



BEGINNERS SAFETY MANUAL FOR LEICA TCS SP8 CONFOCAL SYSTEM

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1. Safety Guidelines

Please adhere to the safety guidelines for your own safety and health. When in doubt, always approach the bioimaging facility for assistance.

1.1 Lasers

Lasers in the confocal facility are class 3B lasers. This means that the lasers are strong enough to cause serious damage to your eyes, including temporary to permanent blindness. Therefore, please always follow the safety guidelines below when using the confocal microscopes:

- Before turning on the lasers, make sure the power connections are all connected properly. If there are any disconnected wires, please reconnect them or inform the facility staff. If you see any exposed wires, do not attempt to use the equipment and inform facility staff immediately.
- For all confocal systems, there is a minimum time for lasers to be switched ON/OFF. Please check the operation procedure for the respective microscopy system that you are assigned to. This is to ensure the lasers have sufficient time to heat up and cool down before running again.
- When using the lasers to image, **NEVER** look directly at the laser while imaging. If a safety shutter is present, make sure it is in the correct position to better protect your eyes.

1.2 Immersion Oil

Modern immersion oil has no known hazards to human beings so far, yet they can cause discomfort to a person if the immersion oil has been left on skin for too long or inhaled. Therefore, please take note of the following safety guidelines for using immersion oil:

- Use the applicator to apply the immersion oil onto the slides for upright system.
- Do not touch the applicator oil directly on the objective lens as this will scratch or break the lens. Instead, allow the drop of oil to contact the lens surface.
- If the bottle containing immersion oil has oil on the sides, wipe down with kimwipes and ethanol. Wash hands immediately after with soap and water.
- Wipe immersion oil off objective lenses after use using lens paper and wash hands immediately after with soap and water.
- Always clean up any spilled oil or residue from oily slides immediately after your session.

1.3 Metal Halide Lamps (MHL)

Metal halide lamps are fluorescence lamps that gives a visible light range of 300 – 650nm, enabling us to observe fluorescence signals such as GFP, DAPI and RFP. Metal halide lamps contain mercury vapours that are extremely toxic to the human body. Therefore, please take note of the following safety guidelines when working with the metal halide lamp:

- Ensure that the metal halide lamp has been off for at least 30 minutes before switching it on. Once the lamp has been turned on, it must remain on for a minimum of 30 minutes to allow sufficient time for it to warm up and run properly.
- Check the timer on the power box to ensure the mercury lamp does not run past 2,000 hours.
- Do not look directly at the MHL when it is switched on as it can damage your eyes.
- Do not attempt to remove the light guide from the MHL, **especially** when it is on. Direct emission of the MHL may set off fires.
- If there are no users in the next 30 mins, MHL must be turned off.
- Never attempt to change a bulb yourself. Please contact facility staff when you notice that a bulb has reached between 2,000 – 2,500 hours or is not functioning properly.

2. Training

Use of confocal microscopy and the online booking system is authorized by facility staff only. All users must attend the confocal training session conducted by the Bioimaging facility staff before they are allowed to access the confocal system.

2.1 To arrange a training session:

- Please email Bioimaging Facility (bioimaging@tll.org.sg) for a training session.
- Fill in the necessary particulars in the form here:
http://microscopy.tll.org.sg/pages/conf_training_form.html
(E.g. Lab, PI, sample, and a brief description of your project)
- We will help you identify the most appropriate microscope for your imaging needs.
- We will then arrange training session according to your availability within a week.
- Training sessions usually last 1 - 3 hours depending on users' previous experience.
- Users receiving training are highly encouraged to bring their own samples so that staff can adjust trainings to your imaging needs, but if not possible, staff have their own samples that they can work with.

Once you have completed the training session, we will grant you access to both the confocal PC and the online booking system. Please keep in mind that only bioimaging facility members are allowed to conduct the training. None of your lab members are allowed to conduct the training for you.

If you need a refresher or some specific advice on anything microscopy related, please approach any member of the Bioimaging Facility for assistance/help.

We also offer training on the various types of image analysis and image processing software available here at TLL, including ImageJ/Fiji, Huygens and Imaris.

3. Online Booking and System Access

- Booking of all Light microscopes prior to use is **COMPULSORY** through microscopy resource booking via TLL intranet (https://intranet.tll.org.sg/App/tll_intranet/booking_searches)
- Users are only allowed to book the confocal system that they have received training on. If they wish to book other confocal system, they have to receive a separate training.
- Users are entitled to advance bookings of up to 2 weeks. They are advised to plan their experiments accordingly to avoid any disappointments.
- During Office Hours (Weekdays from 8.30am to 6pm)
 - Each user is entitled to a **MAXIMUM of 2 bookings** per system per week.
 - Users whom are trained on multiple systems are entitled to a maximum of 3 bookings per week but it has to be shared across the systems that they have received training on.
 - Each booking must not exceed 3 hours.
 - If users have utilized all their entitled bookings for the week, “**24Hr Rule**” can be applied where they can book the system in less than 24 hours in advance according to its availability. If extra slots are booked within the 24-hour period, a note of “**24Hr Rule**” should be made in the booking description.
- During Non - Office Hours (Weekdays after 6pm, Sat, Sun & Public holidays)
 - If users require more slots in a particular week, they can book on weekdays during non-peak hours (*after 6pm*), on weekends (*Sat and Sun*) and on public holidays.
 - If extra time is required, bookings can be extended out of peak hours (*E.g. 3 – 7pm*).
- Bookings exceeding these limits are subjected to cancellation without prior warning.
- Simultaneous bookings of two or more different systems are not allowed. Multiple bookings for the same system on the same day during office hours is strictly prohibited.
- If users cannot attend a booked session for any reason, it is **COMPULSORY** that they cancel their booking through TLL intranet and email (confocal@tll.org.sg) to announce the availability. If they are the last users for the day, they need to check if the system has been switched off completely.
- If users are swapping a session with another user, they must change the booking details accordingly.
- Under any circumstances, users are not allowed to make bookings on behalf of other people. Users who have received training but are yet to gain access to microscopy

resource booking may approach TLL Bioimaging department for booking assistance if they need to use it urgently.

- If a user fails to show up within the first 30 minutes of their booking, the slot is forfeited and is free for any user to use it.
- If any users violate any of these Booking rules, users will be subjected to the 3 strikes policy.

1st offence	A warning will be issued along with the reminder of the rules.
2nd offence	A second warning will be issued and your respective PI will be notified.
3rd offence	Banned from using any of the facility's microscope for 2 weeks.

3.1 Acknowledgements

If you use the TLL Microscopy and Imaging facility and/or have been trained or assisted any of the bioimaging facility members in your research, then this should be acknowledged appropriately in your publications and presentations.

4. Operation Procedures

Every confocal system in TLL Bioimaging Facility has its specific instructional manual which are found in every confocal rooms. Strictly adhere to the correct order of operation for all system. Failure to do so will result in disciplinary action from the facility. Any issues encountered during the operation of the system are advised to seek help from the Bioimaging facility.

Modification, exchange or removal of components beyond this operational manual is strictly prohibited and is only carried out by the manufacturer, Bioimaging facility or by experienced users approved by bioimaging facility. During operation of laser microscopy system, do not look into the laser beam directly as they are all Class 3b and Class 4 lasers.

4.1 Switching ON Protocol

- (a) SIGN IN the Logbook and record your START-TIMING.
- (b) Switch on Laser Key **(1)**, Laser Power **(2)**, Scanner power **(3)** and PC Microscope **(4)**.
- (c) Switch on the Metal Halide lamp **(5)**, if you need it.
- (d) Switch on STED laser module **(6)**, if you need it.
- (e) Wait for PC to start up and log in with your TLL user name.
- (f) Load LASx software and switch on the lasers that you are planning to use:
 - **Diode Laser:** "ON" 405nm laser
 - **White Light laser:** "ON" up to 70% power
 - **Argon laser:** "ON" up to 70% power. (Make sure the argon lasers are ON for a minimum of 2hrs before switching OFF as it requires sufficient time for warming up)
 - **STED laser:** You may switch "ON" up to 100%. (Make sure the STED lasers are ON for at least 1hr before switching OFF.)
- (g) Do not overload the HyD detectors. Keep gain at max 100% as overloading will damage the performance of HyD detectors over time. For best performance of the HyD detectors, set gain at 100% and only use the laser power slider to moderate irradiation intensity.
 - ❖ If the HyD detector still overloads even at minimum laser power, change the detector to the standard PMT detector. Standard PMT detectors are more resilient against heavy emission.
- (h) Finally, fill in the logbook.

4.2 Objective Lens

Objective lens	Immersion media
10x/0.4 HC Plan Achromat	Air
20x/0.75 HC Plan Achromat	Air
40x/1.3 HCX Plan Achromat	Air
63x/1.2 HC Plan Achromat	Water
63x/1.4 HC Plan Achromat	Oil
100x/1.4 HCX Plan Achromat	Oil

4.3 Software initiation

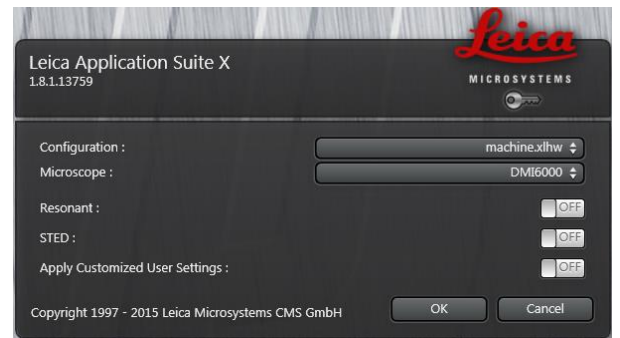
Turn on the machine following the instructions pasted on the wall and inserted in the log book. Wait for complete initialization of microscope and Windows after logging in with your TLL username and password.

Double click on: 

(A) The LAS X configuration screen will appear:


Configuration: Machine .xlhw must be selected.

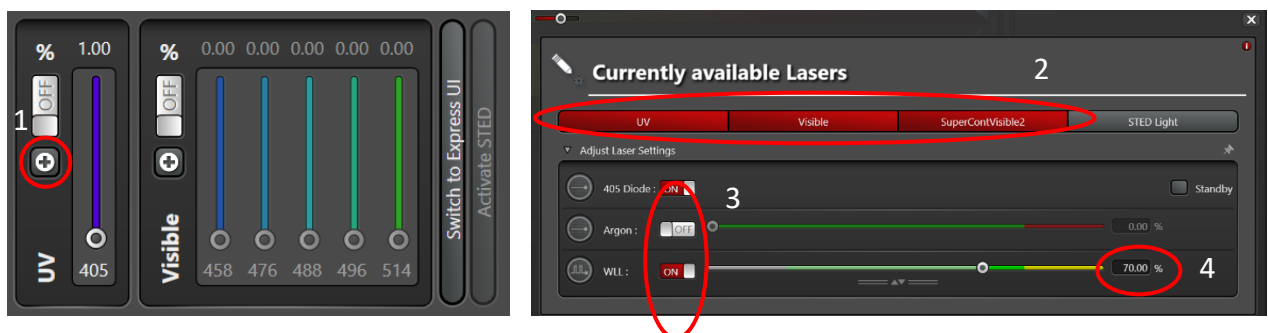
Microscope: Check that **DMI6000** is selected.



Click on **OK** and wait for complete initialization. Do not move anything on the microscope during the initialization process.

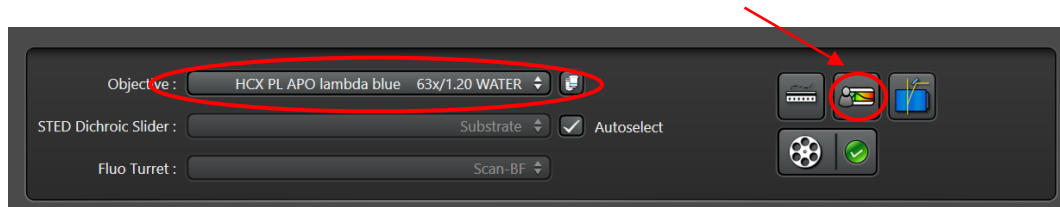
4.4 Turning on the lasers



1. Under the Visible panel, click on the  button to open the laser panel.
2. Click on the UV (405nm), Visible (Argon) and SuperContVisible2 (WLL) bar to observe 405nm, Argon and WLL respectively in the dropdown menu.
3. White light laser (WLL) has excitation wavelengths from 470nm-670nm allowing you to select specific excitation light source. Argon lasers have much higher power than the WLL and has excitation wavelengths – 458nm, 476nm, 488nm, 496nm and 514nm.
4. **Turn on** the laser that you need by clicking on the sliders. Make sure that WLL and SuperContVisible2 are at 70% before carrying on with the next steps.



4.5 Using Dye Assistant tab

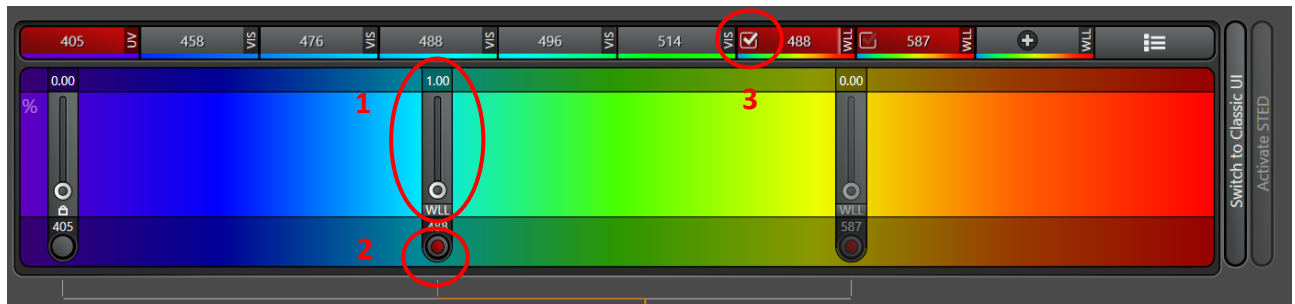
- Select the objective lens that you would like to image with your sample.
- Then select, **Dye assistant** to set up your laser pathway.



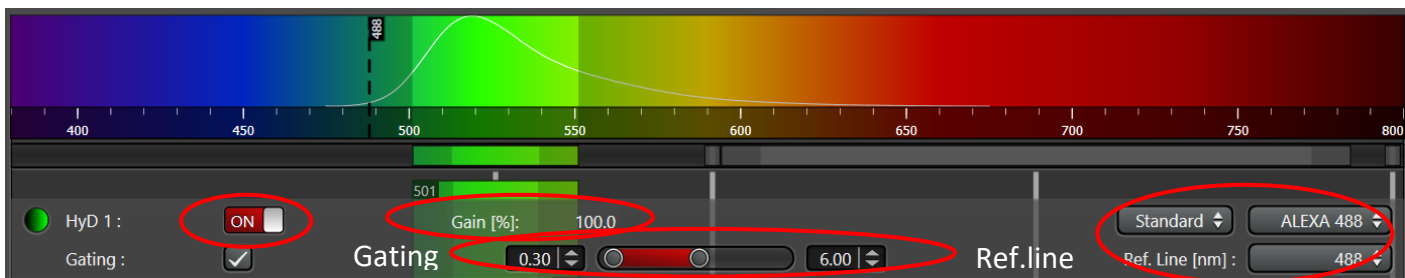
1. Click  to add the number of channels you would like to image.
2. Click  to select the dyes you are using for each channel.
3. Then select the type of detector (HyD or PMT) you would like to image with your dyes. HyD is more sensitive than PMT.
4. The Dye assistant will give you a list of laser settings options to select from. Select the settings which has the least amount of crosstalk. Sequential Scan is always preferred as the amount of crosstalk is lesser. Line sequencing is much faster than frame/stack imaging.
5. Select your preferred laser setting option and click **apply**.



4.6 Setting Laser & Detector configuration

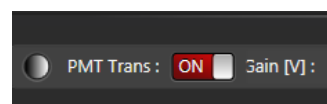


- Based on the no. of dyes, you have selected, it will give you the total no. of seq. (E.g., if you have selected 3 dyes in sequential mode, you will have 3 seqs corresponding to each dye.)
- Select each seq, and increase the laser power to 1% initially. Make sure the laser line is activated by checking both the red button and the laser line shown at 2 and 3.



- Make sure that the HyD/PMT is activated. (Turn on the slider)
- Then, Select the fluorophore you are using. It will give you the emission spectrum of the dye you are using.
- Choose an appropriate emission band to detect your fluorophore. (Important: Make sure that the emission band is at least 8-10nm away from your excitation line.)
- Choose an appropriate detector pseudocolour (also known as a Look Up Table, or LUT).
- For PMT, set the Gain to approx. 700 - 800 and the Offset to 0.
- When using the HyD (2× more sensitive than PMT), default gain is set at 100.
(Important: Do not overload HyD, max is 100.)
- Gating: Removes the first few nanoseconds of the signal as a result of autofluorescence from the sample or mounting medium.
 - Only usable on pulsed lasers such as the WLL. Constant mode lasers like the Argon lasers will not work with the Gating function.
 - Choose appropriate reference laser line for gating.
 - **NOTE:** start with low laser power to avoid damaging the HyD detectors. HyD detectors will shut down if an overload condition is detected.

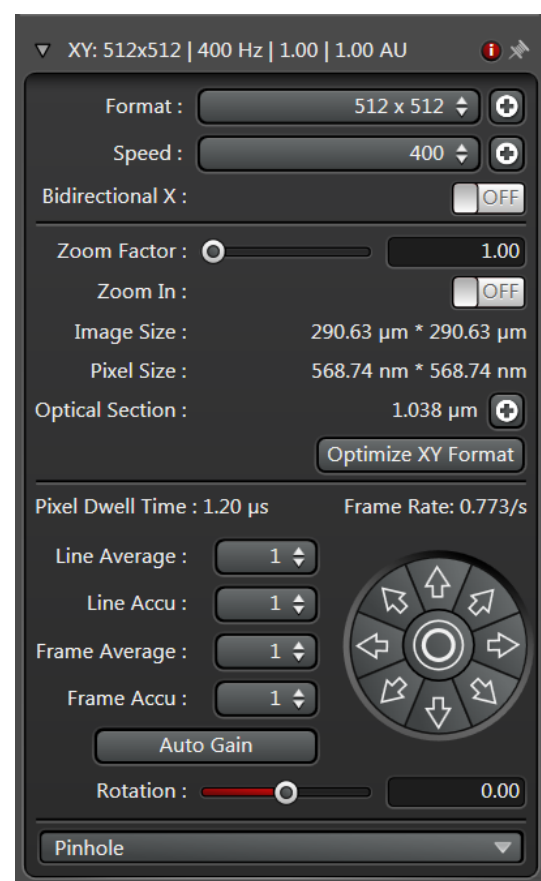
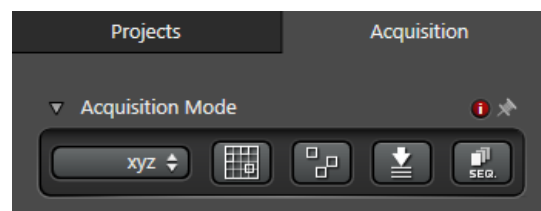
- If you want to also acquire a brightfield image activate the **Transmission channel**.
 - Ensure that there is at least one active laser emission line when you are using this channel



4.7 Using the Acquisition tab

Select the **Acquisition** tab, in which you can set the parameters for your imaging:

- Acquisition Mode:
 - Choose the type of scan xyz (z-stack) or xyzt (time-series) are the most commonly used
 - **Seq**: select this for multi-channel/colour samples
- XY
 - **Format**: the number of pixels in your image (the more pixels the higher the resolution, to an extent, but slower the acquisition). The max is 8000x8000.
 - **Speed**: the slower, the better your image will be (a higher signal:noise ratio; but it takes longer and it bleaches your sample more).
 - **Bidirectional X**: Useful for live imaging unless you require fast imaging (in which case you will need to adjust the phase correction.)
 - **Zoom**: is where you control the zoom parameters, the arrows allow you to nudge the zoomed area within the image. Zooming in can increase your resolution to a point, but can cause more photobleaching/damage.
 - **Average**: averages a given number of scans to get a higher signal to noise ratio. Drawback: slower scanning time and more bleaching. No averaging or Line averaging is recommended if you are doing live-cell imaging. Averaging twice will help you achieve better quality images.
 - **Accumulation**: Is the sum of signal of several images together, hence boosting your signal. Drawback: you also sum the noise and the resulting image may not have improved signal:noise ratio, also has a slower scan time and more bleaching.
 - **Auto Gain**: the program will adjust the gain (beware that this happens only for the active channel if you are in **seq** mode).




Click on **Live** to view a live image. You can now adjust parameters while checking the result directly on the monitor. Be careful though, if you spend too long doing this you will photobleach your sample. Start focusing your sample and then center it moving the stage.

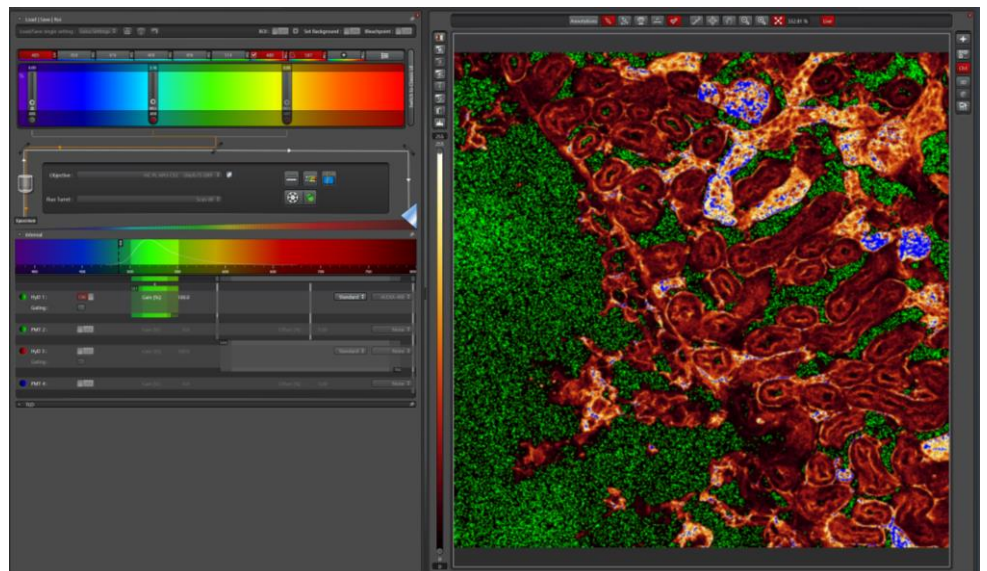
Now, based on the image, you can adjust the brightness in the **Acquire** and **Acquisition** tabs:

- Excitation **laser power**: the higher the laser power, the faster your sample photobleaches. Also, this will alter both the brightness of the fluorescent image and that of the brightfield.
- **Gain**:
 - **Photomultiplier Tube (PMT)**: Keep below 800V during image acquisition to control noise.
 - **Hybrid Detector (HyD)**: Keep at 100% at all times for optimum performance. Change the brightness of your image by changing the laser power only.
- **Offset** (adjusts the background/black level)
- **Pinhole** (the wider the pinhole aperture, the thicker is the optical slice – which means a blurrier, but brighter image). **Make sure you also lower the laser power if you have to increase the pinhole.**

There's no general rule: these adjustments mainly depend on your sample.

4.8 Image Adjustments – Setting the Gain and Offset properly

Check image saturation level by clicking QuickLUT icon . The image will change color to show green, orange and blue. Green indicates that the signal in a given pixel is black, with a value of 0. Blue color in a given pixel means it is saturated (over-exposed) with a value of 255. We need to bring our image back within the limits of our dynamic range, such that the background is black and the sample is not too bright. Different sets of experiments may have different fluorescence intensities.



When using the PMT detectors, we have to adjust our image using a combination of the excitation **Laser power**, **Gain** and **Offset**: Set the Gain at 700 - 800 and reduce it, or the laser

power, until blue pixels just disappear. Decrease Offset until you have a dusting of green pixels in the background where there are no cells.

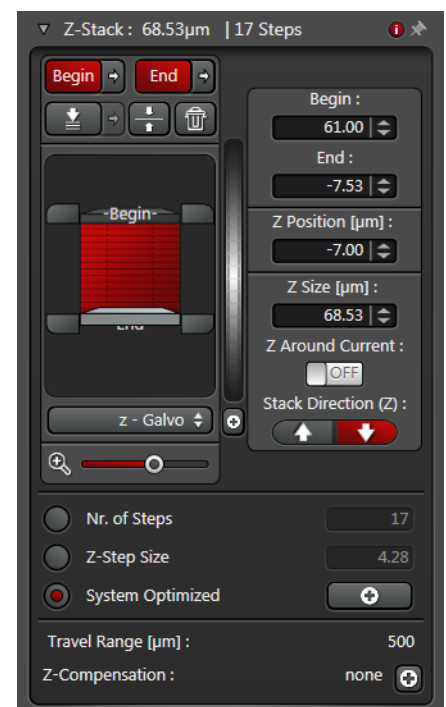
When using the HyD detectors, gain is set at default 100 reduce it or the laser power to prevent oversaturation. Activate gating when using the WLL to remove laser reflection or auto fluorescence. Make sure to choose the correct reference line when using gating.

Click on QuickLUT button twice to switch from range indicator mode to normal colour view.

Click **Capture Image** or **Start** to capture an image.

4.9 How to do a Z stack for a 3D Image

- Scroll down to the **Z-stack** option in the **Acquisition** tab.
- Select **Live** to see the specimen.
- Use the focus knob on the USB control panel or point the cursor in the Z-stack box panel and use the mouse scroll wheel to focus up and down (do not use the microscope's focus knob).
- Move all the way to the top of your sample and click on the **Begin**, turning it red, to mark the start position.
- Use the mouse scroll wheel to move to the bottom of your sample and click on the **End**, turning it red to mark the end position.
- With **system optimized** selected the program will determine the optimal settings for your Z-stack. You can override these settings using the **Nr. of steps** or **z-step size** boxes. Beware that changing these will alter the z-resolution you attain.
- Click the **Start** button to begin the series.



4.10 Saving images and reusing settings

Taking snapshots:

- Right click on the image you would like to take a snapshot of.
- Click on **Snapshot** to get an RGB image of the desired image.

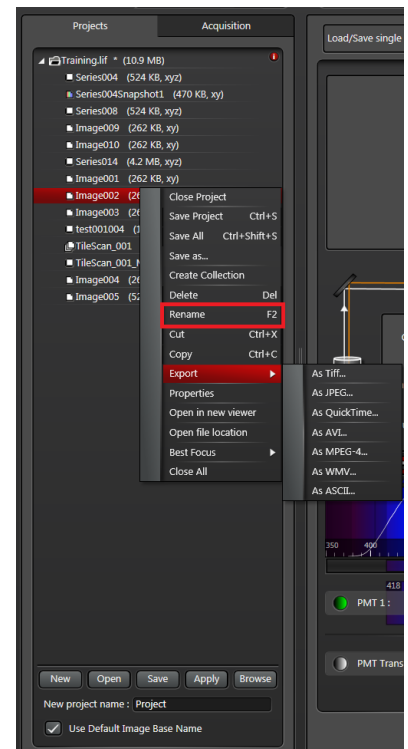
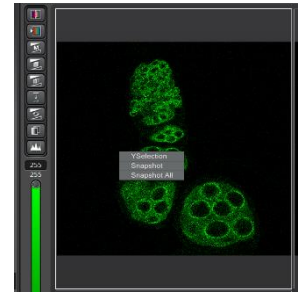
To store an image:

- Select the **Projects** tab and it will list the images you have acquired (either with the **Capture Image** or **Start** buttons. **Live** images are not held in the memory.
- Click on the **Save** button.
- Your images will be saved in a ***.lif** proprietary format.
 - If you turned on Auto-save, your images will be saved in ***.xlef** format. This functions about the same as ***.lif** format in LAS X.
- Please save in your folder. For example:
 - Option 1: *D:/E: → Users → Graham → example.lif*. Do keep in mind that we will delete any images which are older than 3 months. Please make sure to do a backup.
 - Option 2: In TLL research cmn drive folder in your respective lab folders.
 - Option 3: In your own lab drive.
 - Option 4: In your personal hardisk or thumbdrive. Keep in mind that the data transfer will be slow.
- You can also change the name of each image, or delete selected images, to your liking by right-clicking on the name and select **Rename**.
- You could also export your images in the TIFF format. (right-click on the image file)

Important: please do not save any data on the C: drive and remember to back up your data to the network!


To reuse previous settings:

- Open previous images.
- Right-click on the image you would like to repeat and select **Properties**. Here you will see a detailed record of the settings used to acquire that image, and all its properties.
- Click on **Apply Settings** to re-establish the confocal to the same setting.



4.11 Switching OFF Protocol

Check through TLL microscopy resource booking if there is a user immediately after you. If there is a user immediately after you, leave the system on and log off the PC. Otherwise switch off the system.

1. Save your images through TLL common drive folder. Images saved in computer D/E drives will be ***deleted within a month without prior warning***.
2. Lower down the objective lens and carefully clean the objective lenses with Whatman lens paper.
3. Make sure to switch your lens back to the 10x/0.3 objective lens.
4. Clean the sample holder with Kim wipes and 100% ethanol to remove any oil residues.
5. If you have turned on the Argon lasers:
 - a. Open the Lasers toolbar using  in the laser selection bar and move the Argon laser slider to OFF.
 - b. Wait until the cooling fans for the argon lasers shut down (around 5 minutes).
6. If there are bookings after you **within one hour**, even if you did not turn on the MHL:
 - a. Fill in the logbook with the objective lens used and write **LOG OFF**.
7. If there is no one using the system after you for **at least one hour**:
 - a. Exit LAS X software and switch off PC Microscope **(4)**.
 - b. Switch off STED laser module **(6)** if it was used.
 - c. Switch off the Metal Halide lamp if it was used **(5)**.
 - d. Switch off Scanner power **(3)**.
 - e. Turn off Laser Power **(2)**.
 - f. Turn the Laser Key **(1)**.
 - g. Fill in the logbook with the objective lens used and write **ALL OFF**.

5. Troubleshooting

Issue	Explanation & Remedial steps
<p>White Light Laser does not emit laser radiation.</p>	<p>Error occurred with the wavelength setting system (AOBS). The AOBS is part of the laser scanning module (LSM).</p> <p>Confirm that:</p> <ul style="list-style-type: none"> • White Light Laser module interlock is set to ON. • White Light Laser module emission is on. Emission LED should be red. • LAS X shows that the WLL is on, shutter is raised, and there is laser power set on WLL emission lines. <p>If previous steps fail to allow laser emission,</p> <ol style="list-style-type: none"> 1. Close LAS X program. If this issue is a scanner issue, you will not hear clicking as the program is shutting down and performing its pre-shutdown WLL checks. 2. Switch off the LSM (switch 3*, marked Scanner) 3. Switch on the LSM 4. Open LAS X program. If this issue has resolved, you will hear clicking as the program is performing its startup WLL checks. 5. Confirm laser emission
<p>Argon laser does not emit laser radiation</p>	<p>We recommend using the WLL instead of the argon laser <u>unless</u> the sample needs a very strong excitation source.</p> <p>Argon laser may be unable to power on.</p> <p>Confirm that:</p> <ul style="list-style-type: none"> • Argon/405nm laser switch (switch 2) is ON. • Argon/405nm laser fan is on (you hear a continuous loud whirring noise) • LAS X shows that the argon/405nm laser is on, and there is laser power set on argon laser. • If previous steps fail to allow laser emission, • Close LAS X program <p>Switch off the argon laser (switch 2*, marked Laser)</p>

	<ul style="list-style-type: none"> • Switch on the argon laser • Open LAS X program • Confirm laser emission <p>Gating function for removing autofluorescence photons only works for pulsed lasers (WLL). It will not work for continuous mode argon/diode lasers.</p>
405nm laser does not emit laser radiation	<p>Diode laser may be unable to power on.</p> <p>Confirm that:</p> <ul style="list-style-type: none"> • Argon/405nm laser switch (switch 2) is ON. • Argon/405nm laser fan is on (you hear a continuous loud whirring noise) • LAS X shows that the argon/405nm laser is on, and there is laser power set on 405nm laser. • If previous steps fail to allow laser emission, • Close LAS X program <p>Switch off the argon laser (switch 2*, marked Laser)</p> <ul style="list-style-type: none"> • Switch on the argon laser • Open LAS X program • Confirm laser emission <p>Gating function for removing autofluorescence photons only works for pulsed lasers (WLL). It will not work for continuous mode argon/diode lasers.</p>
No signal viewed from the sample	<p>Check that your sample does actually have the signal using the Wide Field eyepiece.</p> <p>If there is signal from the Wide Field eyepiece:</p> <ol style="list-style-type: none"> 1. No laser emission <ul style="list-style-type: none"> • Refer to previous items 2. Wrong detector settings <ul style="list-style-type: none"> • Check detector settings on LAS X 3. Hybrid Detector overloaded <ul style="list-style-type: none"> • Look at the bottom of the LAS X window. If it says the HyD overloaded, use the following steps: <ol style="list-style-type: none"> 1) Turn off Live View.

	<ol style="list-style-type: none"> 2) Ensure that the detection band is set at least 10nm away from any laser you are using. 3) Set the HyD gain to 100% always. Brightness should be set using the laser power only for best sensitivity and minimal photobleaching risk 4) Drop the laser power to the lowest level, then switch on Live View. 5) On Live View, carefully increase the laser power using the mouse wheel until you can see the sample on Live View. 6) Watch the information ticker in case the overload message appears again. <p>4. Out of focus</p> <ul style="list-style-type: none"> • Confocal microscopes are very sensitive to focus shifts. Use the panel knob to carefully shift focus in and out until you see a signal.
<p>Lambda scanning: Error message “LCS: The inactive slider- mirror no. 2 deranges in the scan wavelength range”</p>	<p>This occurs when HyD 1 is set as the detector for lambda scanning. This means that the mirrors for the rest of the scanners will move out of range by the time you reach the long wavelength limit for your experiment if you use HyD 1.</p> <p>Use HyD 3 instead.</p>

*Refer to Troubleshooting Appendix for location of switches

5.1 Troubleshooting Appendix

Switches
(under
computer
terminal)



Power schematic diagram of the SP8

