



Training Guide for Carl Zeiss LSM 5 LIVE Confocal Microscope

AIM 4.2



Optical Imaging & Vital Microscopy Core

Baylor College of Medicine (2017)

1



Verify that main power switches on the front of the laser module are turned to the ON state.

Note: These should always remain in the ON state however since system will not function if they are not - please check!

2



Turn ON 'Components' and 'System/PC' switches on remote paddle.

3



Turn ON X-Cite epifluorescence light source.

Wait for PC to boot into Windows OS and login to 'LSM User' account.

4



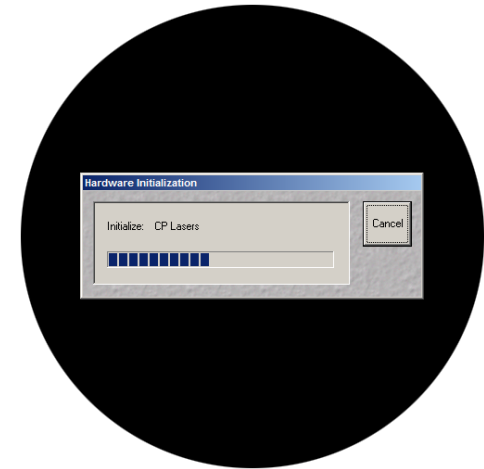
Double click 'LSM 5 LIVE' icon from desktop to start AIM software.

5



From the Switchboard, select toggle for 'Scan New Images' and then click 'Start'.

6

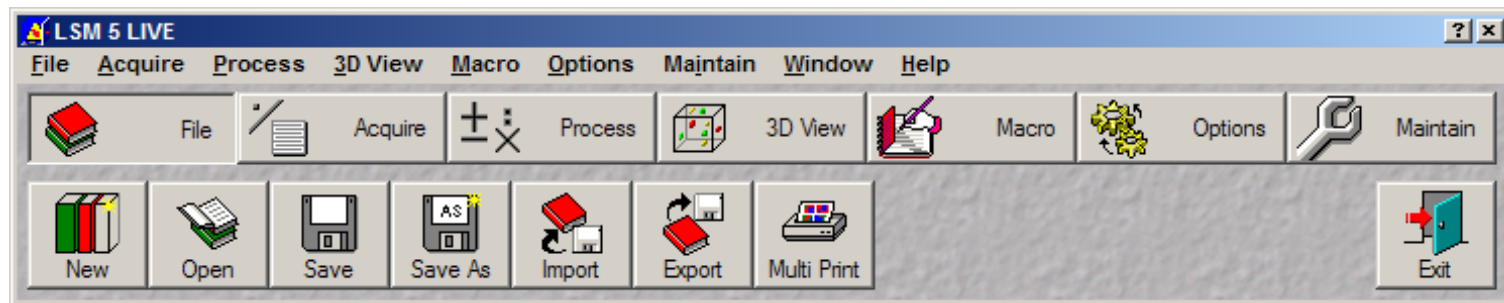


Wait for system to complete its initialization.

Getting Started with AIM 4.2

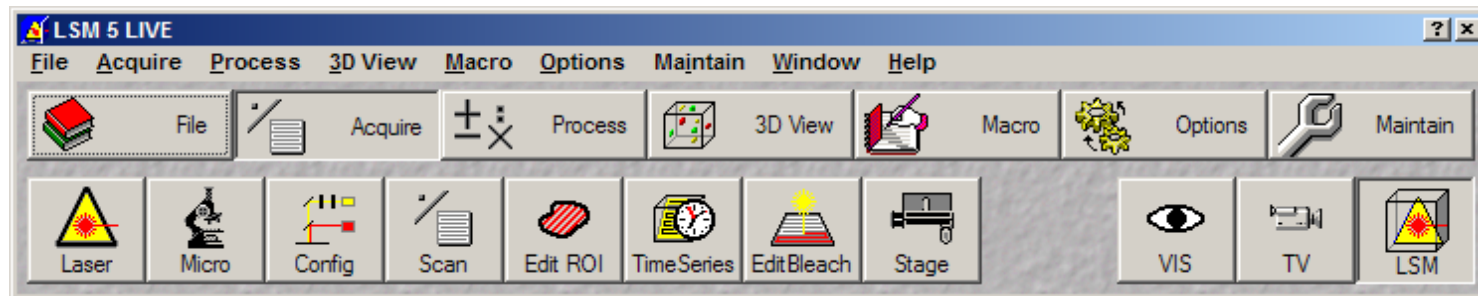


The AIM software is contained within the stand-alone toolbar illustrated below. The first two buttons along the top row of options are the main functions used for image collection. They are:



File

The File Menu contains tools necessary for creating image databases as well as importing/exporting native .LSM files to other formats such as TIFF or JPEG. By default, the AIM software saves natively scanned image data to the .LSM file format. The LSM file format is essentially a TIFF file, but the header of the file contains all the proprietary detail about the settings used to collect the image. When you save a file, not only is an LSM file saved but also a reference to a MS Access database, which defines the location where the image is stored as well as provides a catalog of images and a display of their information.



Acquire

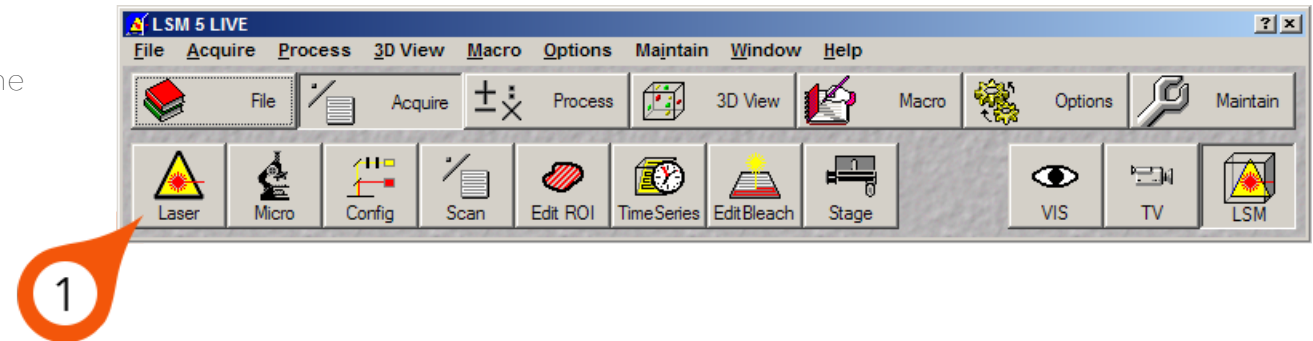
The 'Acquire' menu contains all the functions for defining your microscope settings, configuring your imaging conditions, defining your scan settings and collecting your image(s). It reads left-to-right in the order that you utilize the tools.

Power On Lasers

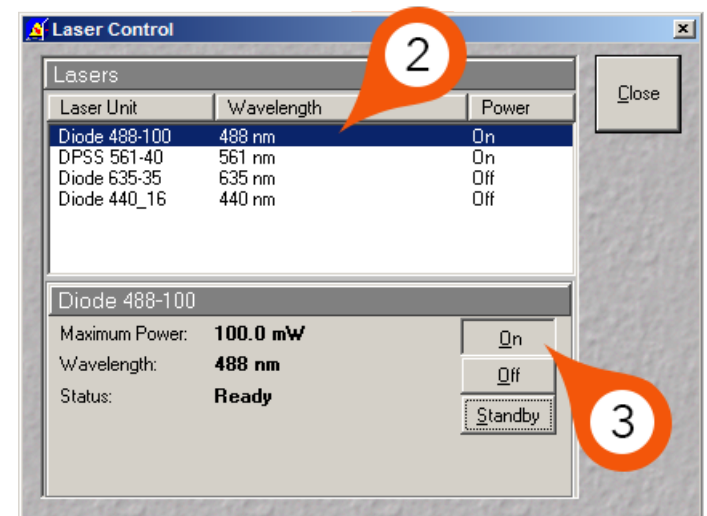


The first thing we need to do before we mount our sample or configure any settings is to power on all the lasers we will require for our experiment. Each laser on the LSM 5 LIVE is a diode or diode pumped solid state (DPSS) laser. These types of lasers have very simple power on/off routines.

1. From the Acquire mode on the main toolbar, click 'Laser'.

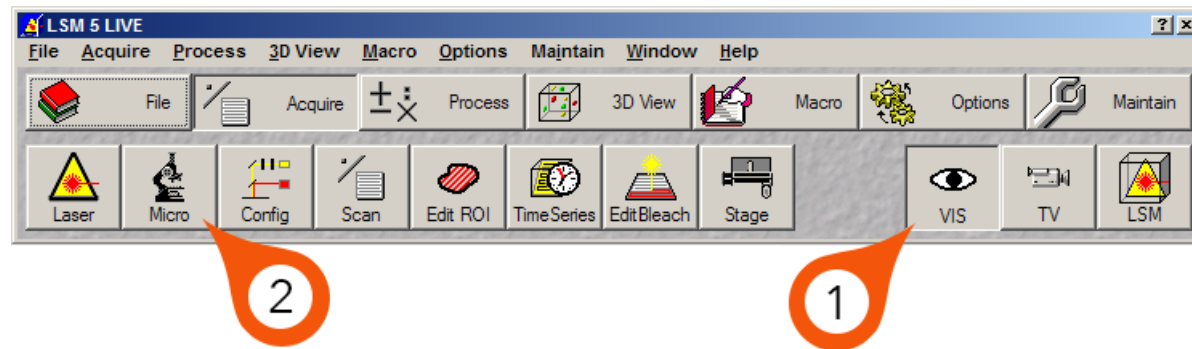


2. Select required laser from the 'Laser Control' dialog.
3. Click 'On'.
4. Repeat steps 2 and 3 for remaining required lasers.



Mounting Sample on the Microscope

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



1. From the Acquire menu on the main toolbar, switch microscope mode to 'VIS' from the default 'LSM'.

Note: Changing the microscope over to VIS mode will close the shutters for all laser lines and put the microscope in a state where you can visualize fluorescence in the eyepieces.

2. Click 'Micro' to open the Microscope dialog.
3. Mount your sample on the stage carrier.

Mounting Sample on the Microscope

- From the 'Microscope Control' dialog, use the shortcut buttons to turn on the fluorescence light path to desired color.

GFP - generic green filter set

DAPI - generic UV filter set

Cherry - generic red filter set

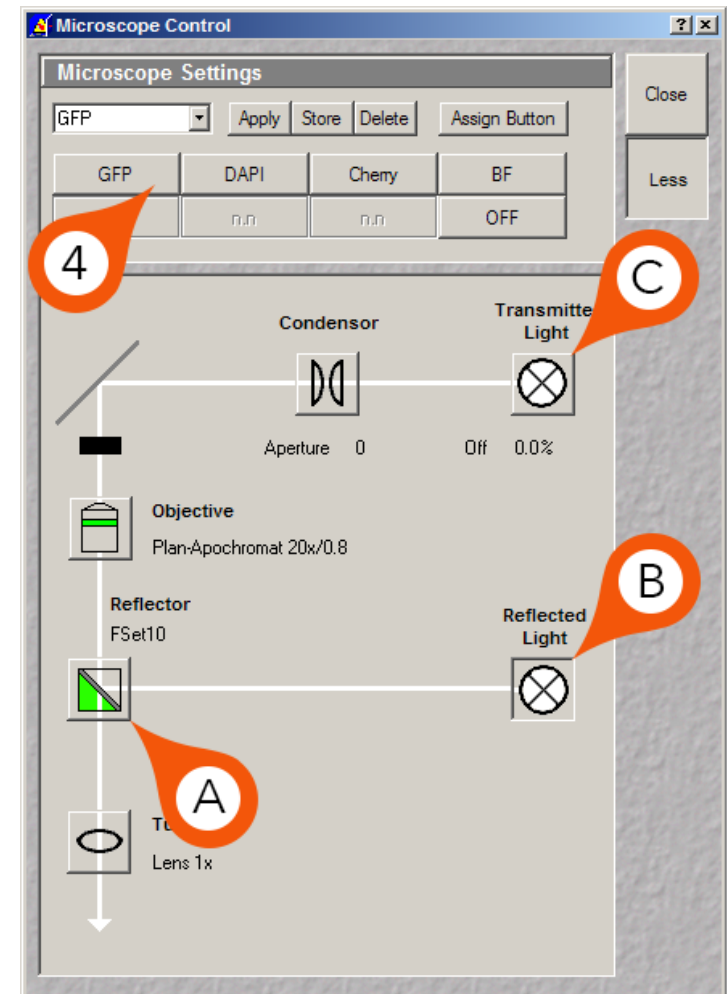
BF - brightfield (transmitted light)

OFF - all light sources off

Note: Alternatively, you can select the filter set (A) manually as well as open/close the epifluorescence shutter (B). There is also control over the transmitted light should you require this (C).

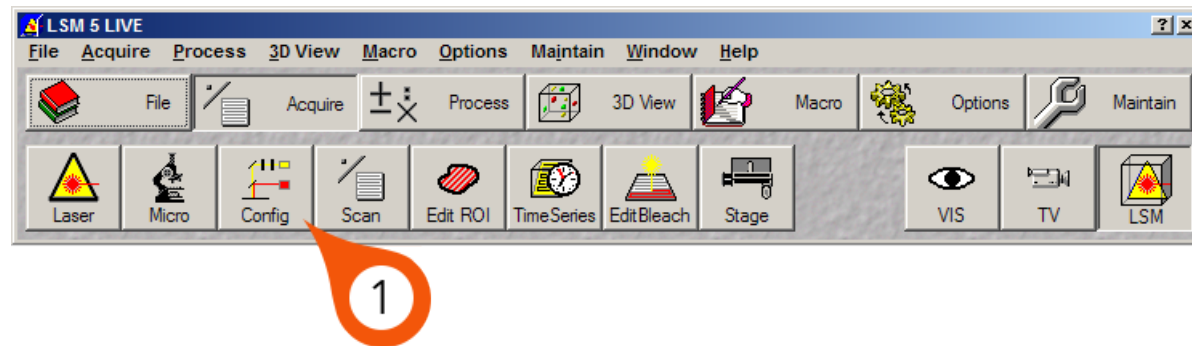
Note: If you open the Reflected Light shutter and no light comes through the objective, make sure to toggle 'Start/Stop' button on front of X-Cite power supply (see Power ON Routine - Step 3).

- Via the microscope eyepieces, visualize your sample and adjust the focus (Z) and stage position (X,Y) accordingly.
- Once your imaging location is found, place it in the center of the field of view.
- To begin imaging, switch back to 'LSM' from 'VIS' in the main toolbar.



Beam Path Configuration

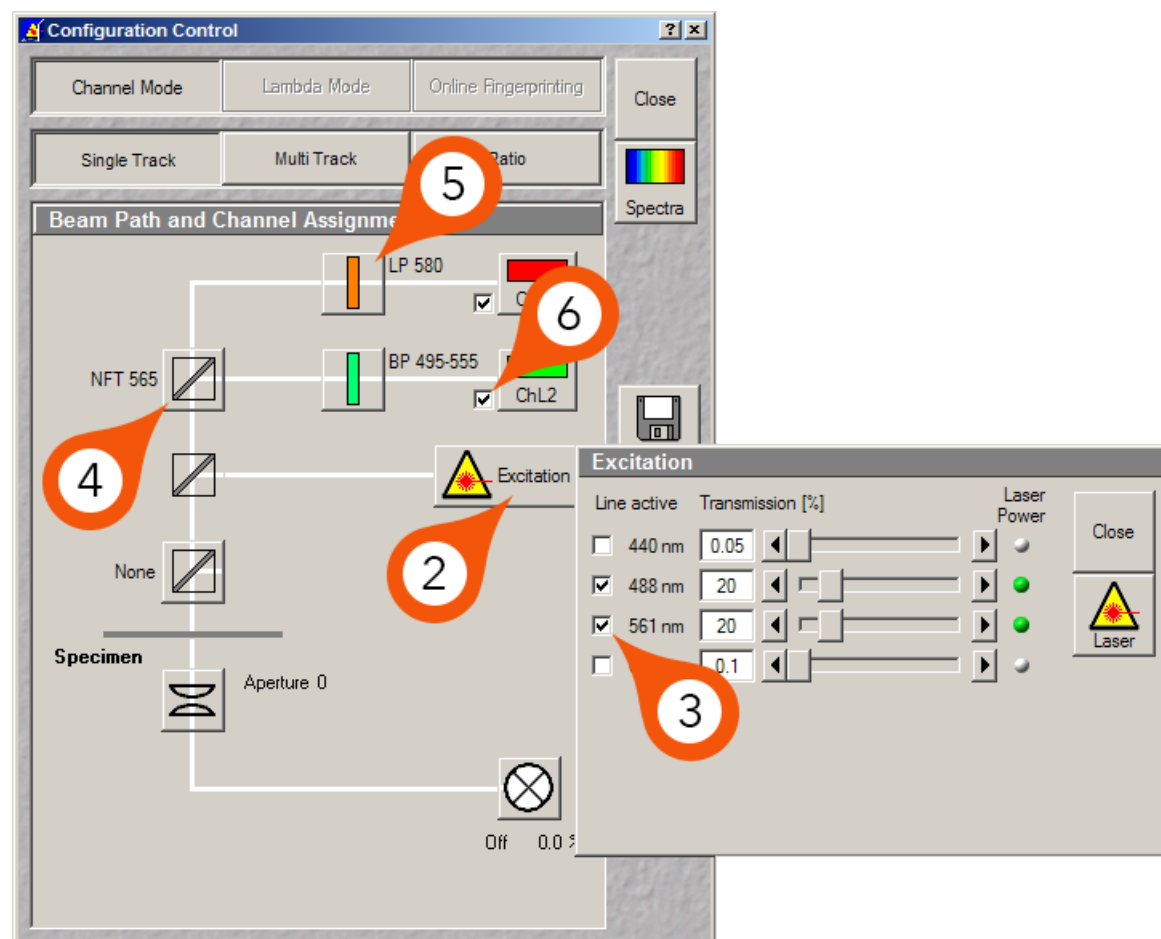
The LSM 5 LIVE is equipped with 2 detectors for 2 color simultaneous or sequential image collection. In this example, we will design a light path for imaging Alexa 568 and Alexa 488 simultaneously.



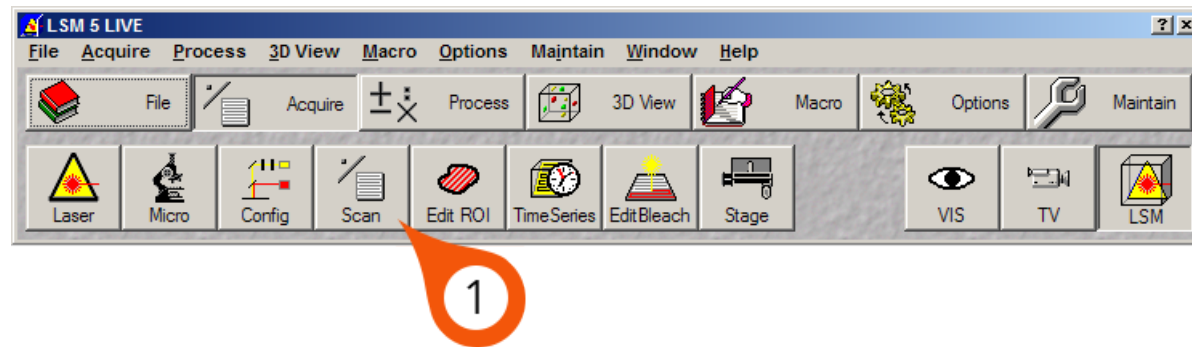
1. Click 'Config' from the main toolbar to open the light path configuration tool.

Beam Path Configuration

2. Click 'Excitation' to expand the laser properties.
3. Place a check in the box next to the laser wavelengths required and set a reasonable Transmission % (laser power). In this example, we will use both the 488nm and 561nm lasers each at 20% total laser power.
4. Select a beam splitter that falls between the emission bands required. In this example, we chose the NFT 565 beam splitter, which will reflect all wavelengths <565nm to ChL2 (for Alexa 488) and transmit all wavelengths >565nm to ChL1 (for Alexa 568).



5. Select an emission filter for each channel. In this example, we have selected a bandpass filter (that will only transmit light within the range of the filter) of BP 495-555 for ChL2 and a long pass filter (that will transmit all wavelengths greater than the cutoff number) of LP 580 for ChL1.
6. Place a check in the box(s) below the detectors required for imaging.



1. Once your configuration has been established, click on the 'Scan' button from the main toolbar.

The Scan dialog contains 2 areas, Mode and Channels, which are selected from the top of the dialog.

Mode

This dialog controls static image settings such as:

Objective – displays currently selected objective as programmed into microscope.

Frame Size – image size in pixels. Default is 512x512 however this can be optimized based on objective and zoom settings.

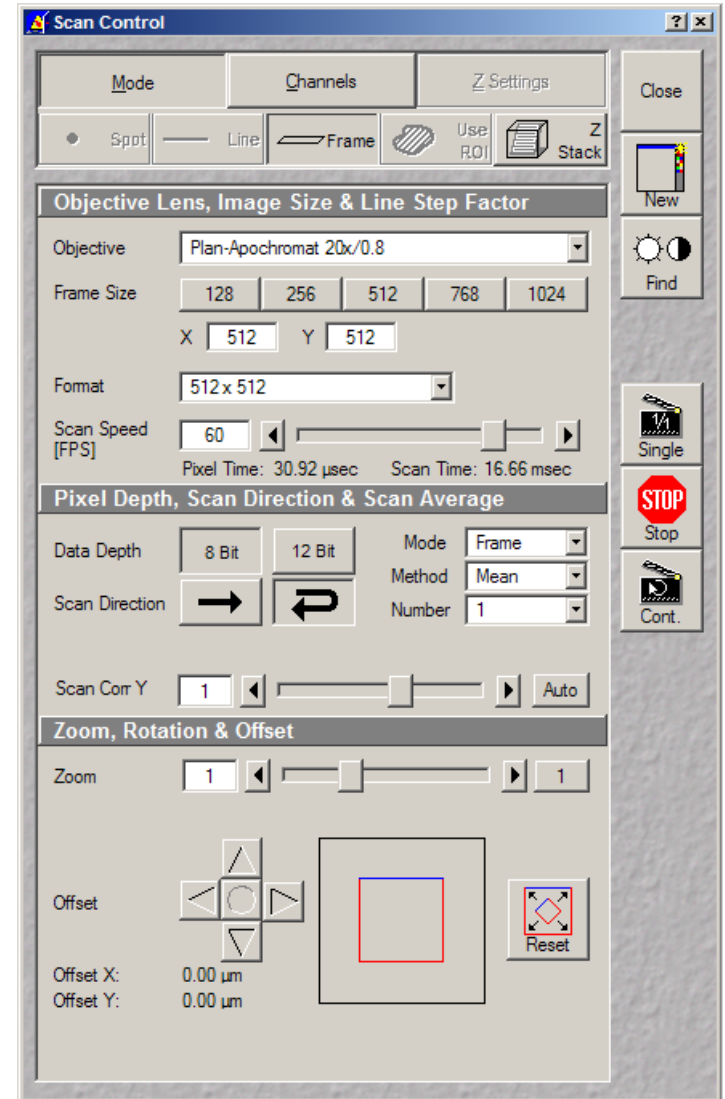
Note: Decreasing Y dimension will allow you to increase dwell time (to increase signal) while maintaining overall speed.

Scan Speed (FPS) – overall speed of the scan during acquisition in frames per second (FPS). Reports pixel dwell time and total scan time. Increasing dwell time (slower scan) increase the signal detected.

Scan Direction – for fast acquisition it is recommended to use the ‘bi-directional’ mode to avoid the longer scanner fly-back cycle time for uni-directional scanning. ‘Scan Corr Y’ will be required when using bi-directional scanning.

Note: Press ‘Auto’ to calibrate bi-directional scanning.

Scan Area – allows scan area adjustments such as X,Y displacement and Zoom to increase magnification without an objective change.



Channels

The Channels dialog controls the dynamic settings for image acquisition of each channel independently.

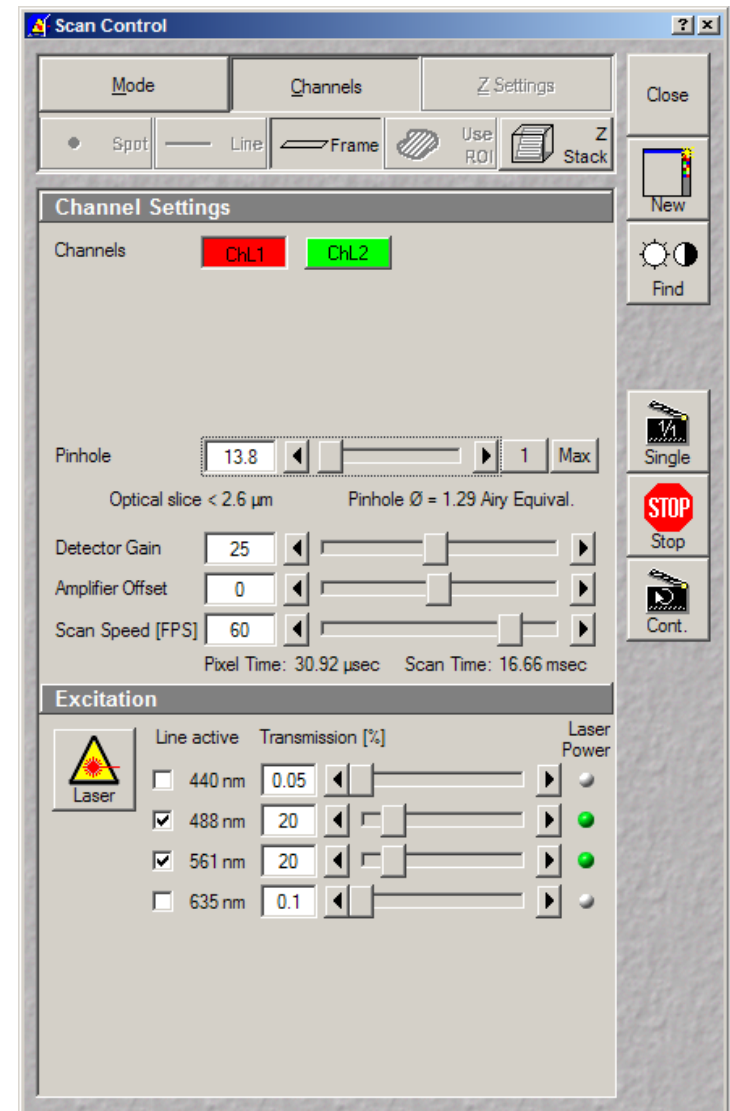
Pinhole – the confocal aperture on the LIVE is a slit configuration (in the shape of a line) which controls the optical section thickness. As a baseline starting point, press '1' to set the aperture to 1 Airy Unit. The larger you make this value the thicker your optical section will be.

Detector Gain – controls the digital amplification of the CCD array. The higher this value the more sensitive the detector becomes to signal AND noise, roughly amplified at the same rate. 25 is the default (no amplification).

Amplifier Offset – controls the dark current offset for the imaging system. When scanning an image with all lasers off this value set to '0' should report background grey values close to/at 0.

Scan Speed (FPS) – overall speed of the scan during acquisition. Reports pixel dwell time and total scan time. On the LIVE, we use scan speed to control not only the speed of acquisition, but it acts as a control for exposure time. Increasing dwell time (slower scan) proportionally increases the signal detected.

Excitation – laser line selection and relative % transmission (power). Check the box next to the laser line and set the Transmission % to 5-10% to start.



Scanning an Image



Once our configuration has been set up, we can scan a new image and adjust our signal level.

1. Click **1** to set the pinhole to 1 Airy Unit.

Note: Setting the pinhole to 1 AU is a compromise between axial resolution and signal level. Reducing the pinhole lower than 1 AU will linearly increase your Z resolution but at the expense of an exponential drop in signal.

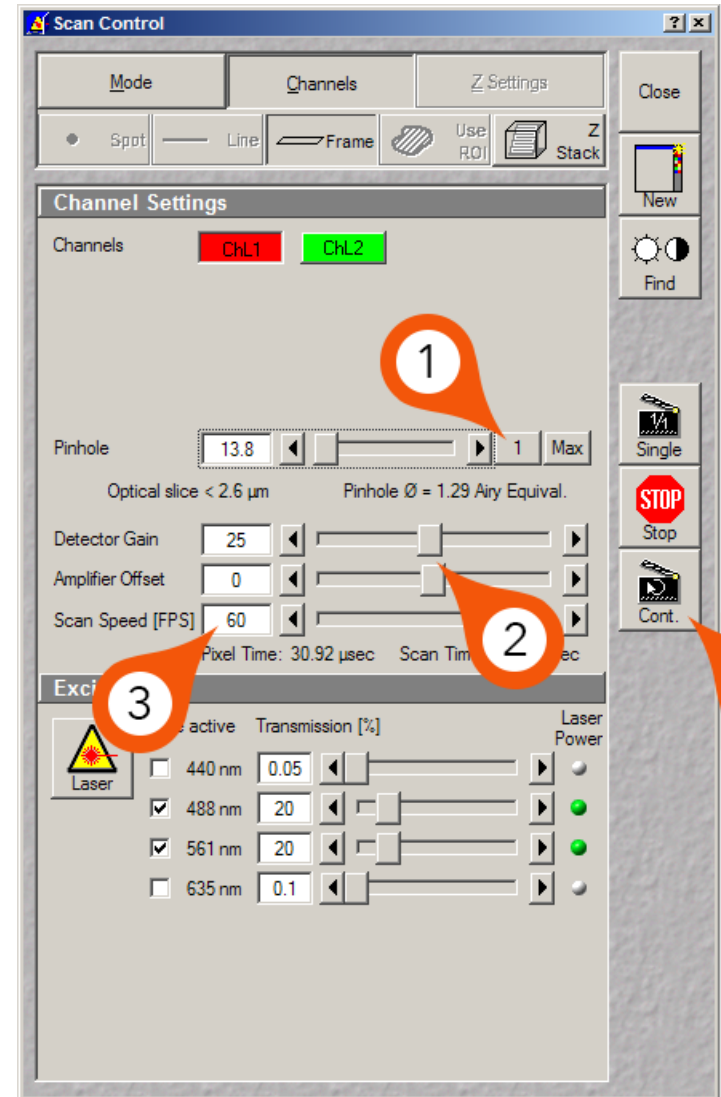
2. Begin with the Detector Gain set to '25' and the Amplifier Offset set to '0'.
3. Set Scan Speed to 10 FPS to start.
4. Click 'Cont.' to initiate a continuous scan.
5. Once you click either 'Cont.' or 'Single' a new image window will appear. Observe the image on screen.

Is the image on screen too dim (underexposed)?

Reduce the Scan Speed one step or increase the Laser Transmission by 5%. Repeat until a reasonable image is found.

Is the image on screen too bright (overexposed)?

Decrease the laser power by 5% or increase the Scan Speed one step. Repeat until a reasonable image is found.



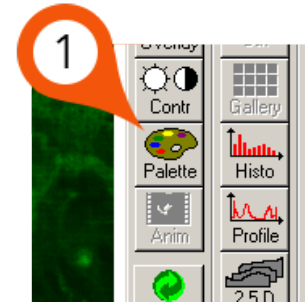
Fine Tuning Image Quality



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

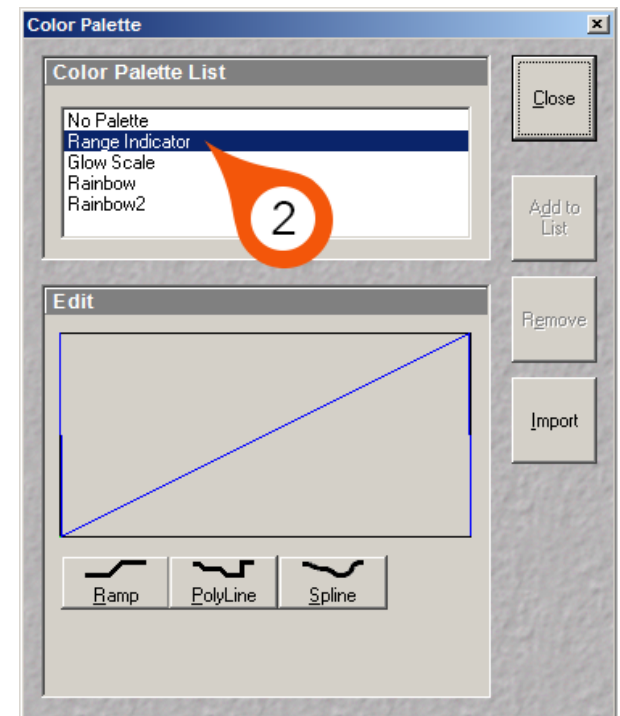
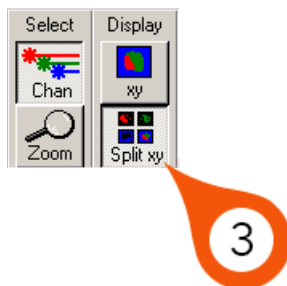
From the new image window that is generated with the scan, there are a series of tools along the right-hand side of the Image Display. Here you will find a button labeled 'Palette'.

In the Color Palette list, you will find 'Range Indicator' which is a lookup table (LUT) that assigns red pixels to areas in the image that are overexposed and blue pixels to background areas that are underexposed.



1. Click on 'Palette'.
2. Select 'Range Indicator' from the list of lookup tables (LUT). Close Color Palette dialog when selection is complete.
3. From the options along the right-hand side of the Image Display, choose 'Split-xy' to view each channel independently.

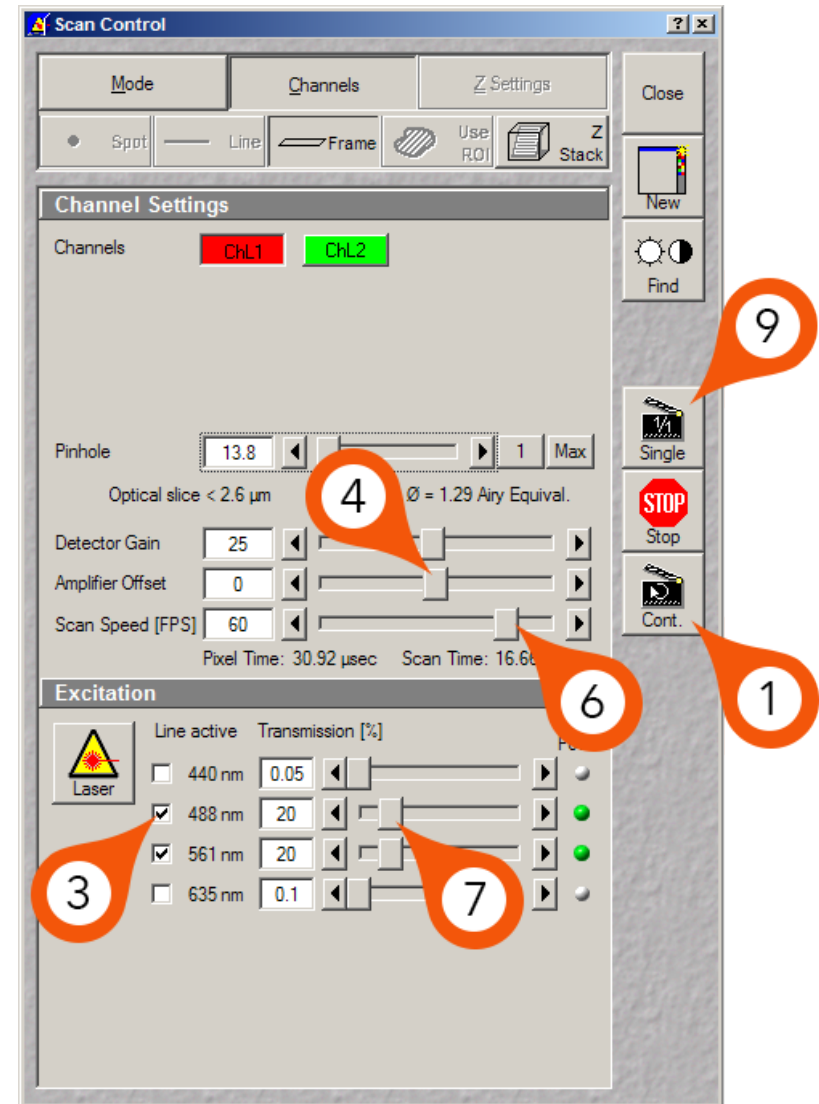
Note: Split-xy is the only way to view the range indicator table on images that have more than one channel.



Fine Tuning Image Quality

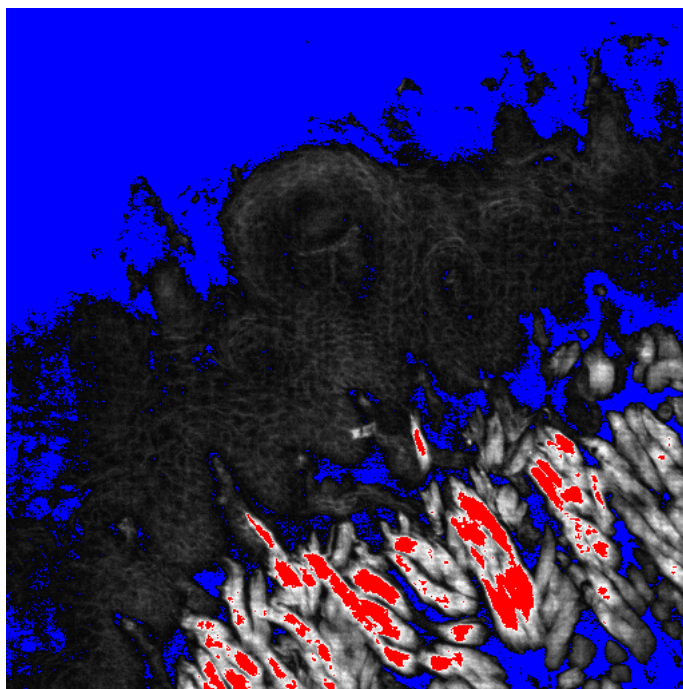
To fine tune the image intensity to ideal levels for final image acquisition use the following steps :

1. Click 'Cont.' to start scanning.
2. Turn on 'Range Indicator' LUT as shown above.
3. While still scanning, deselect laser checkbox.
4. Adjust the Amplifier Offset until background areas are just above the display of any blue pixels.
5. Activate the laser checkbox to re-enable the laser line (3).
6. If the image is too dim, decrease scan speed until you have some areas in the image that are overexposed (red). Fine tune this further by then reducing the laser power in 1% increments until brightest areas in the image are just below the display of any red pixels.
7. If the image is too bright, reduce the laser power in 1% increments until brightest areas in the image are just below the display of any red pixels. If you still have red pixels at the lowest laser power settings then increase the scan speed.
8. Stop Scan.
9. Click 'Single' to collect final image.

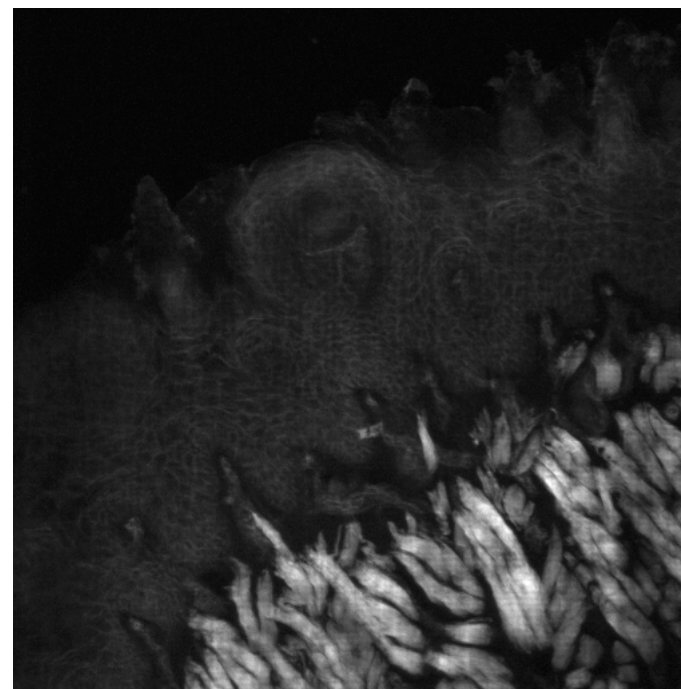


The LSM 5 LIVE system is a bit different from traditional point scanning confocal microscopes whereby the Detector Gain does not play much of a role in image optimization. The Detector Gain setting on the LIVE is a digital control, essentially amplifying signal and noise at roughly the same rate. A setting of '25' is no gain applied to the CCD. You can use this to fine tune the overall intensity range however it often is more effective to try and use laser power/scan speed for this.

Ideally, you do not want any area in the final image to contain red or blue pixels with this LUT. For maximum contrast, have the brightest areas fall just below overexposure (red) and the background areas fall just above underexposure limit (blue). In a properly balanced image you should see no red or blue pixels with this LUT.

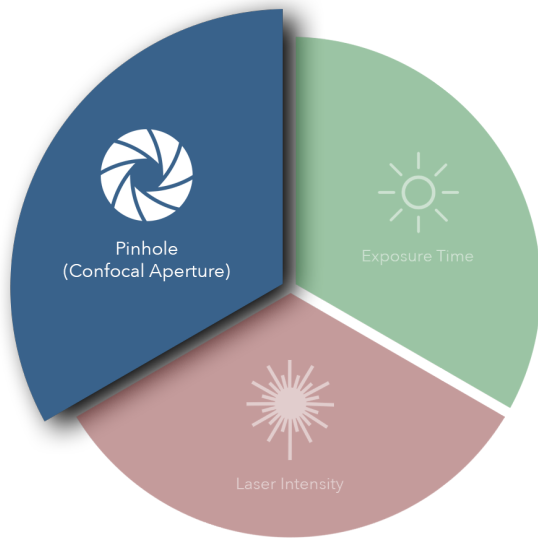


Example of Overexposed/Underexposed Areas in Image



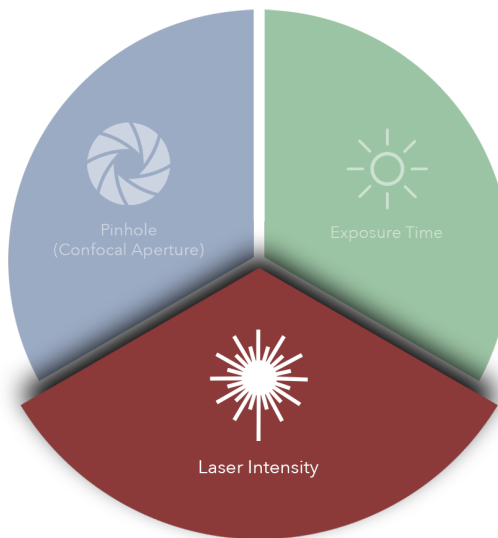
Example of Properly Exposed Areas in Image

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.



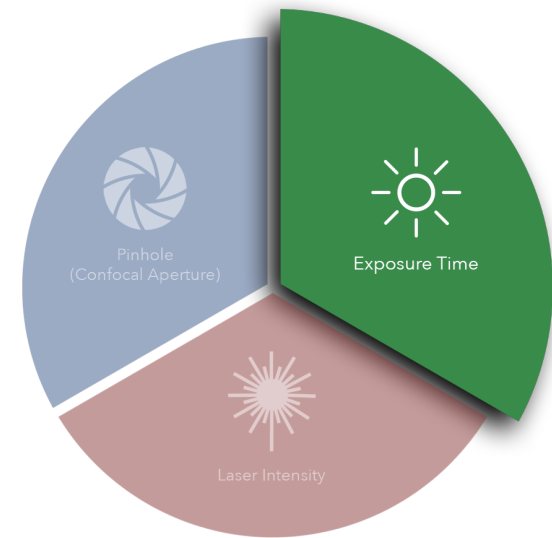
Pinhole (Confocal Aperture)

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.



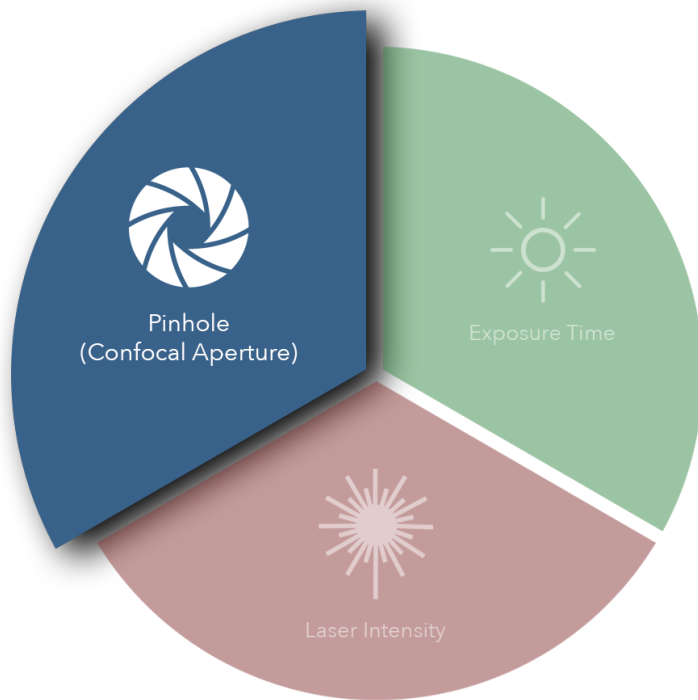
Laser Intensity

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.



Exposure Time

The detector sensitivity of the CCD is largely fixed. There is a gain adjustment that can be applied to the CCD, however the best way to increase signal is to increase exposure time. This is regulated by Scan Speed on the LSM 5 LIVE.



Pinhole (Confocal Aperture)

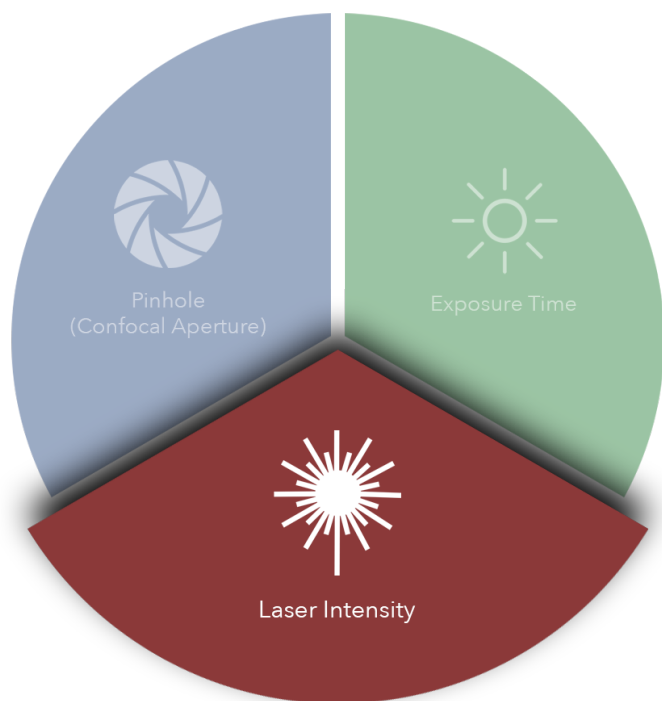
Ideally, the confocal aperture should be set to the size of the structure(s) you are trying to resolve. However, for example, some sub-cellular structures of interest may be beyond Abbe's diffraction limit and therefore beyond the microscope's capability to resolve. The confocal aperture has some practical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

Start by closing down the confocal aperture to 1 AU. You can certainly close the confocal aperture below this value and continue to improve resolution, just understand that below 1 AU 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.

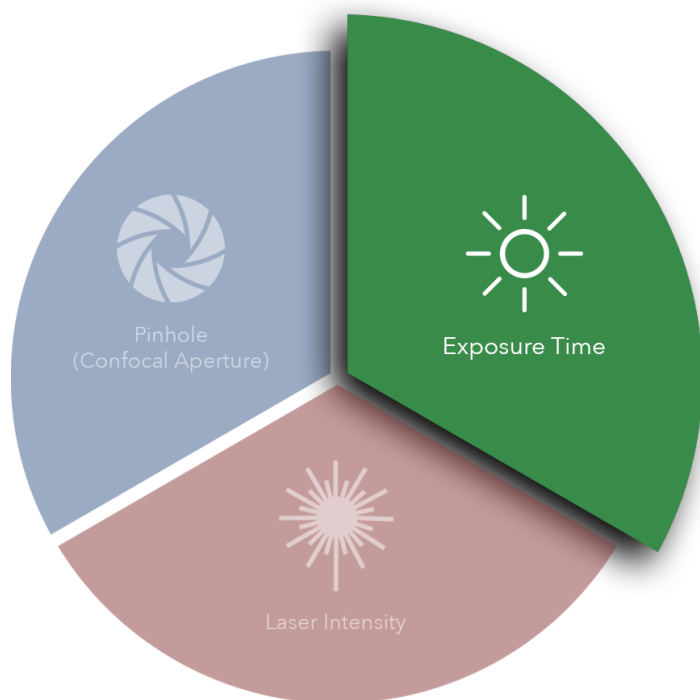


Laser Intensity

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 10% and 20% 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. The point where additional laser power stops increasing signal intensity is the practical laser power limit for that particular fluorophore.



Exposure Time

Exposure time on the LSM 5 LIVE is the most aggressive method for adjusting signal level. The longer the exposure time (slower the scan), the more signal the CCD can integrate thus increasing your signal.

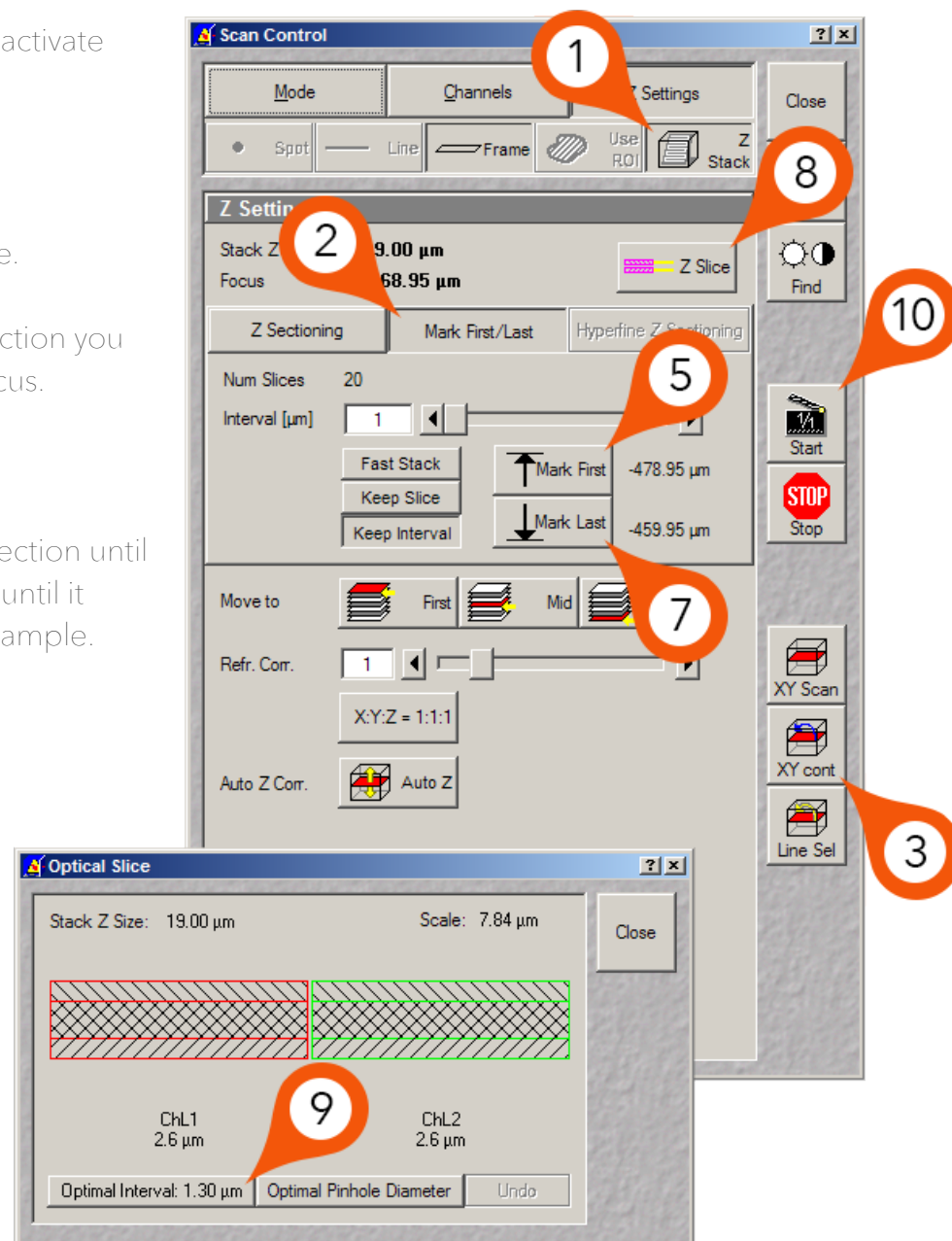
Exposure time is regulated by 'Scan Speed' on this system.

It is recommended to start at 10 FPS and adjust signal from there.

Collecting a Z-Stack

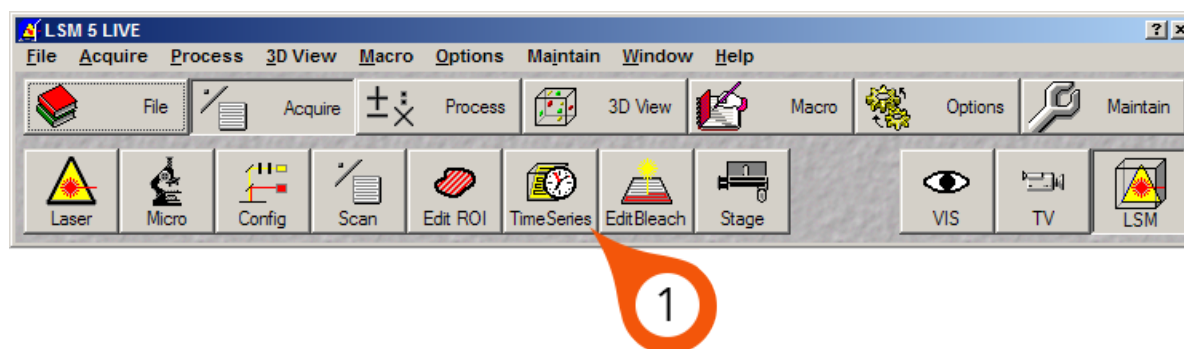
In order to collect serial sections (Z) we need to first activate the Z-Stack tools in AIM from the Scan button on the main toolbar.

1. Click 'Z-Stack' from top of the Scan Control dialog. This will activate the Z Stack settings.
2. Select 'Mark First/Last' from the Z options.
3. Click 'XY Cont.' to begin scanning an actively updated image.
4. Focus the drive in one direction (does not matter which direction you start with) until the image of your sample just goes out of focus.
5. Click 'Mark First' to mark the start position.
6. While continuing to scan, focus the drive in the opposite direction until your sample comes back into focus, then continue focusing until it goes out of focus again. This time on the other side of the sample.
7. Click 'Mark Last'.
8. Click 'Z Slice' to open the slice thickness dialog.
9. From the Z Slice dialog, click 'Optimal Interval' to set the interval size to be equal to $\frac{1}{2}$ the optical section thickness.
10. Click 'Start' to initiate your Z-Stack.



Collecting a Time Series

In order to collect a time series (T), we need to first activate the Time Series tools in AIM from the main toolbar.



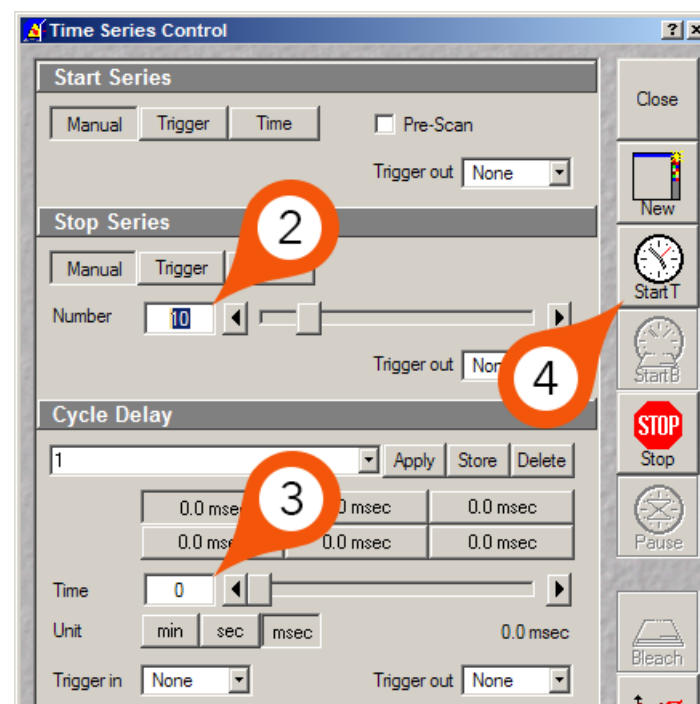
1. Open the 'Time Series' dialog from the main toolbar.

The default mode for 'Start Series' and 'Stop Series' is Manual, which means that the Time Series will start immediately when the user clicks 'Start T' and end when the 'Number' of images has been scanned.

To calculate the required number of time points, divide the duration you require for the series by the interval.

For example, if we wish to collect for 6 hours in total with an interval of 5 minutes between images we would use the following equation $(6 \times 60) / 5 = 72$ images.


2. Adjust 'Number' of images to match your required calculations.
3. Adjust 'Time' Interval to match your required calculations.
4. Click 'Start T' to begin collecting the time series.



Saving Images



Once your image collection is complete you can save your data to the local hard drive.

1. From the Image Display window, click 'Save As'  in the lower right corner of the image. This will open the 'Save Image and Parameter As' dialog.
2. From the Save dialog, select a database from the list of recently opened databases.

Note: If you have not yet created a database click 'Create New Database' and navigate to D:\ and create a folder with your name. Give your database a name and click 'Create' to save the newly created database into your folder.

3. Give your file a Name and enter any description or notes you wish to have saved with the file and click OK.

Note: The image file is saved into the same location as the database by default.

Note: The default format for the AIM system is LSM 5. This file format is essentially a TIFF file that contains all the requisite system information in the file header. This will allow functions such as 'Reuse' to work correctly.

1. Open the Laser Control dialog (See page 6, Power On Lasers) and select each laser you have powered on and turn them off.
2. Close AIM application.
3. Exit from Switchboard.
4. Shut down Windows operating system.

Note: Please wait for operating system to shut down completely before proceeding to next steps.

5. Turn off X-Cite epifluorescence lamp (see Power On Routine Step 3).
6. Turn off 'Systems PC' and 'Components' power switches (see Power On Routine Step 2).