

### **Marianas LightSheet (diSPIM and iSPIM) Module**

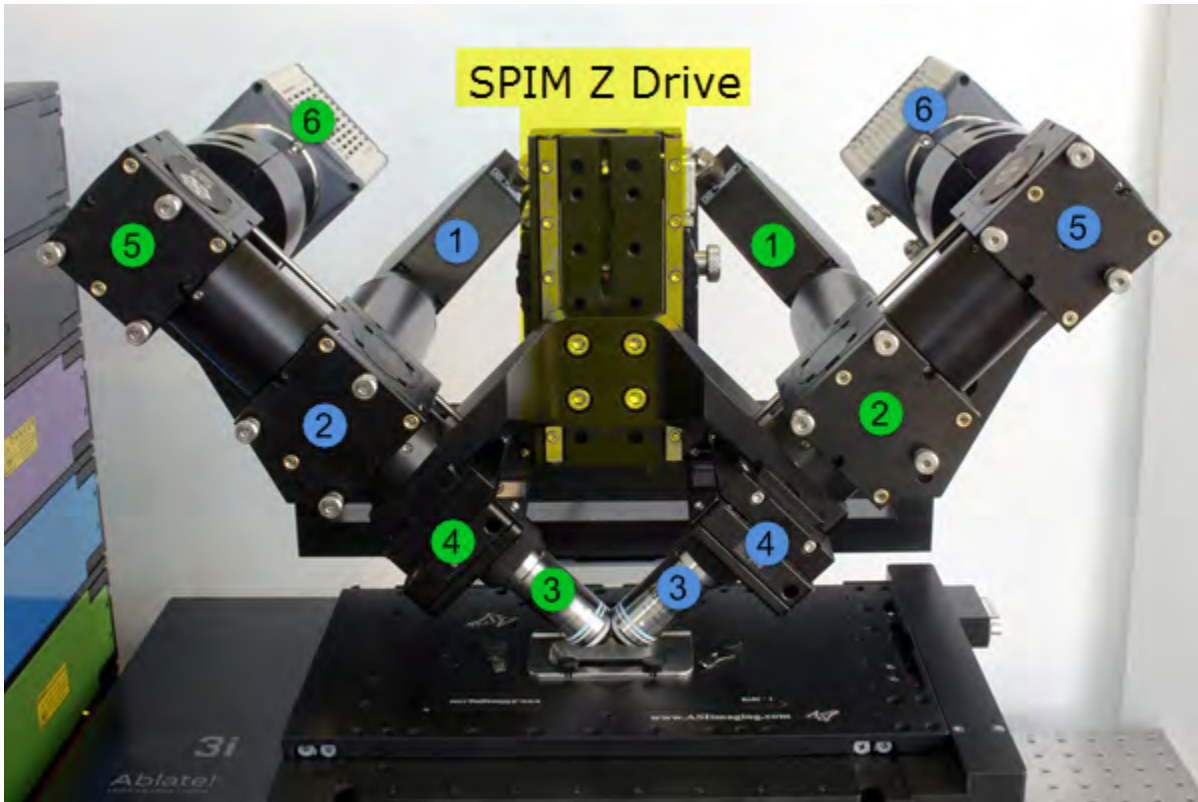
Marianas LightSheet Dual-view Inverted Selective Plane Illumination (MLS diSPIM) is a light sheet method offering low phototoxicity in 3D imaging making it possible to image living cells and small organisms over extended periods of time. It is a flexible light sheet system with an open specimen geometry not requiring special slides or capillaries. MLS diSPIM 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 can be added via the microscope's side-port, such as the Ablate! photoablation system.



## System Hardware

The system consists of two symmetric optical paths (unless in the case of iSPIM 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**, that 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 diSPIM system can be mounted on an inverted microscope frame or the ASI RAMM (Rapid Automated Modular Microscope) system. 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.



## MLS Alignment and Calibration

MLS diSPIM 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:

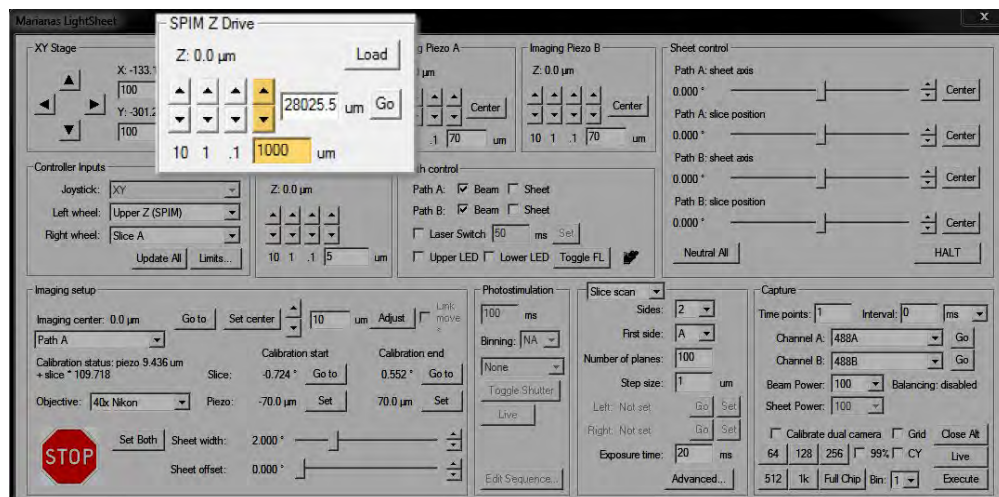
- dye solution (ex. Fluorescein)
- Allen wrench (3/32" size)

## TUTORIAL: Using the SPIM Drive to Introduce Objectives to the Sample Chamber

Lowering the objectives into the sample chamber is a delicate task; it is essential that the sample chamber be centered below the diSPIM objectives.

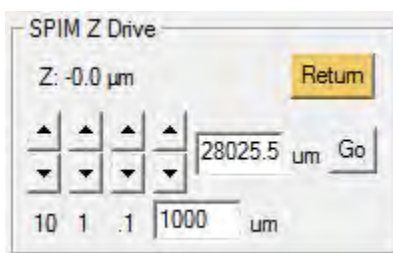
1. Open the **Marianas LightSheet** controls within SlideBook, go to the Home tab, Acquire group >  **LightSheet** > **Marianas LightSheet**.

2. When using the diSPIM system, 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.

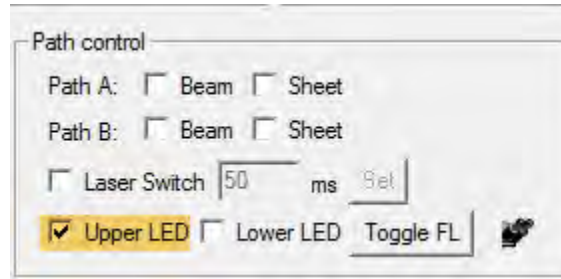


4. Once the sample is in place, you will want to move the SPIM Z Drive so the front lens of the objectives are fully submerged, this configuration will typically put you in position just above the coverslip.

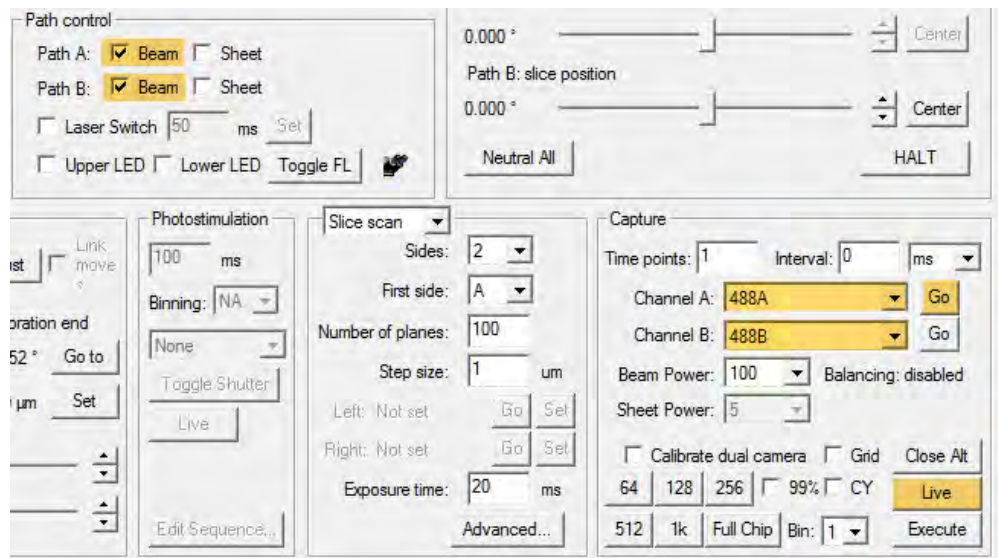
**NOTE:** If you use the **Return** button, the drive will lower to the previous sample position. Make sure your coverslip or sample is not higher.

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

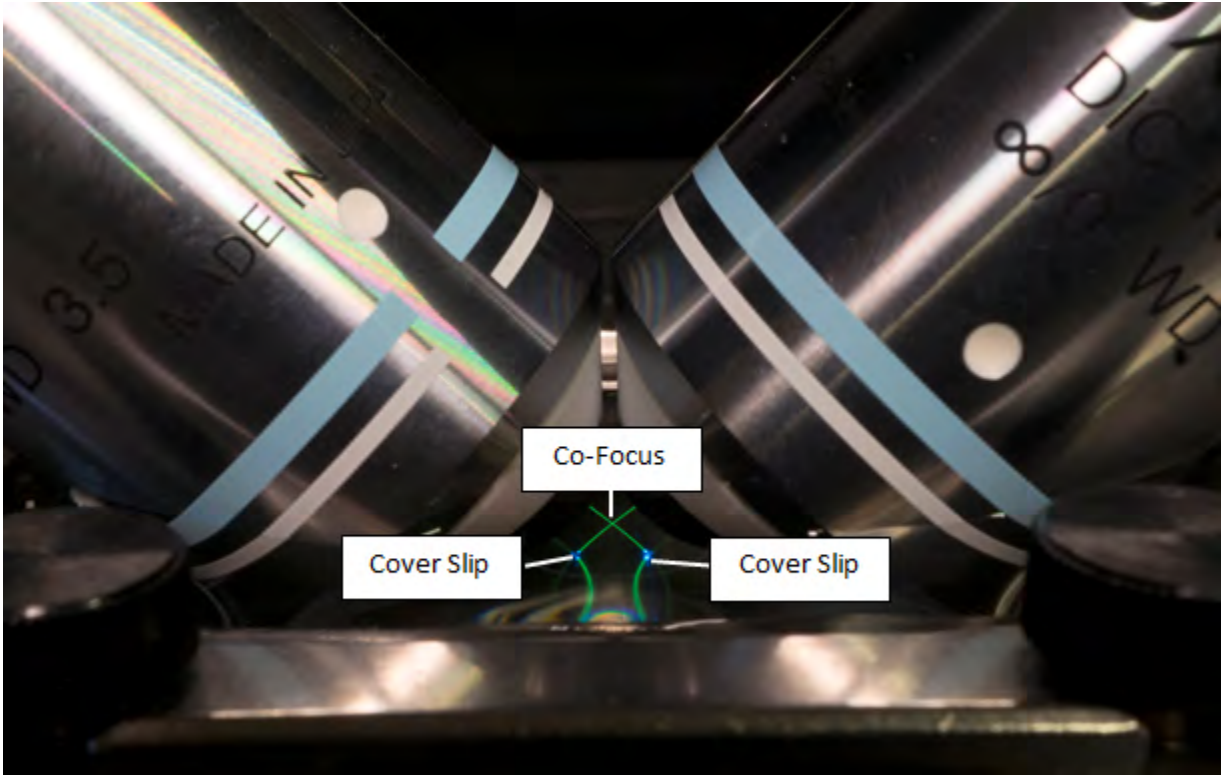


6. In the Path Control group, select a **Beam** for both paths. In the Capture group, select a filter for **Channel A** and **Channel B** respectively. Click **Go** for either of the channels then **Live** to activate lasers.




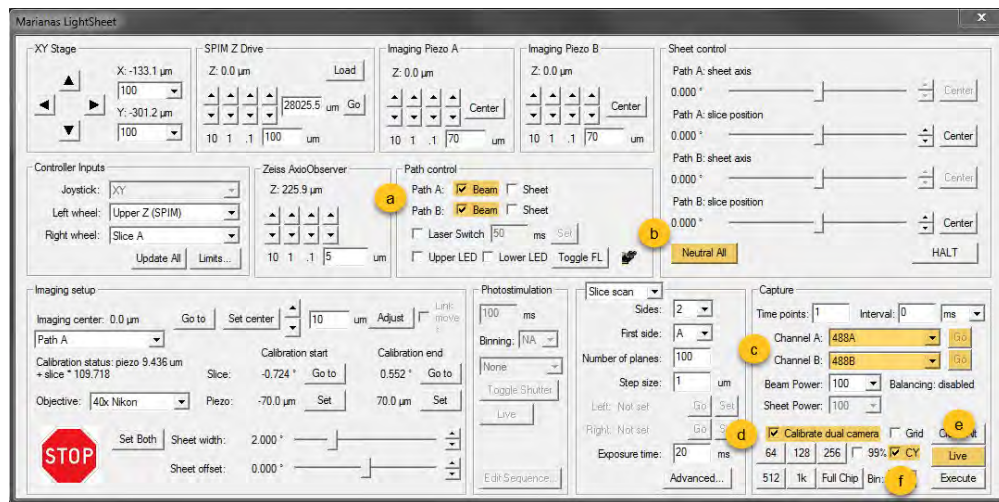
6. The next tutorials will cover co-focusing and aligning the objectives. For imaging samples, you will want both laser paths to be present in the middle of the field of view through the oculars. For alignment, you will want both laser paths to be present on the edges of the field of view through the oculars as shown below.



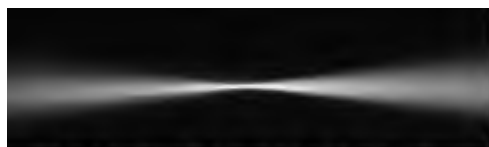
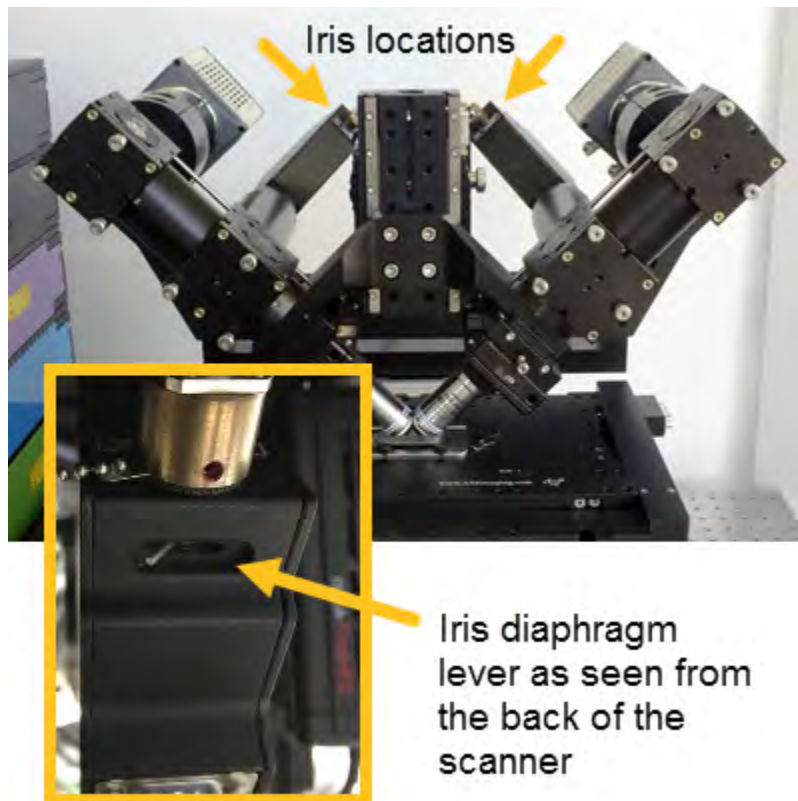


## TUTORIAL: Co-focusing the SPIM Objectives

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 diSPIM Objectives to 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 **Calibrate Dual Camera** 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).



Open iris = Gaussian beam



Closed iris = pencil beam

5. 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 **Calibrate Dual Camera** 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.





6. 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](#).

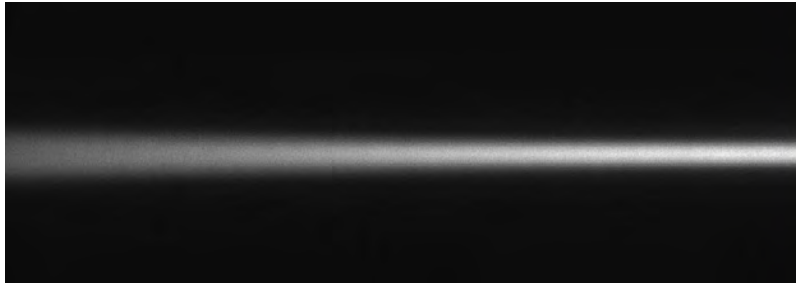


7. After the pencil beams for both channels are focused, you may notice some tilt (either rotational or focus). Please see [TUTORIAL: Adjusting the Tilt of the Pencil Beam](#) to correct for horizontal rotation (YZ tilt) or focus inconsistency (XY tilt).

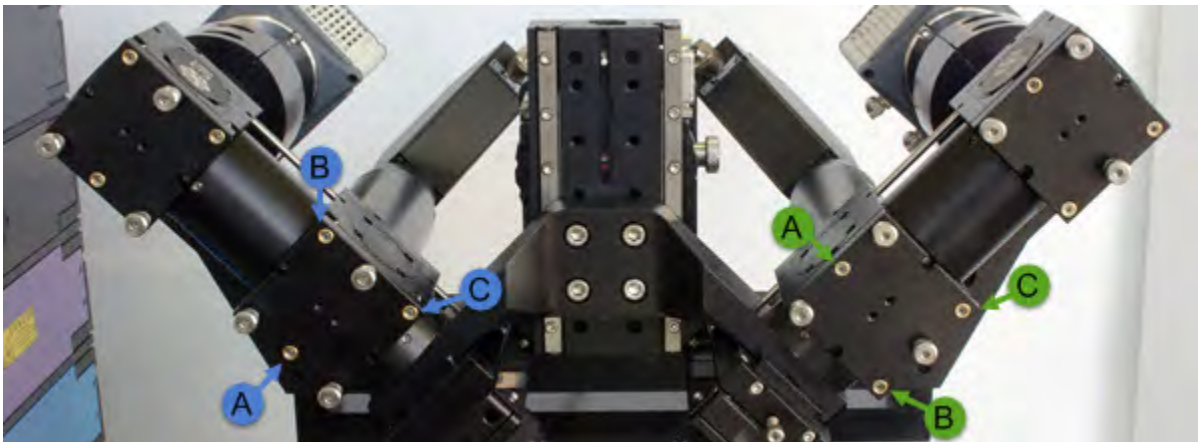
## TUTORIAL: Adjusting the Tilt of the Pencil Beam

### XY Tilt (focus)

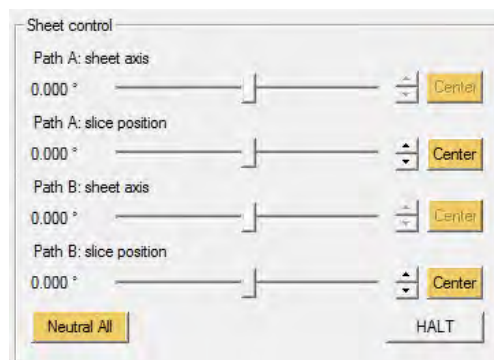
The XY Tilt is apparent when viewing the beam through the camera; if the XY tilt needs to be adjusted, the beam will appear as if it is coming toward (and away from) the camera.



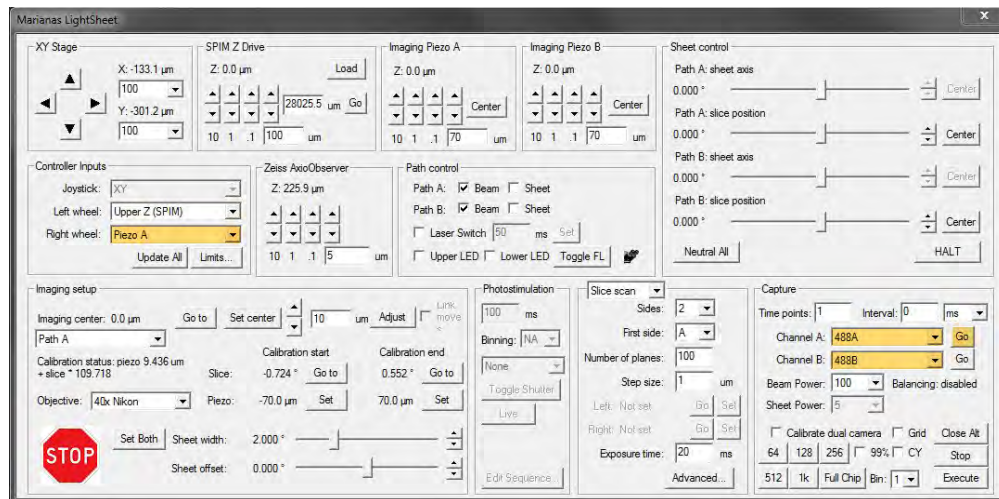
To correct XY Tilt for Channel A, you will adjust the dichroic mirror using an Allen wrench on **Screw A only** or **Screws B & C together** of the **Path A** filter cube. To correct XY Tilt for Channel B, you will adjust the dichroic mirror using an Allen wrench on **Screw A only** or **Screws B & C together** 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.



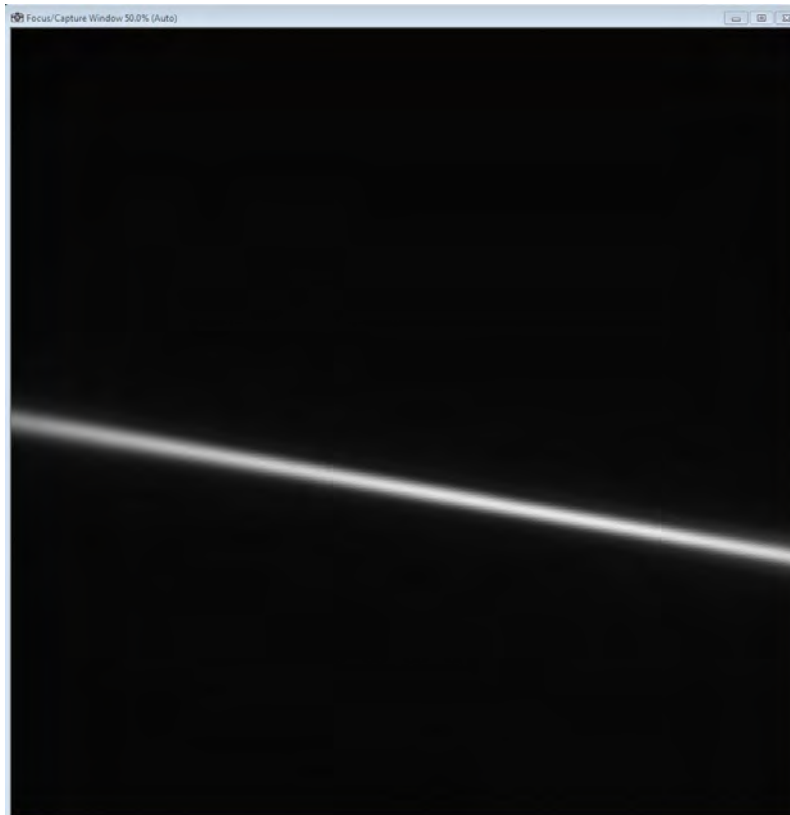
4. Adjust **Screw A** of **Path A** only or **Screws B & C together** on the filter cube manually with an Allen wrench until beam is uniform across the field of view.



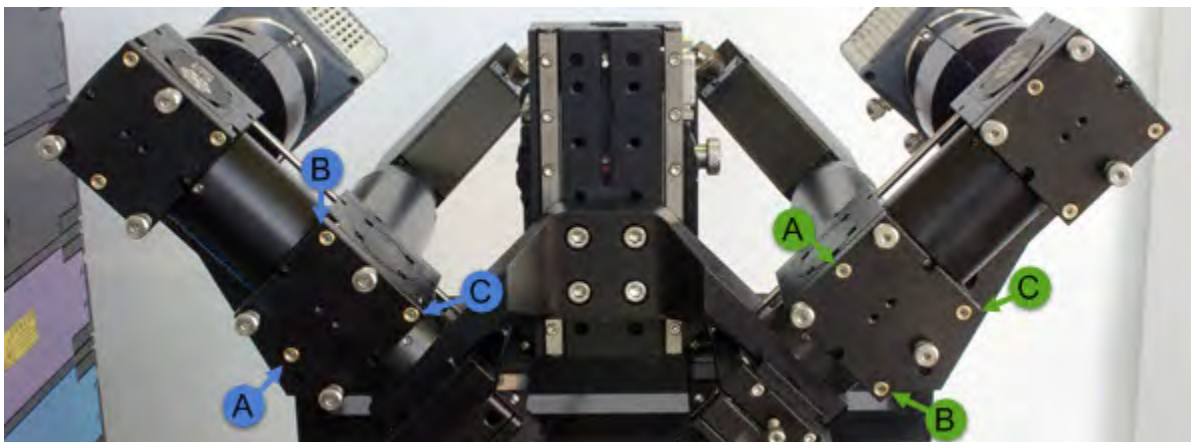
5. Select **Neutral All** to restore the slice position to its neutral position.
6. Refocus both pencil beams using the objective threaded bushings (as described in [TUTORIAL: Co-focusing the SPIM Objectives](#)) until pencil beam is uniform across the field of view.
7. Repeat steps 1-5 for Path B (setting **Right wheel** to **Piezo B**, clicking **Go** for **Channel B**, and using **Screw A only** or **Screws B & C together** of **Path B** filter cube) until both beams are in focus across the field of view.

### 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.

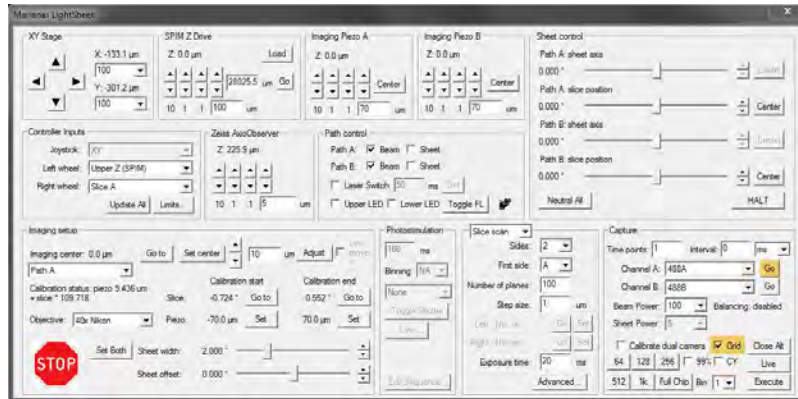


To correct the YZ Tilt, adjust the dichroic mirror using the Screws B and C on the filter cube of the desired path.



1. Turn on gridlines in the live view by checking the **Grid** box, click **Go** for **Channel A** to activate **Path A**.





2. Adjust either **Screw B** or **Screw C** of the **Path A** dichroic mirror.

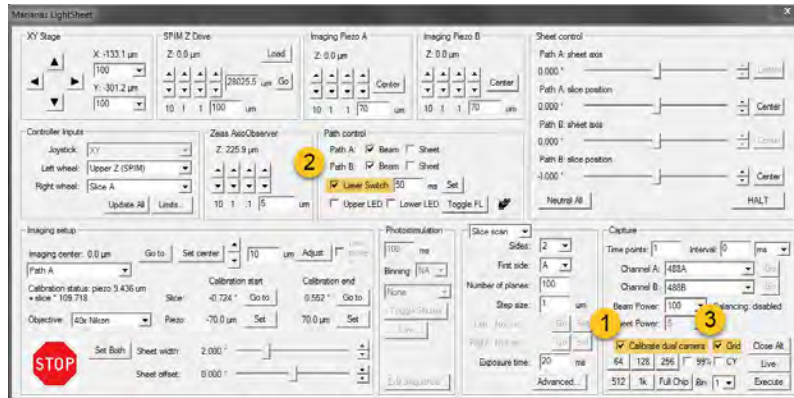
3. Repeat for **Path B** (by clicking **Go** for **Channel B** and adjusting either **Screw B** or **Screw C** of the **Path B** filter cube) until both beams are in focus and parallel with the horizontal gridlines across the field of view. Do not be concerned if beams are not in center of field of view (the beam just needs to be uniform at this point).



## TUTORIAL: Co-aligning the Beams

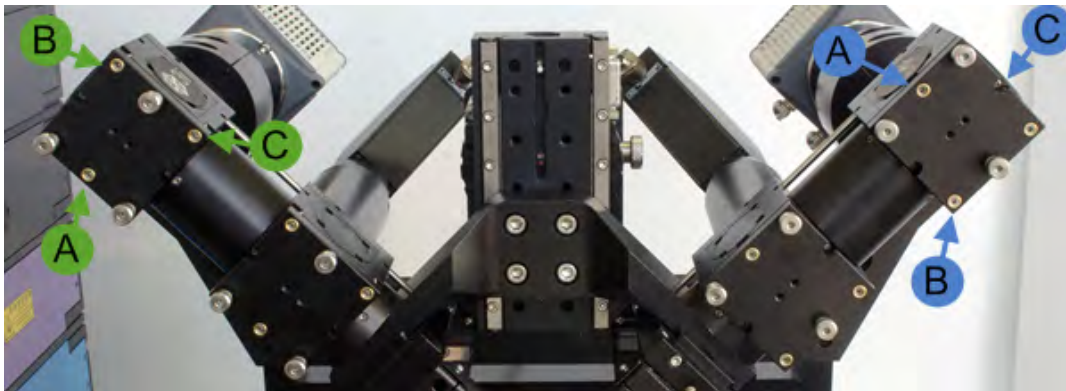
Prepare a sample for the MLS alignment, use the imaging media you will be imaging your samples in (water or PBS are acceptable but not ideal). To better visualize the beams, add a small amount of fluorescein or phenol red. For this tutorial, you will need to show beams and spots from both paths. To do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

- **Calibrate dual camera**
- **Laser Switch**
- **Grid**



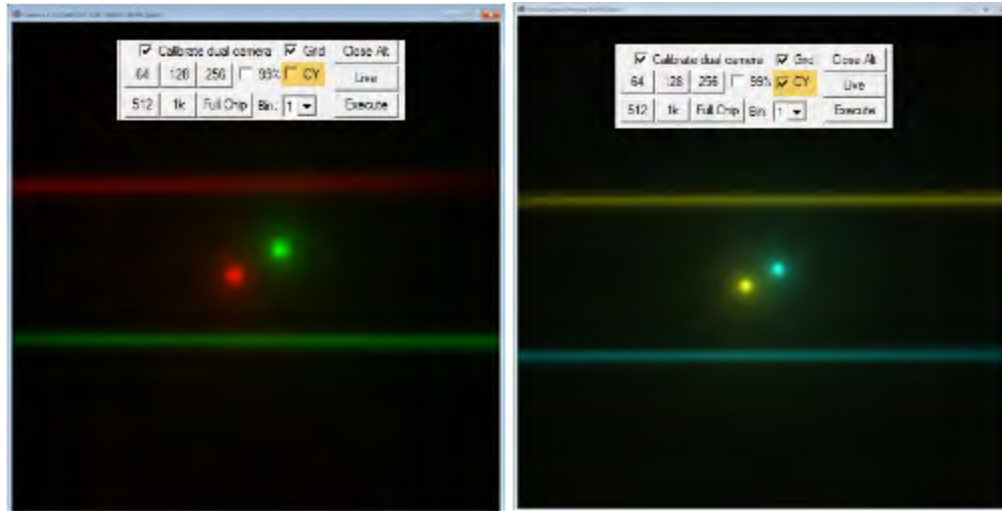
Throughout the tutorial, you will be adjusting the Camera (Upper) Mirror for each path. You will be using an Allen wrench in Screw A (left-right), B, and C (diagonally up-down) of **Path A** and **Path B** as depicted below.

- 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



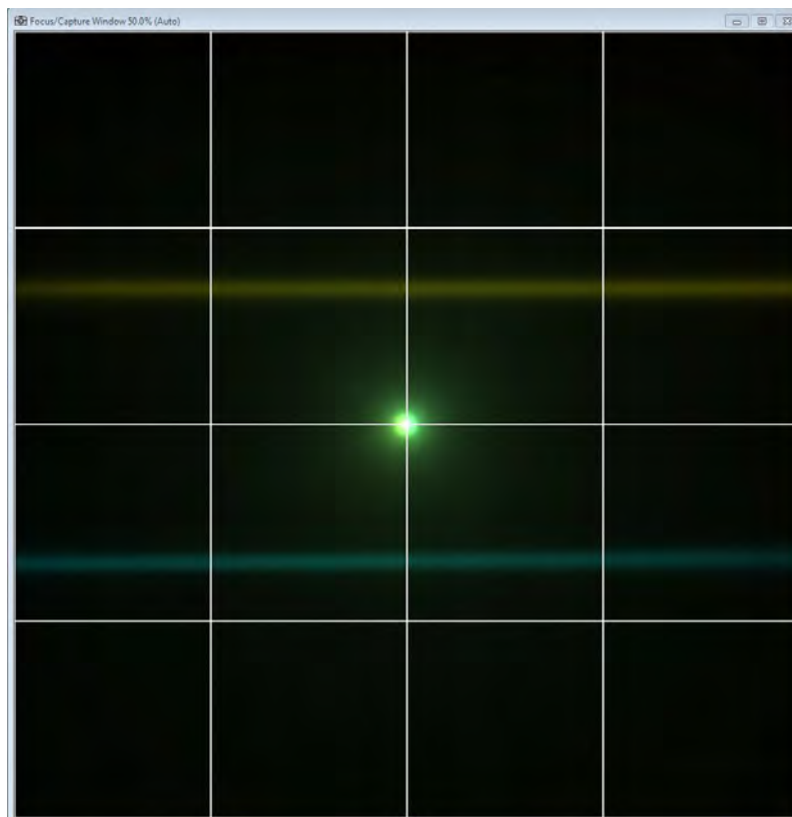
In the live window, **Camera 1 (Path A)** will be pseudocolored red while **Camera 2 (Path B)** will be pseudocolored green.

**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**)

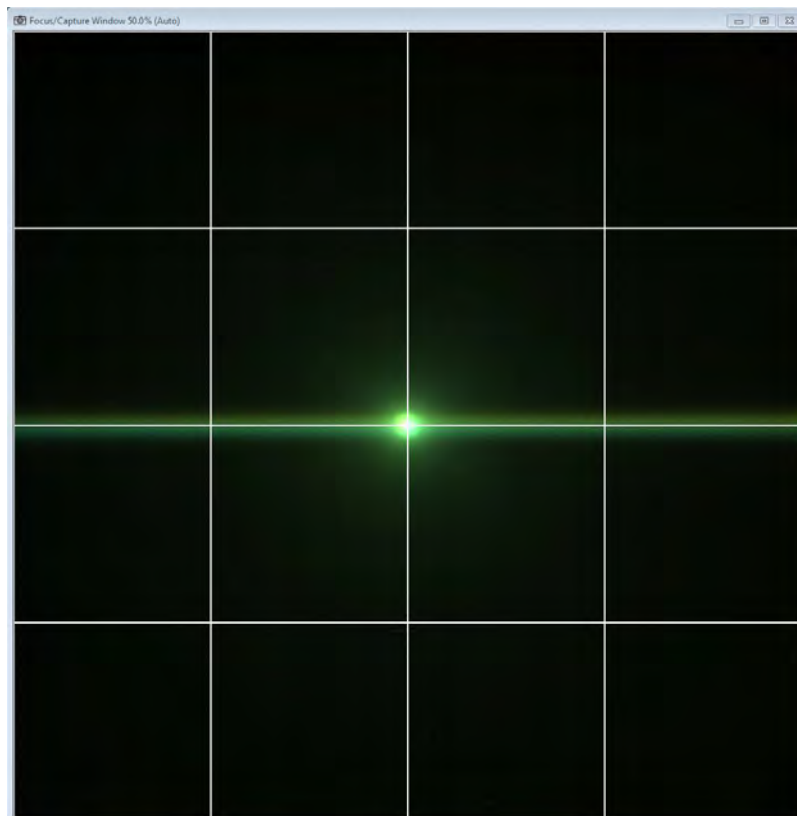
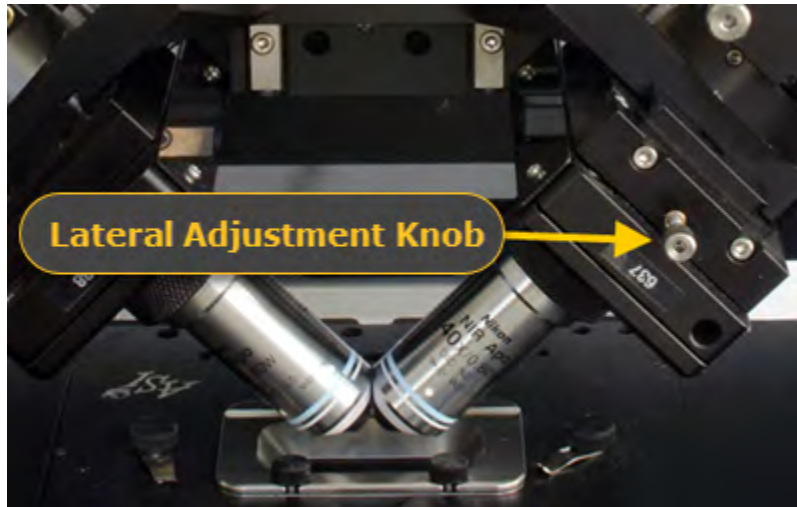


1. Use a combination of screws A, or B and C to move both the green and red spots to the center of the grid. Do not worry about the vertical location of the beams at this point.

**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 **Calibrate Dual Camera** 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**.

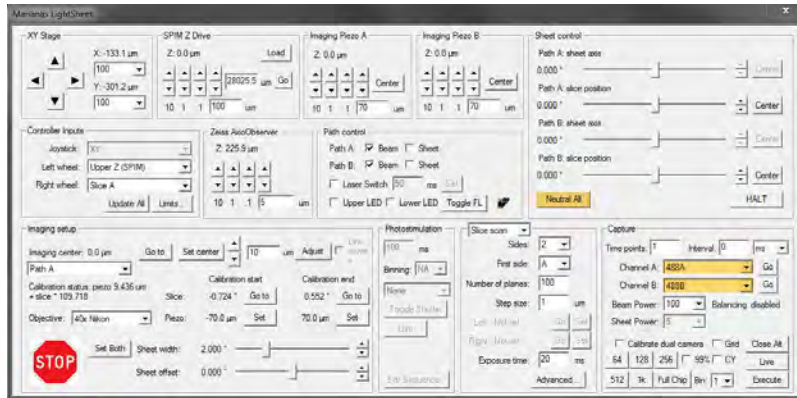




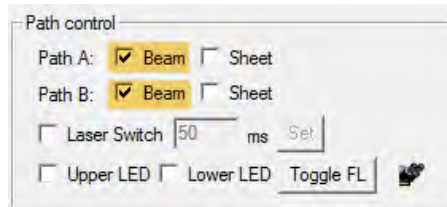
## TUTORIAL: Calibrating the Piezo and Slice Position

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.

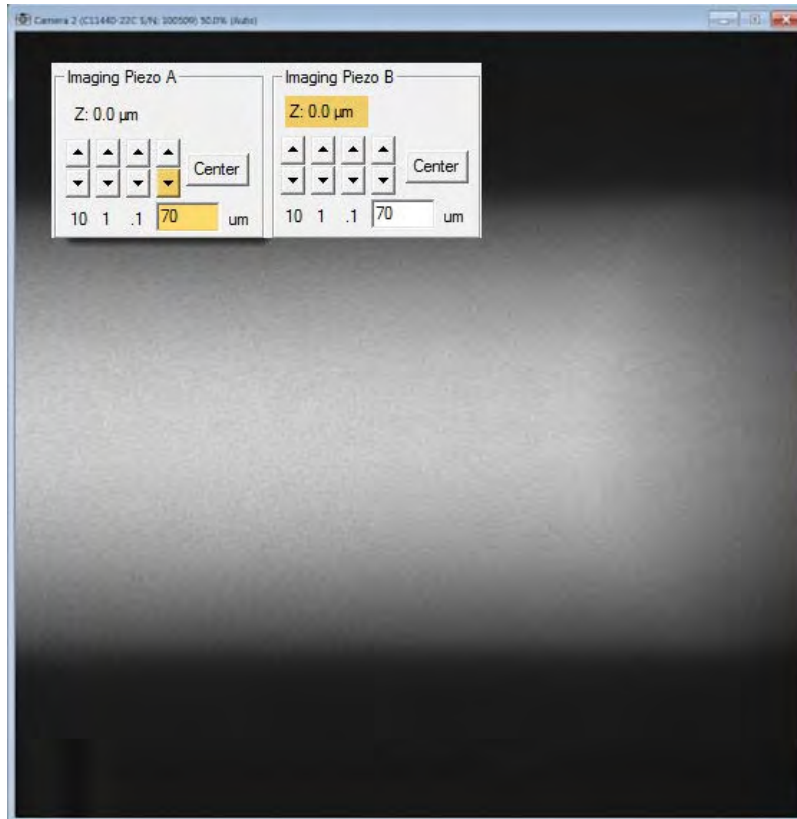


1. Check the box for **Beam** for Path A and Path B.



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.

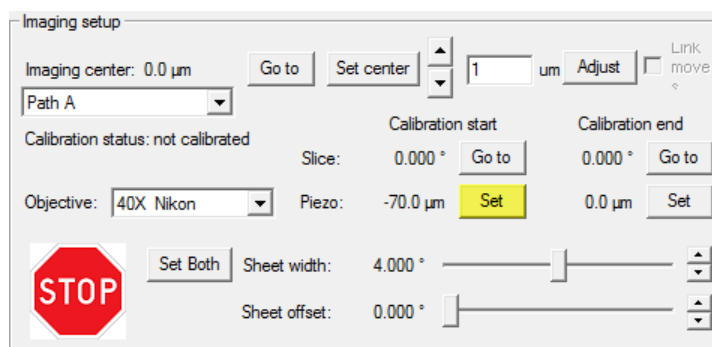
**NOTE:** A Z value is displayed above the arrows, this will indicate the current position of the piezo. For a 150 µm Piezo it will range between -75 to 75 max. Always make sure the opposite piezo is at center position when starting the calibration.



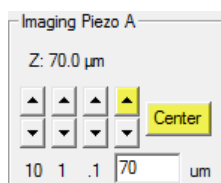
3. Adjust the right wheel on the joystick (confirm Controller Inputs, **Right wheel = Slice A**) until the beam is in focus and uniform.



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.

Imaging setup


Imaging center: 0.0  $\mu\text{m}$      $\mu\text{m}$   ☐ Link move

Path A

Calibration status: piezo -1.#IO  $\mu\text{m}$  + slice \* 1.#IO

Calibration start: Slice: 0.000 °  Calibration end: 0.000 °

Objective: 40X Nikon  Piezo: -70.0  $\mu\text{m}$   70.0  $\mu\text{m}$

  Sheet width: 4.000 °

Sheet offset: -0.017 °

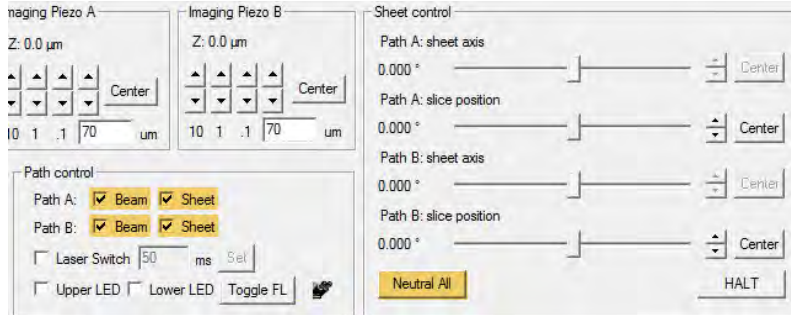
8. Return Piezo A to center by selecting **Center** (as in Step #4) and select Adjust (the beam should become focused again) to check the calibration.

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

## TUTORIAL: Setting Sheet Width and Offset

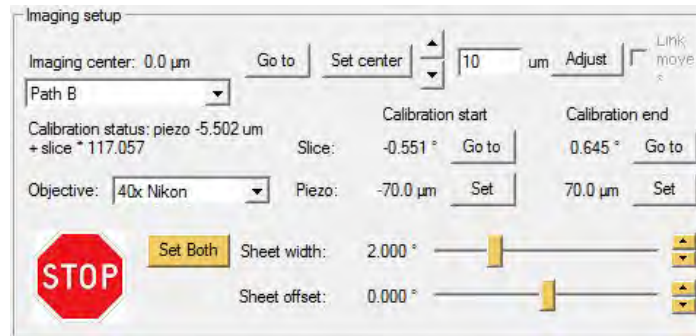
Once the Beams are aligned and calibrated return to the neutral position and continue to set the sheet width and offset.

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

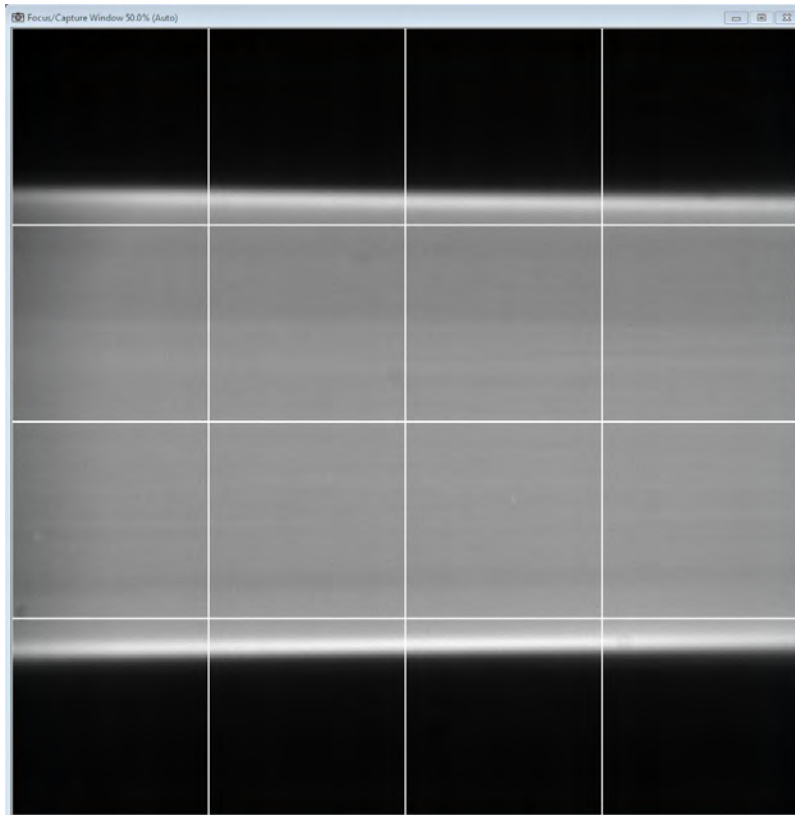


2. Check the box to turn on the **Grid** for easier alignment. Adjust the **Sheet width** (displayed as the vertical height of the sheet) to just outside the field of view. In the following example, the field of view is the center four squares of the grid. If the sheet is not perfectly centered, adjust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

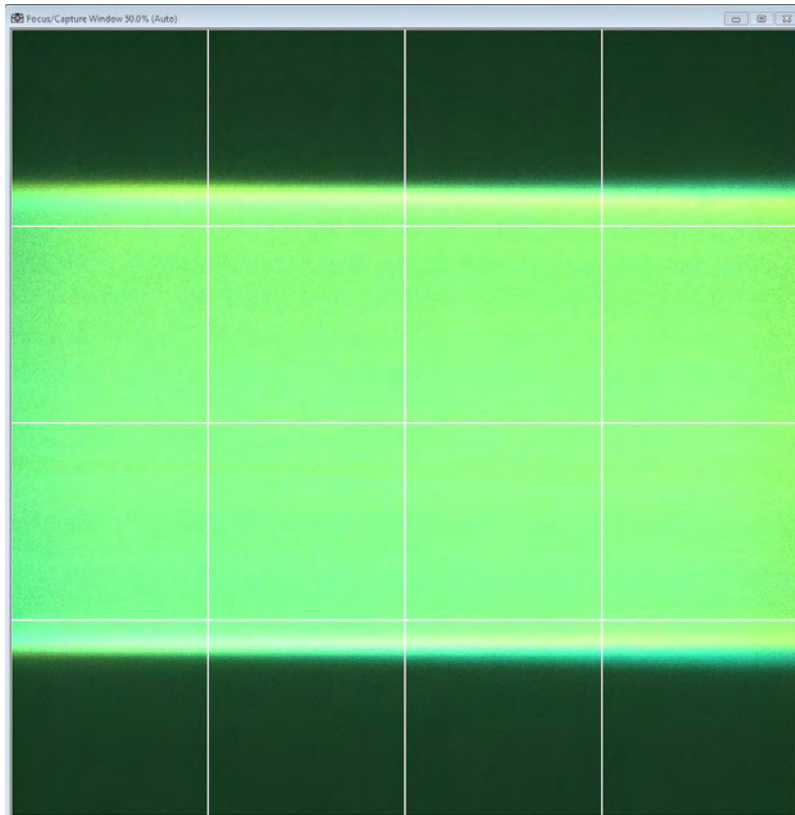
**NOTE:** The arrows to the right of the sliders can be used to adjust sheet width and offset. The step size for a single arrow button click is 0.001 units. Hold **CTRL** while clicking the arrows to use a step size of 0.01 units.



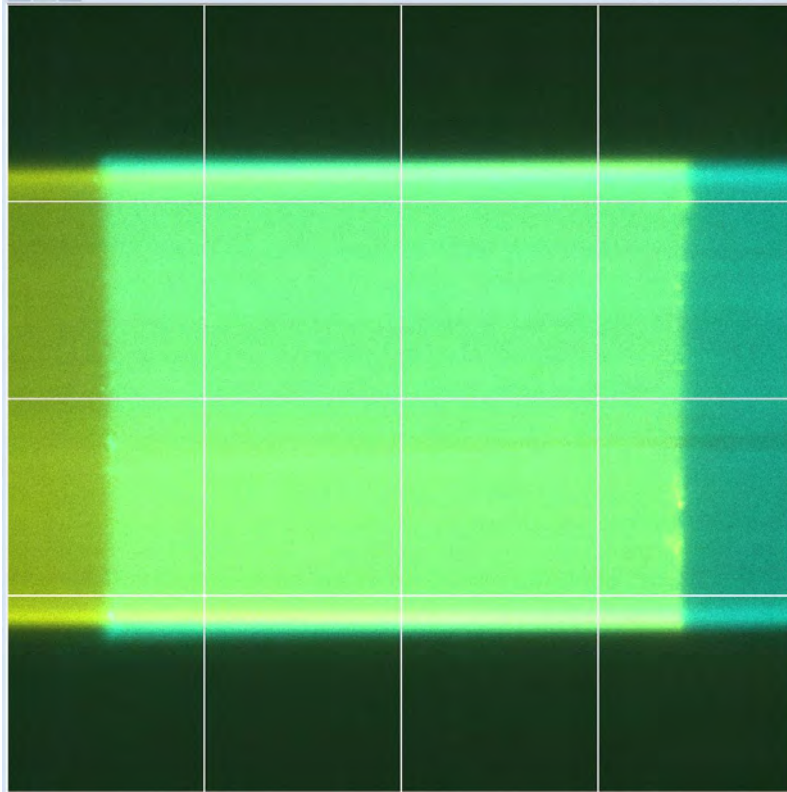




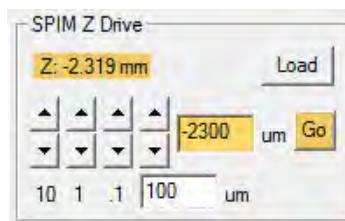
3. Check the box for **Calibrate Dual Camera Mode** to check the sheets are overlaid. When you are in Calibrate Dual Camera Mode, the sheet width and offset will only adjust for the active channel, that is the channel you for which you last clicked Go. Use the Go buttons to toggle between Channel A and 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.



## **MLS Operation**

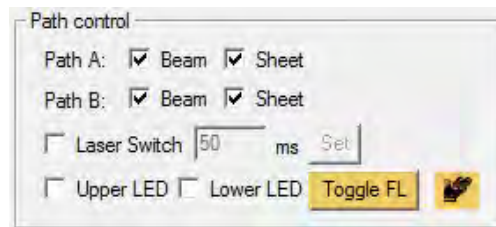
Once the beams are aligned, a sensible sheet width is set, and piezo and slice distances are calibrated, you can start imaging your samples. This section of help pages will walk you through MLS operation from locating your sample to refining the calibrations through to image acquisition.

## Locating and Positioning the Sample

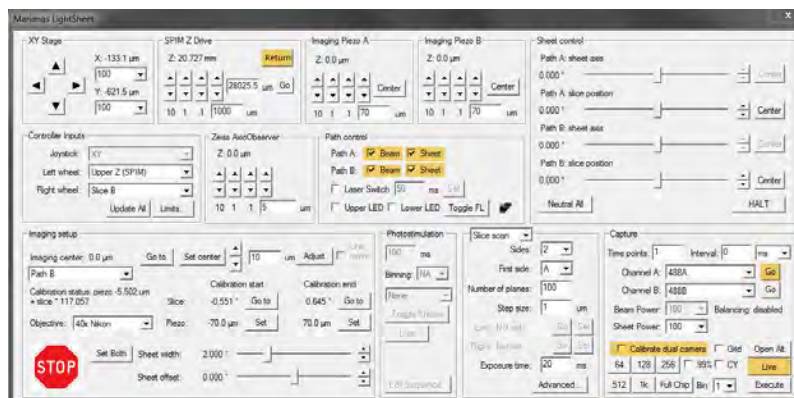
### Locating the Sample

1. Use the **Load** button to fully withdraw the SPIM Z Drive.
2. Load the sample; it should be prepared and placed on a 20 x 40 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.

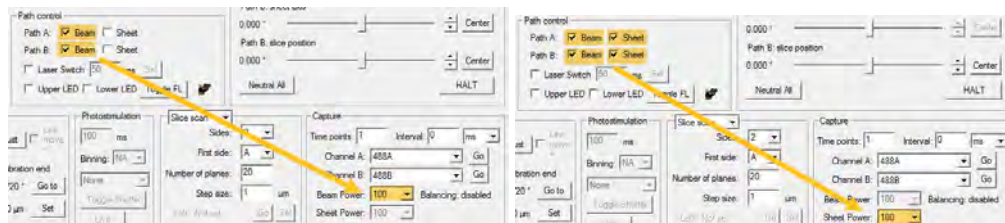


4. Once the sample of interest is located in the center of the field of view through the oculars check the boxes to select **Beam** and **Sheet** for both paths and click **Go** and **Live**. Use **Calibrate Dual Camera** mode while driving the Z Drive down to the sample using the **Return** button.



### Approaching the Sample

1. Check the boxes for **Beam** and turn down **Beam Power** to about 5%. Check the boxes for **Sheet** and turn down **Sheet Power** to about 5%. Leave both sets of boxes checked.



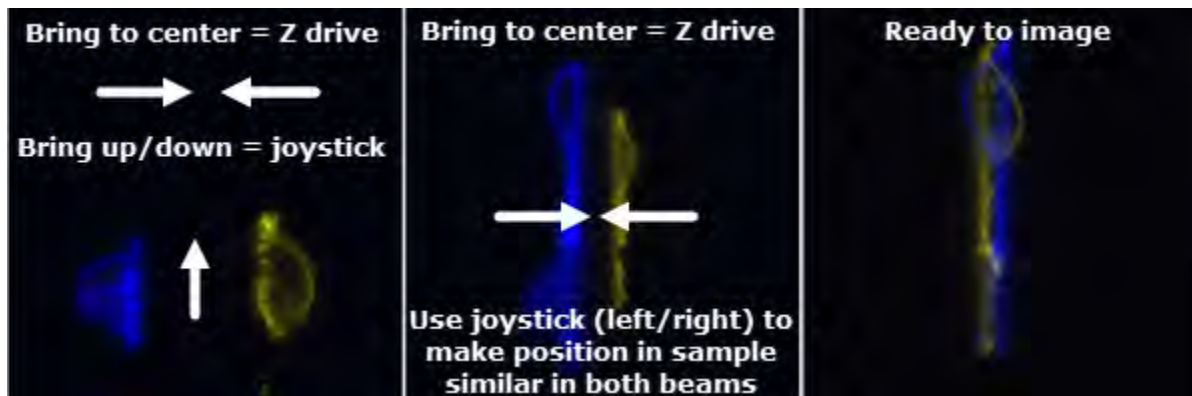
2. Select a filter for **Channel A** and **Channel B** and click **Go** for one path.
3. Lower objective without crushing the sample, as outlined in [TUTORIAL: Using the SPIM Drive to Introduce diSPIM Objectives to the Sample Chamber](#).



#### 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 **Calibrate Dual Camera** function as the diSPIM 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.

4. 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.



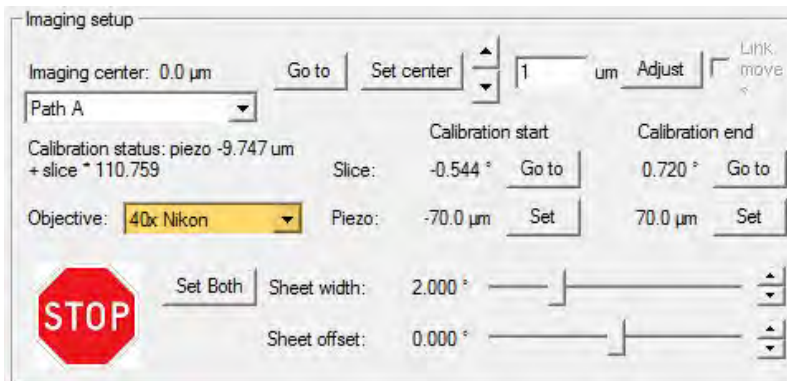
5. To image smaller objects, consider cropping the camera field of view to an appropriate size by clicking 1k (through to 64 x64 pixels). This will save considerable space in data capture. Also consider adjusting the piezo range before capture.

**NOTE:** When imaging a small volume, it is recommended to open the scanner iris slightly in order to narrow the beam waist. Best, adjust the beam width while in Calibrate Dual Camera mode in solution (Z drive is slightly above samples).

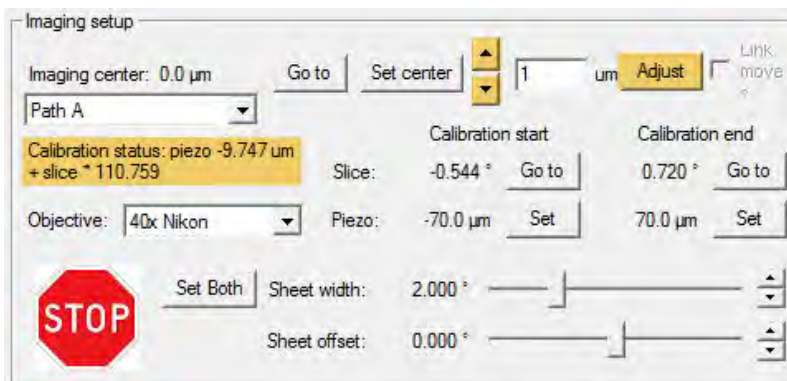
## Before Capture

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.

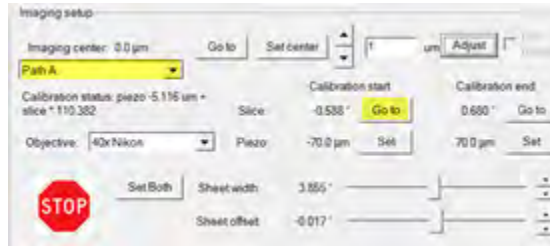
- 1.



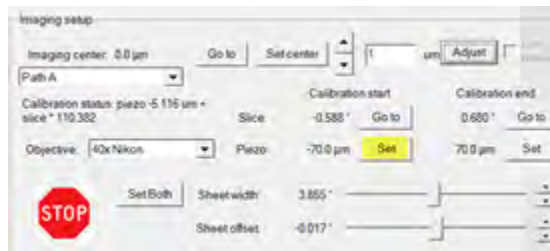
## Refine an Existing Calibration

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.

## Data Acquisition: Slice Scan

1. Have **Beam** and **Sheet** checked for both Paths.

2. In the Capture group:

- Select the (first) filter for each channel A and B respectively.
- Lower the sheet power (5 % recommended).
- Check **Calibrate Dual Camera** and **Live** to view your sample.
- 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:

- Select **Slice scan** from the drop down menu
- Sides** – select 2 for diSPIM, 1 for iSPIM (the second side choice will then be greyed out)
- First Side** - can be either A or B (only significant for iSPIM)
- 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  $\mu$ m Piezo  
Step Size = 1 $\mu$ m, Number of planes =150  
or  
Step size = 2  $\mu$ m, Number of Planes =75
- Set **Exposure time**, minimum recommended exposure is 10ms (at 512 px, 15 ms at 1k px, 30 ms at full chip)  
**NOTE:** Check for the appropriate exposure time in LIVE mode.

The image shows a software dialog box for configuring a 'Slice scan'. At the top, there is a dropdown menu currently set to 'Slice scan'. Below this are several input fields and buttons: 'Sides' is a dropdown set to '2'; 'First side' is a dropdown set to 'A'; 'Number of planes' is a text input field containing '20'; 'Step size' is a text input field containing '1' followed by a unit selector set to 'um'; 'Left: Not set' and 'Right: Not set' are labels next to 'Go' and 'Set' buttons respectively; 'Exposure time' is a text input field containing '100' followed by a unit selector set to 'ms'; and an 'Advanced...' button is at the bottom.

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.

- Check 2, 3, or 4, channel capture and select the filter pairs for additional channels.
- Check **Unique exposure time** to enter an individual exposure time.
- For colocalization assays and multicolour iSPIM, check **Capture all channels at each piezo position** at the bottom of the advanced window.
- Click **OK** to close the Advanced Capture mode window.

☒ Enable 2 channel capture

Channel C: 405A Go

Channel D: 405B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☒ Unique exposure time: 30

---

☒ Enable 3 channel capture

Channel E: 561A Go

Channel F: 561B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☐ Unique exposure time: 100

---

☐ Enable 4 channel capture

Channel G: 405A Go

Channel H: 405B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☐ Unique exposure time: 100

---

☐ Enable stage scan 1 um increment

XY stage settling time (ms): 50

☐ Capture 1 images per piezo positions

☐ Capture all channels at each piezo position

OK

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:

- Select the (first) filter for each channel A and B respectively.
- Lower the sheet power (5 % recommended).
- Check **Calibrate Dual Camera** and **Live** to view your sample.
- 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:

- Select **Stage scan** from the drop down menu
- Sides** – select 2 for diSPIM, 1 for iSPIM (the second side choice will then be greyed out)
- First Side** - can be either A or B (only significant for iSPIM)
- Set the **Step size** (consider the camera crop).
- Set the stage scan extents by moving the stage slightly to the left of the sample, then click **Set**.

f. Repeat for the right end of the sample. The **Number of planes** will be calculated automatically from the set **Step size**. Consider not exceeding 300  $\mu\text{m}$  for data handling reasons.

- Set **Exposure time**, minimum recommended allowable exposure is 10ms (at 512, 13 ms at 1k, 26 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.

**NOTE:** A single stage scan can take up to 5 minutes due to the use of the speed of the XY stage Stepper motor. Plan accordingly.

5. Click **Advanced** to open the dialog to add channels.

- Check 2, 3, or 4, channel capture and select the filter pairs for additional channels.

- b. Check **Unique exposure time** to enter an individual exposure time.
- c. For colocalization assays and multicolour iSPIM, check **Capture all channels at each piezo position** at the bottom of the advanced window.
- d. Click **OK** to close the Advanced Capture mode window.

☒ Enable 2 channel capture

Channel C: 405A Go

Channel D: 405B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☒ Unique exposure time: 50

☐ Enable 3 channel capture

Channel E: 561A Go

Channel F: 561B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☐ Unique exposure time: 25

☐ Enable 4 channel capture

Channel G: 640A Go

Channel H: 640B Go

Beam Power: NA

Sheet Power: NA

☐ Balance laser power

☐ Unique exposure time: 25

XY stage settling time (ms): 50

☐ Capture 1 images per piezo positions

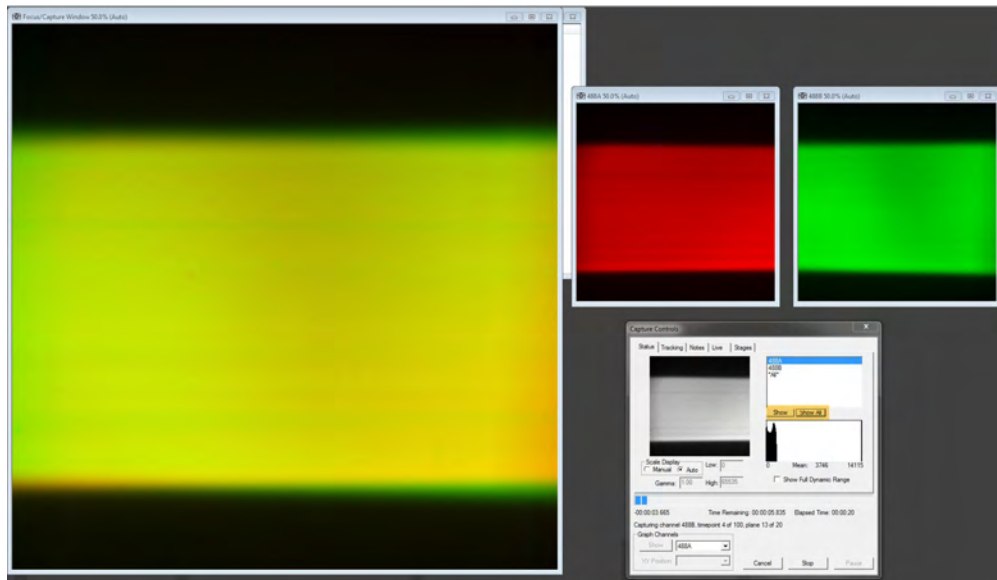
☒ Capture all channels at each piezo position

OK

6. Click **Execute** to start the capture.

## 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**

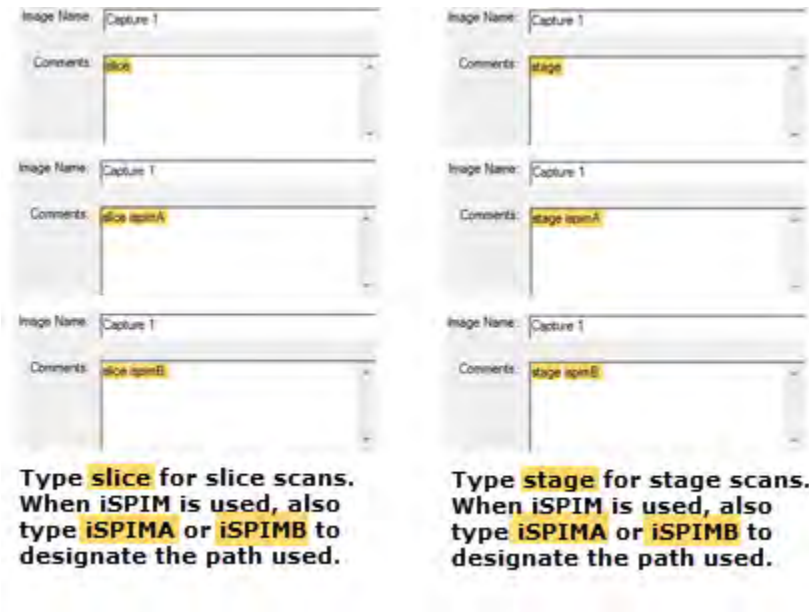
When using diSPIM mode of MLS, the two paths will need to be fused using reconstruction algorithms. In addition to this, any data collected using stage scan mode will need to be deskewed first to put it back into a geometrically correct location. General information about deskewing can be found in [this 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.

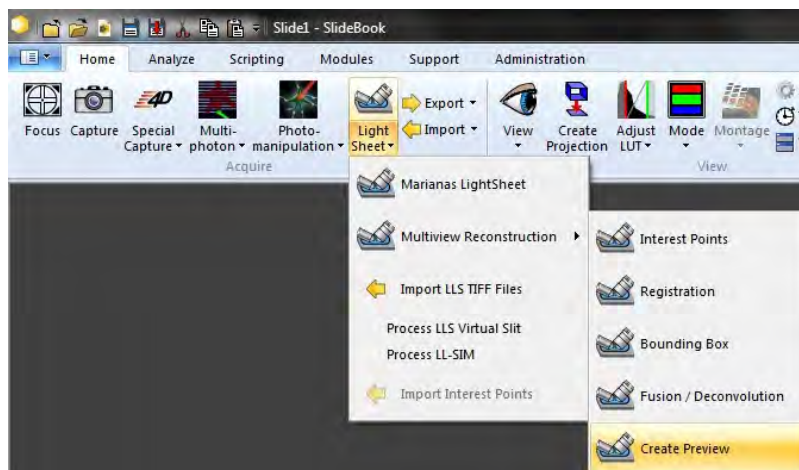
1. Save the **.s1d** before beginning the process.

2. Right click on the image you would like to preview, select **Properties** from the right-click menu displayed. In **Comments**, enter the word **slice** if it is a slice scan. If it is a stage scan the program will have automatically added the info **MLS stage scan**, replace it or add the word **stage** in front of it. If you have an iSPIM capture to look at, add the word **ispimA** or **ispimB** respectively, depending on which path was used for imaging. Click **OK** to apply the changes and close the dialog.



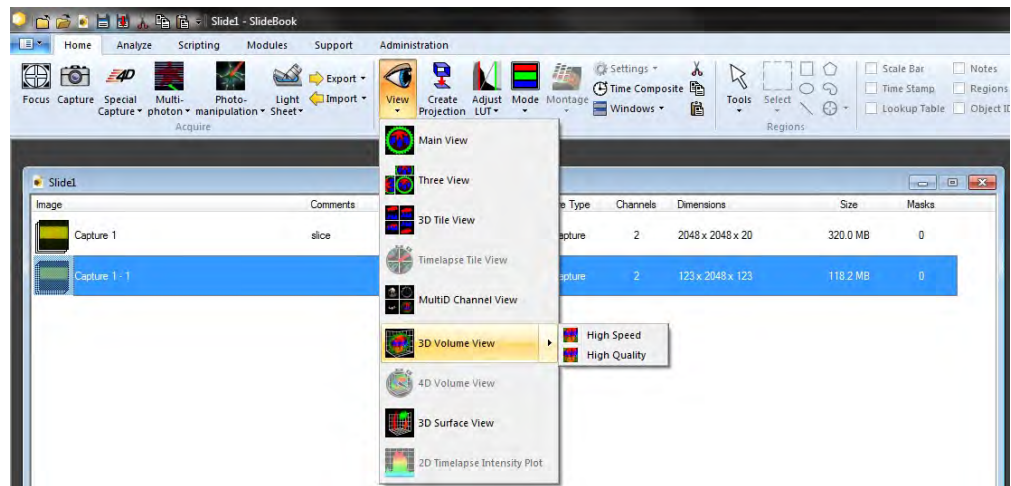
3. With the image still selected, go to **LightSheet > Multiview Reconstruction > Create Preview**.

**NOTE:** This will immediately create a transform using the default settings from previous registrations of the selected picture and will take a while depending on the size of the data set.



4. SlideBook will create a new capture at the end of the **.s1d**. Right-click on the created image, select **Properties** from the right-click menu displayed and erase the comment. Click **OK** to apply the change and close the dialog. **Save** the slide.

5. Select (highlight) the created 3D transform. In the Home tab of SlideBook, select **View > 3D Volume View**. [3D Volume Viewer](#) will open and render a roughly transformed and fused and/or de-skewed picture of your capture in order to decide whether to process it further.

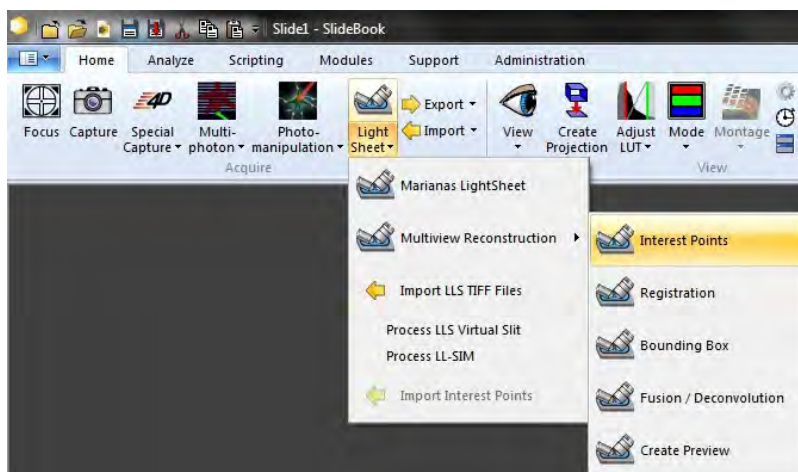




## TUTORIAL: Multiview Reconstruction

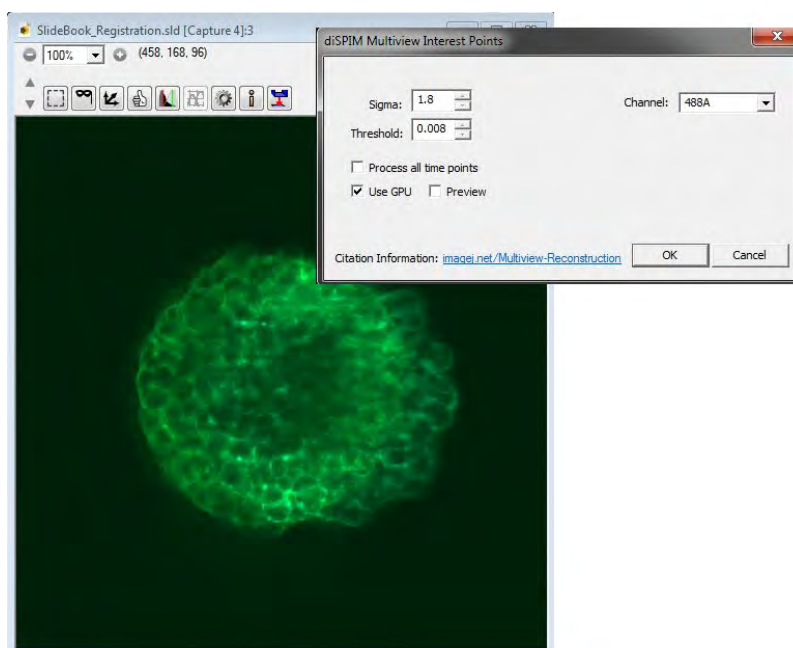
This tutorial will walk you through aligning a two-path diSPIM image, defining a bounding box, fusing, and deconvolving the image.

1. Save the **.sld** before beginning.
2. Select (highlight) or open the image you would like to work with. In the Home tab of SlideBook, select **LightSheet > Multiview Reconstruction > Interest Points**.



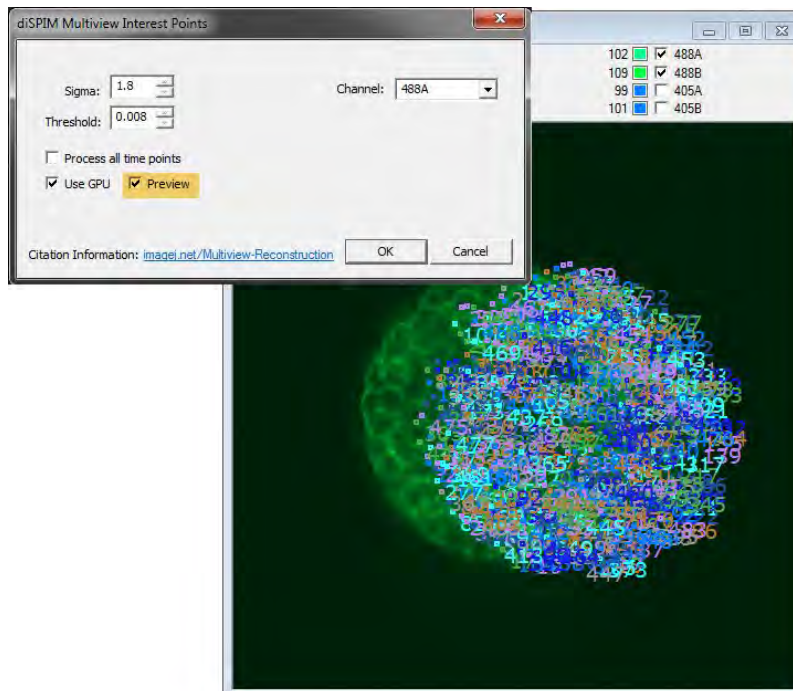
3. SlideBook will load default **Sigma** and **Threshold** values that will define the size and number of regions to interrogate. These values are best for beads, you might want to change these depending on your samples. Small **Sigma** values = small size regions to interrogate; small **Threshold** 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**.

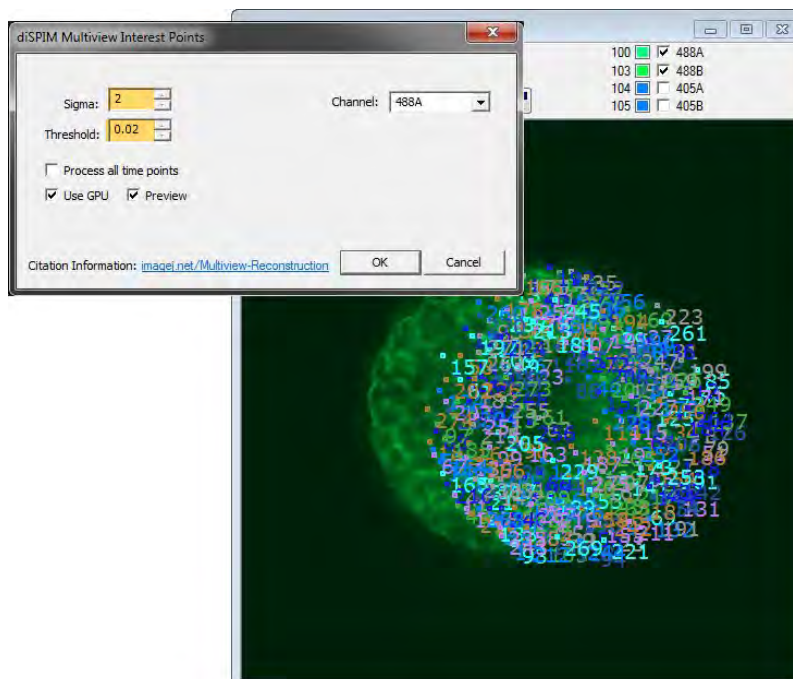


4. Check the **Preview** box to show the regions generated.

**NOTE:** When you check the Preview box, a process will be activated spawning new windows showing the algorithm activity and log. In SlideBook, many small regions will appear in the image.



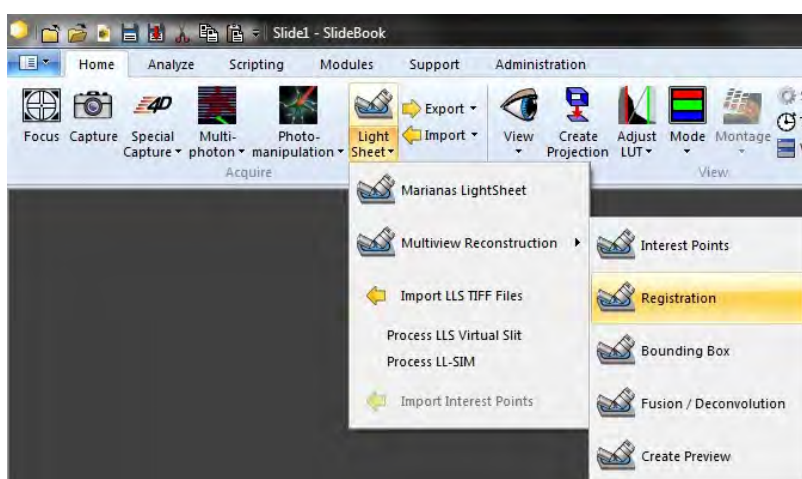
5. Look to ensure that the detected interest points correspond to the "right" features in the channel. There should not be points where there is no structure yet too few interest points will lead to failure during registration. Adjust sigma and threshold if necessary.



6. It is not necessary to check the box to **Process all time points**. A single time point detection should work for all subsequent time points. 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: sldname**.

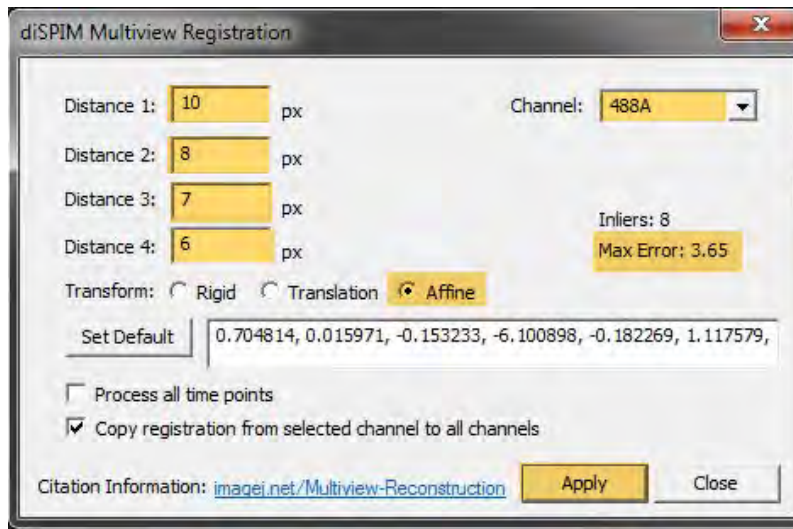


7. Now that interest points have been defined, you can proceed with registration. Go to select **LightSheet > Multiview Reconstruction > Registration**. The diSPIM Multiview Registration dialog will open.



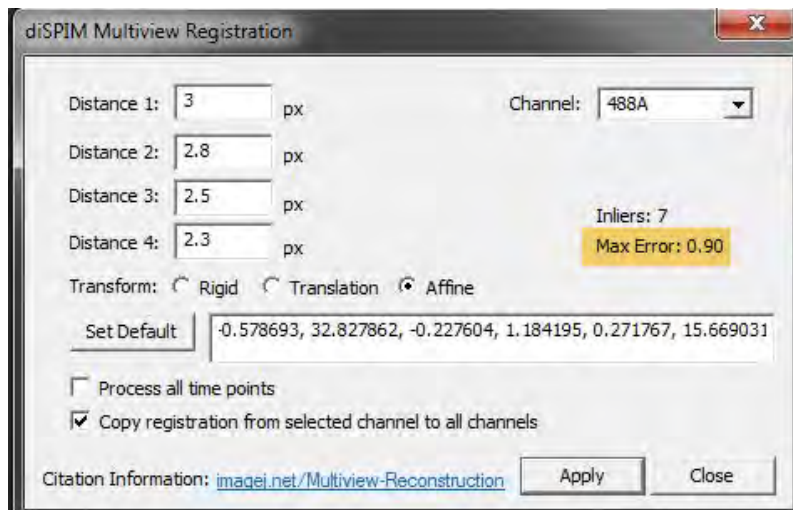
8. Choose the **Channel** for which you detected the interest points in the dropdown. Enter a distance between given interest points in pixels in the first line, to start the registration process. This is an interactive, iterative tool. The default distance to start with, 10 pixels, is best to register beads. For samples with larger structures start with 20-30 pixels. Each distance field is an opportunity for an iteration. Enter values of decreasing values, being sure not to decrease by more than 1/2 of the previous distance value.

For example, the first four values you may wish to enter would be **20-10-7-5**. The next round, you would do **4-3-2-1** then finally **0.8 - 0.5 - 0.3**. Decreasing in smaller steps is preferable to larger steps.



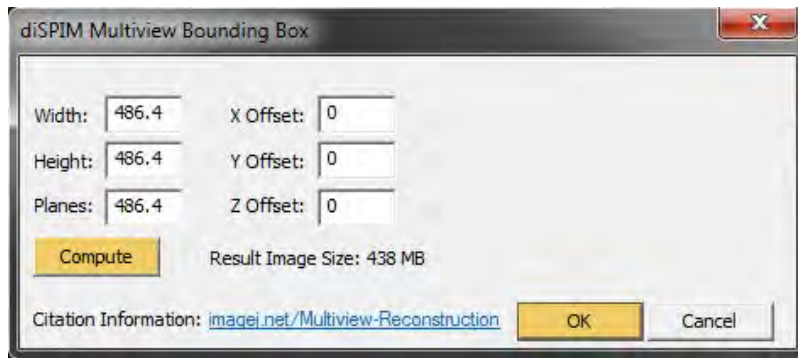
9. After you enter the first four **Distance** values, click **Apply**. then look at the **Max Error** value. The goal is to achieve a Max Error value less than 1. Enter in four more distance values decreasing as described in the example above and click **Apply** again. If the Max Error value decreases, continue decreasing the distance value. If the Max Error value increases, try increasing the distance values and going back down in an iterative process. Use this iterative approach until you achieve a Max Error value less than 1 and a number of inlayers >1.

**NOTE:** For the most successful registration results we recommend to stay with the **Affine** transform type – This is the most flexible registration after Transform only or just a Rigid plane rotation.



10. Next, select **LightSheet > Multiview Reconstruction > Bounding Box**. Click **Compute** to return the maximal bounding box, that is the maximum volume of both paths overlapping. This will set the width, height and number of planes to be interrogated. Click **OK** to set the bounding box.

**NOTE:** It is advisable to leave these settings or decrease them slightly. Cutting too much might end up with cropping important information out of the final picture. The result Image size is an estimate and the final version can be slightly larger.



11. Finally, select **LightSheet > Multiview Reconstruction > Fusion / Deconvolution**.

a. Select one of the three processing options:

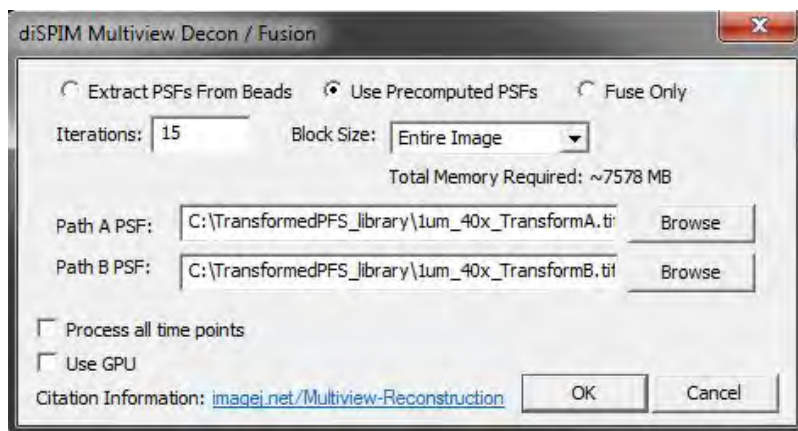
**Extract PSFs From Beads** - Uses beads within the same sample to generate PSFs for deconvolution. This also creates PSF transforms for Path A and Path B which can be used for future deconvolution of data.

**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**. Leave GPU unchecked.



12. 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.

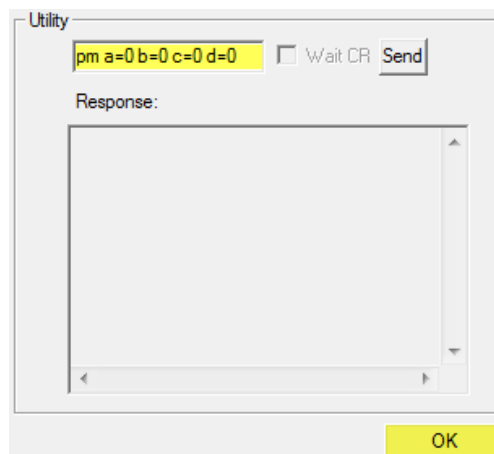
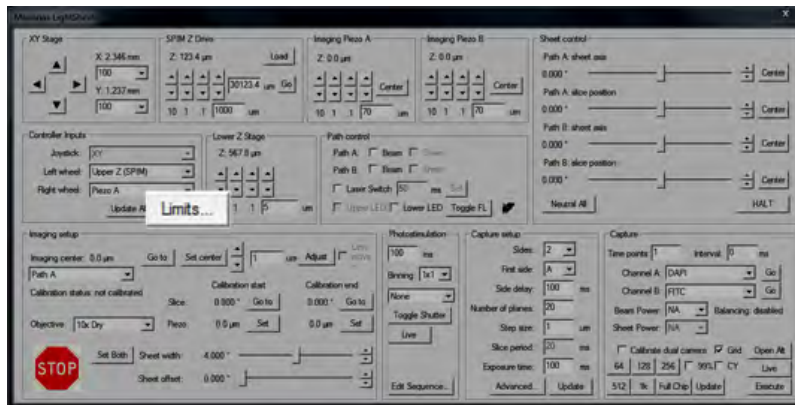
**NOTE:** If you extracted PSF from beads in the image, additional PSF files will be written to the slide. These can be used for future deconvolution. Export those PSF transforms as 16-bit TIFF and save them in a separate PSF library folder.



## MLS Troubleshooting

Below, you will find a list of troubleshooting tips. For additional assistance, please contact [3i Support](#).

- If exposure time is not matched with scan duration, the captured field will be smaller than expected > Recalibrate slice and piezo
- During calibration if you are not seeing all four elements (Path A & B beams and sheets), uncheck and re-check Laser Switch a couple of times
- If you are having trouble aligning the beams, turn everything off and reset the filter cubes and mirror cubes to zero
- Capture is cut off on top for one path, and cut off on bottom for other path - has to do with the 'scan period' (camera exposure was not on long enough)
- Will not always see a display during acquisition, depends on the selected frame rate
- After capture, if the next live image goes to 0, not the calibrated values – need to select 'Adjust' in diSPIM window
- Must re-calibrate and re-align when buffers are switched
- Move joy stick off of air table during operation
- Should you see no signal of one beam and sheet (A or B side), go to Limits to open the ASI Controller dialog. At the bottom of the dialog, in the **Utility** group, type **pm a=0 b=0 c=0 d=0**. Click **Send** then **OK**.





## Adjust Scanner Slice Position Offset

Usually it is possible to get the objectives co-focused with the piezos near their centered or 0 position; if you have achieved this you can skip this step.

If it seems impossible to get the two objectives co-focused with the piezo positions near 0, you have two options: use a non-zero piezo offset or use a non-zero scanner slice position offset.

If the sample of interest occupies most of the piezo range, only the latter is viable; however, using a piezo offset is a simpler approach. In either case start with the objective bushings moved to a middle position that remains fixed.

Using a piezo offset is straightforward: an offset will be computed automatically by the piezo-scanner cross calibration. Simply you will only need to use start and end points of the calibration curve that are not symmetric about 0.

To use a scanner slice position offset, the piezo-scanner cross-calibration will make the offset appear during imaging, but you should compensate for the offset by moving the imaging mirrors so that in the middle of the range the epi-spot is centered in the epi camera.

Establish a focused beam in the dye solution.

Set the Controller Inputs for Path A.

Left wheel controls the "Upper Z (SPIM)" and the right wheel controls the "Piezo A".

Do this by setting the piezo to 0, moving the slice position until the beam is in focus, then adjust the imaging mirror. Repeat for the other side. This should make it so that the epi spot is overlaid with the beam focus.

-Z is 'calibration start', +Z is 'calibration end'

To display the calibrated values, select 'Adjust' in diSPIM Imaging Setup window.

Repeat this process for Path B.