BEGINNERS SAFETY MANUAL FOR LEICA SPE II CONFOCAL

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Changelog

12 Jun 2025	Changelog added Image acquisition and review split from Operation Procedures. Instructions for reusing previous settings split from saving images. Instructions for exporting images added.

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 3 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 kim wipes 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

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 systems, do not look into the laser beam directly as they are all Class 3b and Class 4 lasers.

4.1 Switching ON Protocol

1. SIGN IN the Logbook and record your START-TIMING.
2. Switch on PC **(1)**.
3. Switch on laser Module **(2)** and turn the Safety key to "ON".
4. Switch on Microscope Power Box **(3)**.
5. Switch on Metal Halide Lamp **(4)** if required.
6. Wait for PC to start up and log in with your TLL username.
7. Load LAS X software and switch on the lasers that you are planning to use: 405nm, 488nm, 543nm or 635nm.
8. Fill in the logbook.

4.2 Objective Lens

Objective lens	Immersion media
10x/0.3 ACS Apochromat	**Air**
20x/0.7 HC Plan Apochromat	**Water/Glycerol*/Oil**
40x/1.15 ACS Apochromat	**Oil**
63x/1.3 ACS Apochromat	**Oil**

Correction collar for multi-immersion lens

The 20x/0.7 lens is a multi-immersion lens and can be used with water, glycerol or oil as immersion media with correction done on the lens.

All non-oil-only immersion lens have a correction collar to adjust correction for different cover slip thickness and immersion media. To set correction for this lens:

1. Make sure to use a 0.17mm glass cover slip (standard cover slips are this thick). Correction markings on this lens assume a 0.17mm glass cover slip.
2. Choose immersion medium. Oil is best for most flat samples, glycerol for thick tissue samples (>50µm), and water for samples suspended in water-based culture media.
   - ※ If you need glycerol immersion, please let Bioimaging facility know so that they can prepare the necessary glycerol.
   - ※ We recommend using the silicone lens in the Olympus FV3000 instead if your sample needs glycerol immersion on the SPE II.
3. Carefully rotate the knurled surface with the dot marked on it to the appropriate immersion medium marking. If you are using water immersion, rotate the dot to the 0.17 on the 0.17-W-0 part of the lens. The objective in the picture shown is set to OIL for oil immersion.
4. Place immersion media on the sample, move the turret into place and view your sample.

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 LASX: 

The Start Up splash screen will appear:
Configuration: Select `machine.xlhw`
Microscope: Select `DM5500`.

Click on **OK.**

A separate message asking to initialise stage will show.

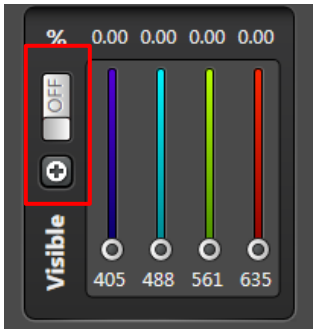
Press **Yes** to initialise the stage.

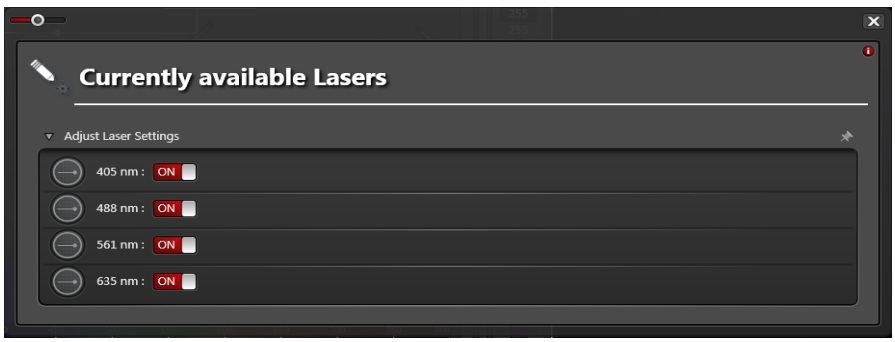
Do not move anything on the microscope during the initialization process.

IMPORTANT: You will need to initialise the stage to be able to control the stage using the USB control panel.

4.4 Turning on the lasers

1. Under the Visible panel, click on the  button to open the laser panel.
2. Turn on the laser that you need by clicking on the sliders.

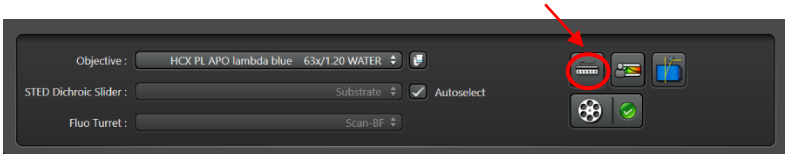




4.5 USB Panel Box

The USB Panel Box is a very useful tool that can be used for quick actions without needing to click on menus. They can even be used to control the stage position and focus distance.

To access the USB Control Panel settings, press this button shown below:



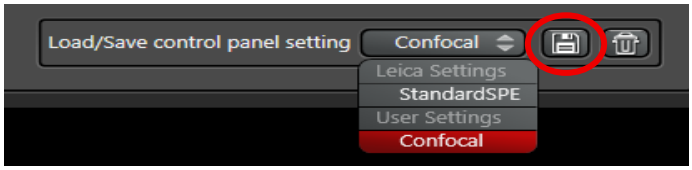
You will see the USB Control Panel settings below. The top dropdown is where you select the setting to be controlled by the knob below it, and the bottom dropdown is to select the speed of the control.

Bioimaging Team recommends the following setting panel for the SPE II:

1. Smart Gain
2. Zoom
3. X Position
4. Y Position
5. Z Wide Position
6. Z Position

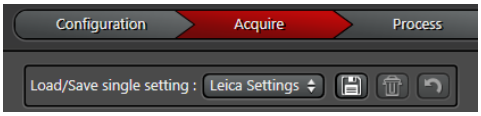
LAS X does not save the control panel state when you restart the system, so you will need to save your configuration. Go to Load/Save control panel setting and press the Save button (circled below) to save your current configuration. Name your configuration in the pop-up window that appears later. If it has the same name as an existing configuration you will be asked if you want to overwrite the configuration. You can either click Yes to overwrite it or No to give a different name to the configuration.

Afterwards you can load your control panel setting by clicking on the appropriate setting in the dropdown menu.



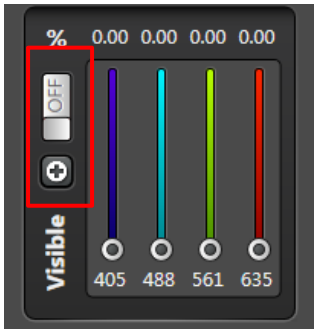
5 Image acquisition

5.1 Using the Acquire tab

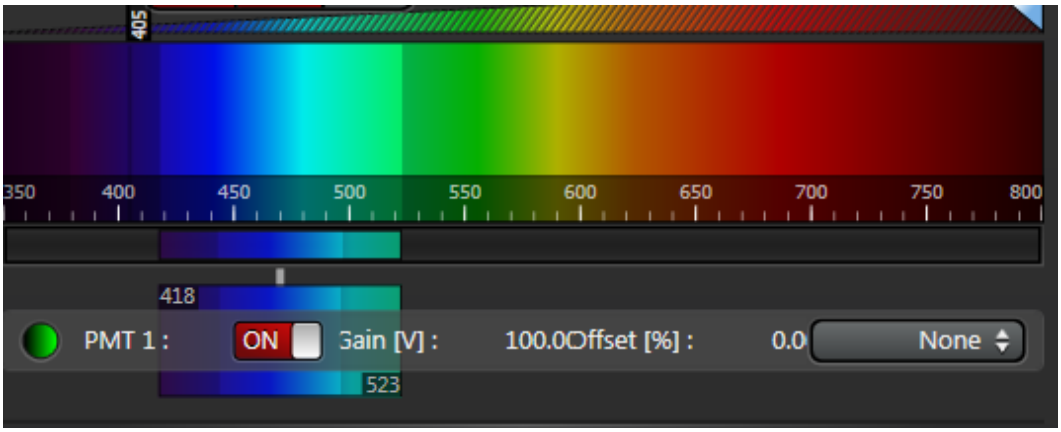


1. The program will display the default screen. Click on the **Acquire** tab to set up the optics for your experiment.
2. If you need to image multiple wavelengths, you need to turn on the **SEQ.** button from the acquisition tab.

3. Within the **Acquire** tab you can load up saved settings (using the **Load/Save settings** box – always double check if they are correct for your sample), apply settings from one of your previous experiments (see **7. How to reuse previous settings**) or manually configure your settings:



4. Activate the lasers by turning on the slider.
5. Turn the laser wavelength you want to use to the required % power. Start off with 5% of laser power.
    ※ 405nm laser is a diode laser, while 488nm, 561nm, and 635nm lasers are diode-pumped steady state lasers. Steady state lasers need some time to warm up after being turned on before they can emit light.
6. Activate the PMT (Turn on the slider).



7. To view the emission spectrum of the fluorophore (eg GFP), select the fluorophore from the option.
8. Select an appropriate emission band to detect your fluorophore. Double click on the bar to input exact figures.
9. Choose an appropriate pseudocolour by pressing on the coloured circle.
10. Start off the Gain with approx. 700 and the Offset to 0.
11. For brightfield images, activate the **Transmission channel**. *Remember to switch the lever on the back of the microscope.*

Switching between eyepiece bright field and laser scanning bright field

You may need to have your confocal scan image overlay on a bright field image. In this case you will need to follow the steps detailed as such:

1. Turn on a laser line. Laser scanning bright field will not work without any laser power.
2. Go to the back of the microscope body and locate the black teardrop-shaped lever as pictured below, then rotate it.

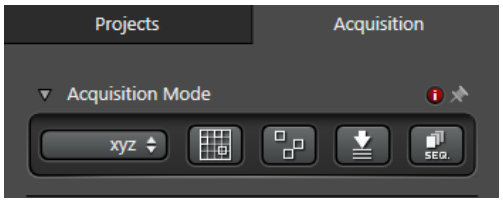
3. Start scanning in Live View, then adjust the PMT Trans up and down to see an image.

To go back to using the eyepiece for bright field,

1. Return to the back of the microscope body and rotate the lever.
2. On the microscope touch screen, switch the output to the eyepiece (eye symbol) from the scanner (camera symbol).

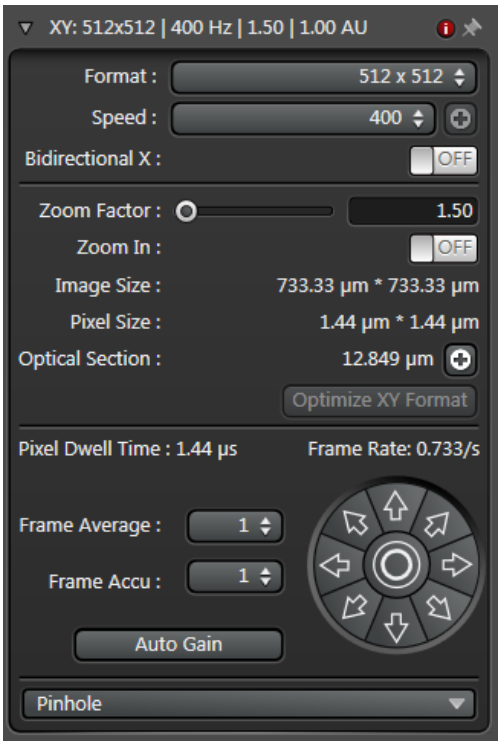
The microscope will automatically switch back to the scanner if you press Live View.

5.2 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 xyt (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 (Nyquist limit), but slower acquisition.
  - **Speed**: the slower the speed, the better your image will be (a higher signal to noise ratio; but it takes longer and sample will be subjected to photobleaching.
  - **Bidirectional X**: Faster live imaging where phase correction is required. Useful for live imaging. Most commonly used setting is monodirectional.
  - **Zoom:** allows a user to magnify a region of interest while maintaining the format size. It can increase the resolution to a certain extent but will result in photobleaching due to concentration of the laser power to a smaller square. Maximum zoom level is also subject to the Nyquist limit which is based on the lens Numerical Aperture.
  - **Frame average**: Averages a number of scans to get a higher signal to noise ratio, at the cost of slower scanning time and more bleaching. No averaging or Line averaging is recommended if you are doing live-cell imaging. Averaging 2 times will help get better quality images if you can afford to do so.



  - **Frame Accumulation:** Adds the signal of several images together, hence boosting your signal. Drawback: you also sum the noise and the resulting image may not have improved signal to noise ratio, also has a slower scan time and more bleaching.
  - **Pinhole**: Sets pinhole size. Pressing **Airy 1** optimises the pinhole size for the thinnest optical section allowed by the Numerical Aperture of the current selected lens. Opening the pinhole will allow a brighter image at the cost of capturing a thicker optical section.
  - **Auto Gain**: the program will adjust the gain for you (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.

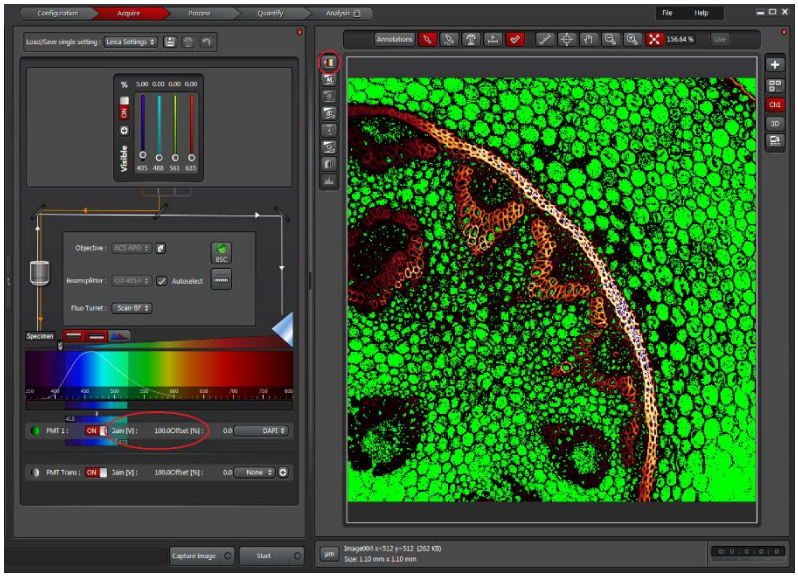
Now, based on the image, you can adjust the brightness in the **Acquire** and **Acquisition** tabs:

- Excitation **laser power** (beware that the higher the laser power, the more you bleach your sample. Also, this will alter both the brightness of the fluorescent image and that of the brightfield)
- **Gain** (keep below 800 for a low-noise image)
- **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).

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

5.3 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 illustrates that the signal in a given pixel is black, with a value of 0. Blue illustrates that in a given pixel means it is saturated (over-exposed) with a value of 255.



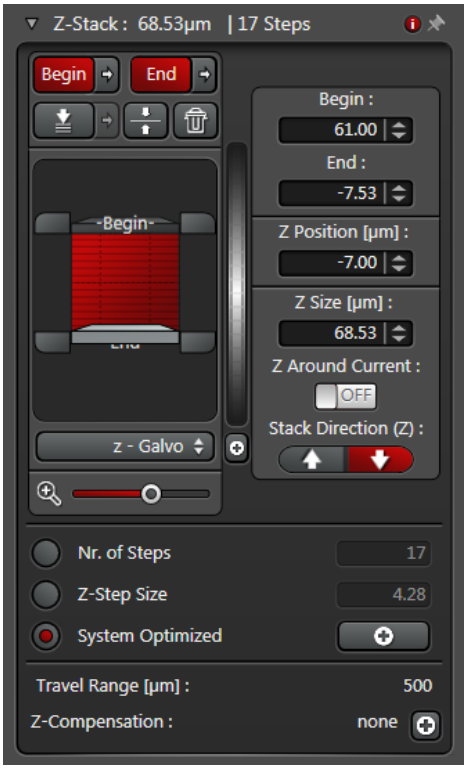
We need to bring our image back within the limits of our dynamic range, such that the background is black and the sample is not too bright. Different sets of experiments may have different fluorescence intensities.

We have to adjust our image using a combination of the excitation **Laser power**, **Gain** and **Offset**:

1. Set the Gain at 700 and reduce it.
2. Reduce the laser power until blue pixels (indicating overexposure) disappear.
3. Decrease Offset until you have a dusting of green pixels in the background where there are no cells.

Finally, click on the QuickLUT button twice to switch from range indicator mode to normal colour view. Click **Capture Image** or **Start** to capture an image.

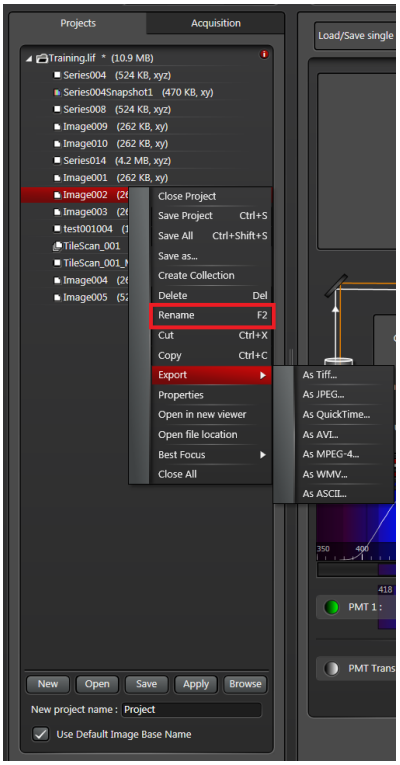
5.4 How to do a Z stack for a 3D Image



- Scroll down to the **Z-stack** option in the **Acquisition** tab.
- Select **Live** to see the specimen.
- Use the z-galvo 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 **Begin.**
- Use the mouse scroll wheel to move to the bottom of your sample and click **End**.
- With system optimized, the selected 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.

6. Reviewing images

6.1 How to save your images



- Select the **Projects** tab, it will list the images you have acquired. **Live** images are not held in the memory.
- Click on the ***Save*** button.
- Your images will be saved in **`*.lif`** proprietary format.
    - **`*.lif` files do not feature autosave, and the version of LAS X used in this microscope does not have an autosave option. Please remember to manually save periodically.**
- Please save in these folders. For example:
    - Option 1: `D:` *or* `E:` → `Users` → `Jing fang`→ `MK cells.lif`. Do keep in mind that we will delete any images which are older than 3months. 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 could also export your images in the TIFF format. (right-click on the image file)

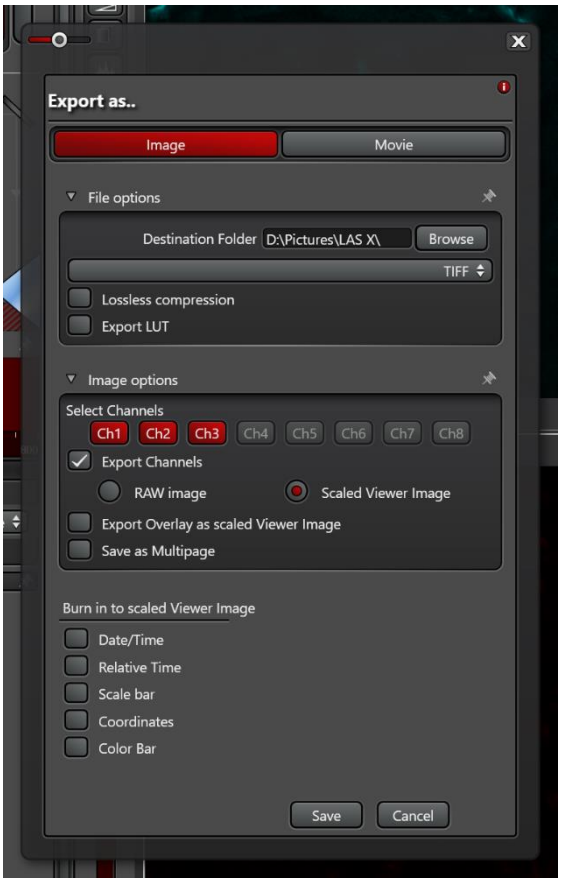
Important: please do not save any data on the C: drive and remember to back up your data to the network!

6.2 How to reuse previous settings

You can upload a configuration from a previous image.

In the **Projects** tab, highlight the image that you would like to use. 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 settings

6.3 Exporting images



1. Right-click on the image you want to export, then click **Export Image**.
2. The window shown on the right will pop up. A few options will be available for you:
   - File options:
     - **File type**: TIFF, ImageJ TIFF, JPEG, BMP, PNG are available. We recommend TIFF or ImageJ TIFF because it loses the least amount of data during saving.
     - **Lossless compression**: The exported image will take up less space, but at the cost of taking longer to save.
     - **Export LUT**: If you have a multi-channel image turn this on to save the individual channels as colour images based on the colour you have set for them.
   - Image options:
     - **Export channels**: Check this and select your channels to export individual channels. Select **RAW image** to export full-range monochrome images. Select **Scaled Viewer Image** to export the image with colour. If you have made any dynamic range scaling before export the dynamic range adjustments will also be reflected.
     - **Export Overlay as scaled Viewer Image**: Exports the combined image of the channels you have selected into one file. This option is separate from Export Channels so you can export both individual and combined channels together.
     - **Save as Multipage**: Only applicable when exporting as TIFF and for experiments with multiple images (time lapse, z-stack, lambda scan). Saves selected channels as a Multipage TIFF so that you can view an entire series as a single file.
   - Burn in to scaled Viewer Image: Only applicable if you selected **Export Channels > Scaled Viewer Image** or **Export Overlay as scaled Viewer Image**. You can burn in additional details for publishing.

7. 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.

(a) Save your images through TLL common drive folder. Images saved in computer D/E drives will be ***deleted within a month without prior warning***.
(b) Carefully clean the objective lenses with Whatman lens paper (use 100% ethanol).
(c) Make sure to switch your lens back to the 10x/0.3 objective lens.
(d) Clean the sample holder with Kim wipes and 100% ethanol to remove any oil residues.
(e) In the event of logging off, you may leave the lasers ON and exit LASX. Otherwise, switch off all lasers within the software.
(f) Shut down PC **(1)** and switch off Microscope Power Box **(3)**.
(g) Switch off Metal Halide Lamp **(4)**, if it was used.
(h) Turn the laser safety key to "OFF" and then switch off the laser module/scanner **(2)**.
(i) Fill in the logbook with the objective lens used.

8. Troubleshooting

Issue	Explanation & Remedial steps
No laser emission	Check: • Key for the laser module is turned to ON. • If not turned to ON, turn the key to ON. If key for laser module is turned to ON: • Wait a few moments. The solid-state lasers used in the SPE II need time to warm up. There is no indication on the laser box itself as to whether they are ready.
No halogen light when viewing wide field	Check: • Halogen lamp intensity is >100 and aperture is set to low (open) on the microscope LCD display. • Lever behind the microscope. Move the lever behind the microscope and see if there is light coming out of the condenser as you move the lever. Remember to move it back if you are using the TL-BF on Confocal mode. 
Error: No Confocal Devices detected during startup	Loss of communication between the computer terminal and the laser module/scanner. 1. Turn the laser safety key to “OFF”, then switch off the laser module/scanner **(2)**. 2. Switch on the laser module/scanner **(2)**, then turn the laser safety key to “ON”. 3. Restart LAS AF. 4. Before imaging, please wait for the lasers to warm up.