




# SlideBook 6

**User's Guide**

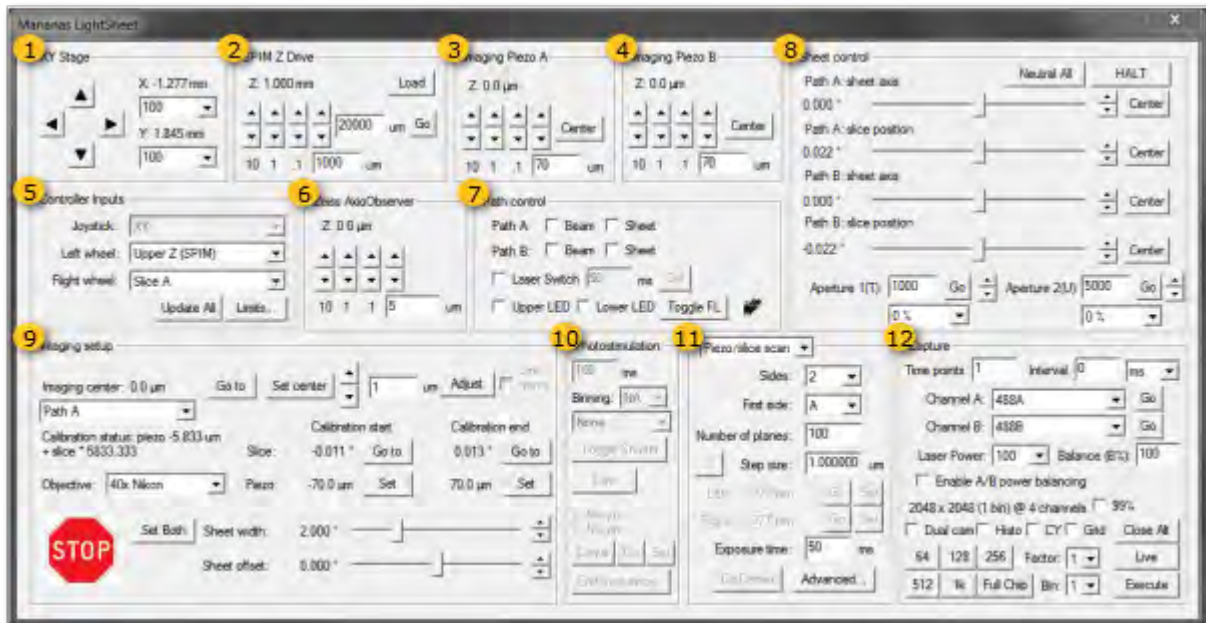
## Marianas LightSheet Window

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 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.

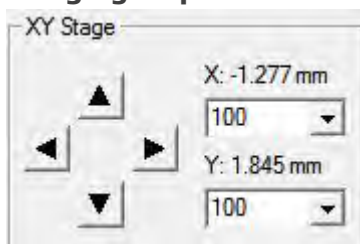
To open the **Marianas LightSheet** controls within SlideBook, go to the Home tab, Acquire group >  **LightSheet** > **Marianas LightSheet**.

See also:

- [Marianas LightSheet Hardware](#)
- [Marianas LightSheet Alignment and Calibration](#)
- [Marianas LightSheet Operation](#)

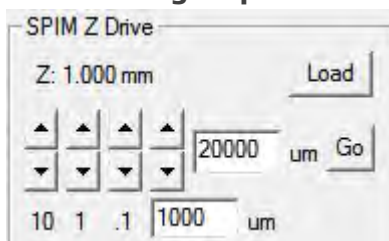


### 1 XY Stage group



- **Arrows** Move the XY stage stepwise in X or Y
- Increment of steps ( $\mu\text{m}$ ) 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

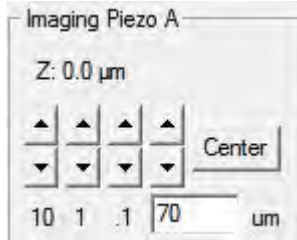
### 2 SPIM Z Drive group



- **Arrows** Lower (arrow down) and withdraw (arrow up) the SPIM drive towards or from the sample for 10  $\mu\text{m}$ , 1  $\mu\text{m}$ , 0.1  $\mu\text{m}$  or a given value (e.g. 1000-50000  $\mu\text{m}$ ) respectively

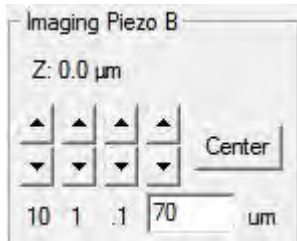
- **Load** brings the SPIM Z drive to its most upper position to enable sample loading. This button will change to Return when a sample height was engaged before
- **Return** will bring the SPIM Z drive to its previously visited lower position at the coverslip
- **Go** brings the SPIM Z drive to the value specified in the field left to the button - **NOTE:** the value is in  $\mu\text{m}$

### 3 Imaging Piezo A group



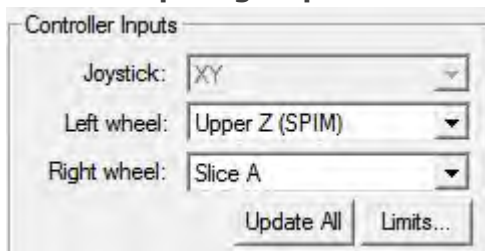
- **Z** indicates the current piezo A position
- **Arrows** Retract (arrow down) and expand (arrow up) piezo A for 10  $\mu\text{m}$ , 1  $\mu\text{m}$ , 0.1  $\mu\text{m}$  or a given value (e.g. 70  $\mu\text{m}$ ) respectively
- **Center** brings the piezo to zero position (0  $\mu\text{m}$ ) - **NOTE:** holding the piezo at zero position is active, piezos will go to sleep/ relax after 5min without activity latest

### 4 Imaging Piezo B group



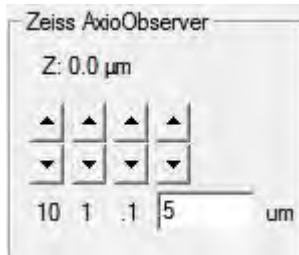
- **Z** indicates the current piezo B position
- **Arrows** Retract (arrow down) and expand (arrow up) piezo B for 10  $\mu\text{m}$ , 1  $\mu\text{m}$ , 0.1  $\mu\text{m}$  or a given value (e.g. 70  $\mu\text{m}$ ) respectively
- **Center** brings the piezo to zero position (0  $\mu\text{m}$ ) - **NOTE:** holding the piezo at zero position is active, piezos will relax after 5min without activity

### 5 Controller Inputs group



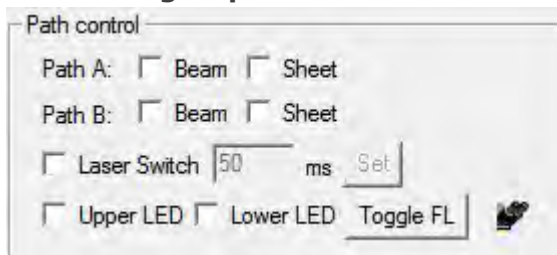
- **Joystick** controls the XY movement of the stage (no alternative selection available)
- Select which device (scanners, piezos, SPIM Z Drive) is controlled by the **Left wheel** of the Joystick controller box
- Select which device (scanners, piezos, SPIM Z Drive) is controlled by the **Left wheel** 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



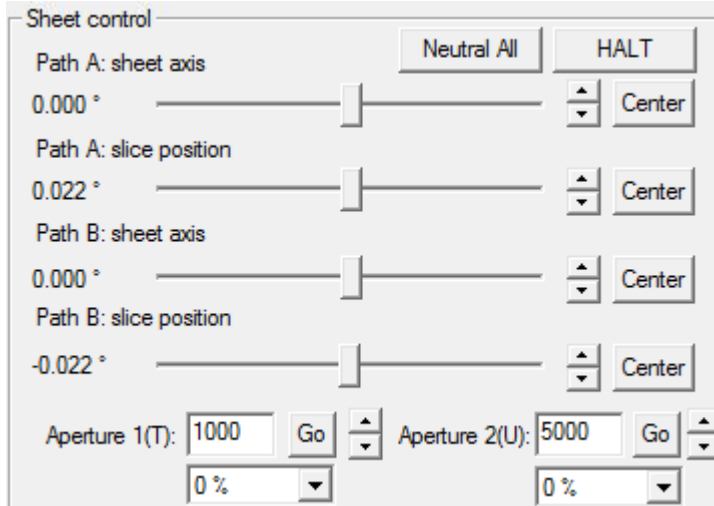
- **Z** indicates the current position of the lower / sub-stage objective, microscope nose piece
- **Arrows** Lower (arrow down) and raise (arrow up) the lower objective for 10 μm, 1 μm, 0.1 μm or a given value (e.g. 5 μm) respectively

## 7 Path control group



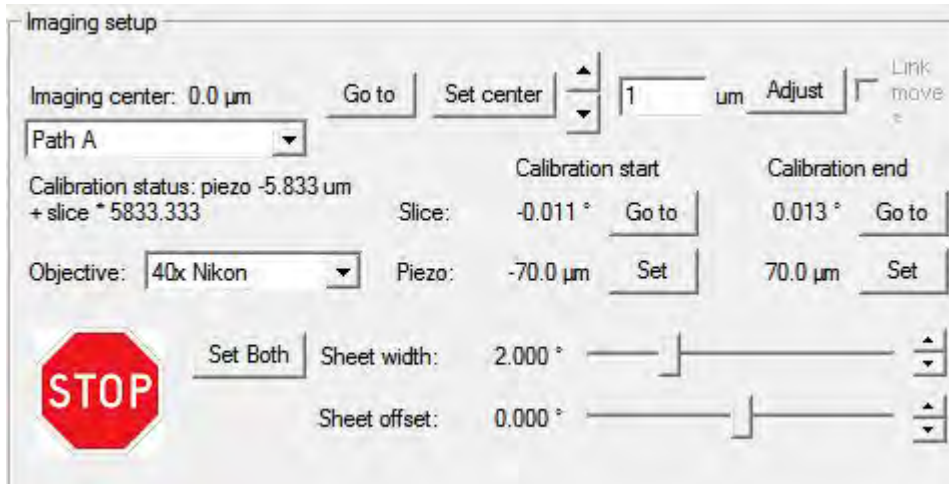
- **Path A:** tick-boxes to enable Beam or Sheet (dithered beam) for scanner of side A
- **Path B:** tick-boxes to enable Beam or Sheet (dithered beam) for scanner of side B
- **Laser Switch:** Select which device (scanners, piezos, SPIM Z Drive) is controlled by the **Left wheel** of the Joystick controller box
- Select which device (scanners, piezos, SPIM Z Drive) is controlled by the **Left wheel** of the Joystick controller box
- **Update All** will update the selection (not required when in live mode)

## 8 Sheet control group



- Adjust the **Sheet axis** or **Slice position** by sliders and arrows. **NOTE:** Changing the slice position by using the joystick wheel is recommended for easier use.
- **Center** will bring each sheet axis or slice position to centre individually.
- **Neutral All** brings the scanner position in all directions as well as the piezos to their center position (initial alignment point for most range).
- **HALT** instantly stops all SPIM Z drive movement, stage movement, scanner and piezo movement.
- 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.

## 9 Imaging setup group



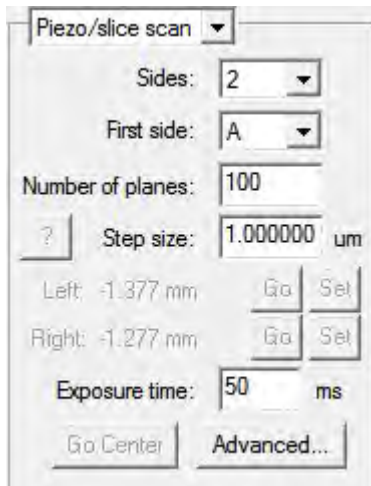
- Indicates coordinates and selection (**Go to**, **Set center**) of the **Imaging center**
- Indicates selected **Path**
- **Calibration Status**: indicates whether a calibration is saved for the path and shows the calibration curve
- Indicates selected **Objective** - **NOTE**: Requires manual change 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**: hold 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**: hold Ctrl while clicking the arrows will move it with 10x increment

## 10 Photostimulation group



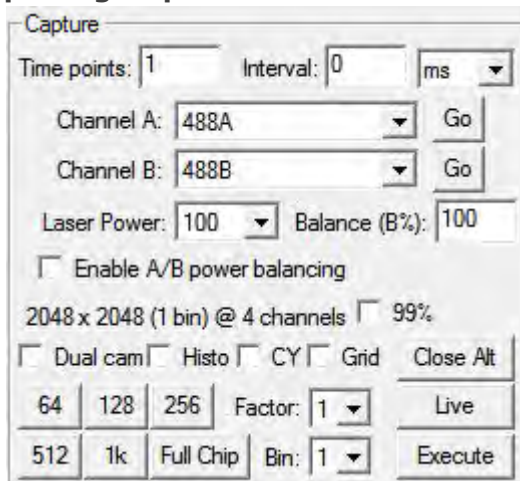
- Select parameters (**Exposure time** in ms, **Binning**, **Channel**) for imaging with the finder Camera
- **Toggle Shutter** opens or closes the selected Epi-port excitation
- **Live/ Stop** enables and disables the Live mode of the camera (separate view window)
- **X & Y** indicate the set stage 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 position
- **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, not 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



## Marianas LightSheet Module

The Marianas LightSheet (MLS) represents our turnkey system to exploit the Dual-view Inverted Selective Plane Illumination Microscopy (diSPIM), a pencil beam light sheet method offering low phototoxicity in 3D imaging making it possible to image living cells and small organisms over extended periods of time. It is a flexible light-sheet system with an open specimen geometry not requiring special slides or capillaries. This Selective Plane Illumination Microscope images a specimen using two perpendicular light sheets from two equivalent optical paths and objectives which are each used sequentially for both illumination and imaging. The resulting 3D datasets have isotropic resolution in X, Y and Z with only two views, resulting in fast image capture with low light dose for extended specimen viability.

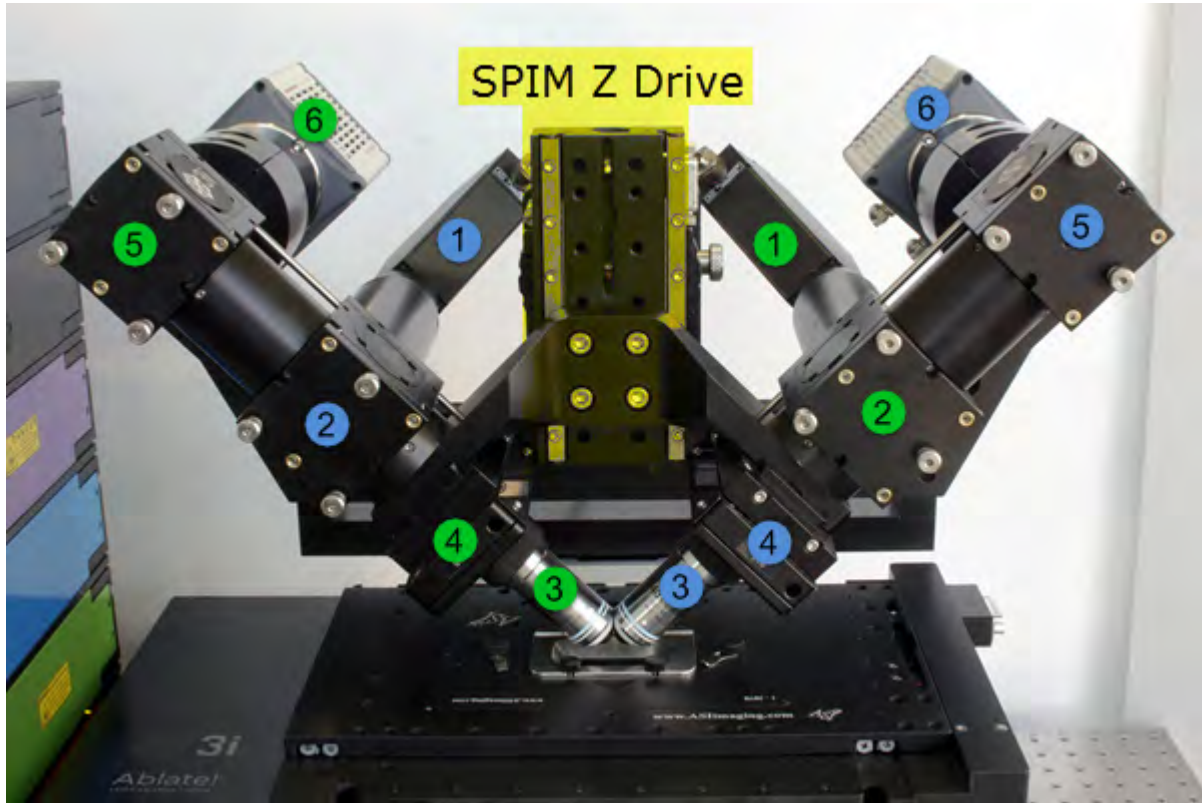
Two matching water dipping objectives can have an NA of up to 0.8 with a working distance allowing efficient orthogonal mounting. Each objective is used for excitation and emission collection via identical high-speed high-resolution sCMOS cameras. A third objective is located below the specimen chamber for locating the sample. Photomanipulation, such as the Ablate! photoablation system, can be added via the microscope's side- port.





## System Hardware

The system consists of two symmetric optical paths (unless 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 created from one objective and then imaged using the other objective. A stack of images is collected by moving the light sheet through the sample. The role of each two objective 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 MLS can be controlled in software or by the accompanying joystick. Review the following diagram to become familiar with the joystick functions. The left wheel (1) controls the SPIM Z Drive. The right wheel (2) controls the Piezo or Slice Position and is set in software. The button on the joystick (3) toggles the XY stage movement between fast and slow.



## **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 alignment steps, and then come back to each step to finish. 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:

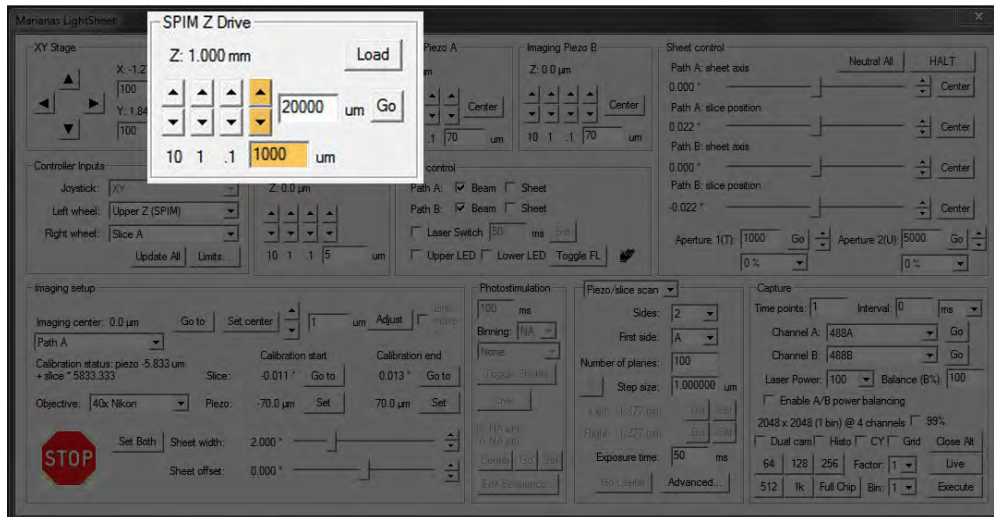
- dye solution (ex. Fluorescein) and/or a dense solution of beads/ fluorescent particles
- Allen wrench (3/32" size) - applies to systems installed before November 2017 only

## 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.

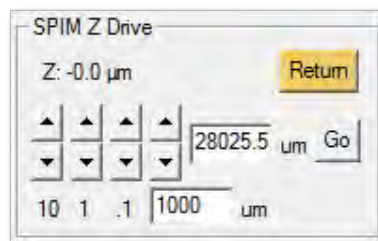
1. Open the **Marianas LightSheet** controls within SlideBook, go to the Home tab, Acquire group >  **LightSheet** > **Marianas LightSheet**.

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 in the lower left of the dialog 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.



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.

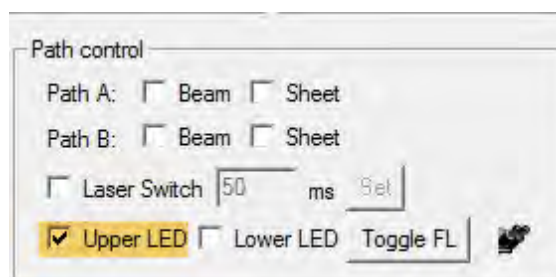


4. Once the sample is in place, you will want to move the SPIM Z Drive so the front lens of the objectives are fully submerged, this configuration will typically put you in position just above the coverslip.

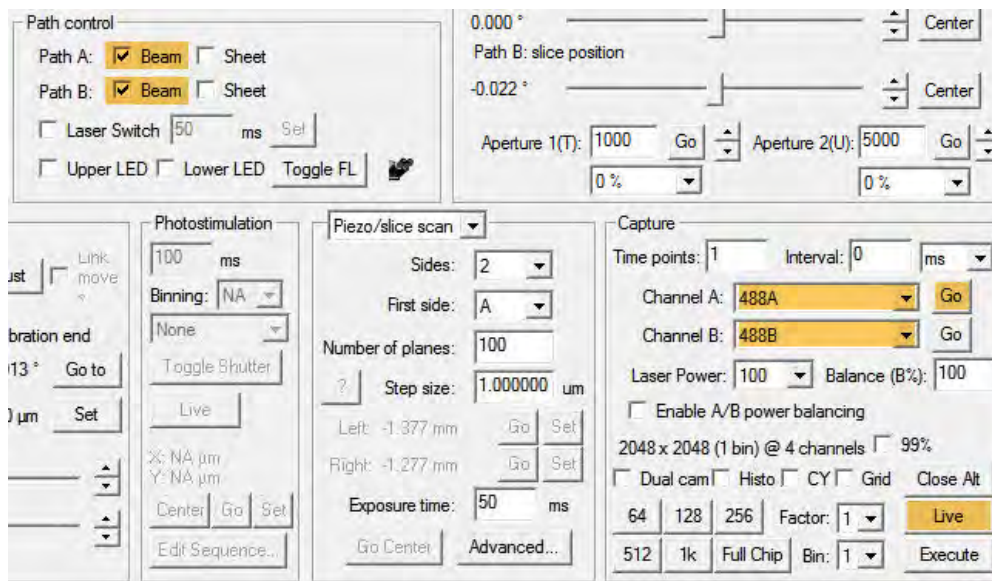
**NOTE:** If you use the **Return** button, the drive will lower to the previous sample position. Make sure your coverslip or sample is not higher.

5. Incrementally step down SPIM Z Drive by entering a value into the **SPIM Z Drive** group as described in step #2.

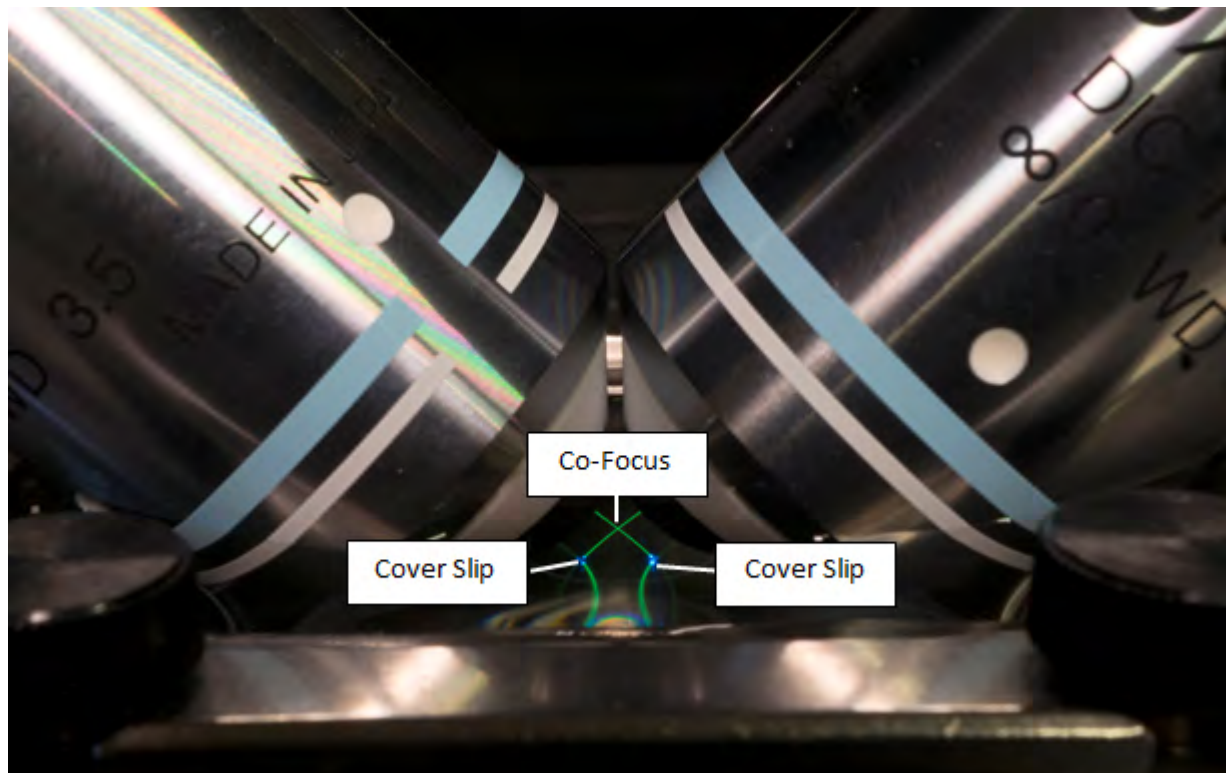
**NOTE:** Turning on the **Upper LED** in Path control may add helpful additional illumination



6. In the Path Control group, select a **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.



6. 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.

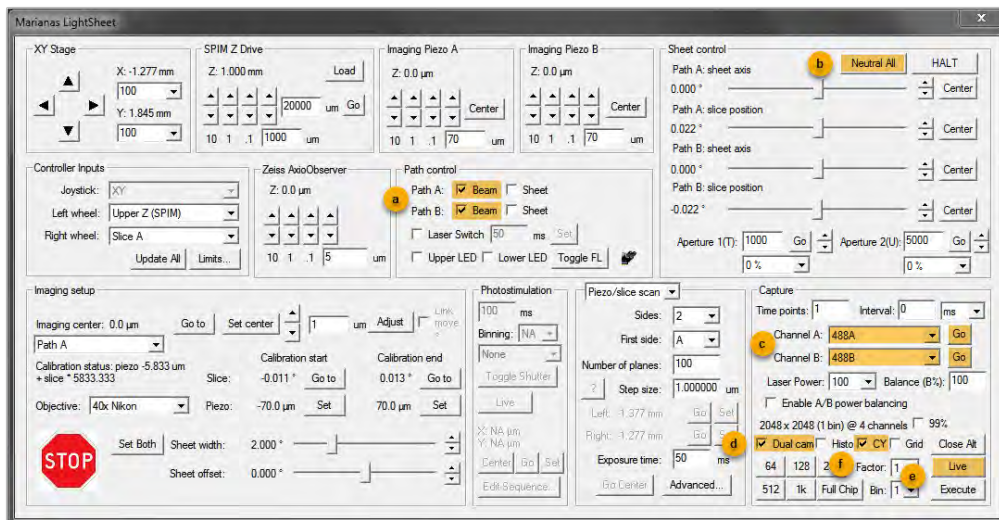




## TUTORIAL: Co-focusing the SPIM Objectives

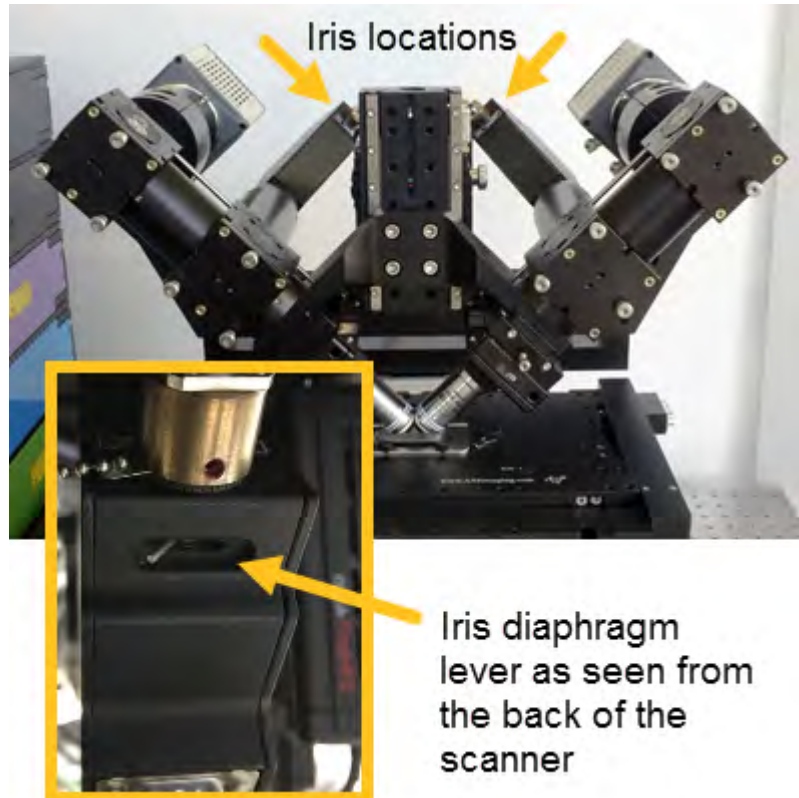


1. Open the **Marianas LightSheet** controls within SlideBook (Home tab, Acquire group > **LightSheet** > **Marianas LightSheet**).
2. Refer to [TUTORIAL: Using the SPIM Drive to Introduce SPIM Objectives into the Sample Chamber](#).
3. To prepare for co-focusing the SPIM objectives, perform the following 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 to **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.

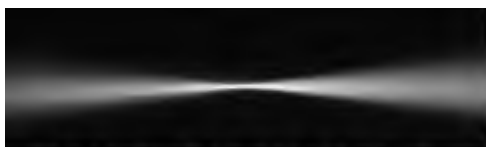
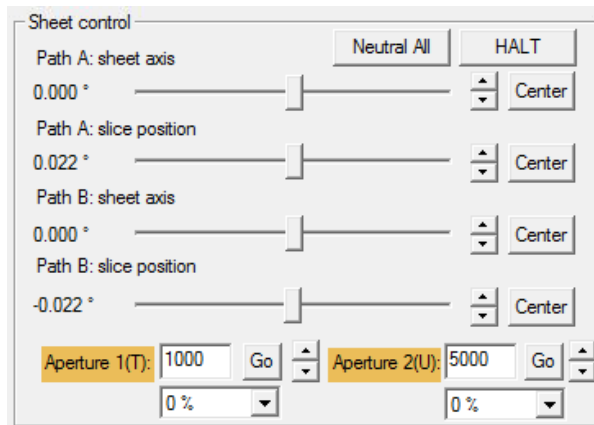


4. Use the iris on the back of each scanner to adjust each beam to a pencil beam. The iris fully closed when the small lever is to the right as seen from the front of the microscope (pictured from the back of the microscope below).
5. When your system is equipped with motorized irises, got to the Sheet control group, type a small value (can be -70000) and click **Go** or set 0% using the drop down menu for each iris **Aperture T** and **U** to achieve a pencil beam.

### Manual Iris



**Motorized Iris**



**Open iris = Gaussian beam**

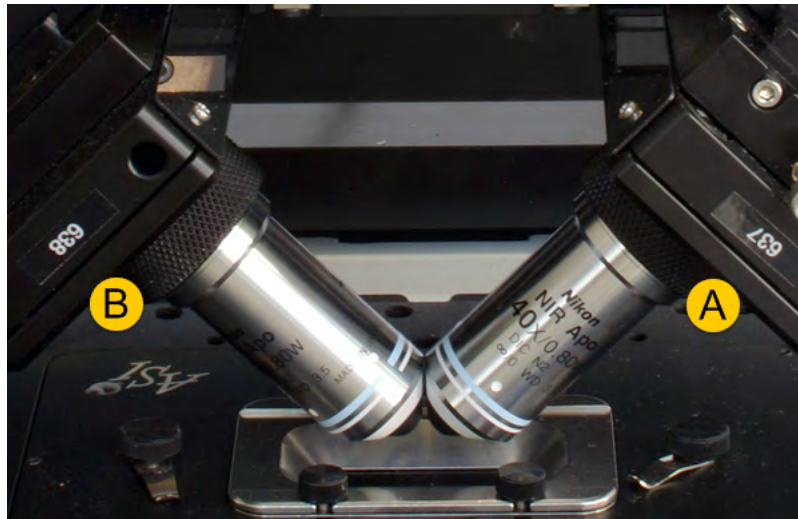


**Closed iris = pencil beam**

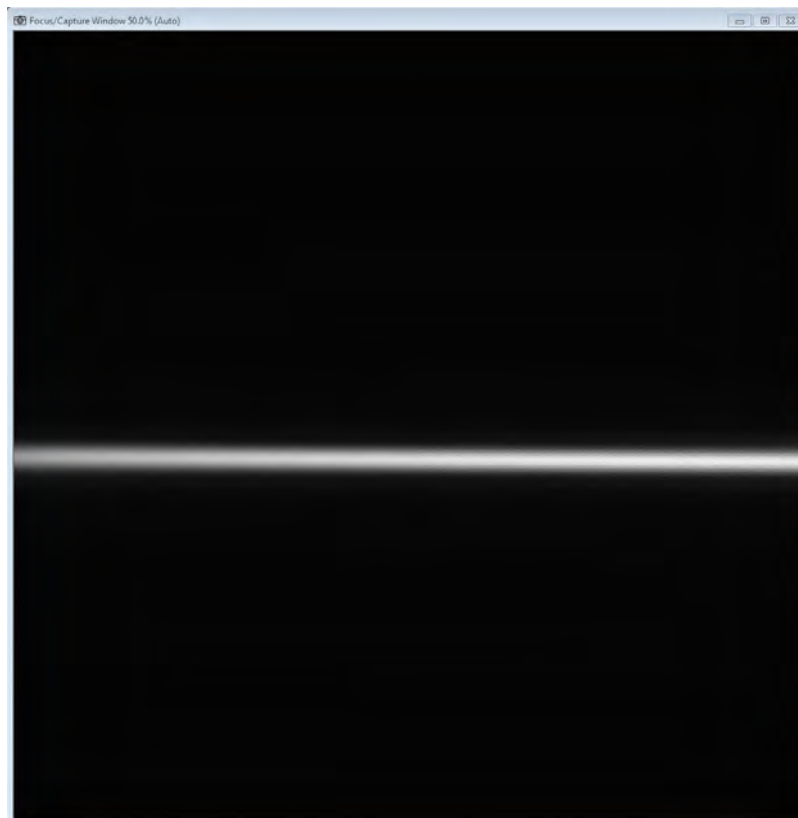
6. Select **Channel A > Go**, adjust the imaging objective's focus on the beam using the corresponding **Objective Threaded Bushing (A)** to focus pencil beam. Select **Channel B > Go**, adjust the beam's focus using the corresponding **Objective Threaded Bushing (B)** to focus pencil beam. Alternate 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 will see an interference pattern present around the beam and can proceed to [TUTORIAL: Co-aligning the Beams](#).

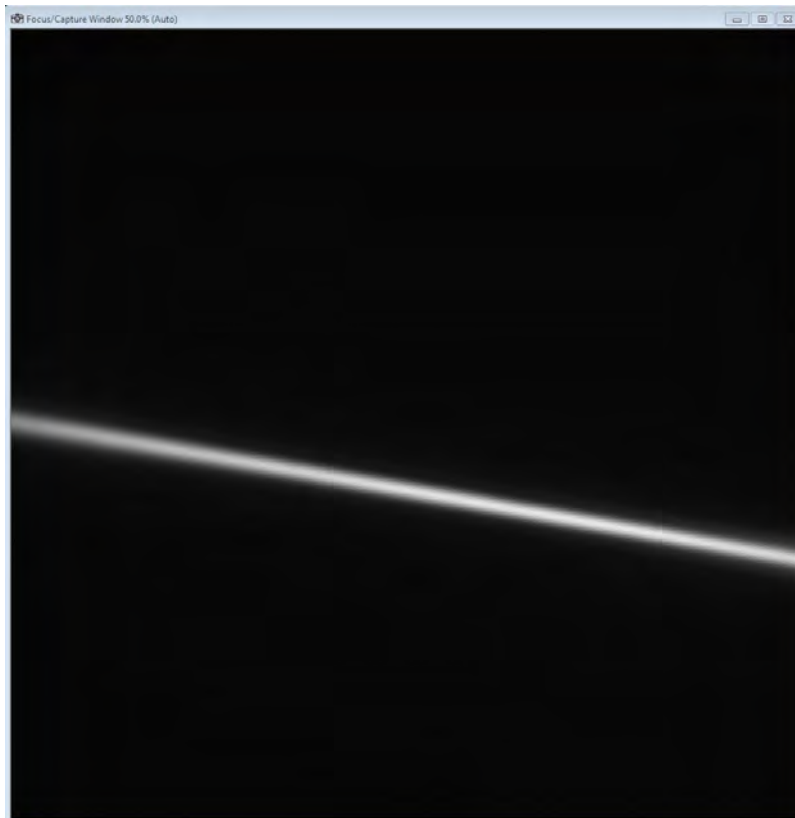


8. After the pencil beams for both channels are focused, you may notice some tilt (either rotational or focus). Please see [TUTORIAL: Adjusting the Tilt of the Pencil Beam](#) to correct for horizontal rotation (YZ tilt) or focus inconsistency (XY tilt).

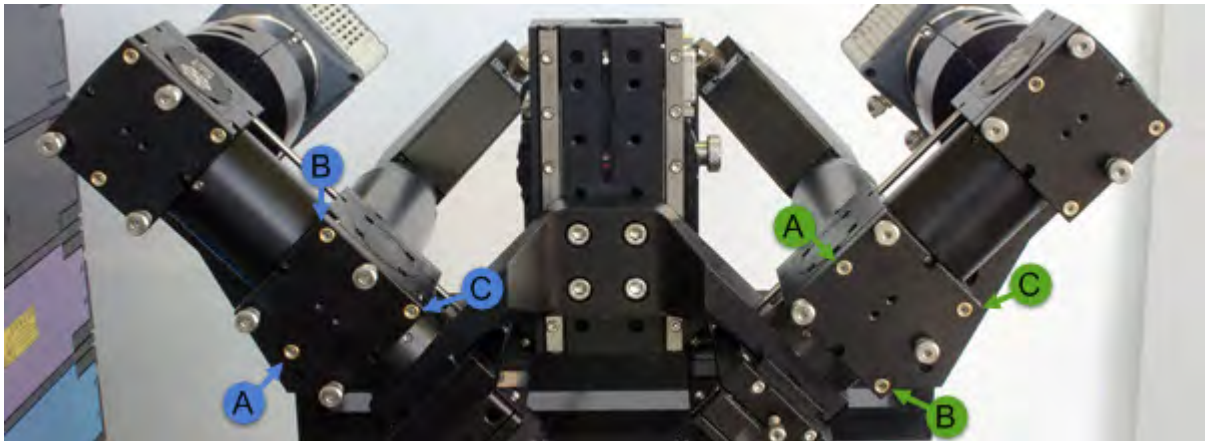
## TUTORIAL: Adjusting the Tilt of the Pencil Beam

### YZ Tilt (rotational)

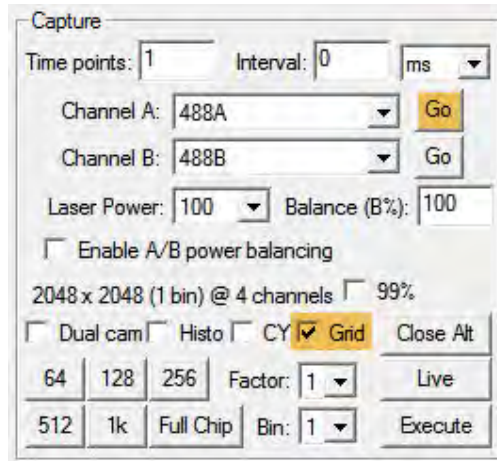
The YZ Tilt is apparent when viewing the beam through the camera; if the YZ tilt needs to be adjusted the beam is slightly rotated in the YZ dimension.



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**.

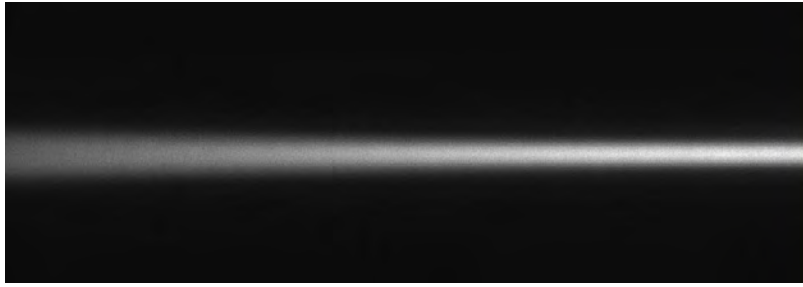


2. Adjust both **Screw B** and **Screw C** of the **Path A** dichroic mirror until the beam is horizontal and parallel to the grid lines.
3. Repeat for **Path B** (by clicking **Go** for **Channel B** and adjusting both **Screw B** and **Screw C** of the **Path B** filter cube) until both beams are in 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).

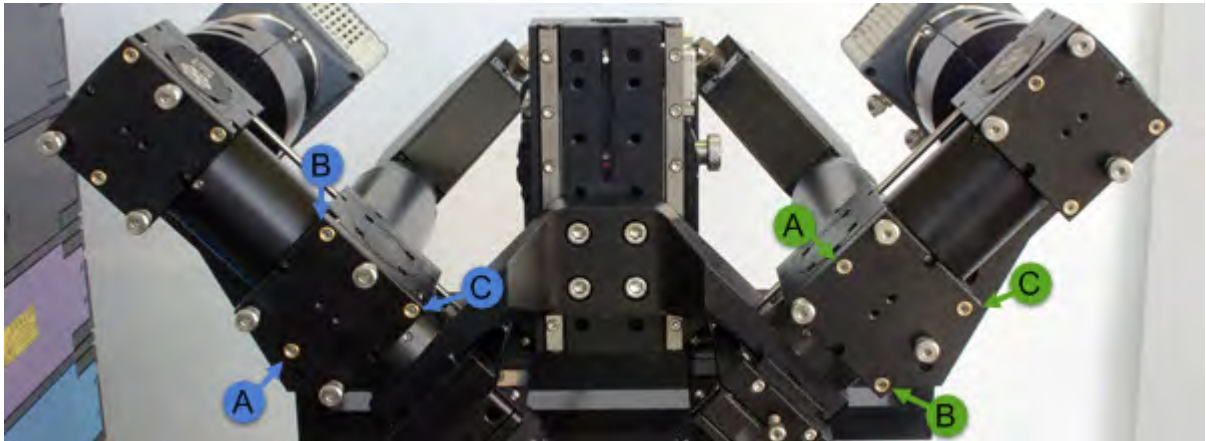


**XY Tilt (focus)**

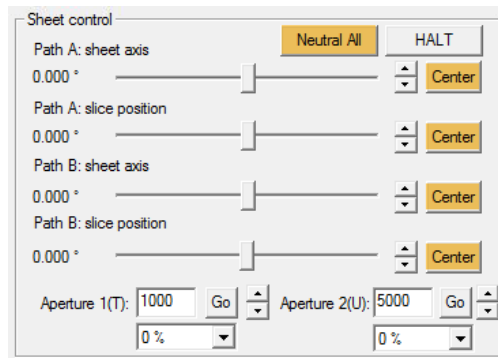
The XY Tilt is apparent when viewing the beam through the camera; if the XY tilt needs to be adjusted, the beam will appear as if it is coming toward (and away from) the camera.



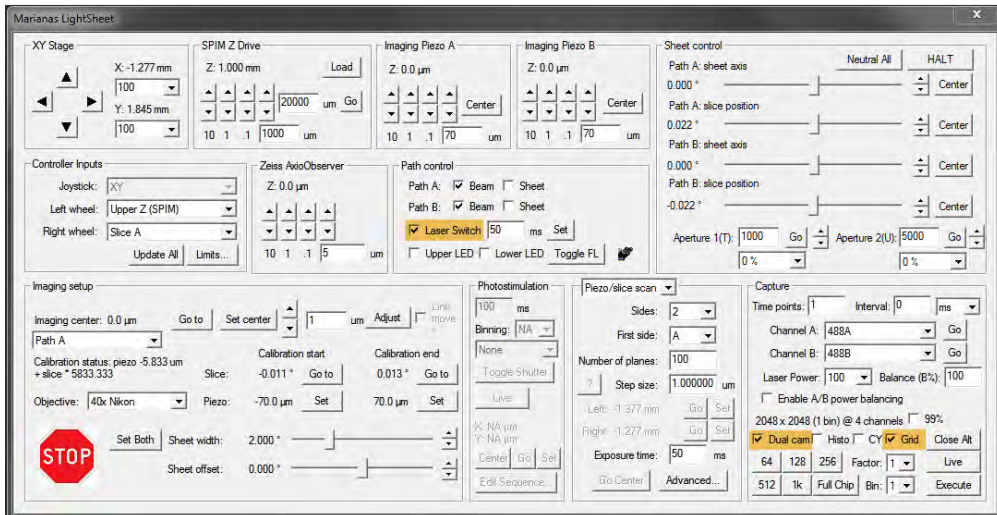
To correct XY Tilt for Channel A, you will adjust the dichroic mirror using **Screw A only** of the **Path A** filter cube. To correct XY Tilt for Channel B, you will adjust the dichroic mirror using **Screw A only** of the **Path B** filter cube.



1. Make sure sheets and slices are centered by clicking **Neutral All** and **Center** for all sheet and slice positions.



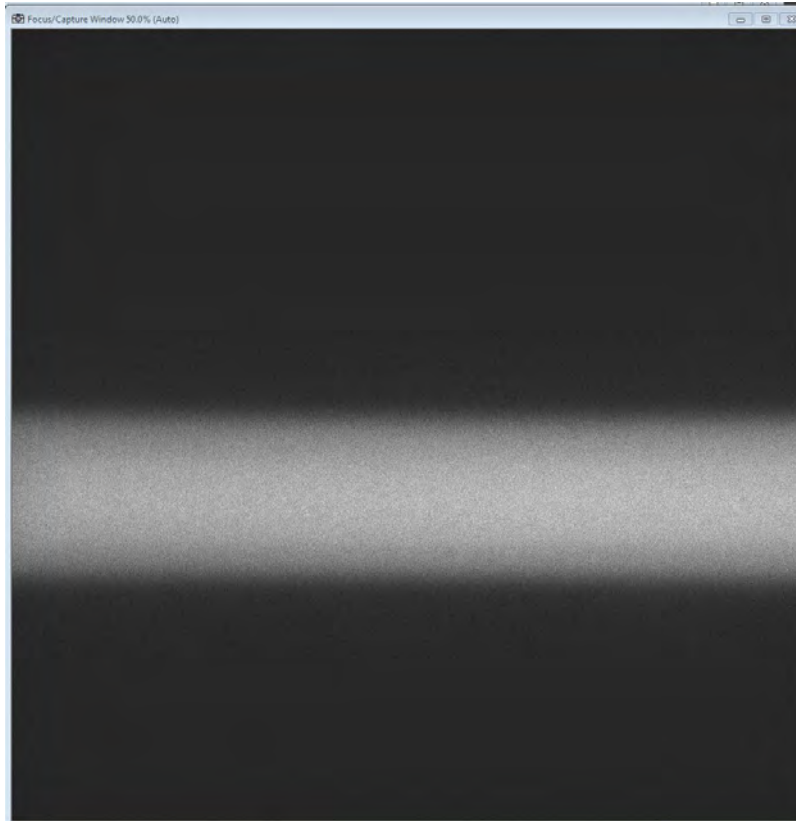
2. In the Controller Input group of Marianas LightSheet controls, set **Right wheel** to **Piezo A** for Path A using the drop-down selection. Confirm that Channel A and Channel B (in the Capture group) are set to the same filter for their respective paths. Click **Go** for **Channel A** to activate **Path A**.



3. Defocus 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.



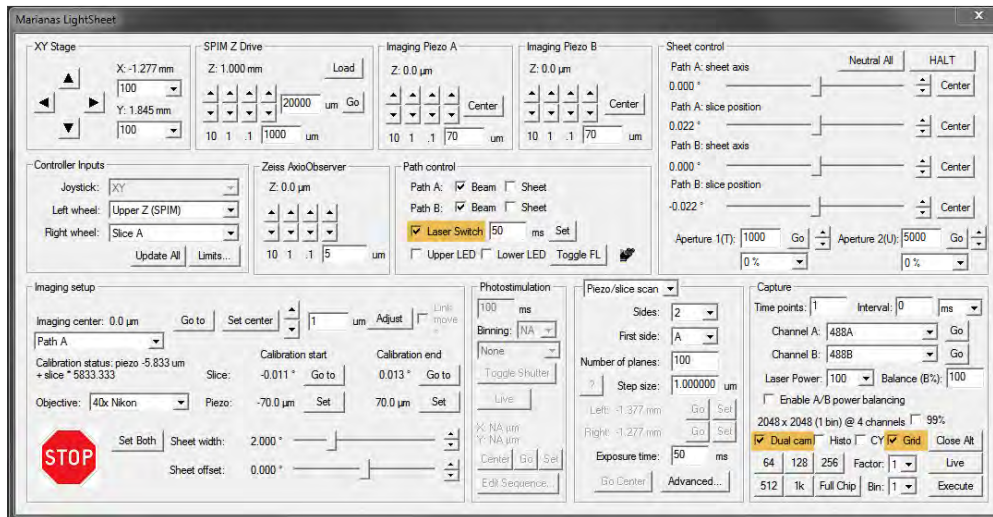
5. Select **Neutral All** to restore the slice position to its neutral position.
6. Refocus both pencil beams using the objective threaded bushings (as described in [TUTORIAL: Co-focusing the SPIM Objectives](#)) until pencil beam is uniform across the field of view.
7. Repeat steps 1-5 for Path B (setting **Right wheel** to **Piezo 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

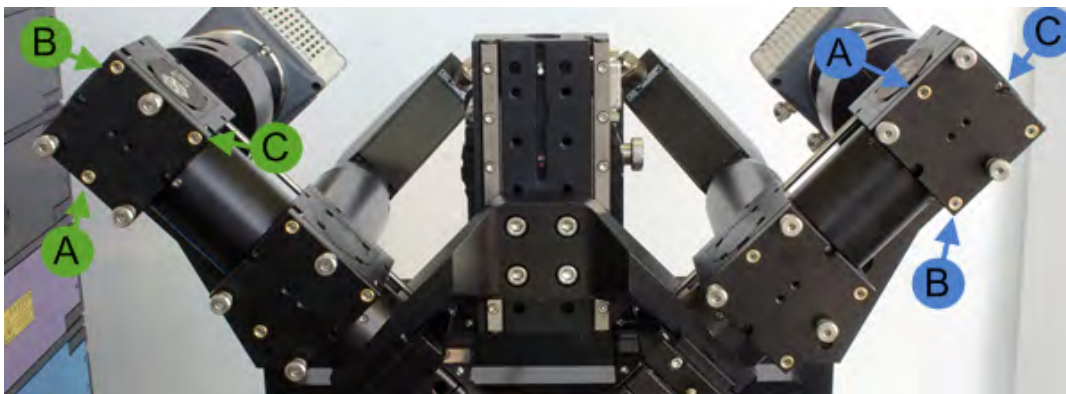
Prepare a sample for the MLS alignment, use the imaging media you will be imaging your samples in (water or PBS are acceptable but not ideal). To better visualize the beams, add a small amount of fluorescein or phenol red. For this tutorial, you will need to show beams and spots from both paths. To do so, check 3 boxes in the Marianas LightSheet dialog:

- **Dual Cam**
- **Laser Switch**
- **Grid**



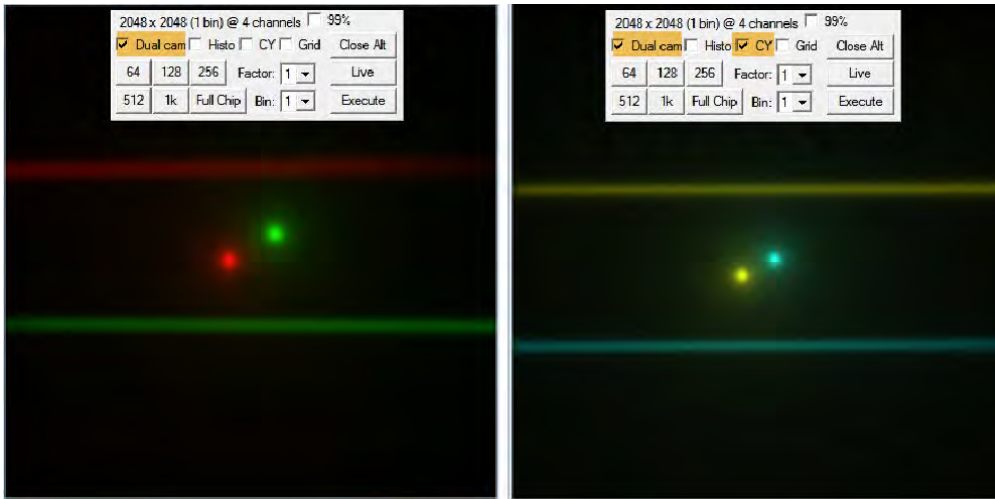
Throughout the tutorial, you will be adjusting the Camera (Upper) Mirror for each path. You will be using an Allen wrench in Screw A (left-right), B, and C (diagonally up-down) of **Path A** and **Path B** as depicted below.

- 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



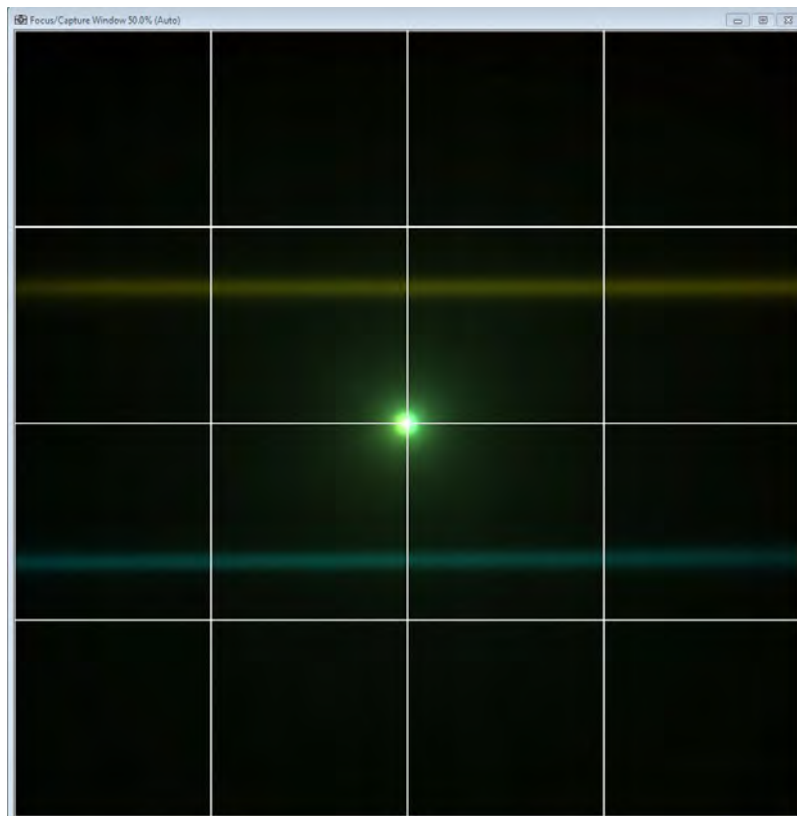
In the live window, **Camera 1 (Path A)** will be pseudocolored red while **Camera 2 (Path B)** will be pseudocolored green.  
**NOTE:** For our color blind users, activate **CY** checkbox (under the Grid checkbox) to view beams and spots as Cyan (**Path A**) and Yellow (**Path B**)



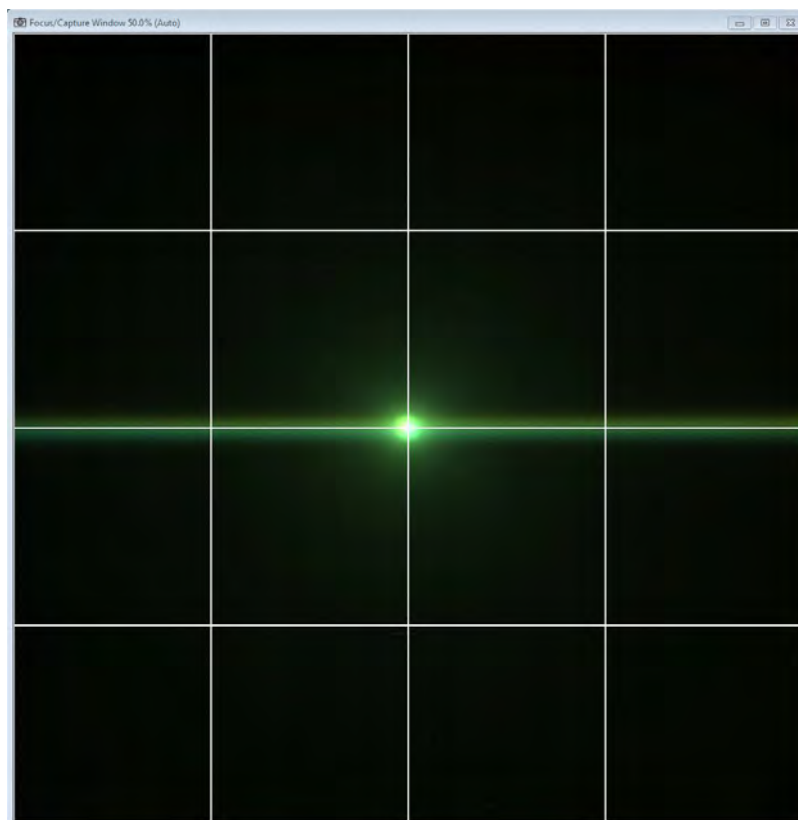
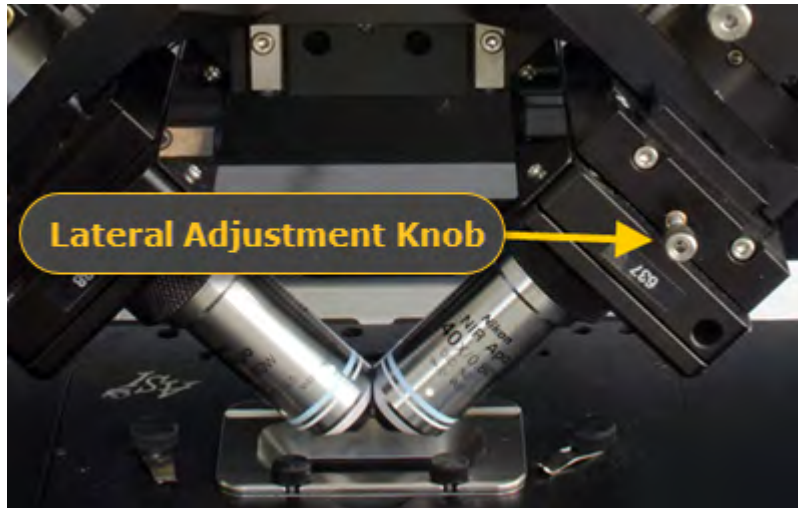


1. Use a combination of screws A, or B and C to move both the green and red spots to the center of the grid. Do not worry about the vertical location of the beams at this point.

**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.

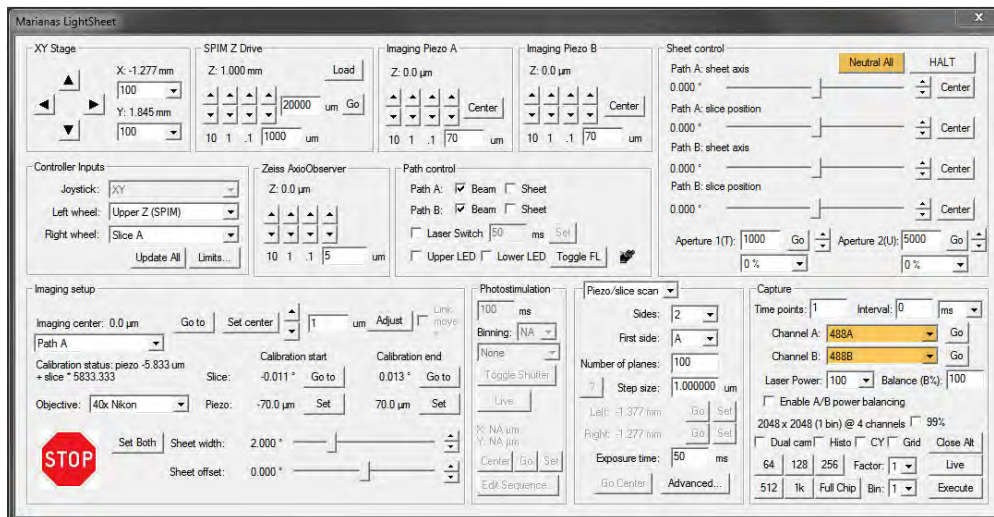
4. To test uniformity, you can use the **Right wheel** to adjust the slice position to see the beams go out of focus uniformly. To do so, click **Go** for **Channel A**, this should set **Slice A** to the Right wheel. Rotate the wheel and check the beam goes out of focus evenly in both directions. Click **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**.



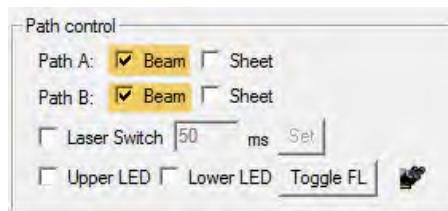
## TUTORIAL: Calibrating the Piezo and Slice Position

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.

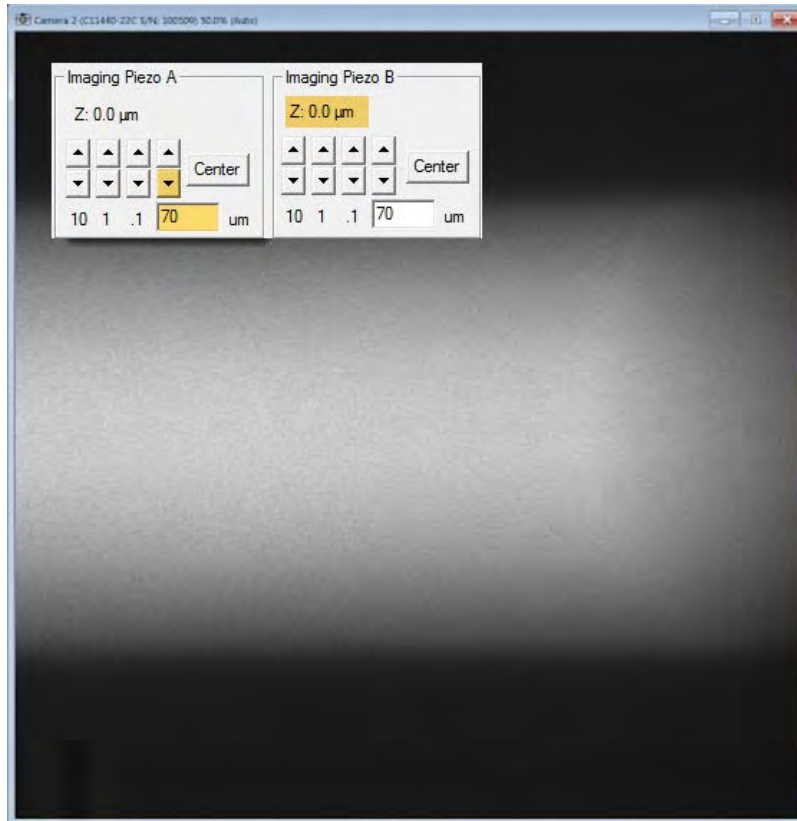


1. Check the box for **Beam** for Path A and Path B.



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.

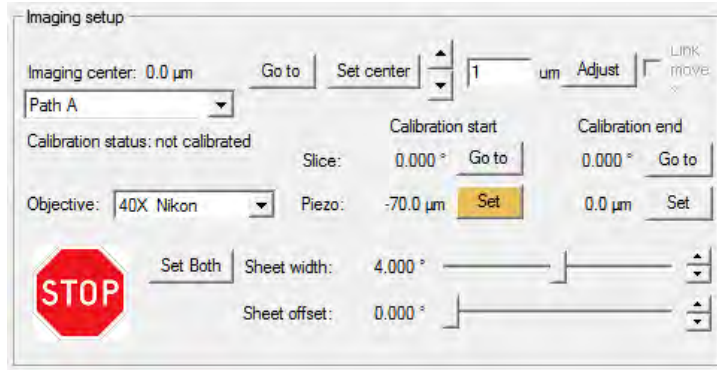
**NOTE:** A Z value is displayed above the arrows, this will indicate the current position of the piezo. For a 150 μm Piezo it will range between -75 to 75 max. Always make sure the opposite piezo is at center position when starting the calibration.



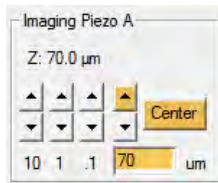
3. Adjust the right wheel on the joystick (confirm Controller Inputs, **Right wheel = Slice A**) until the beam is in focus and uniform.



4. In Imaging Setup, click **Set** to select the Calibration start for the piezo.

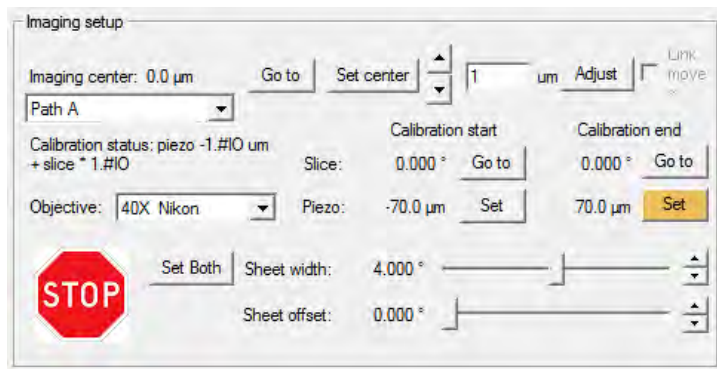


5. In the Imaging Piezo A control, click **Center** to bring the piezo back to 0.0um then click the up arrow to raise it 70um.



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.



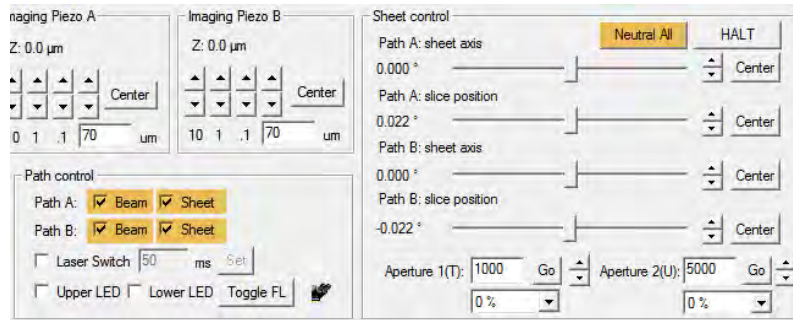
8. Return Piezo A to center by selecting **Center** (as in Step #4) and select Adjust (the beam should become focused again) to check the calibration.

Repeat steps #1-#8 for Path B and Piezo B.

## TUTORIAL: Setting Sheet Width and Offset

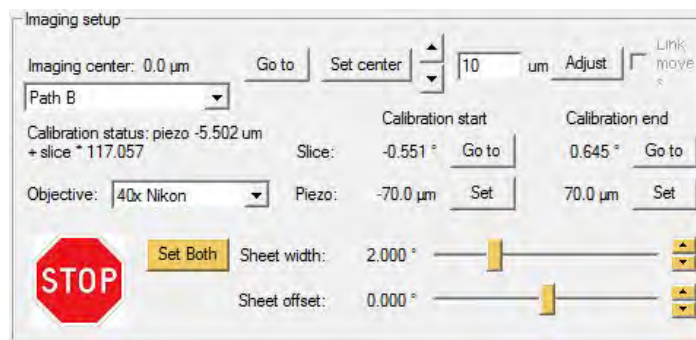
Once the Beams are aligned and calibrated return to the neutral position and continue to set the sheet width and offset.

1. In Path Control, check the boxes for **Beam** and **Sheet** for Path A and Path B. Click **Neutral All**.

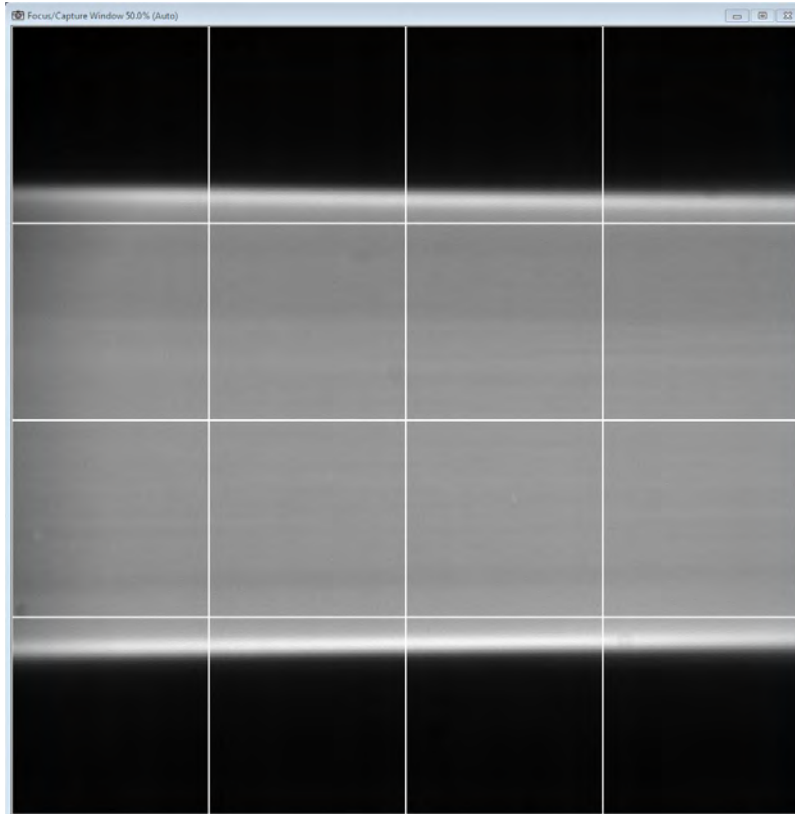


2. Check the box to turn on the **Grid** for easier alignment. Adjust the **Sheet width** (displayed as the vertical height of the sheet) to just outside the field of view. In the following example, the field of view is the center four squares of the grid. If the sheet is not perfectly centered, adjust 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.

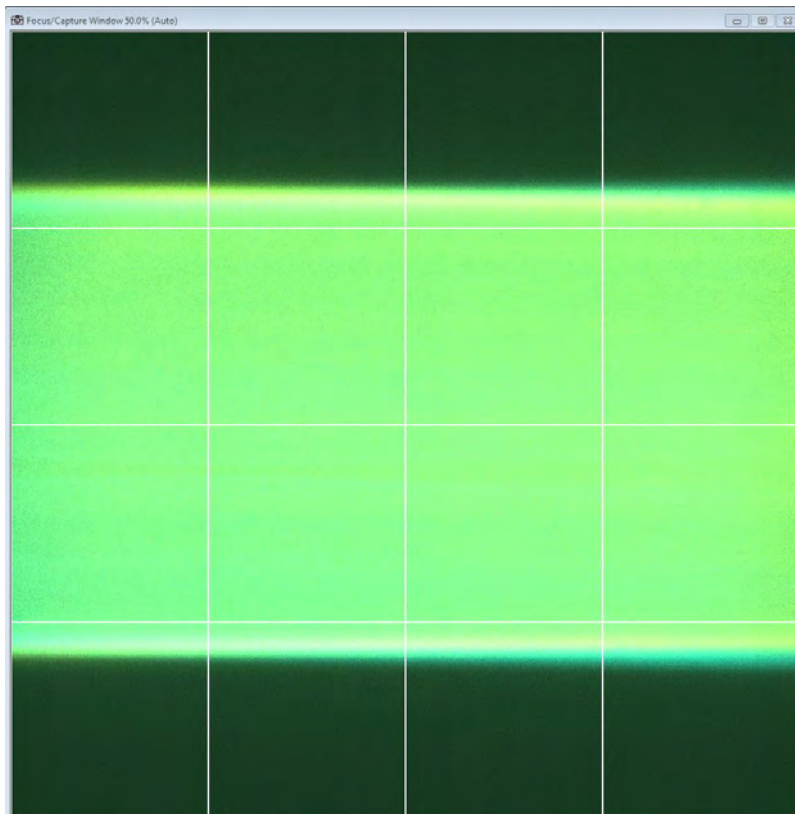
**NOTE:** The arrows to the right of the sliders can be used to adjust sheet width and offset. The step size for a single arrow button click is 0.001 units. Hold **CTRL** while clicking the arrows to use a step size of 0.01 units.



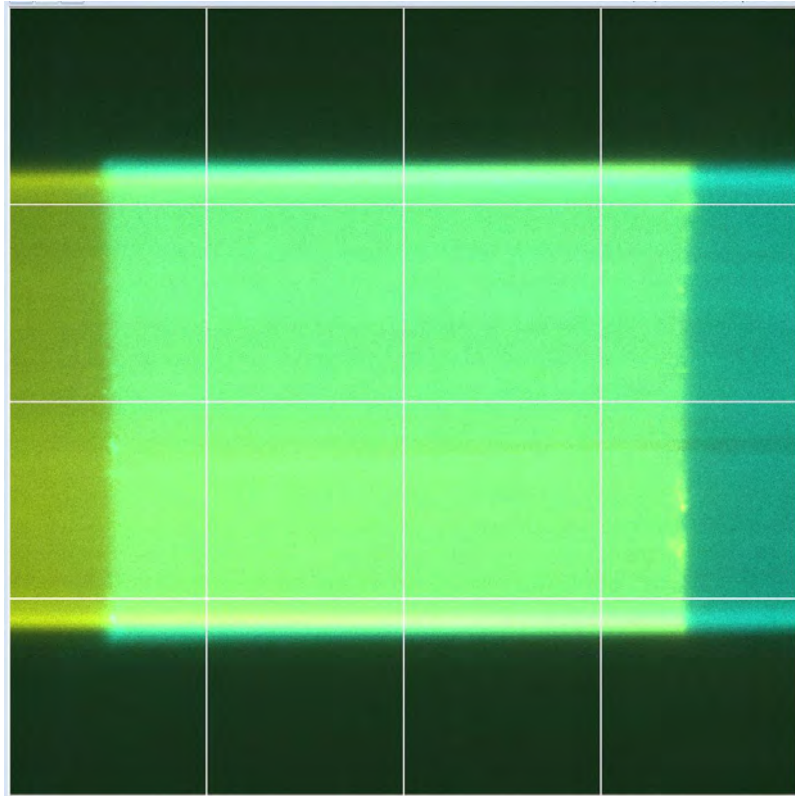




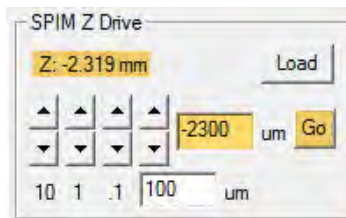
3. Check the box for **Dual Cam** mode to check the sheets are overlaid. When you are in **Dual Cam** mode, the sheet width and offset will only adjust for the active channel, the channel you for which you last clicked **Go**. Use the Go buttons to toggle between Channel A and Channel B while adjusting 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.



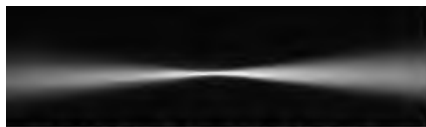
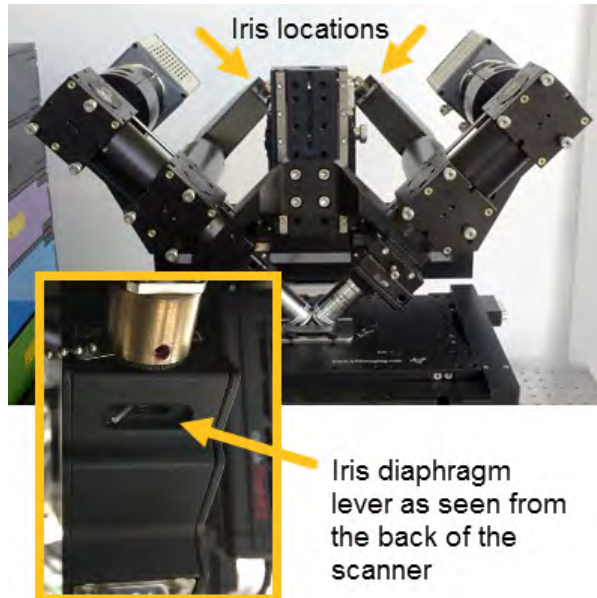
5. Move back up 200-300  $\mu\text{m}$  and write down/remember the Z position. You can later return to this position by typing the value in the text field and clicking Go once your sample is loaded.



## 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.

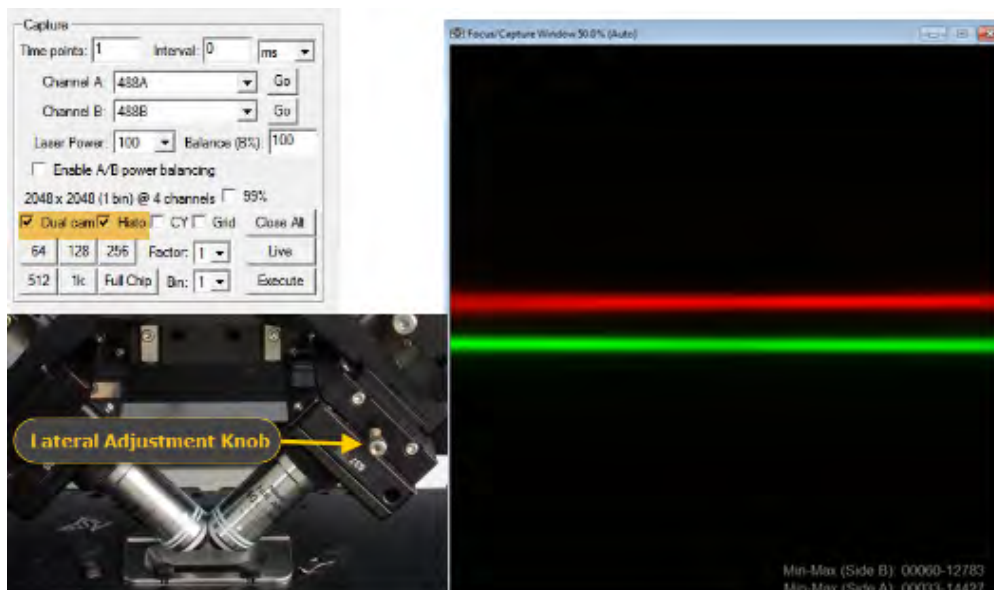


Open iris = Gaussian beam



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. 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.

Min-Max (Side B): 00058-14374  
Min-Max (Side A): 00040-14492

**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**

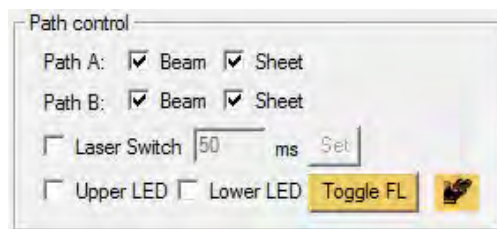
Once the beams are aligned, a sensible sheet width is set, and piezo and slice distances are calibrated, you can start imaging your samples. This section of help pages will walk you through MLS operation from locating your sample to refining the calibrations through to image acquisition.

## Locating and Positioning the Sample

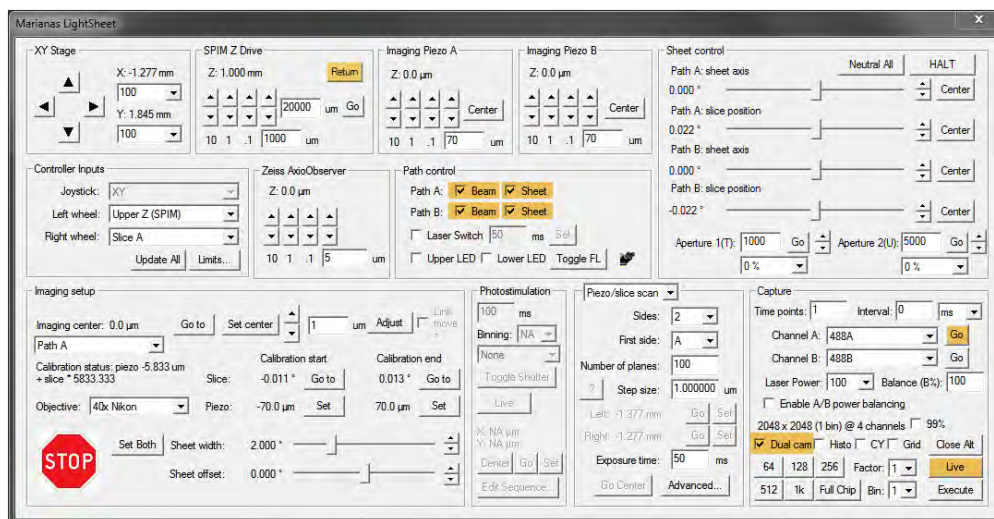
### Locating the Sample

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 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 of interest 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.



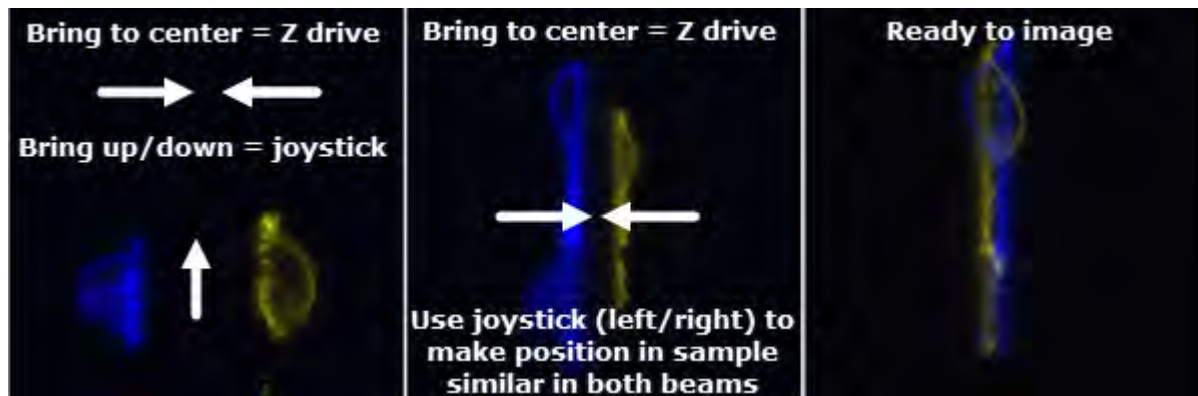
### Approaching the Sample

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 crushing 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 not to crush it.
- 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.



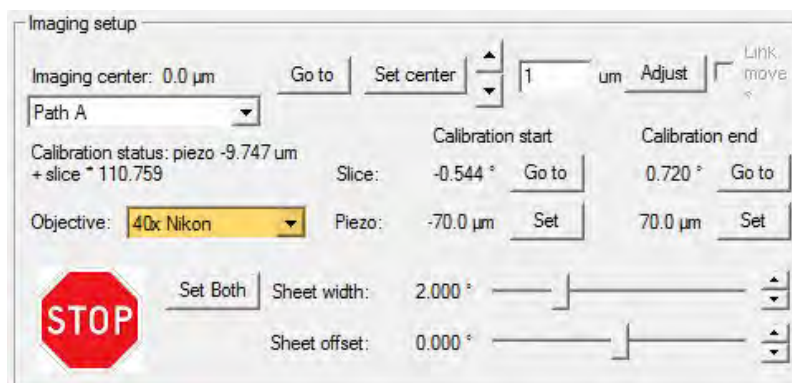
- To image smaller objects, consider cropping the camera field of view to an appropriate size by clicking **1k** (through to **64 x 64** pixels) or narrow the field of view using the **Factor** drop down. This will save considerable space in data capture. And there is also an option (drop down **Bin**) for binning if you are OK sacrificing resolution for signal.
- Also consider adjusting the piezo range to your field of view or vice versa before capture.
- When imaging a small volume (e.g. cells on a coverslip), it is recommended to open the scanner iris slightly in order to narrow the beam waist. Best, adjust the beam width while in **Dual Cam** mode in solution (Z drive is slightly above samples).



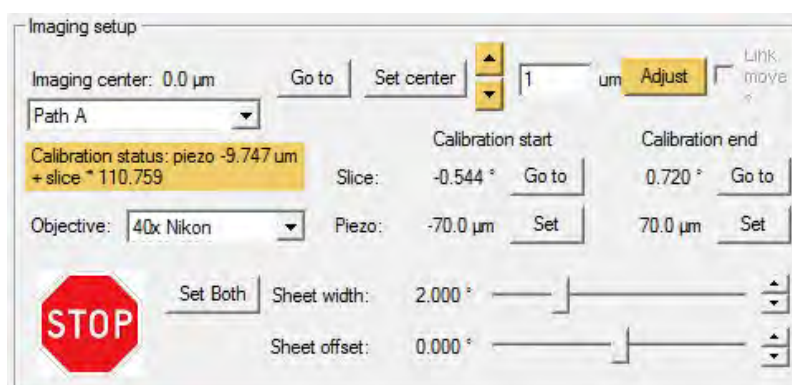
## Before Capture

Before you can 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.

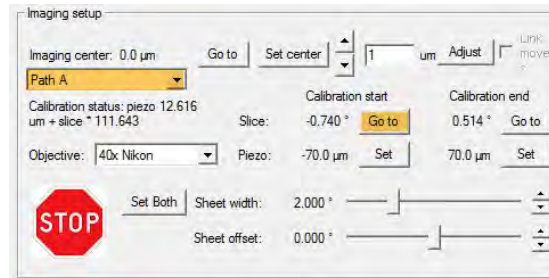
**NOTE:** If refining the calibration is necessary, see [Refine an Existing Calibration](#).

8. Check that the **Imaging center** is set to **0.0 #m** for both sides.

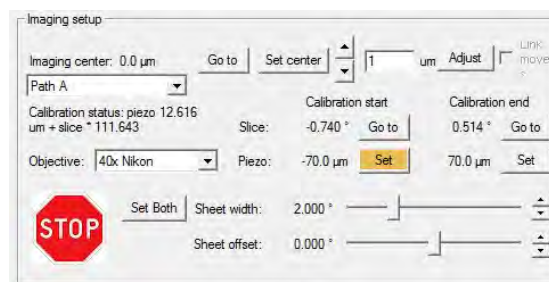
## Refine an Existing Calibration

If an existing calibration has been set and the pencil beams are uniform and centered, you can fine tune the calibration using following instructions.

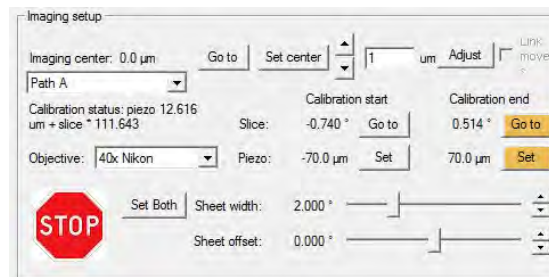
1. Click **Go** for **Channel A**. In Imaging setup, select **Path A** and click **Go to** for Slice, Calibration start.



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**.



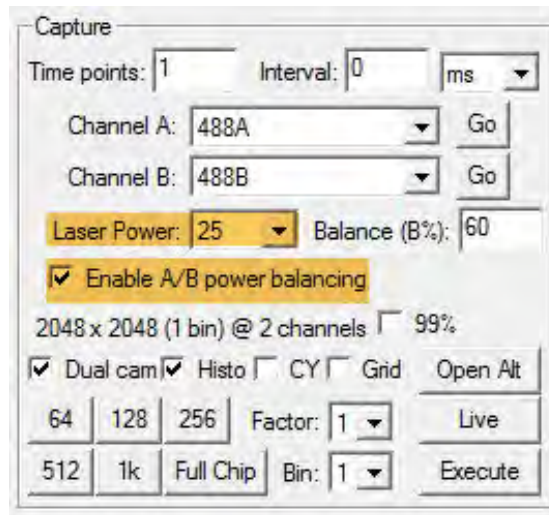
4. Repeat steps #1-3 for Path B.

## 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.

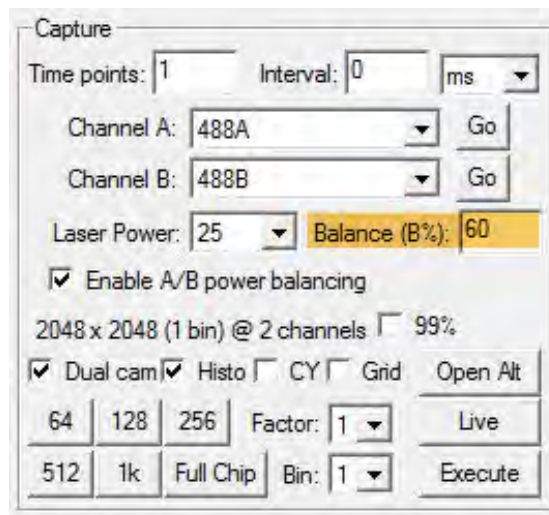


The screenshot shows the 'Capture' dialog box with the following settings:

- Time points: 1, Interval: 0 ms
- Channel A: 488A, Channel B: 488B
- Laser Power: 25, Balance (B%): 60
- Enable A/B power balancing
- 2048 x 2048 (1 bin) @ 2 channels, 99%
- Dual cam,  Histo,  CY,  Grid, Open Alt
- 64, 128, 256, Factor: 1, Live
- 512, 1k, Full Chip, Bin: 1, Execute

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.



This screenshot is identical to the previous one, but the 'Balance (B%)' field is highlighted in yellow to indicate the adjustment point.

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

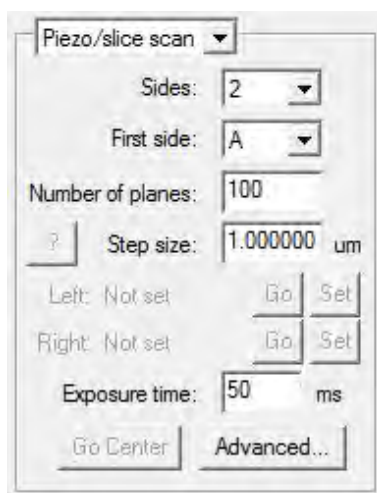
Scan mode: Interleaved (single pass) ▾

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  $\mu\text{m}$  Piezo  
Step Size = 1  $\mu\text{m}$ , Number of planes =150  
or  
Step size = 2  $\mu\text{m}$ , Number of Planes =75
  - e. Set **Exposure time** - For USB3 connected cameras recommended exposure is 5 ms at 512 px, above 15 ms at 1k px, above 36 ms at full chip, for interleaved mode always above 30 ms  
**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.

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

1. 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 one stage scan extents by click **Set**.

Stage scan

Sides: 2

First side: A

Number of planes: NA

? Step size: 1.000000 um

Left: Not set Go Set

Right: Not set Go Set

Exposure time: 100 ms

Go Center Advanced...

- a. Repeat for the opposite end of the sample. The **Number of planes** will be calculated automatically from the set **Step size**.
- b. 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 travel distance the step size as follows:  $\text{Travel Distance}/(\text{Step Size}/\sin(45))$

Stage scan

Sides: 2

First side: A

Number of planes: 72

? Step size: 0.985698 um

Left: 2.887 mm Go Set

Right: 2.987 mm Go Set

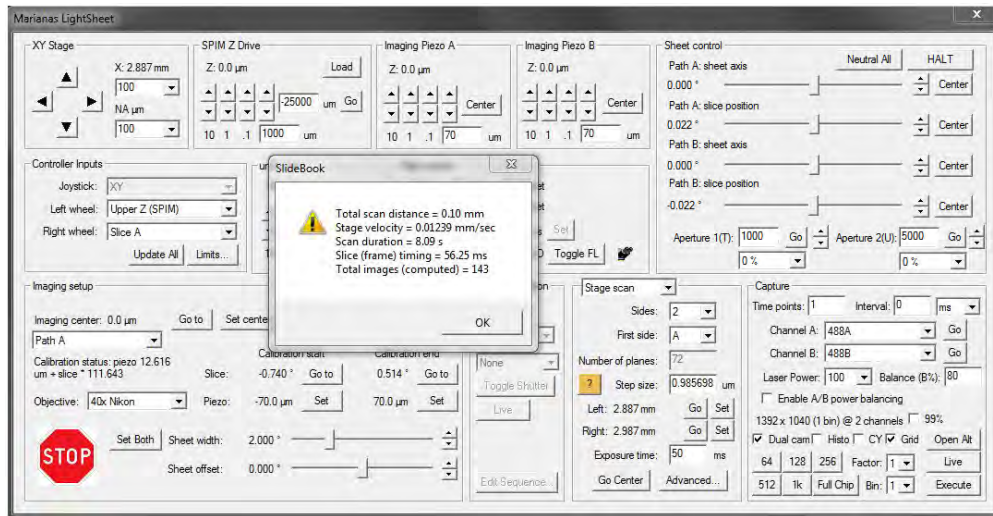
Exposure time: 50 ms

Go Center Advanced...

- a. Set **Exposure time** - For USB3 connected cameras recommended 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.



1. For timelapse acquisition choose the number of **Time points** and **Interval** at the top of the capture group.
2. 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.

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): 50

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): 50

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): 50

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

1. 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 an 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 effected by the event.
5. Select the desired **Photomanipulation filter** from the drop down menu. This filter is usually named by the schema *Wavelength*. For example, the Vector FRAP filter selection for 488 would be called **V488**.

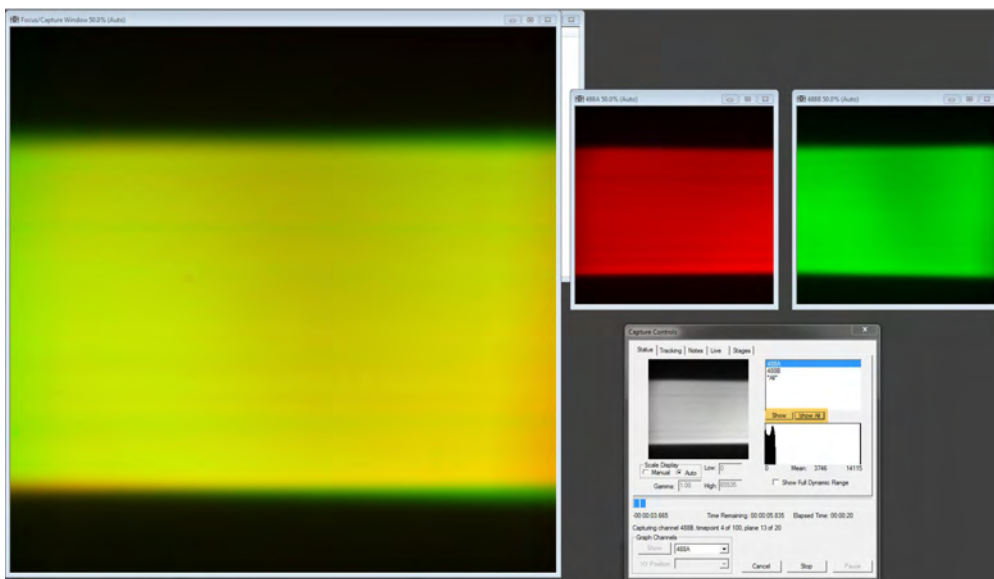
**NOTE:** Always set a specific laser power - leaving it at 'Current' will not initiate the FRAP lasers during MLS capture

The screenshot shows the 'Automated Control' dialog box. At the top, there are input fields for 'Time Point: 1', 'Repetitions: 1', and 'Duration (ms): 1'. Below these are checkboxes for '3D Imaging Plane: 0' (unchecked) and 'In-line' (checked). There are two radio buttons: 'Specific Regions' (unchecked) and 'All Regions' (checked). A 'Region type:' dropdown menu is set to 'Graph regions'. To the right, there are dropdown menus for 'Laserstack Power: Current', 'Power (auxiliary): NA', and 'Filter: V488'. Below these are several buttons: 'Add', 'Add - FLIP...', 'Add - Region...', 'Edit', 'Remove', 'Clear All', and 'Import...'. At the bottom of the dialog are 'OK', 'Cancel', 'Apply', and 'Help' buttons. A table with columns 'Tim...', 'Region', '3D', 'MS', 'Reps', 'Power', 'Filter', and 'In-line' is visible but empty.

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 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**

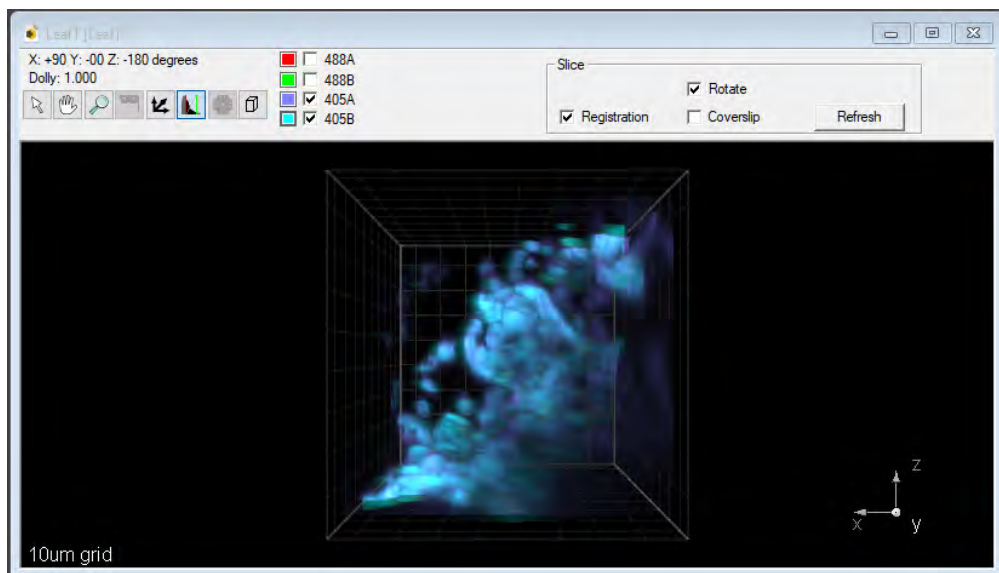
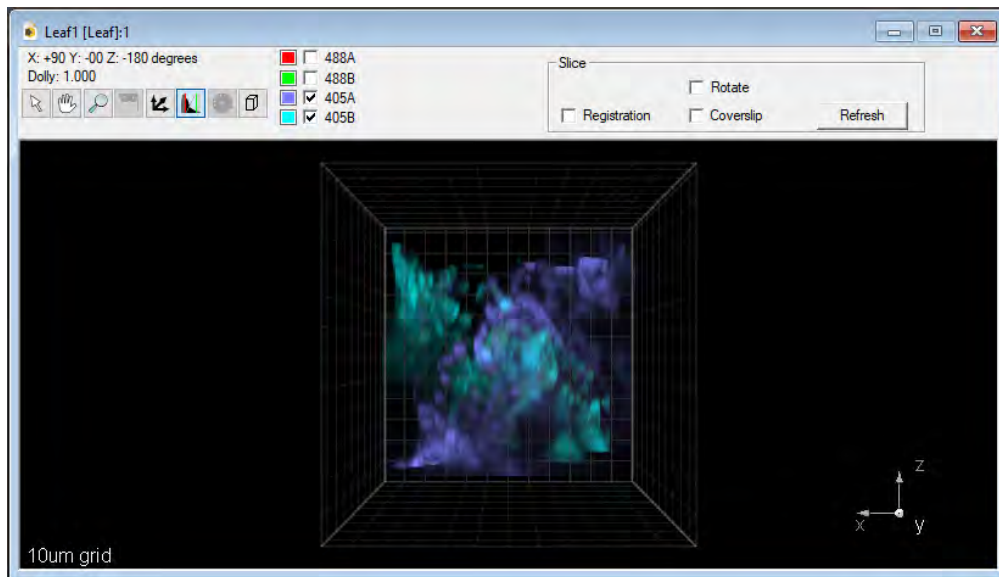
When using Dual View SPIM mode of MLS, the two paths will need to be fused using reconstruction algorithms. In addition to this, any data collected using stage scan mode will need to be deskewed first to put it back into a geometrically correct location. General information about deskewing can be found in [this video](#) about our Lattice LightSheet system. The concept is the same but the acquisition angle is 45 degrees for Marianas LightSheet.

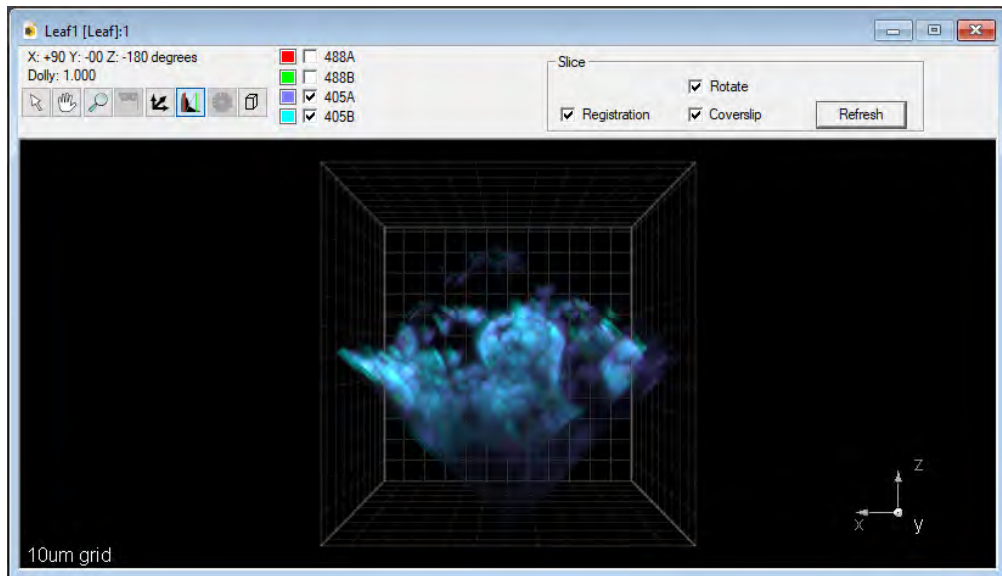


## TUTORIAL: 3D-Transform Preview

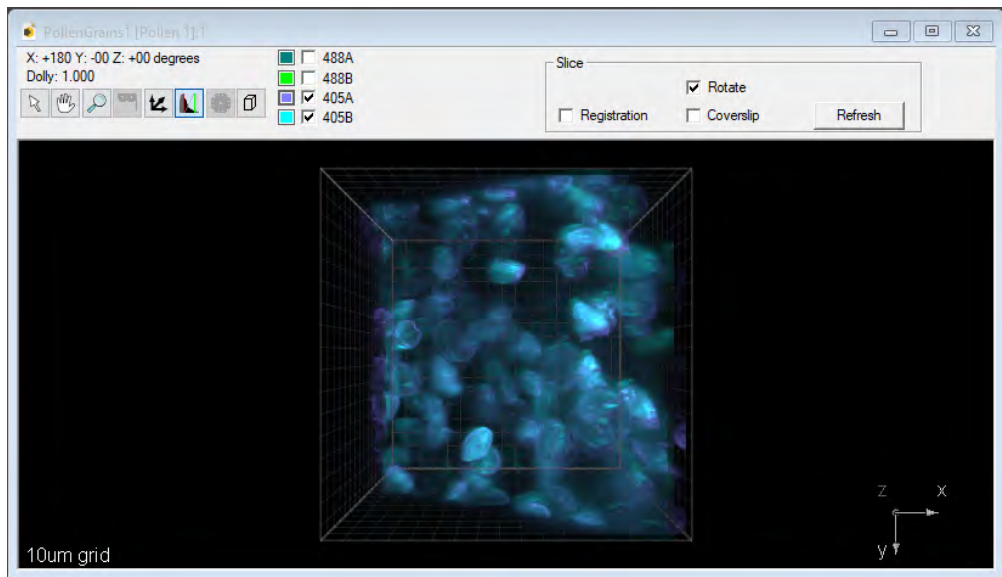
This tutorial will show how to create a 3D-Transform for previewing your data or preparing them for an export as TIF file.

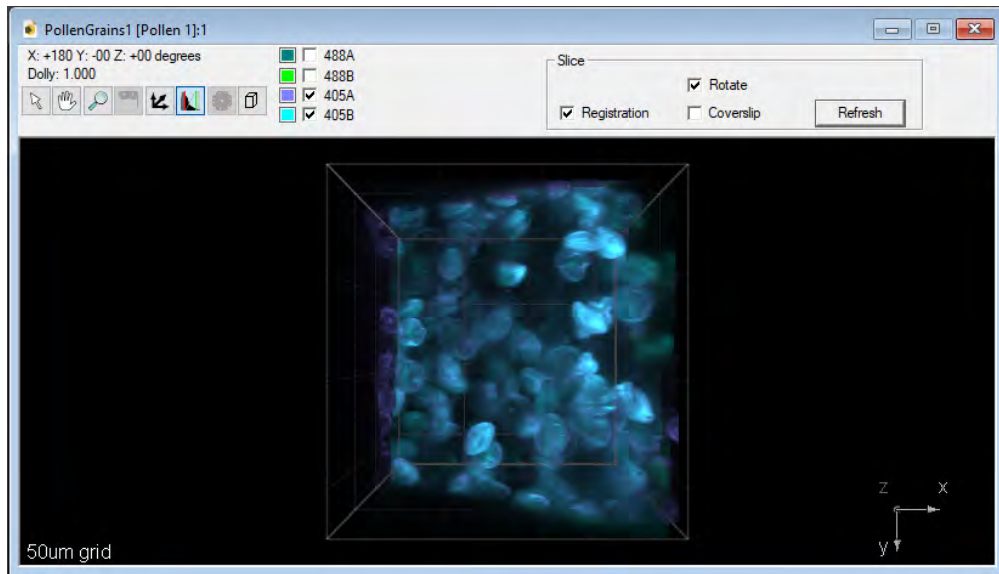
1. Save the **.sld** before reviewing your captures with either the 3D Volume Viewer or the Preview tool.
2. Select (highlight) the captured piezo/slice or stage scan. In the Home tab of SlideBook, select **View > 3D Volume View**. The [3D Volume Viewer](#) will open a rendering of your capture in order 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 ( $90^{\circ}$  rotation).
  - **Coverslip Correct** - This rotates the data another  $45^{\circ}$  so that the sample can be viewed like a conventional Z-Stack.





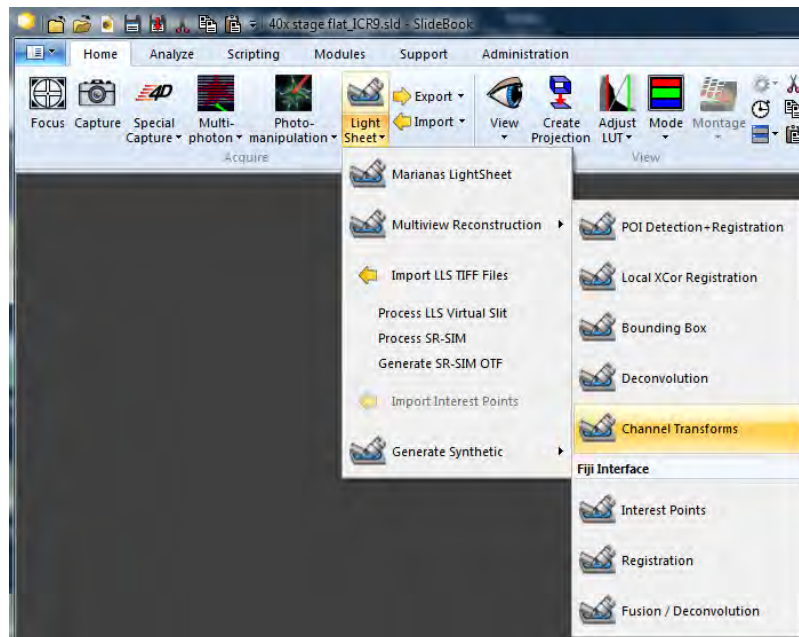
- **Registration** - This applies the default transform saved in SlideBook.  
**NOTE:** Make sure the comment does not contain the word **default**.





**NOTE:** The 3D Volume Viewer will only render a preview of the first timepoint of a time series capture. The 4D Volume Viewer will render each timepoint of a timelapse one after the other. 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 on with [Multiview Reconstruction](#) in SlideBook.

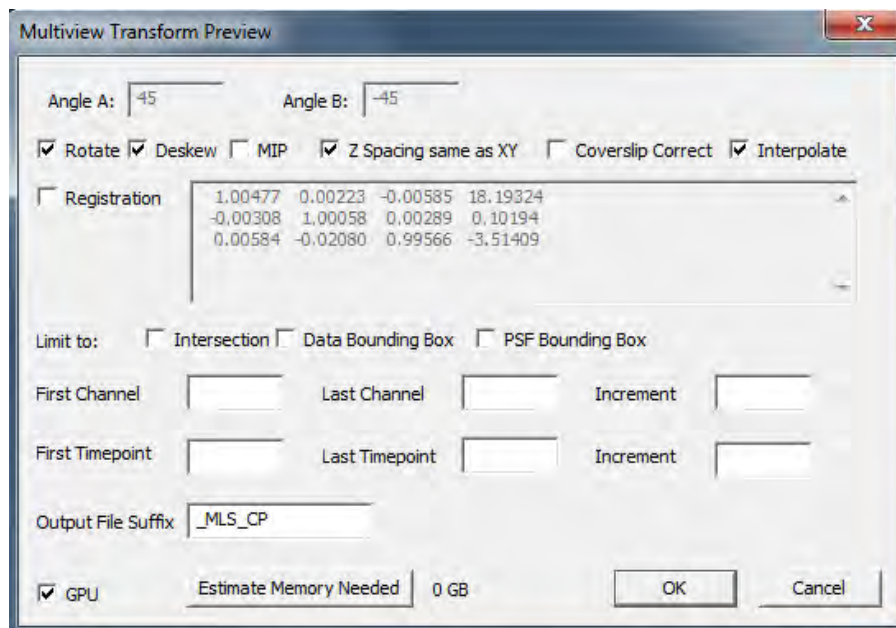
1. Select (highlight) the time lapse or single 3D image of the captured slice or stage scan, go to **LightSheet > Multiview Reconstruction > Channel Transforms**.



2. The Channel Transforms dialogue will open 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** - This gives a high quality image.

- You can also limit the data to:
  - Intersection** - This will create the preview of the volume where both volumes of each side are overlapping.
  - Data Bounding Box** - This will create a preview image of only the volume you assigned with the **Bounding Box** tool (Multiview Reconstruction).
  - PSF Bounding Box** - This will 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 from a 6 channel dual view capture.
- 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.
- If you have a graphic card and CUDA Toolkit installed, check **GPU** for a faster result.
- If you want to use the saved registration (and not only a rigid rotation), check **Registration**.



1. SlideBook will create a new capture in a new **.s1d** file with the suffix **\_MLS\_CP**. You can change this under **Output File Suffix**. If the output file should appear in the same **.s1d** file, delete the suffix. **Save** the slide.

2. Select (highlight) the created 3D transform image. In the Home tab of SlideBook, select **View > 3D Volume View** or **4D Volume View**. **4D Volume Viewer** will open the rendering of your capture. For Single View SPIM captures you can proceed directly to deconvolution with either the raw or deskewed file if desired. For Dual SPIM captures, this 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 CI decon or export for analysis
  - Use raw data and raw beads for PSF (deskew a stage scans afterwards via Create Preview)
  - 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
  - Piezo Slice scan: Best transfer the capture into its own **.s1d** file, save and open in Fiji via the Multiview Reconstruction Plugin (A=0, I B=90)
  - 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](#)).

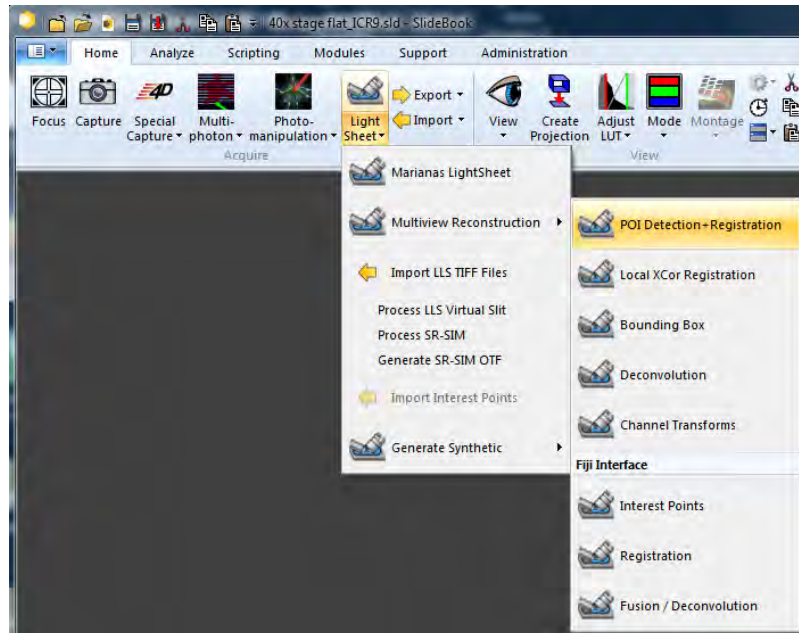


## TUTORIAL: Multiview Reconstruction

This tutorial will walk you through aligning a Dual View SPIM image, defining a bounding box, fusing, and deconvolving the image.

1. Save the **.s1d** before beginning.

2. Select (highlight) 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 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 you 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 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. These artifacts if big enough can be picked as POI and ruin the calculation.
- ii. **Ch1**: The first channel background value (e.g. 488A)
- iii. **Ch2**: The second channel background value (e.g. 488B)
- iv. **Compute Background**: You can click on this button to compute the background. However it might be slow. You can pick the background just looking at the histograms.

### c. Interest Point Detection:

- You can let the program range through a value of sigmas and determine automatically which gives you the smallest error.
- i. 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.
  - ii. Select a **Number of Sigmas** to decide for how many Sigmas in the set range the algorithm detects interest points.
- NOTE:** Testing about 1-3 Sigmas from a Start Sigma: 1 to End Sigma: 4 is a good approach but can be different for individual data. The Program will automatically select from the best combination of interest points and registration accuracy.

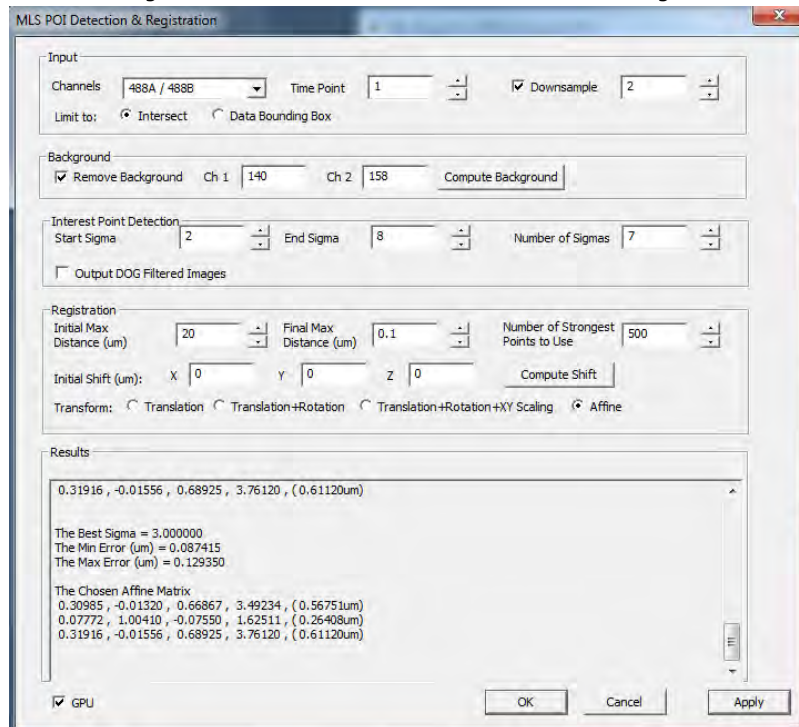
### d. Registration:

- i. Set the **Initial Max Distance (um)** to a distance you want to start approaching the interest points at.



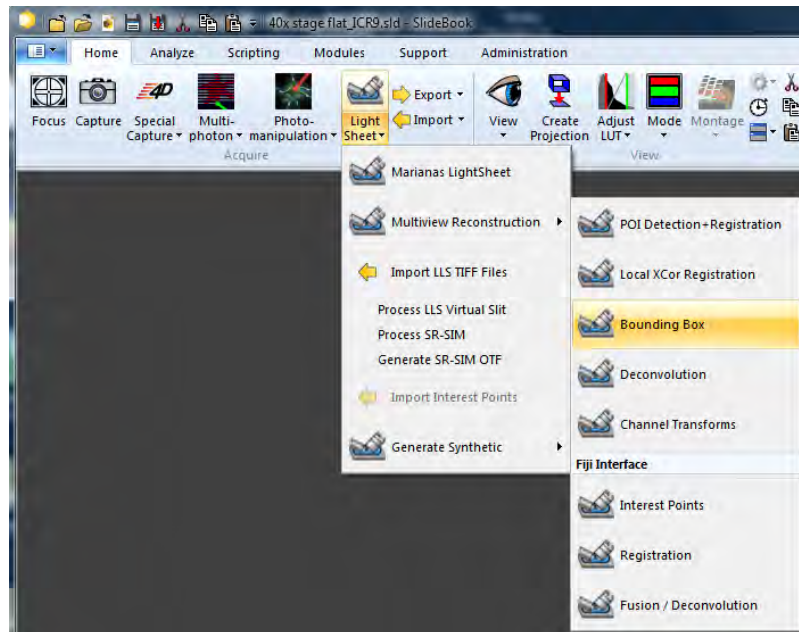
- ii. **Set the Final Max Distance (um)** to the distance between interest points of each side you want to achieve.
  - iii. Set the **Number of Strongest Points to Use** from the detected interest points.
  - iv. **Output DOG Filtered Images:** Check this if you want to look at how the DOG filtering operated. The larger the sigma, the larger become the "blobs".
- NOTE:** Testing about 1-3 Sigmas from a Start Sigma: 1 to End Sigma: 4 is a good approach but can be different for individual data. The Program will automatically select from the best combination of interest points and registration accuracy.

- 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.



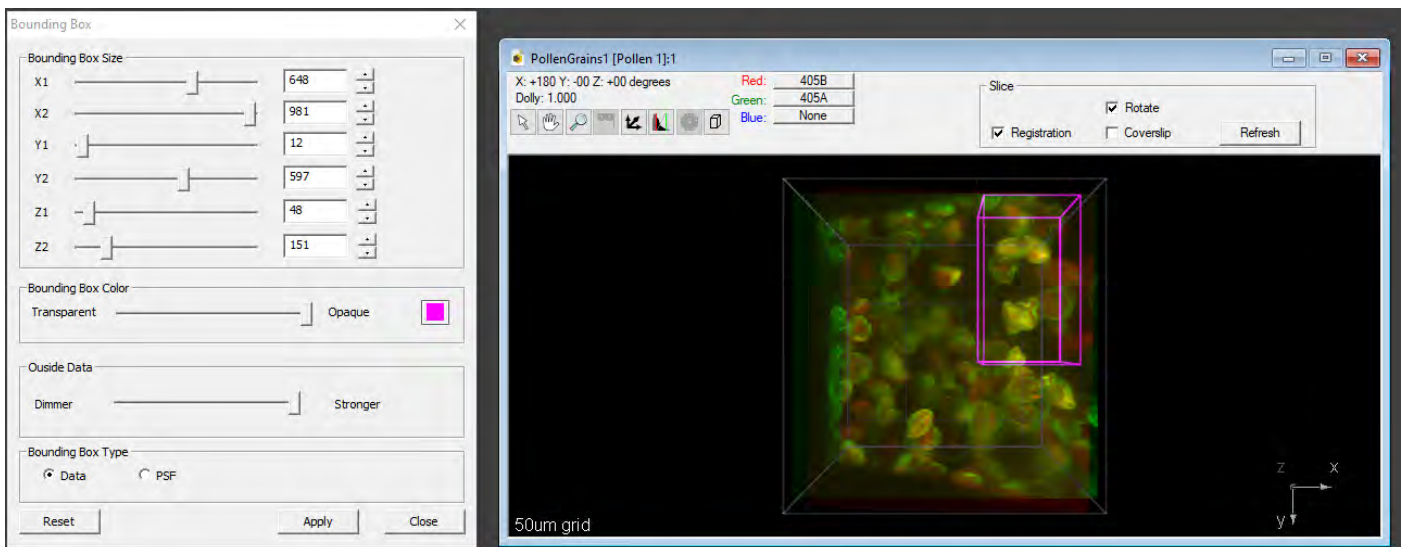
- h. The **3D Volume Viewer** will update and show the 'best' transform when the check box **Registration** is ticked.
- i. If you are not happy with the transform select a new set of parameters and **Apply** again.
- j. If you want to finish the process without saving the transform, click **Cancel**. This will close the Registration dialogue and not update the Default Transform.
- k. Once you are not happy with the transform click **OK**. This saves the registration with the capture metadata once you save the entire **.s1d** file.

1. Next, 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.

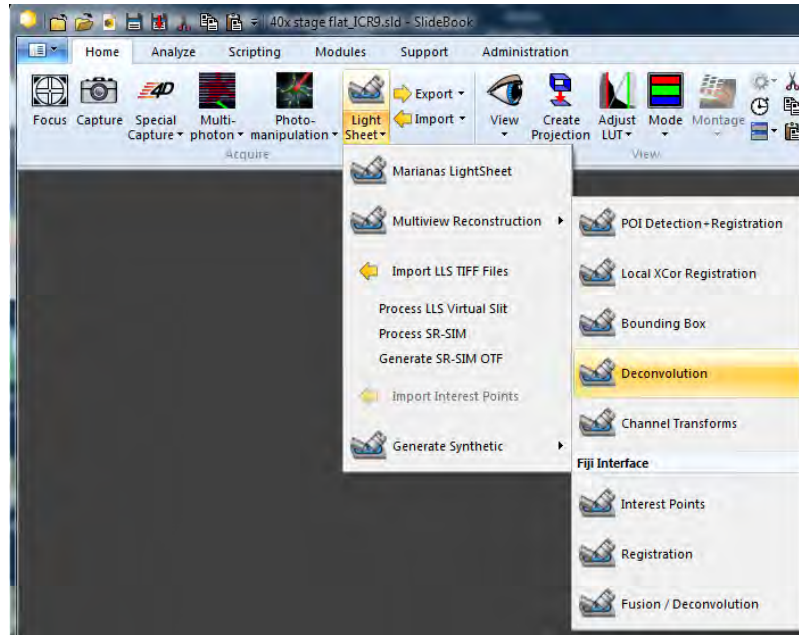


- a. Click **Reset** if you are not happy with the previously set bounding box.
- b. Set the width, height and depth with the sliders.
- c. Select the Bounding Box Type:
  - i. **Data**, to deconvolve the data within the set volume.
  - ii. **PSF**, to use the selection from beads as PSFs in the 3i Deconvolution. 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 **.s1d** file so the bounding box is saved with the capture for further processing.



2. Finally, highlight your capture of interest and select **LightSheet > Multiview Reconstruction > Deconvolution**.



a. **Output:**

- i. **Channel Selection:** Select one or several 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 value or let **Calculate Background** to subtract the background noise before deconvolution. For some captures this can largely improve the deconvolution result.

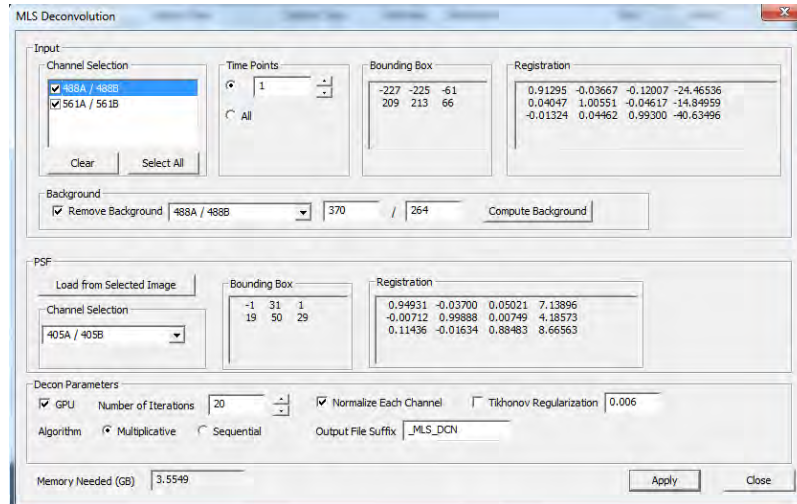
c. **PSF:** You can use a beads file captured with the same magnification as PSF. The beads capture needs to be registered beforehand using the **3i POI Detection + Registration** and a single bead (pair) needs to be selected with as PSF using the Bounding Box tool (tick PSF in this case).

- i. Copy a beads capture, with assigned registration and PSF bounding box into the same **.sld** file as the capture to deconvolve.
- 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.

d. **Decon 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. The required memory for this process will be shown at **Memory Needed** in GB.



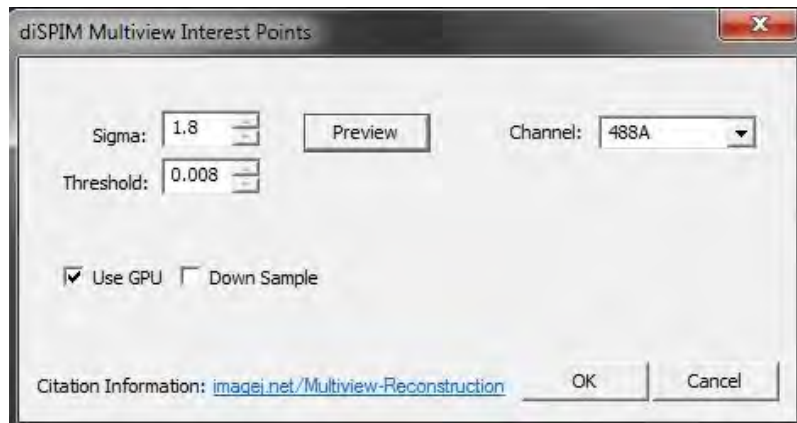
3. Click **Apply**. The deconvolution and/or fusion process can be quite intensive and may take quite a while depending on the computer. When the process is complete, a new image will appear in a new **.sld** file with the suffix **\_MLS\_DCN**. You can change this under **Output File Suffix**. If the output file should appear in the same **.sld** file, delete the suffix. **Save** the slide.

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4. Alternatively you can still use the simplified Fiji interface through Slidebook:

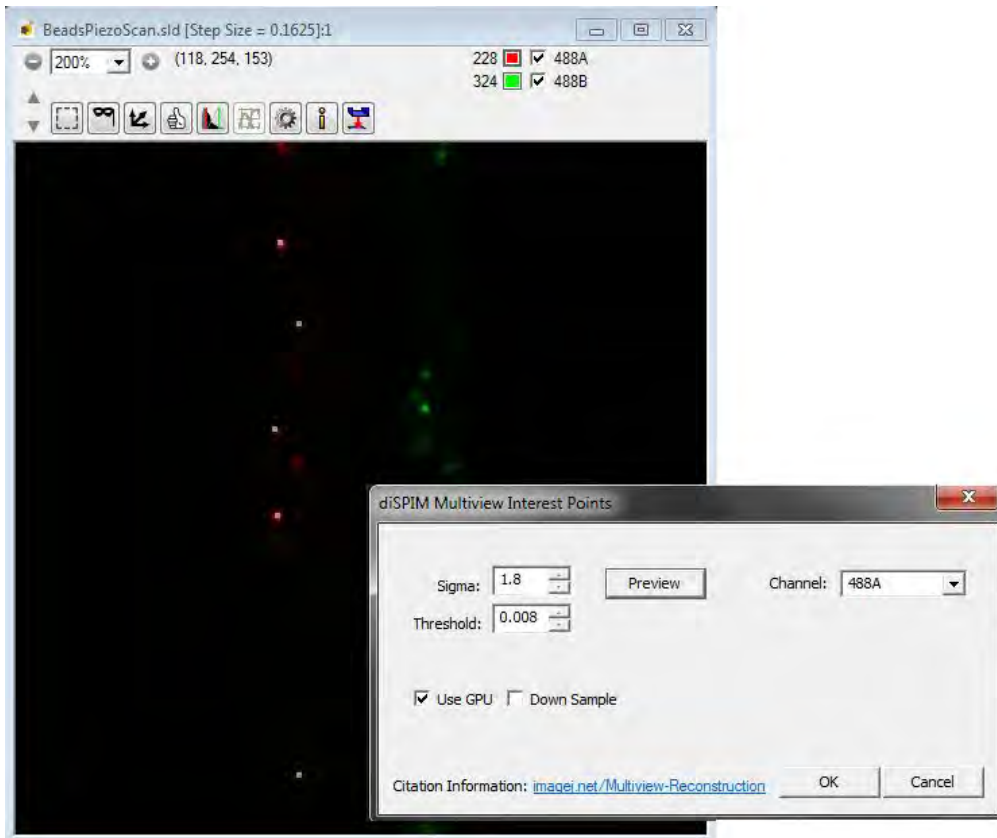
- Select (highlight) or open the image you would like to work with. In the Home tab of SlideBook, select **LightSheet > Multiview Reconstruction > Interest Points**.
- SlideBook will load default **Sigma** and **Threshold** values that will define the size and number of regions to interrogate. These values are best for beads, you might want to change these depending on your samples. Small **Sigma** values = small size regions to interrogate; small **Threshold** values = large number of regions to interrogate. Select a **Channel** from the dropdown menu to interrogate.

**NOTE:** Select a channel that has bright distinct features such as puncta, dendritic spines, or nuclei. Define sigma based on size where larger objects such as nuclei would be a value of **9** and smaller objects such as dendritic spines would be near **1**.

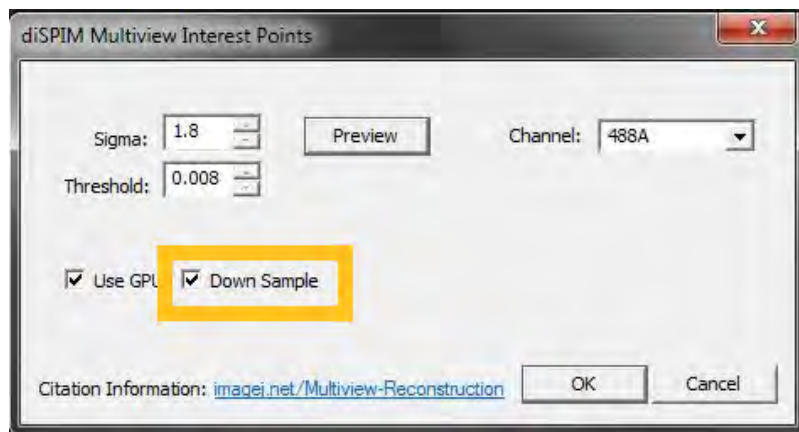


- Click **Preview** to show the regions generated.

**NOTE:** When you click **Preview**, a process will be activated spawning new windows showing the algorithm activity and log. In SlideBook, many small regions will appear in the image. When you scroll through the image, you will need to click **Preview** again to update the regions generated.



- Look to ensure that the detected interest points correspond to the "correct" features in the channel. There should not be points where there is no structure yet too few interest points will lead to failure during registration. Adjust sigma and threshold if necessary. If  $Z > XY$  and you want to reduce the number of interest points, check **Down Sample** to match the XY with the Z resolution during interest point detection.



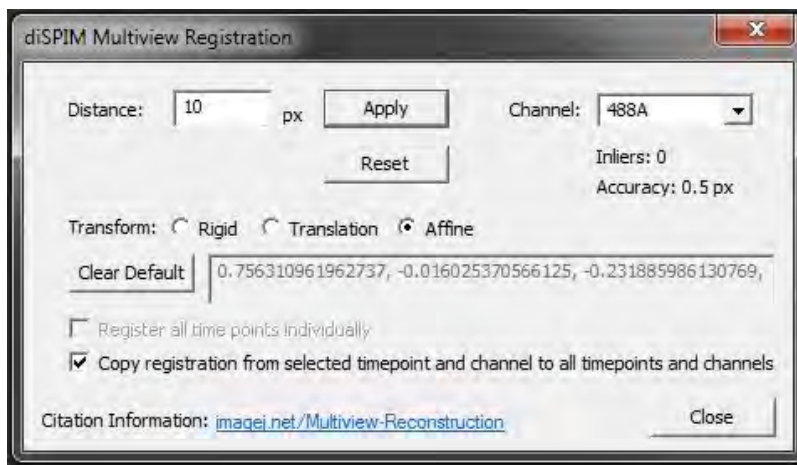
- Check the box to **Use GPU** to use CUDA accelerated processing. Click **OK** to detect interest points. The log file will run through computations. When the process is complete, the regions will disappear from the image view. The log file's last line should read **Closing .sld: slidename**.

**NOTE:** SlideBook will tell you how many interest points were found for the last side. In the log file you will find how many interest points were detected for each side **Found xxxx peaks**. For most data types, values for a good registration lie between 100-5000 interest points per side.





- Now that interest points have been defined, you can proceed with registration. Select **LightSheet > Multiview Reconstruction > Registration**. The Dual View SPIM Multiview Registration dialog and the **3D Volume Viewer** will open.
- Choose the **Channel** for which you detected the interest points in the dropdown.

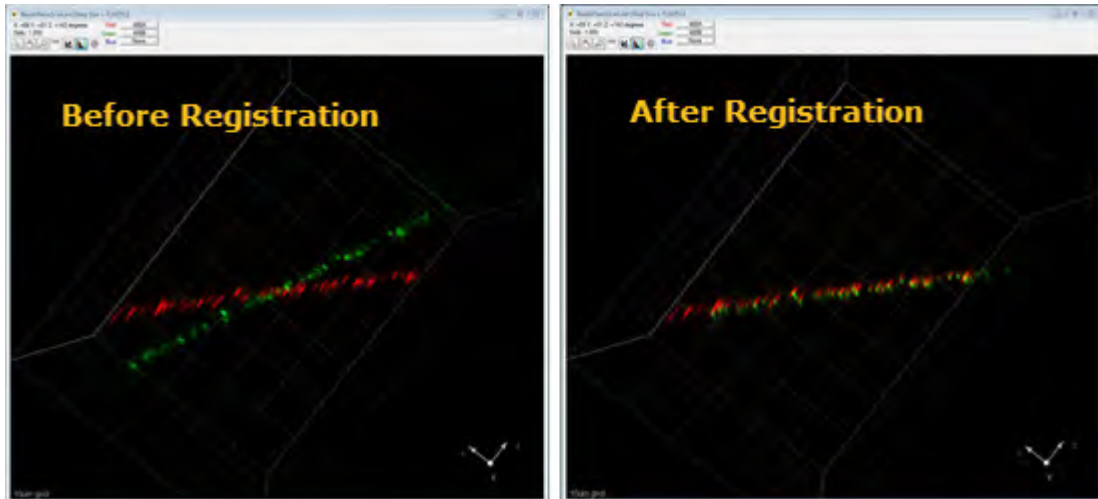


This is an interactive, iterative tool. Enter a **Distance** between given interest points in pixels and click **Apply** to start the registration process. The goal is to achieve an **Accuracy** value (maximum error in px) equal or below 1 pixels. If the Accuracy value decreases, continue decreasing the distance value.

**NOTE:**

- The default distance to start with is 10 pixels with Affine or Translation transform selected, but you might want to increase it depending on your alignment.
- For samples with fewer interest points or larger differences between the structures from each side, start with 50 pixels or higher if necessary and/or select the **Translation** or even **Rigid** transform type for the first registration iterations.
- To approach the best transform (Accuracy <1 px) enter values of decreasing values. A good practice is to choose a value slightly larger than the Accuracy in px of the last transform. e.g. Distance: 10px --> Accuracy: 5.76, next: Distance 6 px.
- You can follow the transformation with the 3D Volume Viewer. For this, have the tick box **Registration** checked in the capture in the **3D Volume Viewer**.





- If the Accuracy value increases or you are not happy with the applied transform, click **Reset** and try anew with try increased distance values and going back down in an iterative process. Use this iterative approach until you achieve an Accuracy value less than 1 and a number of inliers >1.
- If you are happy with the applied transform click **OK**. This will close the Registration dialogue and save the transform as Default Transform in SlideBook to be used on further data if appropriate.
- If you are not happy with the transform and do not want to save it as Default Transform, click **Cancel**. This will close the Registration dialogue and not update the Default Transform

• Next, proceed to **LightSheet > Multiview Reconstruction > Bounding Box** and **LightSheet > Multiview Reconstruction > Fusion / Deconvolution**.

a. Select one of the three processing options:

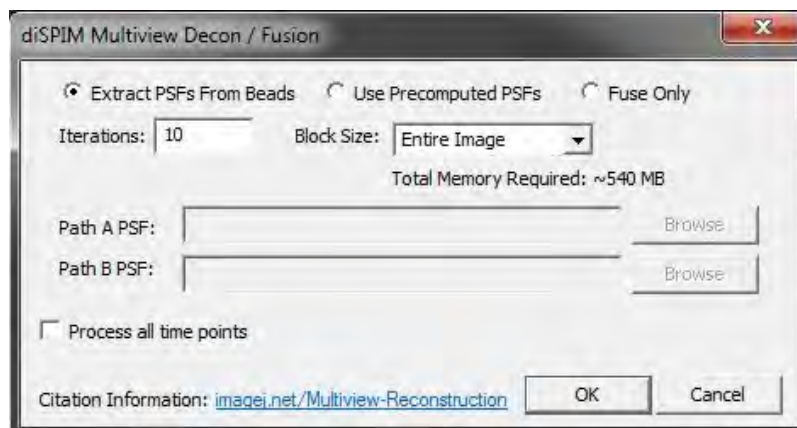
**Extract PSFs From Beads** - Uses beads within the same sample to generate PSFs for deconvolution. This also creates PSF transforms for Path A and Path B which can be used for future deconvolution of data.

**Use Precomputed PSFs** - Uses PSFs previously acquired using the same imaging system and channels. If you select this method, you will need to identify Path A and Path B PSFs from a directory.

**Fuse Only** - Fuses side A with side B, does not deconvolve.

b. Set the number of **Iterations**. The default of 10 usually gives a good result. Increasing to 15 may provide further improvements. For a quick preview, choose 1 or 2 iterations.

c. For time lapse images, check the box to **Process all time points**.



- Click **OK**. The deconvolution and/or fusion process can be quite intensive and may take quite a while depending on the computer. When the process is complete, a new image will appear on the **.sld**. Save the **.sld** before proceeding with analysis or rendering

## MLS Troubleshooting

Below, you will find a list of troubleshooting tips. 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 (middle position of screws), turn the objective bushings in (clockwise) and start the alignment procedure from scratch.
- Will not always see a display during acquisition or live update too slow: This depends on the selected 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.
- Move joy stick off of air table during operation.
- The 3D 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 **.s1d** 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 -> Reformat -> Rotate -> Flip Horizontal**