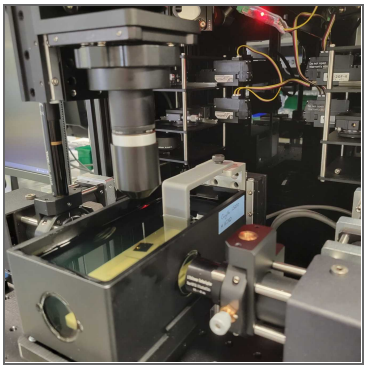

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Protocol status: Working
We use this protocol and it's working

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SmartSPIM setup and alignment

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ABSTRACT

Setting-up and aligning the SmartSPIM light sheet microscope is required before acquiring each dataset. The instrument is capable of imaging whole, cleared, delipidated mouse brains. It utilizes two illumination paths, corresponding to each hemisphere of the brain, can utilize a broad range of objective lenses, from 1.625x to 22.5x, as well as a range of immersion bath solutions, including aqueous, oil-based, and ethyl cinnamate. The microscope utilizes an axially swept light sheet and rolling-shutter camera acquisition to acquire near-isotropic imaging volumes.
This protocol provides instructions to prepare the microscope, to load samples, and to acquire data.

GUIDELINES

Use caution when handling and cleaning objective lens or when translating the objective lens or sample stage on the instrument. All fluorescent samples will bleach -- avoid unnecessarily illuminating your sample with any laser power.

MATERIALS

PROTOCOL integer ID:
81476

Keywords: lightsheet microscope alignment, smartSPIM, whole-brain imaging

Funders Acknowledgement:

Allen Institute

Equipment

4X Super Apochromatic Microscope Objective, 0.2 NA, 17.0 mm WD — NAME

Ojbective Lens — TYPE

Thorlabs — BRAND

TL4X-SAP — SKU

https://www.thorlabs.com/thorproduct.cfm?partnumber=TL4X-SAP — LINK

4x — SPECIFICATIONS

Equipment

SmartSPIM — NAME

Light sheet microscope — TYPE

LifeCanvas Technologies — BRAND

SmartSPIM — SKU

https://lifecanvastech.com/ — LINK

Equipment

CORIO CD-200F	NAME
Refrigerated / heating circulator	TYPE
Corio	BRAND
9012701.03	SKU
https://www.julabo.com/en/products/refrigerated-circulators/refrigerated-heating-circulators/corio-cd-200f	LINK

MACS imaging solution **Miltenyi Biotec Catalog #130-126-335**

Cargille Immersion Liquid **Cargille Catalog #Custom**

SAFETY WARNINGS

LASER exposure warning: The SmartSPIM lightsheet microscope is entirely contained in a light-tight box. However, the front panel must be opened, with a laser ON, during an alignment step. Avoid eye exposure by limiting the laser power (less 20% in 561 nm) and keep side-panels on the instrument. Laser safety glasses are located throughout the lab. If working with ethyl cinnamate, follow SDS instructions and be careful to avoid contact with plastics that may dissolve in the organic solvent.

BEFORE START INSTRUCTIONS

If using a chiller, allow it to equilibrate to its Set Temperature before starting the SmartSPIM acquisition software.

Hardware set-up

1 Turn on the instrument BEFORE starting the acquisition software:

A single surge protector powers both the instrument and chiller.

2 If a water chiller is installed, run the chiller until it is settled at its set temperature of 18 °C . Opera 5m the chiller 24/7 when the instrument is being used, and maintain a temperature of at least 25 °C . The chiller must be running at its chilled temperature before the acquisition software--controlling the camera--is run. Once the surge protector is powered on, press OK to turn it on. The camera may not require a non-pulsatile chiller, but one is installed here to reduce camera vibration for a rotary fan.

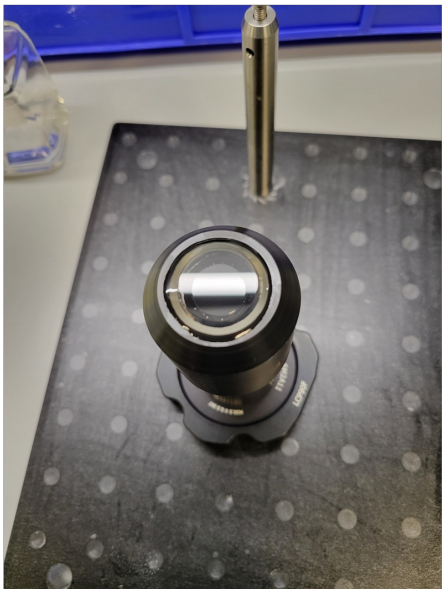
Chiller running at 18 degrees C.

3 Removing and attaching objective lenses: note that the magnetic base has a white line, corresponding 1m the front of the instrument (facing the user). When the objective is inserted into the immersion chamber, visually check for air bubbles underneath the objective.

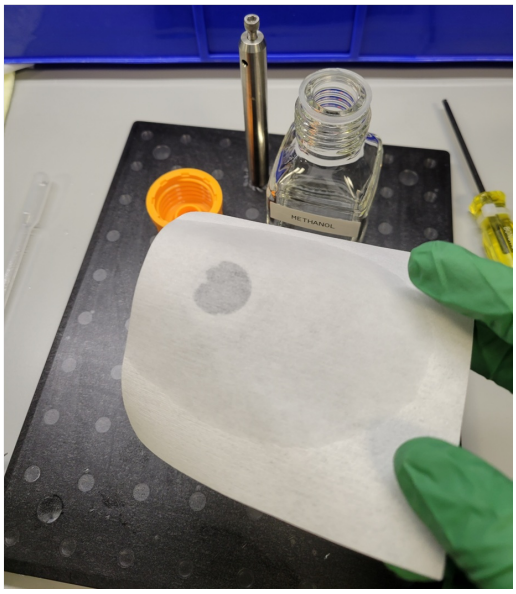
3.6x imaging objective, detached.

4 Cleaning objectives, before attaching to the instrument:

a)



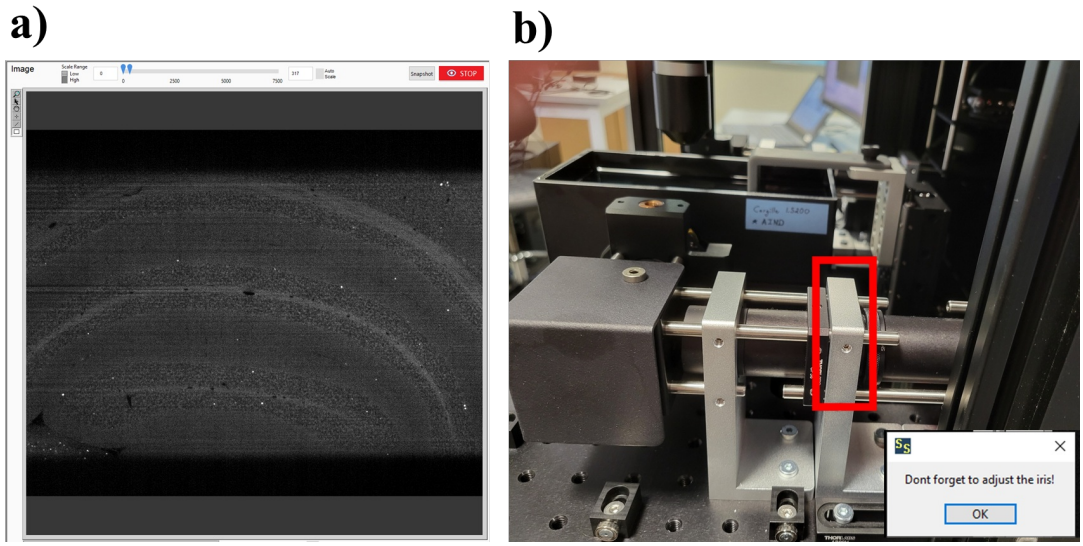
b)



a) Carefully inspect the objective lens. If it is not dirty, then avoid unnecessarily touching it to clean it. b) Use an air-blower to remove dust or dry debris, if it's sufficient to clean the objective. Use reagent-grade methanol and lens wipes to gently clean the objective, by applying a few drops of mentanol and dragging the lens wipe horizontally.

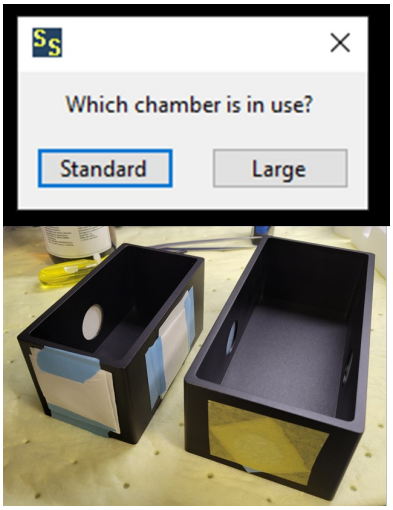
4.1 If the objective is dirty, clean the objective: First use a blower bulb to attempt to remove the dust. If that method is insufficient, place a piece of lens paper on top of the objective (b). Use a clean transfer pipette to drop 100% methanol onto the lens paper, then gently pull the lens paper horizontally, moving any dissolved contaminants and dust away from the center of the len's surface. Avoid rubbing back-and-forth or in circles.

5 Adjusting the iris: both arms of the microscope have an iris that will need to be adjusted. To adjust the iris, rotate it clockwise or counterclockwise until the black bars (a) are gone and the sample fills the entire field of view. The black bars in (a) shows the iris closed too narrow, preventing the sample for being fully illuminated. If the iris is opened unnecessarily wide then sample bleaching may occur in unimaged areas of the sample. Adjusting the iris until the black bars appear, then backing off slightly until they disappear is required to ensure the iris is not open too wide. 1m



a) Adjust the iris if the image is clipped (shown here as black bars on the top and bottom of the FOV). Remember to check the FOV for both the LEFT and RIGHT illumination paths. b) Location of the iris on the RIGHT arm of the instrument.

6 Immersion bath set-up: 10m



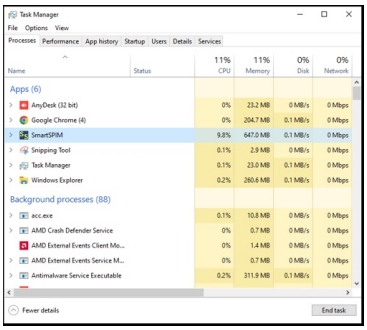
Two immersion bath sizes, holding ~300 mL and 650 mL each.

Acquire refractive indicies for both the sample and immersion bath to ensure close refractive index (RI) matching. The baths have fixed RIs, but the sample may be adjusted. Immersion bath RIs used include 1.52, 1.53, and 1.56.

Software set-up

9m

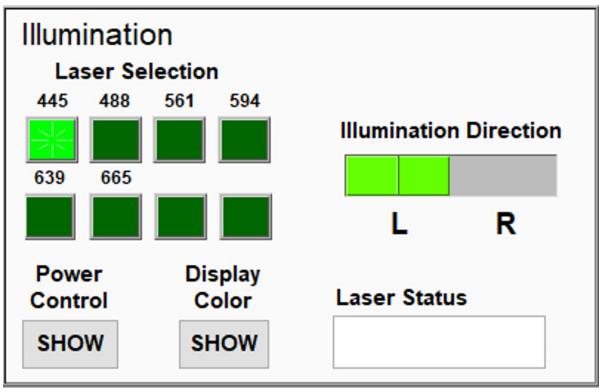
7 Restarting or starting the software: if the software is open from a previous acquisition, close the software and reopen it to begin an acquisition. In rare cases, the software may be frozen, and you may need to use Ctrl-Alt-Delete to restart the software. 1m



In rare cases, if the software is unresponsive, select "SmartSPIM" and "End Task".

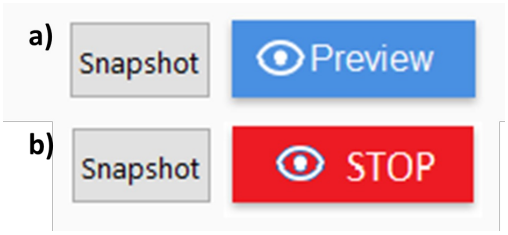
SmartSPIM acquisition software shortcut.

8 Select laser channel and illumination path. In this panel you select the laser that will be turned ON and 1m
OFF in the steps below.



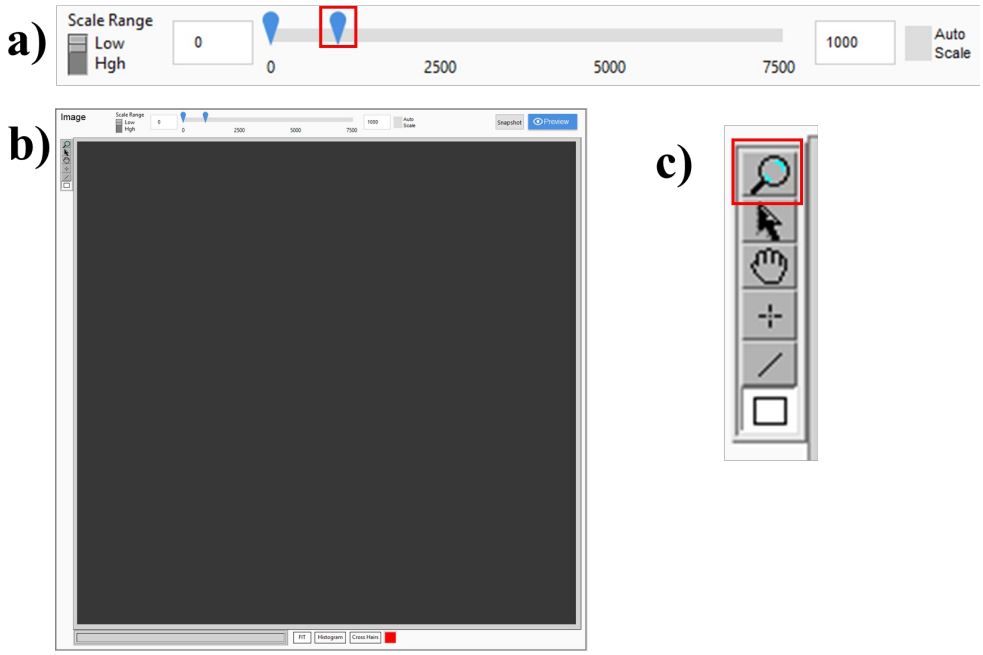
Illumination options: select the laser channel (wavelength in nm) and illumination path (L or R). Adjust Power Control for each laser.

9 Turning ON/OFF lasers: Click "Preview" to turn ON the laser at the wavelength selected. Click "STOP" t 5m
turn OFF the laser.



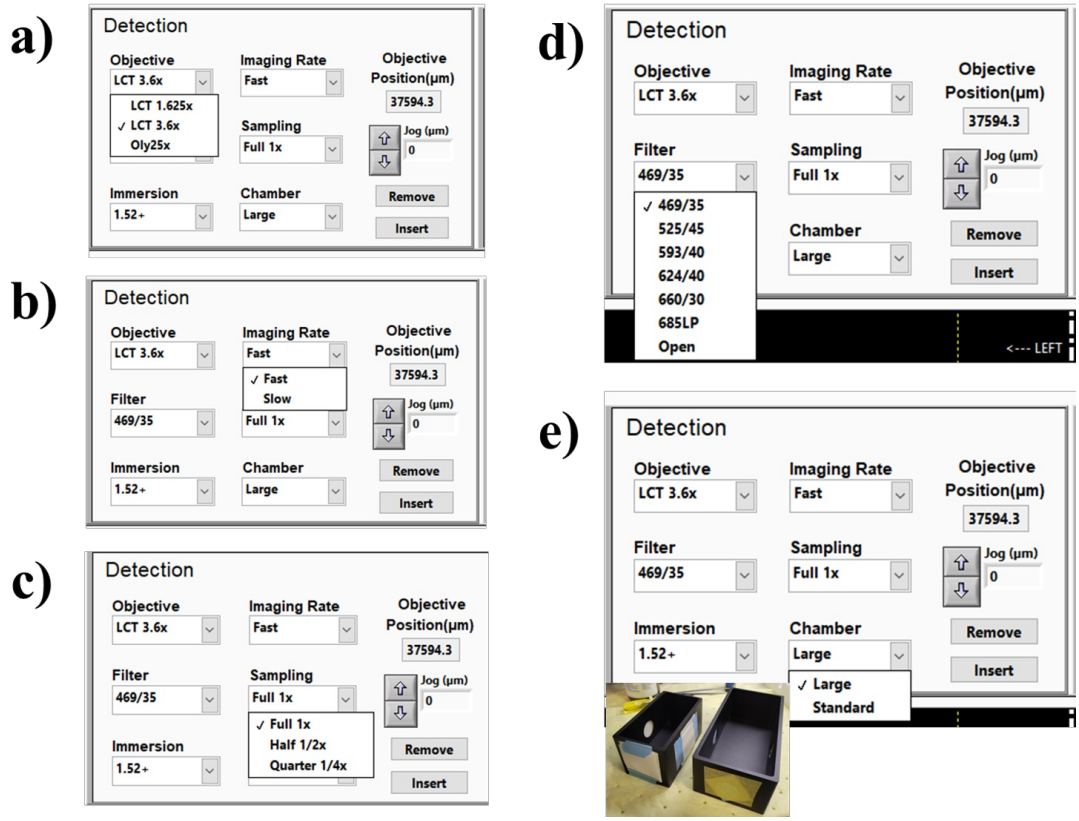
a) The blue PREVIEW button turns on the selected laser. Note that you must select the correct laser channel, illumination path (L or R) and laser power before turning on the instrument. b) When the laser is on, the Preview button is replaced by the red STOP button.

10 Adjust the image field-of-view options. The dynamic range of the camera is 16-bit, or approximately 1- 1m
65,535, so a scale of approximately 0-to-650 --or 1% of the camera's dynamic range--is plenty for setting-up the instrument and focusing on auto-fluorescence and background. Signal from fluorophores should not saturate the detector, 65,535. Lower the laser power if the detector is saturated. Zoom-in to ensure the sample is in focus at the highest possible resolution.



a) Adjust the brightness look-up table by sliding the blue arrows along the horizontal scale. The brightness scale arrow is highlighted with a red box. b) Image field-of-view (FOV) within the acquisition software. c) FOV tools along the left edge: zoom, select, translate, draw, etc.

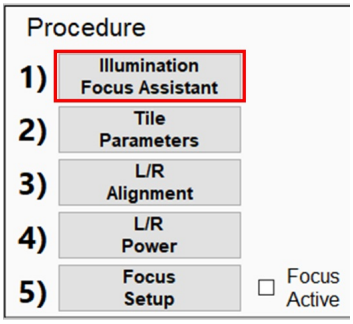
11 Detection menu options: 1m



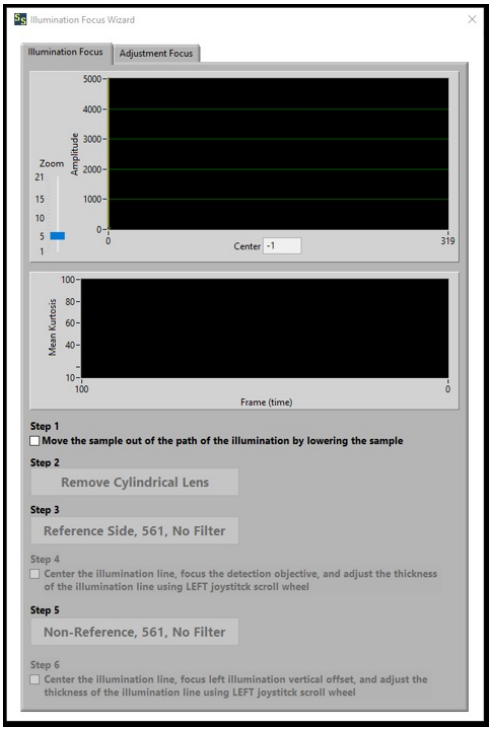
a) Objective lens options. Standard settings are all shown. The objective position ranges from 0 when imaging, to 38,000 when the imaging objective is raised from the immersion bath. Raising and lowering the imaging objective is performed with "Remove" and "Insert." b) Imaging rate options. Note that “Fast” is the typical selection, 2 ms exposure times per image, and “slow” is for high-magnification objectives and has a 10 ms exposure time. c) Sampling, default is 1x, d) Emission filter selection, including the option for no filter in the detection path for alignment steps. e) Chamber options. In the photograph, the right chamber is the Standard size (12.5 x 6.5 x 6.5 cm), the left is the Large size (18 x 9 x 6.5 cm).

Alignment

12 Introduction and overview:
The alignment steps that the user is responsible for are all contained within structured software steps built into the acquisition software provided by LifeCanvas Technologies. Complete Procedure step #1, and the 6-parts within that step.



“Procedure 1)” opens a pop-up menu with 6 additional steps



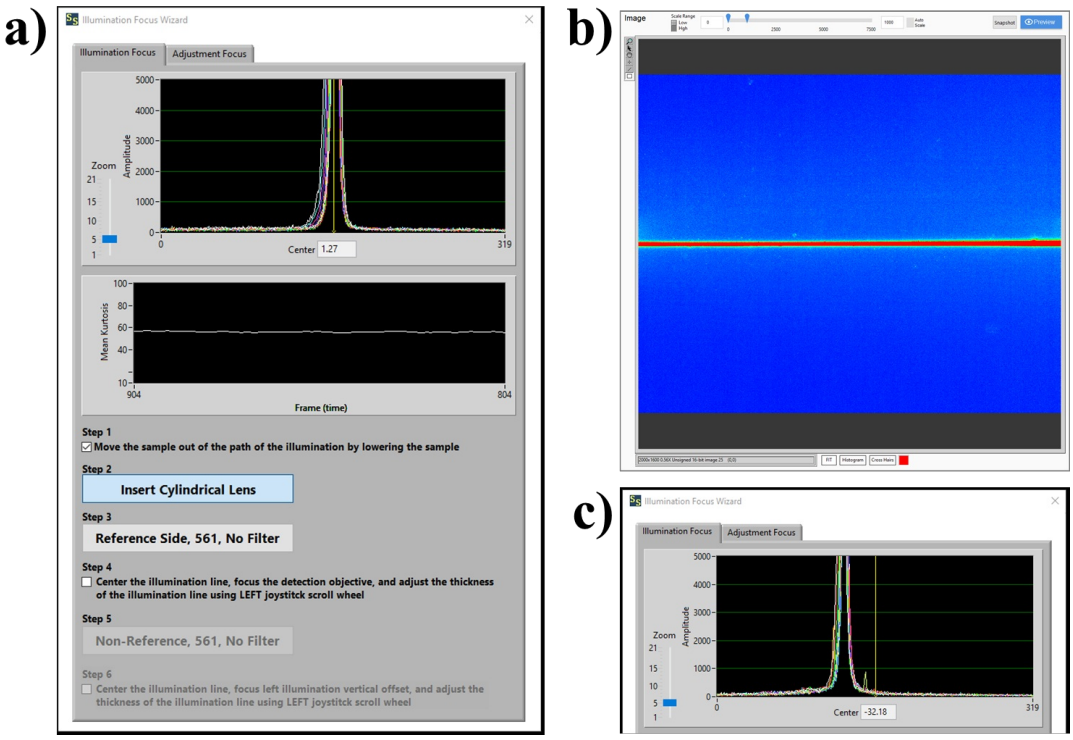
A sample must NOT be in the light path. All of these steps are sample independent.

13 Microscope controls:

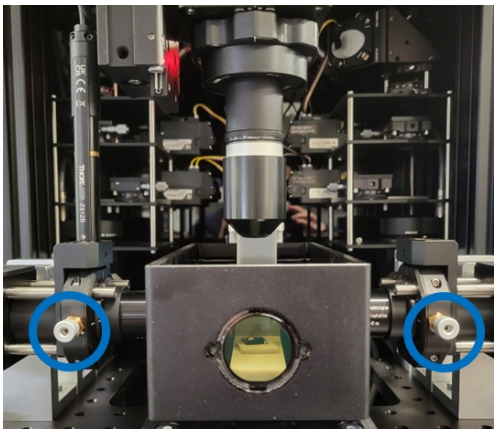
The joystick controls movement in the xy-plane. The left wheel controls the sample stage.

The right wheel controls the detection objective in z, allowing the user to focus on the portion of the sample within the path of lightsheet. The right wheel also controls the actuator which moves the left illumination objective vertically, to align the left and right sheets.

14 Align both the L and R illumination paths using the six Steps outlined below. Note that the imaging objective must be inserted into the imaging bath to obtain in-focus images.



a) Software inferface for Steps (1)-(6). When well-aligned, the intensity is centered, symmetrical, and the Kurtosis is typically around 60 (in Cargille 1.5200 immersion oil). b) The FOV during this step changes from greyscale to a red-to-blue rainbow scale. Correct for any misdirected light (typicaly occurs when there are back reflections or particulates in the oil). c) The laser is typically misaligned for each newly loaded immersion bath or sample.



Adjust the position of the L and R light sheets in Steps (4) and (6) using the knobs near the illumination objectives on each side.

14.1 Step 1: Ensure that a sample is not in the excitation path and click the check box.

14.2 Step 2: Click the button to remove the cylindrical lens. Two audible stage movements should occur.

14.3 Step 3: Click the "Reference Side, 561, No Filter" button to turn on the 561 laser and start a live view of the right-side excitation.

14.4 Step 4: Using the left and right wheels on the joystick, iteratively make adjustments until the laser (as viewed in the live image in figure (b) and/or the profile plot in (a, top)) is at its narrowest and the kurtosis plot in (a, middle) is maximized. Turn the Right mechanical position screw until the plot in (a, top) is centered on 0.

14.5 Step 5: Click the "Non-reference Side, 561, No Filter" button to turn on the 561 laser and start a live view of the right-side excitation.

14.6 Step 6: Using the left and right wheels on the joystick, iteratively make adjustments until the laser (as viewed in the live image in figure (b) and/or the profile plot in (a, top)) is at its narrowest and the kurtosis plot in (a, middle) is maximized. Turn the Right mechanical position screw until the plot in (a, top) is centered on 0.

14.7 Finally: Close the Illumination Focus Wizard. Two audible stage movements indicate that the cylindrical lenses have been re-inserted.

15 Close-up the microscope box:
Close the microscope box to minimize laser exposure to the user and improve imaging. The enclosure must be opened for the adjustments in the previous step and for loading and unloading samples, but otherwise should remained closed.