



Training Guide for Leica SP8 Confocal/Multiphoton Microscope

LAS AF v3.3



Optical Imaging & Vital Microscopy Core

Baylor College of Medicine (2017)

Power ON Routine



1



Turn ON power switch for epifluorescence light source.

This lamp will be used to visualize fluorescence in the microscope eyepieces.

2



Turn ON Laser Power, Scanner Power & PC Microscope green switches then turn Laser Emission key to ON state.

3



Power ON MP-EOM Power Strip.

Wait for Windows PC to boot and Login to User Account.

Power ON Routine – for NLO Only



Turn Chameleon TiS laser key from 'Standby' to 'ON'.



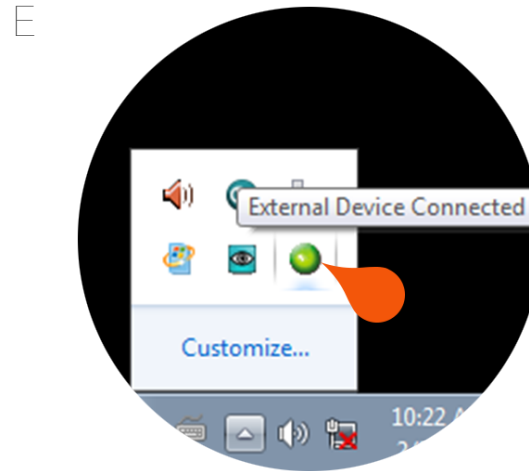
Turn ON power strip for APT.



Toggle Laser selector to position A - 'Leica'.

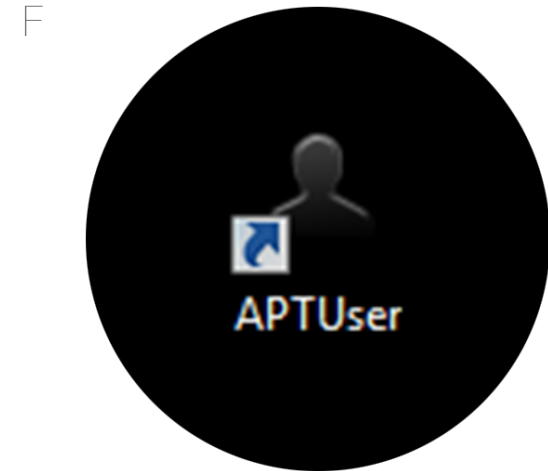


Select USB hub to Position 1.



Once logged in, verify that the USB connection is active for the APT.

Green is active.



Start 'APTUser' application from desktop.

Power ON Routine – for NLO Only

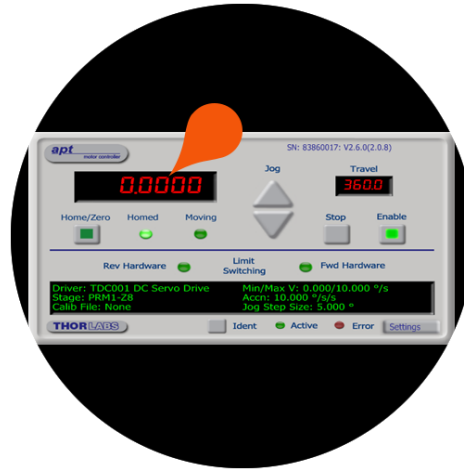


G



Click 'Home/Zero' to home the device.

H



Once homing is complete, click the current position display.

I



Enter '56' into position for Ch1 and press OK.

Minimize APT software.

4



Double-click LAS AF icon.

5



Choose configuration and microscope (With Stage and DM6000 respectively).

Choose toggle for Resonant scanner.

Note: If you do not require high speed scanning – leave Resonant set to OFF. If you turn it on you will not be able to use slower speeds.

Click OK to initialize system.

6



When prompted to Initialize Stage select YES.

Note: Ensure that no samples are mounted or condenser does not interfere with stage travel movement.

Getting Started with LAS



The LAS AF software is separated into four distinct areas.

Image Acquisition & Control Settings

These functions control the static acquisition settings for image collection. Here you can find settings such as Frame Size, Scan Speed, Averaging, Zoom, etc.

Beam Path Configuration

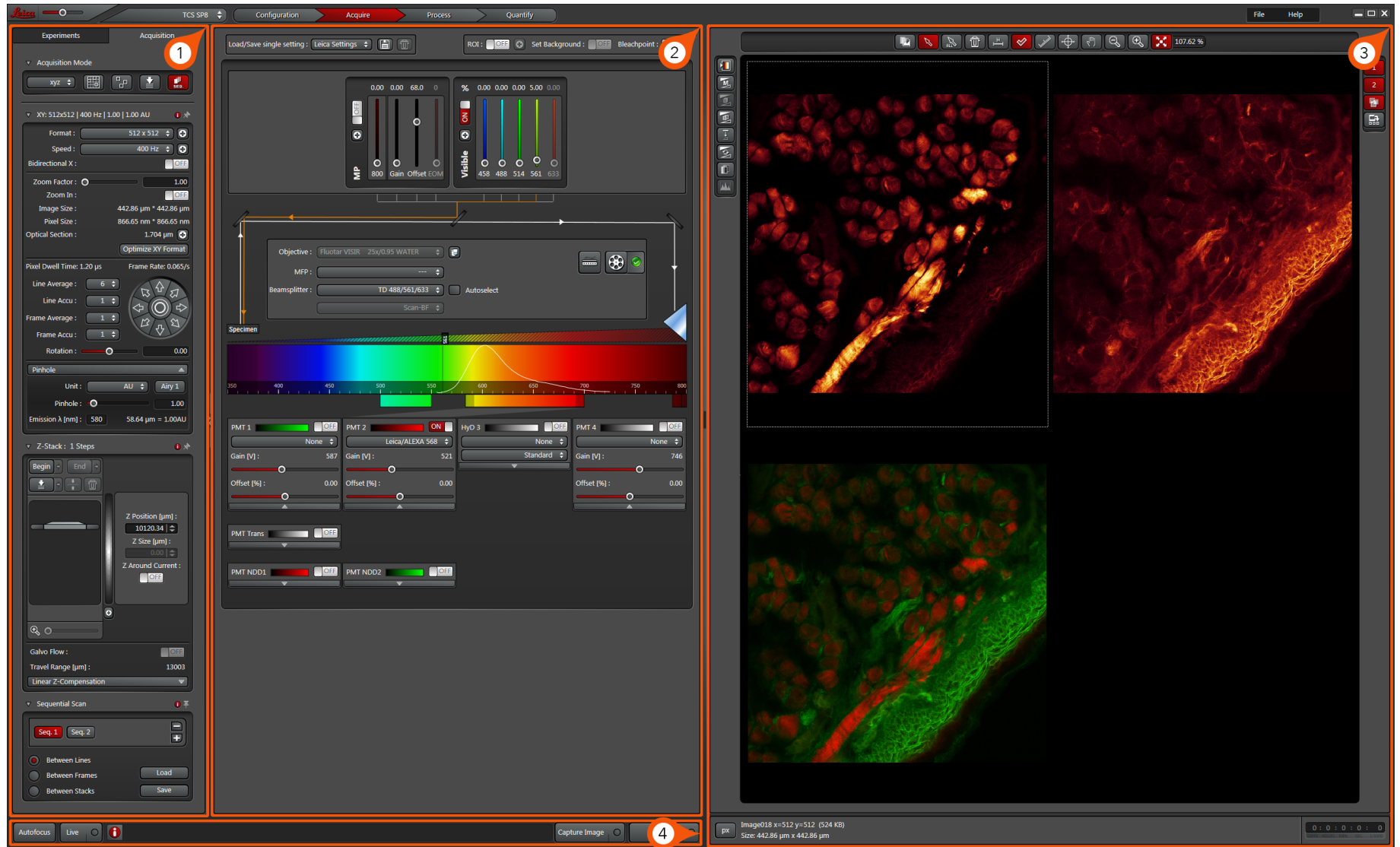
This area is where we set our beam path configuration and control our detector settings to optimize our fluorescence signal.

Image Display

This area is where the image will be displayed. Settings here allow the user to control how the image is viewed on screen.

Scan Action Functions

These functions start/stop scanning and initiate our experiment acquisition.



The screenshot displays the Leica LAS software interface, divided into several functional areas:

- 1. Acquisition Settings (Left Panel):** This panel contains various parameters for the acquisition process, including:
 - Acquisition Mode: XYZ
 - Format: 512 x 512
 - Speed: 400 Hz
 - Image Size: 442.86 μm x 442.86 μm
 - Pixel Size: 866.65 nm x 866.65 nm
 - Optical Section: 1.704 μm
 - Pixel Dwell Time: 1.20 μs
 - Frame Rate: 0.065/s
 - Line Average: 6
 - Line Accu: 1
 - Frame Average: 1
 - Frame Accu: 1
 - Rotation: 0.00
 - Pinhole: 1.00
 - Emission λ [nm]: 580
 - Z-Stack: 1 Steps
 - Z Position [μm]: 10120.34
 - Z Size [μm]: 0.01
 - Z Around Current: OFF
 - Galvo Flow: OFF
 - Travel Range [μm]: 13003
 - Linear Z-Compensation: OFF
 - Sequential Scan: Seq 1 (selected)
 - Between Lines (selected)
- 2. Configuration (Middle Panel):** This panel shows the instrument's configuration, including:
 - Objective: Fluotar VISIR 25x/0.95 WATER
 - MFP: ---
 - Beamsplitter: TD 488/561/633
 - Scan-BF: ---
 - Specimen: A color-coded scale from 250 to 800 nm.
 - PMT 1: None, Gain [V]: 587, Offset [%]: 0.00
 - PMT 2: Leica/ALEXA 568, Gain [V]: 521, Offset [%]: 0.00
 - HyD 3: OFF
 - PMT 4: None, Gain [V]: 746, Offset [%]: 0.00
 - PMT Trans: OFF
 - PMT NDD1: OFF
 - PMT NDD2: OFF
- 3. Image View (Right Panel):** This panel displays the acquired fluorescence images. It shows a multi-panel view with:
 - Top Left: A fluorescence image showing a cluster of cells with red and yellow signals.
 - Top Right: A fluorescence image showing a different view of the same sample, primarily with red and orange signals.
 - Bottom Left: A fluorescence image showing a cluster of cells with green and red signals.
 - Bottom Right: A fluorescence image showing a different view of the same sample, primarily with green and red signals.
- 4. Image Information (Bottom Panel):** This panel provides details about the current image:
 - Image018 x=512 y=512 (524 KB)
 - Size: 442.86 μm x 442.86 μm
 - 0 : 0 : 0 : 0

Powering On Lasers



Before we configure the beam path for the particular fluorophores being imaged we need to turn on the individual lasers we require.

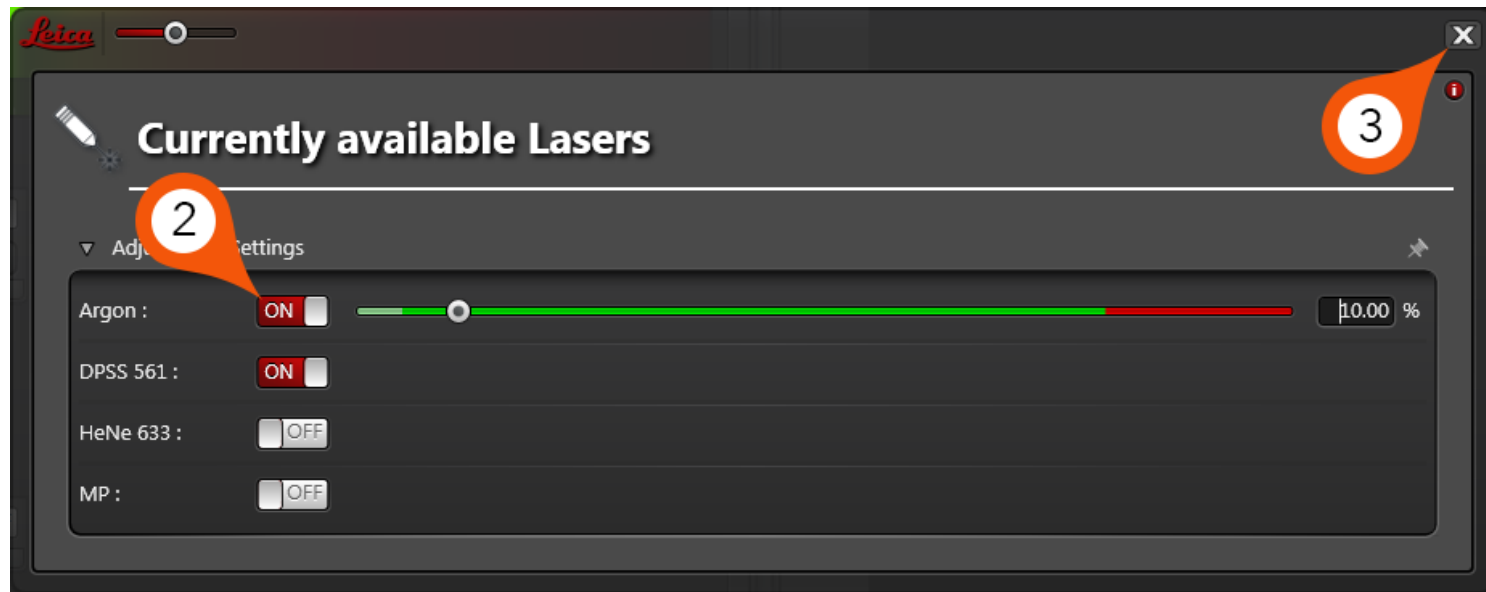
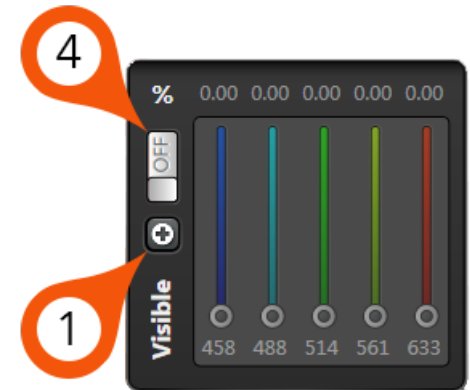
1. Click on the + icon for the Visible Laser dialog.

2. Toggle on each laser required.

Note: For optimal conditions set the Argon laser power to 10% output power.

3. Close Available Lasers dialog.

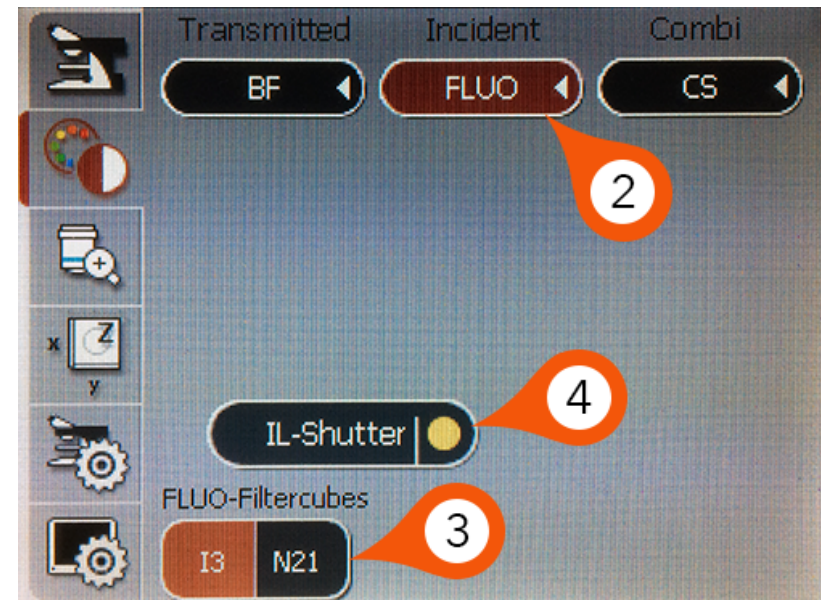
4. To enable Visible Lasers for use - toggle the ON/OFF switch above + to ON state.



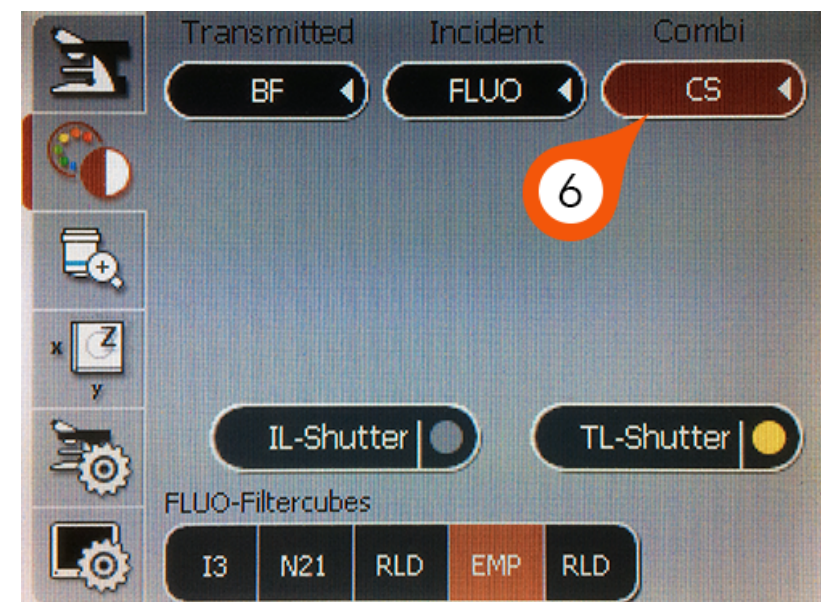
Mounting Sample on Microscope

Once the system has been powered on, we can mount our specimen on the stage below the microscope objective.

1. Raise stage to appropriate height and mount sample.
2. Switch microscope TFT to 'FLUO' to activate the incident path from default 'CS' in Combi.
3. Choose filter (I3=Green, N21=Red).
4. Open/Close reflected light shutter with 'IL-Shutter'.



5. Via the microscope eyepieces, visualize your sample and adjust the focus (Z) and stage position (X,Y) accordingly
6. When sample is in focus and positioned correctly - switch microscope back to CS - Combi to begin imaging with the confocal.



Beam Path Configuration



Once the lasers are powered on we want to configure the beam path to collect a fluorescence signal. In this example, we will design a beam path for an Alexa 568 sample.

1. From the Visible Laser dialog, choose excitation wavelength that matches your fluorophore. In this example, we will use the 561nm laser.

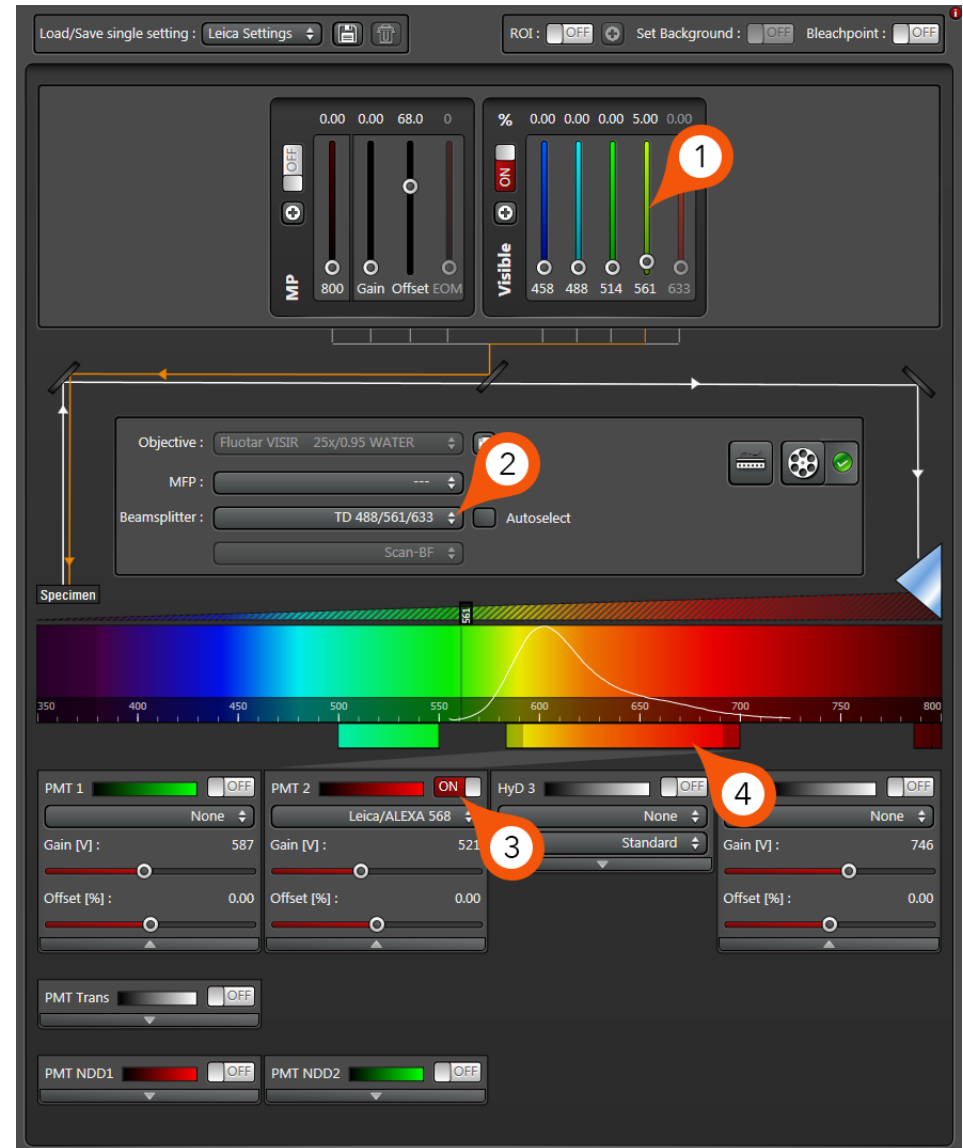
Note: Set the transmission % to a reasonable starting value (this may be changed dynamically depending on signal strength). A starting value between 1-5% is recommended.

2. Choose primary dichroic mirror that matches the laser line you wish to use.

Note: If you will use sequential scanning choose a beam splitter that matches every line you wish to use.

3. Turn on PMT for detection and select LUT (look up table or color) for that particular channel.

4. Define upper and lower emission band thresholds based on the spectrum for your fluorophore.



Beam Path Configuration



Note: PMT selection will be based on where your fluorophore(s) fall in the linear range of the emission spectrum. Meaning that PMT 1 corresponds to the lowest wavelength and PMT 4 corresponds to the longest wavelength fluorophore. In our example, we will be using a sequential scan with Alexa 568 and Alexa 488, so PMT 1 will be used for the Alexa 488 signal and PMT 2 will collect the Alexa 568.

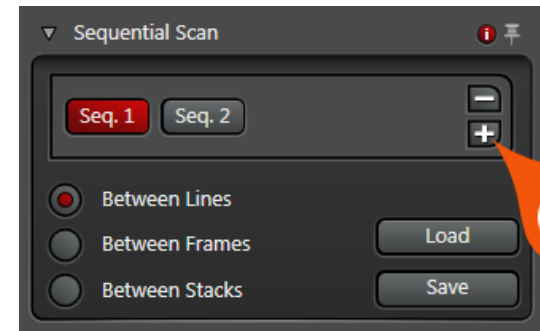
Sequential Imaging for more than one color:

1. Once setup for initial single color is complete (in this example, Alexa 568), ensure the 'Seq' button is selected in your Acquisition control settings (see Acquisition Setup, Step 2, page 14). This will activate the Sequential Scan dialog.

By default, your previously designed single beam path will be Seq 1.

Note: The default selection of 'Between Lines' is recommended so the system will switch between acquisition tracks every line to minimize any potential sample drift.

2. Once these options are selected you can click + to add 'Seq. 2' and repeat steps 1-4 on the previous page to change the beam path settings to match your second fluorophore.
3. Repeat process for total number of channels required.

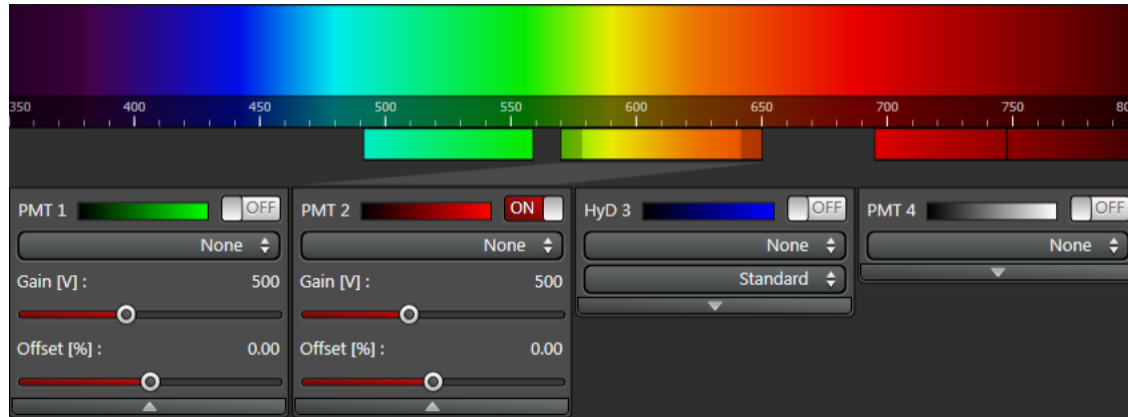


Beam Path Configuration

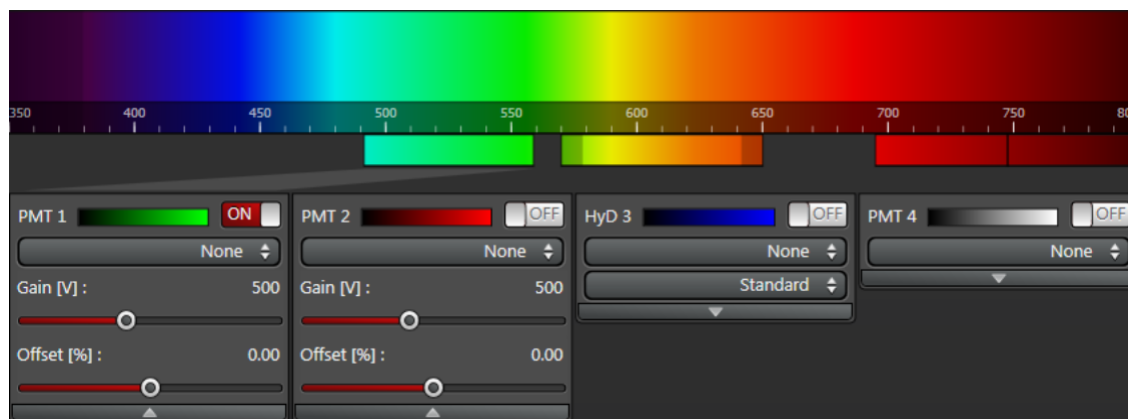


Note: We always recommend that you collect your longest wavelength fluorophores first in order to ensure minimal photo bleaching of lower wavelength fluorophores.

Example Beam path for Seq. 1 - Alexa 568



Example Beam path for Seq. 2 - Alexa 488



Acquisition Setup



The Acquisition Mode parameters located on the left-hand side of the software display contain all the settings available for image optimization and collection.

1. From the dropdown, choose the Acquisition Mode. For example, if you want to collect 3d image stacks, select 'xyz' from the dropdown.



The remainder of the settings in the acquisition mode control dialog can remain at their defaults to start. Depending on sample morphology and signal quality these settings will need to be adjusted later.

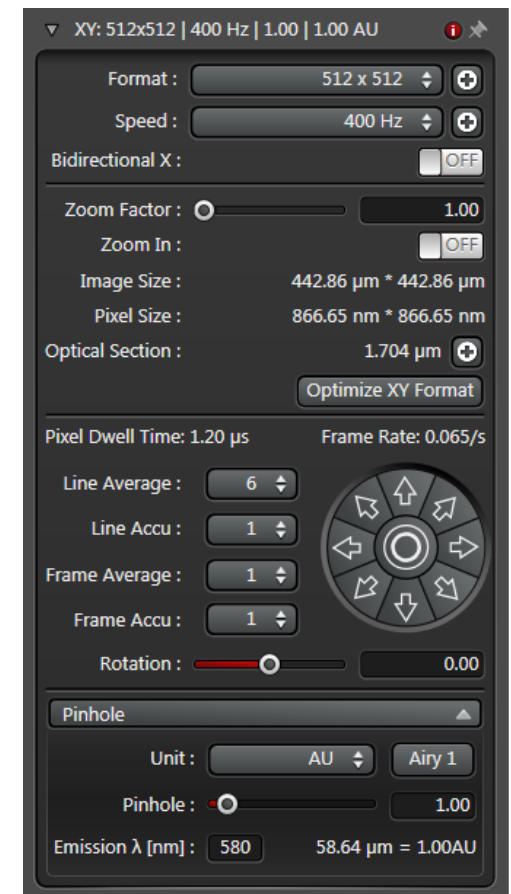
Format - image size in pixels. Default is 512x512 however this can be optimized based on zoom and objective settings. Ideal Nyquist sampling frequency can be quickly selected with 'Optimize XY Format'.

Speed - overall speed of scan during acquisition. Generally, try to run this at the default 400Hz to start and change depending on noise levels.

Zoom Factor - change magnification without changing objective lens.

Line Average - selection >1 will scan each X-line number of times selected then average result. This will be the most effective method for reducing noise in the resulting image.

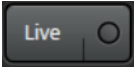
Pinhole - this controls the optical section thickness. To start - select 'Airy 1' button to set optimal pinhole diameter. This can be increased or decreased to adjust image quality.

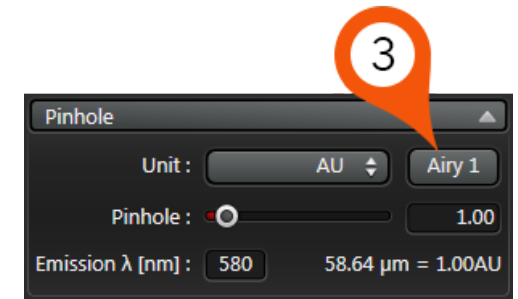


Scanning an Image



Once you have completed the beam path configuration for your fluorophore(s) it is time to begin scanning to optimize the image quality.

1. Press 'LIVE' to begin scanning. 
2. Image will appear in Image Display area (Page 8, Area 3).
3. From the Acquisition Mode dialog, set the Pinhole to 'Airy 1'.

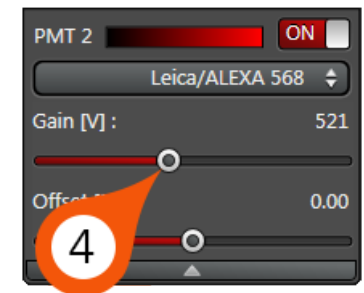


Note: Setting the pinhole to 1 AU is a compromise between axial resolution and signal level. When you start with an open pinhole, you are at a point where your signal is the highest but your axial resolution is at its lowest. As you reduce the aperture diameter you are increasing your Z resolution but reducing your signal level. This is a reasonable trade off until you reach 1 AU. Reducing the pinhole lower than 1 AU will still linearly increase your Z resolution but now signal will start to drop exponentially.

4. Beginning with one channel, adjust 'Gain' in under PMT for selected channel until you can see a reasonable image on screen.

Note: Increasing Gain will effectively increase PMT sensitivity to signal, making your image brighter. This will also increase noise which can be reduced with scan speed/averaging adjustments.

5. Repeat for remaining channels.
6. Stop scan and move to 'Fine Tuning Image Quality' section.



Fine Tuning Image Quality

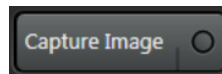
In order to find the optimal gain settings for a particular condition, you must first be able to identify the thresholds of the signal level in the image.

1. While scanning a live image, click Quick LUT and toggle to Glow mode (over & under) in the Image Display.

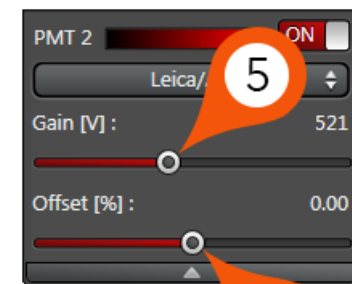
Note: Glow mode assigns a green color to underexposed areas in the image with a grey value of 0 (or less). It assigns a blue color to overexposed areas in the image with grey values >255 (for 8 bit).

2. Switch 'Visible' lasers toggle to OFF.
3. Adjust 'Offset [%]' for PMT to the minimum possible value so the image is just above the display of any green pixels.
4. Switch 'Visible' lasers back to ON state.
5. Adjust the 'Gain [V]' so that your brightest areas fall just below the overexposed limit (blue pixels).

6. Click 'Capture Image' to collect final scan.



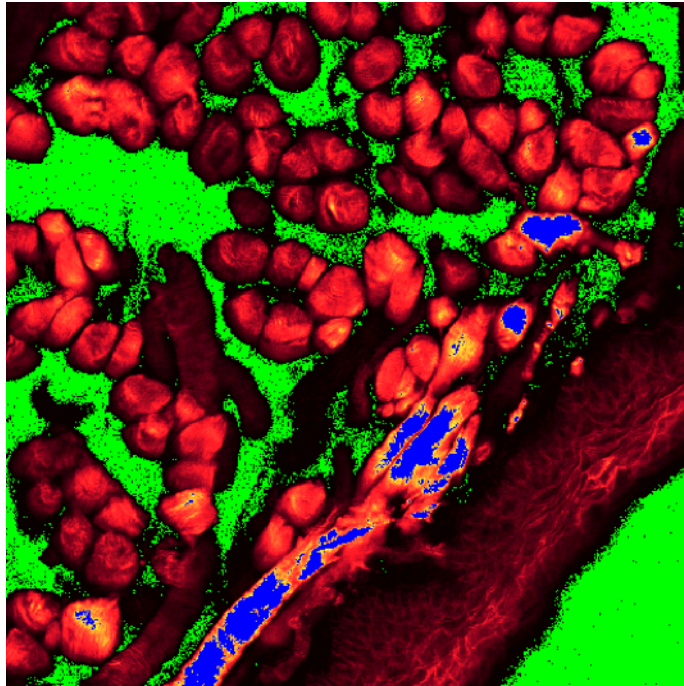
Note: Capture Image will scan with same scan speed and frame size as LIVE, and in addition it will apply any Line or Frame Averaging you have set to control image noise in the Scan Control settings.



Fine Tuning Image Quality

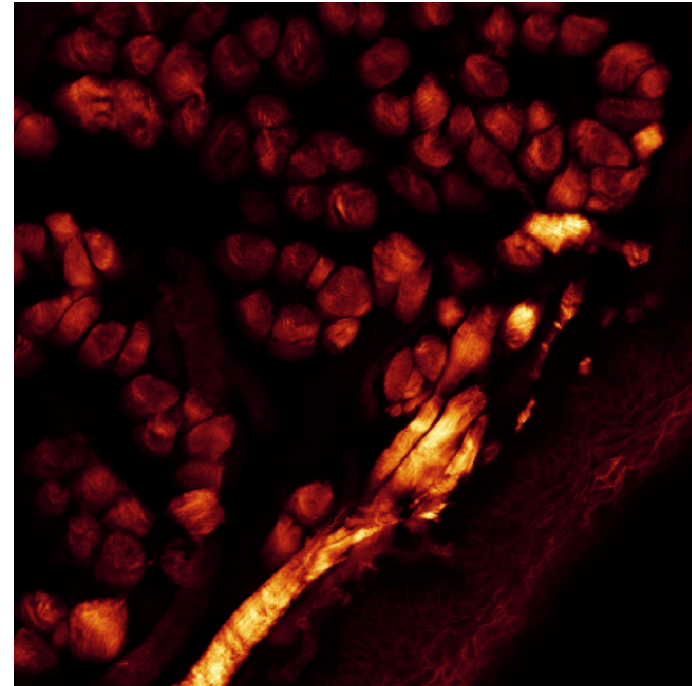


Ideally, you do not want an area in the final image to contain **blue** or **green** pixels with the Glow mode LUT. For maximum contrast, adjust the Gain so the brightest areas fall just below overexposure (**blue**) and the Offset so the background areas fall just above underexposure limit (**green**). In a properly balanced image you should see no **blue** or **green** pixels with this LUT.



Example of Overexposure (**blue**) and Underexposure (**green**)

Solution? Reduce Gain and increase Offset

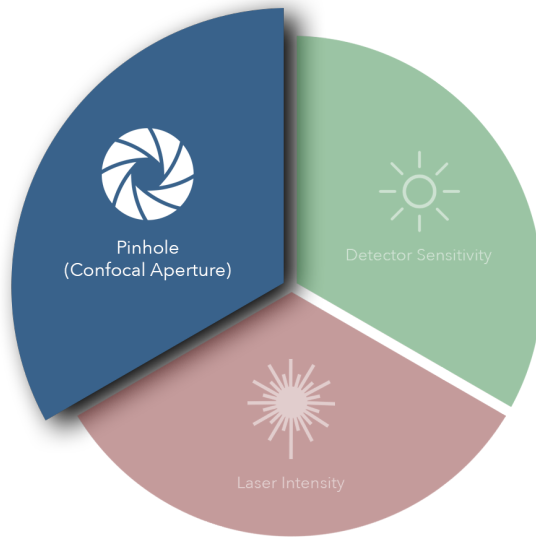


Example of Good Exposure

Understanding Image Quality

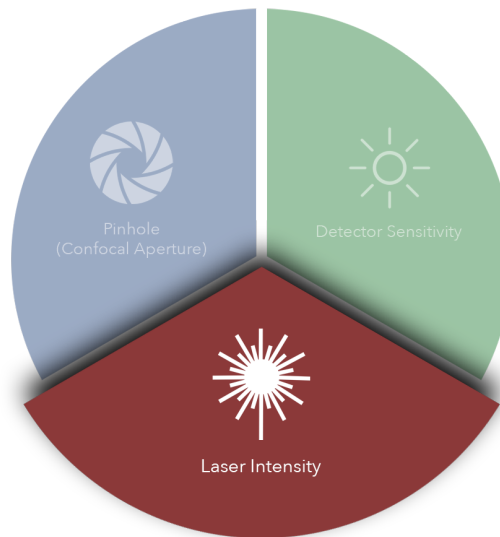


The result of the final scan above may or may not produce acceptable image quality. Therefore, it is important to have an understanding of the three key factors that play a role in image quality.



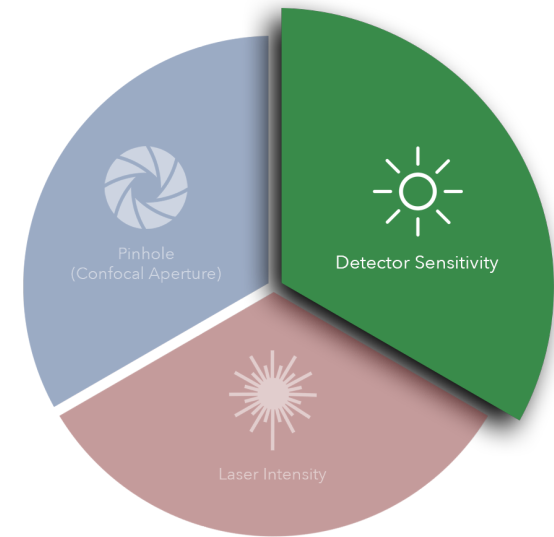
Pinhole (Confocal Aperture)

The confocal aperture controls both axial resolution and signal level. Opening the aperture reduces axial (Z) resolution but increases signal level reported to the detector. Closing this aperture will reduce the signal level but increase axial resolution.



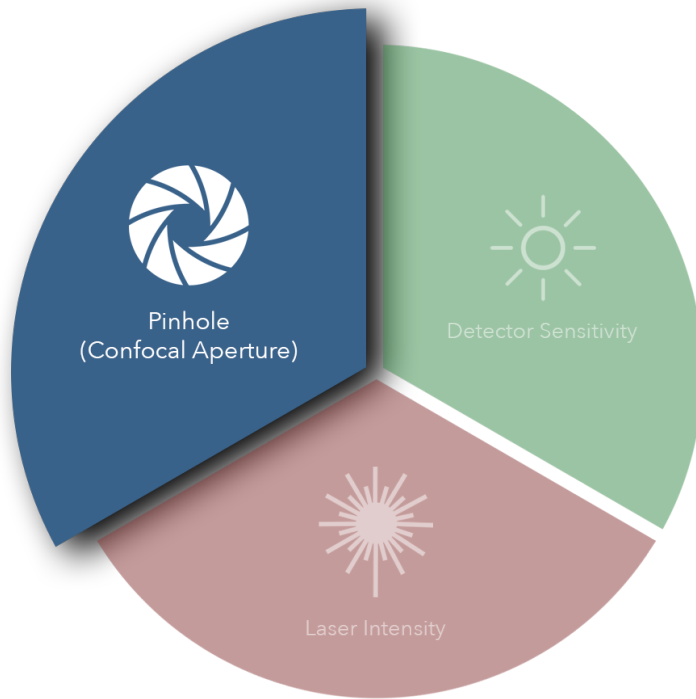
Laser Intensity

The intensity of the laser illumination source has obvious effects on the signal level. Increasing laser power will increase signal levels but may also introduce nonlinear effects such as phototoxicity and photobleaching.



Detector Sensitivity

The detector sensitivity is regulated by the high voltage gain applied to the detector. Increasing the gain directly increases detected signal but also amplifies the inherent noise in the system.



Pinhole (Confocal Aperture)

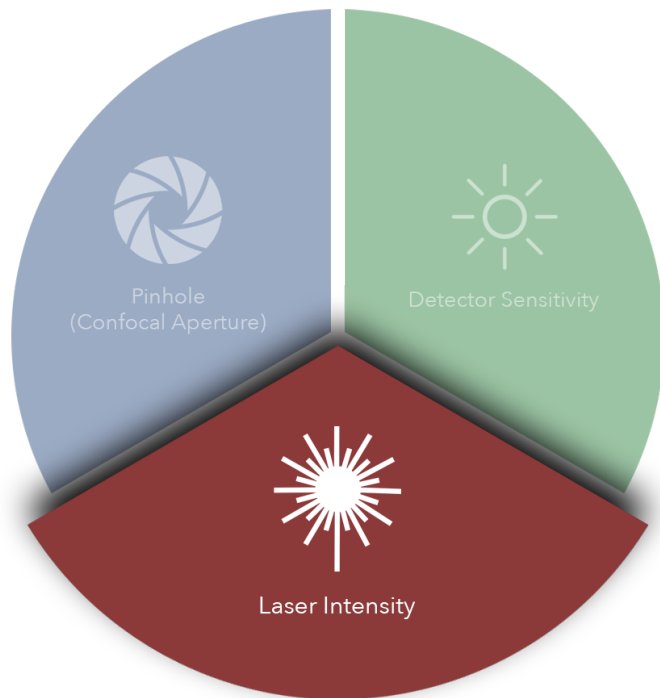
Ideally, the confocal aperture should be set to the size of the structure(s) you are trying to resolve. However, for example, some sub-cellular structures of interest may be beyond Abbe's diffraction limit and therefore beyond the microscope's capability to resolve. The confocal aperture has some practical limits that can be used to guide the usage of this setting.

Start by closing down the confocal aperture to '1 Airy'. You can certainly close the confocal aperture below this value and continue to improve resolution, just understand that below '1 Airy' you will lose signal level at an exponential rate.

Conversely, you can increase the confocal aperture diameter to improve the detected signal level if you can sacrifice axial resolution.

In some cases, the signal level may be so low that increasing the aperture diameter is the only way to lower the gain enough to get a usable image.

In other cases, if the gain is too high (causing excess noise) and the laser power cannot be increased due to photo effects then increasing the confocal aperture is the only option to improve image quality.

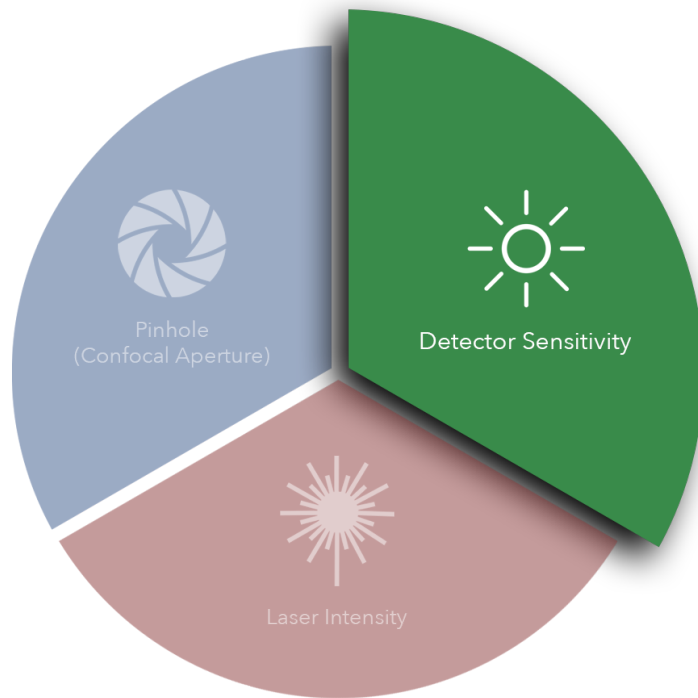


Laser Intensity

The overall power of the laser will directly affect image quality but most importantly it will have the greatest impact on the health of your sample and fluorophore(s). Increasing laser power will yield more signal but it will also induce negative photo effects such as phototoxicity and photobleaching that can harm the sample. Lasers also generate considerable heat when used at higher powers and that may have unforeseen effects on the sample.

For most lasers on this microscope, we recommend laser power settings between 1% and 5% to start. It is recommended to start as low as you can possibly go and still get an image. Then it can be increased as necessary to help balance image quality.

To discover your fluorophore(s) saturation level (i.e. how much laser power you can use before your fluorophore stops absorbing additional photons) you can start imaging at a low laser power and gradually increase the power slider until you reach a point where your image stops getting brighter. Continual increases in laser power will stop yielding more signal. That point is the practical laser power limit.



Detector Sensitivity

The sensitivity of the PMT is a function of the high voltage gain applied to it. The gain setting (as discussed elsewhere in this manual) controls the sensitivity of the detector to photons of light emitted from the sample fluorescence.

Increasing gain provides the most flexible and impactful way of improving image quality, but it has some drawbacks. The most obvious is when you increase the gain you also increase the noise in the resulting image. There are various forms of noise that we won't discuss here, but they are readily apparent when using a gain value $> 500V$.

Below 500V it is very difficult to detect much noise at faster scan speeds, however when using the detector above this value you will find that you will need to make some adjustments to reduce this.

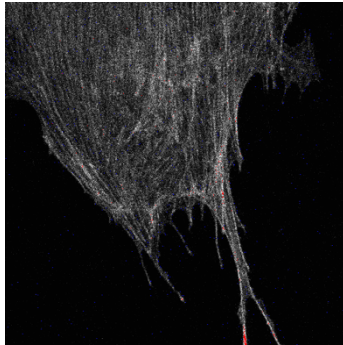
If laser power is high and the confocal aperture cannot be compromised there are a few methods to dealing with noisy signal due to high detector gain.

There are 2 main methods for dealing with high image noise. The first is reducing the scan speed to a slower rate. This will increase the pixel dwell time which will improve the signal-to-noise ratio. The second is signal averaging which will scan the same image multiple times and average the result to produce an image that has an improved signal-to-noise ratio.

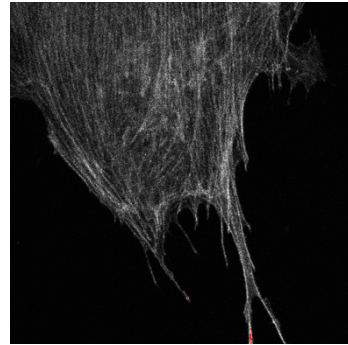
Understanding Image Quality



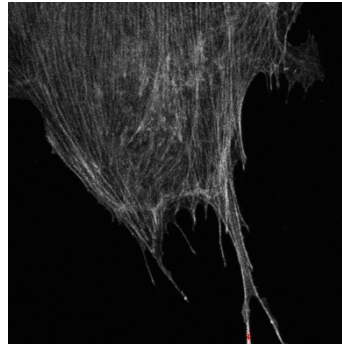
Depending on the quality of the signal level in the final optimized image, you may find that the image is somewhat noisy. To compensate for this, the system has averaging functions in the 'Acquisition Mode' tab (page 14) that can be used to clean up the image. Simply select the rate of averaging (1, 2, 4, 8, or 16x) and rescan.



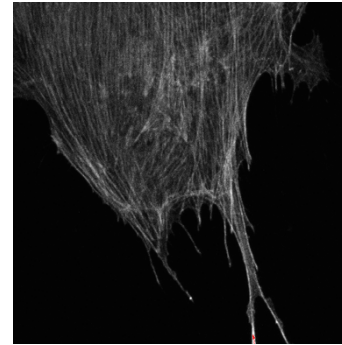
No Averaging



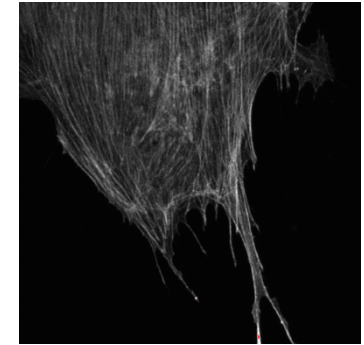
2x Averaging



4x Averaging



8x Averaging



16x Averaging

Note: Averaging signal will increase the acquisition time by the factor of averaging. For example, a 4x average will increase the time to scan the image by a factor of 4.

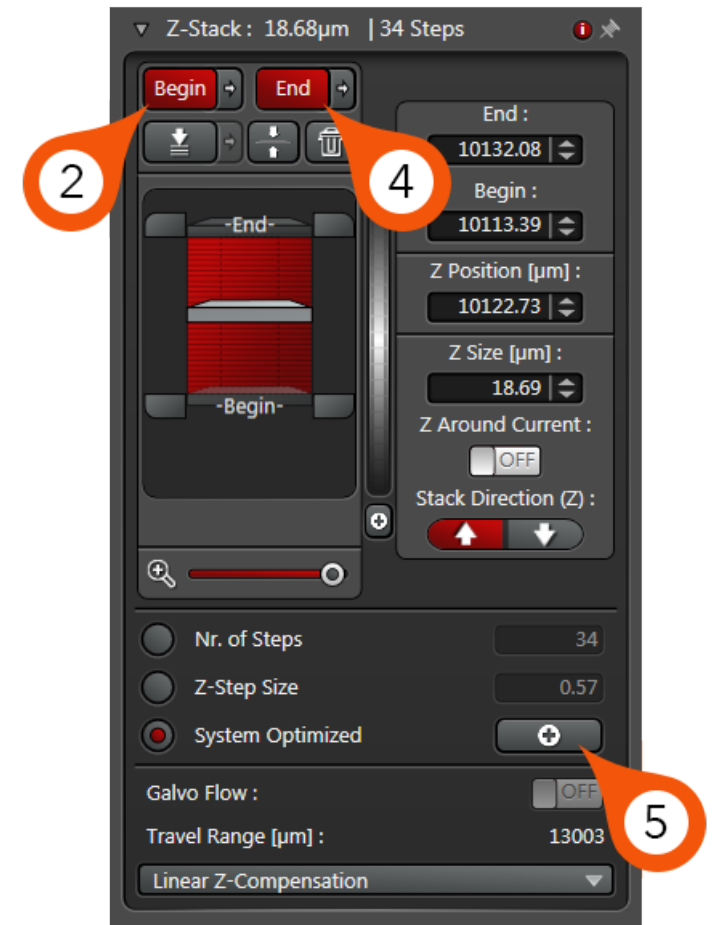
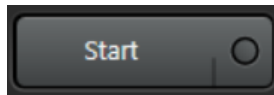
Collecting a Z-Stack

When selected on 'xyz' in the Acquisition Mode, you have access to the Z-Stack utility. Here we can define the settings required for 3d imaging in the Z (or focus) dimension.

1. While performing a LIVE scan, focus your sample in one direction (does not matter which) using the TFT focus knob until it goes almost completely out of focus.
2. Click 'Begin'. This will now mark the starting position for your Z-Stack.
3. Focus back through your sample until you reach a point on the opposite side where the sample begins to go out of focus.
4. Click 'End'. This will mark the end of the stack.
5. By default, the software sets the stack step size to 'System Optimized' using the objective and emission settings from the beam path configuration to calculate the ideal sampling size.

Note: Practical step size will depend on the level of detail required for the stack compared to the signal stability of the fluorescence. If the number of images required from using the optimized settings exceeds the balance between time and exposure, the user can reduce the number of slices manually by toggling to 'Z-Step Size' and increasing the value.

6. Click Start to begin Stack acquisition.



Collecting a Time Series

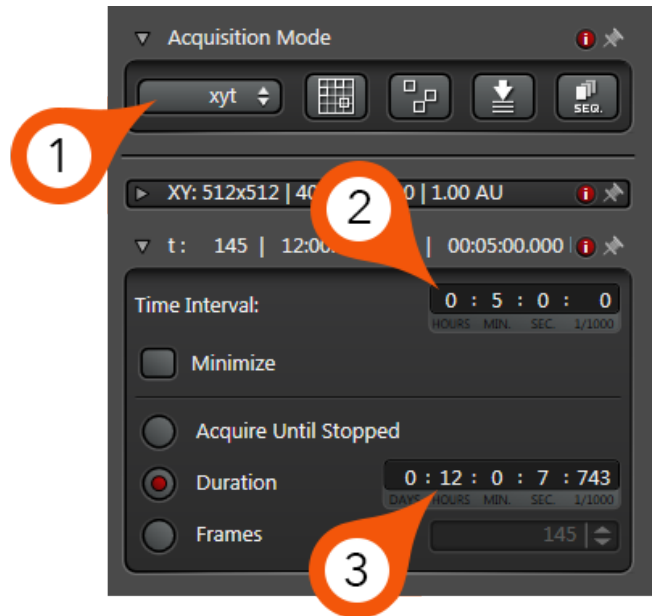
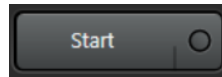


When selected on 'xyt' in the Acquisition Mode, you have access to the Time Series utility. Here we can define the settings required for time-lapse imaging.

1. In the Acquisition Mode - select 'xyt' for 2d time-lapse or 'xyzt' for 3d time-lapse.
2. Select waiting period between time points under 'Time Interval'. In this example, the interval is 5 minutes.
3. Select Duration and enter total time for experiment to run. In this example, the total duration is 12 hrs.

Note: The system will automatically calculate how many images it needs to collect over the interval/duration you set. You can also manually calculate this and entire the values accordingly.

4. Press 'Start' to initiate Time Series.



Saving Experiments

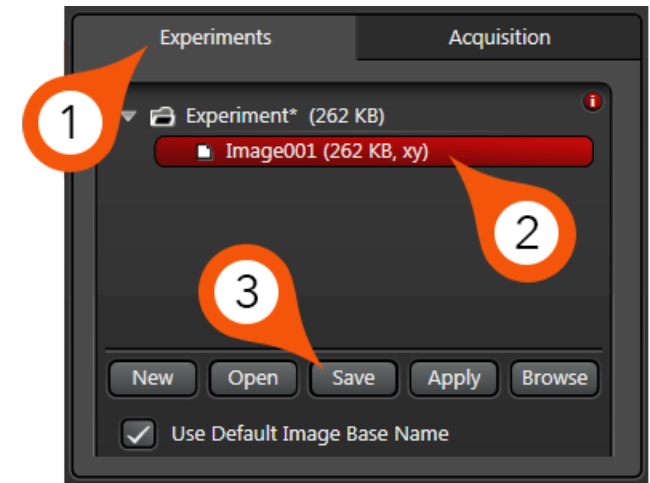


When your imaging is complete, you can save your data to the local hard drive in the Experiments tab. By default, the LAS software saves files in the LIF format, however you can choose the format type before storing data.

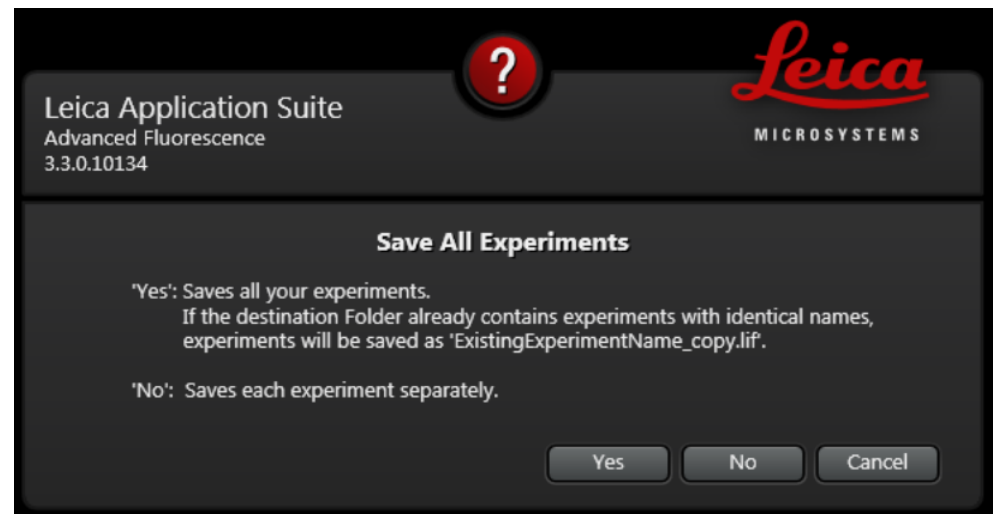
1. Select 'Experiments'.
2. Choose either entire Experiment or individual image.

Note: Selecting on the 'Experiment' folder header will save all images into a single LIF file for archiving. Selecting on each individual image will allow saving files separately.

3. Click 'Save' to store data to hard drive.



Note: After you click save the software will prompt you to choose if you want to save all open experiments to the same location or individual files. Generally, if you saving files one at a time you can click 'No' and save them separately.



Shutting Down System



1. Turn off Lasers via +.
2. Close LAS software.
3. Close APT software (if applicable).
4. Shutdown Windows OS.

Note: Please wait until OS has completely shut down before proceeding to steps below.

5. Power OFF MP-EOM Power Strip (see Power ON Routine – Step 3).
6. Turn OFF Laser Emission Key (see Power ON Routine – Step 2).
7. Power OFF Laser Power, Scanner Power & PC Microscope green switches (see Power ON Routine – Step 2).
8. Power OFF Epifluorescence Lamp (see Power ON Routine – Step 1).

If NLO was used – follow these additional steps:

9. Power OFF APT power strip (see Power ON Routine – Step B).
10. Turn the key on Chameleon TiS laser to 'Standby' (see Power ON Routine – Step A).