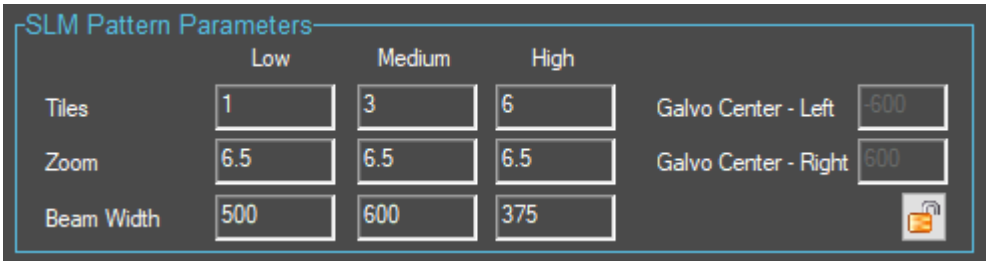
These values set the limits of the stage so that the sample does not collide with the chamber. Please do not alter these limits.

2 Prescan Parameters



These variables set the width of the sheet, FOV in mm for Y and X and the zoom state of the microscope to capture the 2D prescan image. Please do not alter these values.

3 SLM Settings

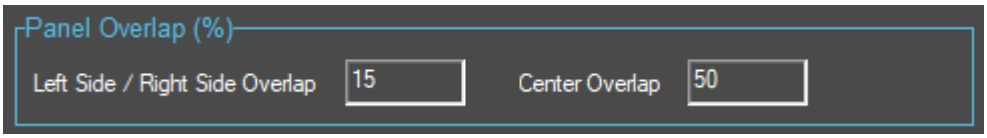


These values describe the number of tiling patterns, the zoom state of the microscope and the width of the LightSheet when the microscope is operated at Low, Medium, or High resolution capture states. Generally speaking, do not alter these values. We will discuss separately creating different patterns and the value of altering the Sheet/Beam width separately below.

4 Setting Stage Speed

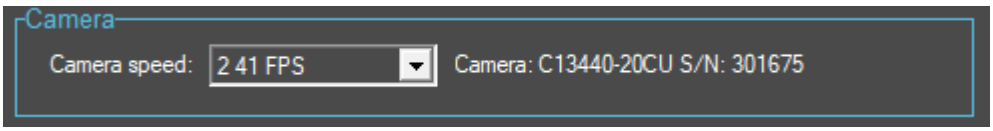
CTLS uses high-speed piezo actuators to translate the specimen. The speed of these actuators may need to be adjusted on occasion for two reasons. First, if the stages are moving at or above 13000Hz they will emit a high frequency tone that may be irritating for some. Reduce the speed to about 8000Hz and the tone is inaudible. Second, some optically transparent tissues, in our experience lack density. Thus, as the sample is translated from region to region they may be subject to "jiggle" or "wobble". Note that there are two speeds for Z. ***Z (Fast)*** defines the rate that the stage is translating as the user operates it manually, to define the top and bottom of the object, for example. This is NOT controlling the acquisition rate. ***Z (Slow)*** defines the rate that the Z-stage is translating as it returns to the bottom of the Z-stack as CTLS prepares to interrogate another FOV. If your sample is subject to "jiggle" or "wobble" alter the ***Z (Slow)*** value.

5 Setting Region x Region overlap



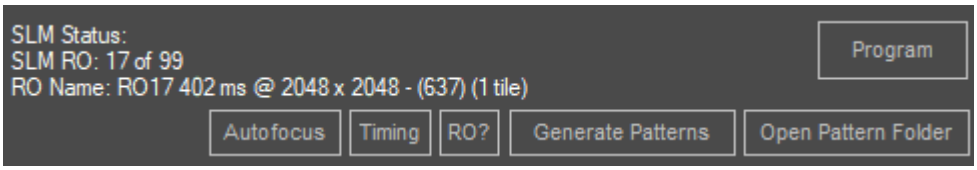
The values here define the amount of overlap SlideBook will effect region x region to minimize striping artefacts in the 3D montage. There are two values to alter. The first variable window shows the amount of overlap in FOVs interrogated by either the Left or Right excitation objective. ***Center Overlap*** defines the amount of overlap that regions interrogated by both the left ***and*** the right laser. This ***Center Overlap*** value could be set to a high value, especially if the center region of the sample is several mms away from the point of excitation, 6-8mms for example.

6 Setting Camera speed



The digitizer speed of the camera may be adjusted to maximize signal to noise. **BE CAREFUL**. You don't want to reduce the speed to a point where the Z-stage is moving faster than the camera is digitizing.

7 Setting SLM Patterns

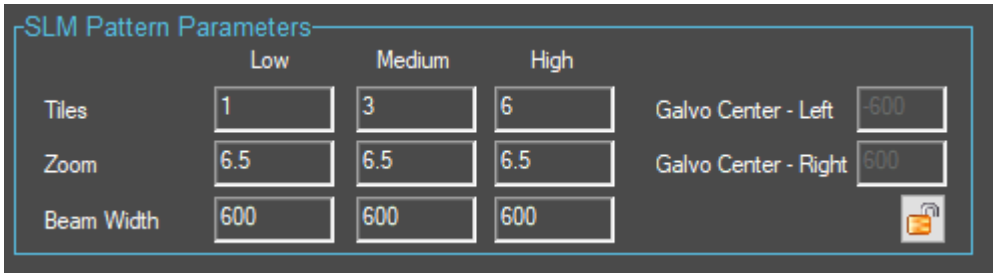


This group contains the button that launches the SLM pattern generator. SLM pattern generation will be discussed separated below.

TUTORIAL: Using the Region Statistics Tool to Maximize photon budget

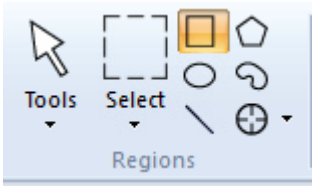
CTLS uses a high-speed galvo to create a light sheet. The width of the light sheet may be modified in SlideBook to match the field of view. At higher magnifications the sheet will cover a small area in the sample. At a given exposure time tis results in more excitation laser light on the same structures in the sample. In this tutorial we'll learn about a tool that will provide you with immediate feedback on signal strength. This tutorial assumes that you are familiar with altering the lightsheet width, explained in detail previously.

To change the width of the beam, navigate to the ***SLM Parameters*** group in ***Advanced Settings***. For the purposes of this exercise let's focus on the ***Beam Width*** values in the Low SLM Pattern column.

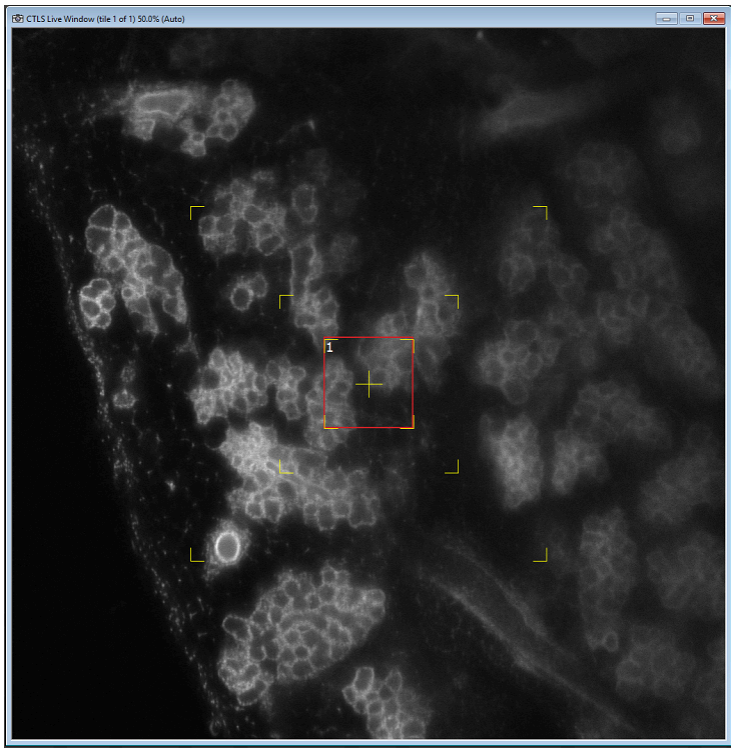


1. Identify a plane in the sample and set the camera exposure time to a low value. For the purposes of this exercise navigate to the Display Options group and select ***Guides.***

2. Next go to the Quick Access Toolbar and navigate to the ***Region Tools*** group***.*** Select a region tool and then draw a region anywhere over the image***.*** The box usually works well.



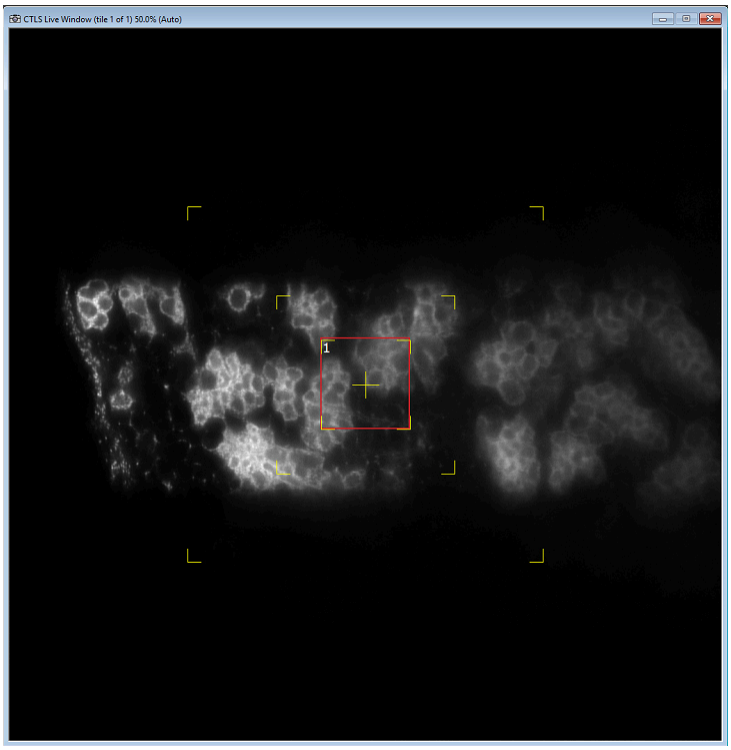
3. In the example below a region has been created over boundaries describing the center of the FOV.



4. Navigate over the ***Advanced Settings*** tab***.*** At the bottom of the tab you'll find a group ***Region Statistics.*** Click the button ***Enable Statistics.*** Click ***LIVE.*** The values next to ***MAX 1*** will update with gray value counts. For the example displayed here, the values we observed were about 1400 counts.

5. STOP the acquisition, navigate to the SLM Parameters group and change the beam width in the LOW column to 100. Click **LIVE** and also note the Region Statistic value. Our value changed from about 1400 counts to about 7300 counts.

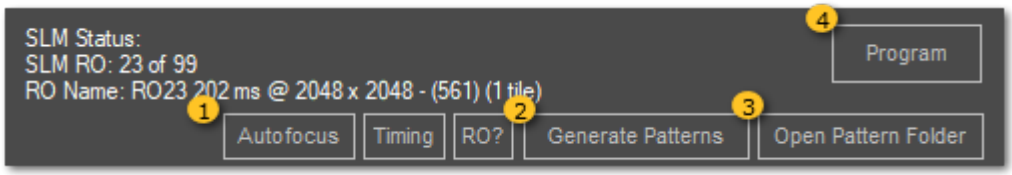
Don't forget to look at the image. By reducing the width of the sheet, we've increased our laser excitation light but at the expense of FOV. For this particular sample altering sheet width may not make complete sense. However, for a smaller sample such as an organoid, or a subset area of a larger sample, the management of beam width could be advantageous, especially if you have a sample with weak fluorescence.



TUTORIAL: SLM Pattern Generation

The centerpiece of the CTLS is a spatial light modulator. The spatial light modulator is used to shape the Gaussian beam to realize numerous solutions including enhanced optical sectioning, enhanced axial resolution, shadow/striping attenuation, etc. Here we will provide a very brief overview of the SLM pattern generator. You will receive a deeper dive into pattern generation at the time of your microscope's installation.

Navigate to the Advanced Settings tab. Towards the middle of this group you'll find this dialog



1 Supporting Resources

These buttons assist our support team in advanced SLM debugging. The ***RO?*** button acts to update the current SLM program

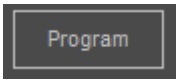
2 SLM Pattern Generator

This button will launch the SLM Pattern Generator interface and will be the subject of our tutorial

3 SLM Pattern Library

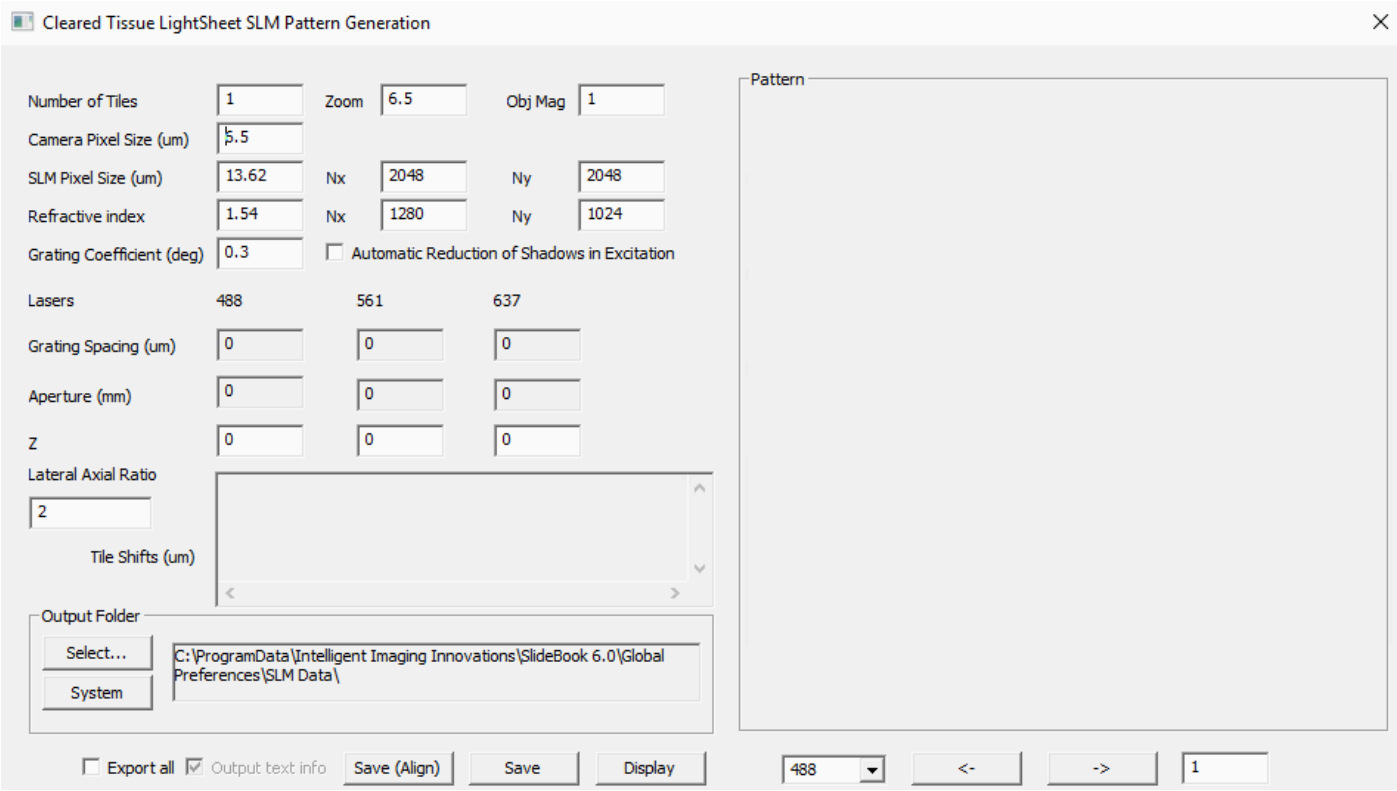
This button will open the folder that contains the library of SLM patterns. A full library of patterns will be created for you when your microscope is installed

4 Load Patterns



Clicking this button will load patterns that you have created in the pattern generator and same them to the Pattern Library

Click on the Generate Patterns button to open up the Pattern Generator interface



You can set the values in the number of tiles variable box to 1, 3, 6, 12, and 20. These patterns will create beams with center-point Gaussian beam widths of 20-microns, 12-microns, 8-microns, 5-microns and 3-microns respectively. The actual waist thickness will vary by a micron or so based on the RI of the index-matching solution.

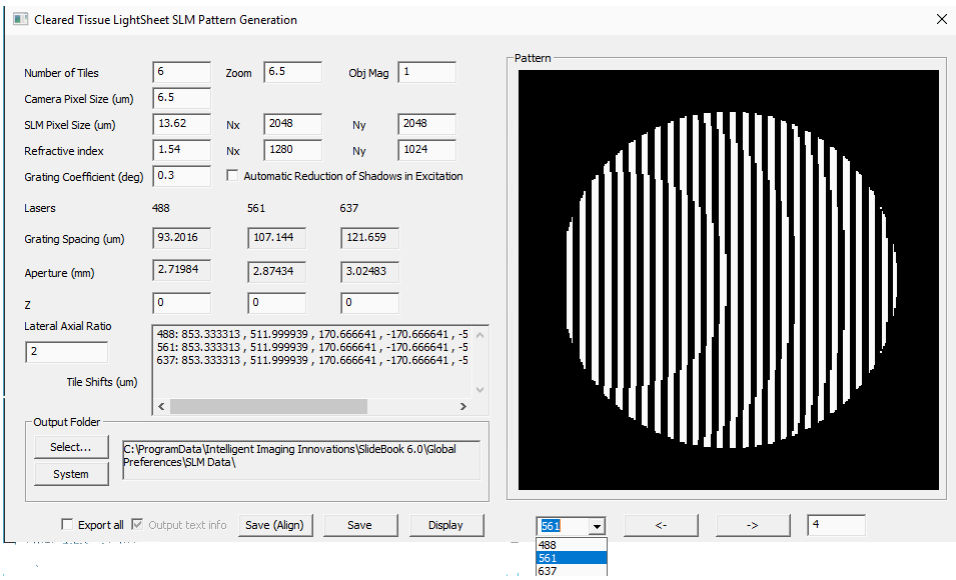
Pay attention to the ***Zoom*** factor of the microscope when creating new patterns. SlideBook will automatically inserted the zoom factor and the Objective Magnification (***Obj Mag***) based on the settings in the CTLS Capture interface.

The ***Camera Pixel Size*** set. Please do not change this value. The ***SLM Pixel Size*** is also set. Please do not change this value. The camera detector array (or pixel number, shown here as number of pixels in a column and row) is automatically read by SlideBook. Please do not change this value.

The value in the **Refractive Index** variable box will be the RI of the index-matching solution.

The ***Grating Coefficient*** (deg) sets the spacing of the grating in the SLM pattern. This may be changed when patterns are manually created to correct for chromatic aberrations.

1. Let's begin by changing the number of tiles to "6" and then clicking the ***Display*** button in the SLM Pattern Generation. A pattern should appear in the ***Pattern*** window



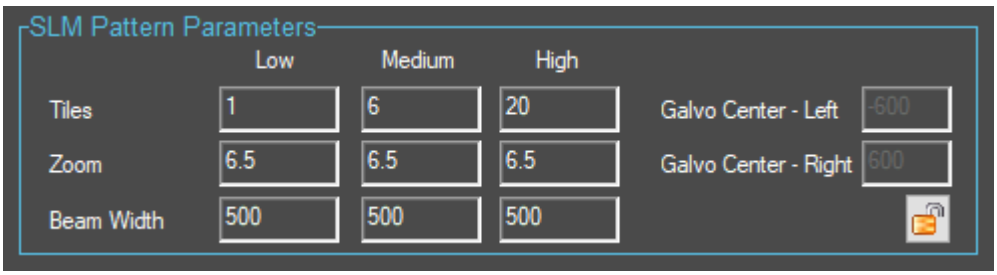
The SLM pattern is composed of an aperture, the grating and a lens. The grating function will depend on the wavelength and the desired exit angle, ***A***. The grating equation is d = wavelength / sin(***A***), where d is the spacing of the grating in microns (wavelength is also in microns). The aperture is just a mask that removes the grating from the area outside the aperture. In the example here, the aperture is circular and centered on the SLM and has only the outer diameter as the main parameter. This parameter defines the length of sheet and determines the thickness of the sheet. Therefore the aperture is dependent on the # of tiles and the field of view (zoom). Finally, the lens determines how the Gaussian beam is displaced over the FOV. You can see the lensing effect by clicking on the ***Left Right*** arrow buttons at the bottom to the Pattern Window. You can also see how the patterns differ per wavelength.

2. Click ***Export All*** and the ***Save*** button to save these 6 patterns.

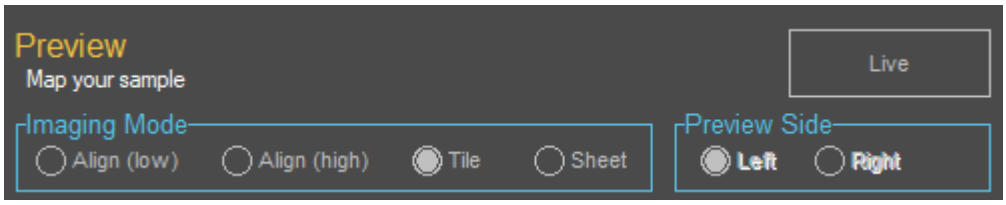
3. Next, change the number of Tiles to 20 then click display. Note that the diameter of the aperture changes but the grating spacing does not.

4. Click ***Export All*** and the ***Save*** button to save these 20 patterns.

5. Program the patterns in the ***SLM Pattern Parameters*** group edit the Tile values: add 6 to the Medium Column, 20 to the High Column



6. Navigate to the ***Preview*** group and choose the ***Tile*** radio button. Set ***Resolution*** to ***Medium***. Click the ***Live*** button



7. In the CTLS Live Capture window you'll be able to see the Gaussian beam's waist march across the FOV. Navigate to the ***Scaling*** group at the bottom of the Prescan window, select ***Manual*** scaling and set the upper value so that you only detect the Raleigh length of the beam. This value may be anywhere between 300 and 3000 depending on the index-matching solution.

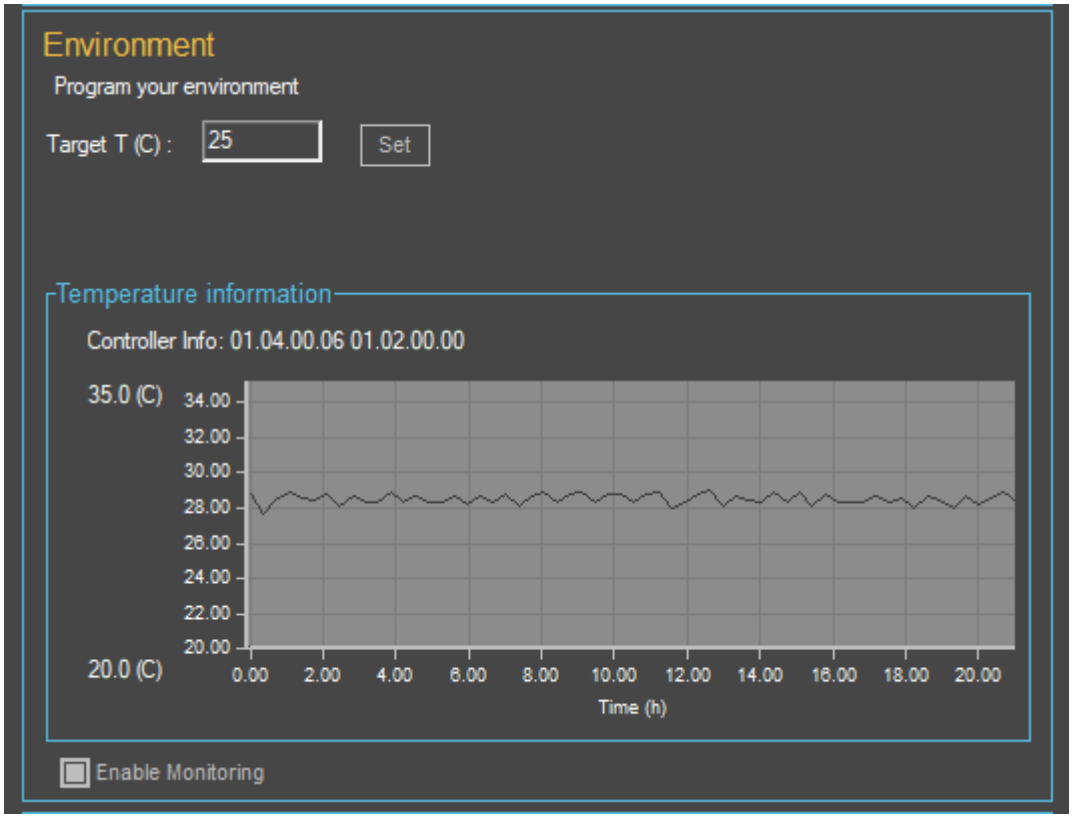
8. Click ***Stop***. Change the ***Resolution*** to ***High***. Click ***Live***. Adjust the scaling as you did in step 7.

Preview is not available

TUTORIAL: Temperature Control

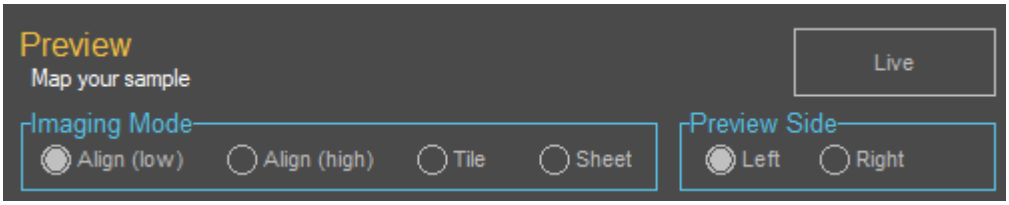
Temperature and Refractive Index are intimately linked. An increase in temperature results in a decrease in RI. The relationship of Temperature and RI is extremely important to manage when imaging tissues in index matching media. Any change in the RI of the index matching media will not immediately translate to the RI of the cleared specimen. The resulting RI mismatch will affect the quality of your image. In this tutorial we'll view the effect of temperature changes on the confinement of the Gaussian beam.

In ***Advanced Settings*** navigate to the ***Environment*** group

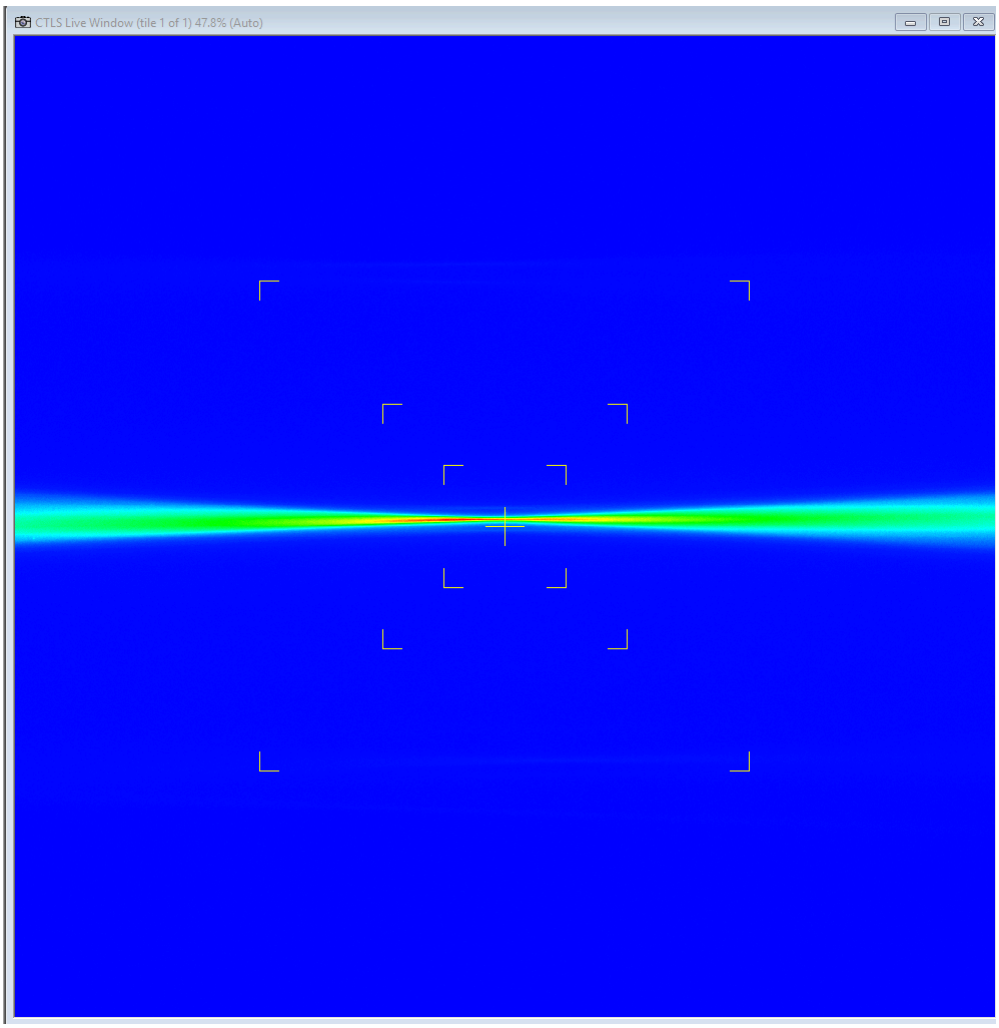


This interface will allow you to adjust the temperature inside the chamber. The value in the variable box is actually the temperature of the thermo-coupled "chair". We've determined that the temperature of the "chair" and the temperature of the media inside the chamber will be off by 1C to 2C depending on the conditions in the room. Rule of thumb: set the temperature in the variable box to be 1C or 2C higher than the temperature of the media. Thus, if you expect to image in media at 25C, set the controller to 26C or 27C. You'll receive continuous feedback on temperature by referencing the graph in ***Temperature Information*** provided that the box ***Enable Monitoring*** has been enabled.

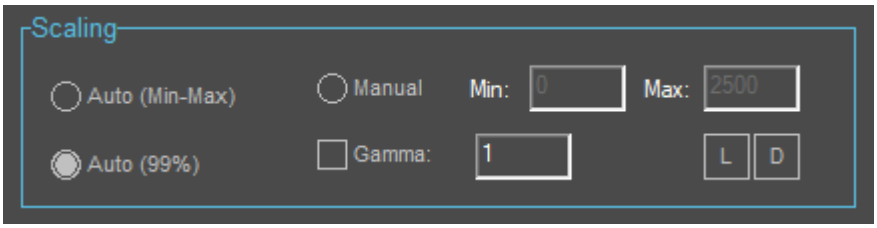
1. Fill the chamber with your index matching solution. Set the temperature of the chamber to 25C and then image and focus on the beam to realize a tightly confined beam waist. In case you've forgotten, go to the ***Preview*** group and select ***Align (low)*** as an ***Imaging Mode.***



Your beam should look something like this.



2. For the purposes of this experiment, be sure to set the display so that it NOT auto-scaling as shown below



3. Wait for about 30 minutes to ensure that the temperature in the chamber is stable. If you want to get sophisticated, you can set-up some thermistors to measure room temperature + bath temperature. Phidgets.com provides a number of very inexpensive temperature sensors.

4. Depending on the conditions of the room, you can either increase or decrease the temperature in the chamber. The effect on beam confinement is pretty dramatic and the beam will lose confinement rapidly. In the video below, we show the last few minutes of an experiment where we reduced the temperature of the media in the chamber to 21C and then elevated the temperature to 25C. The green and pink traces from the Phidget Sensor show the temperature in the room. A Blue milestone at 2:02 marks the time when the temperature is changed. Note the quick response of the "chair" (light blue trace) and the media (red trace) and the affect that the change in temperature has on the beam profile.

Preview is not available

CTLS Trouble Shooting

Below, you will find a list of troubleshooting tips. For additional assistance, please contact 3i Support.

• If you are having trouble seeing the beams, check the laser safety key on the LasesrStack controller. Make sure the key is set to the ***ON*** position.

• If the lasers are on, but you don't see a beam to focus, make sure that imaging mode is set to ***Align*** rather than ***Sheet. TIP:*** set the microscope zoom to a lower value, 1X or 2X and maximize the display scaling to find the beam.

• If the field of view is not uniformly illuminated someone may have changed the width of the lightsheet. Go to ***Advanced Settings*** and check the value of the ***Beam Width.*** The default state should be 600.

• If the 2D prescan map didn't assemble correctly checked your objective setting. Maybe you're imaging with the 1.5X objective, but the ***Objective*** setting in SlideBook has the 1X radio button selected.

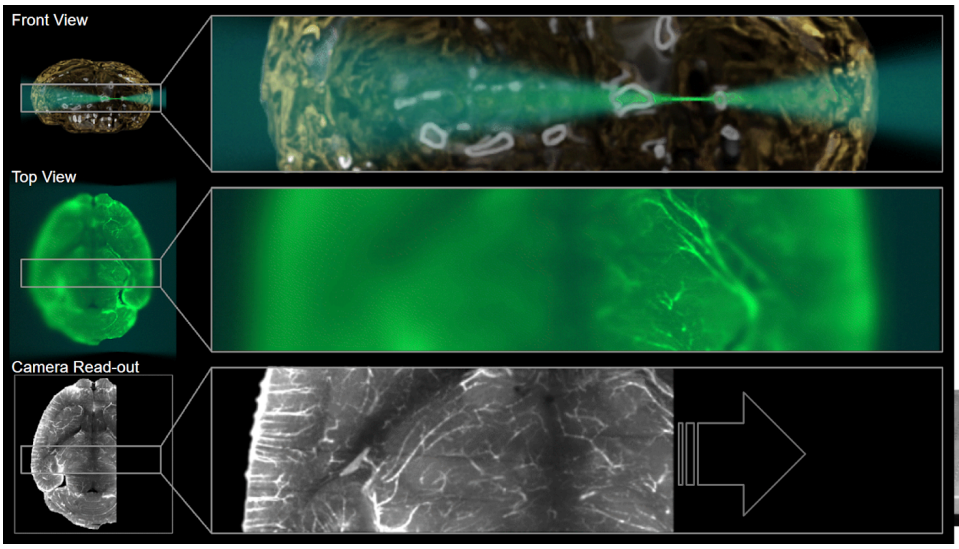
• If SlideBook reports an insufficient memory message upon capture, check your settings: you may have mistakenly set the data to be saved to the desktop.

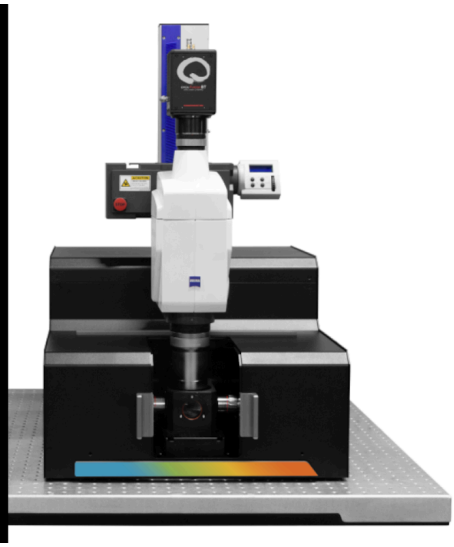
AxL Cleared Tissue LightSheet

AxL Cleared Tissue LightSheet microscope is designed to capture 3D images of large (cm3), fluorescently labeled samples that have been clarified through a process called "tissue clearing". AxL leverages Axially Swept Lightsheet Microscopy (ASLM), first described by Reto Fiolka and Kevin Dean (Dean 2015 BioPhys). AxL seamlessly shifts the 2um thin beam across the entire (up to 10mm) field of view using an electrically tunable lens (ETL) while simultaneously reading out pixels on the camera in rolling shutter mode. As a result, AxL features a 2um thin optical section across a up to 10mm large field of view. This approach offers three key advantages:

- The 2 µm sheet across all magnifications offers true isotropic resolution at resolutions >2um / pixel
- The high NA eliminates striping (shadow) artifacts at all magnifications
- Imaging a single frame is not only faster than acquiring multiple images through tiling methods, it also reduces stitching processes to a minimum

Besides this cutting edge technology, AxL employs a fully automated macro zoom microscope as well as a variety of different sample chambers optimized for samples from <1mm up to 100mm
AxL is powered by SlideBook software, our comprehensive software for beam alignment, image acquisition, stitching, and rendering.





AxL Hardware

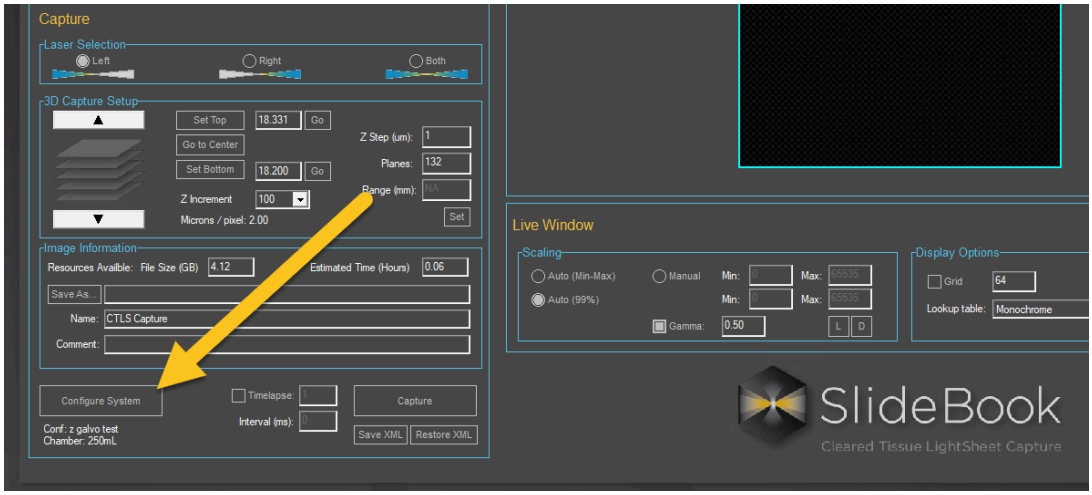
AxL Stage Limits for Different Specimen Chambers

The stage limits ensure that the sample spoon does not accidentally hit the wall of the microscope specimen chamber. The default values for 3i sample holders are:

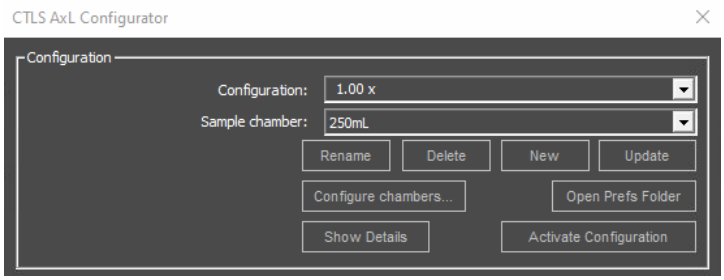
stage limits	S (150mL)		M (250mL)		L (500mL)		XL (1L)	
x	20	30	15	35	15	35	10	40
y	130	148	125	148	100	148	55	145
z	0	15	0	25	0	25	0	25

However, in the case of custom-made sample holders, or if the sample is larger than the boundaries of the sample holder, the limits can be adjusted. To change the stage limits, the best way is to create a new configuration.

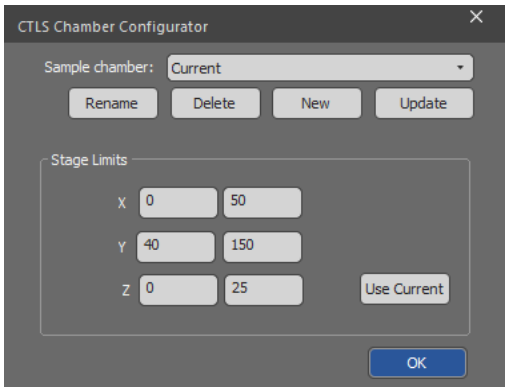
Create a new chamber configuration



1. Open the AxL Configurator, click on **Configure System**



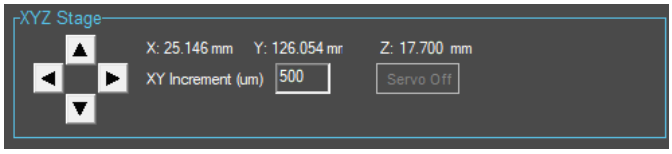
2. Click on **Configure Chambers**...



3. Create a new chamber configuration, click **NEW**
4. Rename the new chamber configuration, click **Rename**
5. In case you need more travel range, define the new chamber to use the maximum travel range. Therefore set the limits for
x from 0 to 50,
Y from 40 to 150 and
z from 0 to 25
6. Apply the new settings, click **Update**
7. To use the new settings, activate the configuration, click **Activate Configuration**
8. The user interface will restart

Define new stage limits

Carefully move the sample holder in one direction and take note of the stage position before the sample hits the wall. Do the same for all directions: left/right, front/back.



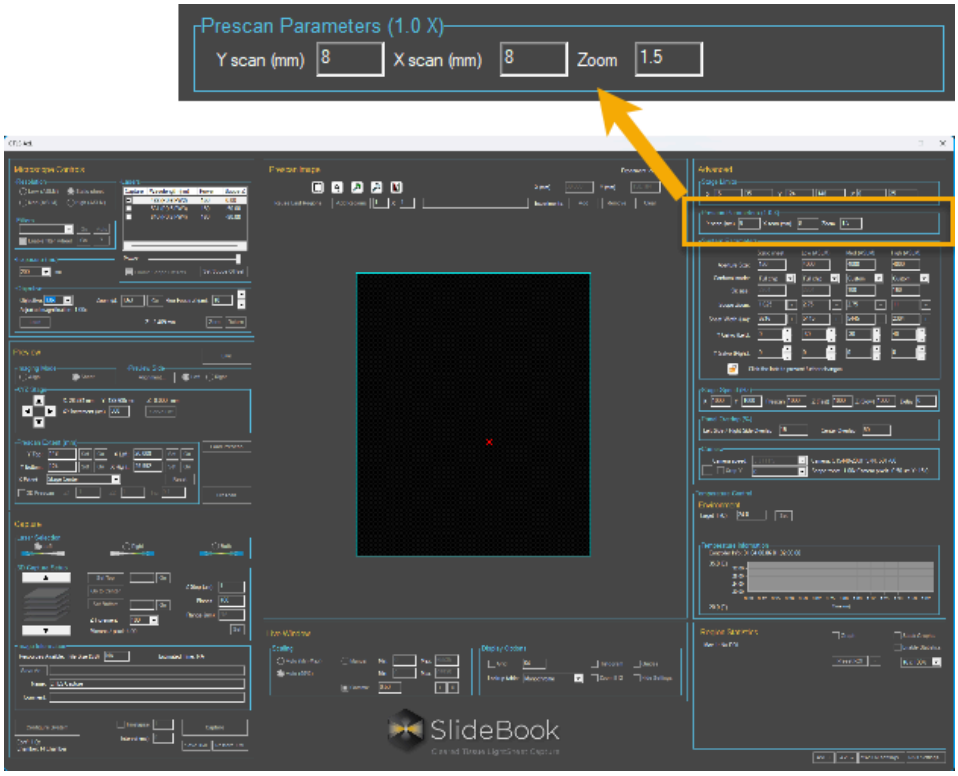
update the limits in the chamber configurator
1. Open the AxL Configurator, click on **Configure System**
2. Click on **Configure Chambers...**
3. Apply the new settings, click **Update**

AxL Prescan Defaults

The prescan is designed to scan the sample area quickly. Therefore, the defaults are set to maximize the field of view. Below are the default settings that should be used in combination with the four different objectives.

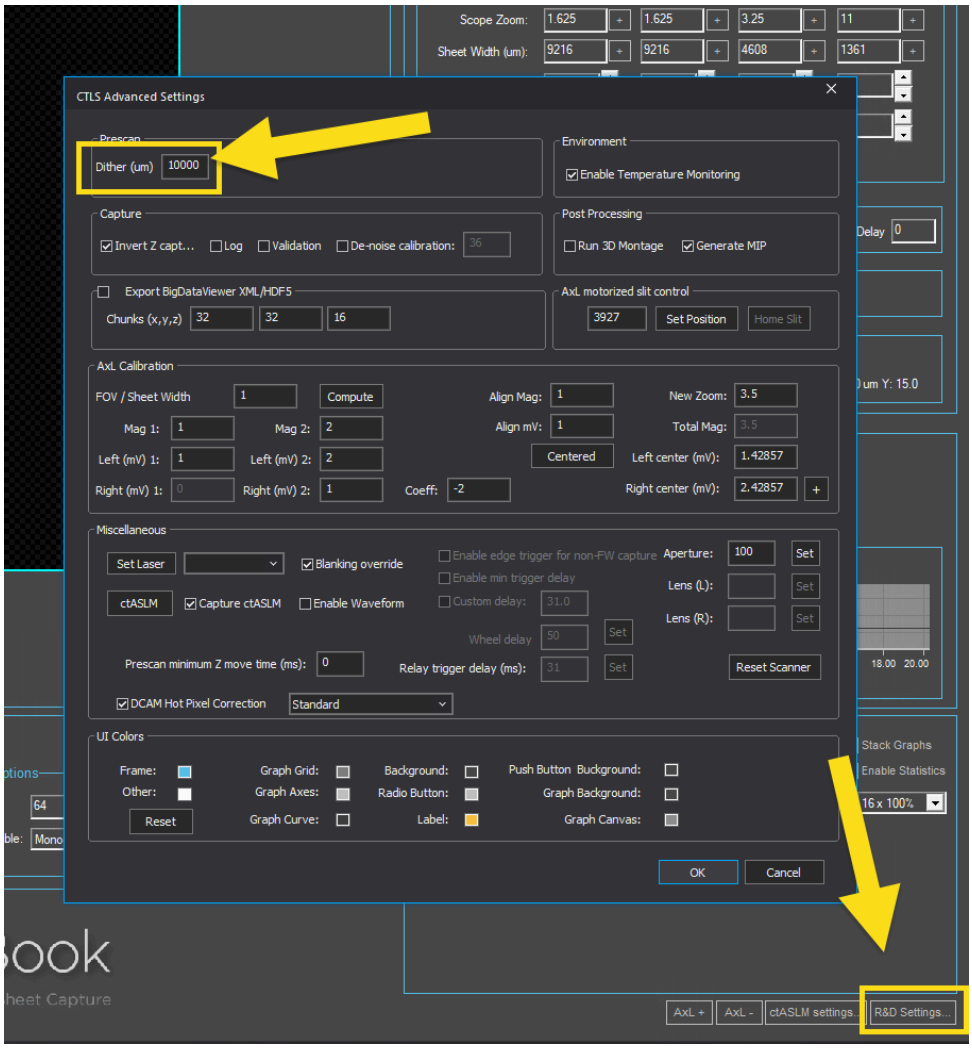
	0.5x Objective	1.0x Objective	1.5x Objective	2.3x Objective
zoom factor	3x	1.5x	1.0x	1.0x
(total mag / FOV)	*(1.5x / 10mm)*	*(1.5x / 10mm)*	*(1.5x / 10mm)*	*(2.3x / 6.5mm)*
x strip size	8mm	8mm	8mm	5mm
y strip size	8mm	8mm	8mm	5mm

The prescan parameters can be changed in the Advanced Settings tab on the right side of the AxL CTLS user interface.

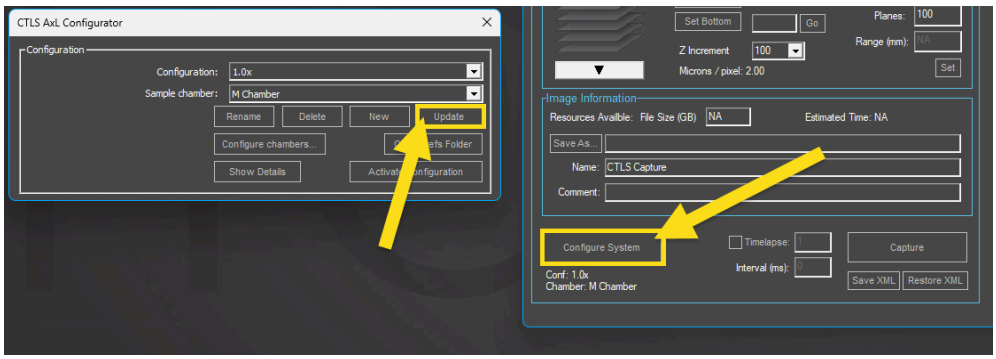


There is another setting that defines the sheet width during the prescan in the R&D settings:

1. Open the **R&D settings** (main UI, bottom right corner).
2. Locate **Dither Prescan** and set it to **10,000**. (This value is stored in each configuration.)



3. In the main UI, click **"Configure System"**.
4. Click **"Update"** to apply the changes.



Other settings, such as **ETL alignments**, are saved automatically when you go **Live** or **Stop**, meaning they update continuously.

Aperture Size (Motorized Slit)

The **Aperture Size** is a useful tool for adjusting sheet thickness. In Static Sheet Mode, the aperture is closed to 100 to effectively reduce the NA of the excitation objective, which increases the sheet thickness and lengthens the beam waist to cover a 10 mm FOV. ASLM imaging modes need the aperture to be all the way open (3990)

For example
Aperture Size Low (ASLM) = 4000 → Lightsheet thickness 2-3um
Aperture Size Static Sheet = 100 → Lightsheet thickness 30um

Confocal Slit and Slit Width Trade-Off

The AxL excitation objectives generate a thin light sheet, typically 2–3 µm thick and ~80 µm long, using the full numerical aperture (NA = 0.14). Images are captured using a rolling shutter readout, where each line of the camera sensor is exposed sequentially. The rolling shutter slit window defines how much of the light sheet contributes to the image as each sensor line is read out, and therefore directly determines optical sectioning and axial resolution.

The per-pixel dwell time is determined by slit width, effective pixel size, and global exposure. Wider slits increase dwell per pixel (higher signal) but reduce optical sectioning. Global exposure can be adjusted independently to boost signal without affecting axial resolution.

Important: Keep laser intensity as low as possible — photobleaching is a concern not only in live-cell imaging but also in cleared tissues. Choosing the right combination of slit width and exposure optimizes signal while minimizing photodamage.

Key Concepts

1. Optical Sectioning
• Narrow slit → better rejection of out-of-focus light → higher axial resolution
• Wide slit → more out-of-focus light → lower axial resolution
• Independent of global exposure

2. Per-Pixel Exposure / Signal
• Wider slit → longer per-pixel dwell → higher signal
• Global exposure can increase overall signal without affecting axial resolution

3. Trade-Offs
• Narrow slit → high axial resolution, lower per-pixel signal
• Wide slit → higher signal, reduced optical sectioning
• Choose slit width based on desired optical sectioning and sample brightness
• To increase overall signal, use longer exposure times rather than higher laser power

Confocal Slit Width Presets in SlideBook

• **Preset**	• **Slit Width**
• Aggressive	• 80 µm
• Optimal	• 320 µm
• Full Chip	• Full 2304 px sensor
• Custom	• User-defined

Slit Width per Magnification and Dwell Times

Global Exposure <u>150 ms</u>

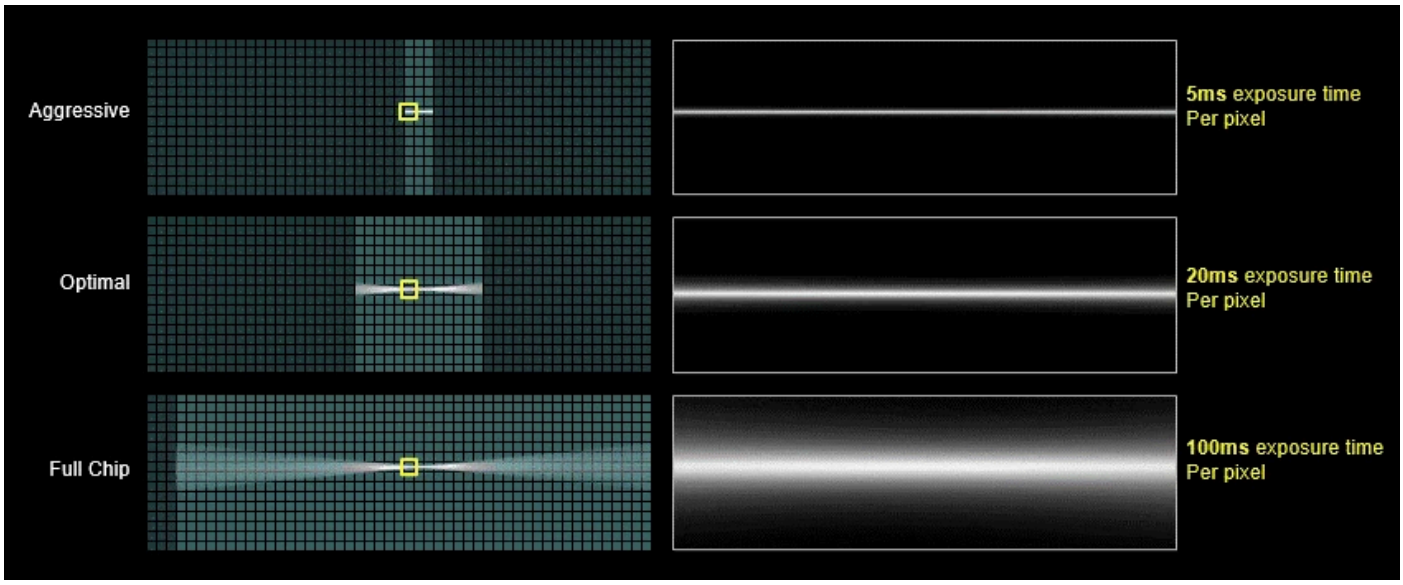
Mag	Eff. px (µm/px)	80 µm Slit (px/ms)	320 µm Slit (px/ms)	Full Chip (px/ms)
26×	0.25 µm/px	320 px / 21 ms	1280 px / 75 ms	2304 px / 150 ms
13×	0.5 µm/px	160 px / 11 ms	640 px / 38 ms	2304 px / 150 ms
6.5×	1 µm/px	80 px / 5 ms	320 px / 21 ms	2304 px / 150 ms
3.25×	2 µm/px	40 px / 3 ms	160 px / 11 ms	2304 px / 150 ms
2.17×	3 µm/px	27 px / 2 ms	107 px / 7 ms	2304 px / 150 ms
1.625×	4 µm/px	20 px / 1 ms	80 px / 5 ms	2304 px / 150 ms

Global Exposure <u>400 ms</u>

Mag	Eff. px (µm/px)	80 µm Slit (px/ms)	320 µm Slit (px/ms)	Full Chip (px/ms)
26×	0.25 µm/px	320 px / 57 ms	1280 px / 200 ms	2304 px / 400 ms

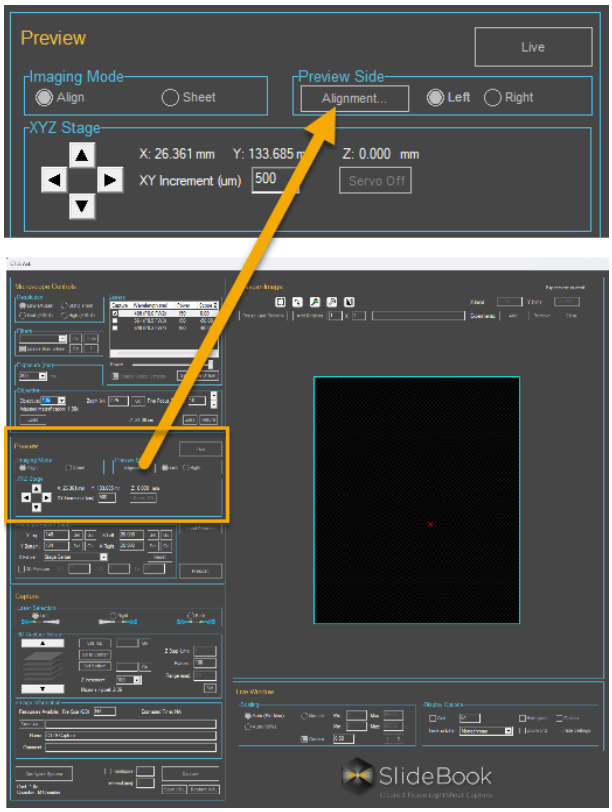
13×	0.5 µm/px	160 px / 29 ms	640 px / 100 ms	2304 px / 400 ms
6.5×	1 µm/px	80 px / 14 ms	320 px / 57 ms	2304 px / 400 ms
3.25×	2 µm/px	40 px / 7 ms	160 px / 29 ms	2304 px / 400 ms
2.17×	3 µm/px	27 px / 4 ms	107 px / 18 ms	2304 px / 400 ms
1.625×	4 µm/px	20 px / 3 ms	80 px / 14 ms	2304 px / 400 ms

The rolling shutter slit window sets axial resolution, while slit width, effective pixel size, and global exposure determine per-pixel dwell time and signal. Choose a slit width that balances optical sectioning and signal, and select global exposure times to minimize laser intensity and photobleaching. For higher overall signal, it is better to increase exposure time rather than laser power.

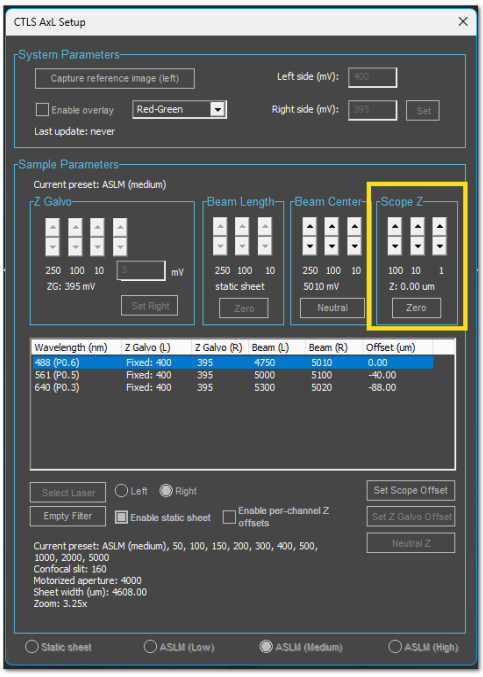


Detection Focus

The easiest way to focus is by using the Scope Z controls in the Alignment window
Open the Alignment Window



This Alignment window looks like this:



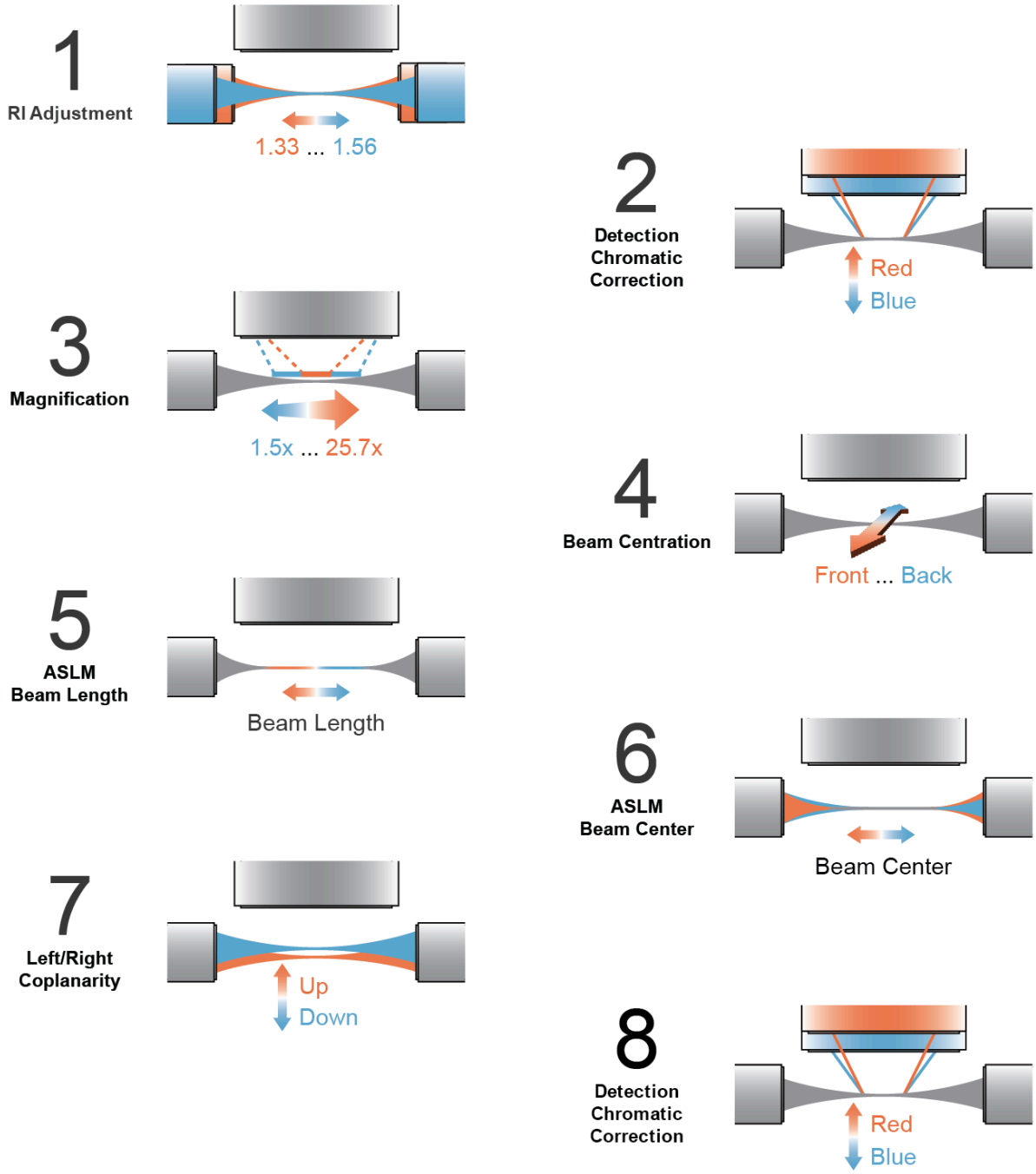
Hovering the mouse cursor over this boxed area enables the use of the mouse wheel to move the microscope up and down.

Each scroll of the mouse wheel moves the microscope by 10 µm.
Hold **Ctrl + mouse wheel** for 100 µm steps.
Hold **Shift + mouse wheel** for 1 µm steps.

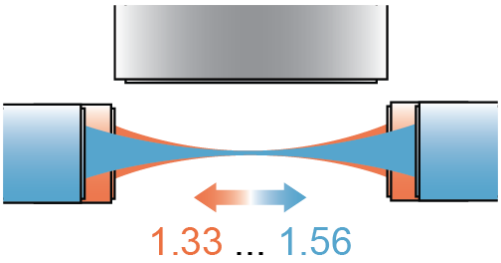
AxL Alignment Workflow

For an efficient workflow, follow the steps below in the listed order.
1. RI adjustment, 2. Detection Chromatic Correction, 3. Magnifications, 4. Beam Centration Y-Axis, 5. ASLM - Beam Length, 6. ASLM - Beam Center, 7. Left/Right Coplanarity, 8. Detection Chromatic Correction

The refractive index (RI) affects the entire system, including the working distances of both the illumination and detection optics, so it's important to check for color shifts. Changing the magnification only affects downstream alignments, like ASLM, without influencing other aspects of the system.
Left/right co-planarity is unaffected by any adjustments, including RI or magnification, but may vary slightly between chambers due to window position differences. Any adjustments are typically minimal and can be addressed through fine-tuning when switching chambers.

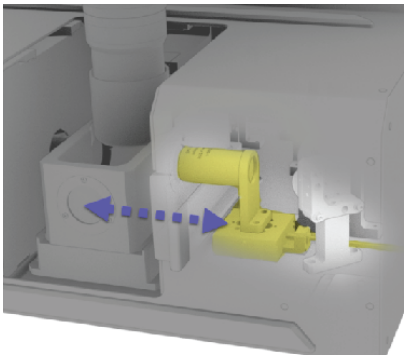


1) RI adjustment

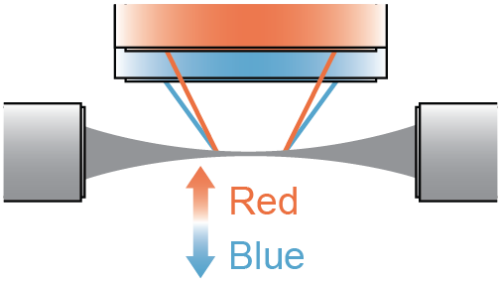


When switching to a medium with a different refractive index, the working distance of the excitation objectives changes. To re-center the excitation focus, the position of the excitation objective must be adjusted. This step should be performed at a low magnification (e.g. 1.625x). Follow these steps:

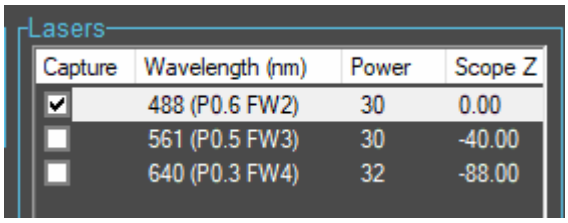
1. Move the sample out of the light sheet.
2. Enable align mode.
3. Enable static sheet mode (to ensure the ETL is in its neutral central position, Beam Center “Neutral” (5000mV)).
4. Set total magnification to 1.625x total magnification.
5. Enable align mode.
6. Use central wavelength (e.g. 561nm)
7. Focus on the beam.
8. Enable guides.
9. Center the beam along the X-axis by either translating the excitation objective using the motorized control in SlideBook or using a 2 mm hex wrench
10. Switch to the other side of illumination and repeat the centering process for the other side



2) Detection Chromatic Correction



To correct chromatic aberration in the detection path, SlideBook allows for storing the focus position for each channel individually. The user specifies a relative offset in the microscope's focus position between channels, called **Z-Scope Offset**. When *Enable Scope Offset* is checked, Z-stacks are acquired sequentially, and the microscope's focus position adjusts between channels. This adjustment may vary with refractive index (RI), or when using objectives with different working distances.

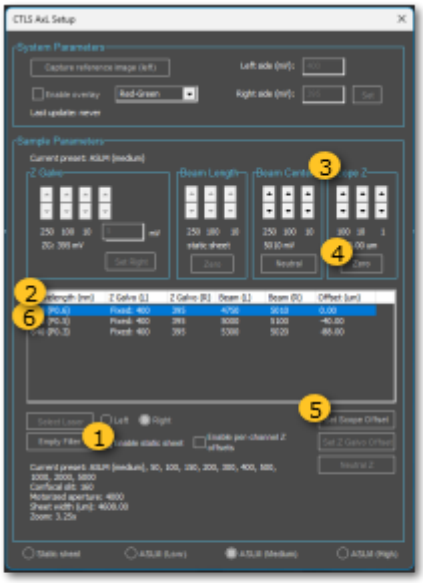


Lasers

Capture	Wavelength (nm)	Power	Scope Z
☑	488 (P0.6 FW2)	30	0.00
☐	561 (P0.5 FW3)	30	-40.00
☐	640 (P0.3 FW4)	32	-88.00

For optimal results, this step should be performed on the static beam at high magnification (e.g., 8x). It is recommended to repeat this step later on the sample in sheet mode (Step 8 of this Alignment Workflow).

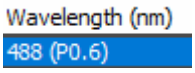
- In the main UI
- Align mode (beam)
- High mag (8x zoom)
- Open *Alignment* window



1 Check enable static sheet

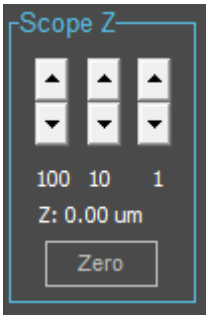
This stops the dynamic sweeping process and ensures that you are focusing on a static beam

2 Select a reference channel



Tip: To ensure the longer wavelengths are acquired first, begin the calibration with the shortest wavelength. AxL captures channels with negative z-positions first, so starting with the shortest wavelength ensures that the longer wavelengths receive negative offsets.

3 Focus

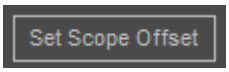


Hovering the mouse cursor over this boxed area will allow you to use the mouse wheel to move the microscope up and down. Each scroll of the mouse wheel moves the microscope by 10 µm. Holding down Ctrl while scrolling the mouse wheel will move the microscope by 100 µm steps, and holding down Shift while scrolling will move it by 1 µm steps.

4 Click Zero

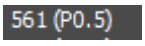
The relative microscope's z-position is set to "0".

5 Click Set Scope Offset



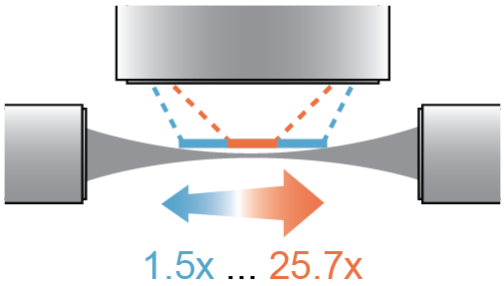
The selected channel receives the current relative offset position.

6 Select the next channel

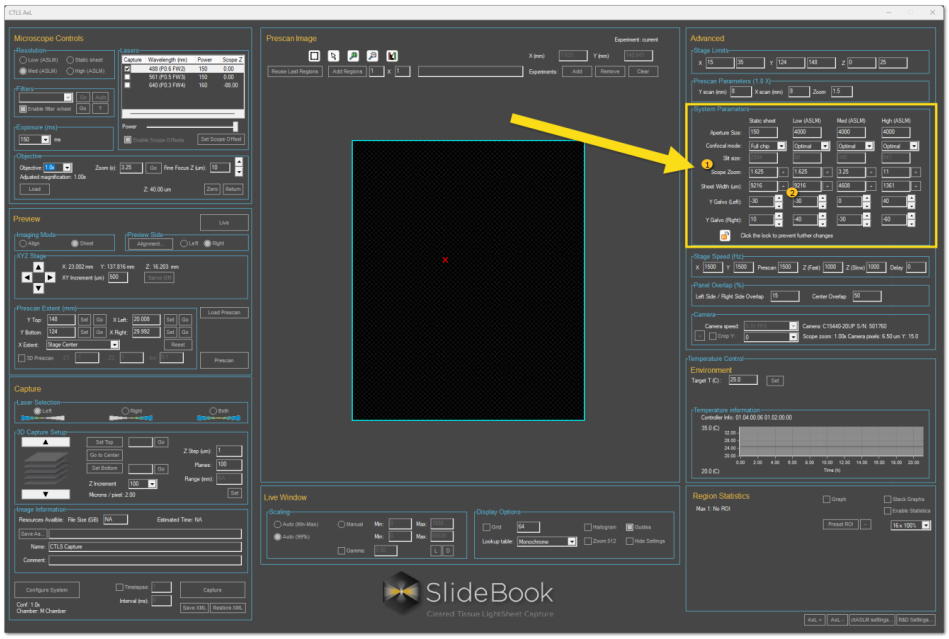


Focus (Step 3) and then click Set Scope Offset to store the relative offset position for this channel

3) Magnifications

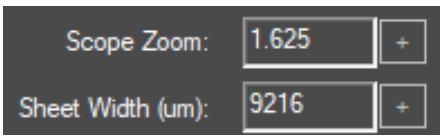


Define the desired magnification for your experiment in low, med or high. Typical magnifications are selected to achieve a final resolution in micrometers per pixel on the camera. The camera has a pixel size of 6.5µm. To calculate the final micrometers per pixel, divide this camera pixel size by the magnification factor, for example 6.5um : 3.25x = 2um/px. Below is a table showing the common magnifications used, depending on the chosen objective



1 Enter the desired zoom factor between 0.7x and 11.2x

2 Sheet Width Calculator

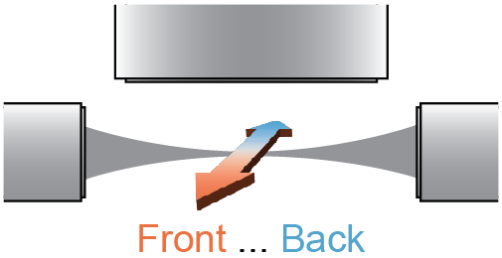


To automatically calculate the correct sheet width, click the "+" button next to Sheet Width (um)

Typical Zoom Factors to achieve a final resolution in micrometers per pixel on the camera

Zoom Factors (X)				
	0.5x Objective	**1.0x Objective**	**1.5x Objective**	**2.3x Objective**
0.25 um/px	out of range	out of range	out of range	11.2
0.5 um/px	out of range	out of range	8.667	5.652
0.75 um/px	out of range	8.667	5.778	3.768
1 um/px	out of range	6.5	4.333	2.826
2 um/px	6.5	3.25	2.167	1.413
3 um/px	4.333	2.167	1.444	0.942
4 um/px	3.25	1.625	1.083	0.707
Field of view (mm)				
	0.5x Objective	1.0x Objective	1.5x Objective	2.3x Objective
0.25 um/px	out of range	out of range	out of range	0.58
0.5 um/px	out of range	out of range	1.15	1.15
0.75 um/px	out of range	1.73	1.73	1.73
1 um/px	out of range	2.30	2.30	2.30
2 um/px	4.61	4.61	4.61	4.61
3 um/px	6.91	6.91	6.91	6.91
4 um/px	9.22	9.22	9.22	9.21

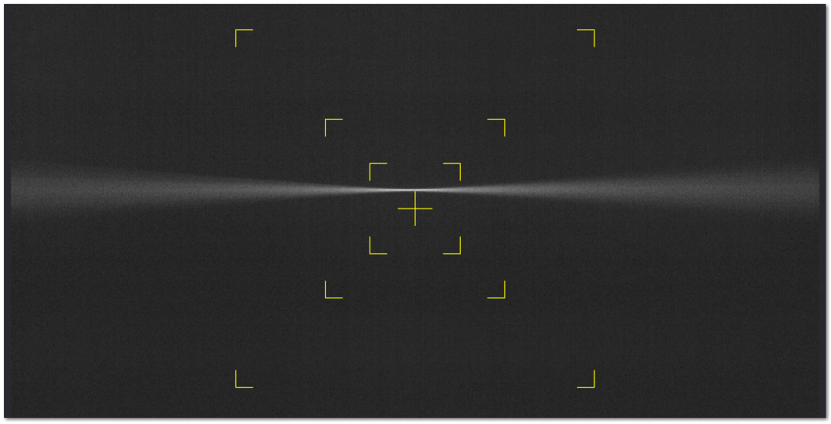
4) Beam Centration Y-Axis

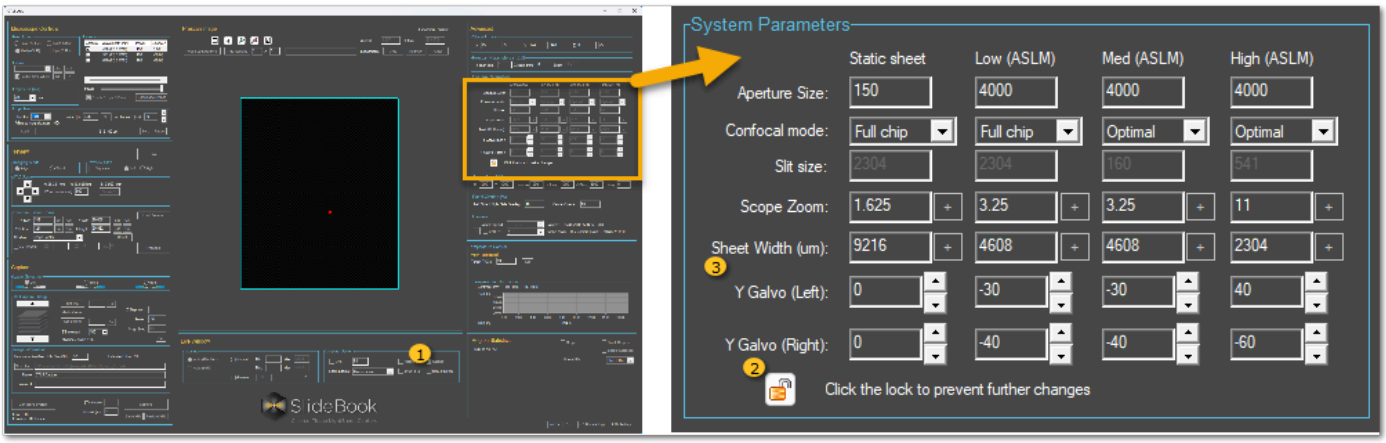


This step adjusts two settings:

1. The center position of the beam/sheet along the Y-axis.
2. The sheet width to cover the field of view.

Both the beam position along the Y-axis (up and down on the camera) and the sheet width are controlled by the Y-Galvo.

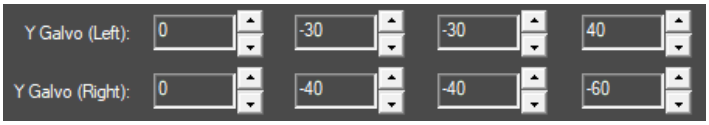




1 Open Advanced Settings Tab

2 Unlock the System Parameters Group

3 Y-Galvo controls

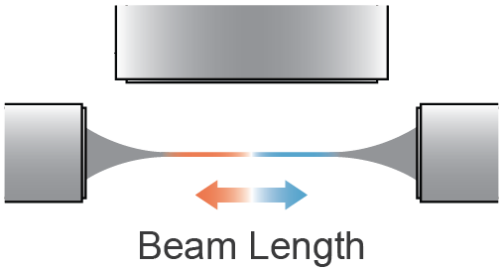


Ensure that you only adjust the Y-position for the current resolution mode (static sheet, low, medium, high) and the selected side of illumination (left or right).

Center position of the beam/sheet along the Y-axis
The y-galvo position can be adjusted in live-view. The unlocked state remains during live mode, and you can click on the arrows to change positions in real-time.
One click on the arrow button shifts the beam by 10 µm.
Hold **Ctrl + click** for a 100 µm step.
Hold **Shift + click** for a 1 µm step

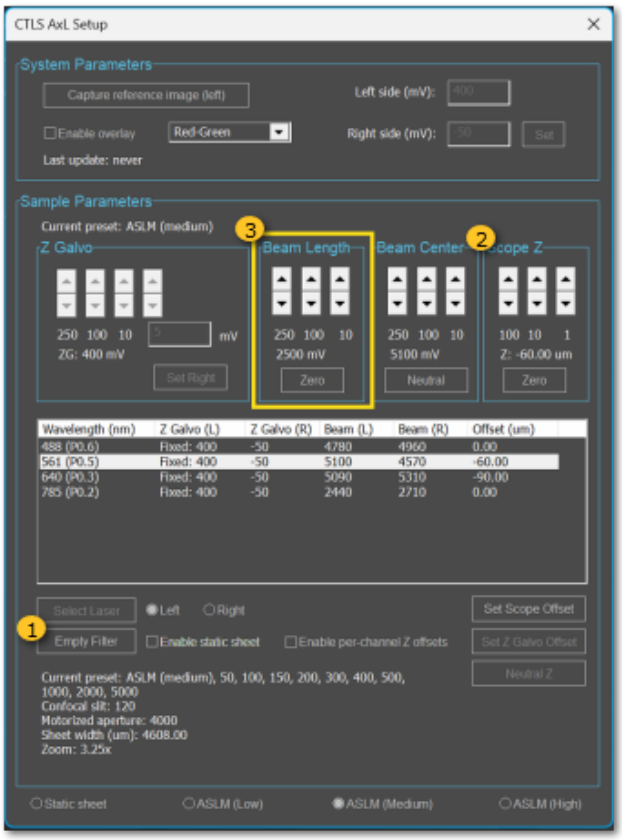
The up arrow button moves the beam up on the screen, and the down arrow button moves it down.

5) ASLM - Beam Length



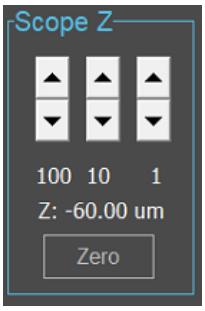
This step (**Beam Length**), along with the following Step 6 (**Beam Center**), is part of the daily alignment process to calibrate the axially swept light sheet in synchrony with the camera rolling. The goal of Beam Length is to adjust the remote focus range to match the field of view. Once set, the beam length remains constant for all colors and both the left and right sides.
The next step, Step 6: ASLM – Beam Center, will offset the beam to correct for chromatic aberrations as well as any differences between the left and right sides.

- **Go live in Align (Beam) mode**
- **Open Alignment window**



1 **If you do not see the beam, click Empty Filter, and enable static sheet (which temporally disables the remote focusing**

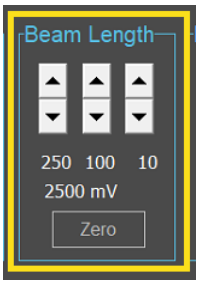
2 Focus on the beam



Hovering the mouse cursor over this boxed area enables the use of the mouse wheel to move the microscope up and down.

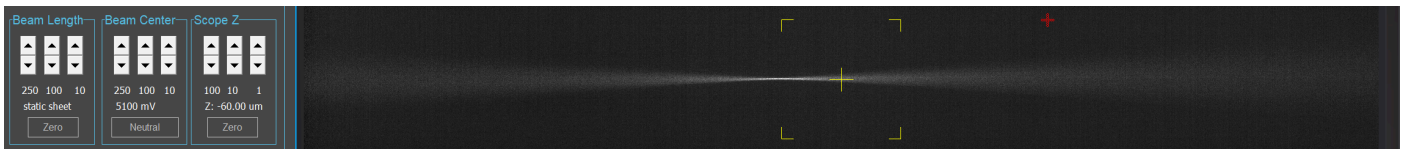
Each scroll of the mouse wheel moves the microscope by 10 µm.
Hold **Ctrl + mouse wheel** for 100 µm steps.
Hold **Shift + mouse wheel** for 1 µm steps.

3 Adjust the beam extent to match the field of view

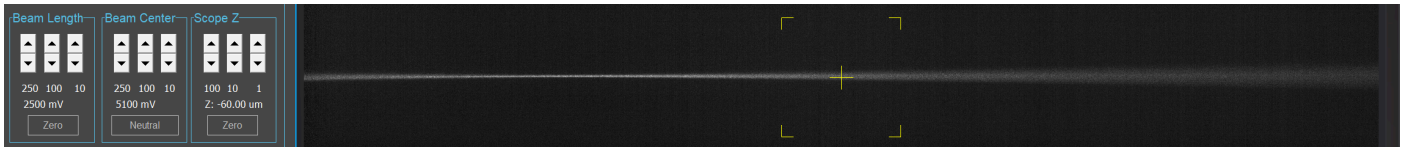


Ensure uniform thickness across it. At this stage, the beam does not need to be as thin as possible, but it should have a consistent thickness throughout.
Clicking "Zero" adjusts it to 0 mV, which is a not-sweeping beam, or static beam/sheet.

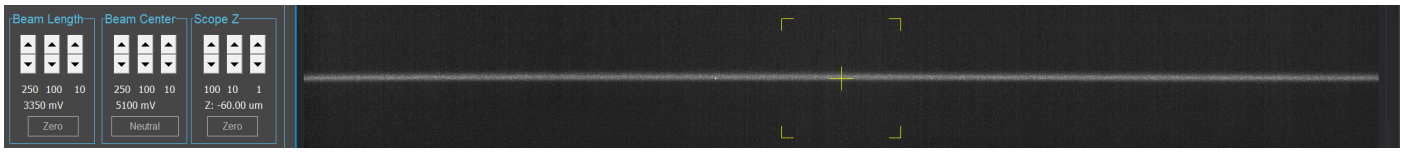
Example for ASLM mode at 3.25x:



Beam Length: static sheet (0mV), Note: It is not important that the waist of the beam is not centered



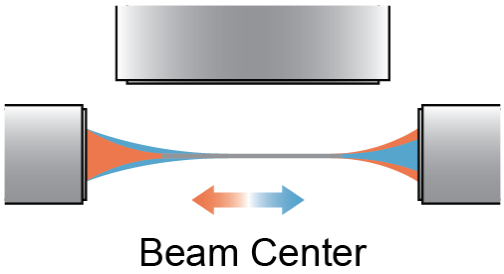
Beam Length: 2500mV, note that the beam is thinner on the left side compared to the right side



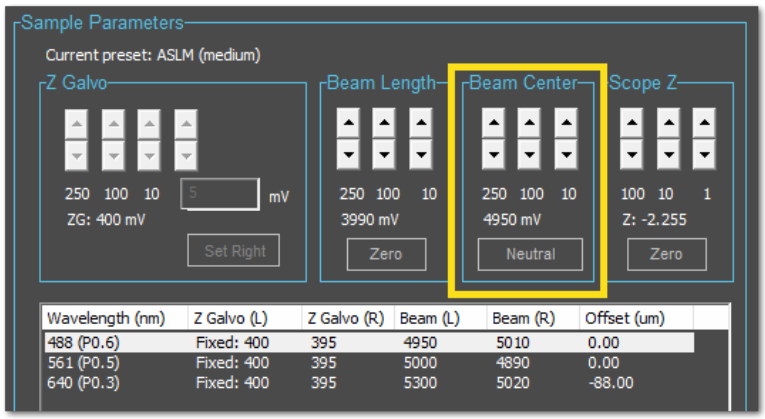
Beam Length: 3350mV, note that the beam has a uniform thickness across the field of view.

That's it! In the next step 6) the beam center offset, will make it as thin as possible

6) ASLM - Beam Center

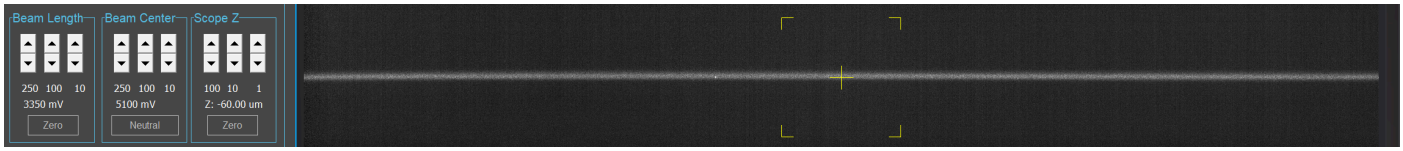


Adjust the beam thickness to be as thin as possible
Apply offsets for each channel to compensate for chromatic aberrations.

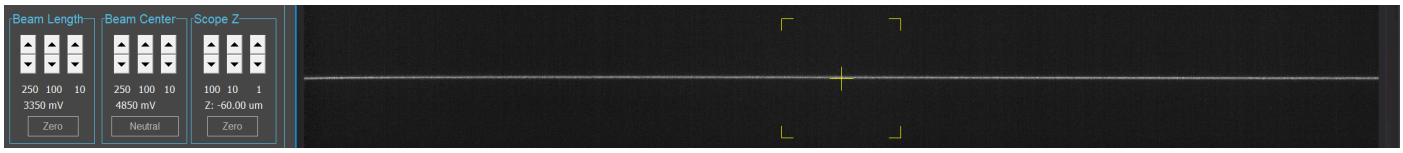


Clicking "Neutral" adjusts the Electrically Tunable Lens (ETL) to its center of range (5000 mV). The ETL has a range of 0–10mV.

Example for ASLM mode at 3.25x

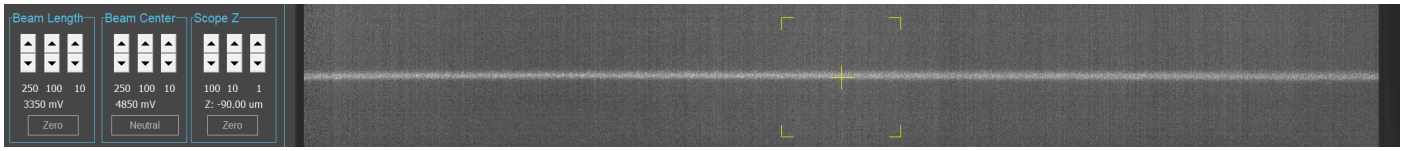


Beam Center: 5100mV, Note: This was the final stage of the Beam Length (Step 5) adjustments.

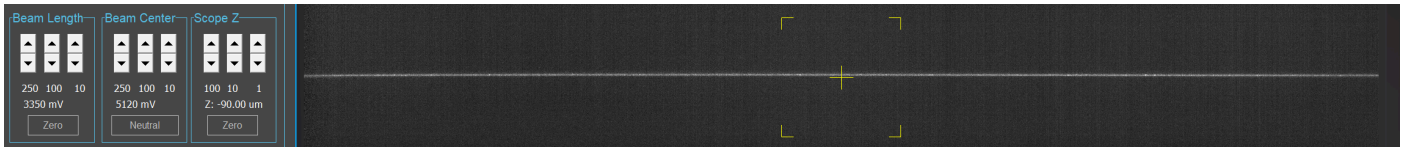


Beam Center: 4850mV, Note: The beam has a consistent thickness throughout

Switch Channels from 561nm to 640nm



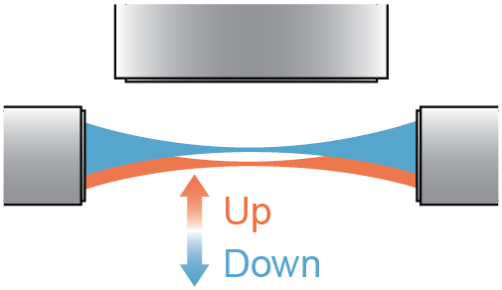
Beam Center: 4850mV, Note: while the beam center position did not change in mV, the beam in 640nm is thicker than in 488nm



Beam Center: 5120mV, Note: The beam has a consistent thickness throughout

Switch to the other illumination side and repeat the Beam Center adjustment to optimize all channels.

7) Left/Right Coplanarity

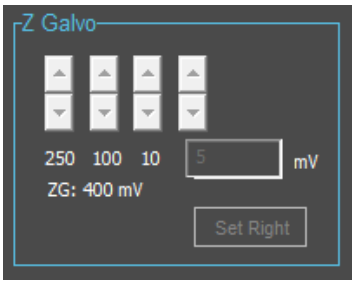


This step aligns both sides of illumination so that the left and right sides are in focus. It is recommended to perform this in sheet mode on the sample. For the best results, move the sample down so that the lightsheets illuminate the surface, ensuring both sides have equal access to the feature.

1 Capture reference image (left)

The button **Capture reference image (left)** takes an image of the current selected channel with the left side of illumination. The system will automatically activate **Enable overlay**, switch to the right side of illumination and go **live**

2 Focus using the Z-Galvo

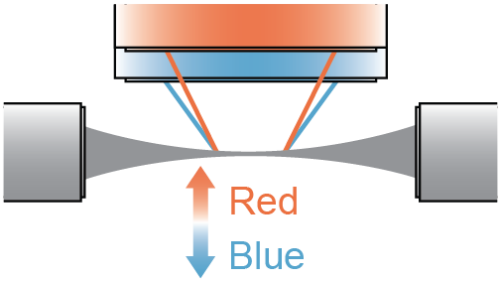


With Enable overlay being active, you can control the Z-Galvo. Adjust the Z-Galvo position until the features are aligned

3 Save and close

Click **Set Right**, which will store the position of the Z-Galvo and close this alignment.

8) Detection Chromatic Correction

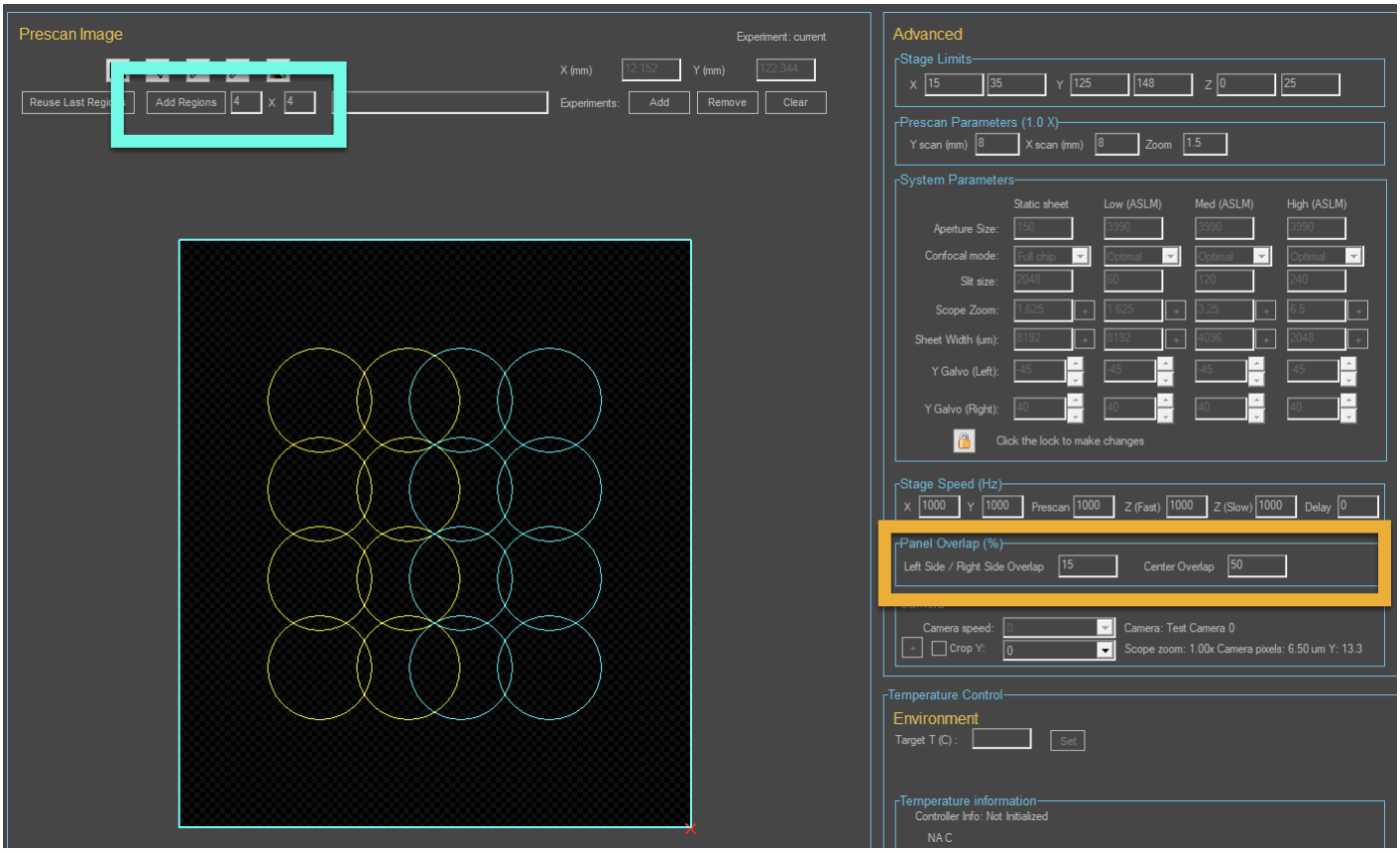


This step repeats Step 2, but in sheet mode on the sample to confirm that all channels are in focus. While Step 2 was performed on the static beam and at high magnification, this alignment should be done the imaging mode and magnification intended for capture.
Ensure you focus on channel-specific fluorescence, not autofluorescence.

- Focus in one channel (e.g. 488nm)
- Click *Zero*
- Click *Set Scope Offset*
- Change channel (e.g. 561nm)
- Focus
- Click *Set Scope Offset*

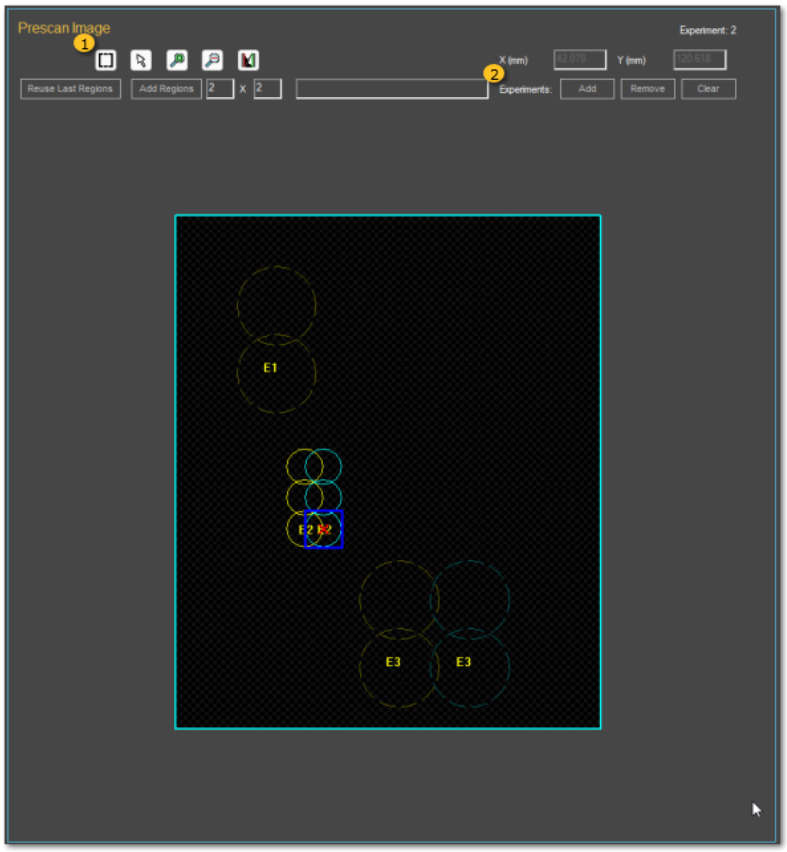
Define Overlaps Between Regions

Regions can be defined either by drawing an area using the **Marquee tool** or by clicking the **Add Regions** button (screenshot below, blue box), which will add the number of regions defined in the box next to the button, in this example a 4x4. The Marquee tool fills the selected area precisely with regions that may have different overlaps. In contrast to this the **Add Regions** function creates regions with defined overlaps. The overlaps are defined in % in the Advanced settings (screenshot below, yellow box). You can move/pan the regions within the Prescan Map by holding down the right mouse button and dragging the region.



Multi-Specimen Experiments

Multiple image acquisitions can be defined with independent imaging parameters. Experiments will be acquired in sequence.



1 Define regions

Define regions by drawing an area with the *Marquee tool* or clicking the *Add Regions* button, which adds the specified number of regions (columns × rows) from the adjacent box. To move or pan regions within the Prescan Map, hold down the right mouse button and drag.

2 Add/Remove/Clear Experiments

Add will create e anew Experiment and allow you to draw new regions and make changes to all other imaging parameters (e.g. imaging mode (low, medium, high), channels, exposure time, z-stack, sides of illumination)
Remove will delete the current experiment
Clear removes all experiments

Typical procedure
1. Define a region.
2. Set up the capture (imaging mode (low medium high), channels, exposure time, z-stack, sides of illumination)
3. Click *Add* Experiment.
4. Define a new region (using either the Marquee tool or *Add Regions*).
5. Click Add Experiment to add more experiments.

Use the arrow tool and click on the experiments to select and switch between them.

Click Remove to delete the selected experiment.
Click Clear to remove all experiments.

To switch between experiments click on the circular regions in the prescan map

TUTORIAL: Deconvolving AxL Lightsheet Data Using Big Data Deconvolution

See also:
Lightsheet PSF Generator
Big Data Deconvolution
3D/ 4D Volume View
Bounding Box

1. Generate the Lightsheet PSF

With the image dataset selected, open ***Analyze*** > ***Deconvolution*** > ***Lightsheet PSF Generator***.
The tool auto-loads imaging metadata from the selected file.

1. Set the refractive index to the actual imaging medium (e.g., 1.56).
2. Click Generate
3. A new file will appear in your Slide File container: [imageName].psf

NOTE: Computation time depends strongly on Slit Width.
Larger slit widths → larger excitation PSF → longer generation (e.g. Full-Chip 2304µm slit width can take several minutes).

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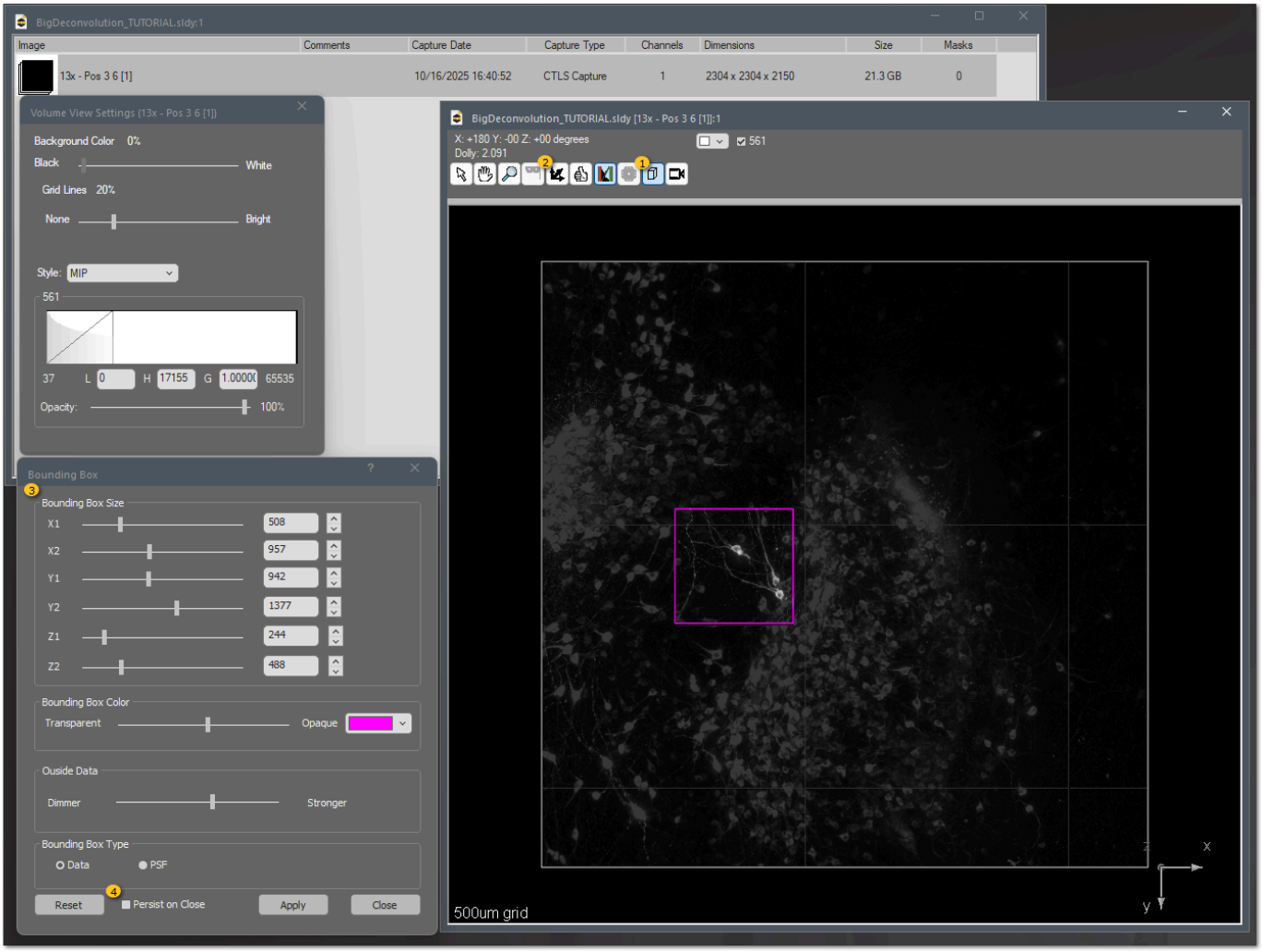
2. Define a Bounding Box for Testing the Deconvolution

It is recommended to test the deconvolution on a small crop of a stitched montage or large 3D image first. Define the Bounding Box using the Bounding Box tool in the 3D/4D Volume View. With the image dataset selected, open ***Home*** > ***View*** > ***3D/4D Volume View***.



1. **Enable Bounding Box**

2. **Switch to Orthographic View (for easier selection)**

3. **Define a Bounding Box**

Use the sliders to create a small magenta cube inside the larger volume

4. **Check Persist on Close and click Apply**

Use this option i you only want to see the selected volume after closing the Bounding Box interface

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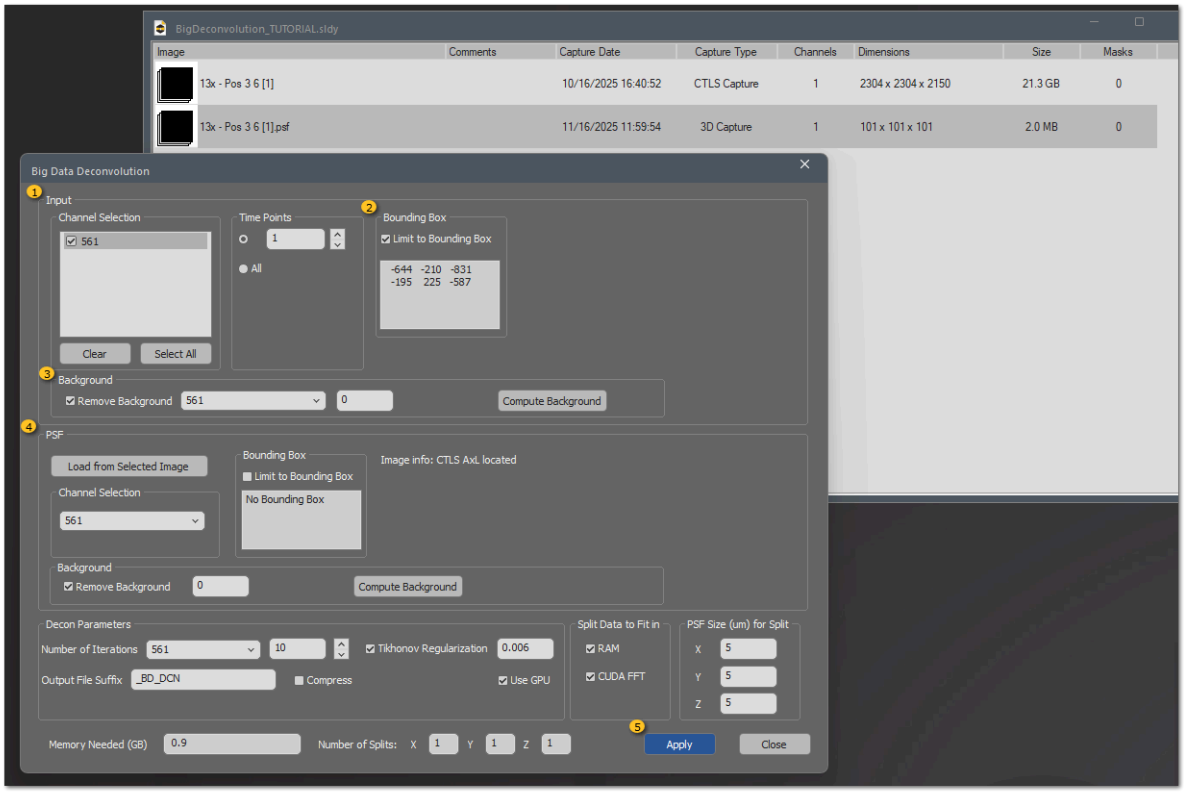
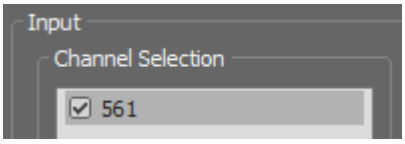
3. Run Big Data Deconvolution on the Bounding Box

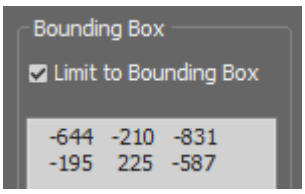
Select the dataset you want to deconvolve and go to ***Analyze*** > ***Deconvolution*** > ***Big Data Deconvolution***.



1 Select the channel(s) you want to deconvolve



2 Enable Limit to Bounding Box

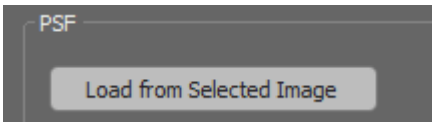


If desired. For the test run it's recommended to reduce the volume and hence time.

3 Remove Background

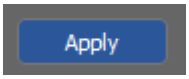
Enter a value or let SlideBook ***Compute Background***
NOTE: Removing Background can enhance the result and allows to reduce (down to half) the required number of iterations significantly and acts as a edge padding equivalent avoid ringing artefacts. Calculation might take long so entering a value is a faster option. A value of 100 is usually used for sCMOS cameras.

4 Load the PSF



- Highlight the PSF capture in the Slide file container
- Return to the Big Data Deconvolution window
- Click Load from Selected Image

5 Click Apply



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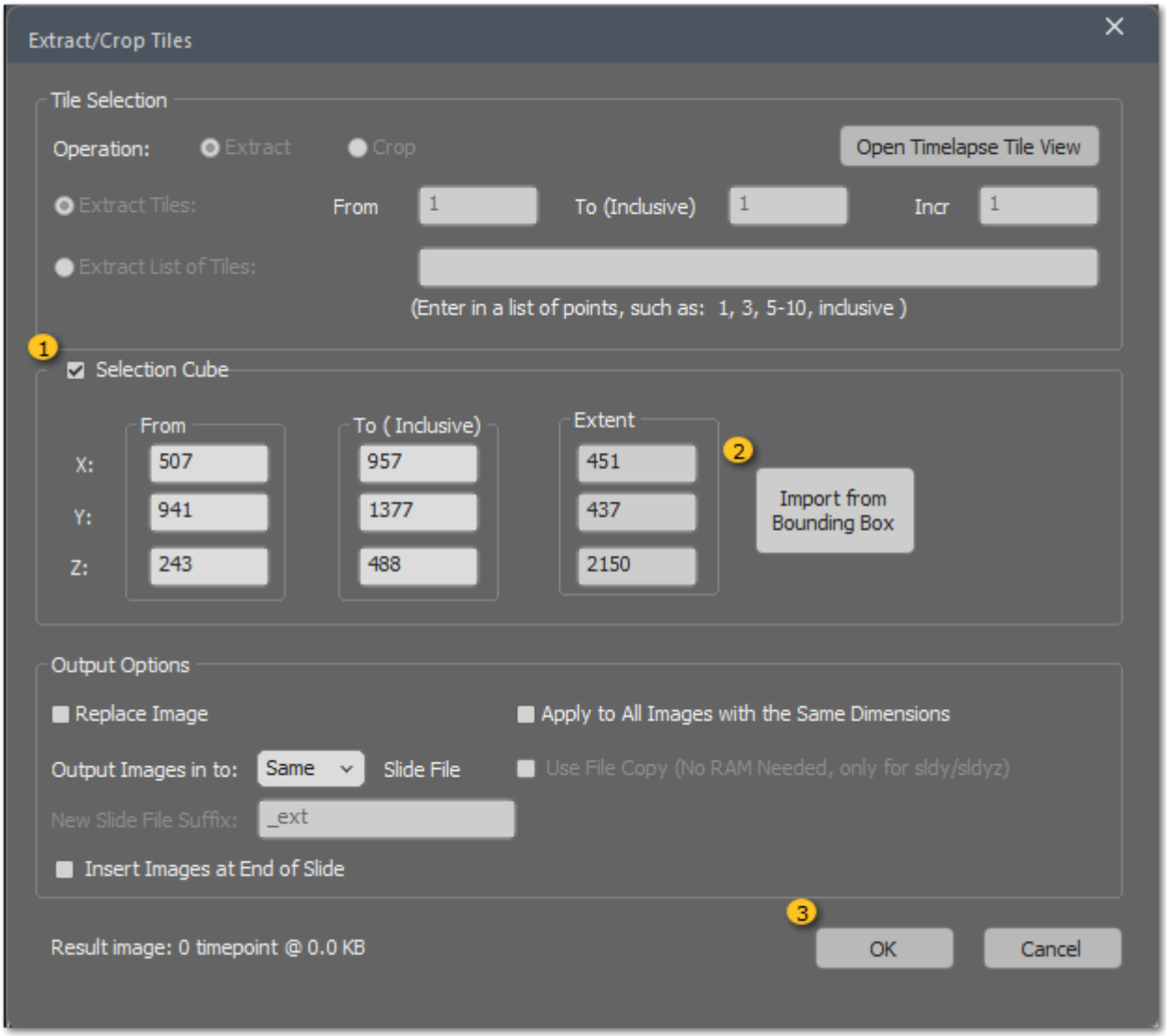
4. Extract the Matching Raw Volume for Comparison

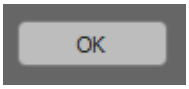
Create a cropped copy of the bounding boxed volume. With the original dataset selected, go to ***Analyze*** > ***Extract/Crop >*** ***Extract/Crop Tiles***.



1. **Enable Selection Cube**

2. **Click Import from Bounding Box**

3. **Click OK**



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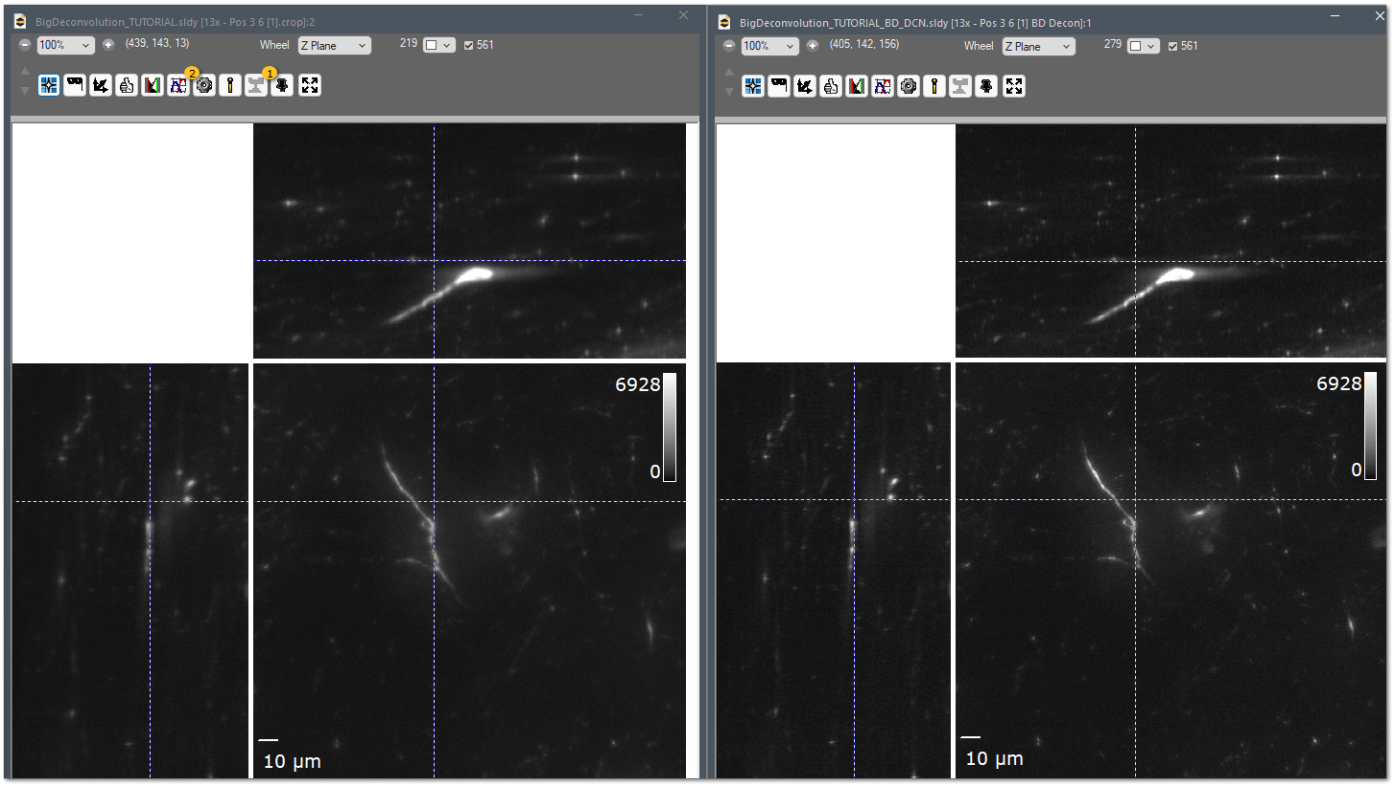
5. Compare Raw vs. Deconvolved Data

Open both datasets in Three View for side-by-side evaluation along all three axes.
If you are not familiar with all tools, it is worth reading about Adjust LUT and Three View. If desired pin the window to keep the window size constant when zoomingand/ or enable the crosshairs.
To evaluate properly, make sure you compare the same orthogonal planes, and adjust the histogram to the same values.



1. **Optional: Enable the pin to lock window size**

2 Optional: Enable Crosshairs

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6. Run Full-Volume Big Data Deconvolution

Once satisfied:

1. Select the full dataset.
2. Open Big Data Deconvolution again.
3. Load the same PSF.
4. Uncheck Limit to Bounding Box.
5. Click Apply.

NOTE: Duration estimate - Deconvolution with 10 iterations on this example dataset 2304×2304×2150 px (~21.3 GB) took 46min on a PC with the following specifications:
• Processor: Intel(R) Xeon(R) w7-3455 (2.50 GHz)
• Installed RAM: 256 GB
• GPU: NVIDIA RTX A6000