

Confocal 1 – Leica SP8

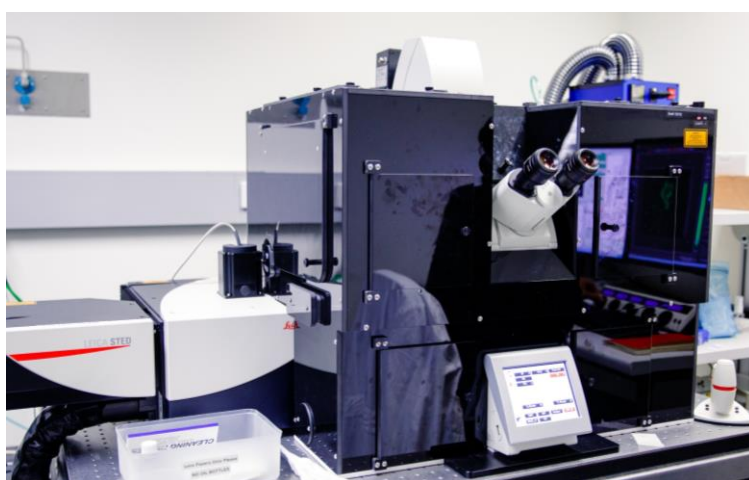
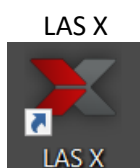
Including STED, Resonant Scanning, FALCON (FLIM), Navigator & Lightning

Quick-start Guide

Location: Room 6.029a

(Updated: 8/12/2020)

Acquisition Software:



Available Objective Lenses: 10x Air, 40x Air, 40x Water, 93x Glyc and 100x Oil

(20x Air & 20x Korr are also available)

Available Laser Lines: 405, 440nm Pulsed, WLL (470-670nm, pulsed), and 592, 660, 775nm depletion

Be mindful of what you are doing at ALL times: The microscope and the lenses are delicate and very expensive and should be treated with care.

** The 93x objective comes with a motorised correction collar (MotKorr) and is controllable within the LAS X software.

Saving Your Data

Save your Data to D:\UserData and create yourself a folder if it doesn't already exist. **DO NOT** save directly to the network, only transfer your files at the end of your session. LAS X saves files as a .lif format which is openable in FIJI/ImageJ provided your Bio-Formats is up to date.

Connecting to Network attached Storage

- Data you acquire on this device should be backed up to a server for future retrieval and use, as the computer is cleaned of data regularly.
- UQ-wide resources:
 - **UQ RDM**
 - Project based shared drive
 - Can be cached locally (<\\data.imb.uq.edu.au>)
 - For more information see <rdm.uq.edu.au>
 - Note this uses UQ-SSO credentials (type uq\username)

Incubation

The incubation on the Leica is turned off by default. If you require temperature or CO₂, the cool-down/warm-up times need to be factored into **YOUR** booking time (and designated as such in PPMS) and you are responsible for ensuring the temperature is back at room temperature **AND** stable before the next user is booked on.

For temperature and CO₂, this can be switched **ON** at the start of your session and set to your desired concentration through both the software and the touch-pad controls. Please make sure to turn the temperature and CO₂ **OFF** again once you have finished your session. The control boxes are located under the air table (Blue = Temp, Red = CO₂).

Slide Cleaning and Preparation:

- Make sure your slide is scrupulously clean – all dust, old oil and mounting media should be removed with a small amount of 70% ethanol.
- If you can use a hard setting mounting media please do so and **DO NOT** use glitter nail polish to seal your slides as this will scatter laser light. Be sure to give your mounting media and sealant at least 8 hours to dry properly.
- For STED always use a #1.5 coverslip with either Prolong Gold/Diamond/Glass
- Do Not Use DAPI for STED

Start-up Procedure

- **Using the Microscope Power Switches, turn on the system in the following order.**
 - Turn on the PC and Microscope switch,
Wait until the PC has started up, then login to the PC before proceeding to the next step
 - Turn on the Scanner Power, then wait 10 seconds
 - Turn on the Laser Power, then wait 10 seconds
 - Turn the Laser Emission key to the ON position
 - Log into the computer using your own credentials



Turn on the Fluorescent Lightsource

- Press the red button on the control box on the computer table to turn on the fluorescent lamp (for the eye pieces) for 30 minutes
- The cooling lamp with flash for 1 minute before turning off the bulb, if you require more time, press the red button again for an additional 30 minutes
- If the yellow cooling mode light is on, the fluorescent bulb will not turn on for 5 minutes.

Start Leica LAS X software

- Note it can take a few minutes for the software to launch.
- When the start-up splash screen appears ensure you turn on the appropriate settings.

Configure = "Machine without climate chamber"


Microscope = "DMI8"

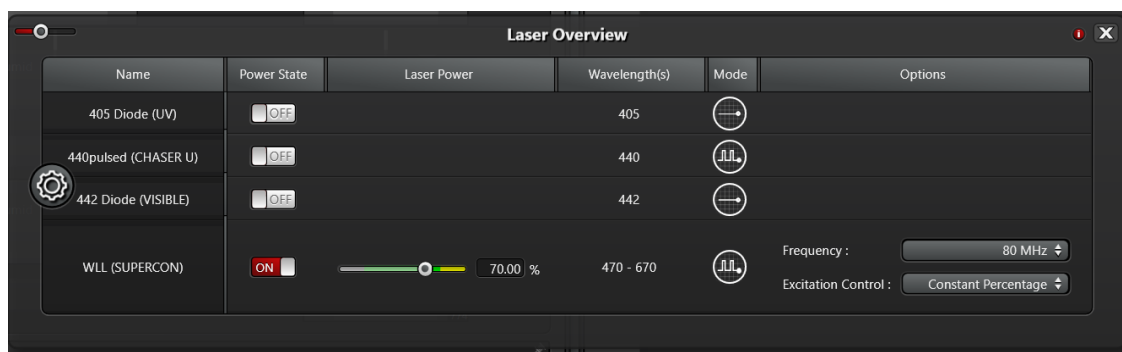
****Note AFC cannot be used with STED**



If you wish to change between modes (i.e. change to Resonant scanning mode) you will need to re-start the software and change these here at the splash screen.

Turn on the Lasers

- Click the  button at the top right of the light path window to open the laser overview panel.
- Note: STED lasers will only be accessible if STED has been activated at start up.)
- Note: STED lasers should be turned on to 100%
- Note: White Light Laser (WLL) should be turned on to 70%

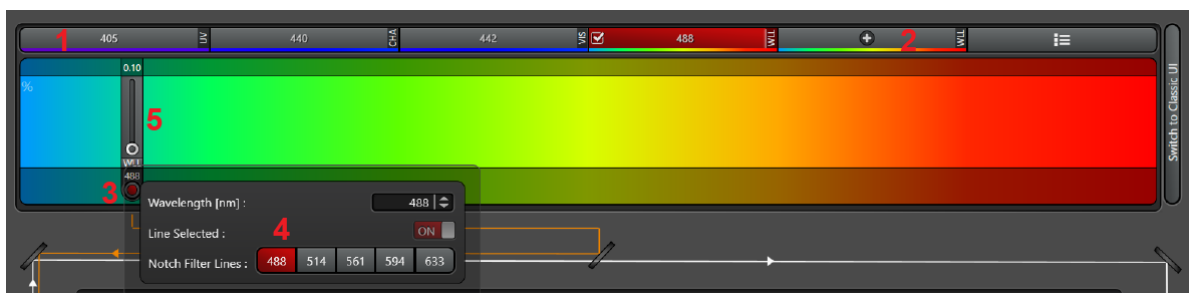


**The WLL does not have equal power across all wavelengths, instead the software by default corrects for this if you have “Constant Percentage” enabled.

- 405 Diode (UV) Laser is suitable for DAPI
- The 440 Pulsed Laser is for CFP FALCON (FLIM/FRET) imaging
- The 442 Diode Laser is suitable for CFP
- The WLL (470-670nm) is suitable for all other fluorophores

Setting up Laser Lines

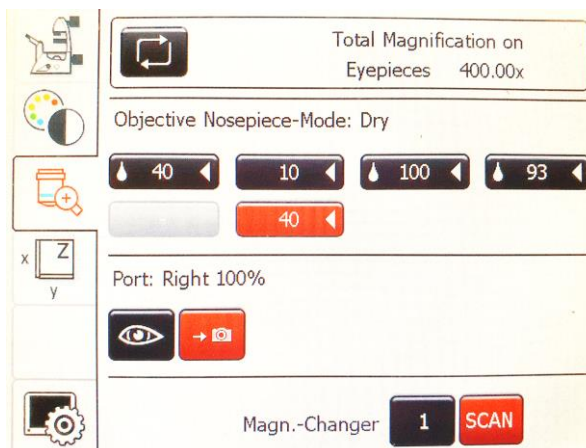
- Adding laser lines to your acquisition can be done by selecting either the diode buttons **(1)** or the “+” button **(2)** for WLL lines.
- To engage the laser the small tick box next to the wavelength indicator must be selected.
- To open the shutter on the relevant laser the small red circle must be selected **(3)**. Note: when laser power is set above 0% and all shutters are open the light path will change from white to orange.



When using the WLL, specific ‘notch filters’ **(4)** exist for common lines which should be utilised for the best performance where appropriate.

Select an Objective

- Objectives – always inspect and clean objectives both **before** and **after** a session:
- Always double check the LCD touchscreen, software or the objective itself to confirm which immersion media should be used on each objective; Dry, Oil, Water or Glycerol
 - Dry (these **never** have media applied): 10x, 40x
 - Water = 40x
 - Glycerol: 93x
 - Oil = 100x
- Do not apply too much oil/water/glycerol to the objectives** and be sure to clean the media off the objective between samples. Apply fresh media for each sample.
- Press the Objective icon on the Microscope LCD display to change to Objective mode.
- Press one of the objective icons on the Microscope LCD display to change objectives.
- If the icon starts flashing this indicates that the immersion fluid for the new objective is different to the current objective. Ensure the current objective is clean, then press the flashing objective icon to continue.



Mount your specimen

- Use the appropriate stage insert (Universal, Slide/Dish or Multi-well)
- Mount your sample, coverslip down
- Press the two front buttons (XY coarse and Z coarse) on the joystick
- Use the front knobs on the joystick to centre your sample above the objective
- Note: Stage Inserts are held in place by screws, talk to staff if you need it changed over.
- Note: The galvanic stage is very sensitive to pressure, **be gentle when changing samples**



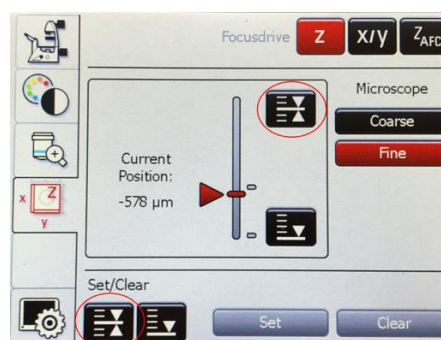
Illuminate your specimen and focus

- Press the FLUO icon on the Microscope LCD display to change to Fluorescence eyepiece mode.
- Press one of the FLUO-Filter cube icons to select a filter cube that matches your sample.
- Press the IL-Shutter to illuminate your sample.
- Alternatively you could use the Transmitted modes to illuminate your sample (BF).

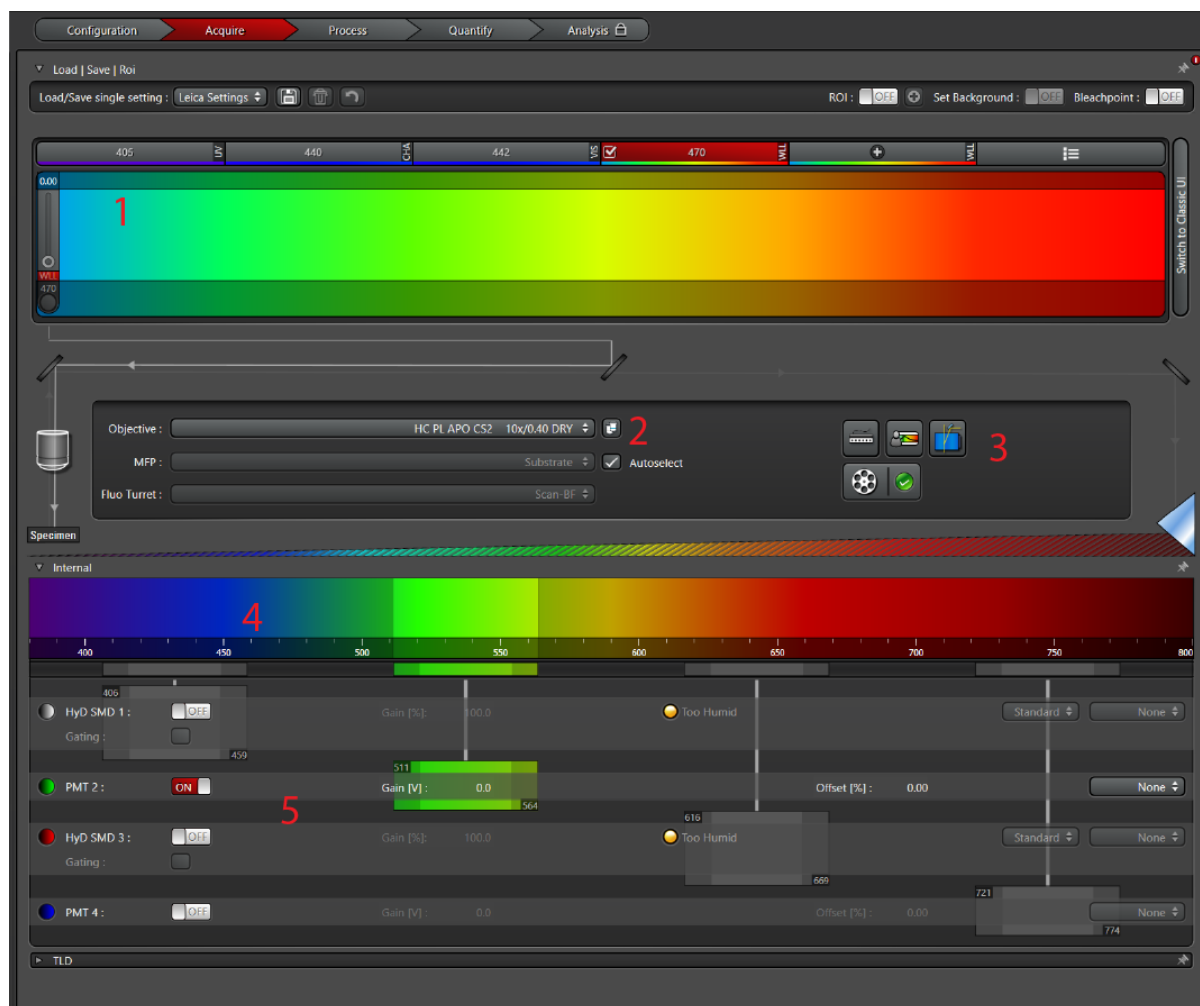


Focus on your specimen

- Use the rear knob on the joystick to focus the specimen.
- Press the Z Fine button on the joystick base, and fine tune the focus of your specimen.
- Press the XYZ icon on the Microscope LCD display to change to Stage mode.
- Press the focus Set/Clear icon to record the focus height, this will enable you to return to focus when changing objectives or similar samples by simply pressing the Focus button.
- Close the IL-Shutter to prevent bleaching of your sample



Software Light path

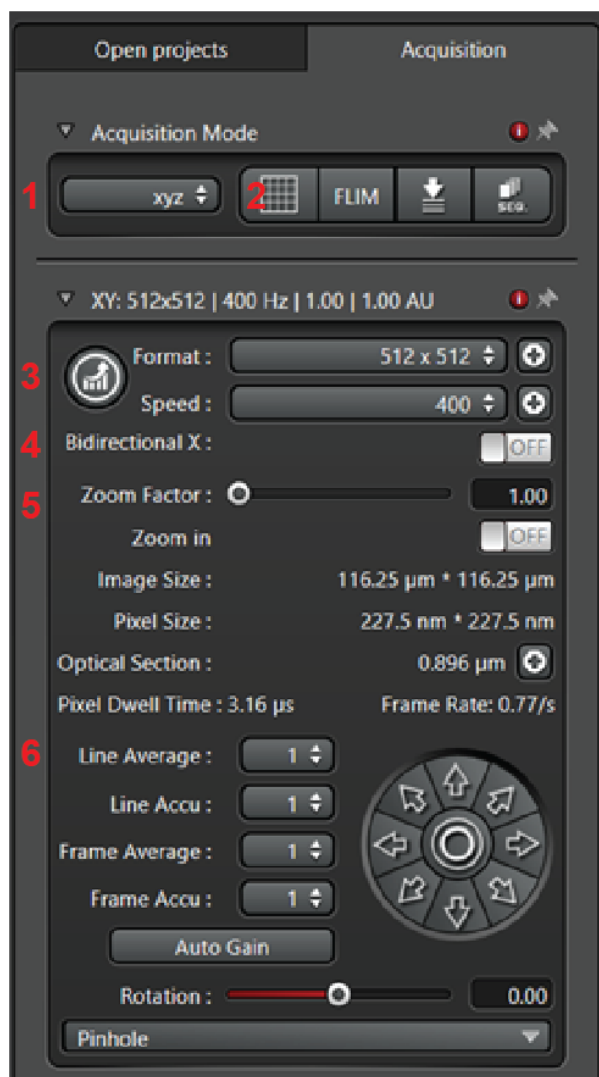


1. **Laser Lines.** To activate a laser line either click on the box corresponding to the relevant wavelength, or the “Plus” symbol for any of the WLL lines.
 - a. Note you will need to drag the WLL lines to the relevant wavelength along the spectrum.
2. **Objectives.** This drop down lists the objectives currently available on the system and also shows the relevant immersion fluid information (eg water, glycerol, oil).
3. **Software Tools.** Here buttons provide shortcuts to the menu for assigning controls to each knob, the Dye Assistant, and filter controls
4. **Emission spectrum.** Active detector gating is visible (and active lasers appear as vertical dotted lines)
5. **Detectors (HyD and PMT).** Detectors are arranged from left to right (HyD1, PM2, HyD3, PMT4, TLD)

Acquisition Parameters

In the top left-hand corner of the LAS X program is the main control panel for setting up acquisition parameters.

- Select the type of acquisition you wish to capture using the drop-down selection **(1)**. Note: for a normal 3D acquisition select xyz. Other modes include time (t) or excitation wavelength (λ) or detection wavelength (Δ).
- If you require 'complex' imaging modes select one of them using the buttons **(2)**. These include *Navigator*, *FALCON*, *Autofocus* and *Sequential Scan*.
- Ensure your final image has enough pixels for Nyquist by clicking the symbol **(3)** to automatically calculate it for you. Alternatively, manually chose a relevant image size from the drop down.
- Scan Speed is measured in Hz in the LAS X program. Meaning the number displayed is the 'number of lines per second.' (E.g. this image of 512 lines will take ~1.2s to acquire).
- Depending on your imaging set up you may wish to acquire bidirectionally. The toggle **(4)** allows you turn this on. (Note: Using bidirectional scanning will decrease your acquisition time by half).
- The field of view can be Zoomed in **(5)** by either adjusting the slider, or by selecting the "On/Off" toggle and drawing a ROI over the image area you wish to acquire.
- To reduce noise, and/or increase signal in your images you may need to perform either averaging or accumulations **(6)**. These can be done on either the line level or frame level. Note you can only perform an average OR accumulation for each acquisition, you can for example do 2 line averages and 3 frame accumulations.



NOTE: Ensure the pinhole is set to 1AU for confocal imaging.

Detector Settings and Adjustments



Generally, detector ranges should need to change between sequences as this is a slow process. For example, the above detector set up is currently suitable for a sample containing **DAPI, Alexa488 & Alexa568**. In this scenario all 3 dyes will be acquired at the same time (which may result in cross-talk). To alleviate this, simply duplicate the sequence and turning off PMT2 and the 488 laser on one sequence, and turning off HyD1 & HyD3 and the 405 & 561 lasers on the alternate sequence.

Setting up this imaging experiment

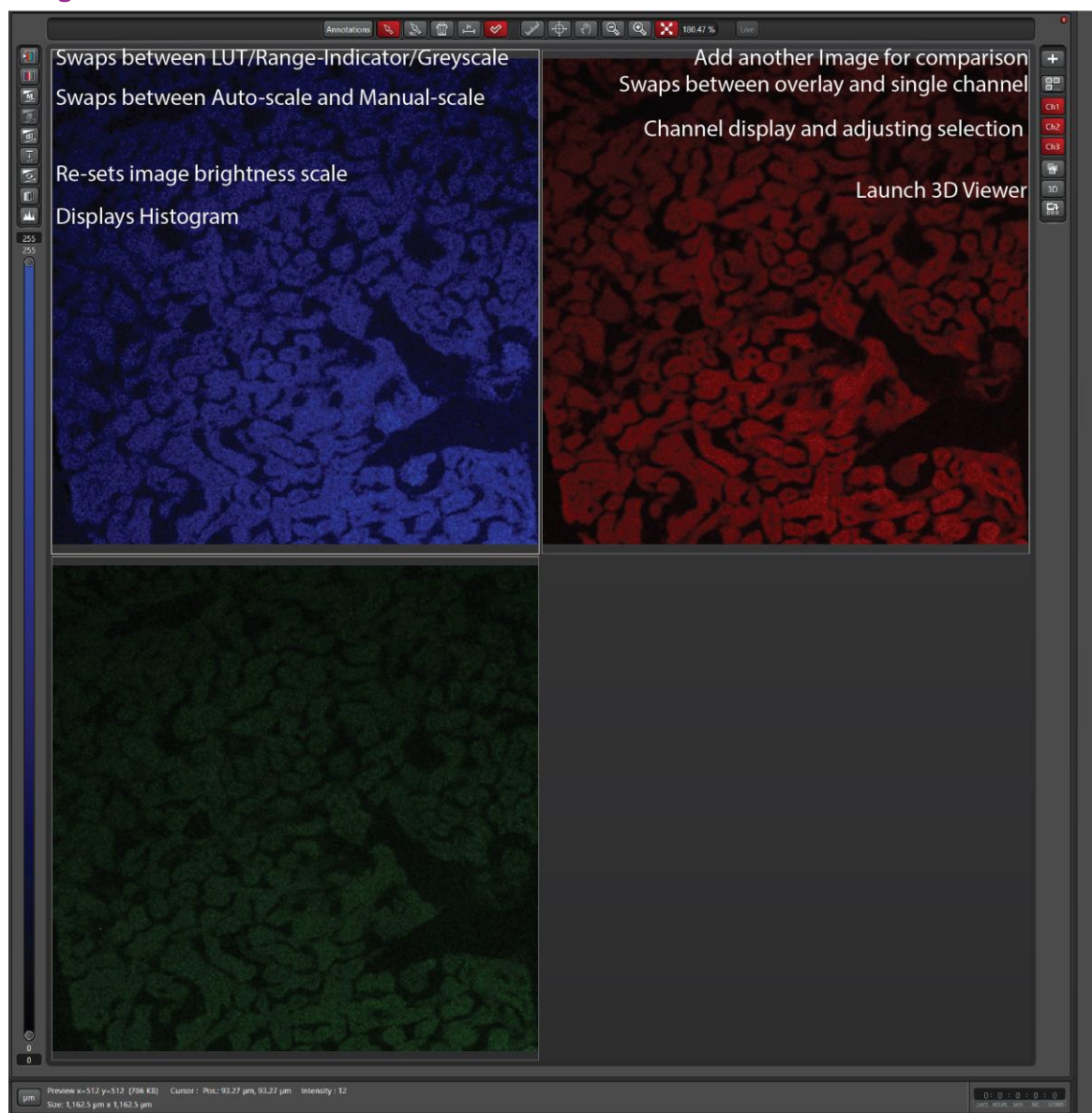
1. Working from left to right (in emission wavelength) and top to bottom (in detector number) turn on HyD1 and set its look-up-table to be blue.
2. On the right hand side of the detector window, set the dye to be **DAPI**
3. Adjust the detector range for HyD1 to cover most of the DAPI emission curve shown above (415-478nm) and set a gain of 100.
4. Turn on PMT2 and set its look-up-table to be green and set its dye reference to be **Alexa488**
5. Adjust the detector range for PMT2 to cover most of the emission curve shown above (498-551nm) and set a gain of 700.
6. Turn on HyD3 and set its look-up-table to be red and set its dye reference to be **Alexa568**
7. Adjust the detector range for HyD3 to cover most of the emission curve shown above (571-747nm) and set a gain of 100.

Note: For this set up lasers have been set to for 405nm, 488nm and 561nm. Leave 10nm either side of the laser for all detectors. **NEVER place a laser line over a detector range.**

Note: HyD1 and HyD3 are gated detectors. Gating the detectors will remove autofluorescence and laser reflection/scattering. When in use select the relevant laser line from the WLL in the drop down on the right hand side.

Note: Gain may need to be adjusted for either a HyD or PMT. If you find you require high laser power (>20%) and HyD gain >400, or PMT gain >1000 speak to microscopy staff.

Image Window Controls



Note: In LAS X the toggle buttons display what you are 'on' now, not what you would like to do. I.e. above on the right hand side the second button displays multi-channel display mode, select this to change to overlay window mode.

Note: When adjusting brightness scale select the window first to choose the active channel to change.

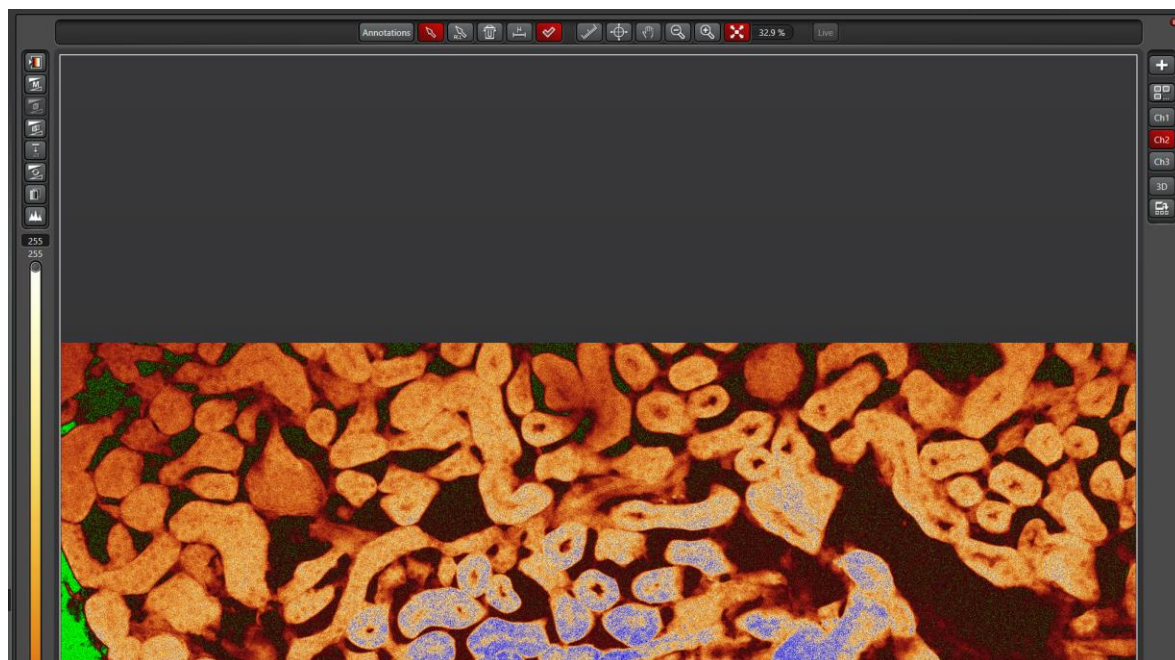
Note: If you need to change the LUT of your channel after acquisition right-click on the displayed LUT on the left hand side of the image display window.

Using Range Indicator to correctly set up your channels

In order to correctly set up your image acquisition parameters the range indicator LUT is a tool which should be used.

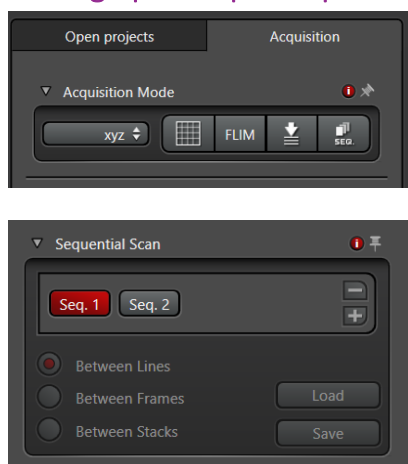
The LUT ranges from Green > Black > Red > Orange > White > Blue

With Green = 0, and Blue = 255 (saturated)



Note: To access this look-up-table select the LUT tool from the top left button in the image control panel above.

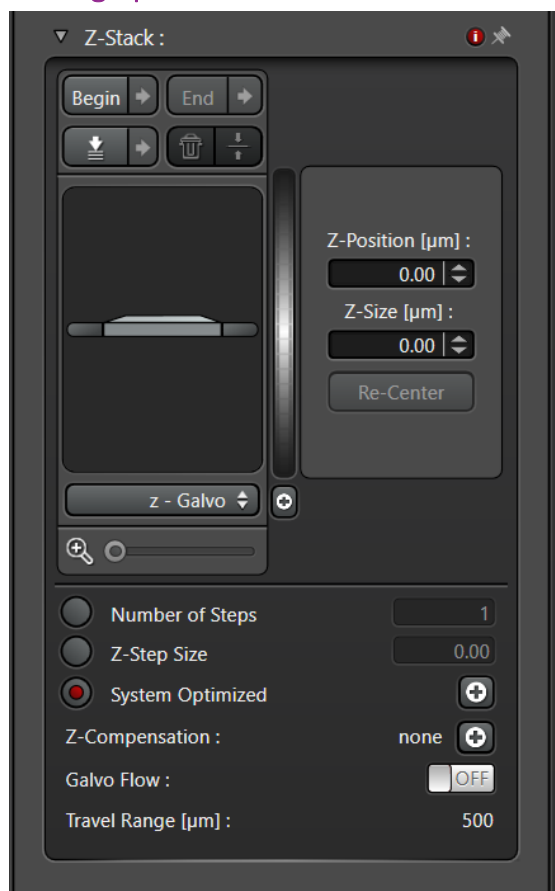
Setting up multiple sequences (tracks)



1. Turn on sequential scanning mode by selecting "SEQ" at the top of the acquisition mode panel.
2. Using Seq. 1 set up your lasers and detector ranges for all channels
3. Press the "+" button in the Sequential Scan tool window to duplicate the current settings
4. Turn on and off relevant detectors and lasers for each sequence
5. Ensure you select "Start" to acquire all sequences.

Note: for STED Between FRAMES/STACKS is required.

Setting up Z-stacks



1. Ensure you have an acquisition setting chosen which includes Z dimension
2. Use the Z-drive (Galvonic Stage) controls on the USB Controller to navigate to either the top or bottom of the sample and select Begin
3. Move to the top of the sample and select End.

Notes:

- You can quickly move to the selecting begin and end points by selecting the arrows next to the respective button.
- System Optimized will chose Nyquist settings for your objective and channels
- Begin and End are independent of Top or bottom.

Leica Dye Assistant

Similar to other vendors' solutions, Leica has a wizard for automatically configuring the light path for you, called Dye Assistant. It is not as thorough, nor as complex as other solutions so use it carefully!

- Select the Dye Assistant icon from the Tools (3 on previous page)
- Add your labels using the "+" symbol. NOTE: if you label isn't in the database please select a similar dye or speak to microscopy staff.
- Once you have selected your dyes you can indicate whether you need a HyD or PMT or either. Remember a HyD is more sensitive so is suitable for dimmer dyes.
- Next select one of the options generated by selecting Apply.

Remember it is important to reduce Crosstalk as much as possible.



The least number of sequences is generally the fastest during your capture, but this option may result in high levels of crosstalk. If more than one sequence is required (most often the case) choosing a "Line Sequential" option will generally be faster than "Frame Sequential." To increase signal, detectors can be set to capture more of the emission range, however these adjustments are slow, and can only be made between frames, during line sequences, detector positions cannot be changed.

Re-using previous settings

1. Load a .lif file into LAS X
 2. Right-click on the project file and select Apply Image Settings
- Note: It wont apply settings such as XY stage position, Z position, however does recall laser power, laser line, z-step size etc.

Shut down Procedure

1. Remove your sample and wipe the objective lens with a clean lens tissue
2. Save your files to the local data drive and begin copying them to a backed-up location
3. Close down LAS X software (note it can take a couple of minutes to perform system checks)
4. Shut down the computer.
5. Turn off the laser interlock key
6. Turn off the switches from Right to left pausing 5 seconds between (Laser Power, Scanner Power, PC Microscope)
7. Leave the room clean and tidy for the next user.

System Hardware Summary

Microscope Stand: Leica SP8 Inverted Microscope with incubation

Objective Lens List

- PInApo 10x/0.45 DIC (WD = 2.74mm)
- PInApo 40x/0.85 DIC (WD = 0.21mm)
- PInApo 40x/1.10 Water Korr (WD = 0.65mm)
- PInApo 93x/1.3 Glyc Mot Korr (WD = 0.3mm)
- PInApo 100x/1.4 Oil (WD = 0.13mm)

Laser Lines Available

- 405nm continuous
- 440nm pulsed (manual adjustment)
- 442nm continuous
- 460 – 670 nm tunable pulsed white light
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Detectors

- HyD SMD tunable, FLIM
- PMT tunable
- HyD SMD tunable, FLIM
- PMT tunable
- TLD transmitted light
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SP8 Light path

Physical Light path

