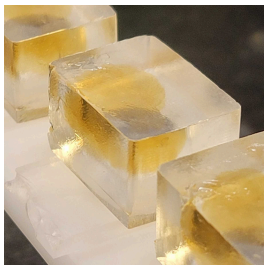
Dec 05, 2023

Imaging cleared mouse brains on SmartSPIM



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We use this protocol and it's working

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Abstract

The SmartSPIM is an axially swept lightsheet microscope that produces uniform axial resolution across the entire imaging volume of large biological samples, such as whole mouse brains. Acquiring these datasets requires well prepared samples, carefully aligned hardware, and a broad knowledge of the acquisition software.

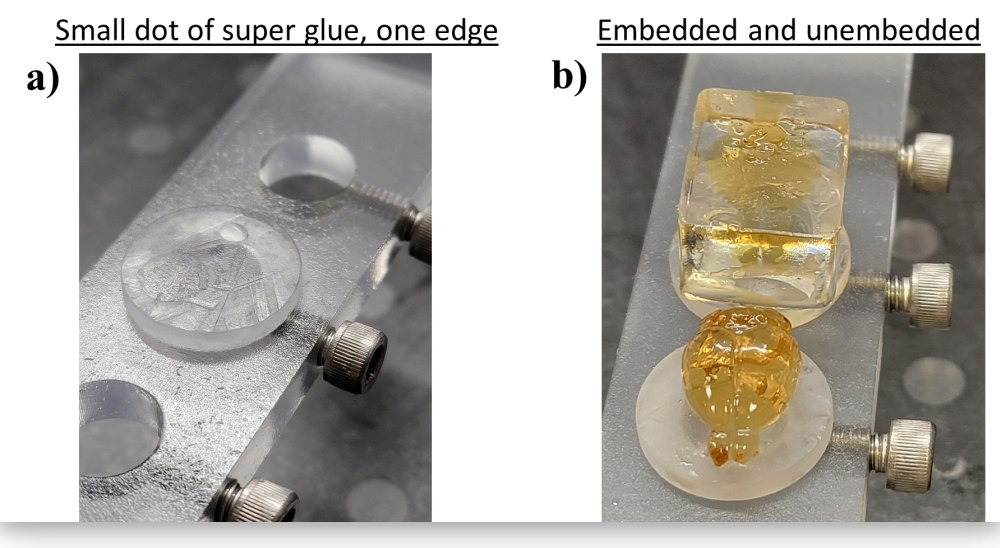
Materials

- Cleared and refractive index-matched mouse brains (optional: agarose embedded samples)
- Sample chamber.
- Immersion media with a refractive index appropriate for the samples to be imaged (Ethyl Cinnamate, EasyIndex, Cargille oil). Follow safety guidelines for all chemicals, especially ethyl cinnamate.
- Sample arm with removable mounting pedestals.
- Super glue or UV glue to adhere sample to sample arm.
- Lens cleaning cloth/tissue.
- Methanol for lens cleaning.
- Razor blade for removing sample from pedestal after imaging.

Troubleshooting

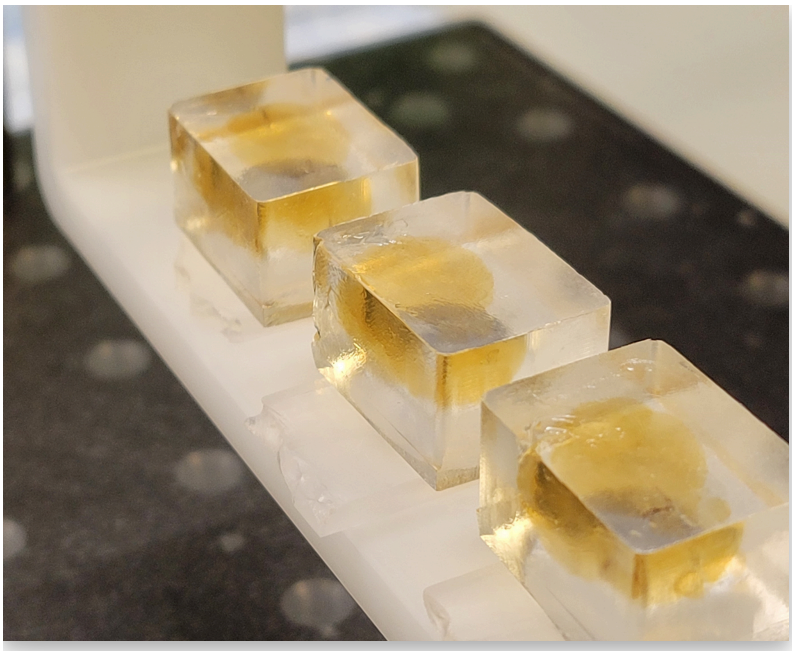
Mounting Samples:

1 Mounting agarose-embedded and unembedded brain samples. A small drop of super glue is sufficient to hold a brain for >24 hrs when submerged in the imaging bath (see figure (a) below). Placing the glue along one edge makes it easier to remove the brain after imaging.



a) A small drop of super glue is shown on a round plastic chuck on the sample arm. A screw is holding the chuck in place in the sample arm. b) Two brains, embedded and unembedded, glued down onto a small round chuck on a sample arm (LifeCanvas provides sample mounting arms and chucks with the SmartSPIM).

2 Mounting multiple samples: up to three brains can be loaded onto the sample arm at a time. The brains are embedded in 10mm x 15mm x 20mm agarose blocks. Align the blocks so the sides are perpendicular to the incident light sheets and the top is perpendicular to the detection path which is orientated vertically upward.



Three brains loaded on a sample arm.

Set-up and align the instrument:

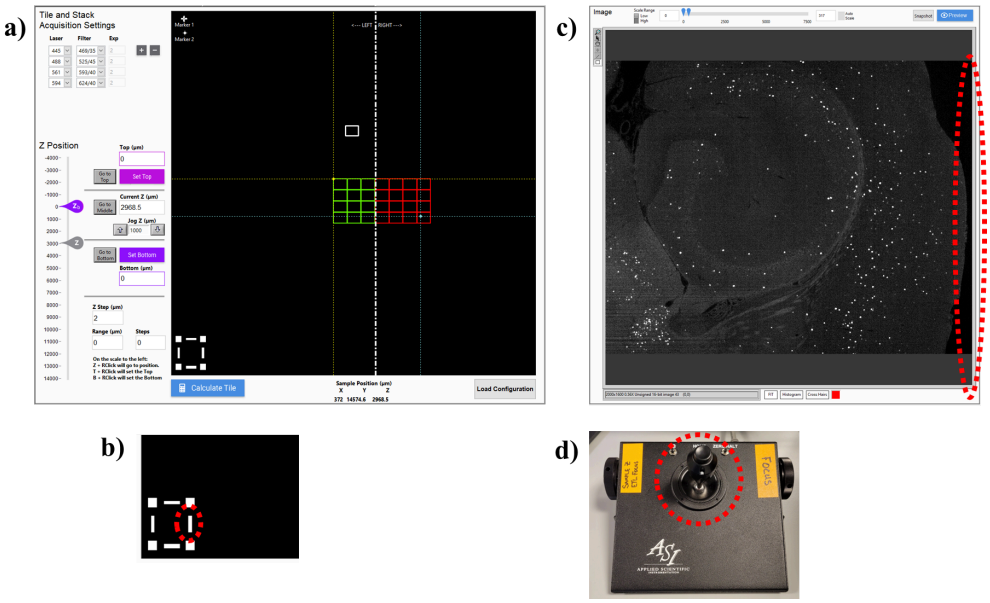
3 To set-up and align the instrument in preparation for a sample, follow the **"SmartSPIM setup and alignment"** protocol.

Configuring the microscope to acquire sample data:

4 Tiling parameters (Procedure Step #2 in the SmartSPIM acquisition software):
Specify the six faces of a rectangular solid surrounding the brain. Center and shift the tiling to align with the midline of the brain. Note that in order to perform these steps, one or more lasers will be ON and potentially bleaching the sample. Turn OFF the laser in between steps to reduce photo bleaching.

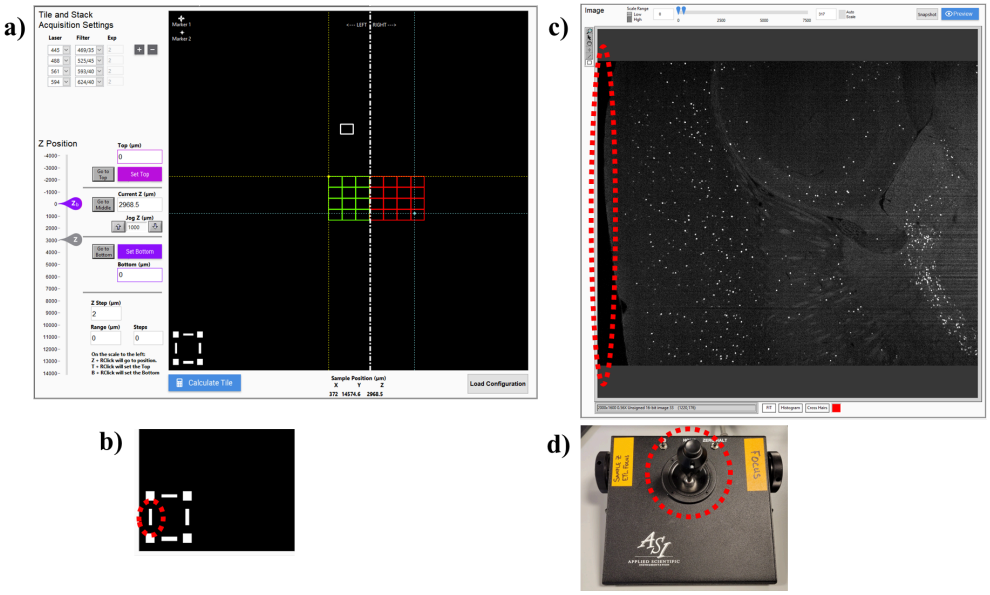
4.1 Aligning the Right(xy) and Left(xy) edges or faces of the sample:

RIGHT(xy): Set the excitation side to "Right," use an excitation laser that is appropriate for the sample (often longer wavelengths like 639 nm are best), and click the "Preview" button to start a live preview. Use the XY joystick and the left control knob to locate the right side of the brain and position it near the right side of the live preview window. Rotate the control knob to move the sample up/down and ensure that the widest part of the brain is bounded by the right side of the preview.



a) Software interface for setting the right(xy) edge of the brain. b) To set the position, click on the right edge of the white box in the lower left corner of the “Tile and Stack Acquisition Settings" panel (circled in red). c) An example of the Right edge of the brain aligned to the right edge of the FOV. d) The joystick is used to move the sample in the xy-plane.

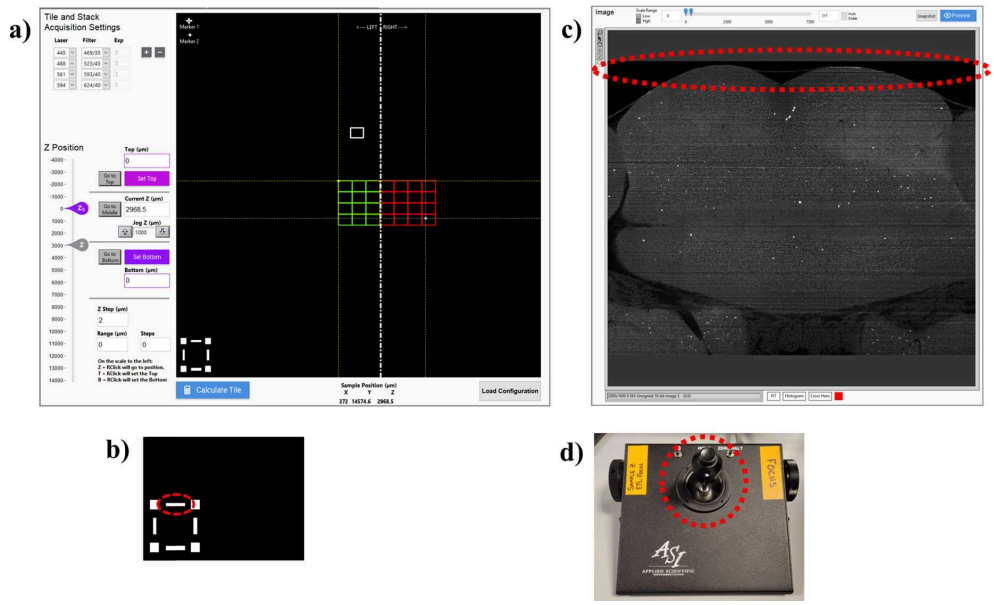
LEFT(xy): Set the excitation side to "Left," and click the "Preview" button to start a live preview. Use the joystick and the left control knob to locate the left side of the brain and position it near the left side of the live preview window. Rotate the control knob to move the sample up/down and ensure that the widest part of the brain is bounded by the left side of the preview.



a) Software interface for setting the left(xy) edge of the brain. b) To set the position, click on the left edge of the white box in the lower left corner of the “Tile and Stack Acquisition Settings panel (circled in red). c) Left edge of the brain aligned to the left edge of the FOV. d) Move in the xy-plane using the joystick.

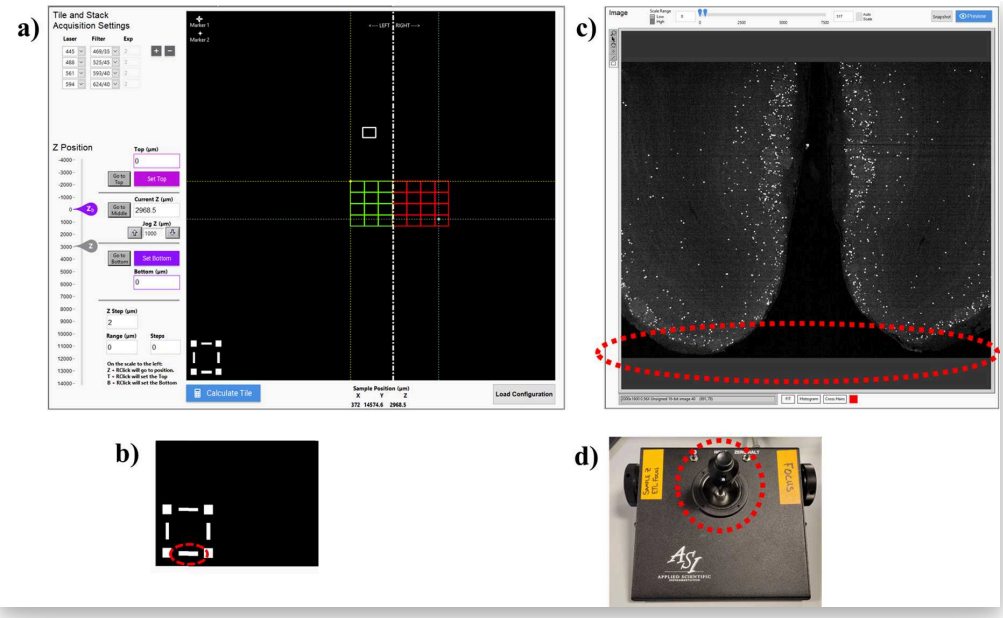
4.2 Aligning the Top(xy) and Bottom(xy) edges or faces:

TOP(xy): Set the excitation side to "Left" or "Right" and click the "Preview" button to start a live preview. Use the joystick and the left control knob to locate the top(xy) surface of the brain and position it near the top(xy) side of the live preview window. Rotate the control knob to move the sample up/down and ensure that the edge of the brain is bounded by the top(xy) edge of the preview window.



a) Software interface for setting the top(xy) edge of the brain. b) To set the position, click on the top edge of the white box in the lower left corner of the “Tile and Stack Acquisition Settings panel (circled in red). c) Top(xy) edge of the brain aligned to the top edge of the FOV. d) Move in the xy-plane using the joystick.

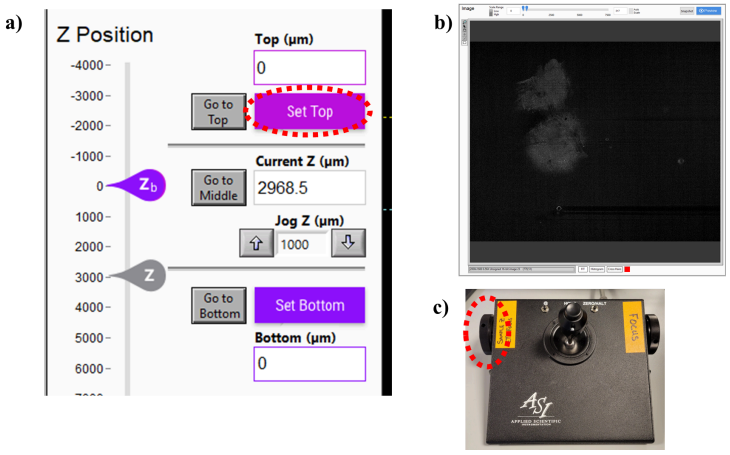
BOTTOM(xy): Set the excitation side to either "Left" or "Right" and click the "Preview" button to start a live preview. Use the joystick and the left control knob to locate the bottom(xy) surface of the brain and position it near the bottom(xy) edge of the live preview window. Rotate the control knob to move the sample up/down and ensure that the edge of the brain is bounded by the bottom(xy) edge of the preview window.



a) Software interface for setting the bottom(xy) edge of the brain. b) To set the position, click on the bottom edge of the white box in the lower left corner of the “Tile and Stack Acquisition Settings panel (circled in red). c) Bottom(xy) edge of the brain aligned to the bottom edge of the FOV. d) Move in the xy-plane using the joystick.

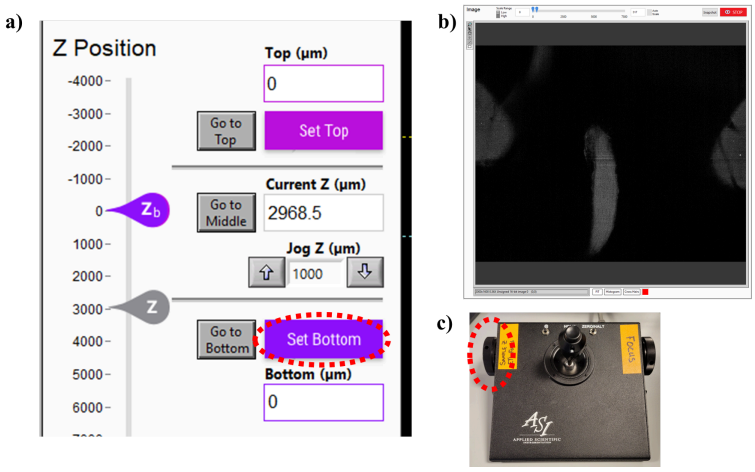
4.3 Aligning the Top(z) and Bottom(z) edges or faces:

TOP(z): Set the excitation side to either "Left" or "Right" and click the "Preview" button to start a live preview. Use the joystick and the left control knob to locate the top(z) surface of the brain anywhere within the live preview window. Rotate the control knob to move the sample down to ensure that the edge of the brain is bounded and fully contained by the Set Top position.



a) Software interface for setting the top(z) edge of the brain. To set the position, click on “Set Top” button, also in the “Tile and Stack Acquisition Settings” panel of the UI. b) Adjust the depth of focus in the z-direction using the right wheel (c), until the top of the brain just drops out of the FOV. Note the irregular shape – brains are often embedded slightly askew. Be sure to scan around the central region of the brain.

BOTTOM(z): Set the excitation side to either "Left" or "Right" and click the "Preview" button to start a live preview. Use the joystick and the left control knob to locate the bottom(z) surface of the brain anywhere within the live preview window. Rotate the control knob to move the sample up to ensure that the edge of the brain is bounded and fully contained by the Set Bottom position.

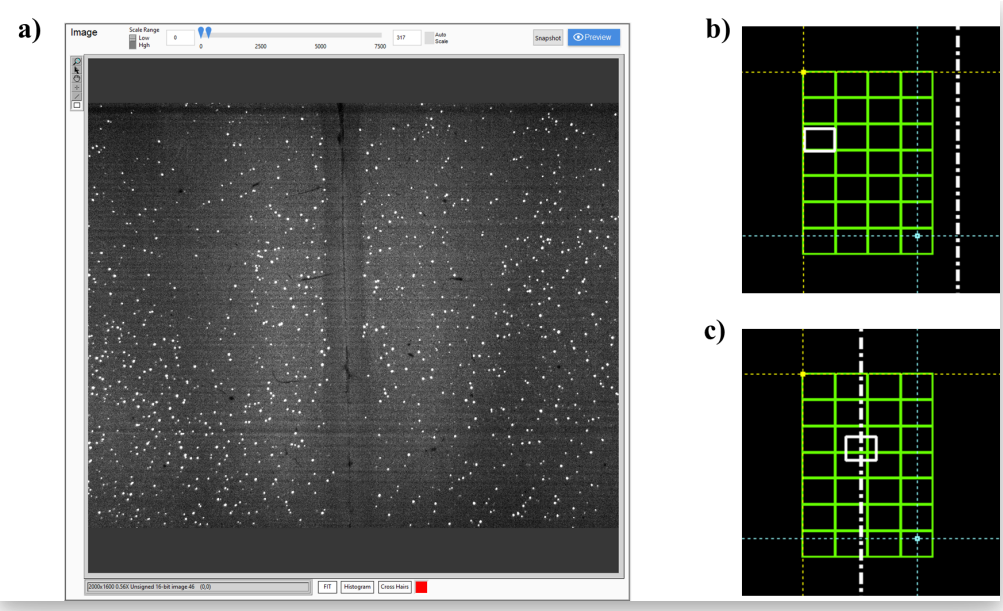


a) Software interface for setting the bottom(z) edge of the brain. To set the position, click on “Set Bottom” button, also in the “Tile and Stack Acquisition Settings” panel of the UI. b) Adjust the depth of focus in the z-direction using the right wheel (c), until the bottom of the brain just drops out of the FOV. Note the irregular shape – brains are often embedded slightly askew. Be sure to scan around the central region of the brain, as well as the front for the olfactory bulbs and the rear for the brainstem.

4.4 Centering the tiling:

Keyboard shortcut: [C] + [Left-click]

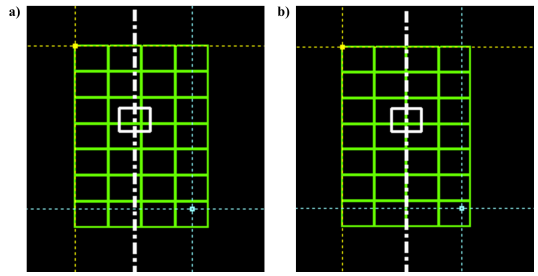
With the live preview active, center the midline of the brain within the preview window (a). Use the keyboard/mouse shortcut [C] + [Left-click] to set this location as the tile grid center. You should see the white dashed line move to the center of the white box (c).



a) Midline of the brain center in the FOV, (b) here the vertical white dashed line is not yet centered in the tiling region (green), (c) after pressing [C] + [Left-click] the vertical white dashed line is now centered within the white box--which represents the current FOV.

4.5 Shifting the tiling:

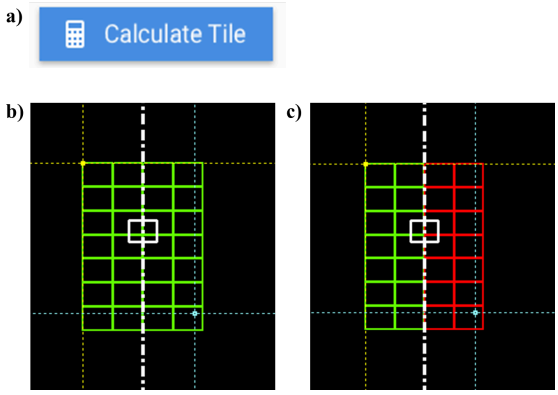
Keyboard shortcut: [S] + [Left-click]



a) Note that the CENTER white line is not aligned to the green tile grid. b) To align the green grid to this vertical, white dashed line, click [S]+[Left-click]. Now, the center of the brain is aligned to the center of the tiling pattern.

Symmetrically dividing the tiling between the illumination paths:

Finally, hit the blue [Calculate Tile] button (a), to (b) update the Left-light sheet (Green tiling) and Right-light sheet (red tiling) positions to now coincide with the centerline of the brain (c).

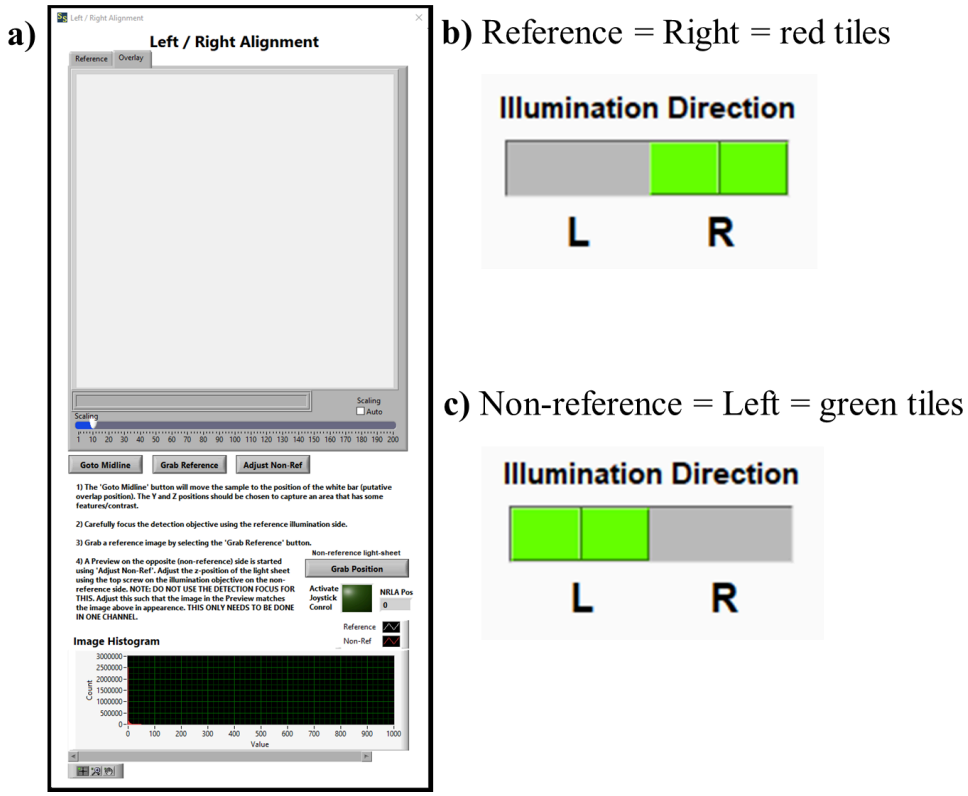


a) The [Calculate Tile] button, b) an example of a tiling arrangement that is not divided between the Left (green tiling) and Right (red tiling) light sheets, and c) symmetrically divided tiling aligned to the centerline of the brain.

5 L/R Alignment of the two illumination paths (Procedure Step #3 in the SmartSPIM acquisition software):

Procedure step 3) opens another pop-up window. There are 4 steps to complete here, listed below, corresponding to the four buttons in the center of the window (three along the middle, and the fourth in the lower right).

First, hit the "[Go to midline]" so both light sheets equally illuminate the region of interest. Be sure to select a location with bright and high-contrast features, and with features that change rapidly in the z-direction. Select a channel where there's fluorescence to ensure high-contrast and low noise for this important set of steps.



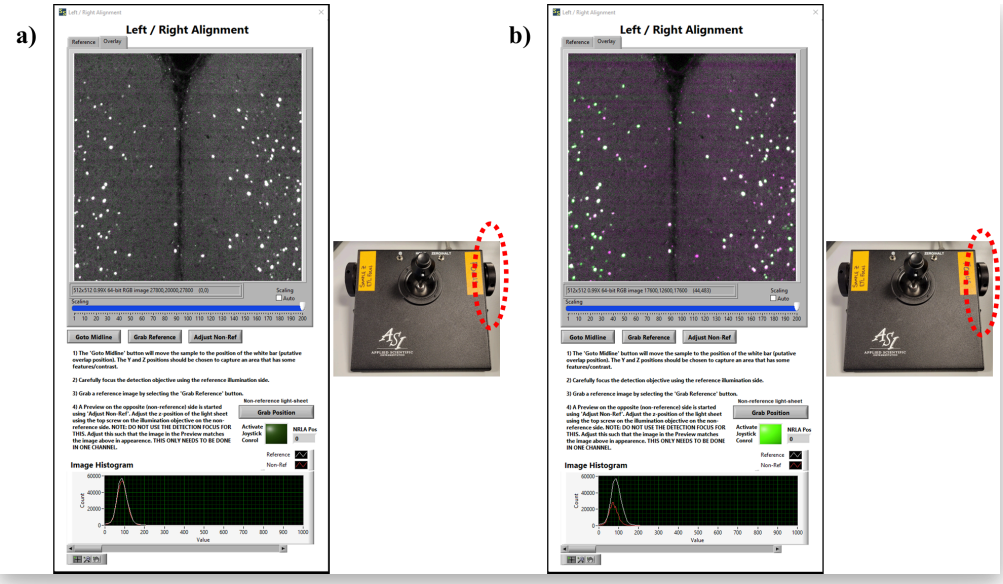
a) Software interface for Procedure Step 3), b) Ensure that the RIGHT illumination path is being used for [Grab Reference]. c) Note that the software will automatically swap to the LEFT illumination path when you click [Adjust Non-ref.].

5.1 Preventing photobleaching:

- Note that during imaging, the exposure time is just 2 ms slit width per plane. Work quickly and carefully, adjust laser power down if you have plenty of signal, and [STOP] the laser as soon as you've completed each step. Completing some steps does turn off the laser automatically, but others do not, and it's easy to bleach the sample during these times. Some fluorophores and dyes will significantly bleach in just a few seconds.
- Be careful to not bleach the sample. Some photodamage is inevitable, because you're working in a channel with fluorescence for high-contrast, low-SNR images to align with. Lower the laser power to reduce the light on the sample, but keep a high enough power to ensure good SNR (signal-to-noise ratio) and image quality.

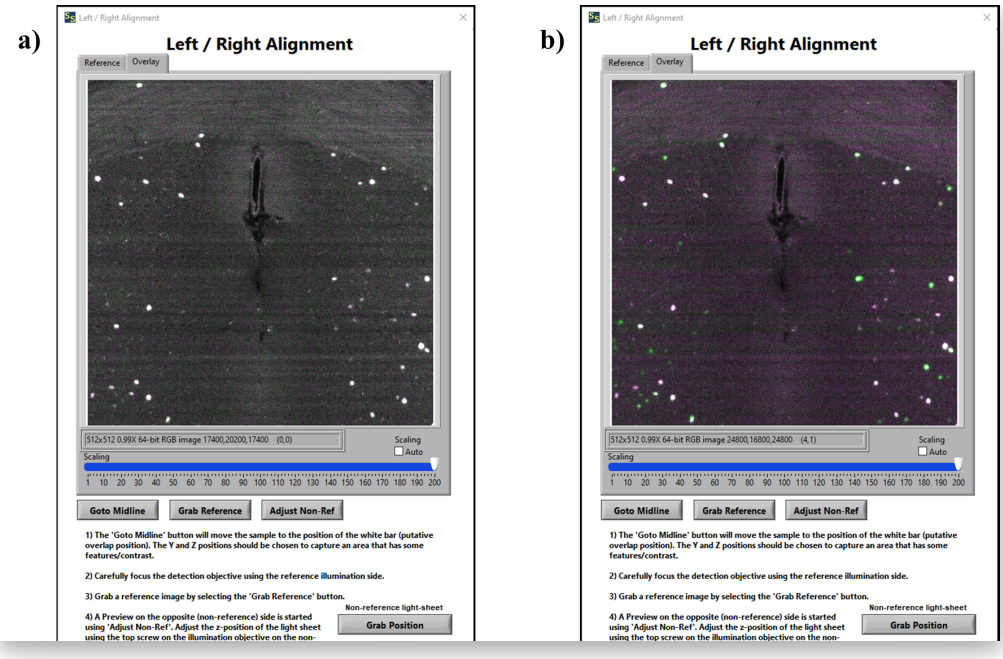
5.2 Acquiring reference images and aligning to the reference:
Goal: capture a position for the "reference" = RIGHT lightsheet, and aligning the non-reference (LEFT lightsheet) to it.
First, turn on the laser and adjust the focus (right wheel) to achieve a tack-sharp image.
Second, click on [Grab Reference]. A static grayscale image appears. Note that for this step the software automatically turns of the laser.
Third, click on [Adjust Non Ref] with the right wheel on the joystick controller.

During this process the reference image (Left lightsheet) is overlayed with the live view of the Right lightsheet. The two images are well-align when green + magenta = white. Look for sharp features, such as small fluorescent cells, to align.



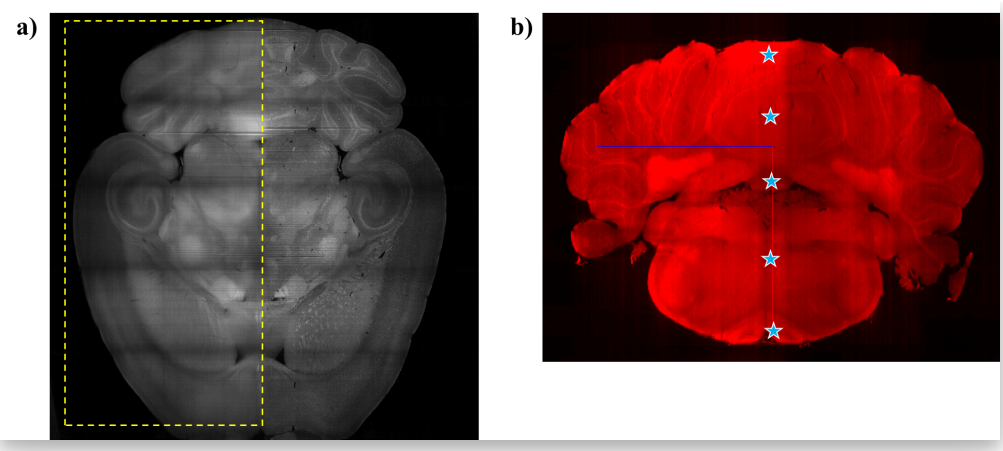
a) Adjust the focus (right wheel) to achieve a tack-sharp image. Next, click on [Grab Reference]. A static grayscale image appears.
b) Next, click on [Adjust Non Ref] with the right wheel on the joystick controller.

5.3 Additional example - Reference images and aligning the non-reference (LEFT light sheet) to it:



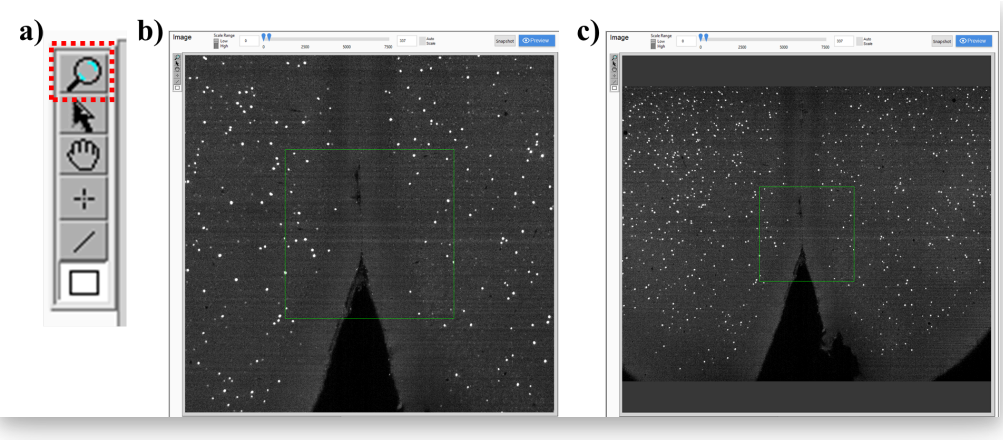
Another example of aligning the left and right light sheets. a) The saved [Grab Reference] image in greyscale, and b) the well-aligned, overlaid images. Note that while green and magenta spots are visible, the majority are white and represent green + magenta.

5.4 Troubleshooting misaligned L/R illumination paths:
Repeat previous alignment steps. a) Note that the left hemisphere is out-of-focus. When this occurs, repeat the L/R alignment before acquiring a dataset of the whole brain. Note that there are several possible locations to perform this alignment along the L/R midline (b). The top of the brain will be the sharpest. Be sure to explore front-to-back as well. Search for high-contrast, sharp features to focus on.



a) An example of improper left-right alignment. b) Possible locations (starred) to perform the L/R alignment step.

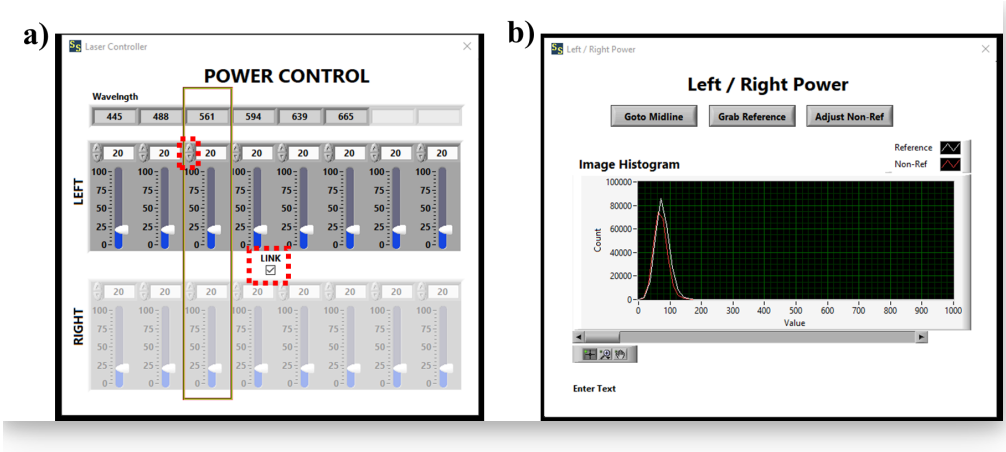
6 L/R Power adjustment (Procedure Step #4 in the SmartSPIM acquisition software):
The last step leaves the FOV zoomed-in. Zoom out before starting these steps:



a) Use the magnifying lens icon in the Image panel to zoom-out, or right-click the Image panel FOV and select “Zoom to Fit” to zoom-out ((b)-to-(c)). This isn’t essential to do, but Step 3 leaves you zoomed-in, and you often want to zoom-out, especially if you’re going to repeat the L/R alignment.

6.1 Adjusting laser power based on the L/R Power histogram:
Note that the relative Left and Right laser powers can be adjusted. By un-checking the “Link” box, each illumination path can be adjusted separately (a). When imaging along

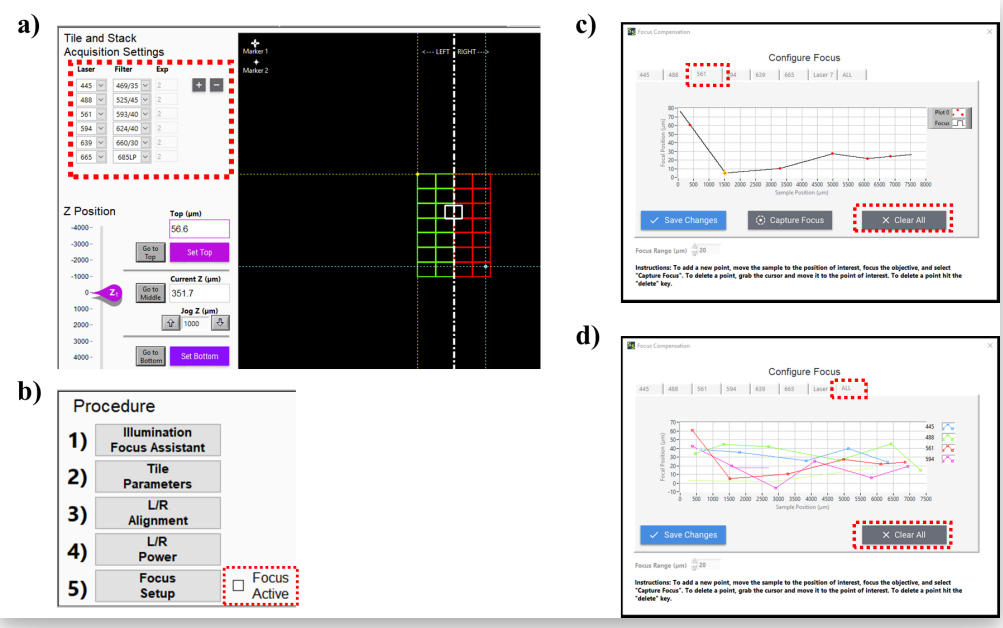
the center-line of the brain, overlap the two histograms (b). Depending on the instrument, this step rarely needs to be performed, because the L/R power is already closely matched.



a) Software interface for adjusting laser power. Red boxes highlighting Up and Down laser power adjustments, and the button to toggle linking or unlinking the Left and Right laser powers when making these adjustments. b) Interface for adjusting the Left and Right laser powers by overlapping two histograms of intensity.

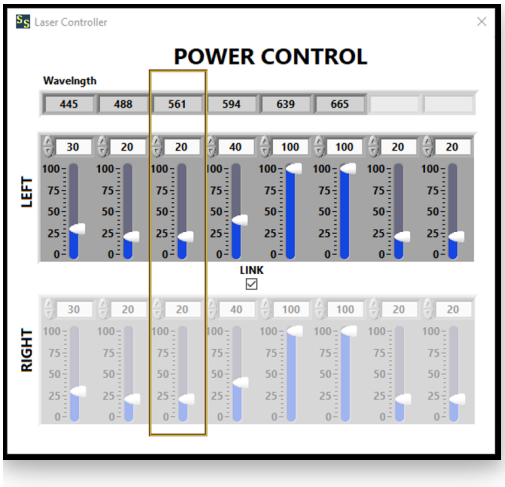
7 Focus setup in "z" (Procedure Step #5 in the SmartSPIM acquisition software):
Make fine adjustments to the position of the detection objective to adjust the focus of microscope throughout the depth of the brain volume.

7.1 "Clear All" channels before beginning each brain (previous parameters are saved):
First, select which channels are required for imaging (a).
Second, uncheck "Focus Active" (b).
Third, use "Clear All" if another brain was previously acquired.



a) Select which channels to acquire with. b) Ensure that “Focus Active” is unchecked. c) If this step (Procedure 5) has been completed earlier (either for a previous brain during a multi-brain acquisition or when acquiring multiple brains individually), hit [Clear All] in each channel, or d) select the “ALL” tab from the top, then hit [Clear All].

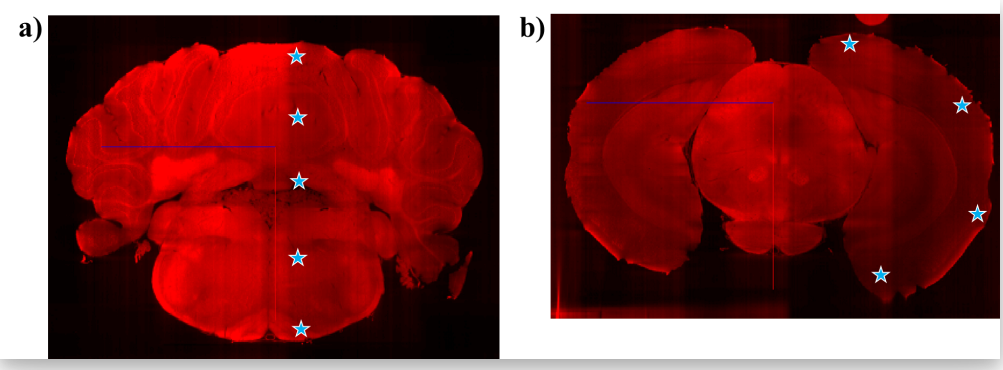
7.2 Adjusting laser power to prevent photodamage:
Especially for brains with endogenous fluorophores, LOWER the laser power during this step to lower the risk of photodamaging the sample. Alternatively, in samples with low SNR (signal-to-noise ratio), you may need to instead boost laser power here to have enough signal to ensure proper focus.



Interface for adjusting laser power.

7.3 Selecting a vertical path through "z":
Recall that the right side of the brain is the “Reference” side of the instrument. Select a path through z that’s near the center of the brain, biased towards the refence side. If the

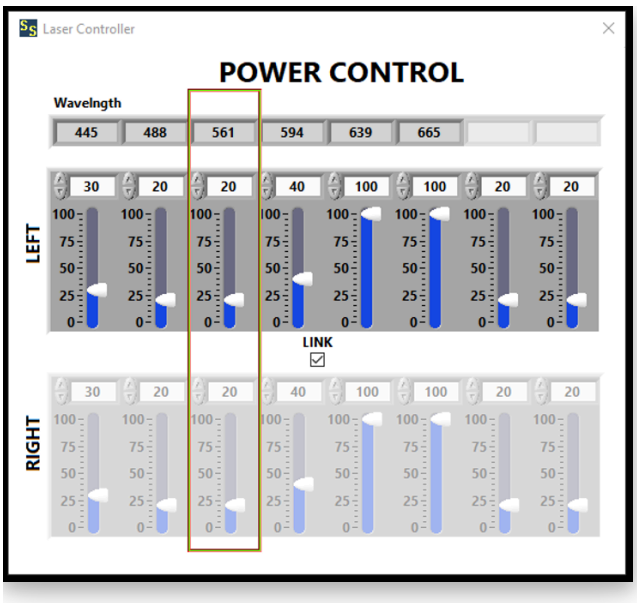
brain is not fully cleared (b), or at short wavelengths where the light is more scattered and the interior of the brain is dim, it may be necessary to follow the edge of the brain for clear enough sample to focus on. Deep regions in z may require you to move to the olfactory bulbs in order to access the sharpness/focus of the image.



a) An ideal path straight down along the midline of the brain, b) for shorter wavelengths or samples with improper clearing, it may be necessary to perform focus adjustments in z along the edge of the brain.

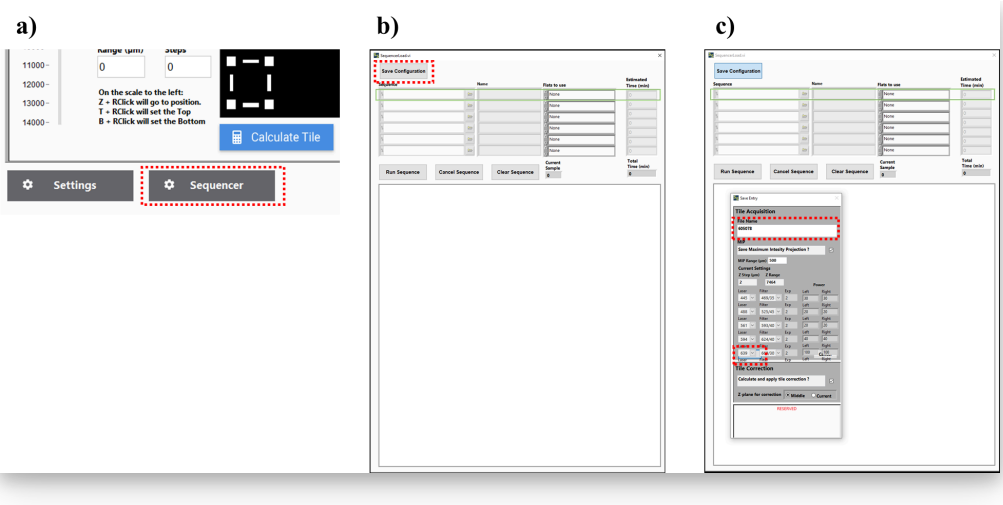
7.4 Readjust laser power:

Now that the manual alignment steps are complete, restore the laser power to a higher value that will be used for imaging. After these steps where the laser must be on for a long period of time (Procedure 3)-5)), be sure to adjust the power to the level you will image at. Typically, lower the power in the channels with fluorescence while doing these alignment steps to avoid bleaching, then increase the power to image, in order to maximize the number of photons reaching the camera.



Software interface for adjusting laser power.

8 Multi-brain acquisitions:
Save configurations and parameters for the first brain before starting a second brain, using the Sequencer option.
a) Once Procedure steps 1)-to-5) are complete, the sequencer allows you to save all of the configurations for the previous sample.
b) Click [Save Configuration] at the top. A pop-up window appears.
c) Name the file, double-check the laser and filter selections that you will be aquiring in. Finally, click [Ok] to acquire a preview single-plane image at the mid-line (along the z-direction) that's stitched.

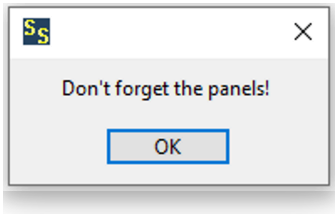


a) Location of the Sequencer button on the software interface. b) Location of the "Save Configuration button", c) Key fields to double-check and adjust. Finally, click [Ok] to acquire a preview single-plane image at the mid-line (along the z-direction) that's stitched. Note: in this example all 6 channels were enabled and the [Ok] button is nearly obscured.

8.1 Save configurations for the first brain:
After clicking [Ok] in the previous step, the sample arm will translate in z to reach the midline (in z), and then translate in x- and y-directions to capture an image for every tile. The position of the rectangular solid around that brain that you set in "Procedure 2)" is now shown in an xy-plane corresponding to the imaging chamber.

Software's user interface showing the tiling location of one brain.

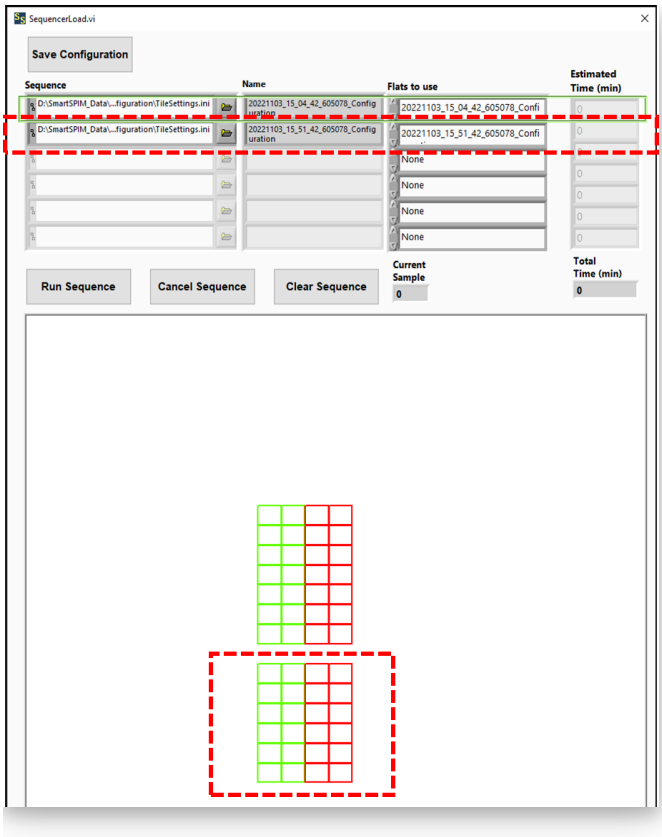
Ensure the microscope's box is closed-up (reattach the removeable panels):



Fully enclose (black-out) the sample and microscope optics by replacing the three removable side panels back onto the instrument.

8.2 Saving configurations for additional brains:

Work through the Procedure 2)-to-5). Now, when you click [Save Configuration] the 2nd brain, its location will be shown, and L/R alignment (Procedure Step 3)) and focus set-up (Procedure Step 5)) will also update for the 2nd brain. These updated paramters aren't visible in the UI, but the xy-plane is a visual indication that everything about the 2nd brain is now saved.

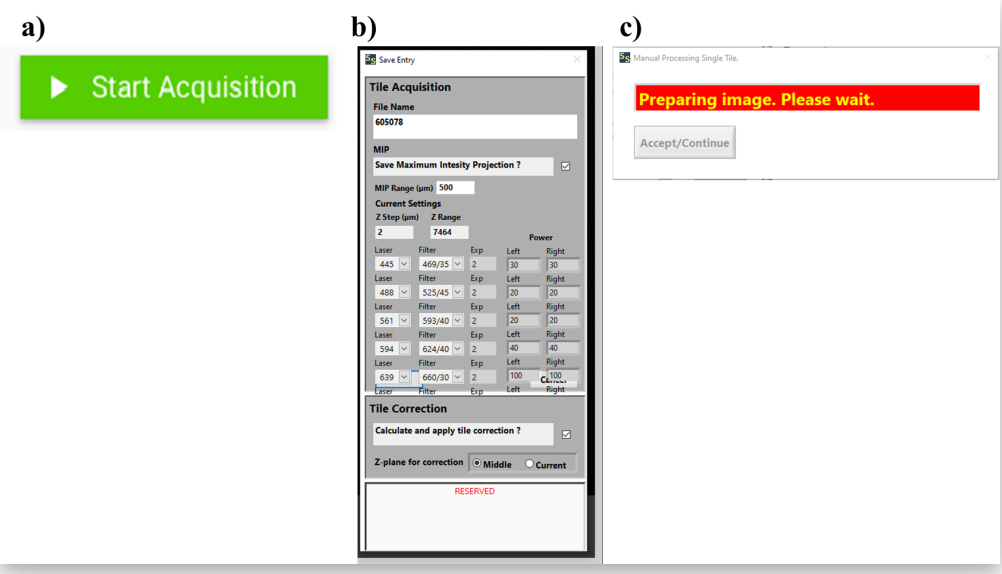


Software's user interface showing the tiling location of a second brain.

Starting an Acquisition:

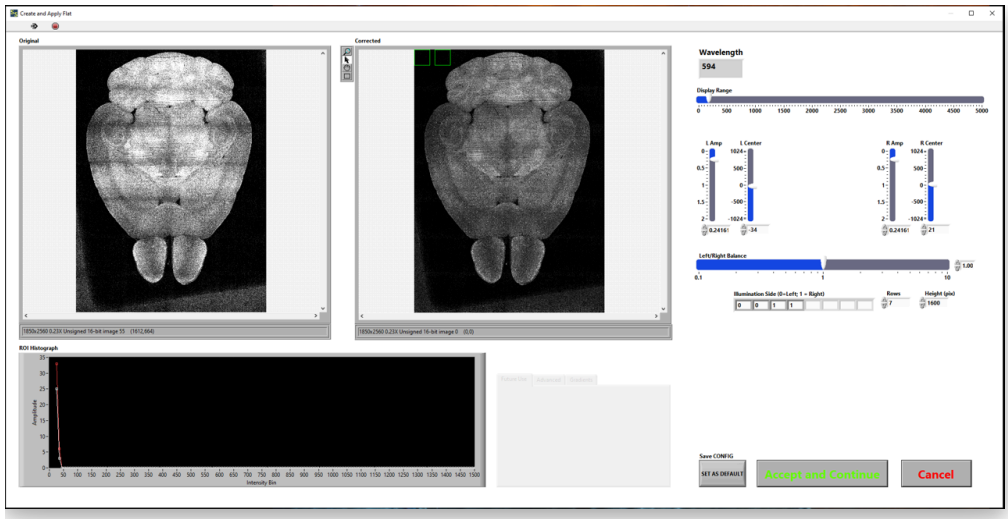
9 Single vs. Multi-brain acquisitions:

9.1 Single brain acquisitions:
a) When not using the sequencer, click [Start Acquisition]. Name the file with the LabTracks ID, and confirm the channels you wish to acquire in are selected. Remember to adjust the laser power of each channel to a level you wish to image with.
b) For the Sequencer (acquiring multiple brains in sequence, up to 4, all mounted on a single sample arm), select [Ok] (blue outline, here it's accidentally obscured by the 5th and 6thchannel information). c) During this step, a pop-up window will appear and flash red-and-white. A time estimate will appear along the bottom of the software.



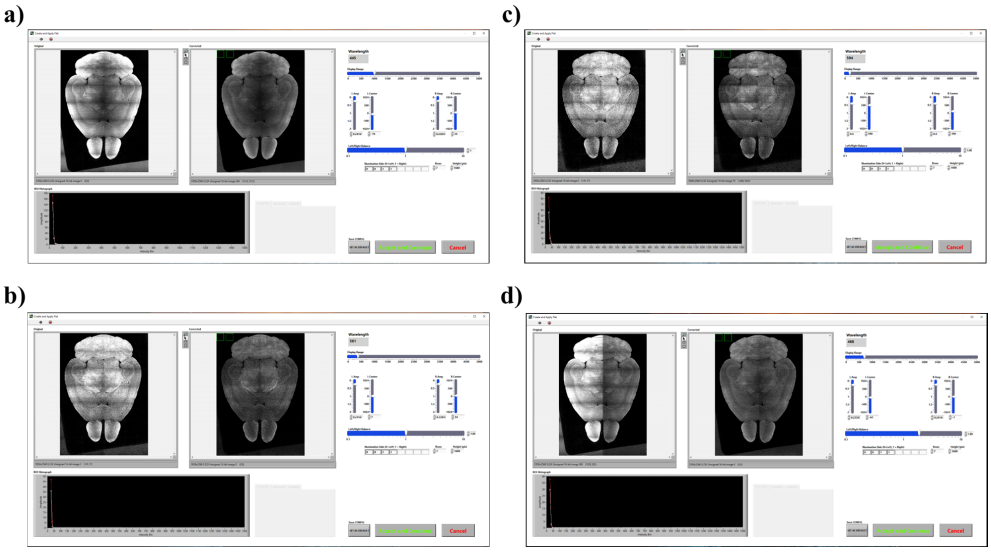
a-c) Software interface for single-brain acquisitions.

9.2 Viewing and adjusting a single plane of the brain. Composite images are stitched in the LifeCanvas software using TerraStitcher.
During this step the software creates and applies a flat to pre-correct the images as they're being saved. The incident laser line that forms a sheet after passing through a cylindrical lens has a Gaussian profile, and the flat corrects for and "flattens" the intensity of the field of view. Warning: this step is not easily reversible, because the flat is applied to the saved data.
The SmartSPIM software, using TerraStitcher, generates a single, 2D, stitched plane image of the whole brain (typically 20-28 tiles). There are six sliders to adjust the brightness (Display Range), the amplitude and center of the tile-overlap adjustments (L amp, L center, R amp, R center), and the L/R brightness (Left/Right Balance). Adjust both the left and right hemisphere to create a clean “corrected” image. The “raw” or “original” image is pictured on the left, and the corrected image is on the right.



Software interface for Creating and Applying flats from a preview image, before beginning the full-brain acquisition.

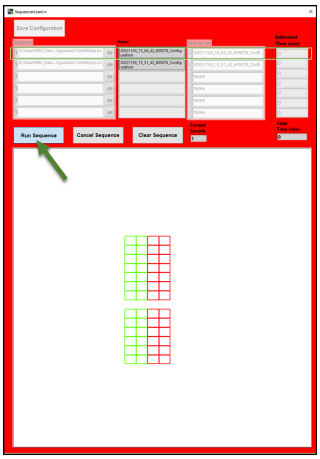
9.3 Additional examples of Creating and Applying flats within the acquisition software:



a-d) Before-and-after images when adjusting the flat correction. a-b) Raw images are on the left (brighter white) and the stitched image with the flats applied are on the right (dimmer grey). A poorly applied flat correction, as in (c), leaves tiling artifacts. The mismatched L/R power in (d) can be corrected digitally in this step, (one hemisphere is brighter than the other in the raw image on the right), however, background noise is also increased in the digitally brightened hemisphere.

9.4 Starting a multi-brain (Sequencer) acquisition:

When imaging multiple brains, you no longer use the “Start Acquisition” button. Note that the Sequencer menu blinks red-and-white during the acquisition.



Software interface of the multi-brain acquisition (Sequencer) window. The green arrow is highlighting the location of the "start acquisition" button.