Leica STED 3X Quick Reference Sheet
ACRF Cancer Biology Imaging Facility
Institute for Molecular Bioscience, UQ
11 April 2017

OVERVIEW

The Leica STED 3X is an inverted compound microscope with multiple automated functions, capable of use as both a fluorescence confocal and transmitted light DIC microscope (10-100x magnification). This microscope also contains a resonant scanner for fast live imaging, Automatic Focus Correction for timelapse imaging, HyD detectors for time gated imaging (FLIM/FRET) and STED lasers for super resolution imaging.

The system can capture tiled images, images at predefined points, and z-stacks suitable for deconvolution.

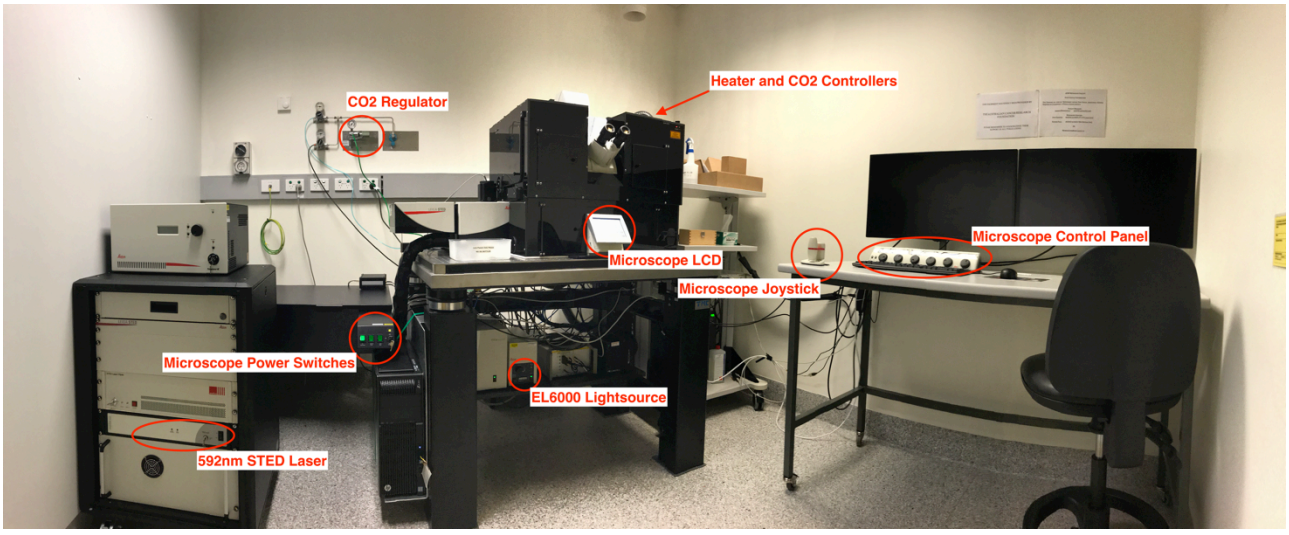
First and foremost: be mindful of what you are doing at all times. The confocal microscopes are delicate and must be treated as such. This Microscope cost ~$1,200,000; using it is a privilege not a right, please look after the instrument. If in doubt about anything, please contact microscopy staff for advice.

AVAILABLE LASER LINES:

- Confocal Imaging: 405nm, 442nm, 470-670nm Tunable Pulsed White Light Laser
- FLIM/FRET Imaging: 440nm Pulsed Laser and 470-670nm Tunable Pulsed White Light Laser
- STED Super Resolution Imaging: 592nm, 660nm and 775nm STED Depletion Lasers

SLIDE CLEANING AND PREPARATION

- Make sure your slide is *scrupulously* clean. All dust, old oil and mounting medium should be removed with a little 70% EtOH.
- If you can use a mounting medium that sets hard, please do so.
- Recommended Mounted: #1.5 coverslip, Prolong Diamond, sealed with nail varnish.
- **Do not** use glitter nail polish to seal your slides as it scatters laser light.
- Be sure to give your slides and sealants at least eight hours to set. 48 hrs for Prolong Diamond



BEFORE STARTUP

- Inspect the scope to be sure that all covers are in place, that there is no oil where it should not be, no medium spilled on the scope, etc.
- Ensure nothing is mounted on the motorized stage, and that it is clear to move freely.

STARTUP

- **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

- **If you will be imaging using the STED Super Resolution imaging method,**

  - Ensure that the SuperK and 775nm laser keys are switched to the ON position (they should never be turned off)
  - Turn the 592nm Laser black switch to the ON position
  - The "SHG Ready" LED will change from Green to Orange and then back to Green, this will take 1-2 minutes
  - Now once it is green again, turn the key to the ON position

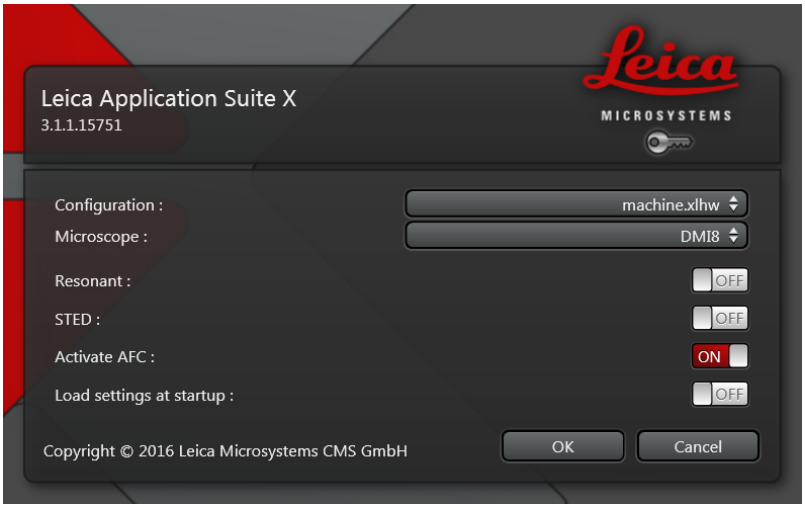
- **Turn on the Fluorescent Lightsource**

  - Check to make sure the EL6000 Fluorescent Lightsource under the microscope table is turned on.
  - Adjust the Intensity knob as required.
  - If performing overnight timelapse imaging, turn off the lightsource when you leave the room at night.

- **Start the Software.**

  - Start LASX by double-clicking the LASX desktop icon. Please be patient: LASX is a large program and does take a while to load and start up. Double-clicking numerous times will not make this process go any faster and may in fact make it take longer. Note that you may also start LASX by right-clicking on the LASX icon and selecting Open from the resulting menu.

  - When the startup splash screen appears, choose the appropriate settings for your experiment.



Note: You cannot use AFC and STED together, and you must restart the software to change the options you have selected.

- The Software will take approximately 2 minutes to finish loading.

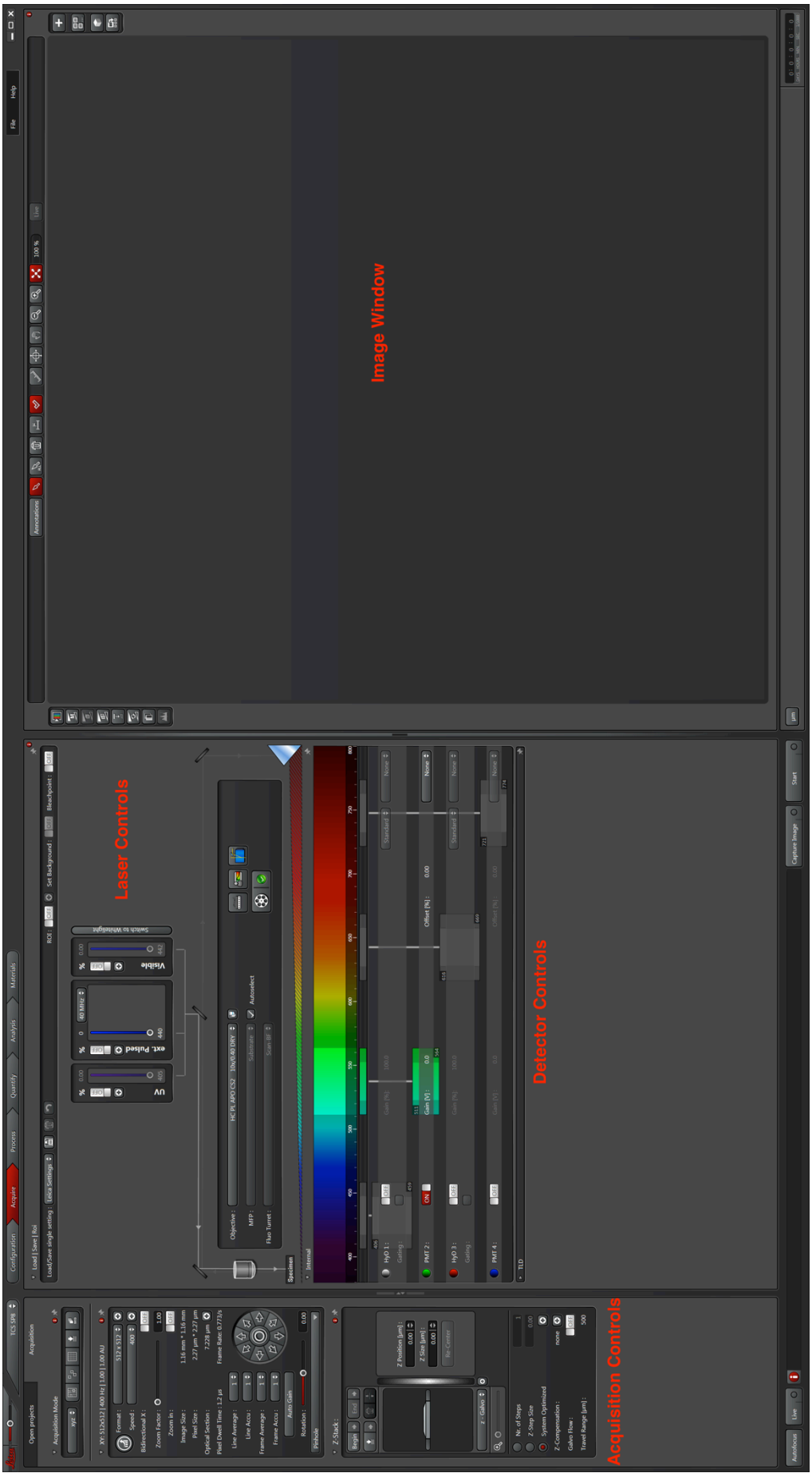
- **If the system will not boot properly or you get a stream of errors, do a *full* restart.**

  - Close LASX.
  - Wait at least sixty (60) seconds.
  - Shut down the computer.
  - Shut the confocal down by reversing the order of the startup sequence
  - Wait at least sixty (60) seconds, but ideally two (2) minutes or more, to allow the components in the confocal to lose any residual charge and reset themselves.
  - Start the system again as described above.

The vast majority of errors are resolved this way, so just be patient and take your time.
The worst thing you can do is rush, as the hardware and software have many things to do and rushing through start up will almost guarantee a fault.

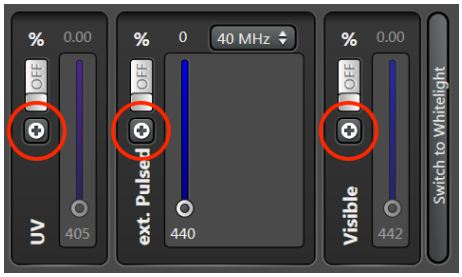
- **If a full restart does not work, contact microscopy staff for assistance.**

- **LASX Software Acquisition Screen**

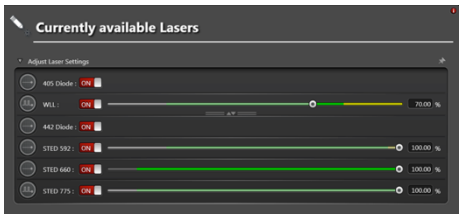


- **Turn on the Lasers**

  - Click one of the “Plus” buttons in the Lasers menu and a window will appear with the currently available lasers. This list will vary depending on whether you selected STED or not.



  - The 405nm Laser is used for imaging DAPI
  - The 442nm Laser is used for imaging CFP
  - The 440nm Laser is used for CFP/YFP FLIM/FRET
  - The White Light Laser (WLL) is used for imaging all other fluorophores.
  - Do not set the White Light Laser (WLL) past 70%
  - Set any other required lasers to 100%
  - The lasers may take up to an hour to stabilise



- **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

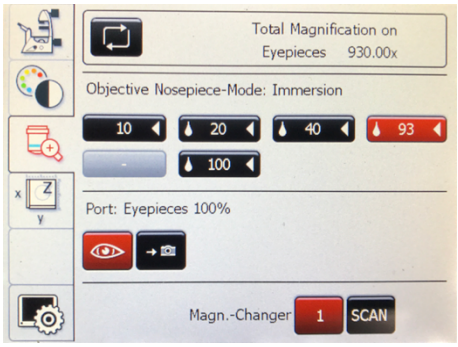
    - Dry (these **never** have media applied): 10x
    - Imm = Oil/Water/Glycerol = 20x (use the immersion fluid that best matches the RI of the mounting media in your sample) you will need to adjust the correction collar on the objective to match the immersion media.
    - Water = 40x
    - Glycerol: 93x
    - 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.

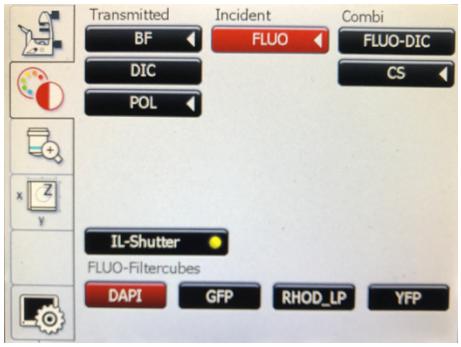


- **Mount your specimen**

  - Use the appropriate stage insert (Universal, Slide/Dish or Multiwell)
  - 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

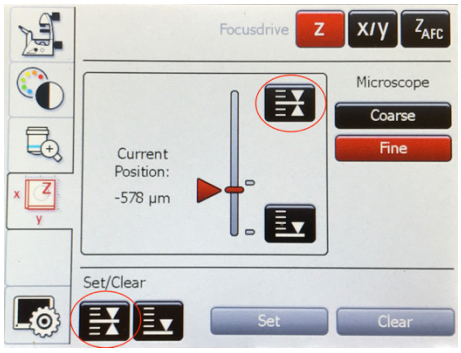
- **Illuminate your specimen and focus**

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



- **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



ACQUISITION MODE

- **Set Diode Laser Power**

  - Open the shutters for the lasers you require to image your sample, and set the power to 2%.

  - For example, DAPI requires the 405nm laser, CFP requires the 442nm laser.

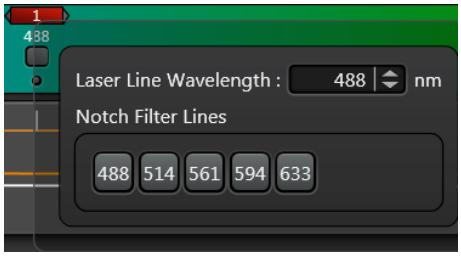
Set WLL Power

- Click on the “Switch to WhiteLight” icon in the Laser Controls section.

- Select 1-8 laser lines on the WLL as required to illuminate your sample.

- Drag the laser lines to the appropriate wavelength for your fluorophores.

- If possible use the notch filters to reduce laser scattering in your image.



- To enable the notch filters, double click on the wavelength number (eg: 488 or 561),

- Then click on the appropriate notch filter for your sample.

- Ensure each laser line is ticked on and the Shutter is open

- Set each laser line to 2%

- **Simultaneous Acquisition**

  - Turn on the same number of detectors as laser lines you have turned on. Try and use HyD detectors if possible as they are generally more sensitive with lower noise than PMT detectors.

  - Turn on Gating on the HyD detectors to remove unwanted background light. Gating can be also be adjusted to remove autofluorescence.

  - Adjust the Gain as pictured below. Recommended HyD gain between 50 and 100, PMT gain between 600 and 800. For HyD compared to PMT you will need roughly 1/3 the laser power.

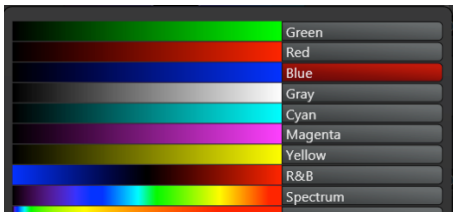
  - Select a Fluorophore from the menus on the right to adjust your detector ranges appropriately

  - Drag the detector ranges as pictured above, but matching your laser lines and fluorophores.

  - You should try and leave a 10nm gap between the laser line and the detector start and end range.

  - Click on the coloured round circle next to the Detector name to change it's colour

  - Double click on a suitable colour to select it.

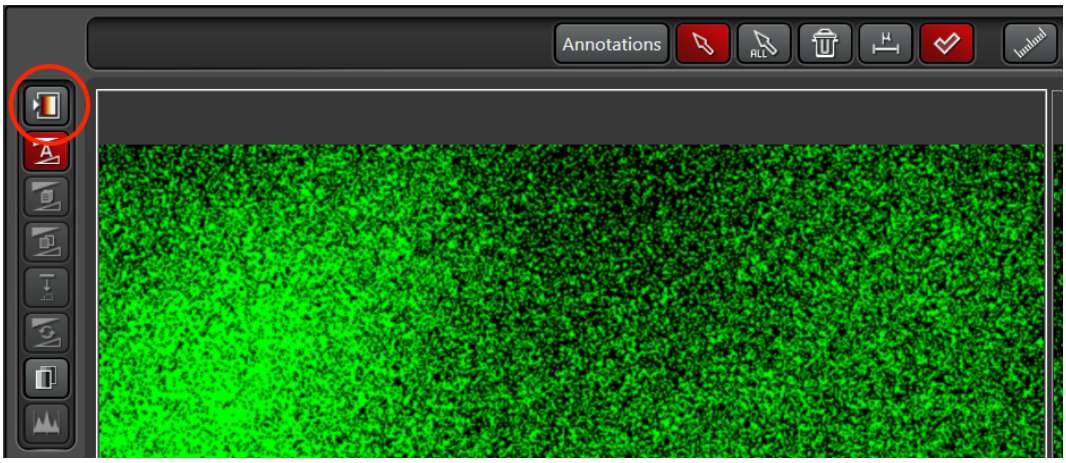


  - An example of BAD Detector settings

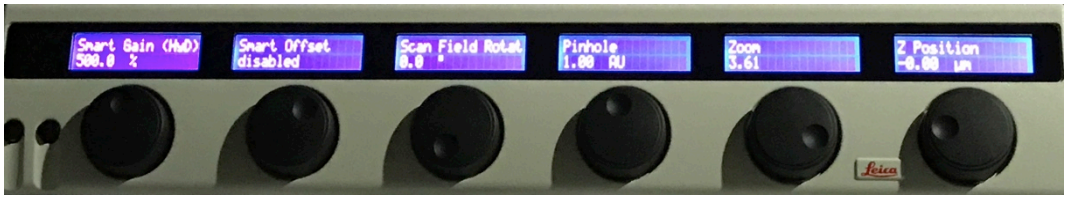
  - The Detector range of HyD 1 overlaps with the 488nm Laser line, and the Gain is set quite high.

  - The detector should never overlap the laser line, as the bright light may damage the detector. Only in rare cases will we use reflection mode and this MUST be performed with PMT, never with HyD.

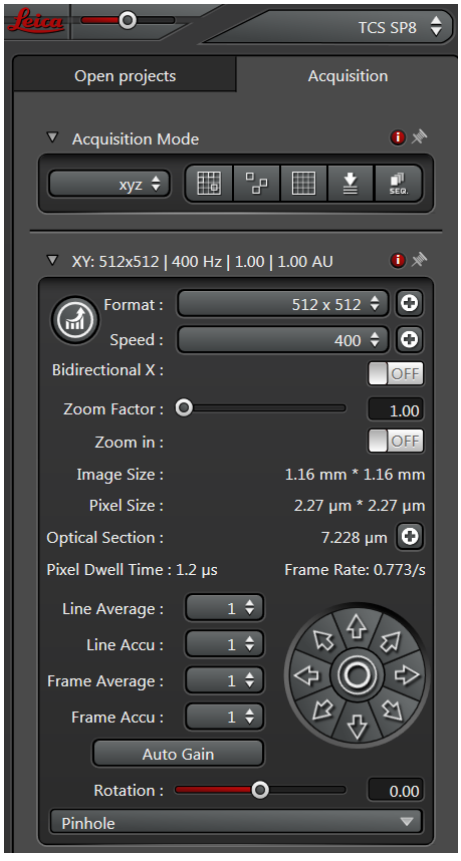
- Press the Live icon at the bottom left of the screen to start acquiring an image.
- Click on the LUT icon at the top left of the Image window to show the Glow Over/Under image.



- Click on the top left image to highlight it (white border), then use the Microscope Control Panel to adjust the Detector Gain.



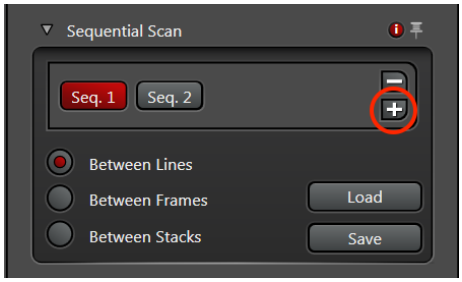
- Adjust the Gain and Laser power until you have an image with no saturated pixels.
- Adjust the focus using the Z Position knob on the control panel to get the brightest image possible.
- Repeat the adjustment of Gain and Laser power ensuring there are no saturated pixels.
- Adjust the Offset if using a PMT detector using the Smart Offset knob on the control panel.
- Repeat the steps above for each of the image windows.
- Click Stop.
- Adjust the Image Acquisition settings as required.
- Click “Start” to acquire an image.



- **Sequential Acquisition**

  - Click the Sequential Scan icon

  - Add a Track by clicking the “Plus” button, this duplicates the current track settings, so ensure they are correct before you click “Plus”. Right Click on the track if you wish to delete it.



  - It is common practice to image Blue and Red channels together to prevent overlap of fluorescence excitation and emission, and then image the Green channel separately.

  - Click on Seq. 1, then turn off the Green detector, and set the 488nm laser power to 0%

  - Click on Seq. 2, then turn off the Blue and Red detectors and set the 405nm and 561nm lasers to 0%

  - Choose when you want to change between sequences

  - Between lines – fastest method, captures a line with first sequence, then repeats for each sequence, before imaging the next line.

  - Between frames – Captures an image (frame) with first sequence, then repeats for each sequence

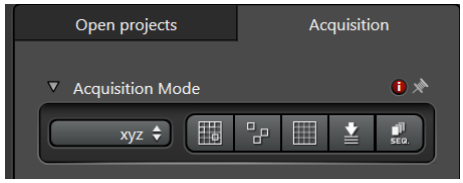
  - Between Stacks – Captures an entire Z-stack with first sequence, then repeats for each sequence

  - Click “Start” to acquire a sequential scan.

  - If you wish to remove a sequence and you are not working in that sequence (not highlighted in red) then right click and select delete to remove the sequence.

- **Z-Stack Acquisition**

  - Ensure you have selected an Acquisition mode that contains a Z dimension such as “XYZ” or “XYZT” etc



  - Choose whether you will use the Z-Galvo or Objective focusing(Z Wide). Z Wide has a much larger travel range, Z-Galvo is fast and facilitates XZ scans.

  - Whilst you are scanning with live mode, adjust the focus using the “Z-Position” knob on the control panel. Mark the “Begin” point, then rotate the “Z-Position” knob in the other direction and mark the “End” point of the z-stack.

  - Alternatively find the centre of your sample, and type in the “Z-Size” you wish to scan.

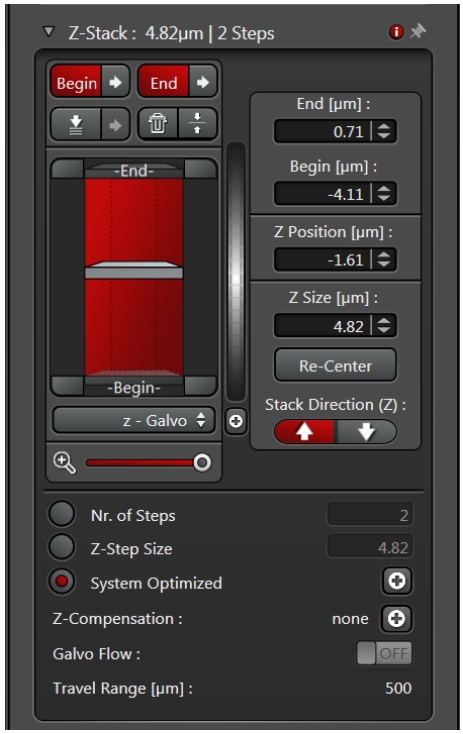
  - Ideally use “System Optimised” to sample your Z-stack at the optimal intervals, or you may manually set the number of steps, or Step Size.

  - Galvo Flow will continuously scan the Galvo Z-stage whilst capturing, enabling very fast Z-stack captures, however you may obtain a dimmer image with possible artifacts.

  - This image shows a Z-stack that has been correctly setup with a range of 4.82 microns.

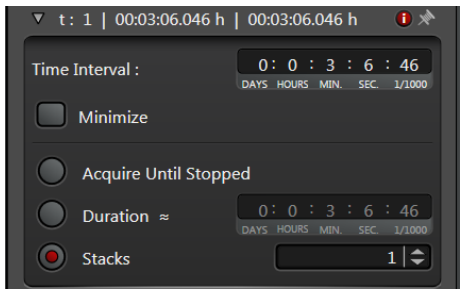
  - Click “Start” to capture the Z-stack.

  - Click on the 3D icon on the far right of the image window to view the dataset in 3D.



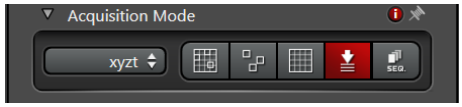
- **Timelapse Acquisition**

  - Ensure you have selected an Acquisition mode that contains a T dimension such as “XYT” or “XYZT” etc
  - Set the number of Stacks (Images) or total duration required
  - Click the Start Icon

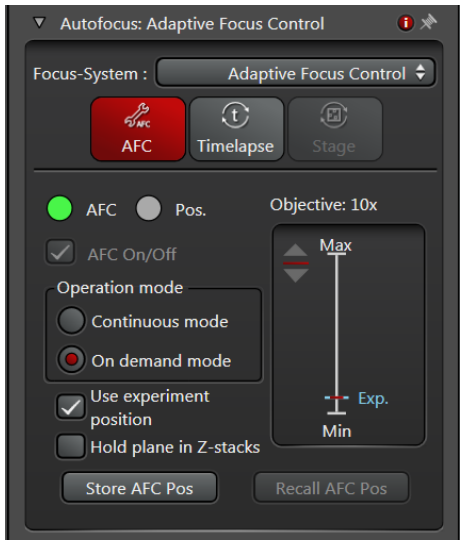


- **AFC Acquisition (Autofocus)**

  - Click the AFC icon



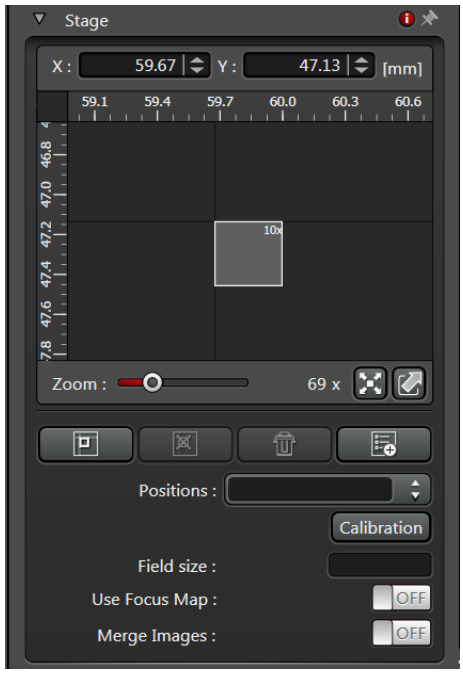
  - Ensure the AFC indicator is Green. If it’s not then you probably have a Refractive Index issue.
  - Focus on your sample.
  - Click the “Use Experiment Position” tickbox to record that position.
  - The AFC system will ensure that your sample is in focus before acquiring each image.



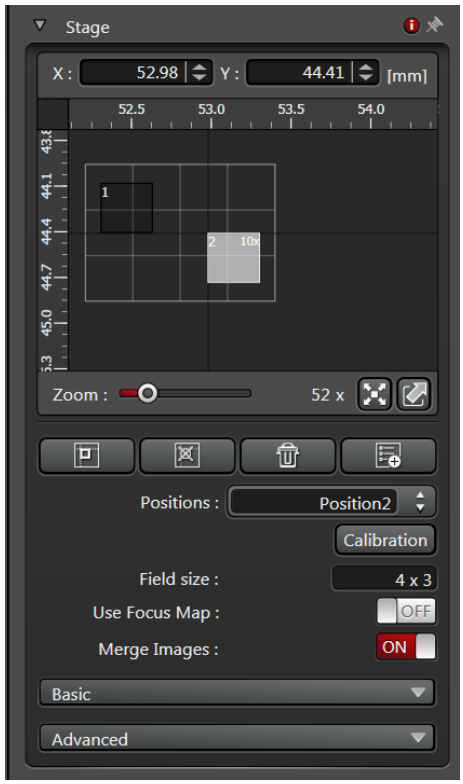
- **Tile Acquisition**

  - Ensure you have selected the Tiling icon

  - Click the Mark position button
  - Use the Joystick to find the end point of your sample
  - Click the Mark position button



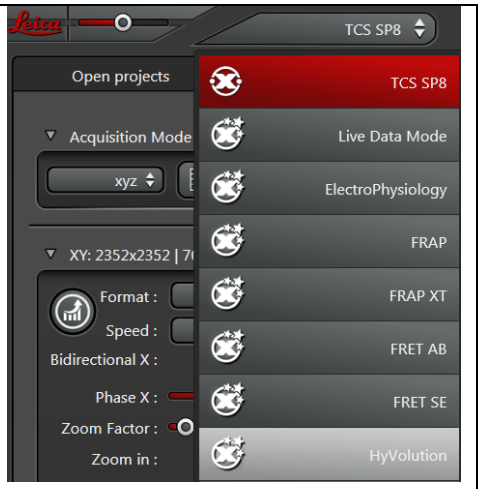
  - The Tile map will now be displayed
  - Turn off “Merge Images” if you wish them to be saved individually for later processing.



- **Mark and Find Acquisition**

  - Ensure you have selected the Mark and Find icon
  - Click the Mark position button
  - Use the Joystick to find the next mark and find position
  - Click the Mark position button
  - You can select position in the drop down menu
  - Either select ‘same stack for all’ or uncheck to adjust individual Z stacks
  - The ‘Centre’ (use Re-Centre) will attach to the focus job of your choice (AFC and/or Software autofocus)

- **HyVolution Acquisition**

• Select HyVolution from the TCS SP8 dropdown menu	
• Select the appropriate mounting media you have used in your sample from the drop down menu	
• Adjust the quality vs speed slider	
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- **Manual Deconvolution Processing**

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- **CO2 Incubation and Heater Control**

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- **2D STED Acquisition**

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- **3D STED Acquisition**

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