

LEICA Stellaris 8 Fluorescent Confocal Microscope SOP



WWU Scientific Technical Services

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For any questions about additional methodology, imaging techniques, or operation of the instrument, please contact the fluorescent confocal microscope instrument manager.

Initial Start Up

1. Log in to your FOM scheduled session using your WWU credentials.
2. Fill out the sign-in sheet on the provided clipboard.
3. Remove the microscope cover and hang it on the wall.



4. Gently tilt the transmitted light arm up.



5. Once the computer has fully turned on and logged in, turn on the microscope.
 - a. Flip switch #1 to turn on the microscope.



- b. Wait for touch pad on the microscope stand to fully load.

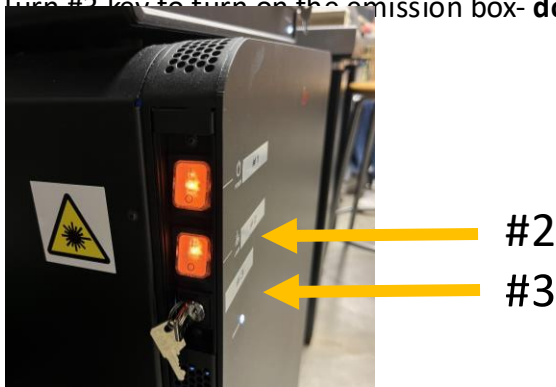


Loading in process



Fully loaded screen

- c. Flip switch #2 to turn on the white light laser (WLL).
 - d. Turn #2 key to turn on the emission box- **do not remove the key!**

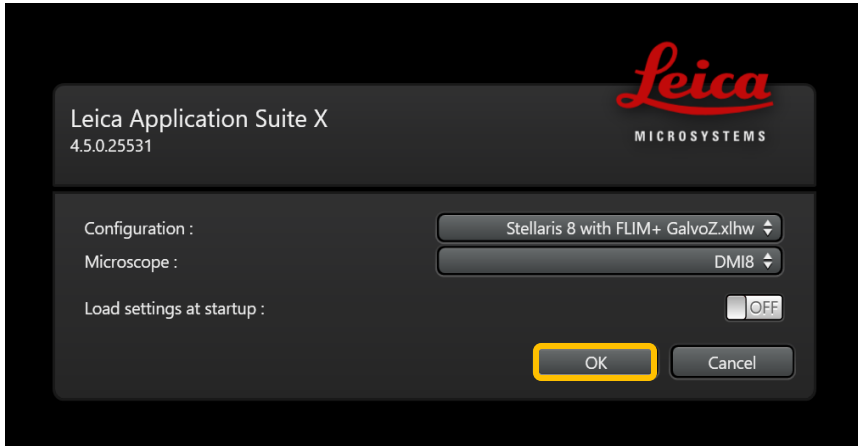


6. Open the 'LAS X' application from the upper right of the desktop.



7. Confirm the configuration settings

- Configuration: Stellaris 8 with FLIM+ GalvoZ.xlhw
- Microscope: DMI8
- Load settings at startup: OFF
- Click “OK”

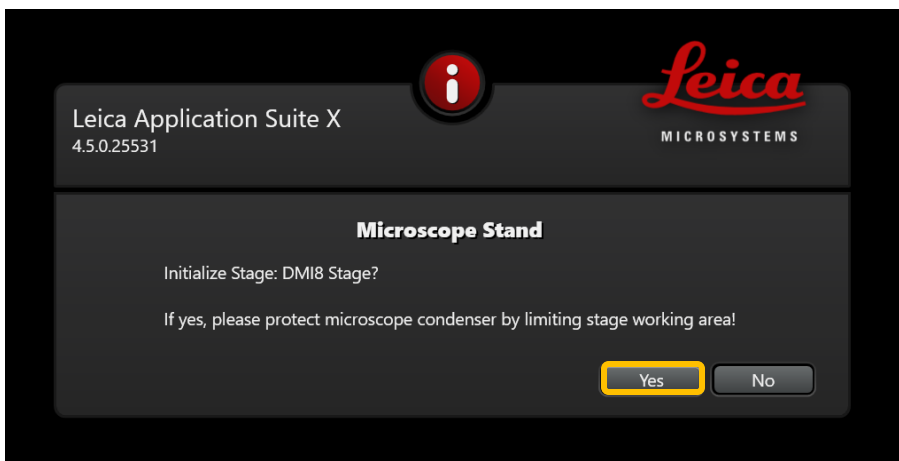


8. Ensure transmitted light arm is tilted up as directed in step 4 and the sample stage is clear (i.e. no sample holder, no objects resting on the sample stage, no wires sticking out from around the objectives)

DO NOT EVER MOVE OBJECTIVE TURRETT MANUALLY



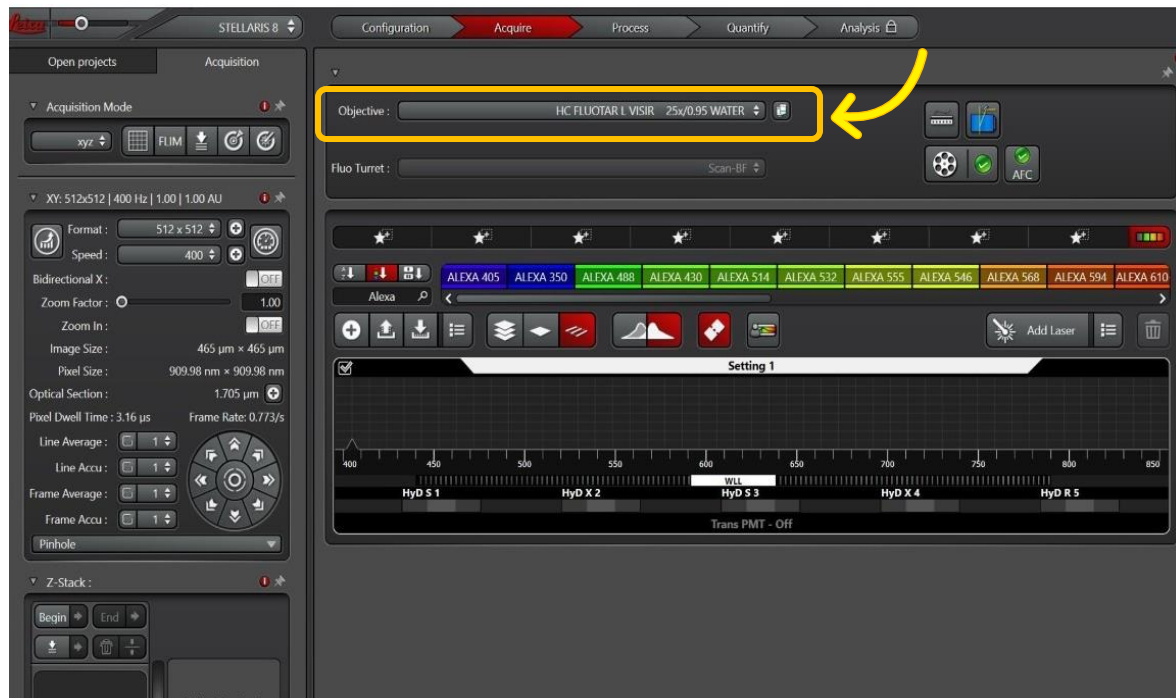
9. Click ‘yes’ to calibrate the DMI8 stage- stage will start moving



Setting Objective and Sample Loading

1. Select objective lens in “Objective” drop down menu.

Always use the software to select your objective- NEVER use the screen on the microscope to choose your objective!



2. Place proper stage mount on the scope and secure screw to finger tightness.

3. Place sample coverslip facing down in the stage mount with the coverslip facing the objective. (If using an immersion objective, do not forget to add the appropriate immersion liquid to the objective before placing down your sample)

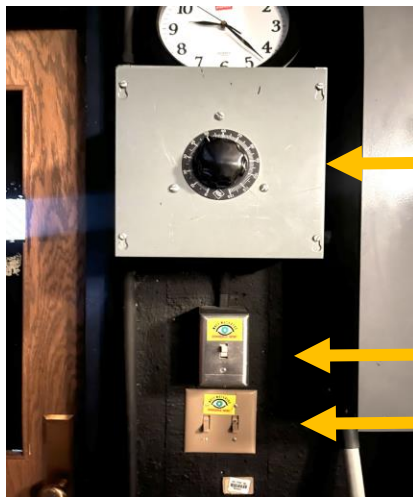
4. Center sample to approximate area of interest using the 'XY FAST' setting. Once centered, switch to the 'XY PRECISE' setting. (if using an immersion objective, use the coarse focus to raise the objective to the point that the immersion liquid touches the coverslip).



5. Gently lower the microscope arm back down.



6. Turn on the dimmable lights and turn off the main track lighting. Please be courteous and give to those sharing the space with you a heads up that you will be turning off the main lights.

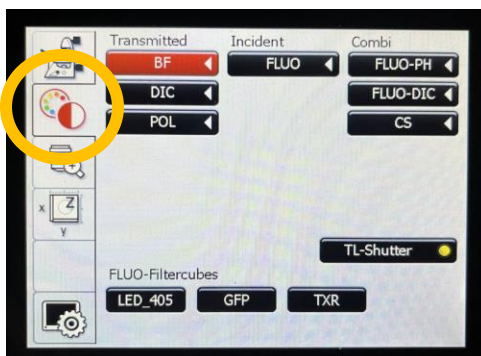


Dimmer Dial (usually set 40-60%)

Dimmable lights

Main track lights

7. Select light and colors option on microscope screen.



8. Choose a light setting to focus your sample.

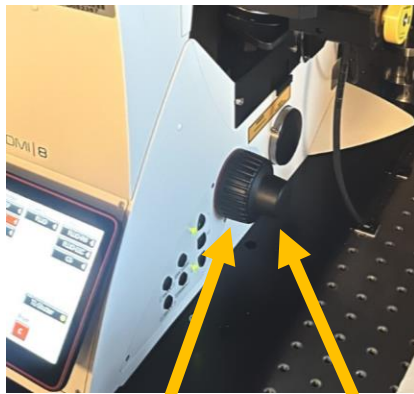
Suggestion:

Use BF if sample has visible pigment.

Use DIC if sample is primarily translucent.

Use FLUO if sample is best viewed under fluorescence.

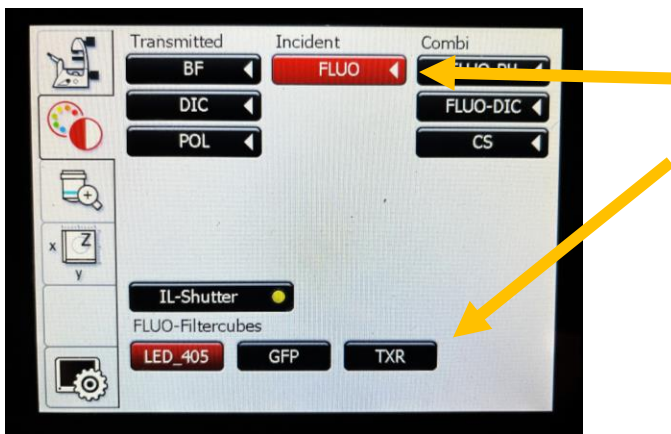
10. Focus sample using focus knobs through the eyepieces by adjusting the coarse and fine focus knob on the side of the microscope.



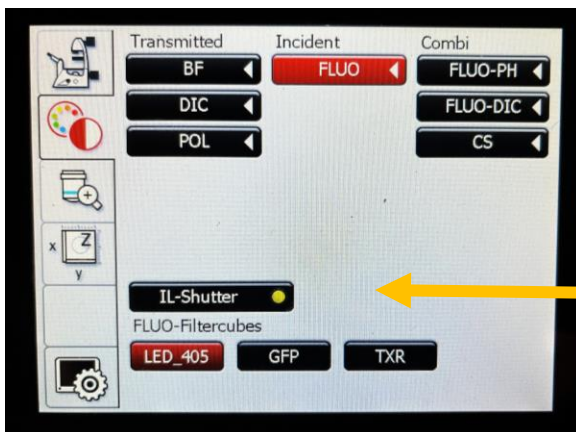
Coarse Fine

11. Center sample area of interest as best as possible. The view field in the software will be much smaller than the view field through the eye pieces.

12. If you have not checked fluorescence already, switch to a fluorescent filter cube to ensure your sample is fluorescing as expected. Select the 'FLUO' button under incident, choose one of the filter cube options nearest your sample dye, and turn on the IL-Shutter (the dot will appear yellow on the screen when on).

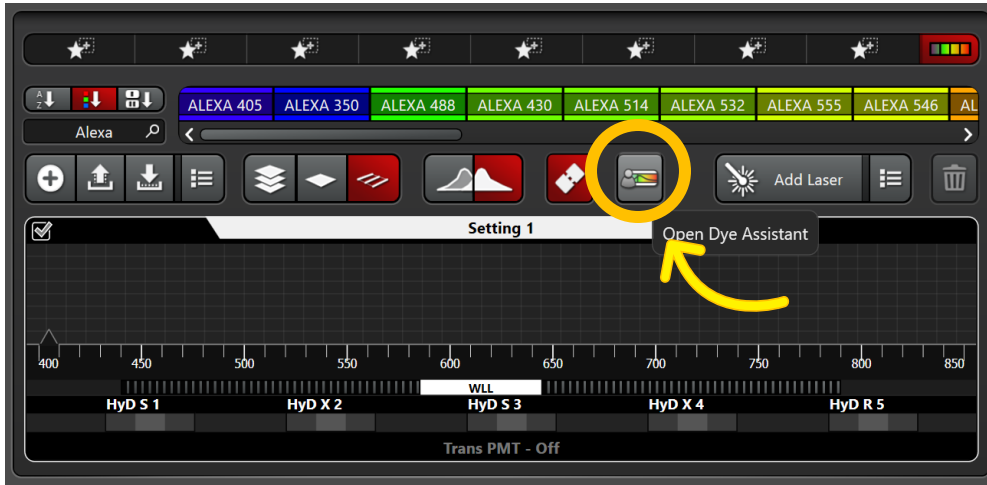


13. Once your sample is in focus and centered, turn off the shutter to limit photo bleaching of your sample.

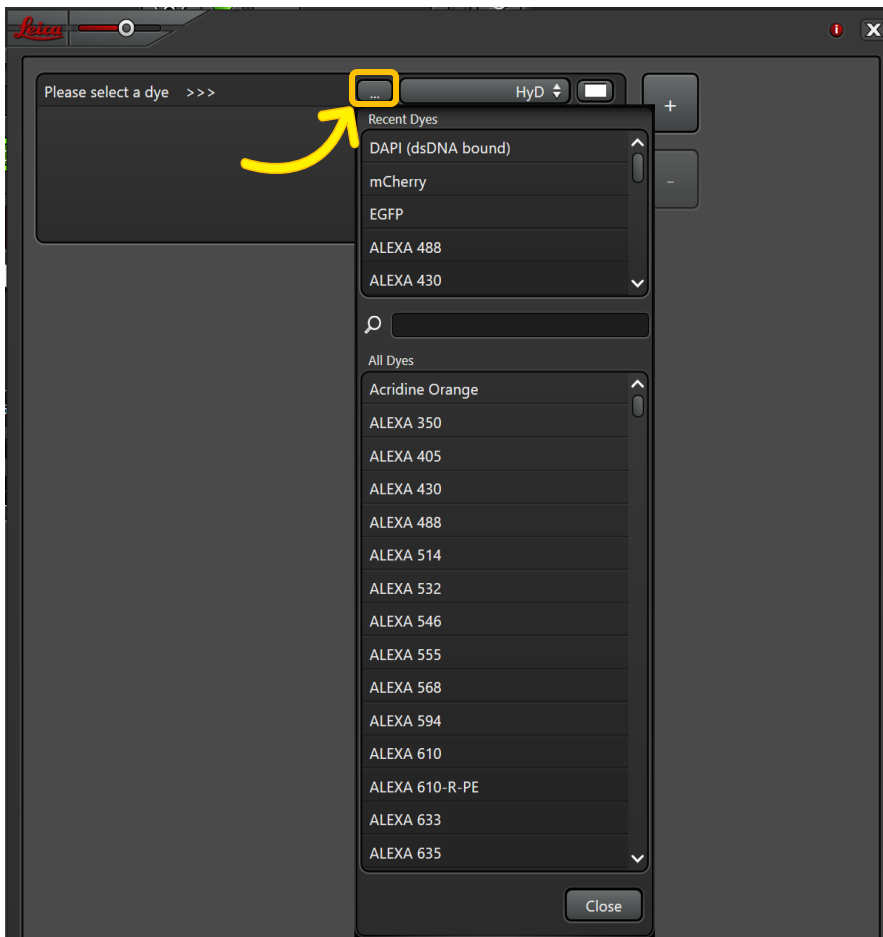


Selecting Dye Conditions

1. Open dye assistant.

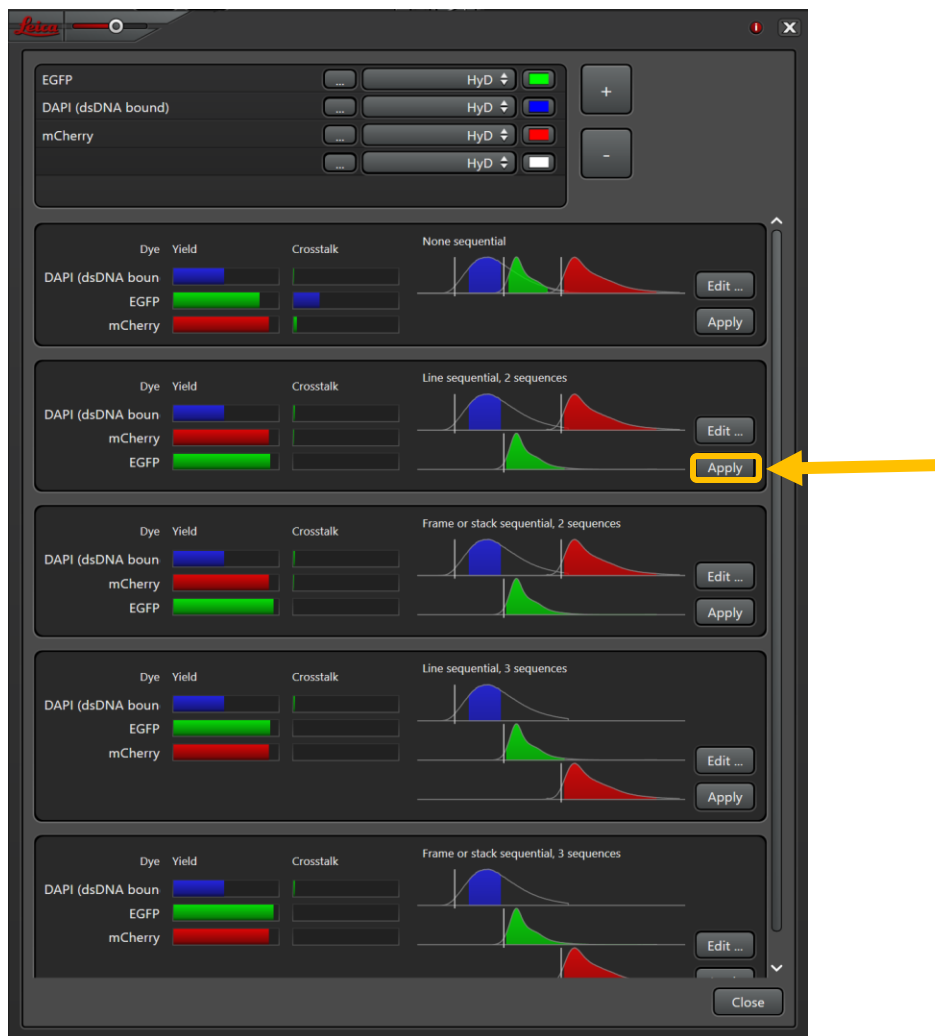


2. Click ellipse to open dye select options. Select your dye(s). Ensure 'HyD' is selected in the accompanying dropdown menu to auto assign optimal detectors to each dye of interest.

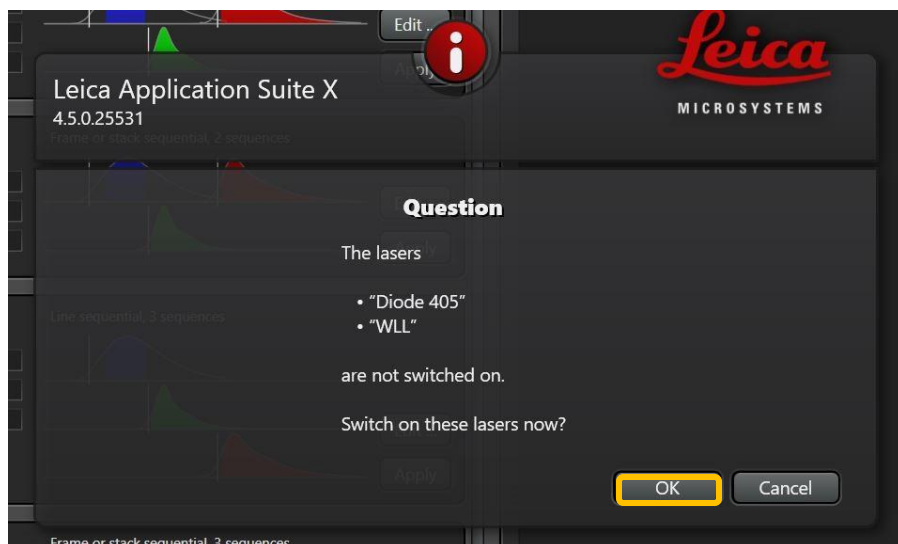


*Note:
No more than eight
dyes can be selected
at once.*

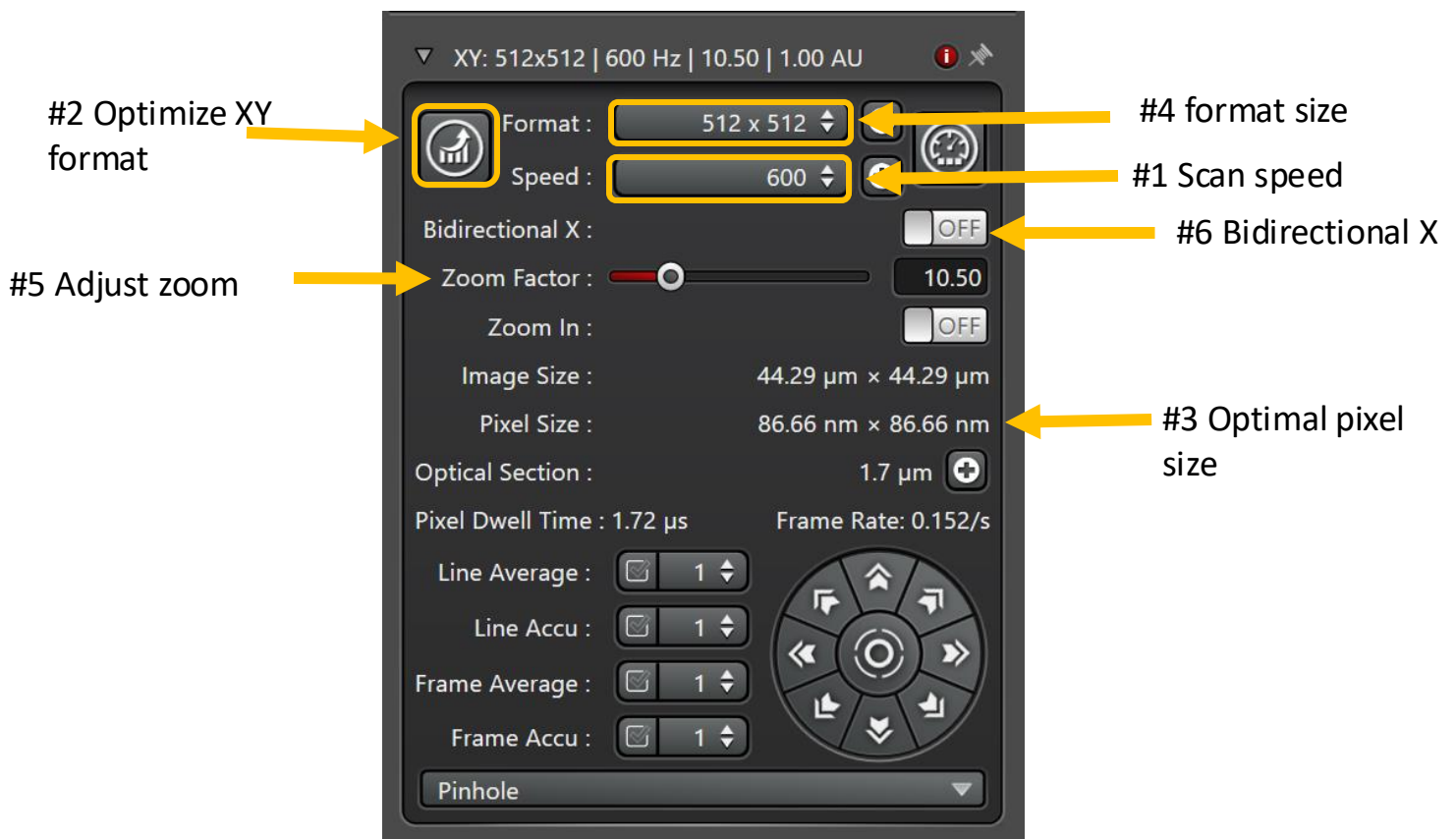
3. Click 'Apply' to choose your scanning sequence. *Selecting the line sequential with the least crosstalk and lowest number of sequences is recommended.*



4. Select 'Okay' to fully turn on lasers.



Optimizing Image Format



1. Select desired scan speed (typically around 400-700 Hz).
2. Select optimize XY Format button in the upper left of the window.
3. Record the pixel size value on a piece of paper.
4. Pixel optimization will result in your format size changing automatically. Select desired image size in the 'Format' drop down menu (typically 512 x 512 or 1024 x 1024) .
5. Adjust the zoom factor until the pixel size matches the optimal pixel size you recorded in step 2.
6. Turn on Bidirectional X.

Note: This is necessary to ensure highest possible pixel resolution for your raw data.

See appendix for additional guidance on additional format settings such as line averaging/accumulation and frame averaging/accumulation.

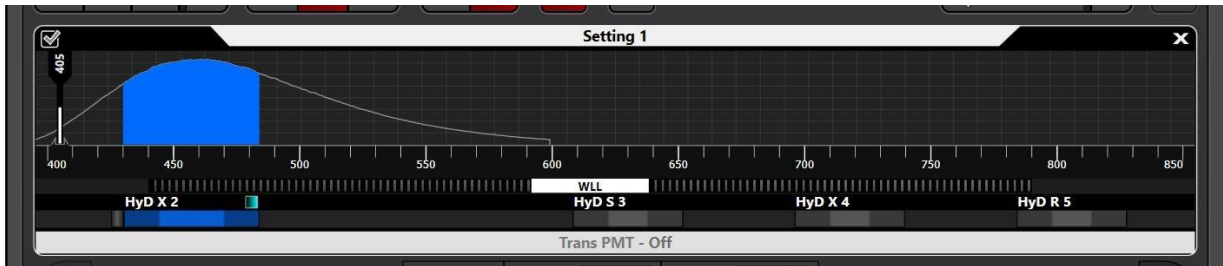
Laser Conditions Overview

1. Currently selected dye setting will be outlined in white. You will only be able to view one setting at a time while in 'Fast Live'.
 - o 'Live' scanning will allow you to view all setting channels at once, but at a slower scan speed than 'Fast Live' which puts your sample at higher risk of photo bleaching
2. Select the excitation flag to open laser intensity settings.
 - o Laser intensity can go as low as 0.01% and should not exceed 15%. (If a sample does not demonstrate enough fluorescence at 15% laser intensity, staining procedures should be re-evaluated.)
 - o Users should not click and drag the intensity slider to change the setting (this results in too quick of a change and causes problems for the sample/detectors most of the time). Instead, use the arrows, hover the mouse over the slider and scroll up on the mouse scroller, turn intensity dial on the physical control panel (though I do not recommend using this if there are multiple dyes within the same setting window because the control panel does not always 'talk' to the correct dye), or type in the box manually (typing in the box manually is the only way to adjust the laser setting below 0.1%)

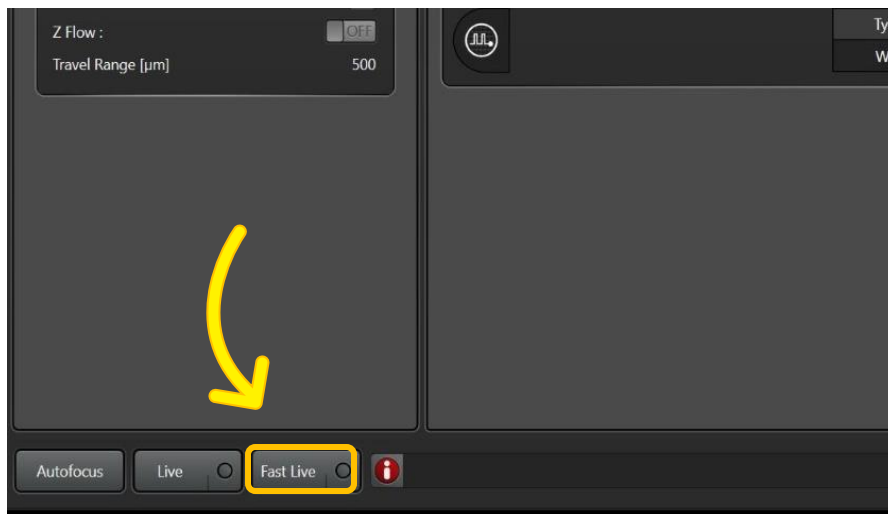


Optimizing Laser Conditions

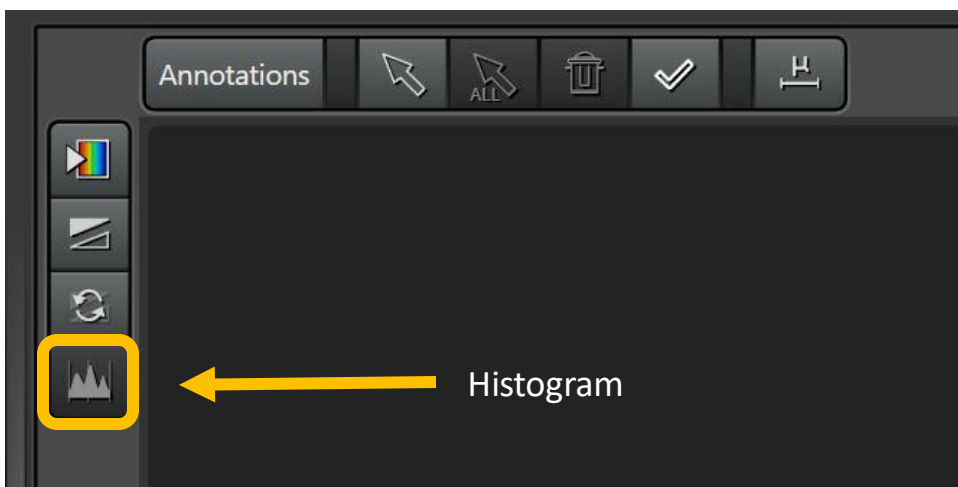
1. Currently selected dye setting will be outlined in white.



2. Click 'Fast Live'.



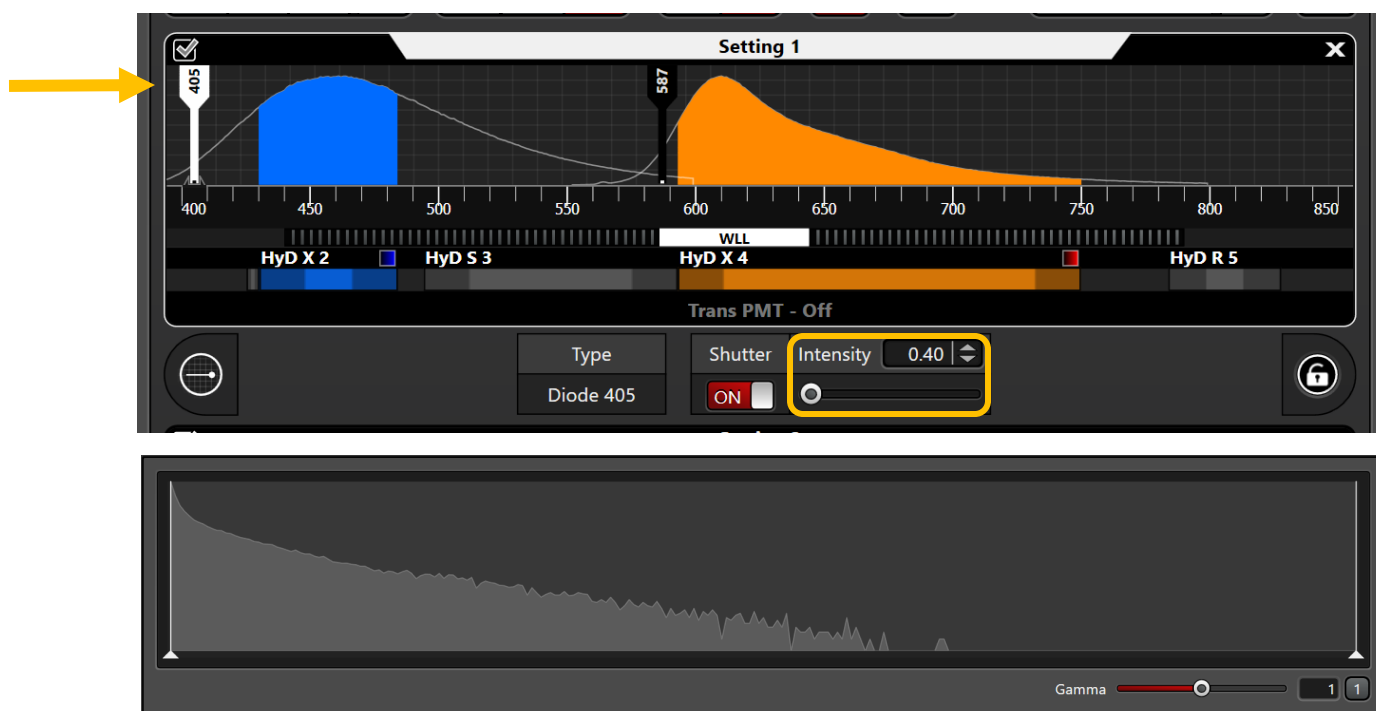
3. Open the histogram.



4. Focus your sample by adjusting Z position to where your sample is brightest (i.e when the histogram extends the farthest.)



5. Click the excitation flag to open laser intensity setting. Adjust laser intensity until the histogram extends about half-way to the full extent across the entire bar. (do not increase laser intensity past 15%)



6. Stop 'Fast Live' once you have optimized the laser setting and repeat for any additional dyes.

Detector Conditions Overview

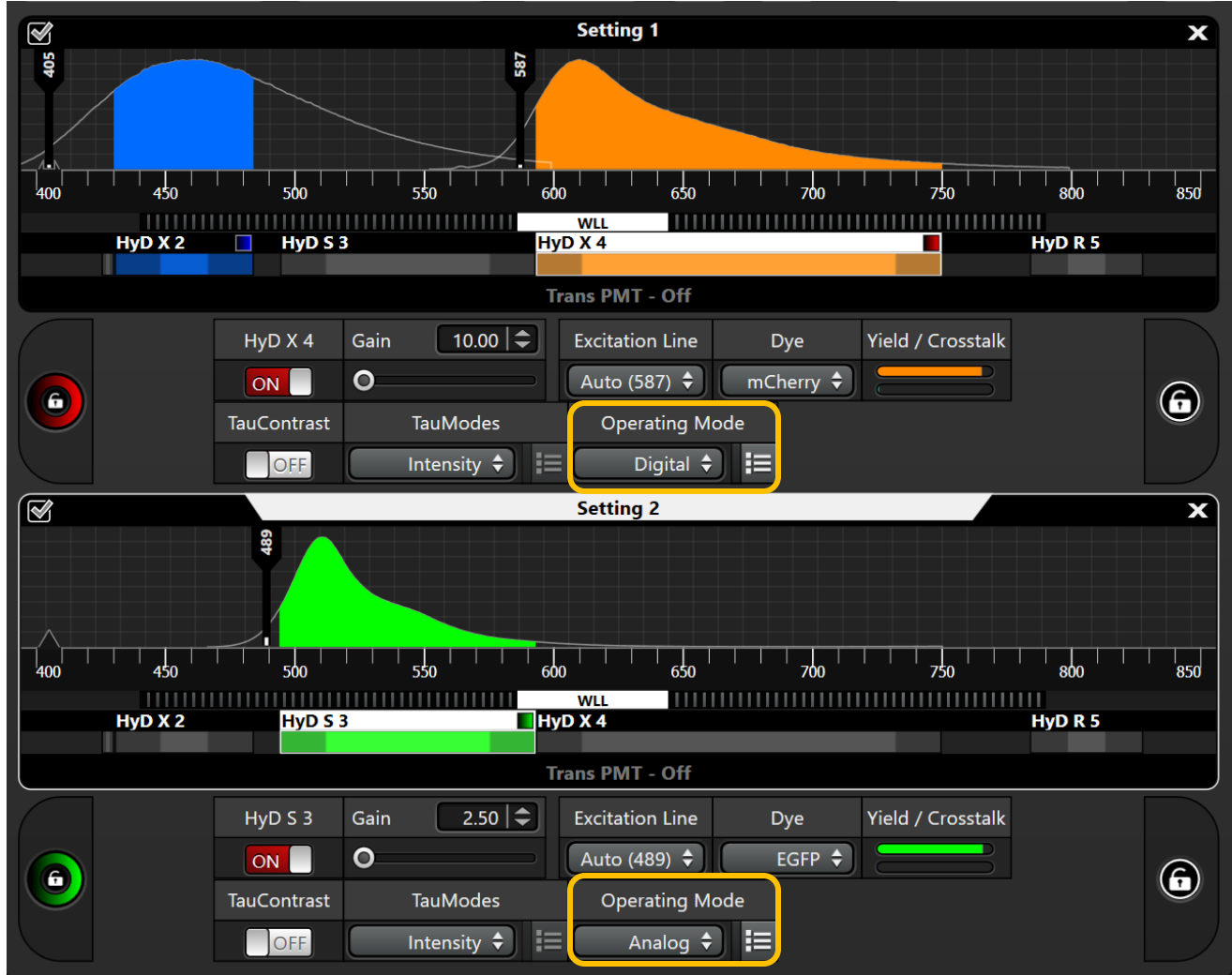
1. Select the detector bar to open detector settings

- Operating mode will automatically default to 'Counting mode' which does not allow you to increase gain from its base setting. This setting is most efficient at capturing all fluorescent signal emitted.
- HyDS detectors have a minimum required gain value of 2.5.
- HyDX detectors have a minimum gain of 10.0.



2. You can also alter the pseudo-color by selecting the colored ring on the left side of the detector settings.

- More color-blind accessible color schemes would be cyan (instead of blue), yellow (instead of green), and magenta (instead of red).



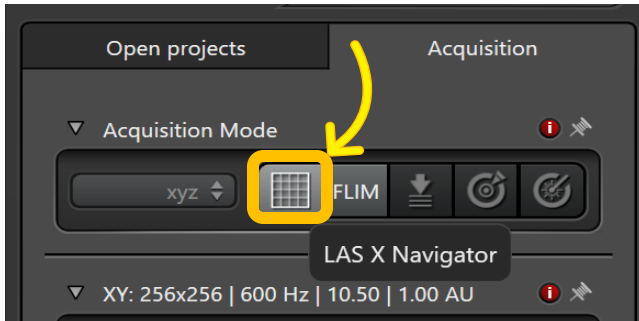
3. Each detector can be switched to 'Digital; or "Analog' operating modes to enable gain settings

Gain has no upper limit value; however, it acts like a multiplying factor, so increasing it results in exponential increase in signal

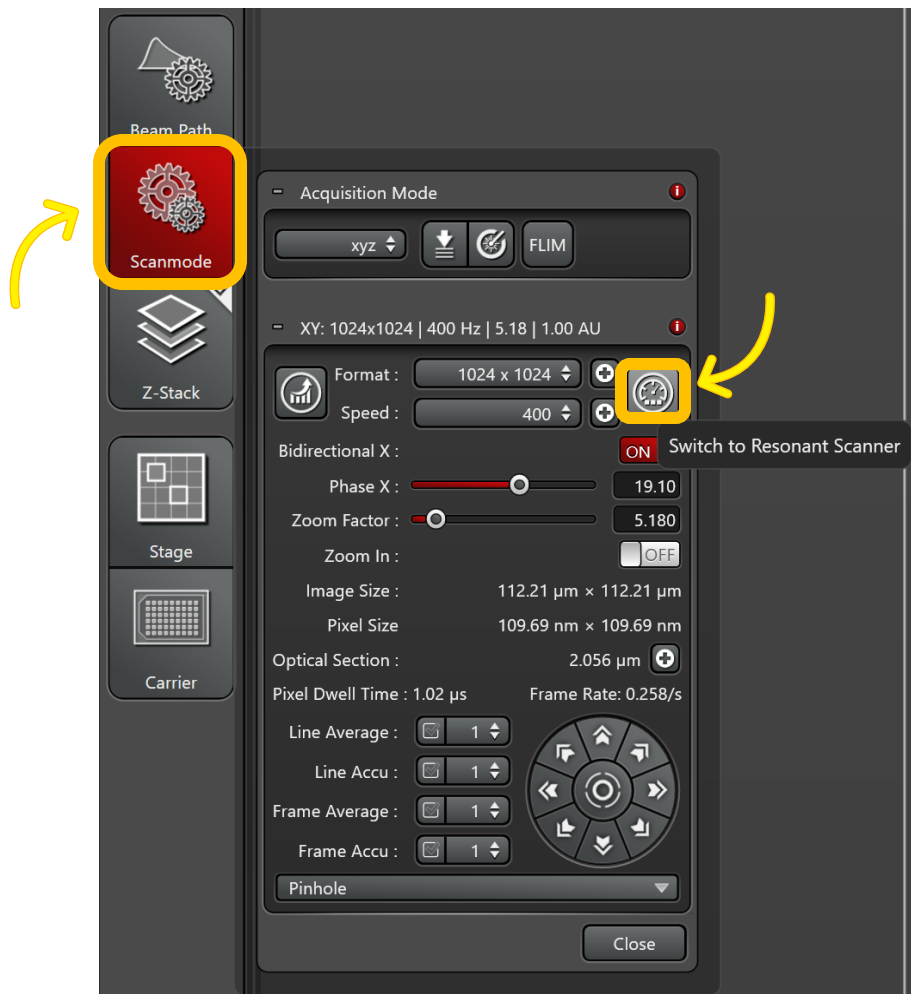
Gain is the voltage applied to the photo-multiplier tube (PMT) after the laser has already hit the sample, so it increases fluorescent signal without causing increased photobleaching. It does, however, also amplify noise, so your signal to noise ratio will get worse as you increase the gain.

Using Navigator/Mapping your Sample

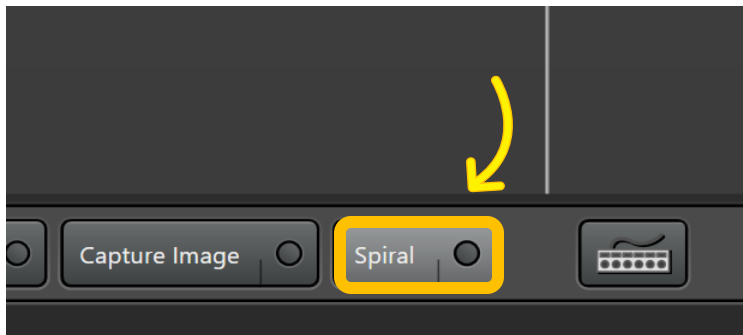
1. Open 'Navigator'.



2. Open 'Scanmode' and turn on 'Resonant Scanner.'



3. Select 'Spiral'.



4. Turn on 'Auto Range'.



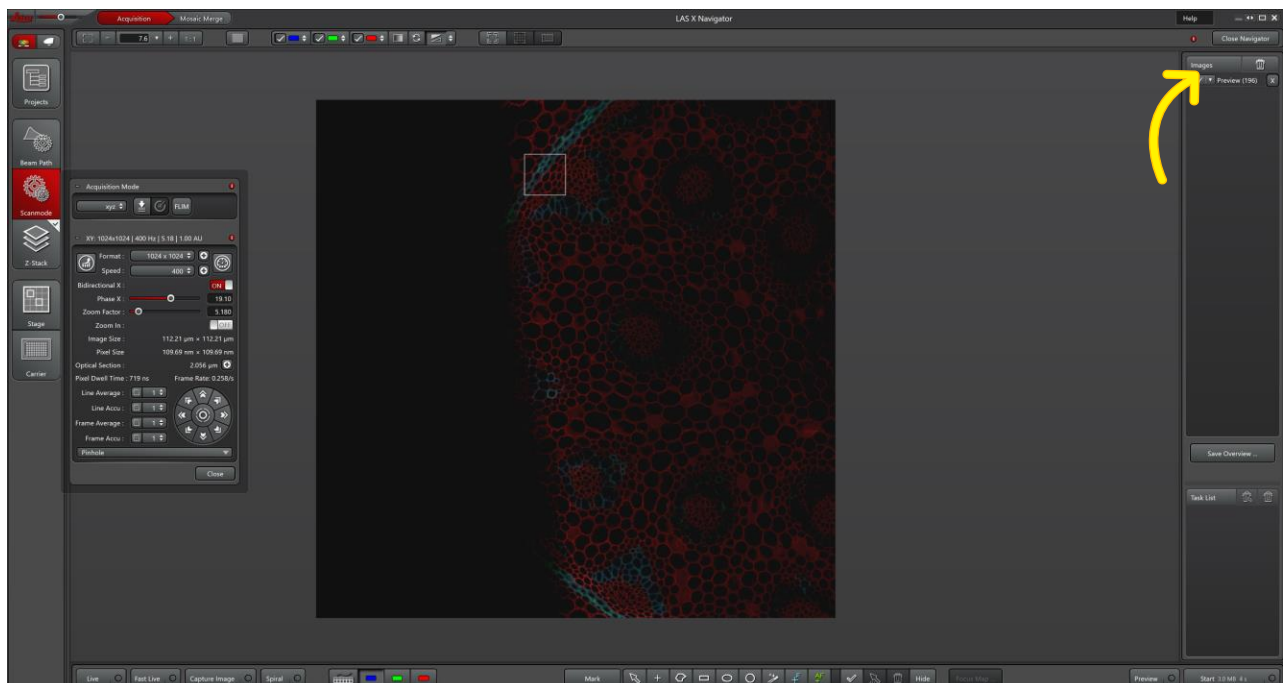
5. Let the spiral run or select 'Stop' (where the spiral button was originally) when it has mapped enough of the sample.

6. Use the xy-joysticks to navigate around your sample and center on your region of interest.

You can also double click; however, this result in an image being scanned at normal speed that is not saved anywhere. It results in the sample being exposed to the laser unnecessarily, so it is not recommended. Turn off 'Resonant Scanner'.

7. Turn off Resonant Scanner.

8. Select 'Close Navigator'.



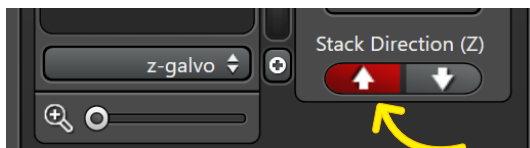
Setting a Z-Stack

1. Turn on 'Fast Live' for whatever setting has the dye that is best representative of the thickness of the sample.
2. Turn z-axis knob clockwise until sample is fully out of focus (the histogram is nearly extinct) and click 'Begin'. Then rotate the z-axis counterclockwise until fully out of focus and click 'End'.

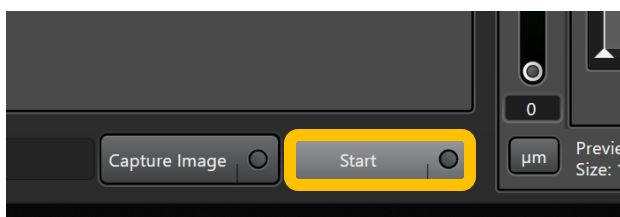


3. Turn off 'Fast Live'.
4. Select upward stack direction.

This is important for avoiding photobleaching of the layers closest to the laser source and avoiding excess noise in your first image,

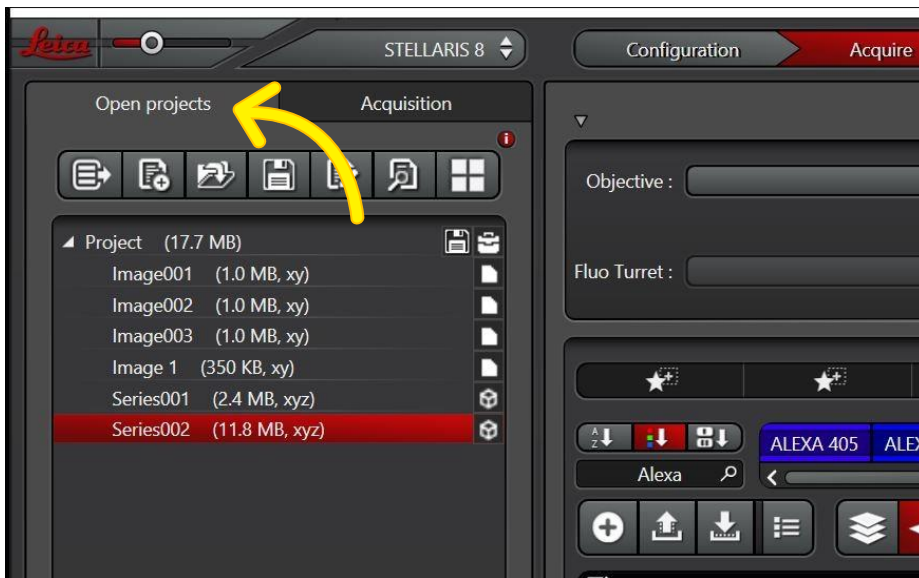


5. Keep 'System Optimized' or choose a determined step size by selecting 'Z-Step Size' and typing in a new value.
6. Select 'Start' to begin your z-stack. (Note that if you are doing a tile scan, you will need to press start from the navigator window.)

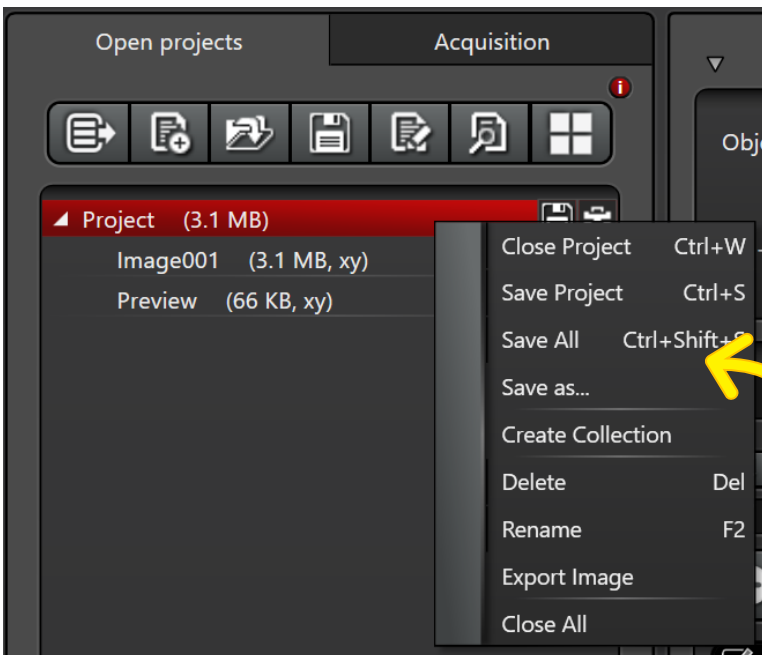


Saving Your Project

1. Open projects to view captures.

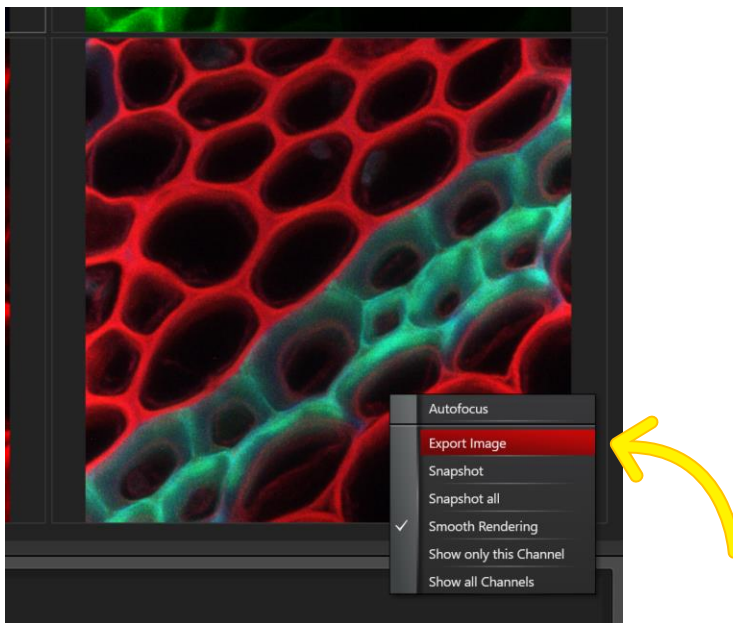


2. Right click on project title and select 'Save as...'.
 - a. Files will be saved to E:drive/image data/your folder as a Leica Image File (.lif)
 - b. Upload files from local E:drive to OneDrive, SharePoint, or thumbdrive to access from other devices.

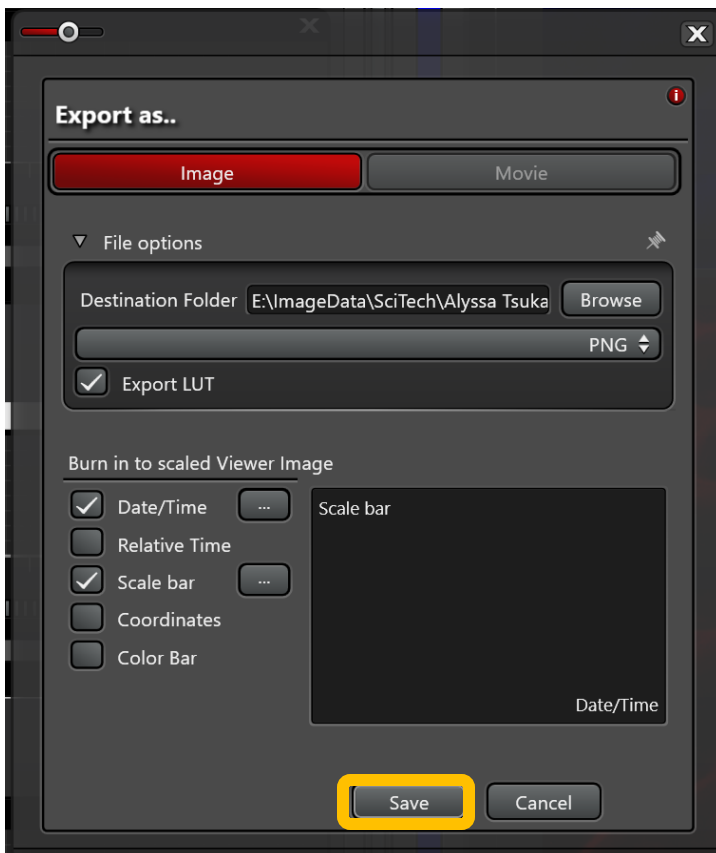


Exporting Individual Images

1. Right click on the view field you would like to export and select 'Export Image'.



2. Ensure the image is being saved to the proper folder in the desired format and select 'Save'.



Shut Down

1. Ensure all scanning is turned off.
2. Tip transmitted light arm up.
3. Lower the objective.
4. Remove sample.
5. Store sample holder in proper drawer.
6. Clean objective if necessary.
7. Lower microscope arm back down
8. Close out LasX software.
9. Shut off the #1, #2, and #3 switches – no need to wait in between each one.
10. Place cover back over the microscope.
11. Log out of your FOM session.
12. Be sure you filled out the sign-in sheet for your session.