BEGINNERS SAFETY MANUAL FOR
OLYMPUS FV3000 UPRIGHT CONFOCAL (#02-19)

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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 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 (MHL)

Metal halide lamps are fluorescence lamps that emit 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. The lamp has an enforced cooldown of 300 seconds (5 minutes) after shutdown where the lamp cannot be switched on until the 300 seconds is up.

- Check the timer on the power box to ensure the mercury lamp does not run past 2,000 hours. The timer indicator on this particular lamp power box blinks when the timer crosses 1,900 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 the time indicator is blinking.
- **Important!** If you hear an explosion and see the burner indicator turn red from blue, **save your work and evacuate immediately.**

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 the Master switch on the wall **(1)** & PC **(2)**. Wait until windows icon pops up.
(c) Switch on CBH controller **(3)**.
(d) *(Optional)* Turn on the Metal Halide lamp **(4)**, if needed.
(e) Press the Touch Panel Controller (TPC) power button at the back **(5)** and wait for 5s for the TPC to light up.
(f) Switching ON Lasers,
   - To use 405/488/561/640nm, switch on the power button on the main combiner **(6)**, then turn the key to "ON" **(6A)** and finally turn on the individual lasers that you need.
   - 445/514nm lasers are on separate control units with their own individual interlock keys. Turn on their switches and rotate their individual keys to "ON" of you need to use them
(g) After confirming that the TPC has loaded up (is showing a button that says "Start Operation"), load the FV31S-SW software and check if the model is "PRIOR H10F". Then click OK. Wait for complete initialization and **<u>DO NOT</u>** move any components on the microscope during the initialization process.

Last updated on Sep 2023

4.2 Objective lens

Objective lens	Immersion media
1.25x/0.04 Plan Apochromat N	Air
10x/0.40 U Plan X Apochromat	Air
20x/0.80 U Plan X Apochromat	Air
40x/1.40 U Plan X Apochromat	Oil (**Blue** Bottle)
60x/1.42 U Plan X Apochromat	Oil (**Blue** Bottle)
100x/1.45 U Plan X Apochromat	Oil (**Blue** Bottle)
30x/1.05 U Plan Super Apochromat*	Silicone (**Green** Bottle)
60x/1.3 U Plan Super Apochromat II*	Silicone (**Green** Bottle)

*Important!

If you need to use the silicone lens, please let Bioimaging know. Staff will assist in taking the silicone lens and changing the application settings.

30x silicone lens is now fixed in the FV3000 Upright. Please let us know if you need it in a different system or if you want to swap it for the 60x silicone lens.

4.2 Software Initiation

Wait for complete initialization of microscope and Windows after logging in with your TLL username and password.
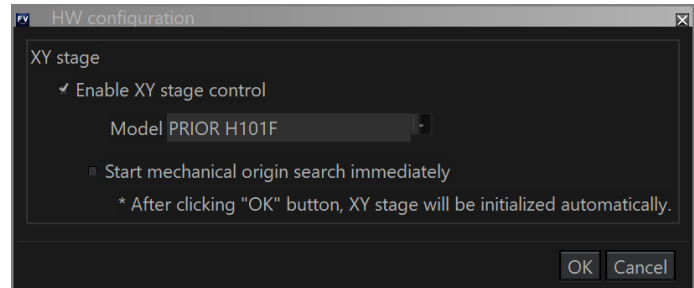
Double click on: FV31S-SW

The HW configuration screen will appear:

Select Enable XY stage control.

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

4.2.1 New user setup

If this is the first time you are using the computer terminal, even if you have been trained in an FV3000 system before, you will need to follow the steps below.

You will only need to run this once.

1. (If your monitor is a 4K monitor) Right click on the desktop > select "Display settings" > set **Display scale** to **125%**
2. Start up FV31S-SW
3. Click on "**Layout**" > Select "**Advanced Workflow and Viewer Visualization**" > Click Apply.
4. In **PMT Setting** tab
    i. Turn on **Sequential Scan**: change from "None" to "Line" or "Frame"
    ii. Activate **Pinhole "Auto"** adjustment
        ※ Pinhole defaults to the smallest setting if you don't press this button.
5. In menu **Tools** > **Configuration** > **Microscope Link** tab, check the following:
    i. Enable **Parfocal correction**
    ii. Enable **Parcentral correction**
    iii. Enable **Condenser interlocked with objective lens**
    iv. (upright only) Enable **Automatic insert of condenser top lens**
    The following two options can be selected depending on your preferences:
    v. **Turn the TPC backlight off during scanning**: Check to turn off TPC screen during scanning (live view or acquisition)
    vi. **Turn the LED indicator off during scanning:** Check to turn off LED indicators on the microscope body during scanning.

4.2.2 TPC controls
You can switch to and control the wide field ocular mode by using the TPC.

Fluorescence mode

1. Press the button at the top right corner of the TPC until it switches to the eye symbol.
2. Press FL1 to switch to fluorescence mode and see the fluorescence controls.
3. Press the appropriate button on the Mirror section to switch excitation wavelengths.
    *   U-F**U**NA: **UV** cube – use for DAPI
    *   U-F**B**NA: **Blue** cube – use for GFP, Alexa Fluor 488
    *   U-F**G**WA: **Green** cube – use for RFP and mCherry
    *   MIRROR: Mirror cube for confocal imaging only. Not for ocular viewing.
    *   IX3-F DICT: DIC cube for DIC viewing.
4. Cycle the Attenuator to adjust the excitation brightness. The number shown is the amount of light shining on the sample as a percentage of the total from the MHL.
5. Press the Shutter button at the lower left corner to open the shutter for sample excitation to view and focus your sample.
    *   Keep the shutter closed until you actually need to view the sample. Even if you are looking away from the sample, make it a habit to turn off the shutter to minimise photobleaching.

Bright field mode

1. Press the button on the top right corner of the TPC until it switches to the eye symbol.
2. Press BF to instantly see white light controls.
3. Press the « < > » buttons to change the brightness of the LED light. « » changes the brightness by 10 while < > changes the brightness by 1.
4. A preset of 130 is defined. To instantly go to that brightness, press Apply.

4.2.3 Wide field observation

Alternatively, on the FV31S-SW software, you can press on the ocular tab to observe the specimen using either bright field/DIC or fluorescence mode.

- For Brightfield imaging, select **Ocular>Ocular>DIA.**
  Select Cube turret #1: **Empty/IX3-F DICT**
  Under DIA, **ON** both TD shutter and Trans lamp. Increase intensity of Trans lamp for specimen viewing.
- For Fluorescence imaging, select **Ocular > Ocular > EPI.**
  Select Cube turret #1**:** U-F**U**NA/U-F**B**NA/U-F**G**WA
  Select the appropriate EPI ND Filter to control the fluorescence intensity.
 Then EPI shutter: **Open**
  ※ Keep it **Closed** until you are actively viewing your sample.
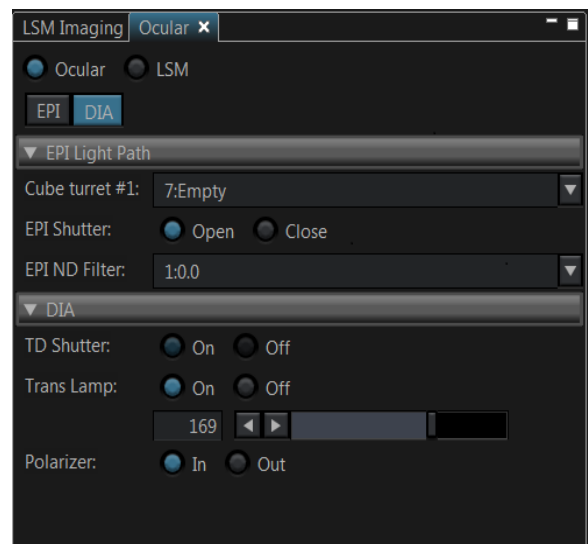
4.3 Setting up directory to save images

If this is your first time using the FV3000, you need to change your default folder away from the Fluoview software default, which is in the C: drive.

- Go to Configuration → Preference tab → File/Folder
- Uncheck Use default folder
- Press the Browse button to change the folder to either inside the D: or E: drives.
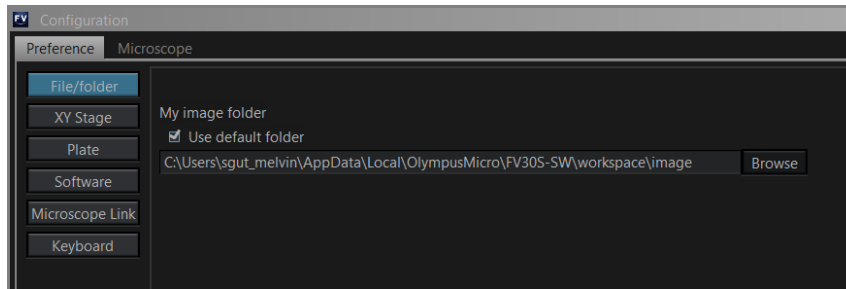
To save an image:

- Under **Acquire tab**, select the **folder icon** to select your directory to save all your images.
- Please save in your folder. You have several different options on where you can save your images.
  - Option 1: D: or E: → Users → shaalini (create your own folder)→ BPAE cells.oir. 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.
- Key in the title of your image. Subsequent images will be saved in a running sequence no.
- Your images will be saved in *.oir proprietary format.

<mark>Important: please do not save any data on the C: drive. Images saved in C: drive may be subject to deletion without warning!</mark>
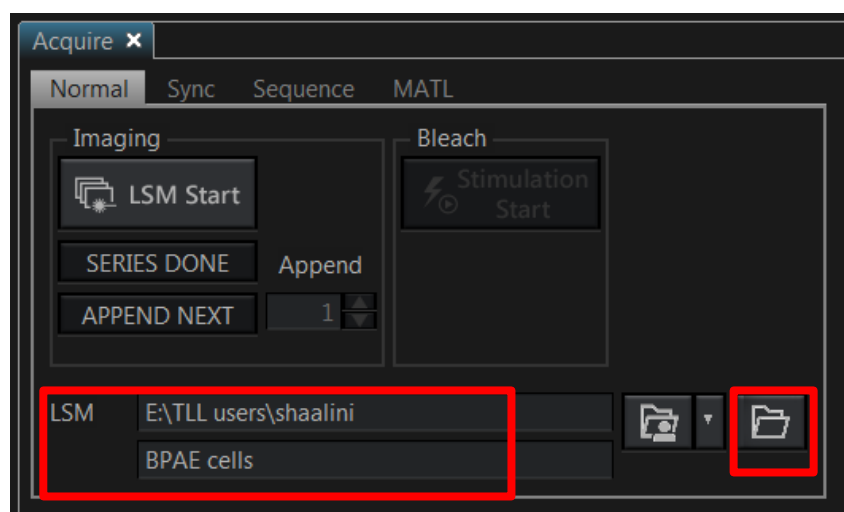
4.4 Laser Configuration Settings

Under **PMT Setting tab**, click on **Dye and Detector**. A list of commercially available dyes along with its excitation and emission values are available for selection.

1. Choose the appropriate dye.

2. Select **Add**.

3. It will immediately be reflected under phase 1 where each dye will be allocated to its respective channel. A transmitted light channel will also be shown by default.

4. Click **OK**.

- Under Laser ND filter: Select 10%.
  - ※ If you need more than 10% laser power you can switch off the ND filter, but most imaging will not need that high power of a laser.
- Set your laser power for all your channels at 1%.
- Emission range could be altered to include a broader range of signal otherwise you could use the default settings.
- For detectors:
  - **HSD**: keep HV within 350-500. More than 500 will produce intense background noise.
  - **SD**: keep HV within 600-800. More than 800 will contribute to intense background noise.

Under PMT settings tab:

- **Mode**: Select *VBF*
- **Average**: average a given number of scans to get a higher signal to noise ratio.
  - In *Line mode* the averaging will be done on a line-by-line basis.
  - In *Frame mode* the averaging will be done frame-by-frame. Drawback: slower scanning time, more bleaching, double exposure effect if your sample moves. Averaging of 2 times will help you to get better quality images if you can afford to do so.

  No averaging or Line averaging is recommended if you are doing live-cell imaging.

- **Sequential scan:** Select *Line* to prevent crosstalk among the fluorophores. If you select *None*, simultaneously imaging is done where cross talk is prominent.
- **Confocal Aperture**: press **Auto** button to make sure pinhole size is set to optimal confocal imaging at Airy Disk ×1. This gives the most optimum size for the thinnest optical slice while not sacrificing imaging quality. Increasing the size of the Airy Disk from

Last updated on Sep 2023
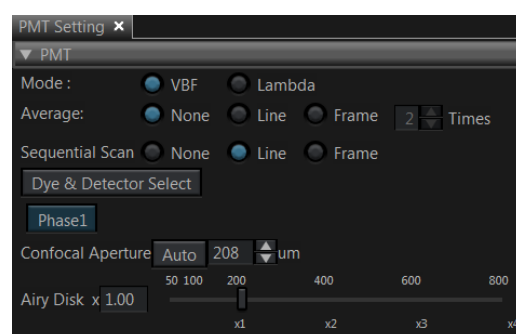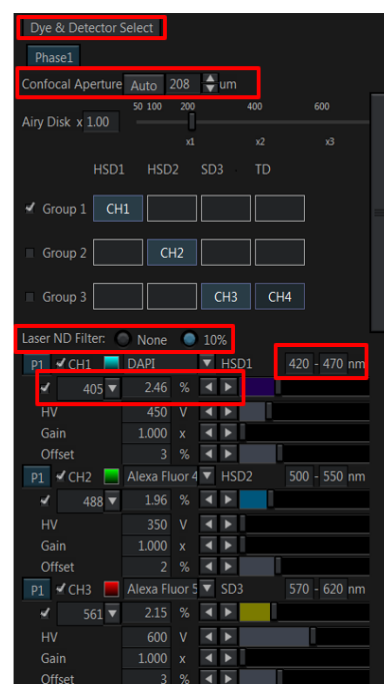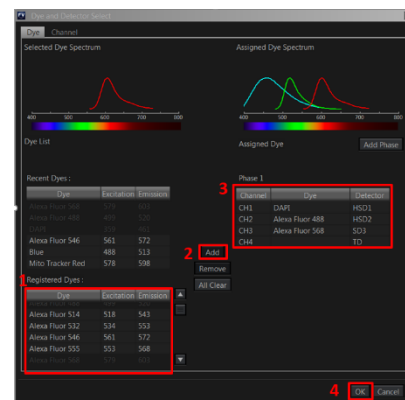
1× gives a brighter image while making the optical slice thicker, while decreasing the size of the Airy Disk from 1× gives a darker image while not making the optical slice thinner.

4.5 Using the LSM Imaging Tab

Under **Scan Settings** tab, in which you can set the parameters for your imaging:

- **XY**
  - ➢ **Type:** normally, Galvano is used. For live imaging, resonant imaging is preferred for its fast imaging but resolution will be lower.
  - ➢ **Mode:** Normally, mono-directional mode is used. For fast imaging, roundtrip can be selected where phase correction has to be adjusted.
  - ➢ **Interlace:** Normally OFF is preferred. If 2x is selected, it would capture the image by skipping every 2 lines of the image.
  - ➢ **Speed**: the slower, the better your image will be (a higher signal: noise ratio; but it takes longer and its more prone to photobleaching of your sample).
  - ➢ **Scan Size**: the number of pixels in your image (the more pixels the higher the resolution, to an extent, but slower the acquisition)
  - ➢ **Zoom:** allows you to magnify your region of interest, the arrows allow you to zoom images only in the center. Ideal zoom which satisfy the Nyquist limit can be achieved by zooming till the red arrow, any zoom beyond it will result in poor resolution and result in more photobleaching/damage.
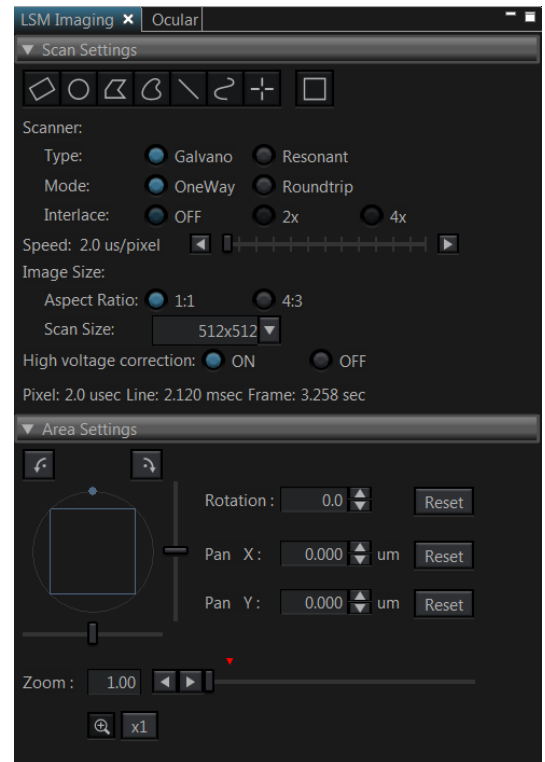
Click on **Live** (*images according to your scan setting configuration*)**/Live 2x** (*2x faster imaging*)**/Live 4x** (*4x faster imaging*) to view a live image. You can now adjust parameters while checking the result directly on the monitor. Be careful though, if you spend too long doing this you will photo bleach and damage your sample. Start focusing your sample and then center it moving the stage.

Now, based on the image, you can adjust the brightness using:

- **Excitation laser power:** The higher the laser power, the higher the risk of photobleaching. Also, this will alter both the brightness of the fluorescent image and that of the transmitted light detection.
- **Gain:** This adds computer processing to the signal from the detectors into the image to increase/decrease its brightness. For optimum signal to noise ratio, keep at 1.00×.
- **HV:** Keep below 500/800 for SD/HSD detectors 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.

4.6 Image Adjustments – Setting the Gain and Offset properly

Check image saturation level by clicking Hi-Lo icon. The image will change color to show red and blue pixels. Blue indicates that the signal in a given pixel is black, with a value of 0. Red indicates that the signal in a given pixel is saturated (over-exposed) with a value of 4095.

For optimal performance, the image needs to be so that then entire image is set to the limits of the dynamic range. Different sets of experiments may have different fluorescence intensities.

Adjust the image using a combination of the excitation *Laser power*, *HV* and *Offset*: Reduce either the HV or the laser power, until red pixels disappear. Increase Offset until a dusting of blue pixels in the background are observed.
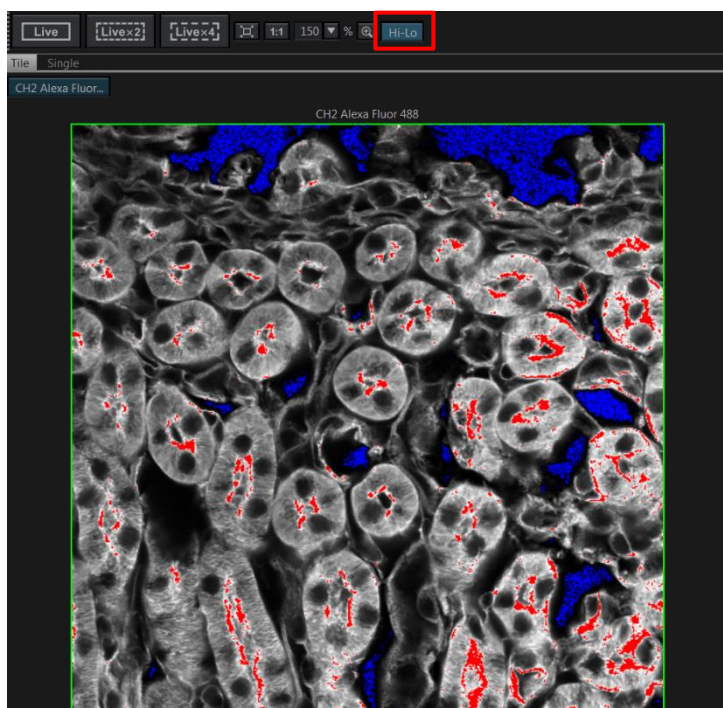
Click *LSM start* to capture an image.

4.7 How to do a Z stack for a 3D Image (xyz)

1. Under Z Series: **ON** for Z.
2. Select **Live 2x** or **Live 4x** to see the specimen and click **Register** under origin tab to save your focal plane where your image is focused. After completing the Z-stack. **Move** can be selected to move back to the focal plane of focus.
3. Turn the focus knob clockwise and move all the way to the top of your sample. Click **Register** under start tab.
4. Turn the focus knob anticlockwise and move all the way to the bottom of your sample. Click **Register** under end tab.
5. With **system optimized** selected, the program will determine the optimal settings for your Z-stack. You can override these settings using the **slices** or **z-step size** boxes. Beware that changing these will alter the z-resolution you attain.
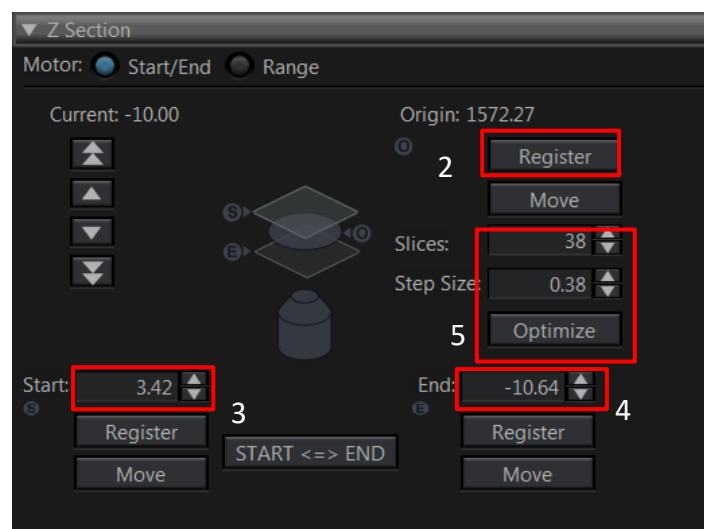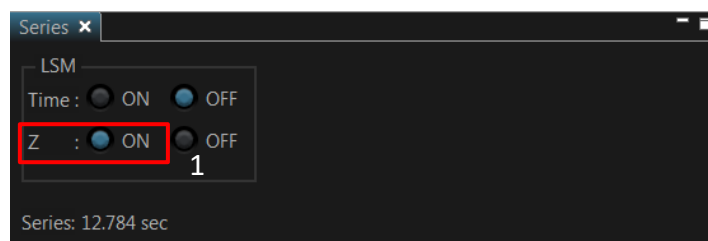
6. Click the **LSM Start** button to begin the series.

4.8 For Time-lapse (xyzt)

- Under Series: *ON* both Z and Time.
- Register Z stack before registering Timelapse.
- LSM Total: indicates the total time it would take to complete your imaging according to your Timelapse settings.
  - ➢ **Cycle**: is the number of times you would like to repeat the imaging during your timelapse experiment.
  - ➢ **Interval**: "FreeRun" continues to capture images until it has completed your selected cycle. You could always choose an interval of your choice.
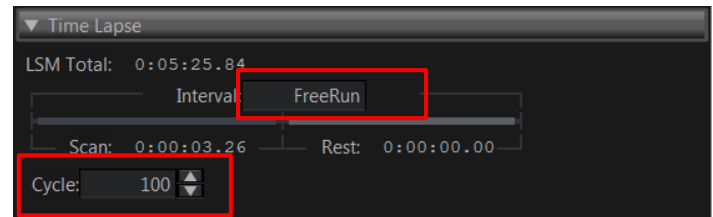

4.9 Reuse previous settings and export images

- Reusing previous Settings:
  - ➢ You can upload a configuration from a previous image. **File>Open>Image**.

  - ➢ In your image tab, select the arrow and click **property** where it displays a detailed record of the settings used to acquire the image, including its properties. Click on the **load acquisiton parameters** icon of the image to reuse your previous image settings.
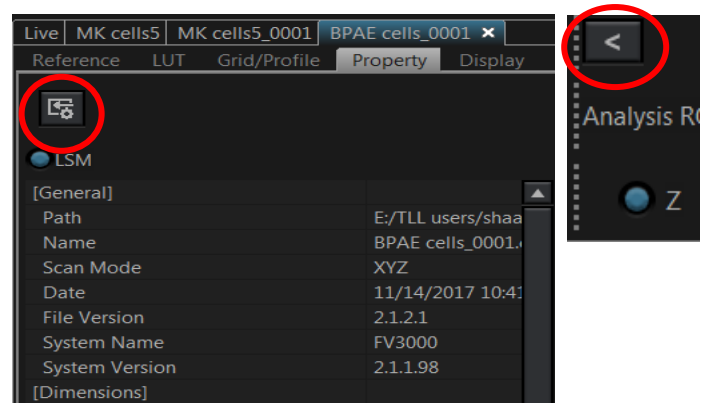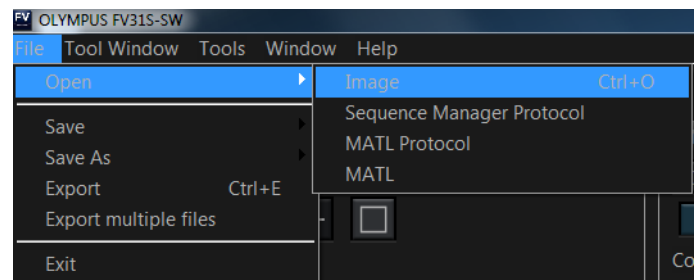
- Export Images:
  - Right click on the image and then export. An Export windows will pop up.
  - Select your directory where you would like to save your TIFF images into.
  - Save as type: TIFF (*.tif, *.tiff)
  - Under Output format, select RGB Color with merge and 24bit Full color.
  - Click Save.
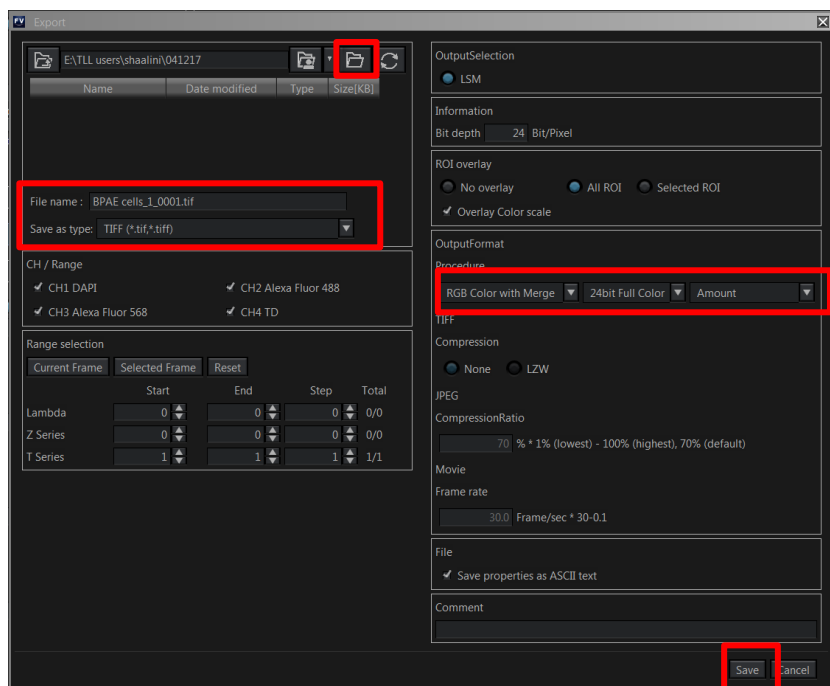
4.10 Switching OFF Protocol

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) Lower down the objective lens completely. *(Z value= 0)*

(c) Remove the specimen holder with the sample and return the stage to central position.

(d) Carefully clean the objective lens with Whatman lens tissue using 100% ethanol.

(e) Switch lens back to 10x/0.3 objective lens.

(f) Exit the FV31S-SW software and shut down PC **(2)**.

(g) Press "OFF" on the touch screen panel and wait for 5 secs for the light to disappear. Then, switch OFF the power button at the back of touch screen panel **(5)**.

(h) Switch off the CBH controller **(3)**.

(i) Switch off the HBO/Mercury lamp if used **(4)**. (Record down the lifespan hours in the logbook.)

(j) Switching OFF lasers,
  - To turn off 405/488/561/640nm, turn off the individual laser lines and turn the key to "standby", **(6)**. Switch off the main combiner **(6A)**.
  - To turn off 445/514nm, turn off the individual laser lines and turn the key to "standby".

(k) Turn off the incubator **(12)** if it was on.

(l) Finally, Switch off the master switch on the wall **(1)**.

(m) Fill in the logbook with the objective lens used.

5. Troubleshooting

Issue	Explanation & Remedial steps
No laser emission	Check: • Laser emission switches (6, 6A) for the appropriate lasers are on. • On the laser switch box (6A), the key to the laser is turned to the ON position. • Lasers are properly warmed up: Look at the back of the laser switch box. Blinking white light means laser is still warming up, solid white light means laser is ready for emission. • Appropriate channel that uses the laser needed is set to emit laser. If all laser settings are confirmed and there is still no laser emission, please contact Bioimaging Group.

No signal during confocal scanning	1. Use the transmitted detector to check. If the specimen is thick and non-transparent, the specimen should be removed to first check for laser pass through to transmitted detector. For easier verification, standard specimen slides can be used as confirmation check.
	2. Check to see if scanning spot can be spotted on the specimen.
	3. If there is no scanning spot:
	    I. Check that main laser key is switched on and the appropriate laser is switched on.
	    II. Check the laser rack to check the laser status light. Blinking light on the diode laser (which indicates laser starting up) has turned to a solid white or coloured light (sometimes red light might be indicative of error).
	    III. Restart the main controller right at the bottom if none of the steps taken work, especially if the machine has been left on by the previous user.

Signal image not visible/too dim even at high laser power	**Check**: If fluorescence signal is visible on wide-field eyepiece. **Check**: PMT settings if fluorescence can be seen on wide-field Ideal base settings are • **HV**: 350-500V for HSD, 600-800V for SD. *You can set the HV higher for quick sample spotting, then when you find the sample you can tune the HV lower and increase laser power.* • **Gain:** 1x. *Decreasing Gain reduces brightness and increasing Gain doesn't increase the sensor's sensitivity anyway.* • **Offset:** 0%. *Adjust it only after you find your sample.* • **Pinhole:** Auto. *When set to manual mode it may default to the smallest size which will make the signal very dark.*
Control panel switches off when Live View is activated	This is a feature by Olympus to allow a fully dark room during imaging that can be switched on/off when needed. To check status, go to Configuration > Preference tab > Microscope Link and check **Turn the TPC backlight off during scanning** and **Turn the LED indicator off during scanning**. You can set them to your preferences.

Startup popup error code: TPC_LOGIN_ERROR	Connection between computer and the TPC was broken on the computer side and cannot be recovered. Restart computer terminal.
Startup popup error code: TPC_PORT_NOT_ INTERFACE	Connection between computer and the TPC was not established or otherwise interrupted. 1. Close the FV31S-SW program. 2. Shut down the TPC and restart it. 3. Wait for the "Start Operation" button to appear, then wait another 30s before opening the FV31S-SW program again. 4. The program may show a LAST_OPERATION_NONCOMPLETED error when the program is restarted after the previous steps are followed. Press OK and restart the program as normal (see below item).
Startup popup error code: LAST_OPERATION_ NONCOMPLETED	 The last user did not shut down the program properly, or the program encountered a fatal error during the previous session and the last user closed their session without restarting the program. • No action required. Press OK and restart the program as normal.