

Leica TCS SP8 Instructions

Leica True Confocal Scanning-Spectral Photometric 8



RCB Advance Microscopy Facility
Key number 102 B

Regional Centre for Biotechnology

NCR BIOTECH SCIENCE CLUSTER

DEPARTMENT OF BIOTECHNOLOGY

SOP created on 11 Feb 2017

LASER SAFETY & GUIDELINES

Do not defeat interlocks.

Do not open panels.

Do not detach light guides.

Prior doing Confocal Imaging Keep the Temperature of room at 21- 22⁰ C.

While during imaging do not disturb both the Microscope & PC Laser table.

Imaging room should be kept dust free.

There should be no vibration or shaker kind of instruments should be running near the Imaging room.

Do not open any external part of the Instrument.

Imaging should be made under Technical Observation only for non certified users.

Make proper Google calendar booking.

Use of cell phone is prohibited inside microscopy room.

Submit duly filled sample submission form prior to experiment.

SWITCH ON PROCEDURE

Only people who have received introduction instrument training and passed certification test can use instrument alone and on holidays are allowed to operate the system! If you need intro please write to surajtevari@rcb.res.in, sivaram@rcb.res.in, or sjmathew@rcb.res.in or call at 0129-2848800 ext.: 786 (Microscopy facility)

1. **Check** the microscope if everything looks clean and normal. If not report it in the logbook.
2. Turn on the power switches with **green 1** (wait for 2 minutes till camera light stops blinking), **red 2** and **yellow 3** flagged in sequence.
3. **Switch on** all the three green buttons marked as **PC Microscope**, **Scanner Power** and **Laser Power** on the right side under the table are. Switch these on in this order and turn the **Laser Emission key** 90 degrees clockwise.
4. **Switch on** the **fluorescent lamp** (white box on the left side of the microscope desk).
5. **Switch on** the **thermostat** (blue box on the small desk left of the microscope) if you want to do live imaging with temperature control.
6. **Login** to the computer with your account.
7. **Wait** 1 min so the system is fully booted.
8. **Click** on the **LAS X** software icon on the desktop. Shortly you will see the **LAS** start screen. Check under configuration that **machine** is selected. Press **OK** to start the system.
9. If live cell setup is needed turn on all the 4 power point and select **machine-okolab** from the configuration.
10. At the end of the starting process it will ask you to initialize the stage. Click **YES**.
11. **Switch on** those lasers that you want to use in the **LAS X** software under the **Configuration\Laser menu**.
12. If you switch on the argon laser put it on 2% always. **Note** that the **405 and argon lasers** need at least 20 minutes warm up time for proper use.
13. The machine is ready to use.

TURN ON HARDWARE

- A. Switch on button pad – left to right then turn key on.



- a). **First** switch on PC Microscope button

*** You do not have to start the operating system – it starts automatically when you switch on the computer.**

- b). Log On the Computer Under “USERS”

- c). **Second** switch on Scanner Power button

Wait for 2 minutes

- d). **Third** switch on Laser Power button

***The power supplies and fan of the system have been started.**

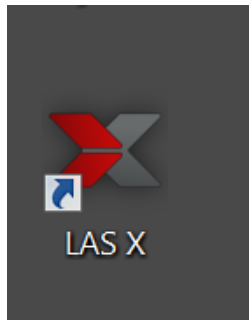
- e). Turn the Laser Emission key towards On-1

Wait for 2 minutes

START SOFTWARE

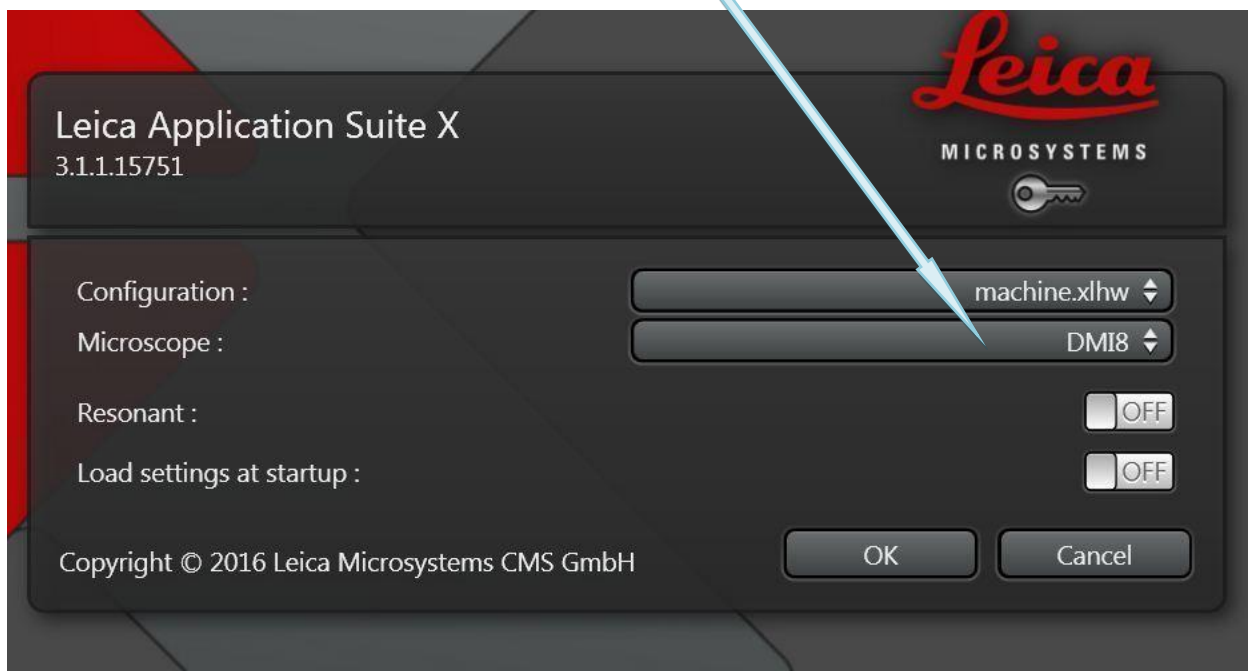
***Note :- HARD to SOFT**
(Confirm all Hardware's *ON* then go to Software)

Activate the Leica *LAS AF Confocal* software with the icon on the desktop.



***Before the system start or start of the LAS X, the objective must be moved lowered, because during initialization, it can come into contact with the objective nosepiece and damage the objectives**

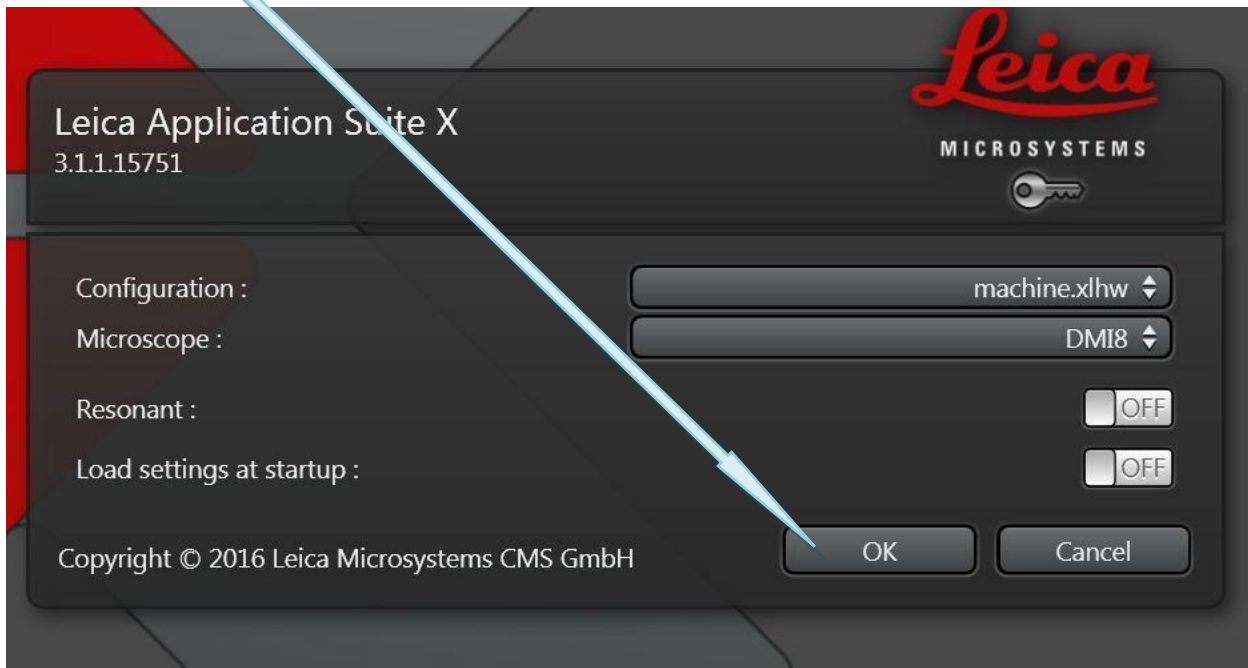
Look for the settings DMI 8 in Microscope Stand



Look for the settings Machine in Configuration mode



Click **OK** in the splash-screen.



After clicking on **OK** button Software will start analyzing the configuration for the Instrument.

Software will ask for Stage Initialization, make sure the stage is moved downward & then press **YES**.



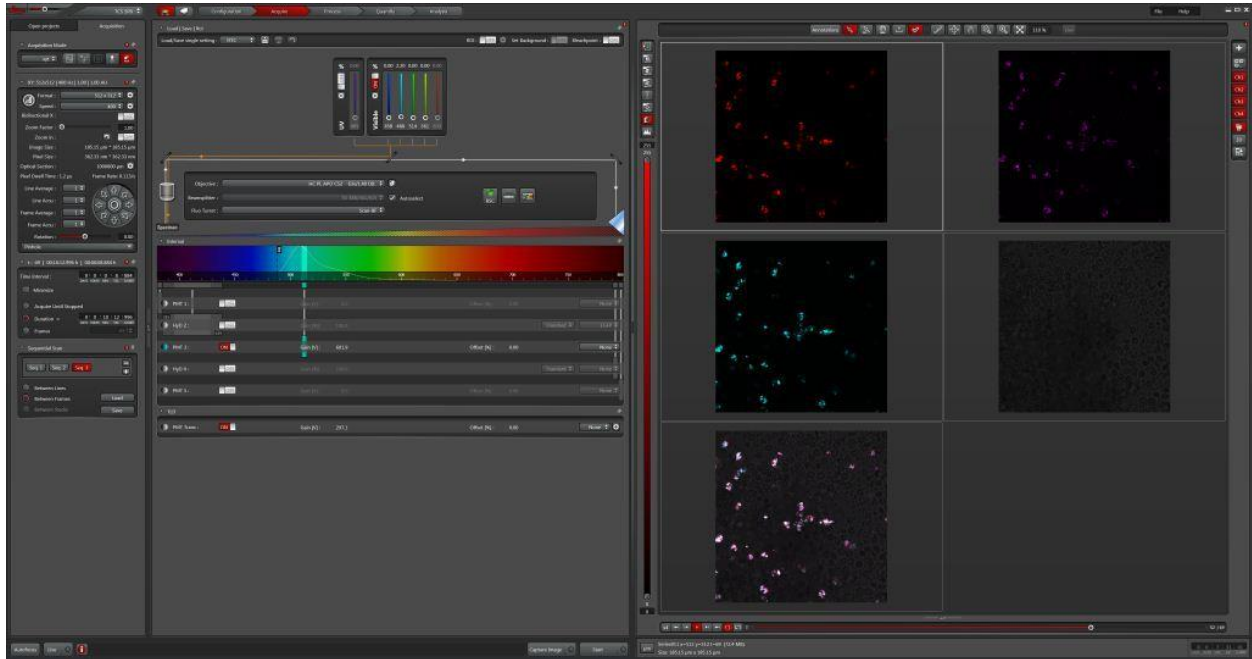
IMPORTANT: BEFORE proceeding.

When the **Microscope Stand** windows appears, select **No** unless you plan to use the **Tile** or **Mark and Find** functions for the motorized stage.

NOTE: If you need to initialize the stage, lower down the objective, because the motorized stage might possibly hit the objectives during initialization.

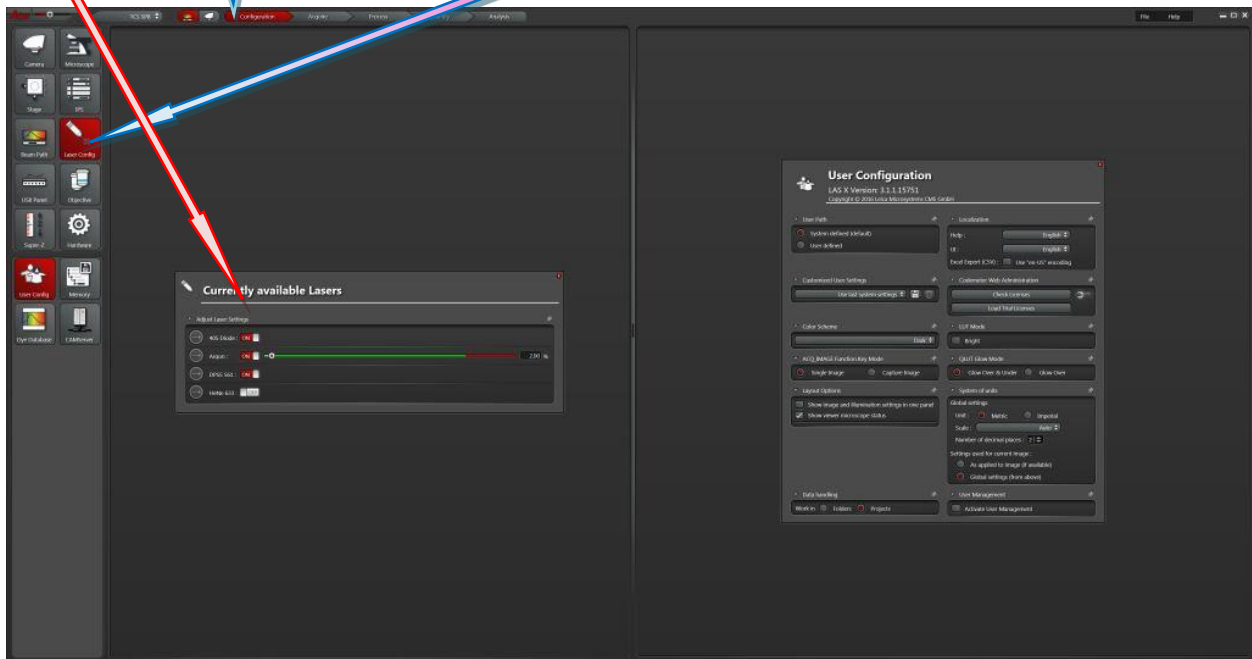
Once the software is initialized, the screen will pop up & will appear as follows.

Where Position 1, Position 3 and Position 5 are PMT and Position 2 and Position 4 as HyD.



Select the **Configuration** tab and then the **Laser setup** window.

Turn On the lasers you require. If you are using the Argon ion laser, drag the slider to 11 %.



MOUNT SAMPLE

Gently tip the transmitted light arm backwards for easy access to the stage.

Add a small drop of Type F immersion liquid to your sample or the objective and mount your sample. Raise the objective by activating coarse focus on the axis controller unit and focusing up using the microscope's focus wheel until the objective just touches the immersion liquid.

Gently return the transmitted light assembly.

(NOTE: - While moving the light arm do not apply extra pressure or force)

Find sample through eye piece.



Fine

Course

OBJECTIVE

Choose an objective lens from the drop down menu.

10x air 0.40 NA

20x air 0.70 NA

40x air 0.85 NA

40x oil 1.3 NA

63x oil 1.4 NA

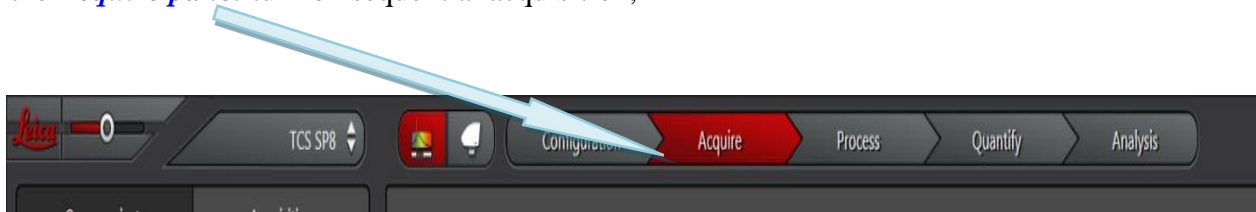
100x oil 1.4 NA

Place the appropriate immersion oil (EXCEPT for the air lenses 10x, 20x and 40x) on the objective lens. Place your sample on the microscope stage. **BE VERY GENTLE**--the stage is very delicate. Position the sample over the lens using the stage controller on the **SmartMove** and focus using the Z axis wheels (either **Fine** or **Course** settings).

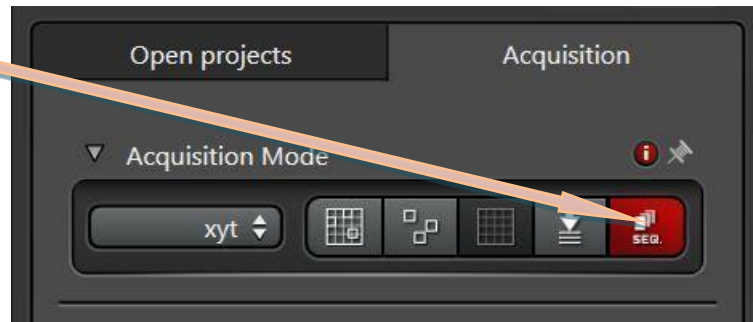
CONFIGURE HARDWARE

A). Dye settings

In the *Acquire panel* turn on sequential acquisition,



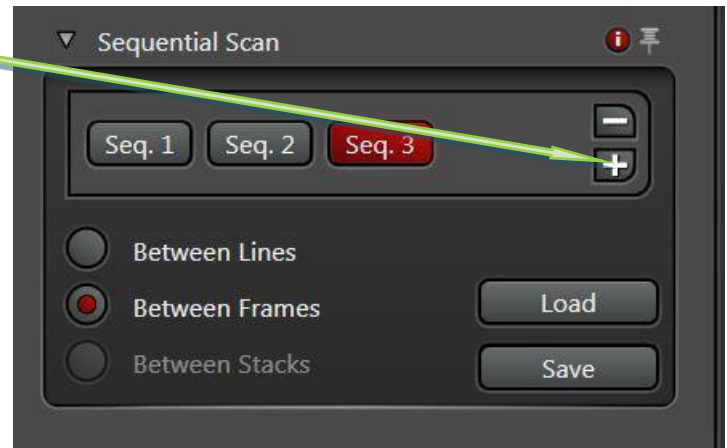
click the *seq.* button on the *Acquisition mode* window. The sequential control panel should now appear at the bottom.



If you already have a sequential acquisition protocol saved you can **load** this now.

If you need to configure a new protocol, add the number of channels you wish to acquire by clicking on the **plus button**.

The **Sequential Mode** menu appears at the end 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".



B). Scan speed

While you configure the detectors, set *Acquisition Mode* to XYZ.

Set the scan speed to high by activating the *XY dialog* and selecting *400Hz* from the Speed drop down box for observation.

You can speed scanning further by using the *Bidirectional X* scan option although this sometimes results in a de-interlaced image. This will give you a noisy image but allow faster setting of the detectors with less bleaching of the sample.



C). Pinhole

The pinhole determines the thickness of the “optical section” you acquire. The thinner this is the sharper your image, but the fewer photons in your image (i.e. it will be noisier). The optimal size of the pinhole is determined by the objective’s numerical aperture and the wavelength of light you are acquiring.

Configuration Window

UV: 405 diode laser

Visible Lasers:
Argon with multiple laser lines 458, 488, 514 nm
Yellow diode with 561 nm
Red diode with 633 nm

You can choose a preconfigured setting

To open a laser shutter, click on the radio button (red when open)

Laser power is adjusted using the slider bar

Choose an objective

Objective : HC PL APO CS2 63x/1.40 OIL
Beamsplitter : TD 488/561/633
Fluo Turret : Scan-BF

Internal

400 450 500 550 600 650 700 750 800

PMT 1 : OFF Gain [V] : 0.0 Offset [%] : 0.00 None
HyD 2 : OFF Gain [%] : 100.0 Standard EGFP
PMT 3 : ON Gain [V] : 601.9 Offset [%] : 0.00 None
HyD 4 : OFF Gain [%] : 100.0 Standard None
PMT 5 : OFF Gain [V] : 0.0 Offset [%] : 0.00 None

TLD

PMT Trans : ON Gain [V] : 297.1 Offset [%] : 0.00 None

There are 2 HyD detectors and 3 photomultiplier tube (PMT). Turn on the box to activate the laser

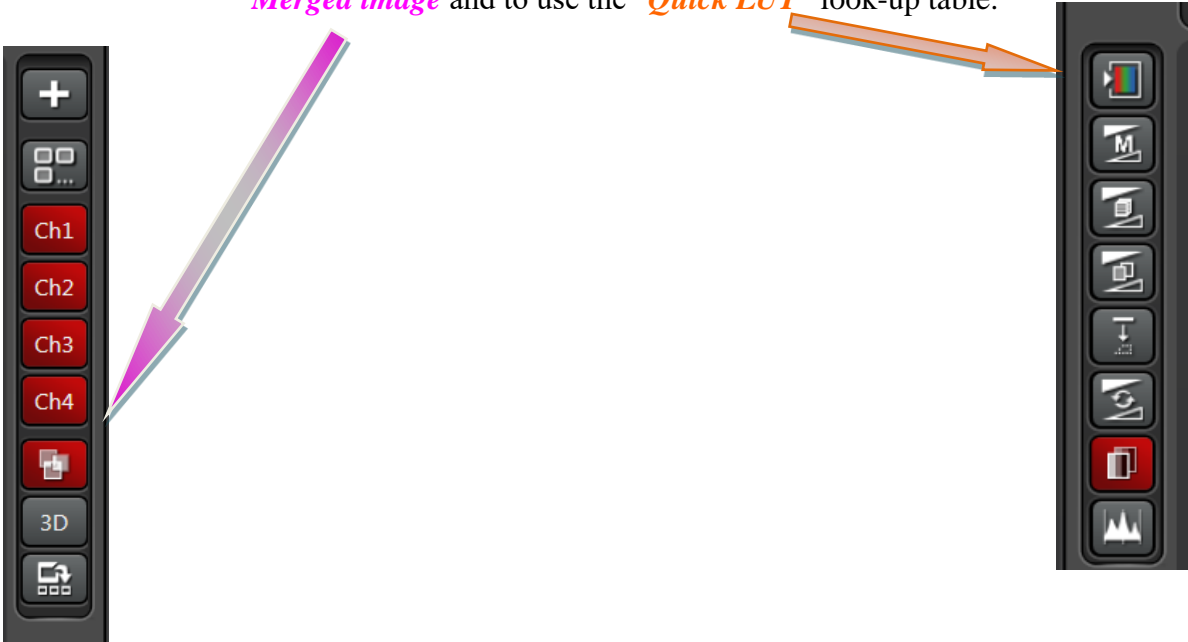
Transmitted light detector

Spectral detection (emission) range is adjusted here

ADJUST DETECTOR SETTINGS

A). Prepare display

To aid adjustment of the detectors, the display should be set to show the *Merged image* and to use the “*Quick LUT*” look-up table.



Click the Quick LUT button until it cycles to the range set LUT.

This LUT will display bright-green when the grey level equals zero and bright blue when it saturates. Where blue means over-saturated area immediately reduce AOTF range. Green means undersaturated area with HyD there will be great signal to noise ratio so green range will not be a problem.

B). Adjust Offset

When there is need to change the offset or gain, always click Live and after adjustment click Stop. The adjustment should make in such way so that they should not make the area under observation go Bleach out. When not in observation go Stop.

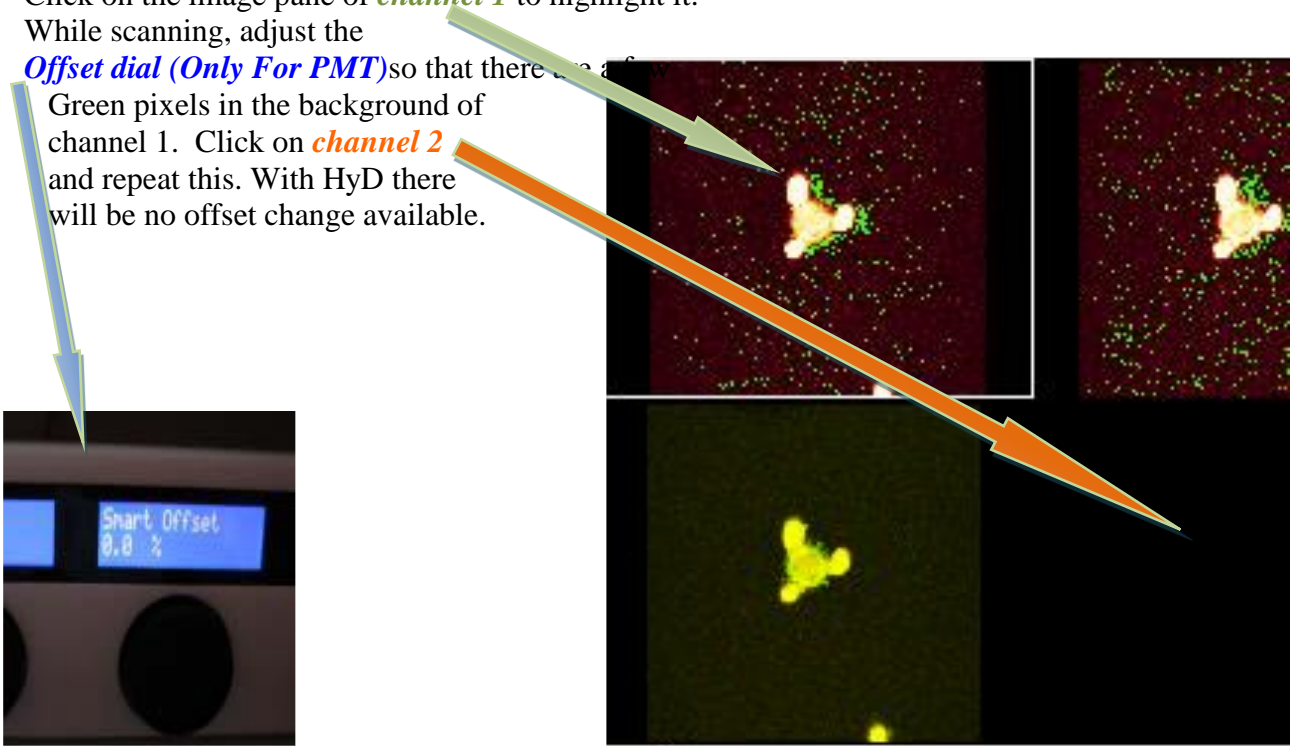
NOTE: The spectral range should always be at least 10 nm greater than the excitation laser line.

Example: If using the 405 nm laser, then the minimum in the spectral range should be at least 415 nm.

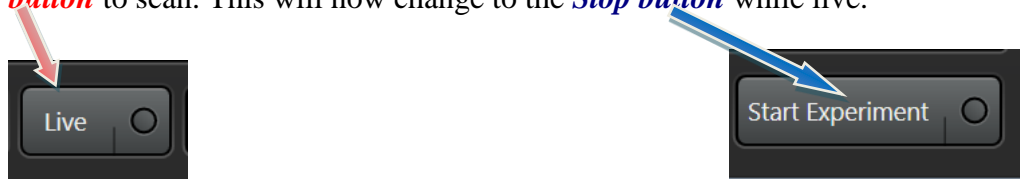
Adjust the focus until you have the brightest focal plane of interest. Click on the image pane of **channel 1** to highlight it.

While scanning, adjust the **Offset dial (Only For PMT)** so that there are a few

Green pixels in the background of channel 1. Click on **channel 2** and repeat this. With HyD there will be no offset change available.



Click the **Live button** to scan. This will now change to the **Stop button** while live.



C). Adjust Intensity

The intensity of the image is dependent on two things at this point: the number of photons generated (**laser power**) and the amount they are amplified by the detector (**detector gain**). The optimal settings need to be determined empirically. Adjust the intensity with these two parameters until there are a few speckles of bright blue pixels indicating that you are using the full range of the detector.

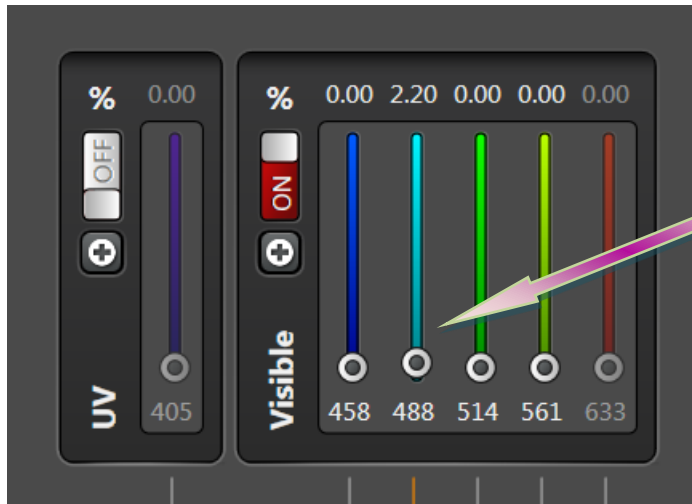
D). Detector Gain

You can increase the signal amplification using the **Gain dial** but this will increase noise. The gain dial will max out at 1000 V but extra gain up to 1250 V can be achieved using the software control. If you increase gain a long way, you may need to reset the offset. Try not more than 700 V.

E). Laser Power

Visible continuous wave (CW) lasers. You can increase AOTF values in % the number of photons generated by adjusting the *laser powers* but this will increase bleaching and will cause overloading in detector. Try keeping lower values to begin with like 0-3%.

Ultra Violet (UV) lasers . You can increase the UV laser as per the requirement.



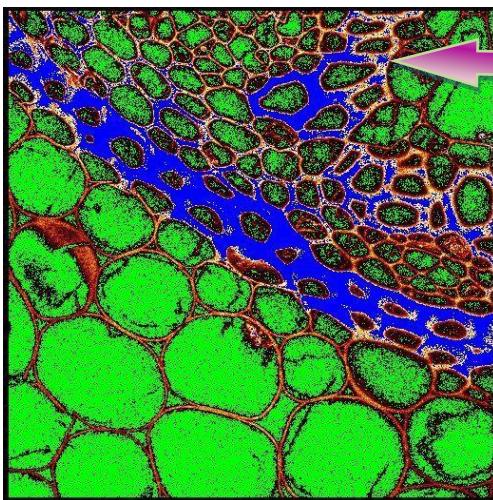
Set the **laser power** with the slider. Start with 3%.

REMEMBER: The HyD detectors are very sensitive.

Make sure all the other lasers are at 0% power.

For imaging use PMT first. If image comes with PMT do not try to go for HyD.

If HyD is needed in cases for imaging then lower down AOTF values to 3% and the open LUT to see saturation level. If oversaturation appears reduce AOTF



saturated

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.

The range-finding LUT is different to the one used by LSM.

LAS X uses green for below range, blue for above range, and red for within range.

SET FINAL SCAN PARAMETERS

A). Frame size

Before setting the frame size, crop the image to include only the field of view of interest. Select **Zoom in** from the XY panel, then select the rectangular **region of interest (ROI) tool** and drag this around the area you want to acquire.



You can resize and reposition this ROI.

Activate Live scan to ensure you have the correct field of view selected, then stop acquisition. The frame size should be set to ensure optimal **pixel size**.

Pixels smaller than the optimal will result in over sampling, increased bleaching, increased noise and no extra spatial information.

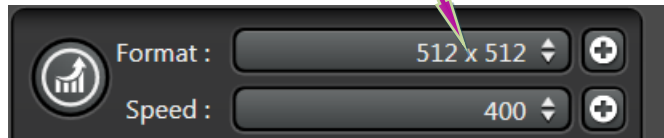
Pixels larger than the optimal will result in lower spatial resolution. This may or may not be important to you.

Objective Optimal pixel size (nm) with 488nm laser

63× 1.3NA 100

20× 0.7 NA 200

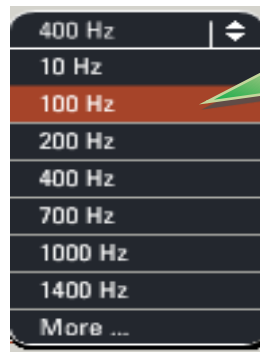
Adjust the frame size by clicking the **Format** drop down box and selecting **More**.



SCAN SPEED

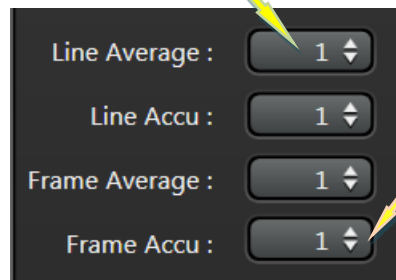
The noise in a confocal image is primarily intrinsic photon shot noise. The Signal to noise ratio is proportional to the number of photons sampled. So you get less noise if you sample more photons. Reducing the speed of the scan effectively increases the photon sample time per pixel – the “pixel dwell time”. It does reduce the frame rate so a slow scan speed is not suitable for adjusting the detector settings or high speed biological events.

For fixed samples where maximum resolution is required, set the scan speed to **100Hz**.



AVERAGING

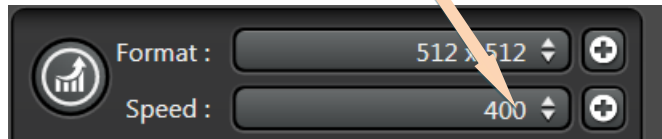
Another way to improve the signal to noise is to sample and average each pixel a number of times. The microscope can be configured to average a number of frames and/or lines. Or for weak signals, these values can be added to ‘accumulate’ the signal.



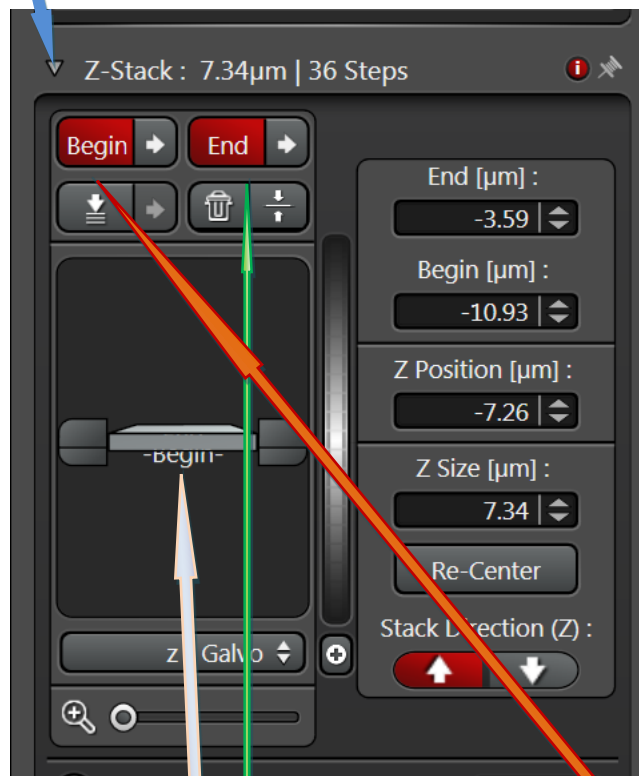
NOTE: Averaging removes “noise” detected by the PMT. Generally, less averaging is required when the Gain is set low.

ACQUIRING A Z-SERIES

Set Z-range First, change the Speed to **400Hz** to facilitate setting the z-range.



Activate the **Z-stack** drop down window



Activate **Live** acquisition

Click the beige **plane indicator** to select it. It will get an orange border once it is highlighted

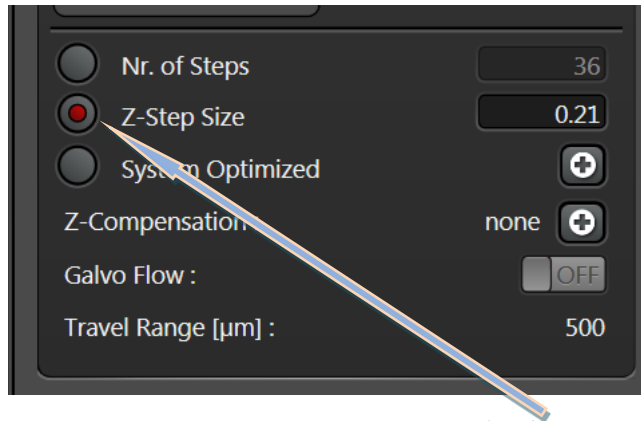
Use the mouse wheel to scroll to the top of the sample. Then click the **Begin** block-arrow which will then enter the z-position.

Select the **plane-indicator** again and scroll down until the bottom of your sample appears in the live image window. Click the **End** block-arrow to set the end of the z-range.

Set Z-step

By default, the software calculates the optimal z-step and the number of steps required for the range you have set.

However, often, we do not need to sample with such high frequency.



To override the default settings, select the **top radio-button** and enter the step size, or number of slices you wish to acquire.

Acquire Z-series

Change the scan speed back to your original slow speed then select **Start**.

Your z-series will then be sent to your experiment and need to be saved.

Using the Dye Assistant to set up beam paths

The **Dye Assistant** is a wizard to simplify beam path setup. It is activated using the button. It allows selecting one or more dyes from the dye database, and a preference of detector. It then suggests settings for acquisition, showing fluorescence yield and crosstalk.

The suggestions can be edited, or applied directly. Applying a setting turns on lasers, sets emission sliders, activates detectors, etc.



SEQUENTIAL SCANNING

To minimize the potential for cross-talk between fluorophores, each channel can be acquired in sequence. This means that when you are collecting the red emission, the green dye is not being excited so does not appear in the red-channel.

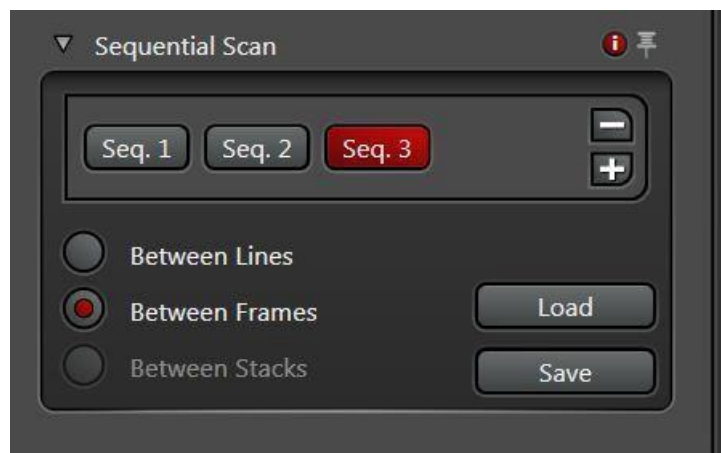
This is of critical importance for colocalisation studies but does cause problems with live cells as the sample may move between channel acquisitions. It is best to acquire channels in the order ‘longest-wavelength-first’, i.e. red, then green, then blue. Channels can be obtained sequentially in a number of ways.

Between lines is quickest and allows for fewer issues with movement artifacts, but does not allow radical changing of the filter settings.

Between Frames allows filter sets to be completely reshuffled between channels for maximum cross-talk prevention, but has a significant time delay between channels and prone to movement artifacts.

Between stacks. When acquiring a sequential channel z-series, the delay between frames and duration of acquisition can be very long if you change settings between frames (i.e. each z-slice).

This option speeds acquisition of z-stacks but can result in movement artifacts and z-registration issues. These registration issues can arise if the z-focus does not return to exactly the same position for slice 1 of channel 2. This can cause errors, especially in colocalisation or ratio-metric analysis.



Set channel settings

Select the first channel and configure as you would a simultaneous acquisition (Section 5). Click Scan 2 button and repeat for your shorter wavelength dye. Repeat for all channels. Adjust the final scan parameters.

Save these settings to reuse them next time using the **Load** button.

REMEMBER: The minimum in the spectral range should be 10 nm greater than the 405 nm laser line.

FOR HyD DETECTORS, THE SMART OFFSET IS ALWAYS 0.0%, so you can only adjust the gain.

Alternate Acquisition Modes - Tile Scan

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

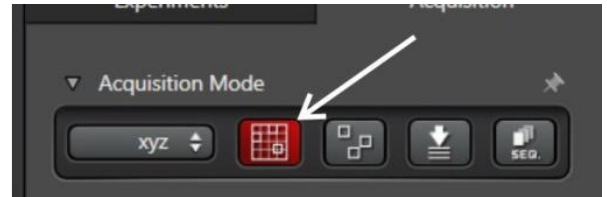
Note: Tile Scan should not be combined with image rotation or erroneous results will be obtained.

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

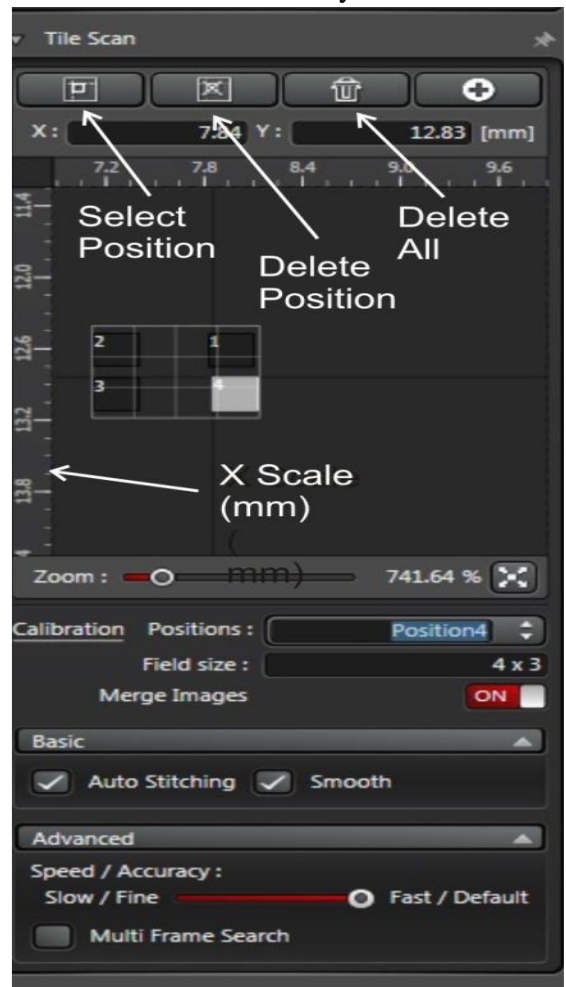
The simplest method is to select Auto Stitching, with Smooth off.



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. 25



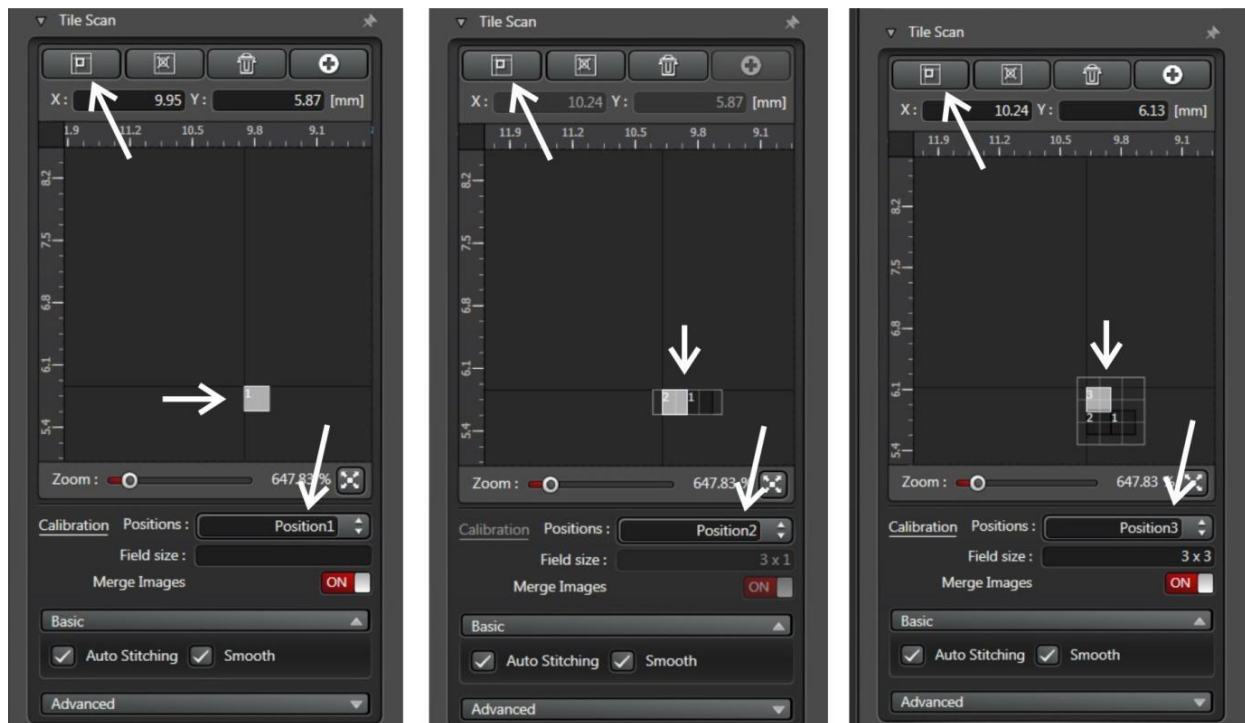
Setting up a Tile Scan The approach to setting up a tile scan is different.

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. 26

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 as 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.

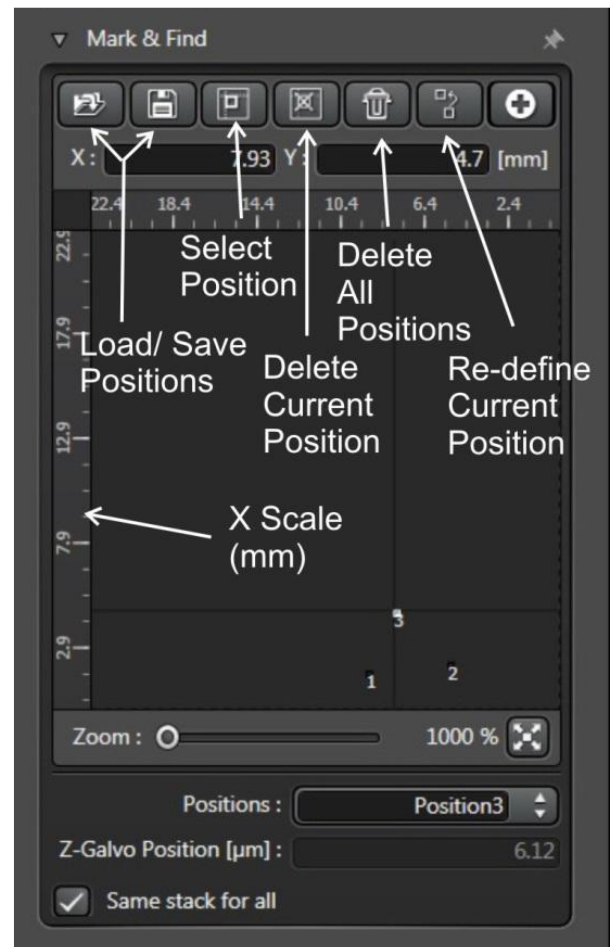
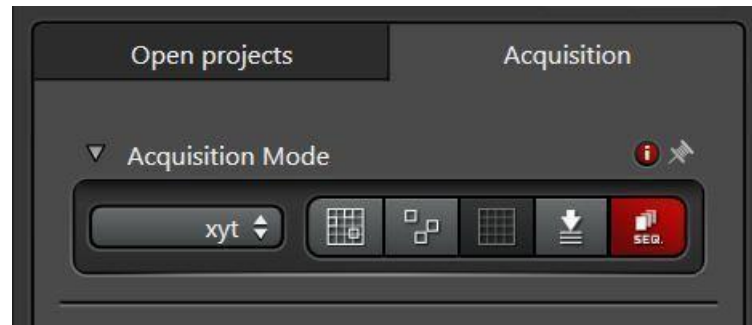
Use the pull-down list of position numbers to select a new position. The stage will move to that position and the focus position will be adjusted accordingly

Combining Tiling with Sequences

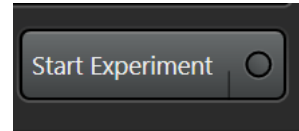
When defining a sequence involving changing X-Y position, remember to select the appropriate position number when defining the sequence event.

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.



EXECUTE EXPERIMENT



A). Acquire

To acquire a single XY image (actually XY as each pixel has a finite z-depth) click the **Start** button.

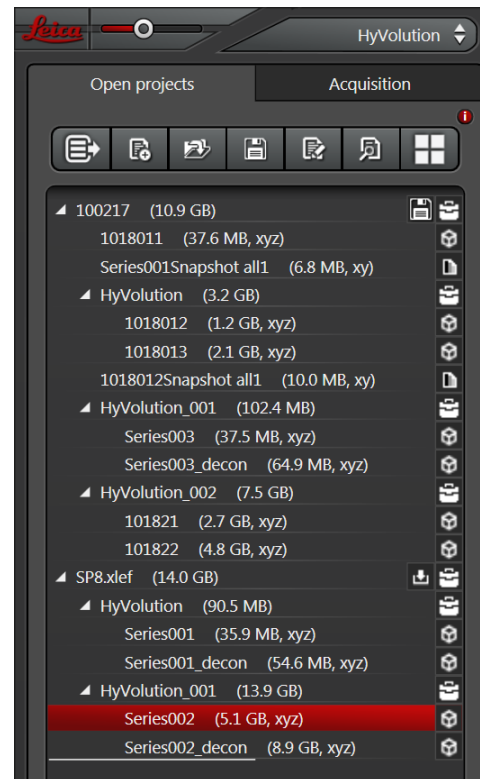
This will put the XY image in the **Experiments tab**.

B). Save

Your data is saved in a single experiment file (*.LIF). This file contains the multiple image series (xy, xyz, xyt etc.) that you acquire whenever you hit the **Start** button. Save your experiment by right clicking it and selecting **Save Experiment As** from the menu.

Rename the current image (SP8) by right clicking it and selecting rename. Subsequent acquisitions will be added to this Experiment, but not automatically saved. You need to right click the series rename to rename it something more memorable than "Series002".

Then right click select **Save Experiment...** to save the data.



COPYING DATA

The saved data then can be copied into cd/dvd's. Data should be properly labeled and stored with proper entries.

Avoid use of USB in all condition (This probably can cause serious damage to the installed software for imaging).

Camera Mode Imaging



Leica TCS SP8 is with **Hamamatsu ORCA Flash 4.0** connected with Cam link for high speed read out. Select the camera sign from the main menu bar.

There will be system display user interface. From this desired filter can be added as per need (DAPI, FITC and Rhodamine) by selecting +.



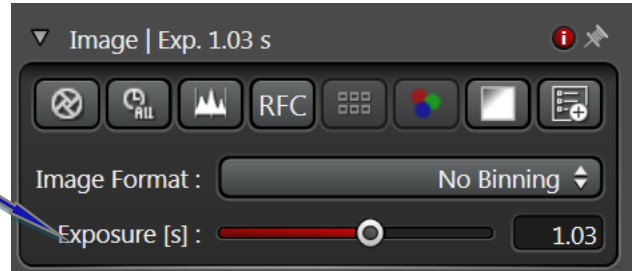
Objectives can be changed as per the experiment demand.

Focus on the specimen using transmitted light (DIC), if possible

Switch to the desired filter in the software (e.g., FITC)

Adjust the exposure time from the slider.

Click Live to observe the image and adjustments.
Click Start to capture the image.



Illumination Settings

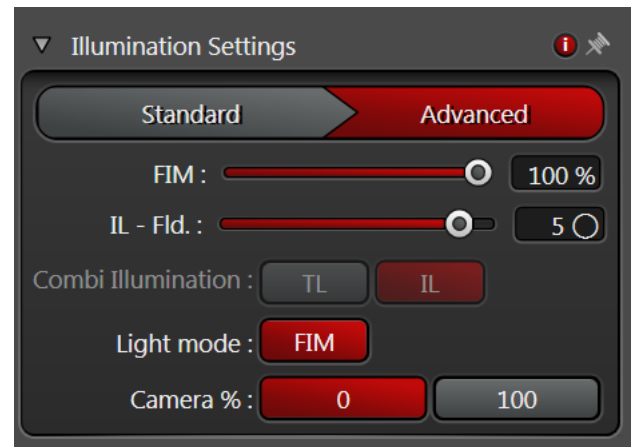
In this dialog, you can configure the lighting for image acquisition with the camera.

Intensity

You can control the intensity of the illumination of the specimen under Intensity.

If you are working with transmitted light (TL), you can set the intensity of the transmission light source.

If you are working with incident light and have activated the (IL) Fluorescence Intensity Manager (FIM) function, you can reduce the intensity of the excitation light. Two fixed increments (100 %, 55 %, 30 %, 17 %, 10 %) for the intensity of the excitation light are available. You can use this button to open the advanced illumination settings.



IL-Fld.

You can use this slider to control the field diaphragm.

Combi Illumination

Here you can set the illumination with which you want to work: it is as FLUO-DIC



Touch Screen Control of Microscope Function

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)

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 (labelled 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. 6

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

Z -mode:

The Home position and an approximate focus position are marked. When Z is selected, press and hold the Home button to raise the nosepiece to its maximum. Press and hold the up/down button to return the objective to an approximate focus position.

X-Y Mode:

Use of this mode is NOT recommended. When X-Y 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.

Screen 5: Not normally needed by users.



SWITCH OFF PROCEDURE

When you are finished with your study do the following:

1. **Save** your **files** to your folder. It is recommended to save the data file (.lif) which contains all the settings of your experiment. You can reuse these settings later when you do imaging with similar samples.

2. **Clean** all the **objectives**, the **condenser** and the **stage**.

IF YOU DO NOT CLEAN THE OBJECTIVES PROPERLY, YOUR MICROSCOPE PRIVILEGES WILL BE TAKEN AWAY.

3. **Swing in** the **lowest magnification objective**.

4. **Close LAS X** software and log-out from your windows account. If you are not the last one of the day (check in the booking calendar!), leave all the lasers ON.

If you are the last user of the day (check it in the booking calendar), follow with these steps to shut down the microscope:

5. **Switch off all lasers** in the **LAS X** software (Configuration\Laser, uncheck the boxes) before exit from the software.

6. **Switch off** the **OKO LAB setup** (Whire box on the small desk left of the microscope, if used).

7. **Turn the Laser Emission key** anti clockwise to off. Wait 5 min while the lasers cool down. You can hear when the ventilation switches off automatically.

8. **Shut down Laser Power, Scanner Power and PC Microscope** buttons in this order. Do not switch off the **Laser Power** button until you can't hear that the laser ventilation turned off (or if you waited 5 minutes)!

9. Turn off the power switches with **yellow 3, red 2 and green 1** flagged in **sequence**.

10. *Use the **logbook** and write in any problems and comments occurred during your microscope session! If you signed the logbook and did not report any problem, it means you left the machine in perfect condition.*

11. **Switch off** all the lamps in the room and close the door submit the keys back.