User Guide to the IBIF Leica TCS SP8 MP Confocal Microscope

This version: 1.5.17.

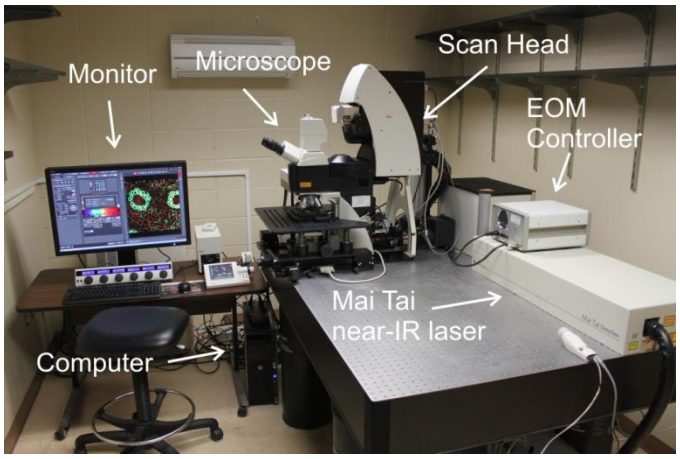
Introduction

The IBIF Leica confocal microscope is made available on a fee-for-use-hour basis to all users who have been trained. It has a number of hardware features, specifically the objectives, the stage, and the condensers, that can be added or removed by the user, with the permission of the Imaging Specialist. To minimize inconvenience to other users, and to reduce the risk of inadvertent damage to the equipment, we ask that users obey two rules:

1) Leave the confocal as you found it. Replace and/or reset everything that you modified.
2) Be aware that other users may have failed to obey rule 1. Be alert, especially in regard to mechanicals such as stage translation, condenser height, and objective rotation.

Thank you, and we wish you every success in your imaging. We are here to help.

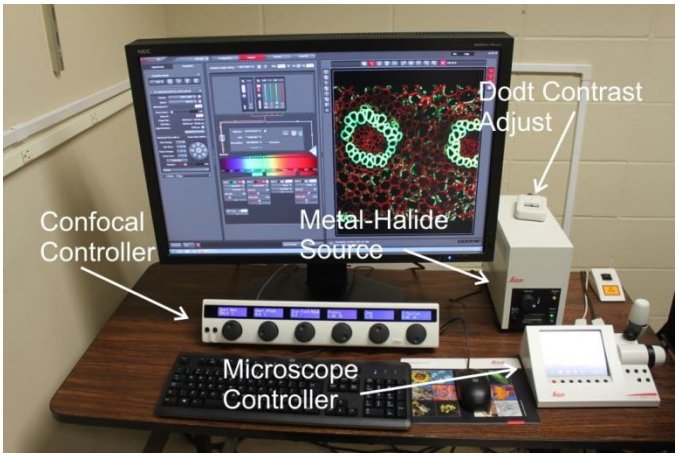
The Confocal Microscope and Accessories

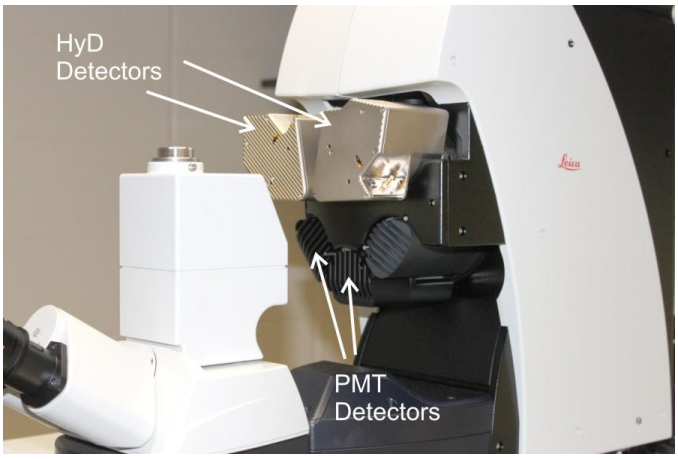


The instrument is a Leica TCS SP8 MP confocal microscope, equipped for multi-photon confocal microscopy. The components are the microscope body, two automated stages, the scan head, the electro-optical modulator (EOM), the monitor and computer, and the Spectra-Physics Mai-Tai tunable near-IR laser.

The items on the desk (along with the monitor, keyboard, and mouse) consist of a confocal controller, a microscope controller, a metal-halide power source for inspection of the specimen via the eyepieces, and a controller for the Dodt contrast enhancement system (which replaces the Nomarski contrast enhancement method of the previous system).

The unlabeled switch is a shutter for the Mai Tai laser and should only be needed in emergencies in which the beam is exposed.



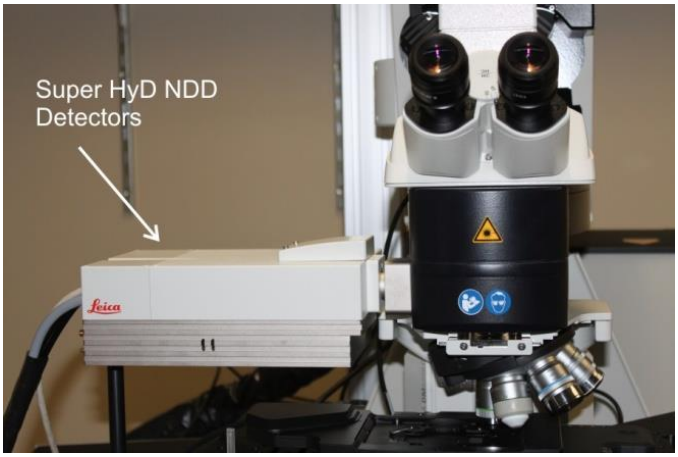


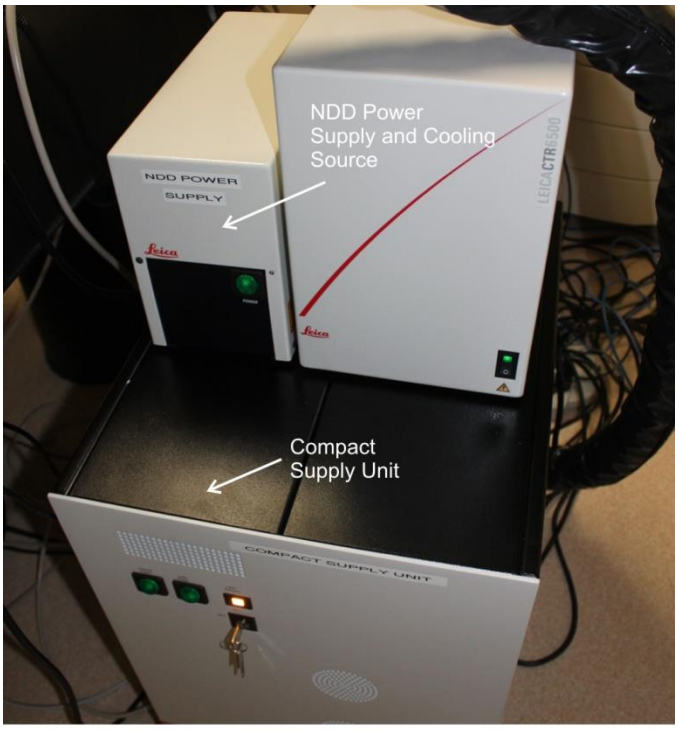
Two photomultiplier tube (PMT) detectors and two hybrid-PMT (HyD) detectors are available for conventional (single-photon) confocal microscopy.

Some cell-phone carriers use frequencies that interfere with the HyD detectors. Switch your phone off or leave it at the front of the room.

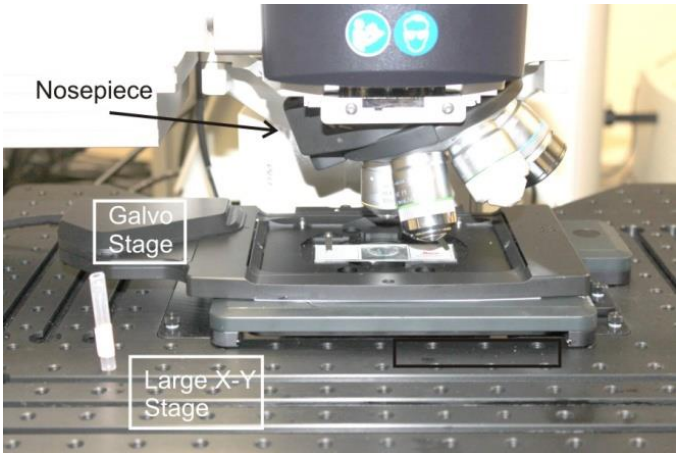
Two high-sensitivity Super HyD detectors are installed for non-descanned (multi-photon) confocal microscopy. These detectors are electrically- and liquid-cooled for decreased background. They should only be used in a darkened room and with the microscope covered by a dark room cloth (available).

The Super HyD detectors are also vulnerable to cell-phone interference. Please turn off your cell phone or leave it at the far end of the room, close to the door.





Behind the air table at the rear are three boxes of instrumentation. The lower box is the Compact Supply Unit, which houses the visible light lasers. The smaller of the two boxes on top of the Compact Supply Unit is the power supply and cooling unit for the Super HyD non-descanned detectors.



The microscope has no manual focusing adjustment. Instead, it has two stages and two computer-controlled focusing mechanisms. The large X-Y stage performs lateral translation in all modes. The nosepiece has its own focusing drive, controlled from the microscope controller, or the confocal controller, or from the software.

The smaller Super Z Galvo stage rides on top of the large stage and includes the slide holder. It is used for stacks. The large translation stage can be used without the super X Galvo stage, primarily for whole animal work or specialized experimental chambers. *Contact us for instructions on how to do this.*

System Specification

Lasers

The system has four visible-light lasers: 405 nm, 488 nm, 514 nm, 552 nm, and 633 nm. The 405 nm should be used to excite DAPI or similar blue-emitting dyes, therefore use of the multi-photon laser will not be necessary. The 488 nm and 552 nm lasers are for FITC and rhodamine bands respectively. The 514 nm laser can be used to excite YFP, while the 633 nm laser is for longer wavelength dyes such as Cy5. All of the lasers are semiconductor types and can be switched on and off without special care.

All of the lasers are very powerful (20 mW or more). Users will find that only a few percent excitation is required in most circumstances.

Optics

The available objectives (illustrated above, from left to right) are as follows:

Position	Objective	Magnification	n.a.	Working distance (mm)	Medium
1	HC PL Fluotar	10x	0.3	11.0	air
2	HC PL Apo	20x	0.7	0.59	air, coverglass
3*	IRAPO	25x	0.95	2.5	water
4	HC PL Apo	40x	1.30	0.24	oil
5*	IRAPO	40x	1.10 Corr	0.65	water
6	HC PL Apo	63x	1.40	0.14	oil

*available on request

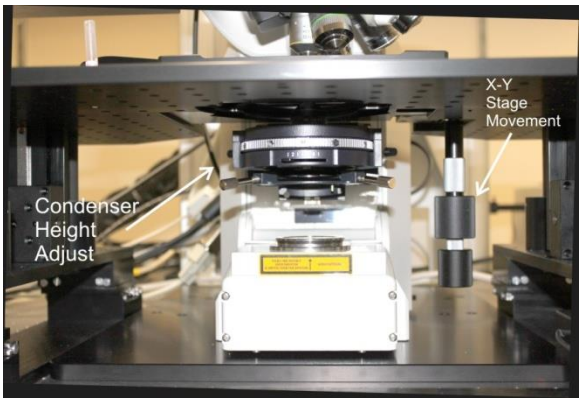
The air and oil objectives (1, 2, 4, and 6) are similar to those you have used in the past. The two water objectives are especially good for deep-tissue imaging and are corrected for multi-photon excitation. *Note: the 40x and 63x objectives have spring-loaded front ends that can be inadvertently twisted and locked in the withdrawn position. Handle them with care.*

The two IR APO objectives are for use as water-immersion objectives, typically for multi-photon studies. They are locked up and can be used only by prior arrangement.

Disk Drives

The computer has two drives, a 120 Gb solid-state drive (C:) and a 1.8 Tb disk drive (D:). All data should be saved to the D: drive.

Condenser



A transmitted light condenser is available on request if high-quality visualization of unstained tissue or cells is required. However, there is enough unfocussed light available in transmitted light mode to enable simple localization of specimens without a condenser. The Imaging Specialist will demonstrate the use of the condenser if requested.

The condenser has a short working distance (1.0 mm) and very few options. Condenser centering is via two screws, as in Zeiss microscopes. The condenser focus knob is to the left side. The condenser cannot normally be brought into focus, but as long as it is properly centered it will function satisfactorily for the acquisition of transmitted light images. The Dodt contrast system does not use the condenser.

If you have brought the condenser near to focus (for example, if trying to visualize unstained cells), please remember to lower it before using the large X-Y stage, otherwise damage to the condenser may result. For the same reason, install the condenser *after* the large X-Y stage has been initialized.

The condenser must be removed after use.

The Microscope Controller

Control of Stage Movement

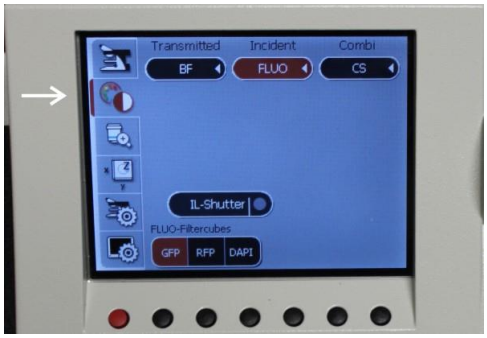
Manual x, y and z stage control is via the knobs attached to the microscope controller, or by the manual X-Y stage control on the stage itself. The microscope controller moves the large X-Y stage only. The knobs allow much finer control than the manual X-Y stage control.

Touch Screen Control of Microscope Functions

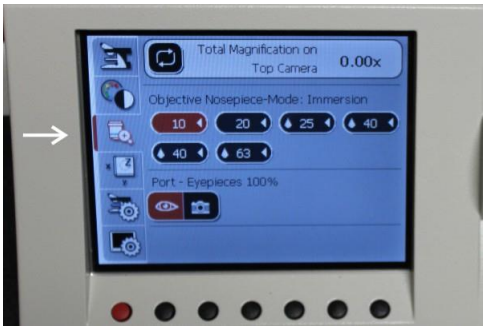
The touch screens on the microscope controller are as follows:

Screen 1: Status and Illumination. Use to adjust illumination intensity, and the aperture and field stops in transmitted light (TL). These are in the microscope body, not the optional condenser.

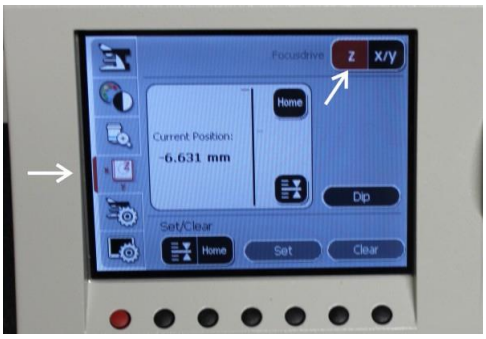
Screen 2: Illumination Method. Select TL or RL (Reflected Light, i.e., epifluorescence). Open and close the shutters to either path. In RL, select the filter cubes (labeled GFP, RFP and DAPI) for green, red, or blue emissions.



Screen 3: Objective selection. Some selections will pause to allow time for oil or water application or removal. The operation is completed by pressing the selection again.



Screen 4: Nosepiece (i.e., objective) Z-control or stage X-Y control.

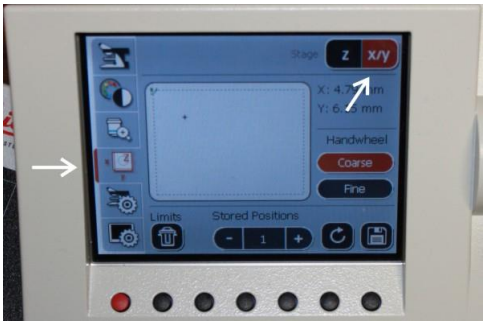


Z-mode: The Home position is the highest point to which the nosepiece can currently be raised. It is usually set at the upper limit of travel of the nosepiece. The Focus Stop position is the approximate focus position (actually, a little above) for the 10x objective. Both positions are indicated by – marks. The current position of the nosepiece is indicated by the = mark and its distance from the Focus Stop is indicated in mm on the display.

Press and hold the Home button to raise the nosepiece to the Home position. Press and hold the up/down button to move the nosepiece to any position. *Note that this button will allow the nose piece to travel below the focus position. Careless use of it may drive the objectives into the specimen.*

The Home and Focus Stop positions should not normally be modified. See the section below for a procedure to set or re-set them.

X/Y Mode: When X/Y mode is selected, the sensitivity of the X-Y control knobs can be selected (coarse or fine). Positions in the X-Y plane can be stored for future use. Maintained touch in the screen will cause the stage to translate to that position. *If the condenser is installed, be sure to lower it first.*



Screen 5: Allows programming of any of the black buttons for specific functions or combinations of functions. *See us before you program any of them.*

Screen 6: Not normally needed by users.

Setting and Restoring Home and Focus Positions (Screen 5).

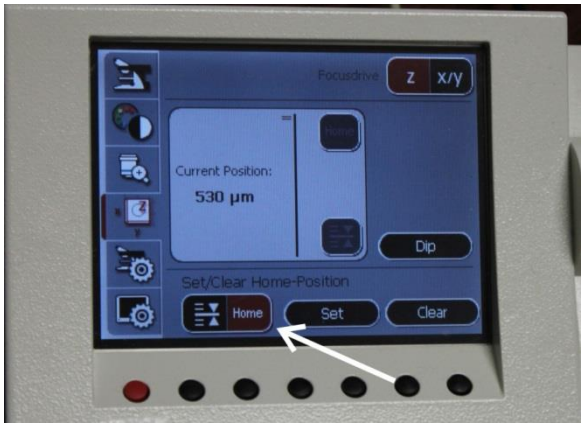
In some situations, for example when using the large X-Y stage in a lowered position, it may be desirable to adjust the Home and Focus positions. Here's how to do it.

First, select Screen 3 on the microscope controller display and select the 10x objective. Then select Screen 4 and select Z-mode (top right).

Setting the Home position

Using the controls on the microscope controller, raise the nosepiece all the way to the top, or to a level that allows you to change your sample without interference from the objectives. Note the Set/Clear Limits button (bottom left) which has two parts, the Up/Down symbol and the word Home Home .

On the Set/Clear Limits button, push the Home button on the Set/Clear Limits button (it will turn red). The button will now be labelled Set/Clear Home Position.



To set the Home position, hit the Set button (it will turn red). The Home position indicator will appear.

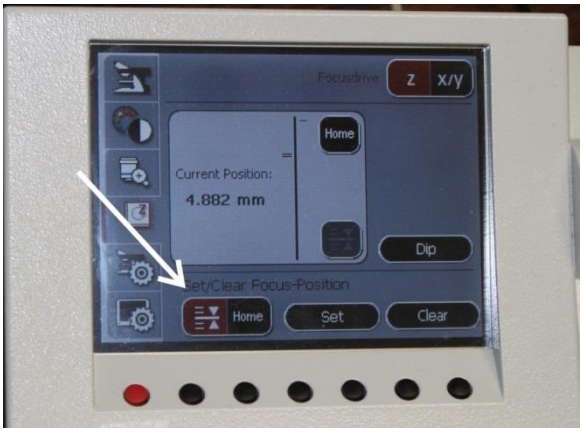
To clear the Home position, hit the Clear button. It will also turn red. The Home button will go gray.

Setting the Focus Stop position

Raise the nosepiece and set a glass slide specimen on the stage. If you do not have a suitable slide, there are some H & E stained slides in the top drawer of the desk. The slides are autofluorescent to GFP or YFP excitation, so you may use fluorescence as an alternative to transmitted light.

Manually lower the nosepiece until the specimen is in focus through the eyepieces with the 10X lens. Raise it a small amount.

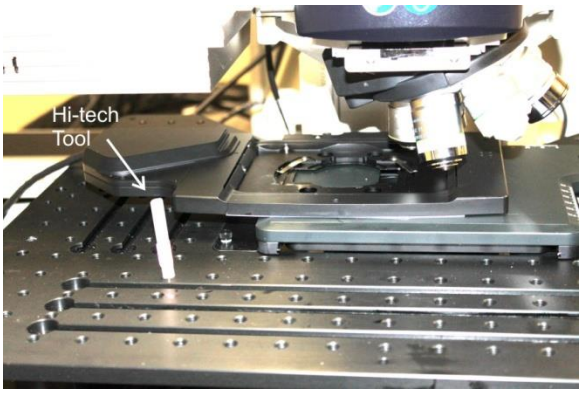
On the Set/Clear Limits button, push the Up/Down button (it will turn red). The button will now be labelled Set/Clear Focus Position.



To set the current nosepiece position as the Focus Stop position, hit the Set button (it will also turn red). To clear the Focus Stop position, hit the Clear button. It will also turn red. The Focus Stop position indicator will appear.

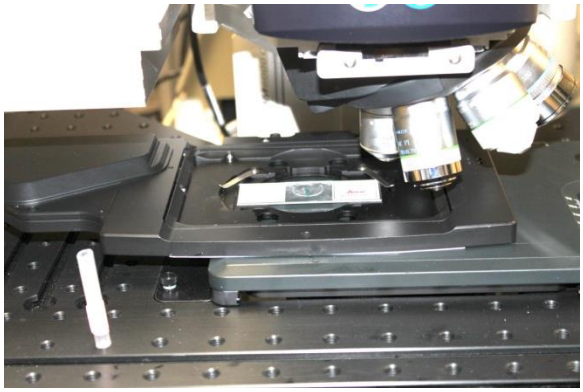
If you have changed either setting, remember to restore the Home and Focus positions for the next user.

Positioning a Slide



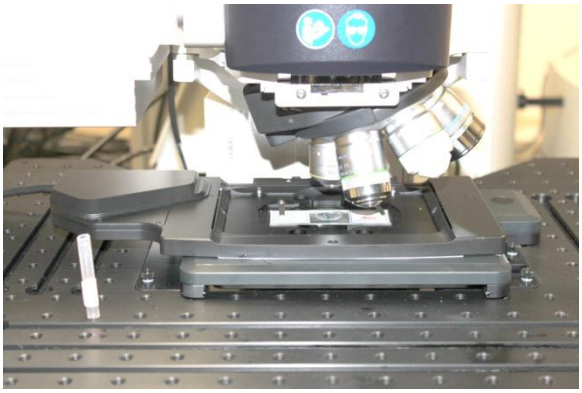
Raise the objectives using the microscope controller. Displace the stage to the left manually or using the stage X-Y manual control. For extra room, rotate the objectives manually so that the 10x is over the stage. Be sure that the clips are positioned at the edges of the slide recess. If the clips are hard to move, use the hi-tech tool provided.

Place the slide in the slide recess. Placing the right side first is the most effective method.



Place the clips over the slide, using the tool if necessary.

Manually reposition the stage under the objectives.



Note: dishes can also be accommodated, subject to the limitations of objective size and working distance. Ask us for details.

Confocal Operation without Multi-photon

Users will not need multi-photon microscopy for DAPI excitation because of the availability of the 405 nm laser.

Metal-Halide Power Supply

Be sure that the metal halide power supply shutter control button is set to *remote* (out).

Application Start

Turn on the computer, monitor, confocal power strip, and metal-halide power supply, in any order. Double-click on the desktop LAS AF icon. *Do not start LAS AF if the confocal power strip is not on.*

There are two questions to be answered during startup.

Optional selection of multi-photon laser. Select MP_LASER_OFF.

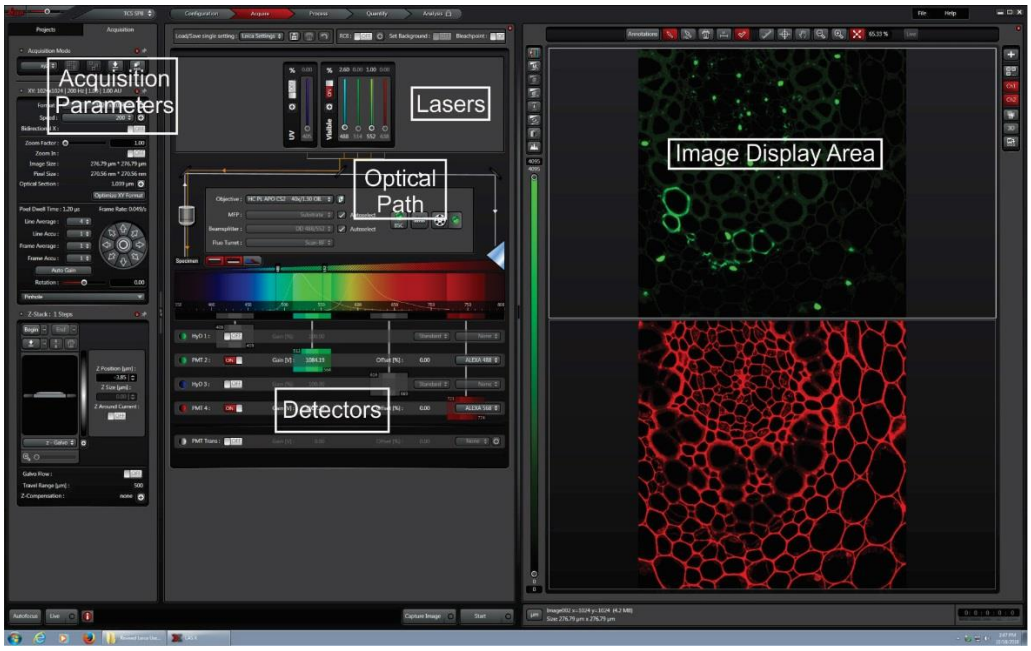
Initialization of the large X-Y stage. Usually, select No.

Initialization of this stage is not necessary unless you anticipate using the stage for tile scans. The manual X-Y controls can still be used for specimen positioning.

If tile scans are to be used, protect the large X-Y stage (raise the objectives, lower the condenser if present), then select Yes. The large X-Y stage will execute a series of X and Y movements before returning to its home position.

LAS AF Application

The application screen will start up in the Acquire pane and will look like this:



Buttons and Sliders

Most functions are controlled by sliders. Click on the slider button to change the slider value. Drag the button or, for more precise adjustment, use the mouse wheel. Some functions also allow keyboard data entry.

Confocal Controller

The confocal controller has six knobs that control acquisition functions and two buttons that toggle the current image/ detector combination. The knobs and buttons can be operated while acquiring in *Live* mode. Modifications to them will be reflected in the Acquire pane. From left to right they are:

Left button – move the current detector/image one step to the left.

Right button - move the current detector/image one step to the right.

Smart Gain – adjust the gain of the current detector.

Smart Offset - adjust the offset of the current detector (if it is a PMT).

Scan Field Rotation - self-explanatory.

Pinhole Diameter (in Airy units)–*use sparingly*.

Zoom –a large range is available (0.7 to 40). An audible warning will sound when the usable zoom has been exceeded.

Z position (in µm) – particularly useful when setting up stacks.

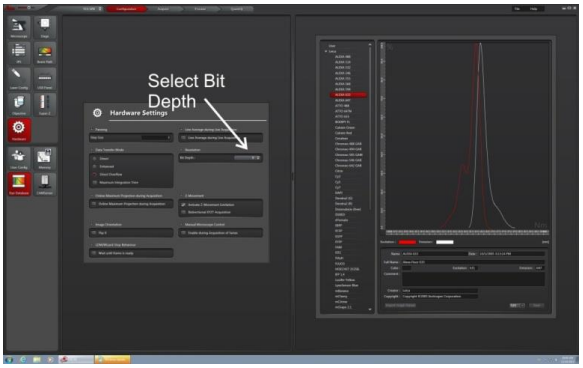
Program Panes

The program has four panes – *Configuration, Acquire, Process*, and *Quantify,* selected by the tabs at the top left of the screen. We will not discuss the *Process* and *Quantify* panes in this document. Contact us if you want help with the functions available in those panes.

Configuration Pane

The *Configuration Pane* allows setting of multiple hardware parameters. Many parameters can also be set or modified from dialogs in the *Acquisition Pane*, so users do not need to use its features often.
The *Laser Configuration* panel may be used to turn on or off lasers. The lasers may also be set to Standby mode if not required for a period.

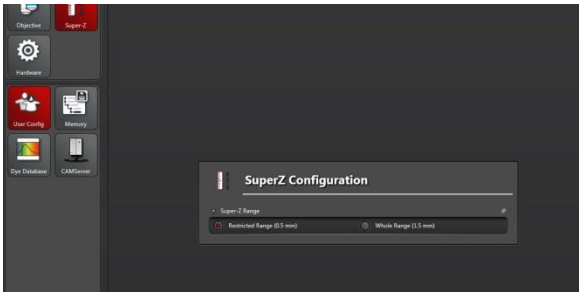
The *Hardware* dialog is useful to select the acquisition bit depth. The default is 8-bit, which is adequate for image acquisition. If quantitative analyses are to be performed, we recommend 12-bit acquisition. 16-bit acquisition is available, but only for PMT channels.



The *Configuration Pane* also has a comprehensive dye data base that is valuable when setting up novel experiments.

The *Objectives* panel presents a window with useful information about the objectives, such as numerical aperture and working distance. However, it cannot be used to change the working objective.

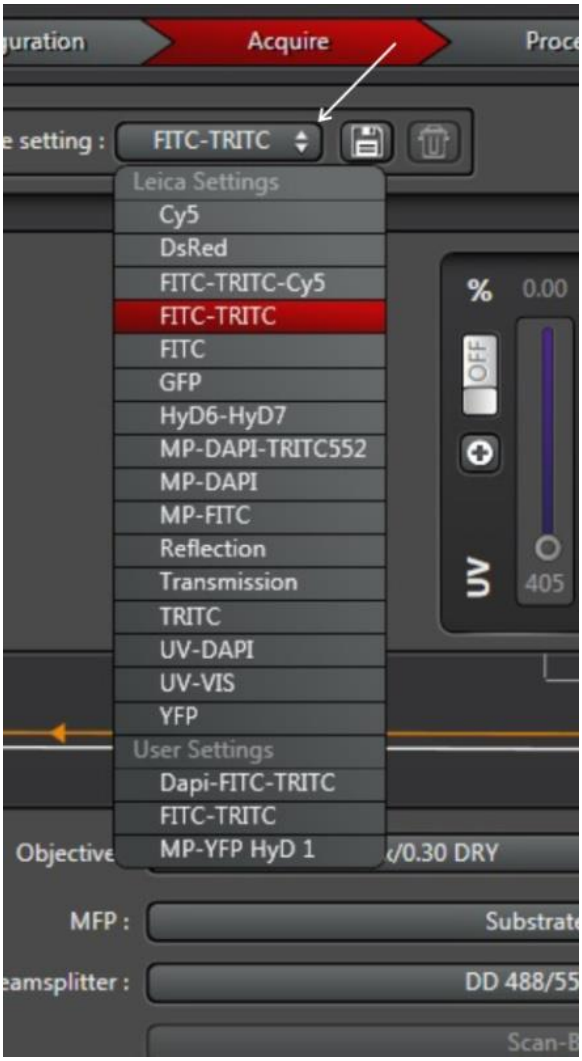
The Super-Z button allows selection of the working range of the Super –Z Galvo stage. We recommend leaving it at 0.5 mm.



Acquisition Pane

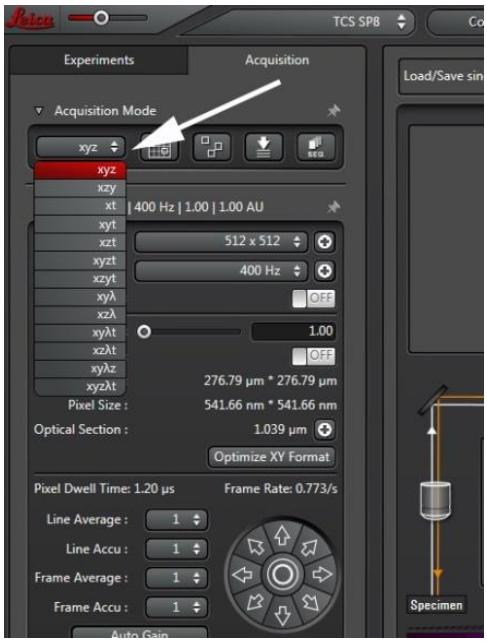
Selection of Configuration

Sixteen standard configurations are available to all users from the pull-down menu above. Please don't modify them! You can also create your own configurations and store them.



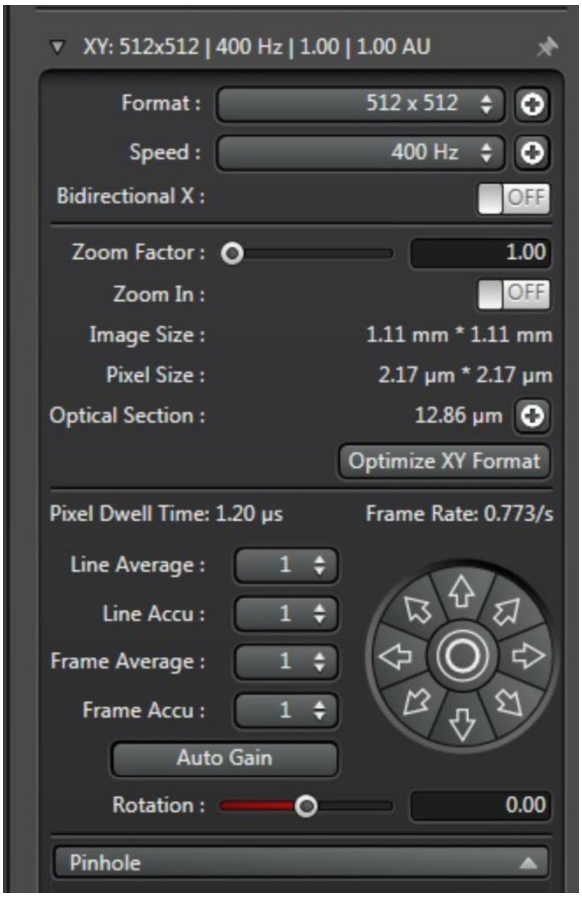
Acquisition Mode

The acquisition mode defaults to xyz, with z = 1, that is, a single frame. Other modes are discussed later in this document.



Acquisition Rate

The acquisition rate window selects the acquisition scan parameters, including the number of pixels in the image and the scan speed, via pull-down menus. We recommend 400 Hz as an initial rate.



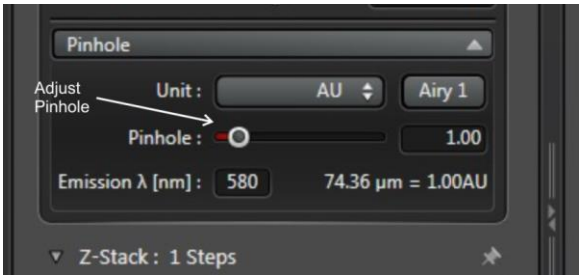
The image scan may be zoomed down to 0.7 and up to 40.

Averaging may be by line or by frame, using pull-down menus – we recommend line averaging and no more than 4 averages. Accumulation may be used instead for weak signals. It is also used when the HyD detectors are in photon-counting mode.

The scan orientation may be controlled using the rotation slider. The image area may also be translated in the directions indicated by the rosette arrows. Large lateral translations at low zoom will move the scan range off the optical axis and are therefore not recommended. Move the stage instead.

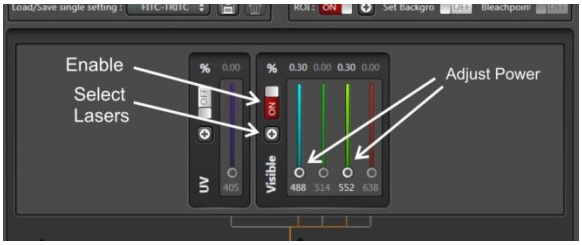
Pinhole

There is only one pinhole. Its diameter is determined by the longest emission band and defaults to 1 Airy disk. It may be modified by clicking and dragging on the pinhole button.



Lasers

The visible light lasers must be enabled by clicking on the ON/OFF button. The lasers may be selected using the laser menu in the *Configuration* Pane, or by clicking on the '+' sign. Click and drag on the button to adjust power level.

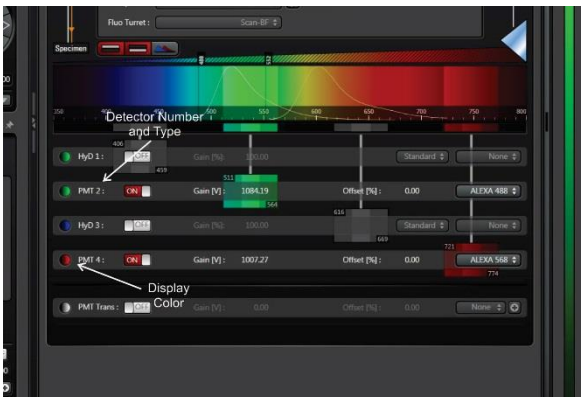


Objectives

Objectives may be selected by the pull-down menu. The objectives will be raised and lowered as part of the rotation. The multi-function plate (MFP) should be set to *substrate* for most applications. Use *Autoselect* determination of the beam splitter.

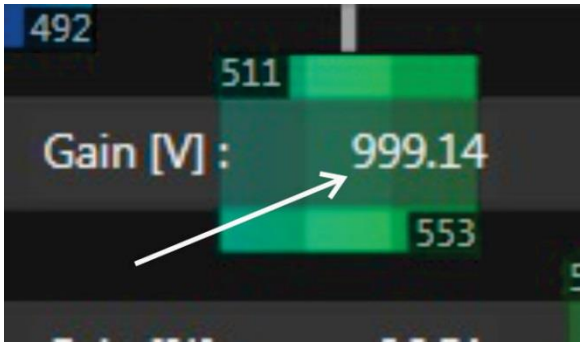
Selecting the Fluorescence Detectors

The type and properties of each detector are represented by horizontal strips in the detector window. Up to four fluorescence detectors may be selected simultaneously. The detector type (HyD or PMT) is indicated at the left of each detector. The detector is selected by clicking on the *on/off* button. The color of its display output is selected from a pull-down menu next to the type indicator.



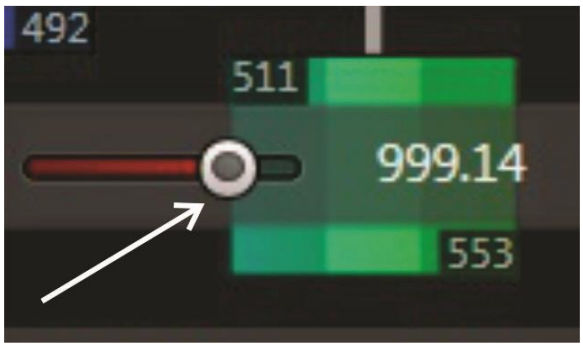
PMT Detector Gain

PMT detector gain is indicated numerically to the right of the on/off button.



Modifying PMT Detector Gain

To modify the gain of a PMT detector, place the cursor over the word *Gain* (clicking is not necessary). The word *Gain* will disappear, and the gain slider and button will appear instead. You can then manipulate the gain by dragging the button or by holding the button and manuipulating the mouse wheel. The range is 0 to 1250.

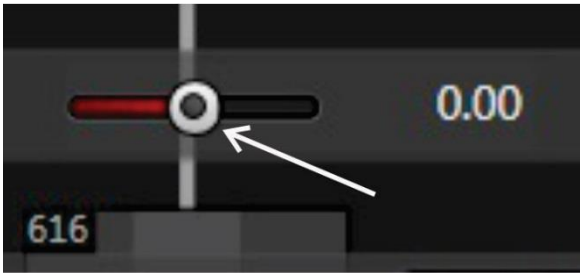


PMT Detector Offset

PMT offset defaults to zero (% of full range). Setting it to a negative value may be used to remove background fluorecence and PMT detector noise. Leica recommends a starting offset of -0.3%. *Note that, since offset is calculated as a percentage of range, it may have to be adjusted if you change the detector gain*.

Modifying PMT Detector Offset

As with detector gain, bring the cursor to the word *Offset*. The word *Offset* will be replaced by the offset slider, which you can modify as before.



Modifying HyD Detector Gain

HyD detectors are photon-counting detectors rather than analog light intensity detectors. Thus the detector returns an integer number instead of an analog value. HyDs have three distinct modes of operation. The mode is selected from a drop-down menu at the far right of the detector line.

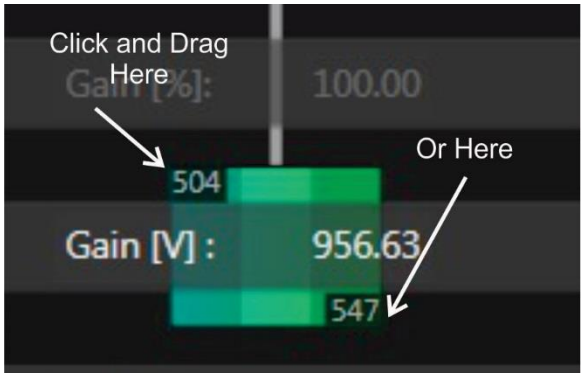
Standard Mode: In Standard Mode the photon count is scaled by the Gain value (0-500). If the Gain is set to a high value, the pixel values will be highly quantized and the imaged will have abnormal gradations of scale. We recommend you set the HyD gain to no more than 100 in this mode, even for a bright signals

Counting Mode: In Counting Mode, the Gain is disabled and the raw photon numbers are returned. For multiple presentations, you must also use Line Accumulation and Frame Accumulation The image, initally dim, will gradually improve in quality as the photon counts increase over rmultiple presentations. *We have found that this method produces very sharp images if used properly*.

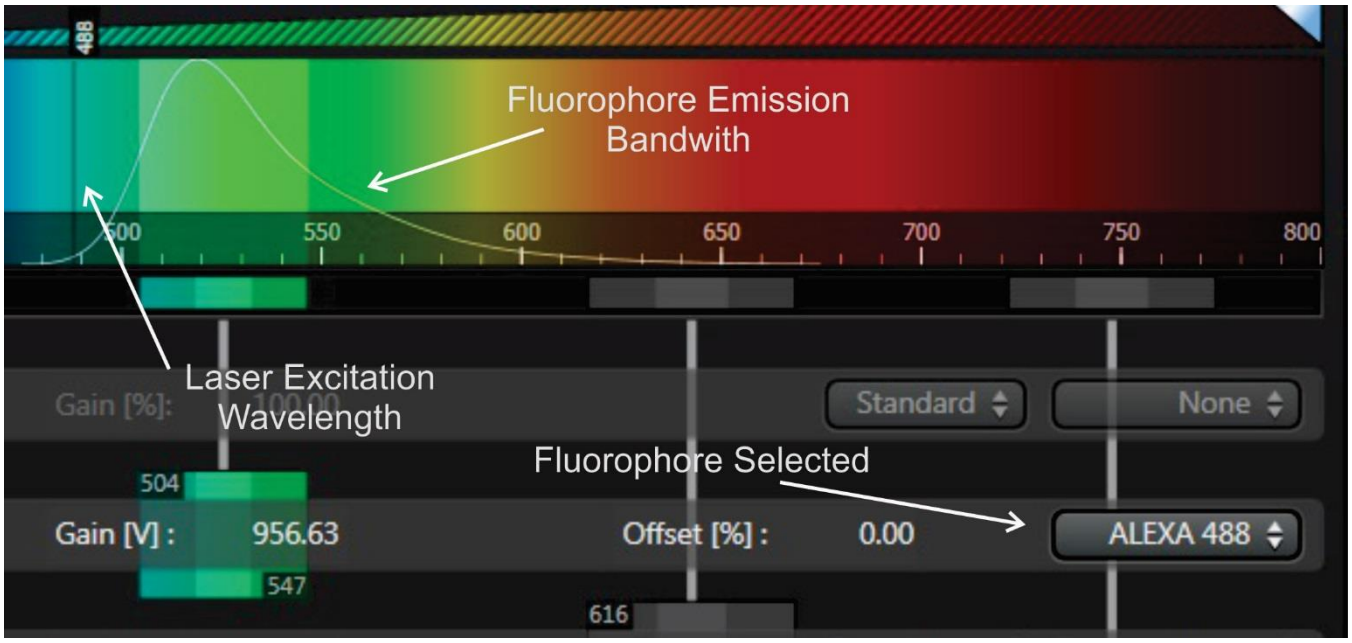
BrightR Mode: We do not understand this mode and suggest you not use it.

Modifiying Detector Passband

Detector passband adjustment is the same for both detector types. Up to four fluorescence detectors may be employed at any time. Each may have an individually tailored bandwith. The detector bandwidth may be modified in either direction by simply clicking on and dragging its edges. The minimum bandwith is 5 nm. Maximum detector passband width is 350 to 800 nm. However, adjacent detector bands may not overlap, which lmits the maximum detector bandwidth slightly.

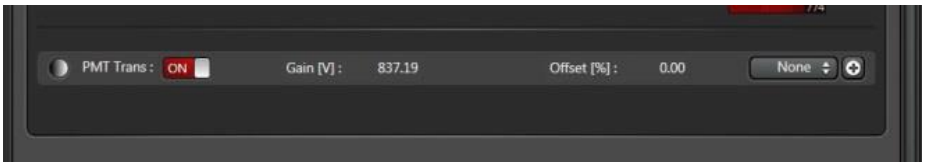


Optimizing Detector Bandwith



At right on each detector's line is a button. Select it and it rveals a pull-down menu of fluorphore names. Select the fluorophore of choice. Its emission bandwidth will appear above the detectors, along with bars corresponding to the detector bandwidths. The bar for an active detector will be be bright, as above. Use the emission bandwidth diagram to optimize the detector bandwidth. Concentrate on wavelengths around the peak of the emission bandwith. Admitting light from the weaker wavelengths of the emission tail at longer wavelengths increases the chance that spurious background emission will be be collected. If a laser is activated and set to a level above 0%, its excitation wavelength will also be shown. *Be careful not to include the laser excitation wavlength in the detector passband!*

Selecting the Transmitted Light (TL) Detector



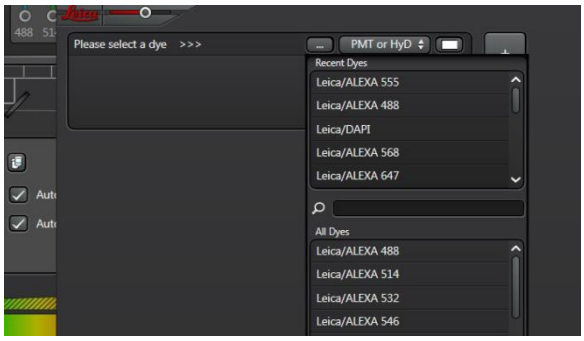
The TL detector is in the last row of detectors. It is selected by clicking on its *on/off* switch. It is a PMT and therefore it has gain and offset controls that function the same as for the fluorescence PMTs. However, it has no bandwidth setting. Instead, it is responsive up to 900 nm. It may be used in conjunction with as many as four fluorescence detectors. However, since it is constructing an image using fluorescence excitation light passed through the specimen, it may not make sense to use it with more than one fluorescence detector.

The condenser should be installed for optimum use of the transmitted light detector (see earlier in this document). Proper centering of the condenser aperture is required. The condenser need not be focused but it should be close to the specimen.

Use the Dodt controller to modulate the contrast. The optimum Dodt setting when viewed with the eyepieces is not necessarily the same as the optimum setting when using confocal.

Dye Selection Guide

At left, below the pathway diagram, there are three buttons. The leftmost button selects or hides display of the fluorescence detectors. The middle button selects or hides display of the TL detector (here, hidden). The rightmost button (arrow) brings up a most useful tool, the *Dye Selection Guide*. This can be used to select the most appropriate acquisition mode and to warn against possible emission band overlap



Clicking on the button brings up a panel from which you can select a fluorescence detector type (PMT or HyD) and the name of a dye, either one used recently or one from the Leica dye database. Click on the + sign and a second selection of dye and detector becomes possible.

In this example, DAPI has been selected as the dye for a PMT detector and Alexa Fluor 488 has been selected for a HyD detector. The chart shows three possible acquisition modes:

- *Non-sequential* (i.e., simultaneous)
- *Line Sequential*
- *Frame or Stack Sequential*

In *Non-sequential* acquisition, both channels are acquired at the same time. The diagram in the middle shows the emission bands of the two fluorophores, DAP in blue and Alexa Fluor 488 in green, the excitation wavelengths (the vertical white lines), and the practical filter settings (recall that filter bands may not overlap). The emission band of DAPI overlaps the emission band of Alexa Fluor 488, and this is indicated in the horizontal bars labeled *Crosstalk* by blue bar in the Alexa Fluor 488 strip. Running these filter settings for DAPI and Alexa Fluor 488 in non-sequential acquisition would therefore yield some DAPI emission in the Alexa Fluor 488 channel, which is of course undesirable. Note also the bars under the heading *Yield*. These bars indicate that not all of the DAPI emission would be captured by the standard filter settings on the DAPI channel.

In *Line Sequential* mode, each channel is acquired separately, line by line, using the standard filter settings. Each fluorophore is excited separately. However, there is not enough time between lines to changes the filter settings, so the filter bands remain the same. The advantage of this mode is that DAPI is not being excited at the same times as Alexa Fluor 488, so no DAPI emission ends up in the Alexa Fluor 488 band, and therefore the *Crosstalk* is zero. However, not all of the DAPI emission is captured

by the DAPI filters, hence the yield of DAPI photons is less than optimum. Also, the requirement to scan each line twice means that the time for acquisition of the complete image is approximately doubled.

In *Frame or Stack Sequential* mode, frames (or stacks) for each fluorophore are captured sequentially. Again, DAPI is not being excited at the same times as Alexa Fluor 488, so no DAPI emission ends up in the Alexa Fluor 488 band, and therefore the *Crosstalk* is zero. However, there is now time to adjust the filter settings between frames (or stacks), so the filters bandwidths can be optimized. Note that the DAPI yield is 100% in this mode.

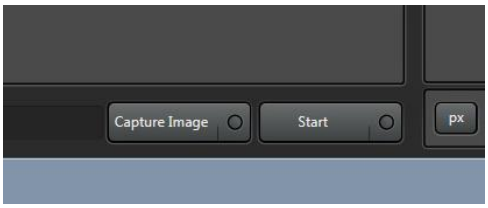
The bandwidths may be adjusted by selecting the *Edit* button, within the limits imposed by the requirement that bands do not overlap in *Line Sequential* or *Frame or Stack Sequential* modes.

Once a mode has been selected, press the *Apply* button. The selected mode, detector type and bandwidth will be applied.

Acquisition Start Buttons

At the base of the pane are four action buttons. At left, the *Autofocus* button should not be used. The *Live* button initiates scanning at the indicated rate and format until it is pressed again.

At right, the *Capture Image* button is used to acquire a single image. The *Start* button is used to initiate acquisition of a stack or a sequence.

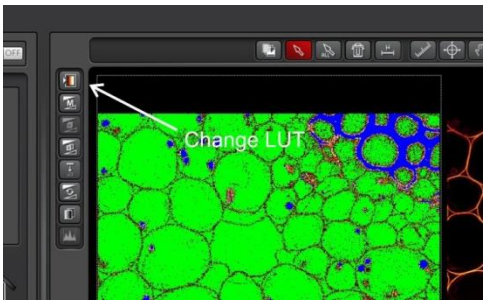


Display

The displayed image is rotated 90° anti-clockwise with respect to the image seen in the objectives (which is inverted top-to-bottom). Thus x-axis stage translation moves the image vertically on the screen, and y-axis stage translation moves the image horizontally.

Displaying Results

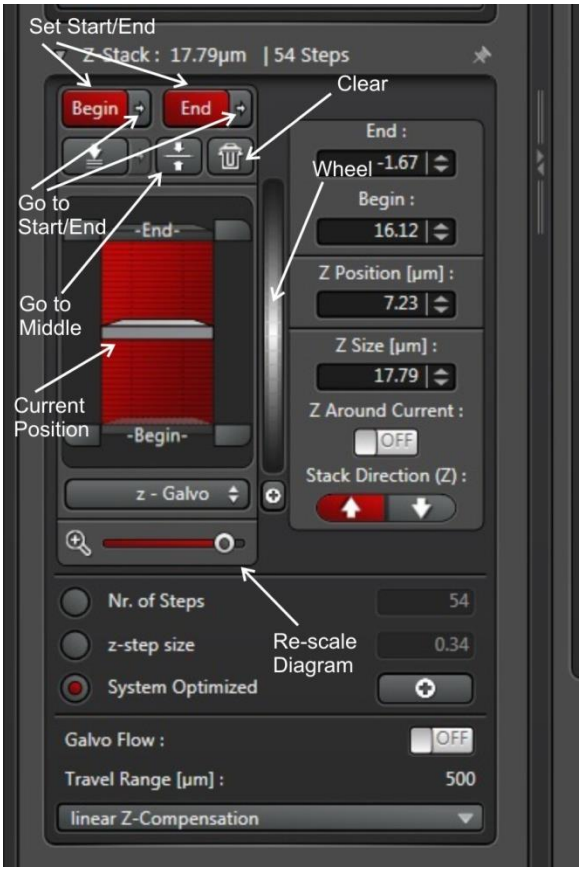
The look-up table LUT used to display the results may be changed using the button at the top left of the display window. The first click selects a range-finding LUT for the active window that can be used to set gains and offsets. The second click changes the display in all active windows. A third click returns the LUT to the normal type.



Range-finding in the LAS AF uses green for below range, blue for above range, and red for within range.

below range	within range	above range

Programming a Stack



Programming a stack requires the following steps:

1. With Live acquisition going, adjust the focus to the top of the stack. Use the Confocal Controller. Fine adjustments may be made using the clickable wheel.
2. Click on *Set Start*.
3. Re-adjust the focus to the bottom of the stack.
4. Click on *Set End*.

The number of steps and the step size will be automatically computed based on the longest wavelength emission band. It can be modified by clicking on *Nr. of Steps* or *z-step size*.

The diagram indicates the size and spacing of the stack and the current stage position. It may be re-scaled for better viewing using the slider provided.

Clicking on *the double-vertical arrow* button at the top moves the stage to the middle of the stack.

Clicking on the *garbage can* icon will delete the stack. This should be performed before creating a new stack.

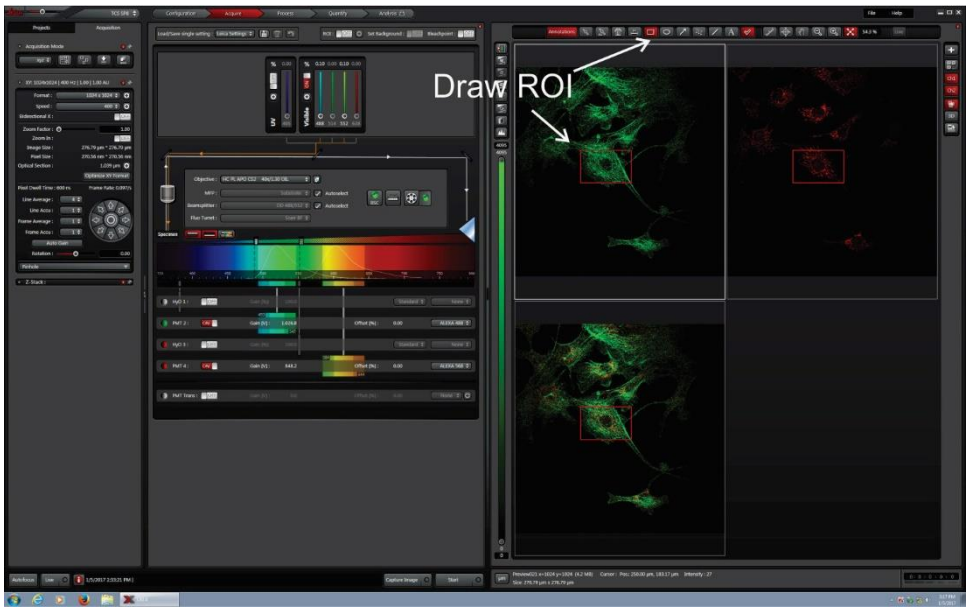
Use of the z-Galvo stage or the nosepiece for vertical movement is controlled by the pull-down menu. The z-Galvo stage is more precise.

Leave *Galvo Flow* off.

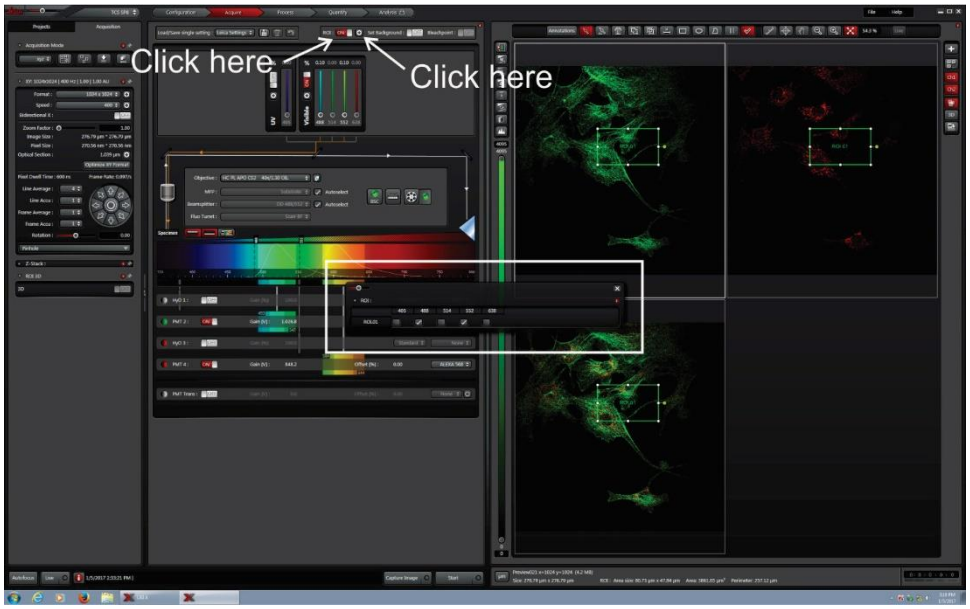
Acquiring from a Region of Interest

Images can be acquired from a region of interest (ROI) alone, or from an ROI with different conditions between the ROI and the background. Acquiring from an ROI does *not* speed up acquisition.

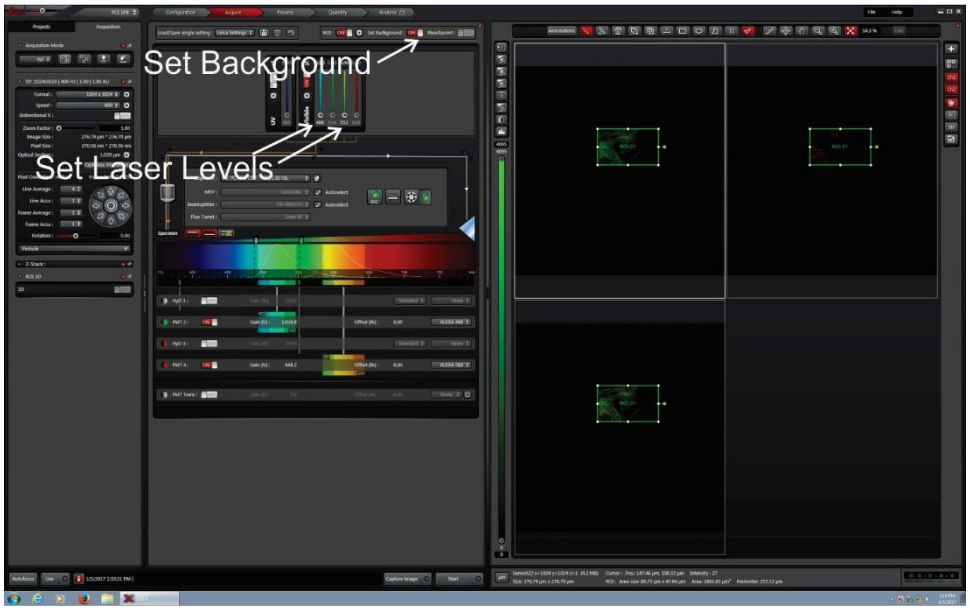
First, acquire a full-field image at the desire settings (laser intensity, detector gain, &c.). Draw the desired ROI using the drawing tools. You may select more than one ROI.



Click on the *ROI* button to set the foreground conditions. Acquisition conditions within the ROI will be as specified in the *Detectors* panel. Click on the + sign next to the *ROI* button and a panel will appear. This panel shows which lasers will be used.



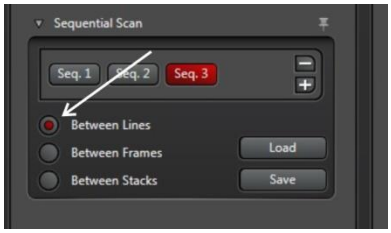
To set the background conditions, click on the *Background* button. Set the laser levels and any other acquisition conditions. In this example, the background excitation is set to zero.



To acquire the image, click on *Start*.

Alternate Acquisition Modes – Sequential

Click on the right-most icon in the *Acquisition Mode* menu.

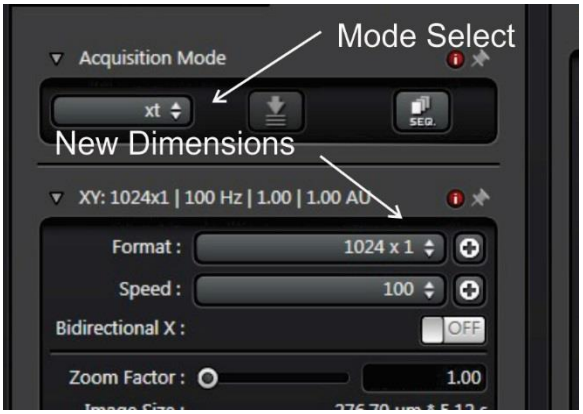


The Sequential Mode menu appears at the bottom of the acquisition parameters window. Click '+' to add sequential events, '-' to remove them. Up to six sequential acquisition events are possible. Events may be any of the available modes, e.g., xy, xyz, xyt, xyλ. Events may be acquired line-by-line (indicated by arrow). This is the preferred method, because it involves less switching of the scan components. However, if the detector bandwidth or the average or accumulation number changes between events, use *Between Frames*. If one or more event is a stack, use *Between Stacks*.

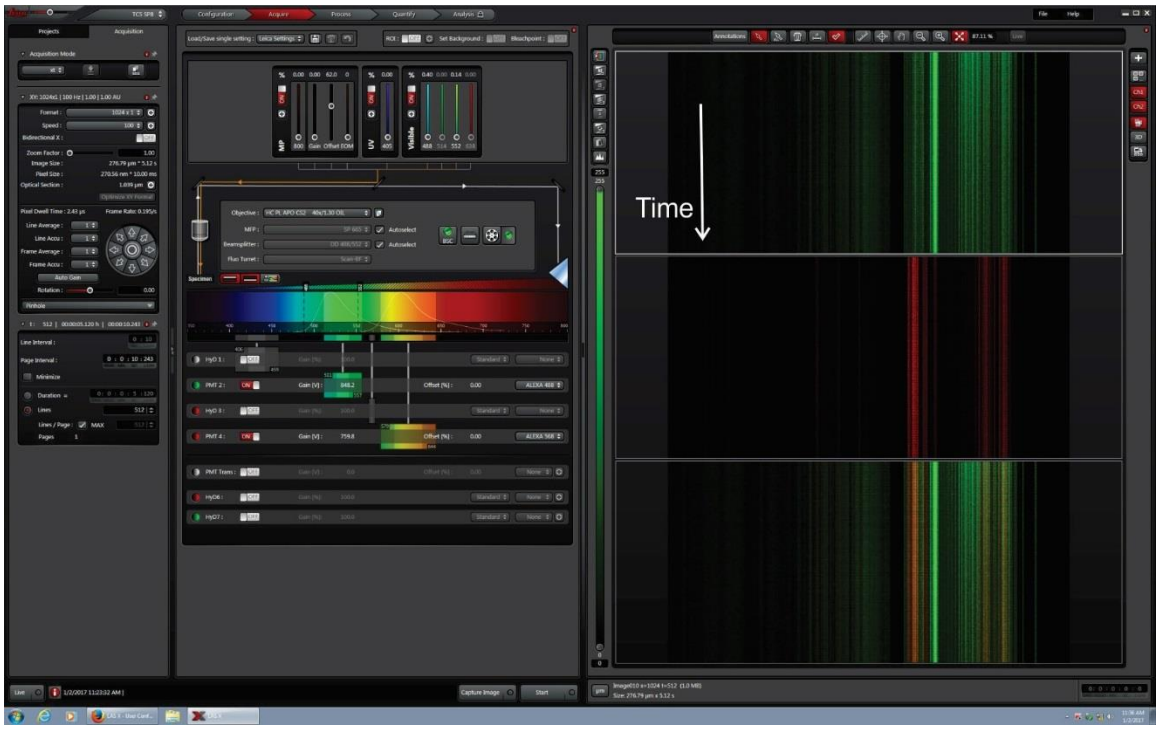
Alternate Acquisition Modes - Line Scan

Line Scan Mode is used for acquiring a time series from a very small area at high speed. The confocal scans only in the x-direction (which is the y-direction at the specimen, unless image rotation is used), thus it is much faster than x-y scanning. It is useful, for example, in monitoring a calcium current at a small cluster of ion channels as might be found at a synapse or spine.

To start *Line Scan Mode*, select *xt* as the acquisition mode. Note that in the acquisition parameters menu, the y-value automatically defaults to 1. This can be set to another value (2, 3, 4, &c), while preserving at least some of the speed advantage of *Line Scan Mode*.

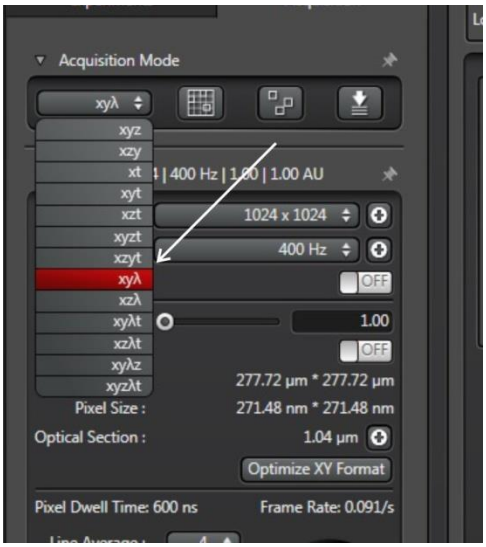


The image generated consists of a series of streaks, increasing in time from top to bottom of the image. If the signal were time-varying, such as an ionic current, the streaks would change to reflect the temporal variation.



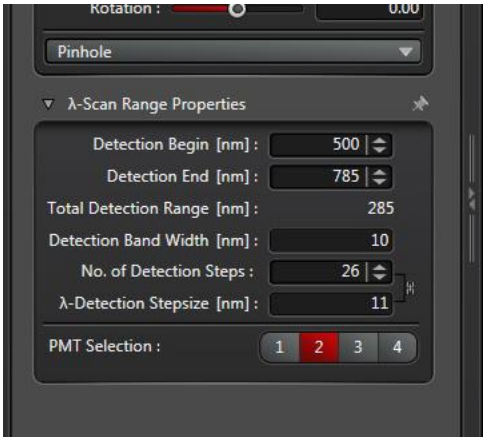
Alternate Acquisition Modes - Spectral

Select *xyλ* or similar acquisition mode.



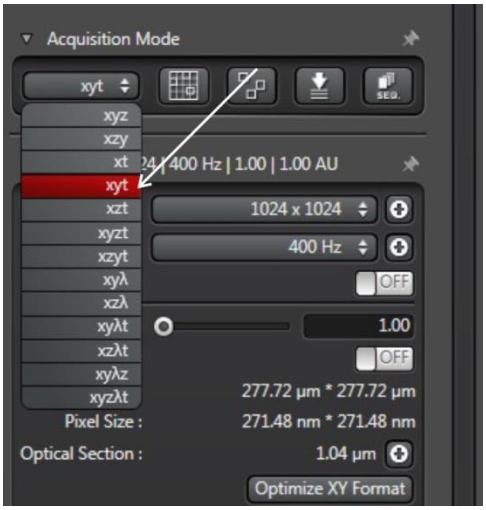
The laser and detector section of the acquisition pane will appear as shown here.

The number of bands and the bandwidth is set in the *λ-Scan Range Properties* window. The maximum spectral range is 380 nm to 785 nm. The shortest-wavelength laser is 405 nm, so the shortest practical wavelength is 420 nm, unless multi-photon excitation is used. The minimum bandwidth is 5 nm. The minimum step between bands can be less than 5 nm. At 5 nm spacing, the maximum number of bands is 81. Any one of the four descanned or the two non-descanned detectors may be selected as the detector.

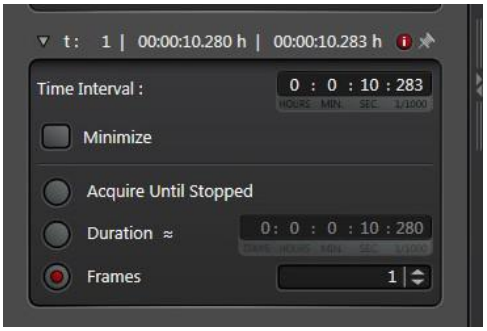


Alternate Acquisition Modes - Time Series

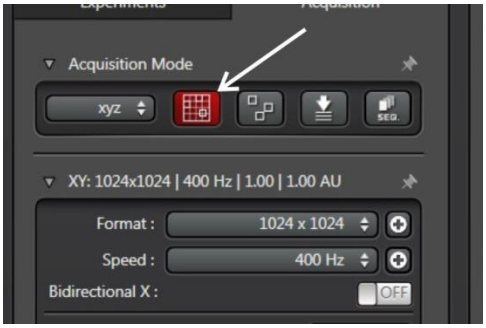
Select *xyt* or similar acquisition mode.



Under *Time Interval*, select the number of minutes, seconds and milliseconds between images. Click on *Minimize* if you want the fastest possible under the current image size, number of channels and scan speed. To determine the length of the time series, select either *Acquire Until Stopped* (with a Carriage Return), *Duration* = and select the length of the series in hours, minutes, seconds and milliseconds, or select *Frames* and set the number of frames to whatever suits.



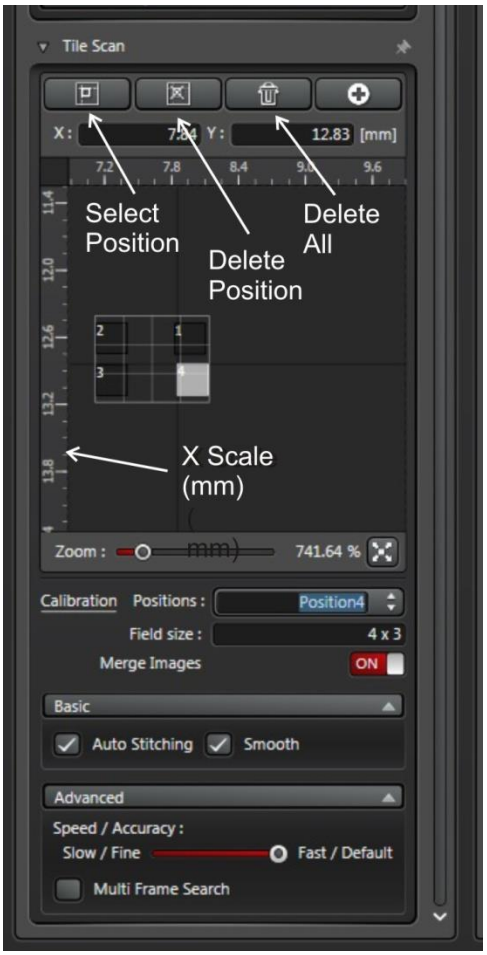
Alternate Acquisition Modes - Tile Scan



Select *Tile Scan* in the *Acquisition Mode* menu. The *Tile Scan* window will appear below in the Acquisition window.

Note: Tile Scan should not be combined with image rotation or erroneous results will be obtained.

Tile Scan Window

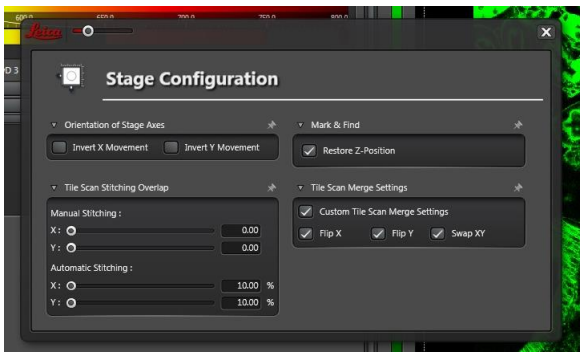


The *Tile Scan* window features an X-Y map showing the current position of the stage. The scale is in mm, and the X-axis is vertical, as for the image display. The map scale can be expanded or contracted using the button and slider below the map.

Above the map are four buttons. The left-most button selects the current position for storage. Multiple positions can be stored. The current position number is reflected below the map. The second button deletes the current stored position, as reflected by the position number below the map. The third button deletes all currently-stored positions. Clicking on the right-most button opens a stage configuration menu

The simplest method is to select *Auto Stitching*, with *Smooth* off.

Stage Configuration Menu

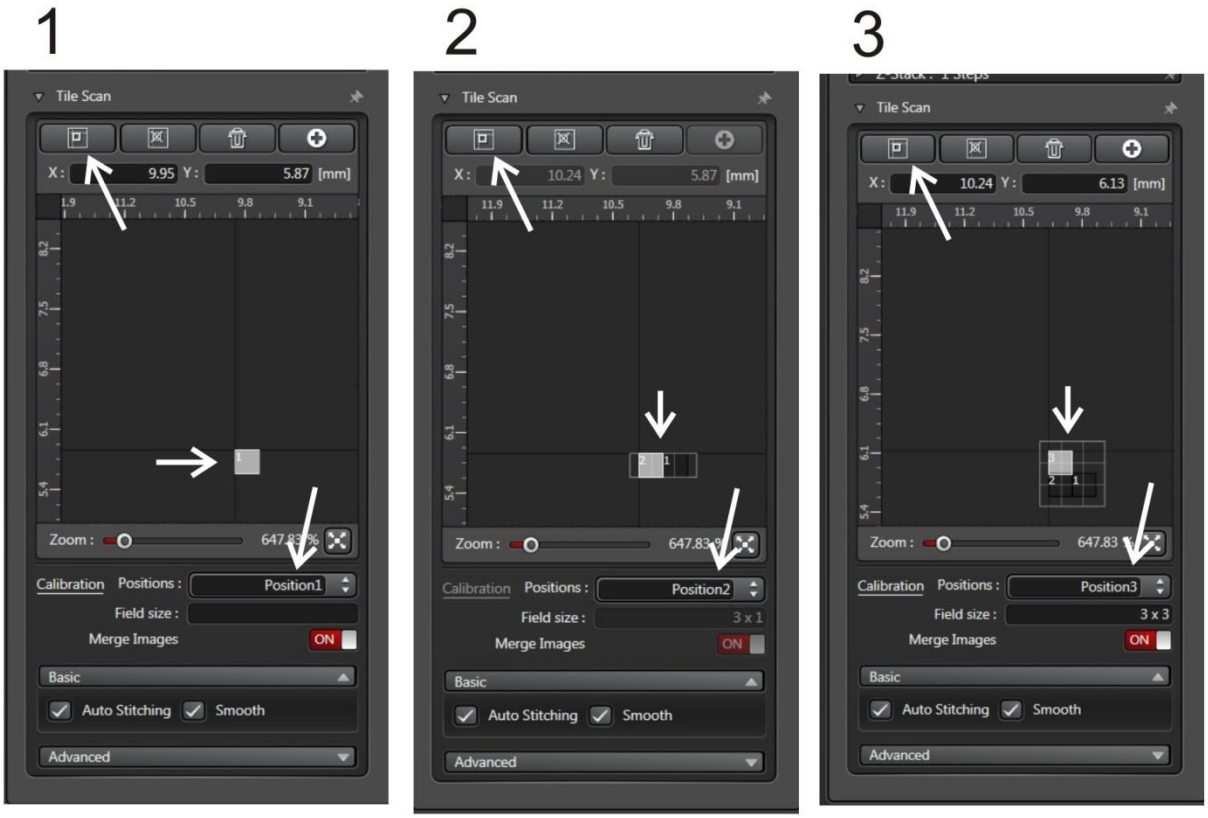


Note that in the default condition, the X- and Y- axes are inverted in sign (corresponding to the inverted microscope image), and that the X and Y axes are exchanged.

When *Auto Stitching* is selected, the percent of overlap is selected in this menu. We find that 10% overlap works satisfactorily without requiring excessive sampling or computation time.

Setting up a Tile Scan

The approach to setting up a tile scan is as follows:



Step 1: Click on the *Select Position* button to select the current position as Position 1.

Step 2: Move the stage to a position at one extreme of the area to be tiled. Use the microscope controller X-Y controls to move to the new position, while observing through the eyepieces or observing the display in *Live* mode. Click on the *Select Position* button to select the current position as Position 2. The map will display a map indicating the number of tiles to be scanned. In this example, a 2 x 1 tile map is to be acquired.

Stage 3: To expand the tiled area, move the stage to a new position at an orthogonal extreme, as before. Click on the *Select Position* button to select the current position as Position 3. The map will display a revised map indicating the expanded number of tiles to be scanned. In this example, a 3 x 3 tile map is now to be acquired.

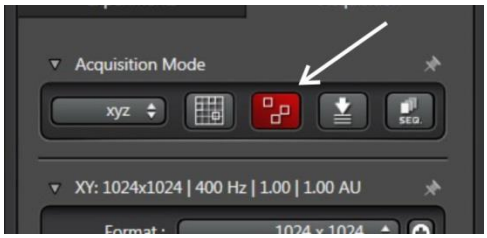
Click on *Start* to begin acquisition of the tiled scan. If *Auto Stitching* is selected, after acquisition is completed the images will be automatically stitched together to form a composite image.

Combining Tiling with Stacks

Stacks may be combined with tiles and with averaging. Be sure to check that the stack dimensions are appropriate at all locations of the tile scan.

Alternate Acquisition Modes – Mark and Find

Mark and Find enables you to store multiple locations on your specimen so that you can return to them individually. It functions much like the way you store locations in the X-Y plane using Screen 4 of the Microscope Controller. However, it has the advantage that the stored locations can be employed in a sequential acquisition.



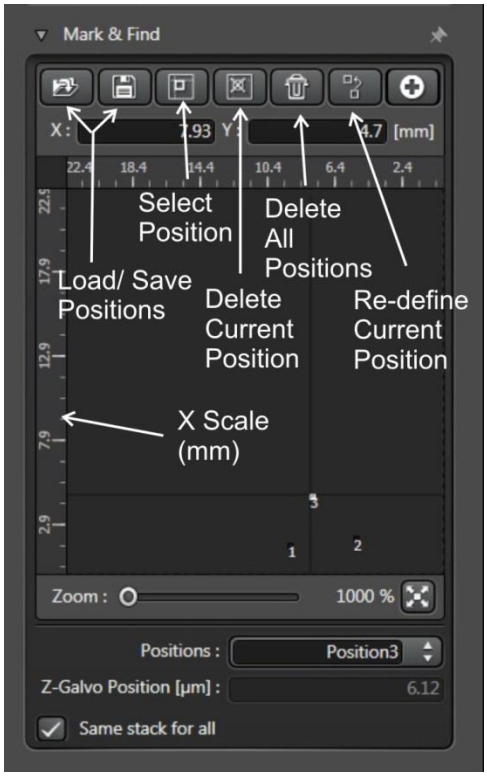
Setting Up a mark and Find List

The window shows an X-Y map, as in *Tile Scan*. Remember that the X-axis is vertical, as in the Display. Move the stage using the Microscope Controller X-Y controls, observing either via the eyepieces or in *Live* mode.

At each position, find the appropriate focus. Click on *Select Position* to store the position. The *Position Number* window will increment. You may delete the current position using *Delete Current Position*, or delete all currently-defined positions using the trash can. You may also re-define the current position.

A family of positions can be named and saved using the floppy-disc icon (second from left). The left-most icon enables you to recall a saved list of positions.

Use the pull-down list of position numbers to select a new position. The stage will move to that position and the focus position will be adjusted accordingly



Combining Tiling with Sequences

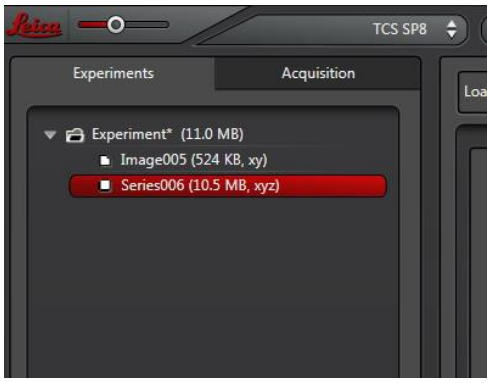
When defining a sequence involving changing X-Y position, remember to select the appropriate position number when defining the sequence event.

Combining Tiling with Stacks

A stack acquisition may be defined in a sequence acquisition. The stack may be different for each position in a sequence. If the stack is to be the same, check the *Same Stack for All* box at the bottom of this window.

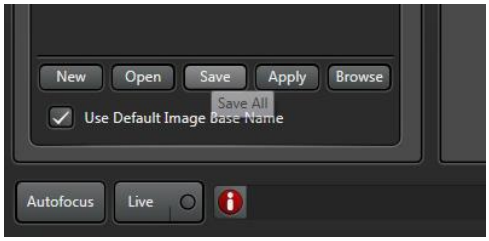
Storing Experiments

Click on the *Experiments* tab to show a list of completed experiments.



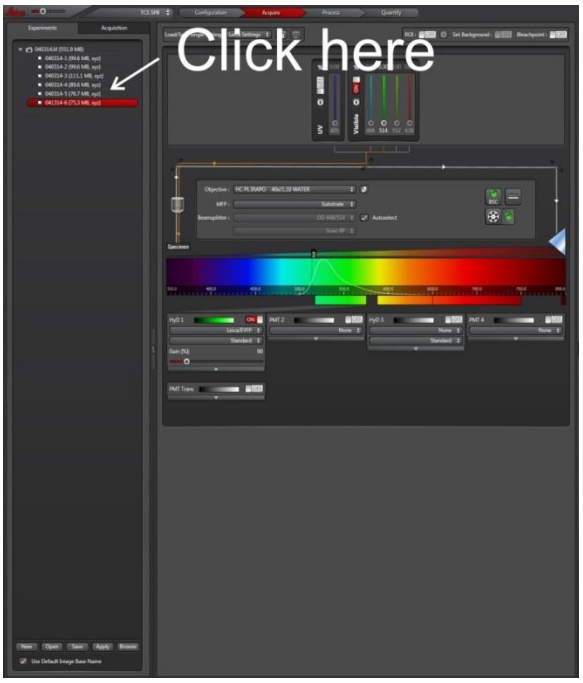
Double-click on an experiment to inspect the result or analyze it. Right-click on an experiment or sub-experiment to change its name.

Use the *Save* button below in the *Experiments* window to save experiments. You will also be prompted to save unsaved experiments on shutdown.



Using the Experiment Metadata

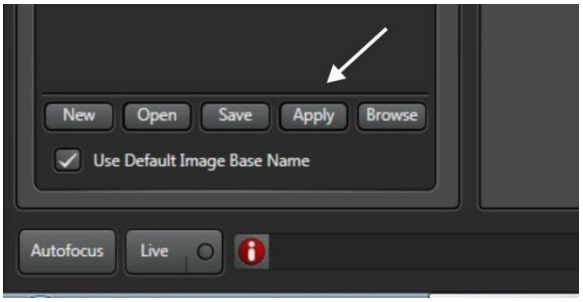
If you wish to repeat the acquisition parameters of a previous sub-experiment, you can do so by either of two different methods.



If you are confident that the sub-experiment represents the correct parameters

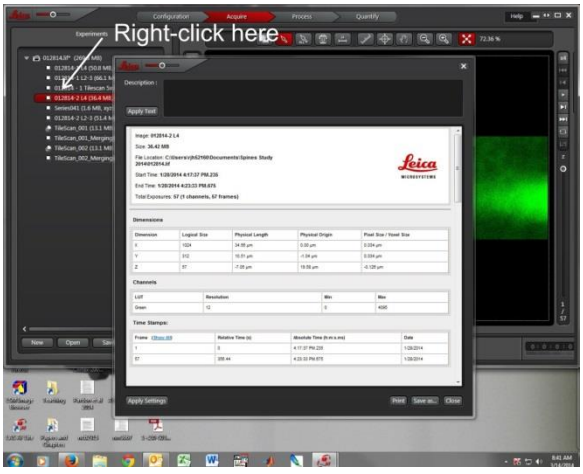
Load the experiment if it is not already loaded. Click on the sub-experiment.

Select the *Apply* button at the base of the *Experiments* window to apply the parameters.

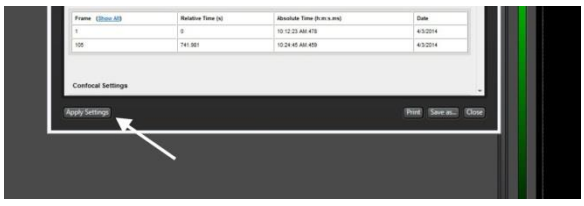


If you wish to check the parameters first

Select the file, and right-click on it. A panel will appear displaying the acquisition parameters. You may scroll down the list of parameters to inspect them.



Click on the *Apply Settings* button at the bottom left of the window to apply them.



Note - Not all parameters will be applied.

These parameters are applied from an image file:

- Laser status, including multiphoton (assuming multiphoton laser is on)
- Multiphoton gain and offset
- All detector settings (including acquisition type for HyDs - Standard vs Photon-Counting)
- Pinhole diameter

These parameters are NOT applied:

- Bit depth (important – use the Hardware selection in the *Configuration* pane)
- Format (pixel-by-pixel size)
- Acquisition speed
- Zoom
- Number and type of average
- Stage position
- Z-axis position
- Stack parameters
- Tiling parameters

Shut Down

Raise the objective using the nosepiece Z-control (screen 4 of the Microscope Controller).

If you have used the oil or water objectives, select the 10x objective (screen 3 of the Microscope Controller). This will make the oil or water objectives easier to clean.

Clean any oil objectives you have used with lens paper and ethanol.

Clean any water immersion objectives with high-Q water.

Remove your specimen from the stage.

Close the LAS AF application – you will be prompted to save the files that you want to save. Save to folders on drive D: *only*.

Log off the computer.

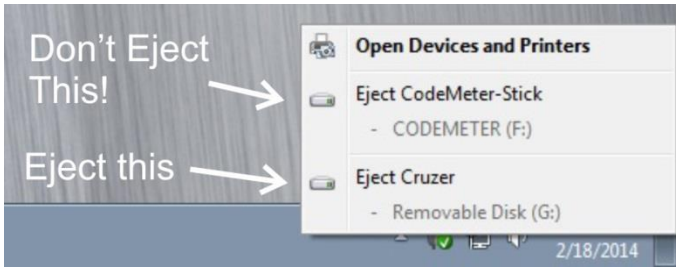
Switch off the confocal at the power strip.

Switch off the metal halide illuminator power supply.

Don't forget to restore the Home and Focus levels if you have changed them. Return the condenser and any water immersion objectives to john or, if after hours, leave them in the drawer.

Ejecting Storage Devices

When ejecting your backup memory device, be careful not eject the F: drive. The F: drive is the license dongle that permits operation of the LAS AF software. Ejecting it will prevent other users from using LAS AF.



Confocal Operation with Multi-photon

Detector Filters and Barrier Filter

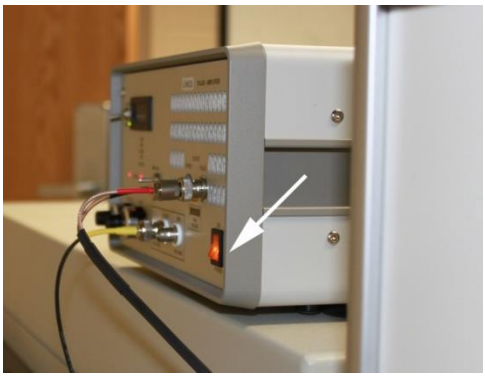
The Super HyD detectors have a 620 nm barrier filter in front of them to protect against reflected IR light. For bandwidth selection, you will need a suitable filter in front of the detector or detectors. Ask us how to set this up.

Start Up

It is not necessary to power down the computer or the microscope when switching from MP_LASER_OFF to MP_LASER_ON operation. However, certain steps must be followed for the correct sequence.

Switch on the Electro-Optical Modulator.

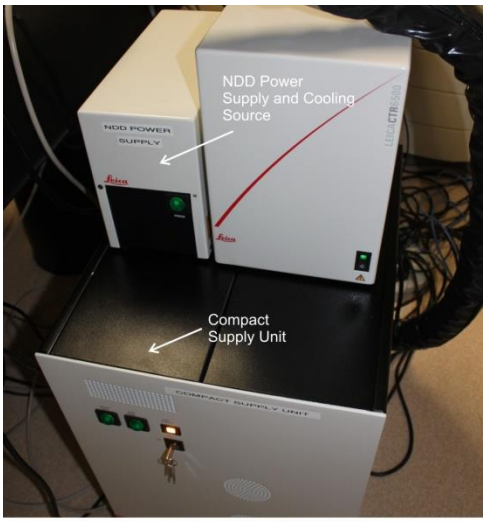
The modulator is ready for use immediately, but does not achieve full modulation depth for about 15 minutes. Switch it on *first*.



Switch <u>off</u> the Compact Supply Unit at the back of the table.

Switch <u>on</u> the Super-HyD detectors power and cooling unit.

Switch the Compact Supply Unit <u>back on</u>.



Application Start - Select **MP_LASER_ON.**

Initialization of the large X-Y stage. Initialization proceeds as for single photon imaging. Usually, select *No*.

Initialization of this stage is not necessary unless you anticipate using the stage for tile scans. The manual X-Y controls can still be used for specimen positioning.

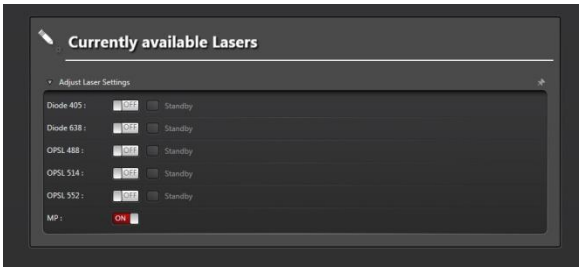
If tile scans are to be used, protect the large X-Y stage (raise the objectives, lower the condenser if present), then select *Yes*. The large X-Y stage will execute a series of X and Y movements before returning to its home position.

Pinhole

The pinhole is not in the emission path for the non-descanned detectors, so it can be ignored. Operation of the confocal with multi-photon excitation and descanned detection is ineffective and should be avoided.

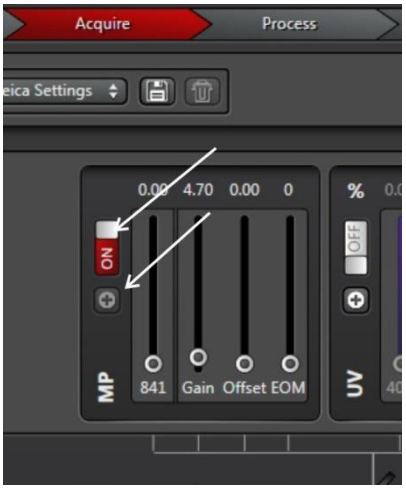
Enabling the MP Laser

Unlike the semiconductor lasers, the MP laser must be turned on in the *Configuration* pane.



Setting up the MP Laser

In the *Acquire* pane, first click MP on. Then click on the + button to open the MP laser window.

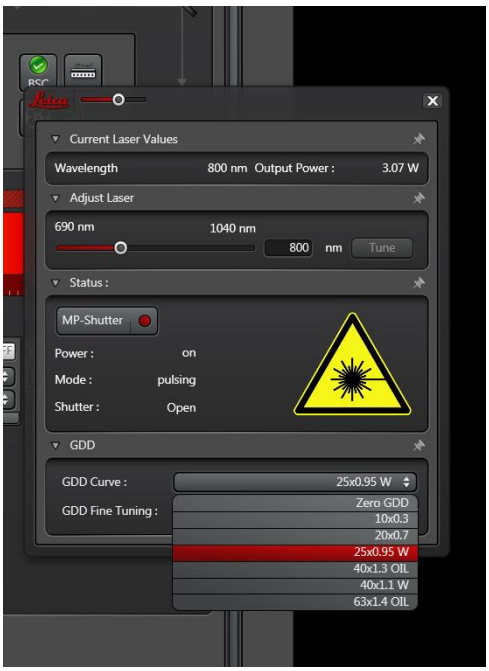


The MP Laser Window

Select the laser wavelength. The laser will take 2-3 minutes to go from ‘CW”, with little or no power, to ‘pulsing’, with full power (2-3 W). To open the shutter, press and hold the shutter button.

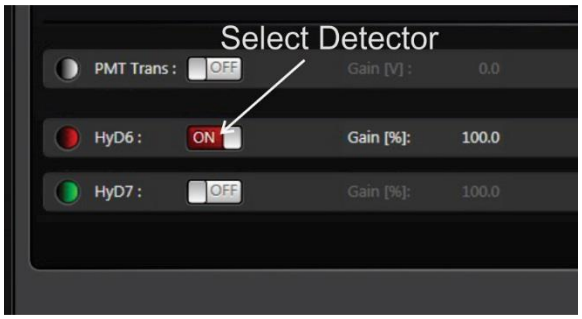
Dispersion Compensation

Dispersion compensation of the near-IR laser is set in the GDD pull-down panel. Select the appropriate objective from the table. The default (zero GDD) is adequate for multi-photon operation. The fine tuning slider can be adjusted for special circumstances (e.g., operation in deep tissue).



Setting the NDD Detectors

Starting up in MP_LASER_ON mode will enable the NDD Super HyD detectors, HyD6 and HyD7. They appear as strips below the single photon detector strips. Click on the box at left to activate them. Click on the colored circle at left to change the color rendering.



The gain is controlled using a single gain slider. The NDD detectors are *very sensitive*. Use low gain and low laser power initially. Operate only in a darkened room. A darkroom cloth is available to mask stray light.

Setting the Detector Modes

As for single-photon acquisition mode, the HyD detectors can operate in three modes:

Standard Mode: In Standard Mode the photon count is scaled by the Gain value (0-500). If the Gain is set to a high value, the pixel values will be highly quantized and the imaged will have abnormal gradations of scale. We recommend you set the HyD gain to no more than 100 in this mode, even for a bright signals

Counting Mode: In *Counting Mode*, the Gain is disabled and the raw photon numbers are returned. For multiple presentations, you must also use *Line Accumulation* and *Frame Accumulation*. The image, initally dim, will gradually improve in quality as the photon counts increase over multiple presentations. *We have found that this method produces very sharp images if used properly.*

BrightR Mode: We do not understand this mode and suggest you not use it.

Managing MP Laser Power

The EOM window, which controls MP laser power, has four slider controls.

On the right, the EOM slider must be set to 1 to permit near-IR excitation to reach the specimen. The offset parameter is set by the application as a function of wavelength and should not be modified.

On the left of the window are two controls that are misleadingly labeled. They are, effectively, coarse (left) and fine (right) control of laser intensity at the specimen. The number at the base of the left slider is the excitation wavelength, in nm.

Best practice is initially to set the coarse slider to zero and the fine slider to a low value. Using the *Live* mode, increment the coarse slider slowly until an image is obtained. Adjust the fine slider to obtain the optimum image.

Shut Down

Switch off the EOM controller and the Super HyD power supply, if used.

Raise the objective using the nosepiece Z-control (screen 4 of the Microscope Controller).

If you have used the oil or water objectives, select the 10x objective (screen 3 of the Microscope Controller). This will make the oil or water objectives easier to clean.

Clean any oil objectives you have used with lens paper and ethanol.

Clean any water immersion objectives with high-Q water.

Remove your specimen from the stage.

Close the LAS AF application – you will be prompted to save the files that you want to save. Save to folders on drive D: *only*.

Log off the computer.

Switch off the confocal at the power strip.

Switch off the metal halide illuminator power supply.

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