Leica Stellaris 8 Confocal with Falcon

Start-up Procedure

1. Start the system using the two *switches* on the large black box. Do not turn any keys, press any buttons, or flip any other switches.
2. Check the chiller (black box on the floor to the left). If the light is on, tell Dr. Kubow immediately and do not use the system.
3. Log-in to the computer and start LAS X.
    1. The configuration should be machine.xlhw
    2. The microscope should be DMI8

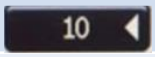
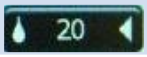
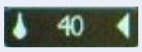
Shut-down Procedure

1. Clean-up any immersion media.
2. Focus the objectives down as far as they will go.
3. Engage the 10x objective.
4. Exit LAS X.
5. Log-off the computer but do not shut it down.
6. Check the chiller (black box on the floor to the left). If the light is on, tell Dr. Kubow immediately.
7. Turn off the system using the two switches.
8. Cover the microscope.

If you just want to use the microscope for viewing/analyzing images…

1. Don't turn on the microscope system.
2. Log-in to the computer and start LAS X.
    1. The configuration should be Simulator.xlhm
    2. The microscope should be DM Manual-6
3. When you're done, just exit LAS X and log-off the computer (don't turn it off).

Important notes about objectives and immersion media!

Objective	Symbol on touchscreen	Immersion medium
10x/0.40 DRY		None (air)
20x/0.75 DRY		None (air)
20x/0.75 IMM		Oil (type F), distilled water, or glycerol (type G)
40x/1.25 GLYC		Glycerol (type G)
63x/1.40 OIL		Oil (type F)

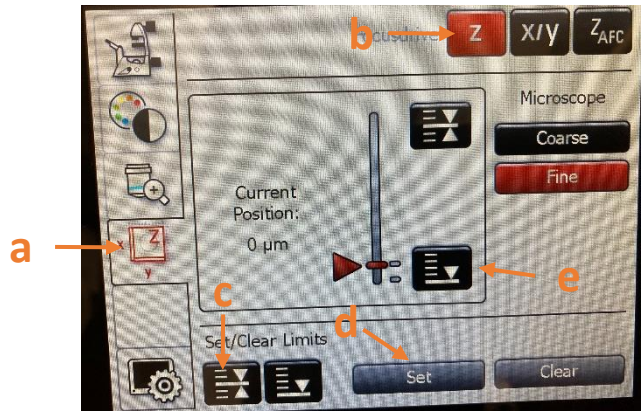
- Do NOT use the incorrect immersion medium.
- You may NOT use the 20x multi-immersion lens unless you have specific permission from Dr. Kubow.
- You MUST clean immersion media from the objective **before** switching objectives – *even objectives using the same medium*. Ask Dr. Kubow if you need to do this.
- The 20x IMM and 40x have correction collars that must be set correctly to get good images.
- If you put the wrong immersion medium on an immersion objective, simply clean it off with lens paper, followed by lens paper wetted with cleaning solution. Then tell Dr. Kubow.
- If you get immersion medium on a dry objective, tell Dr. Kubow immediately.

More information about immersion media on the back of the binder…

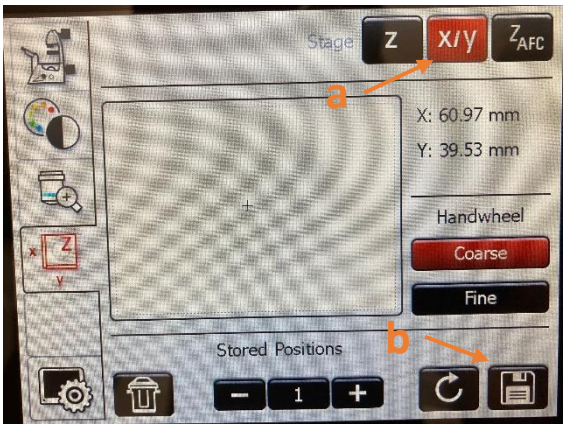
How to switch to an immersion objective when you're already in focus with a dry objective and you don't want to lose your spot…

If you are already using an immersion objective, **STOP**. You must clean off all immersion media from the slide and objective before continuing.

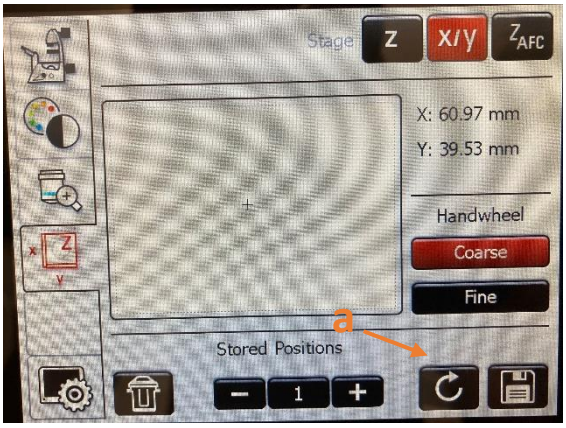
1. Record your z-position (focus point).
   a. Go to this panel.
   b. Select Z.
   c. Select the "set center" button.
   d. Push set. The Current Position should now be 0.
   e. Move the focus all the way down by pressing and holding the "go to bottom" button.



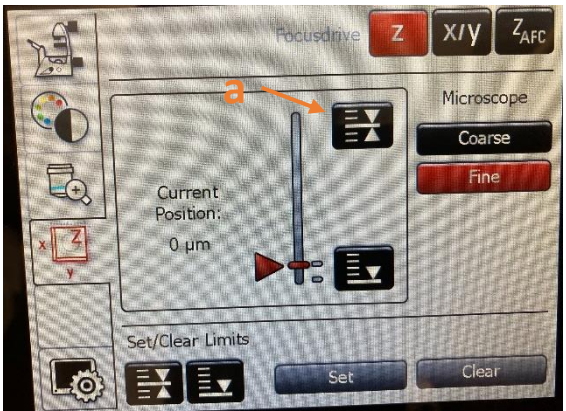
2. Record your x-y position.
   a. Select X/Y.
   b. Click the Save button.



3. Switch to the desired immersion objective.
4. Move the stage forward or backward so that the objective is no longer under the slide, but peaking-out on the side.
5. Add the correct immersion medium.
6. Return to the X/Y position.
   a. Push the "return" button.



6. Return to the z-position.
   a. Press and hold the "go to center" button.



Main Control Panel

Do not press buttons that you do not understand!

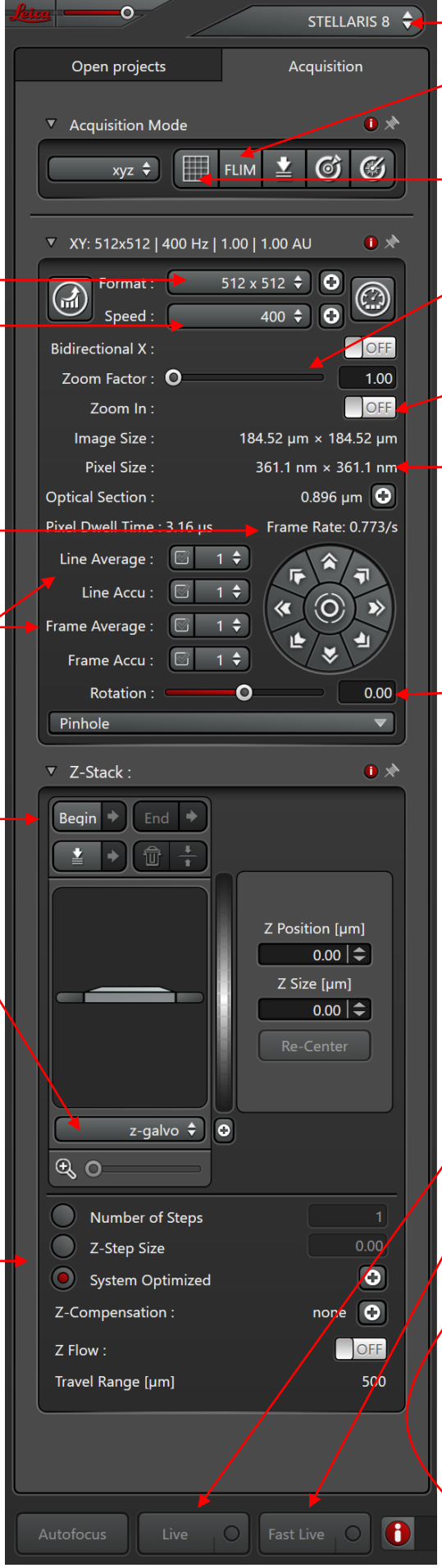


Image dimensions (in pixels)

Imaging speed (in lines/s); higher number = faster

Frame Rate: influenced by image dimensions ("Format"), imaging speed, and averaging/accu.

Line and Frame Averaging: use to reduce noise in images. Use line averaging for live imaging; otherwise, frame averaging.

Set beginning and ending positions for Z-stacks. Press Begin/End buttons to set. Press arrows to move to set positions

Set focus mechanism. There are two mechanisms. This must match the mechanism you are using to change the focus. The focus knob on the control box controls "Z-galvo." The focus knobs on the microscope control "Z-wide." If your z-stack is > 500 µm, you must use Z-wide.

Set z-step size. System optimized will adjust to ~1/3 the optical section size. You can enter a different size in Z-Step Size. It is generally poor practice to set the number of steps.

Click here to switch to **Lightning**

Click here to switch to **Falcon** (quantitative FLIM)

Click here to switch to **sample finder** and large image stitching

Zoom. Note that 0.75 and not 1.00 indicates fully zoomed-out; speeds higher than 600 will force you to zoom-in

Activate to **zoom to a ROI** that you draw on the image window

Pixel size: influenced by image dimensions ("Format") and Zoom. Should be ~1/3 of the optical resolution for best possible resolution for normal confocal imaging (not Lightning)

Rotation: rotate the FOV

Live: start live image

Fast Live: start live image with reduced resolution (faster)

Capture Image: capture a single of exactly what you're looking at (1 slice).

Start: capture a full set of images (e.g. z-stack)

Dye Configuration Panel

Do not press buttons that you do not understand!

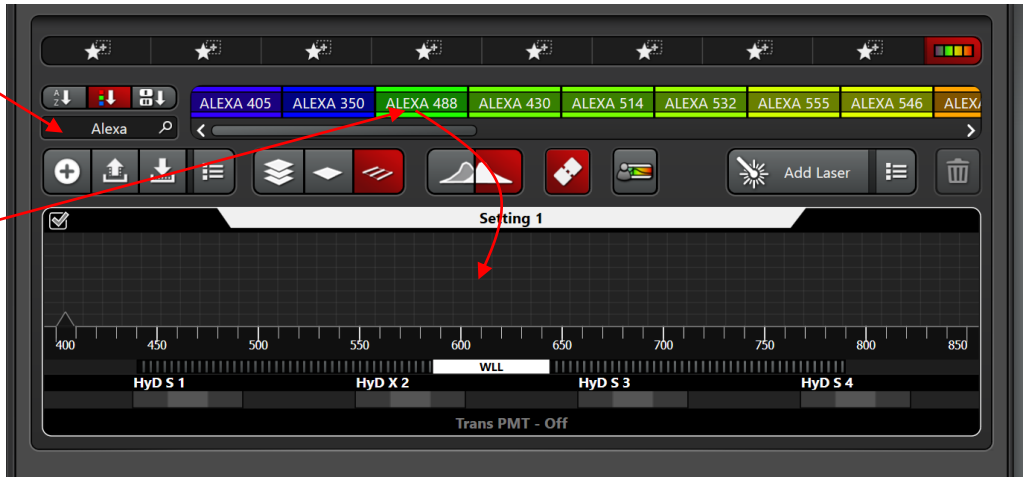
Setting-up you dyes

1. Search for a dye by name

2. Drag the dye onto the plot area. The computer will automatically assign it a laser line and detector.

3. Do this for every dye in your sample.

To remove a dye from the plot area, drag it off.



Click to add a new plot window (Setting 2, etc.). This allows you to set-up sequential imaging.

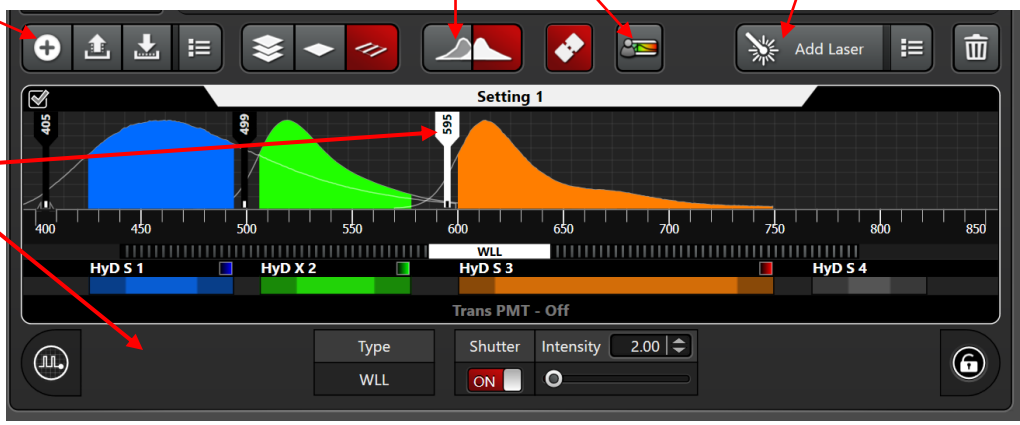
Click to display excitation spectra

Click to open **Dye Assistant**

Drag to plot area to add laser line manually

Click on a laser line to see its controls.

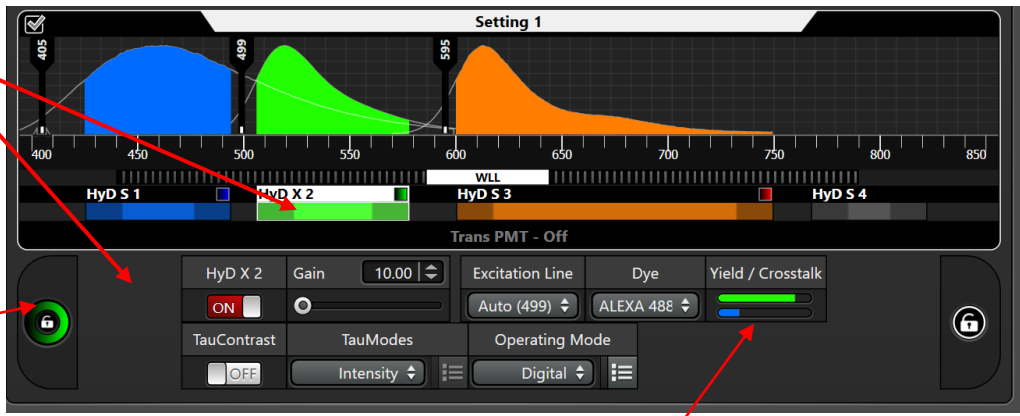
The primary control is the laser intensity, but it's easier to use the control box knob to adjust.



Click on a detector to see its controls.

The primary control is the gain, but it's easier to use the control box knob to adjust.

Click the colored ring (not the lock) to change the color this dye's image is displayed in (its look-up-table; LUT)

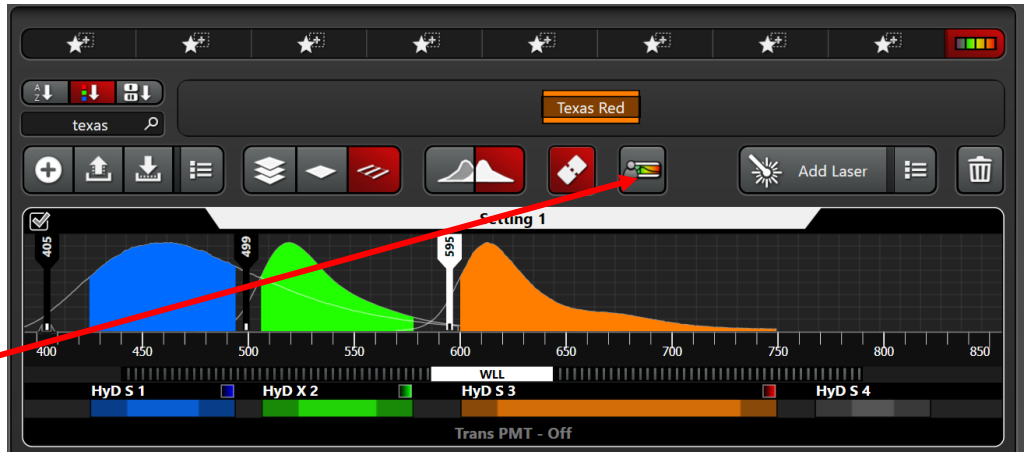


Yield (how much light is collected) and crosstalk are shown here. See the next page for more about this.

Using Dye Assistant to Optimize Dye Excitation and Acquisition Settings

Example: set-up for DAPI (blue), Alexa 488 (green), and Texas Red (orange)

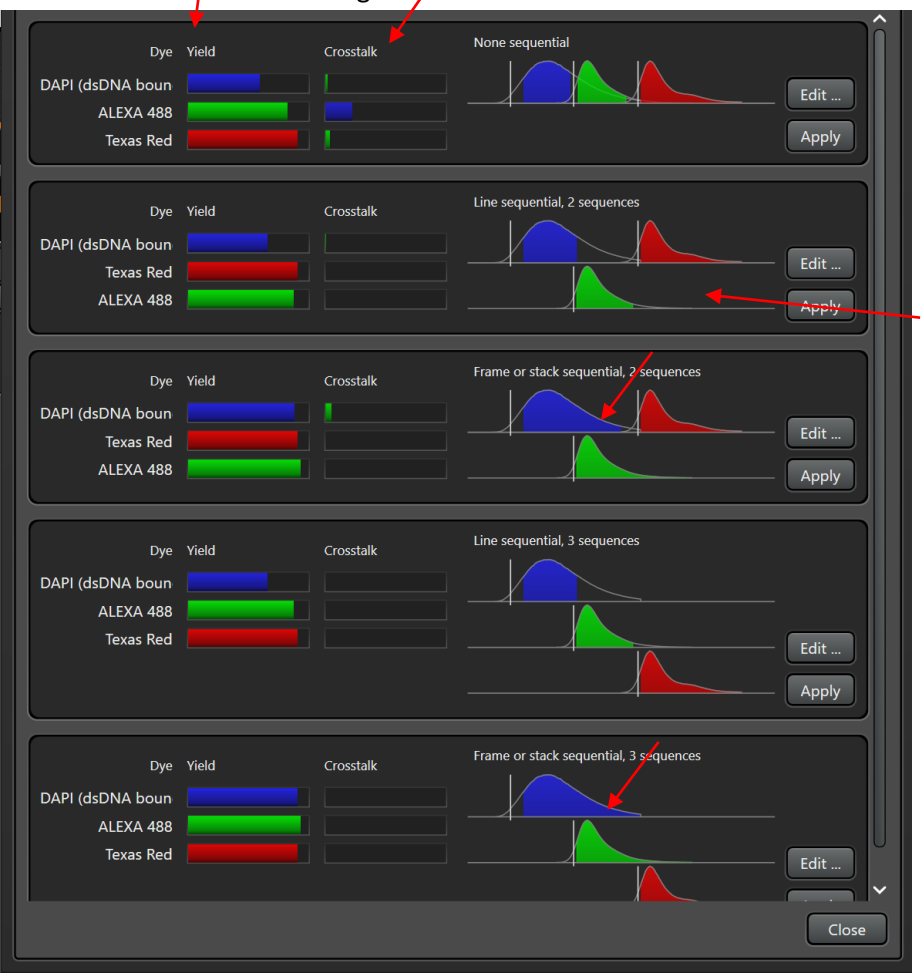
There are many different ways to configure the parameters including the laser wavelengths, detector wavelengths, and whether to acquire the dyes simultaneously or sequentially. You can explore the different possibilities using the Dye Assistant.



Dye Assistant Window

Yield: how much light will be collected. You want this high.

Crosstalk: you want this low, preferably zero.



This is the current set-up. Note the high crosstalk from DAPI into the Alexa 488 channel. This is bad. The rest of the potential set-ups provide options for reducing crosstalk and increasing yield.

This one acquires DAPI and Texas Red simultaneously, but Alexa 488 sequentially to eliminate crosstalk. Note the Alexa 488 plot is on a second line.

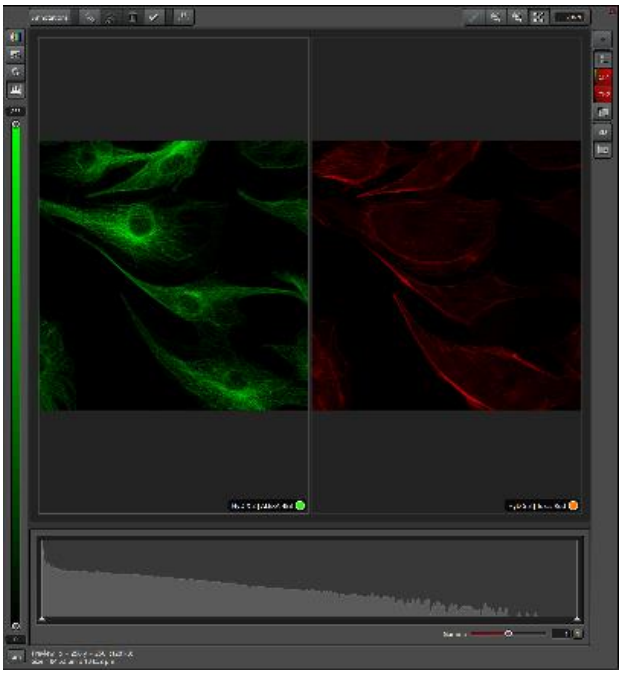
This one is the same as #2 but increases the DAPI Yield by increasing the range of its detector. However this also increases Alexa 488 crosstalk.

This one is the same as #2, but acquires all the channels sequentially and honestly isn't really an improvement.

This one is the same as #4, but increases the range of the DAPI detector. This option has the highest Yield and lowest Crosstalk, however because all the channels are acquired sequentially, it will take 3x as long to image as option #1 and 1.5x as long as option #2.

Click Apply next to the option you want to use. You can, of course, configure everything yourself using the controls on the main dye window. In case you're wondering, I would have picked option #2. It is the best balance of minimizing crosstalk, maximizing yield, and not taking too long to image. However there are different experimental situations where each option might be the best pick.

Image Window



Click on a channel's image to use the control box knobs to adjust that channel's laser intensity and detector gain.

Click to **toggle between color schemes (LUTs)**. Cycles between in color (currently shown), saturation indicator (all channels shown in a red/orange/yellow LUT with saturated pixels shown in blue), and grayscale (all channels shown in black and white). Recommended: use the saturation LUT to help optimize channel intensities.

Autoscale histogram (not recommended)

Reset histogram scaling

Display histogram

Toggle between showing each channel individually and showing an overlay only

Toggle whether each channel is displayed

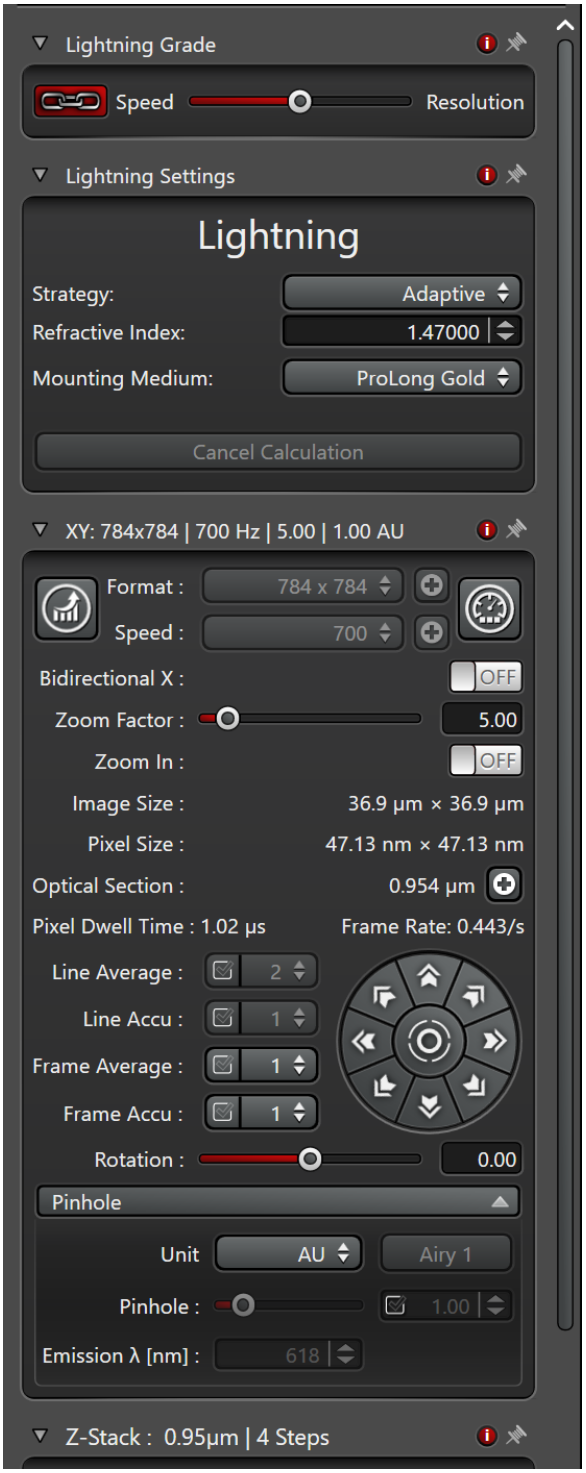
Display overlay in addition to individual channels

Simple 3D rendering

Lightning Mode

Click here to switch to **Lightning**

Lightning mode increases image resolution by 1) making the confocal pinhole smaller and 2) deconvolving the image. Closing-down the pinhole increases your optical resolution, which means that you also need to increase your digital resolution (i.e. decrease your pixel and z-step sizes). Smaller confocal pinholes also decrease the amount of light collected, so you also need to adjust your acquisition parameters to increase your signal and decrease your noise.



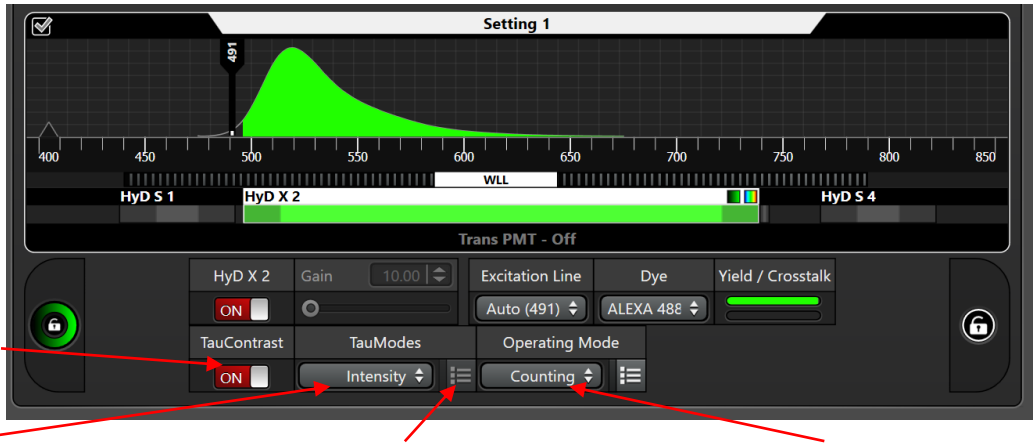
The Leica software will adjust all these parameters for you automatically if you use the Lightning Grade slider. As the slider suggests, there is a trade-off between speed and resolution. Higher resolution = slower imaging. You can set all the parameters yourself if you unclick the chain icon. A description of the parameters changed by Lightning Grade is shown in the table, below.

Because deconvolution depends on the refractive index of the mounting medium (and sample), **it is essential that you specify this parameter as accurately as possible**. You can either select the mounting medium from the drop-down menu or input a numerical value in the Refractive Index box.

Parameter	As you move the Lightning Grade to higher resolution…
Pinhole	Decreases, down to a minimum of 0.5 Airy Units (AU). This increases resolution but also excludes more light making the image dimmer.
Pixel and Z-step size	Decreases to enable correct Nyquist sampling for the optical resolution given by the pinhole size. Pixel size is decreased by increasing the “Format” – i.e. increasing the number of pixels in the image. The zoom is not changed.
Speed	Decreases to increase the amount of light collected and compensate for the decrease in light collection due to the smaller pinhole.
Line Averaging	Increases to reduce noise that is introduced because of the lower light collection due to the smaller pinhole.
Detector Gain	Unchanged
Laser Power	Unchanged

TauSense Fluorescence Lifetime Imaging

TauSense is not strictly quantitative and is best used for separation of different lifetime species in an image. If you need quantitative lifetime measurements, use Falcon FLIM.



Select the detector you want to use and then switch the TauContrast switch on.

Then select the TauMode you want to use from the drop-down menu.

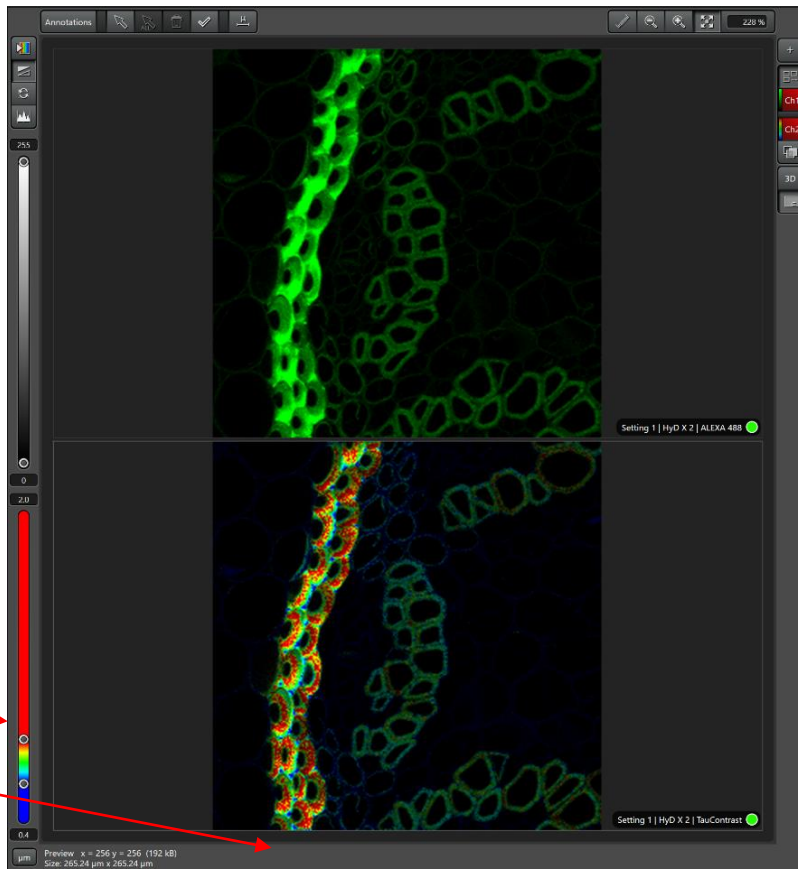
Click the menu button for options for most TauModes.

The detector is set to Counting mode. **If you switch TauContrast off, you'll need to manually set this back to Digital**, which is the normal setting for imaging.

TauMode: Intensity

This mode shows you a normal intensity based image for the channel in addition to the lifetime image.

Intensity based image. Note that because the detector switched to Counting mode, you may need to decrease your image intensity by decreasing laser power or gain.



Lifetime image. The colors correspond to fluorescence lifetimes. You will need to activate autoscaling

or adjust the display range manually to be able to differentiate the different lifetimes in the image.

If you hover over the image, the lifetime of the pixel you are on is displayed at the bottom of the window (in nanoseconds, ns).

TauMode: TauSeparation

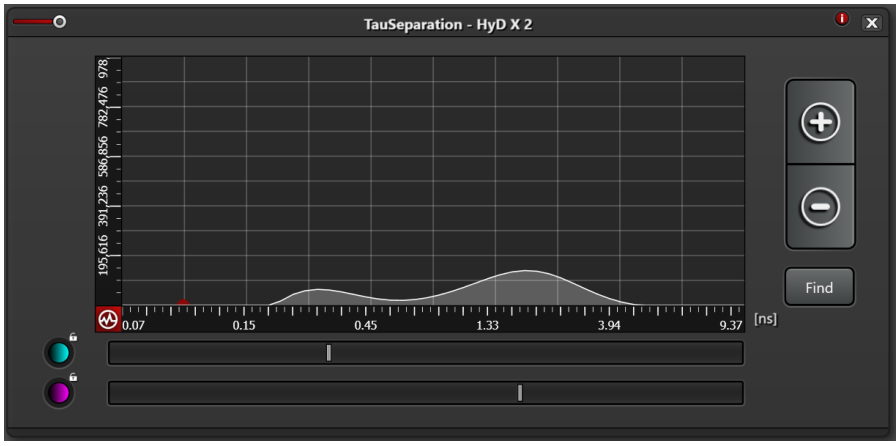
This mode allows you to separate two or more different lifetime populations in an image. For example, the previous image showed a sample in which some features had high lifetimes and others had low lifetimes. We could use TauSeparation to separate these two sets of features into different images.

1) Switch to TauSeparation mode and click the menu button to display this window.

2) If the plot is empty, start a live image (you can stop it once you've got an image).

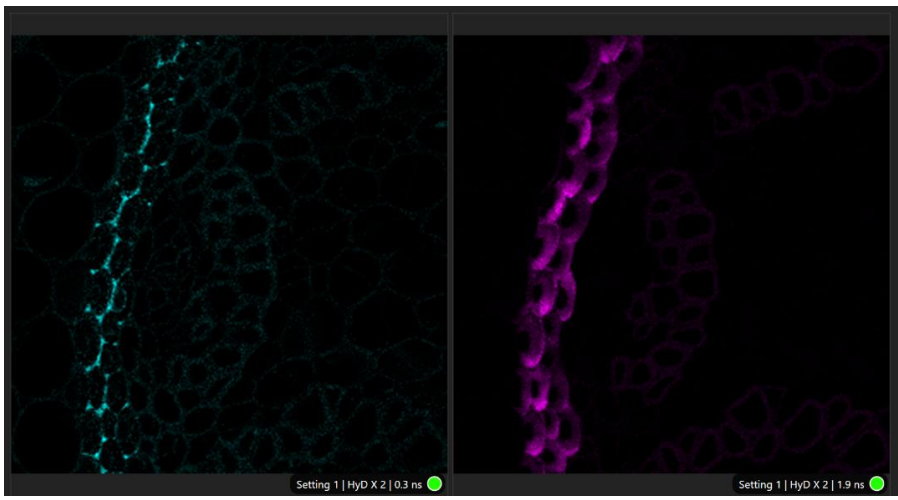
3) The plot is a histogram of lifetimes (x-axis, in nanoseconds). The plot in the image shows two clear populations.

4) Add enough channels (indicated by the bars below the plot) to match the number of populations you wish to separate by clicking the +/- buttons.



5) Click Find to have the computer identify the peaks of each population. Or you can do this manually by dragging the vertical lines for each channel

The computer will computationally separate the different lifetime populations into different fluorescence channels. The images on the left and right show the features with the low and high lifetimes, respectively.



TauMode: TauGating

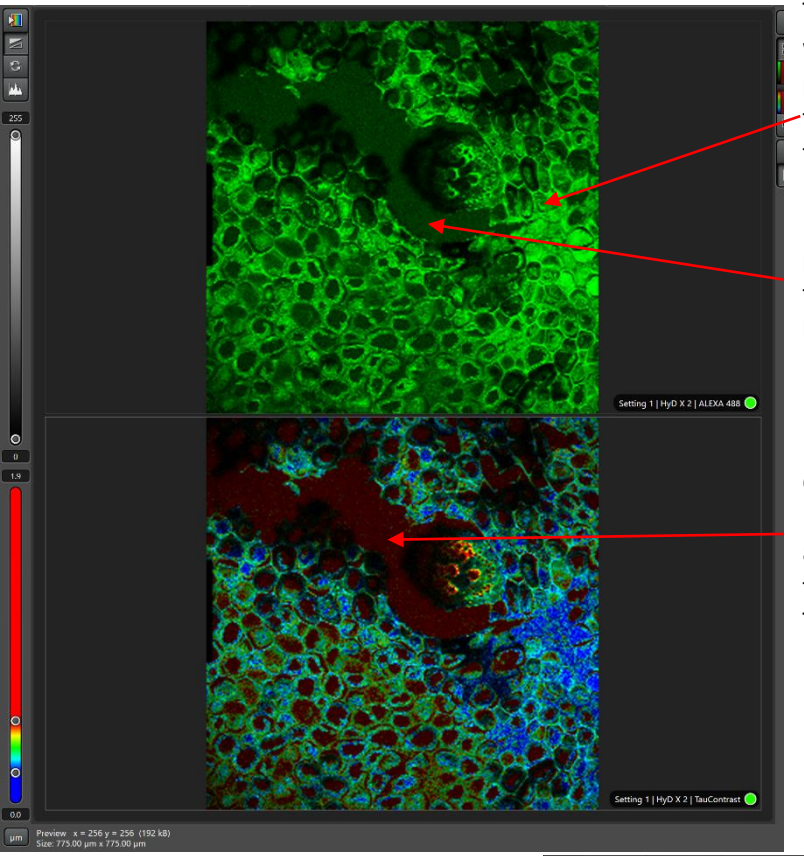
This mode allows you isolate features with specific ranges of lifetimes. It's basically the same thing as TauSeparation except it's completely manual and the different lifetime populations are not computationally unmixed, they are divided based on rigid cut-offs. In general, I would use TauSeparation for most purposes and only use TauGating if there were a very well-defined and well-separated lifetime population that I wanted to capture or exclude (e.g. autofluorescence).

1) Switch to TauGating mode and click the menu button to display this window.

2) Add enough channels to match the number of populations you wish.

3) Adjust the gray bars for each image to identify the ranges of lifetimes to be included in each channel. It can be useful to use TauSeparation to visualize the lifetime distributions first as an aid to identifying where to place your cut-offs.

Example: Using TauSense to remove autofluorescence



This is a prepared slide of a fern cross-section that was prepared for brightfield microscopy. Like many plant tissues, the fern cross-section has an intrinsic fluorescence and can be visualized using fluorescence microscopy.

Unfortunately, the mounting media used to prepare the specimen also has an intrinsic fluorescence (autofluorescence) that produces a high background signal.

Let's investigate the fluorescence lifetimes of the different image features using the **Intensity mode**. Luckily for us, the mounting media autofluorescence has a much higher lifetime than the fern fluorescence. This means that we can use TauSense to remove it!

Using **TauSeparation**, we can see the distribution of the lifetimes in the image. The lower peak corresponds to the fern; the higher peak corresponds to the mounting media. TauSeparation separates these two components into two different channels (fern on the left).

It's likely that you don't want/need an image of the mounting media autofluorescence. In this case, you can use **TauGating** to select only the lifetimes corresponding to the lower peak. You will get an image of just the fern and the fluorescence from the mounting media will be eliminated.