. Uppsala University . Platform . Biological Visualization .

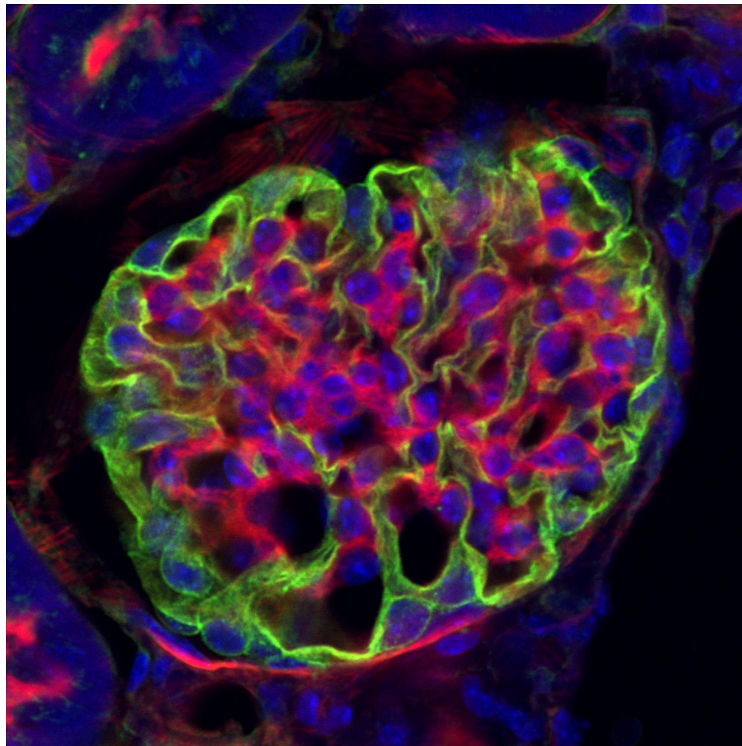
BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

LEICA SP8 DIVE

Multiphoton microscope

Manual/Quick guide

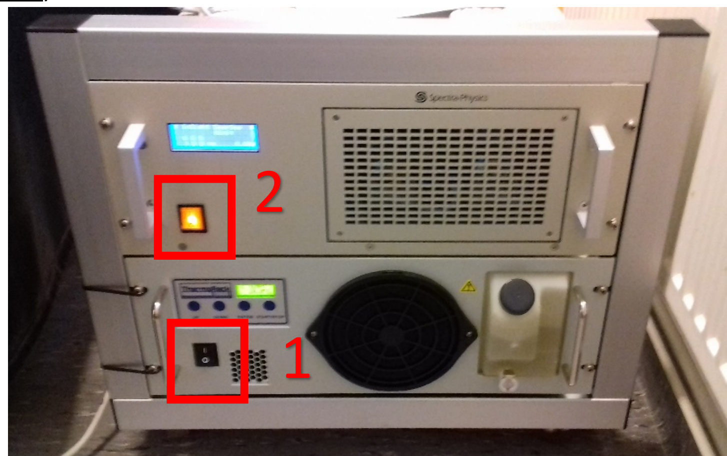


Matyas Molnar, Biovis 2020

Starting the microscope

- IF it's not ON: Turn ON the chiller for the NDD detector under the optical table (<u>THIS IS HANDLED BY BIOVIS, IT SHOULD BE ON BY DEFAULT</u>)

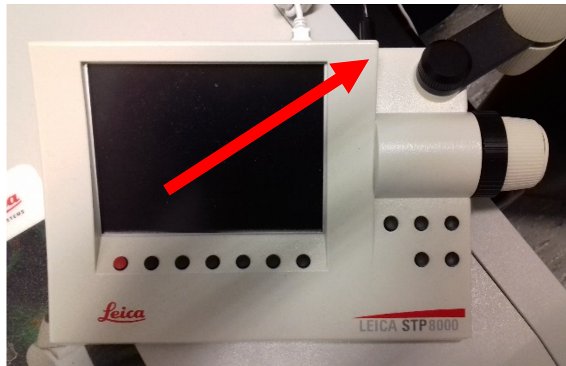
- IF it's not ON: Turn ON the Ti:Sapphire laser chiller (<u>THIS IS HANDLED BY BIOVIS, IT SHOULD BE ON BY DEFAULT</u>)



1. **Turn ON** the computer, wait till Windows is booted

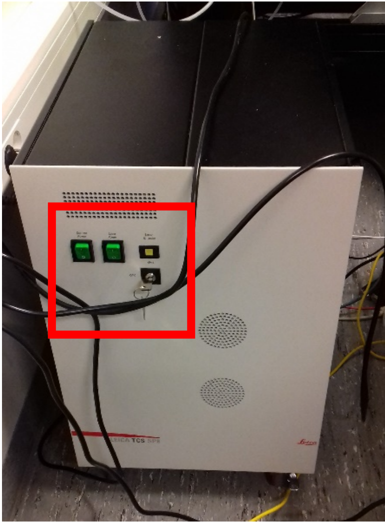
----- After Computer is ON, the following steps are not strict, the order can be changed -----

2. **Turn ON** the TFT screen on the PC desk (button on the back)



3. **Turn ON** the microscope stand controller, white box next to the curtain

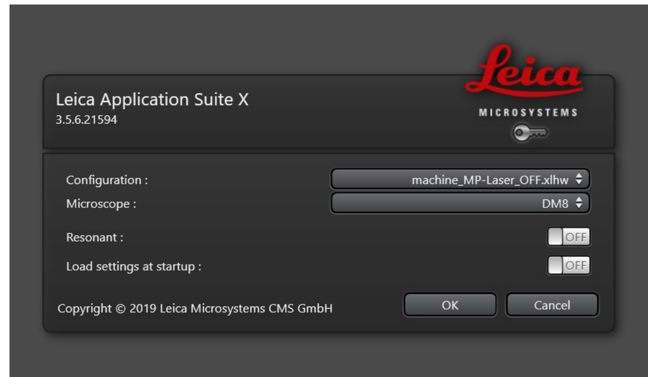
4. **Turn ON** the confocal box behind the PC desk



   a. Scanner power
   b. Laser power
   c. Laser Emission key

5. **Turn ON** the fluorescence lamp if needed

6. **Start** the LAS X software



Options:

- Start LAS X software with or without the multiphoton laser (Configuration: MP-Laser_ON-OFF)
- Microscope: DM8 to be selected
- Resonant (fast scanning) mode ON or OFF (if ON, normal better quality scanning is not activated)
- Load a previously saved settings ON-OFF

Before stage initialization **always remove the stage arm**, so that there is nothing close to the objective that can break it when moving. Also when any process is started in the software that can involve stage movement (e. g. Z-stacking, loading old settings and positions, etc.) always take extra care for the stage and the objective, and remove the stage arm from the way!

Laser safety

Always be extra careful when working with lasers, especially with the infrared femtosecond Ti:Sapphire (multiphoton) laser.
The multiphoton laser has several dangers, including:

- **It can cause permanent eye damage – please note that you have no blinking reflex against infrared radiation, the direct beam or even scattered radiation can cause blindness in a fraction of a second.**
- **It can start fire if combustible material crosses the beam path, and it can burn your skin.**

The multiphoton laser is a CLASS 4 laser (highest laser class), everyone who is working with it MUST understand the risks and take extra measurements to eliminate accidents.

- **Only trained users can operate the instrument and can stay in the room**
- **Always close the door and window when working with the microscope**
- **Don't wear rings, watches, jewelries or anything that can reflect the beam**
- **During scanning, never open the curtain and never look behind the curtain**
- **Don't cross the beam with combustible or any material except your sample**
- **After you are done with the imaging, always turn OFF the laser and verify with the laser's indicator lamp that you leave the room with the multiphoton laser's emission OFF.**

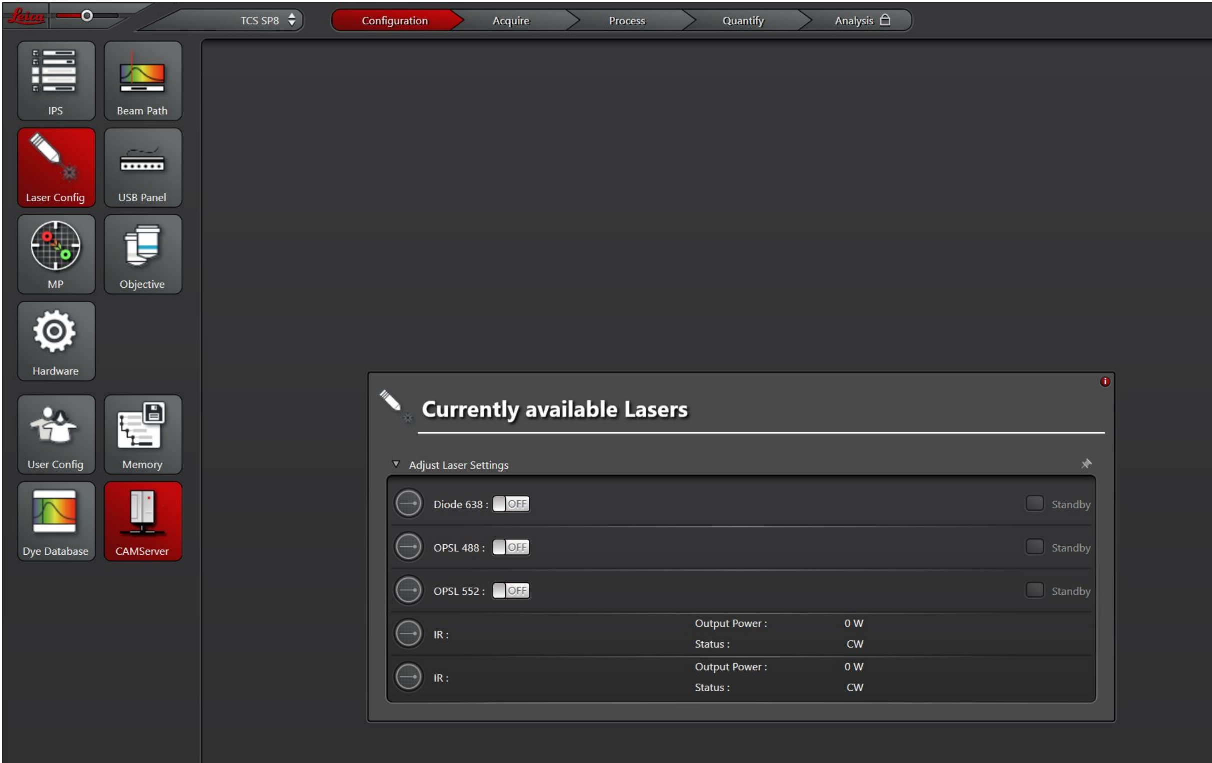
Settings to see fluorescence with the eyepiece

The microscope is equipped with a triple filter that enables us to find our sample and fine-tune the focus in blue-green-red channels. If you don't see any signal through the eyepiece, make sure that you are using the following settings on the TFT screen:

Using the LAS X software

In LAS X, hover the mouse on any button to get info about it.

Configuration tab

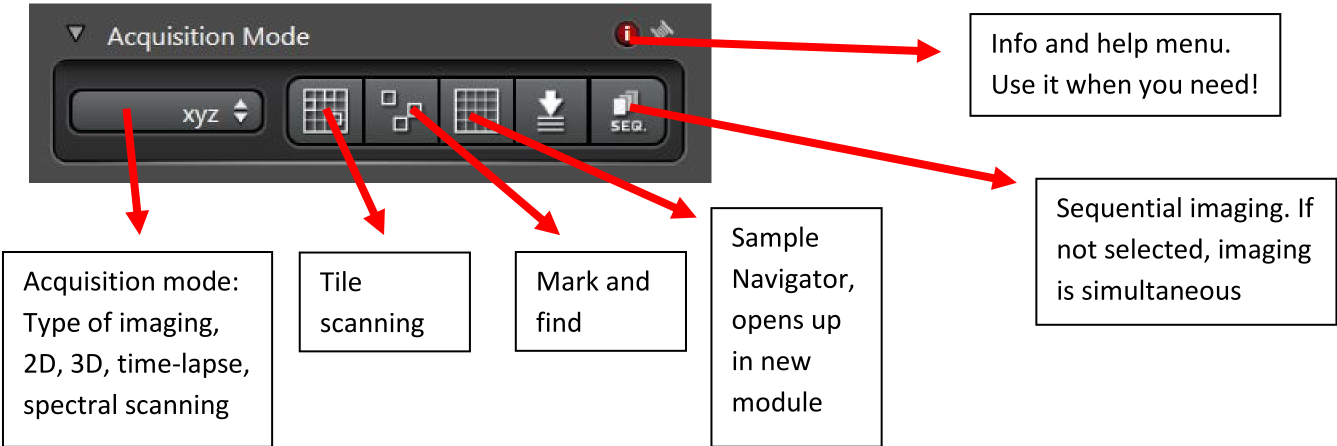


Under the configuration tab, several options can be changed and lasers can be turned ON and OFF.

- In the **Laser config** menu: laser can be turned ON or OFF
- In the **Hardware** menu: dynamic range can be set to 16-bit (the default is 8-bit)

Acquire tab, confocal imaging

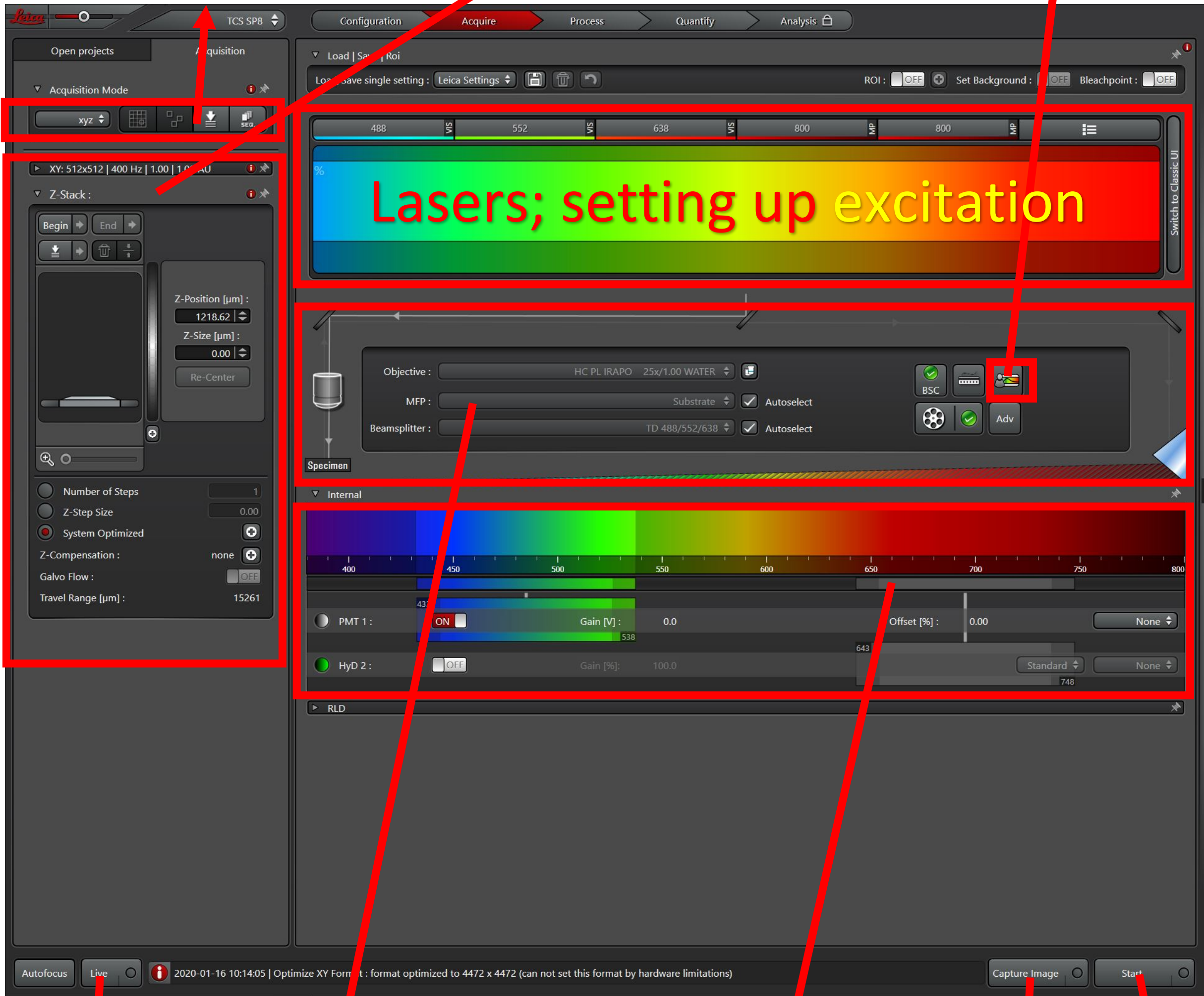
This is the most used tab, here the imaging setup can be configured and imaging can be started.



Acquisition mode:
Type of imaging, 2D, 3D, time-lapse, spectral scanning, simultaneous or sequential imaging

Acquisition menus

Auto mode. If you are unsure what you are doing, start setting up your imaging here



Objectives and beam splitters. Objectives can only be changed manually for this instrument. If unsure, leave all other options on autoselect.

Detection configuration. Two detectors (one PMT and one HYD) can be turned ON-OFF, and their parameters such as detection range, gain and offset can be modified.

Start to acquire 3D; time-lapse; or spectral scanning, etc.

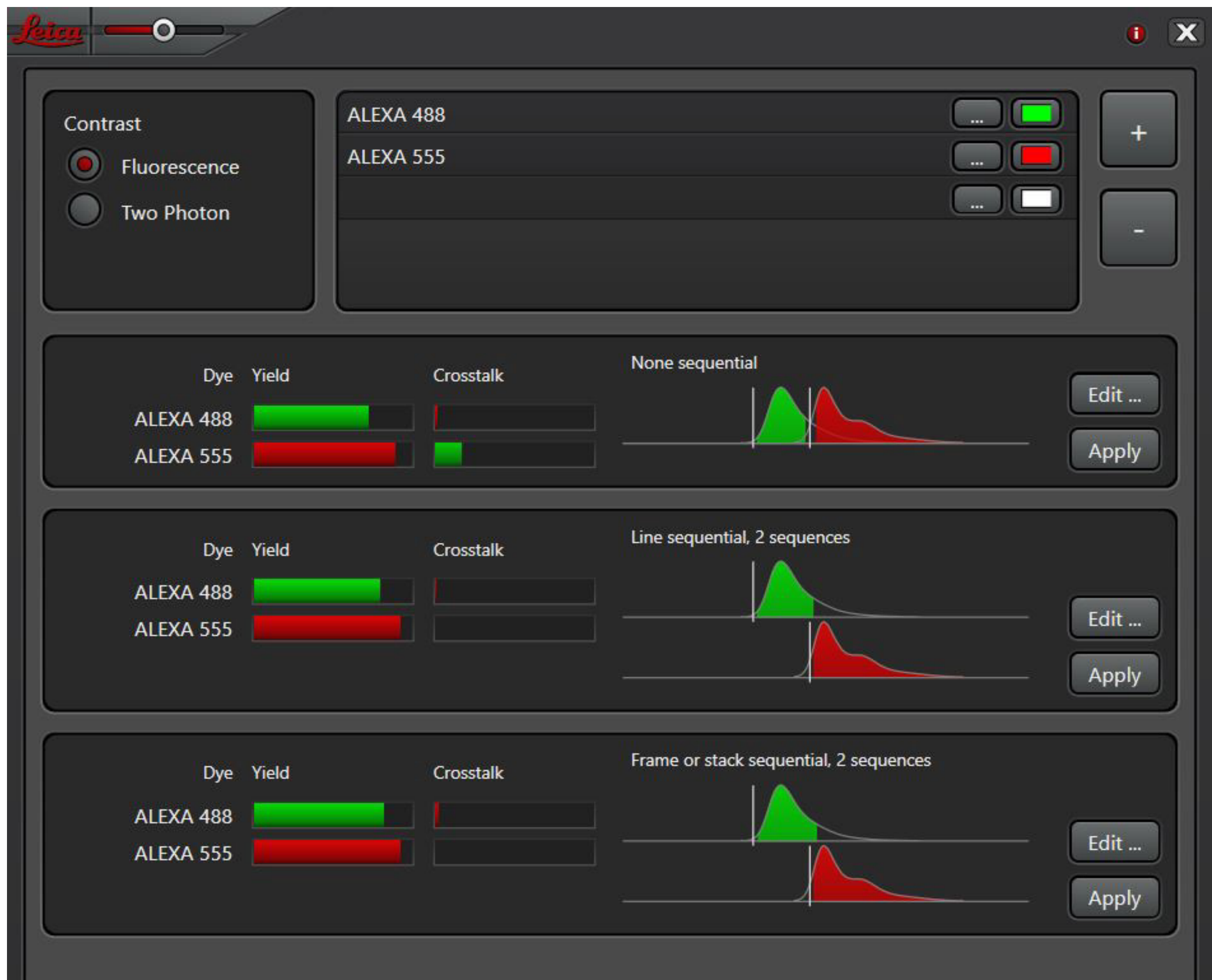
Live mode to check the sample and find locations

Capture an image

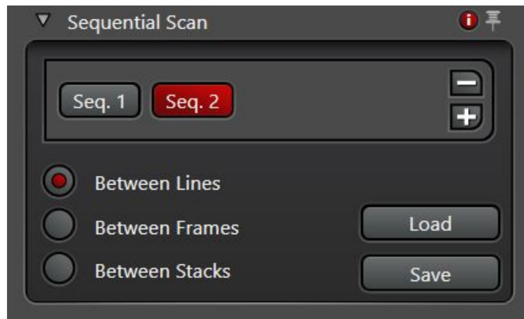
Auto mode

Auto mode is a quick and easy way to create the settings for fluorophores and channels. Unexperienced users should start with the auto mode. Please note that auto mode might not work perfectly in special cases (like multiphoton) or has to be fine-tuned manually.

- Select your fluorophores
- Select **fluorescence** or **two photon**
- Select between **simultaneous** or **sequential** imaging, **line** or **frame**

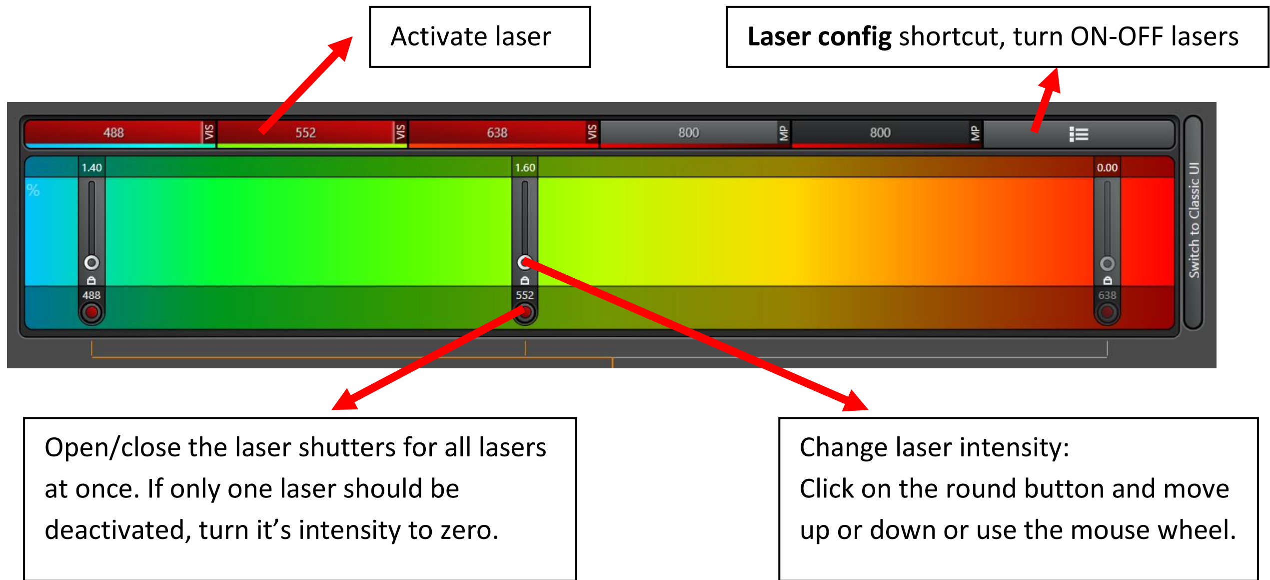


Sequential mode



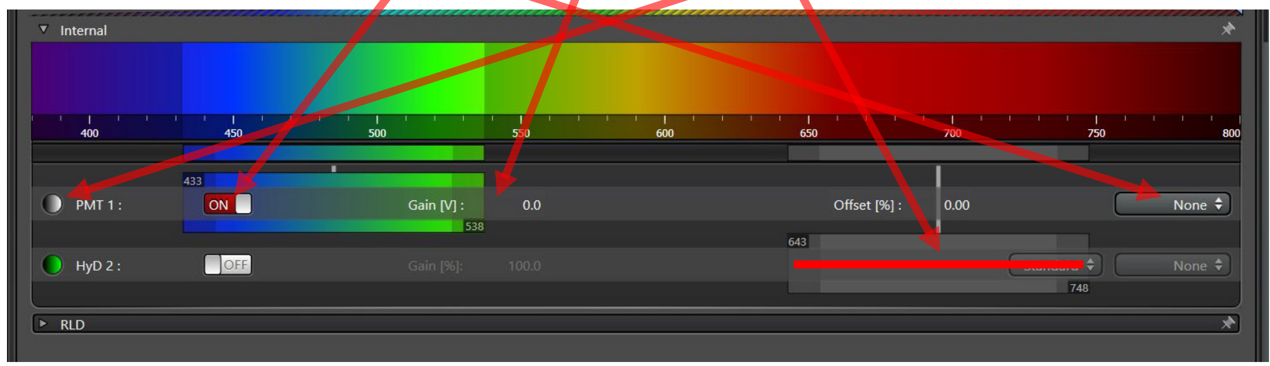
If sequential mode is activated, a new menu will pop up in the menus where the parameters of the sequential scanning can be configured. Here the excitation and emission settings for the different sequences (seq.1; seq.2, etc) can be checked and modified by clicking on any of the sequences.

Lasers



Detection configuration

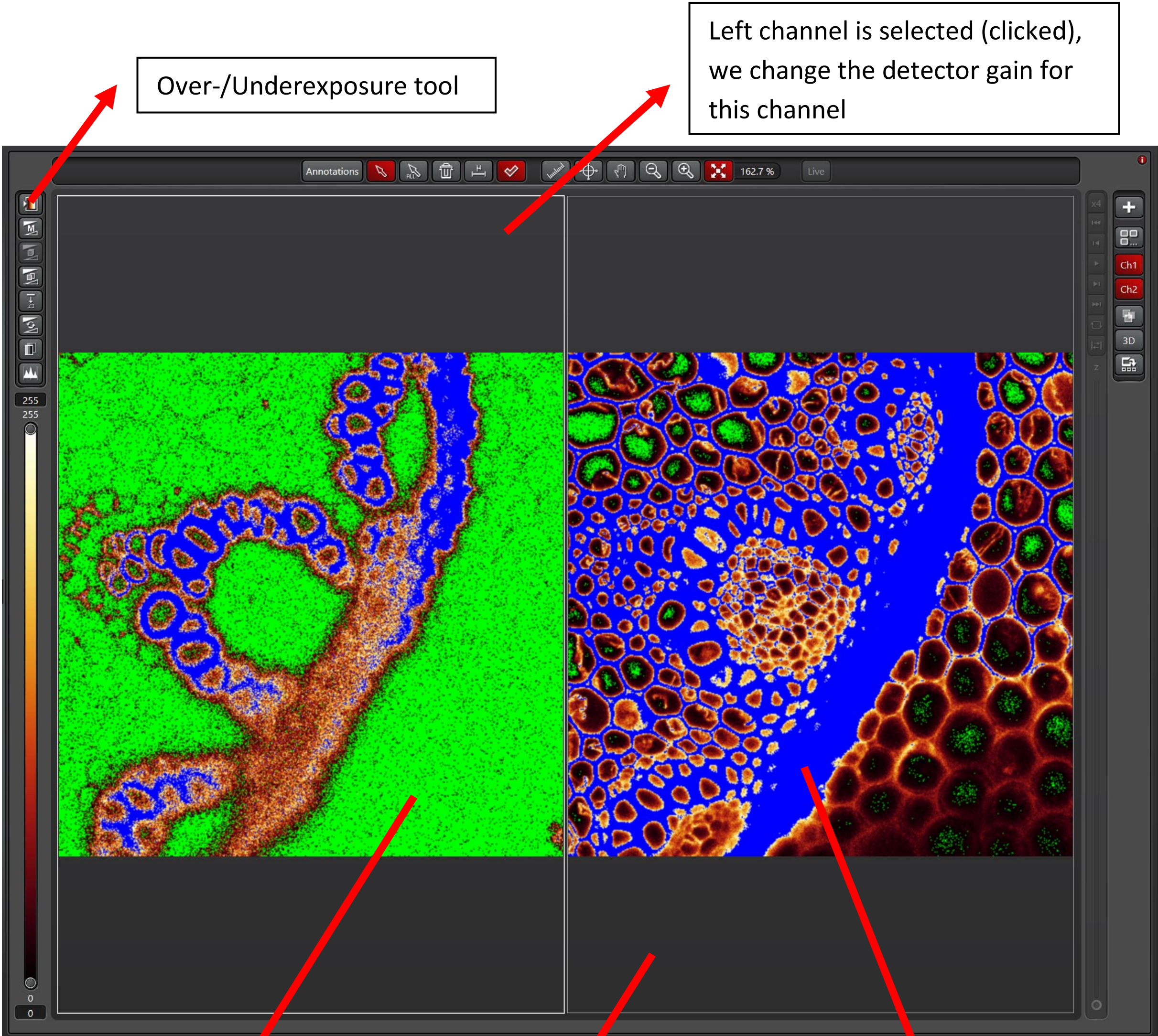
Here the different detectors can be activated; their spectral ranges can be specified; the virtual color of the channel can be changed; and intensity (gain) of the signal for the different channels can be adjusted. If needed, the spectrum of a selected fluorophore can be visualized to help setting up the channel.



Adjusting the correct intensity with the Detection configuration menu or the knobs

The best way to adjust the correct intensity of a channel is to use the Over-/Underexposure tool on the left side of the image window (first button). If Over-/Underexposure tool is activated we see the lowest intensity pixels (pixel value: 0) in green; and the overexposed pixels in blue. The overexposed pixels are out of the detector range, their intensity information is lost. We must avoid seeing overexposed (blue) areas. By changing (decreasing) the detector gain (voltage) or the laser intensity, we can move the overexposed areas into the range of the detector. For multichannel simultaneous imaging, be sure to click on the image of the channel you want to change, the other channel's detector is not changing meanwhile. Which one should we change, the laser or the detector? There are no rules here, both have advantages and disadvantages. If we use high laser power with low gain we see a good quality image with low noise, but we can bleach the sample, so as always, COMPROMISE between quality and time/bleaching! Don't use a default laser or gain settings, always change them freely to get the best image without ruining the sample.

Note that if you change the pinhole or spectral range, the signal is collected in a different Z/spectral range (intensity is changed), therefore new intensity adjustment is needed.



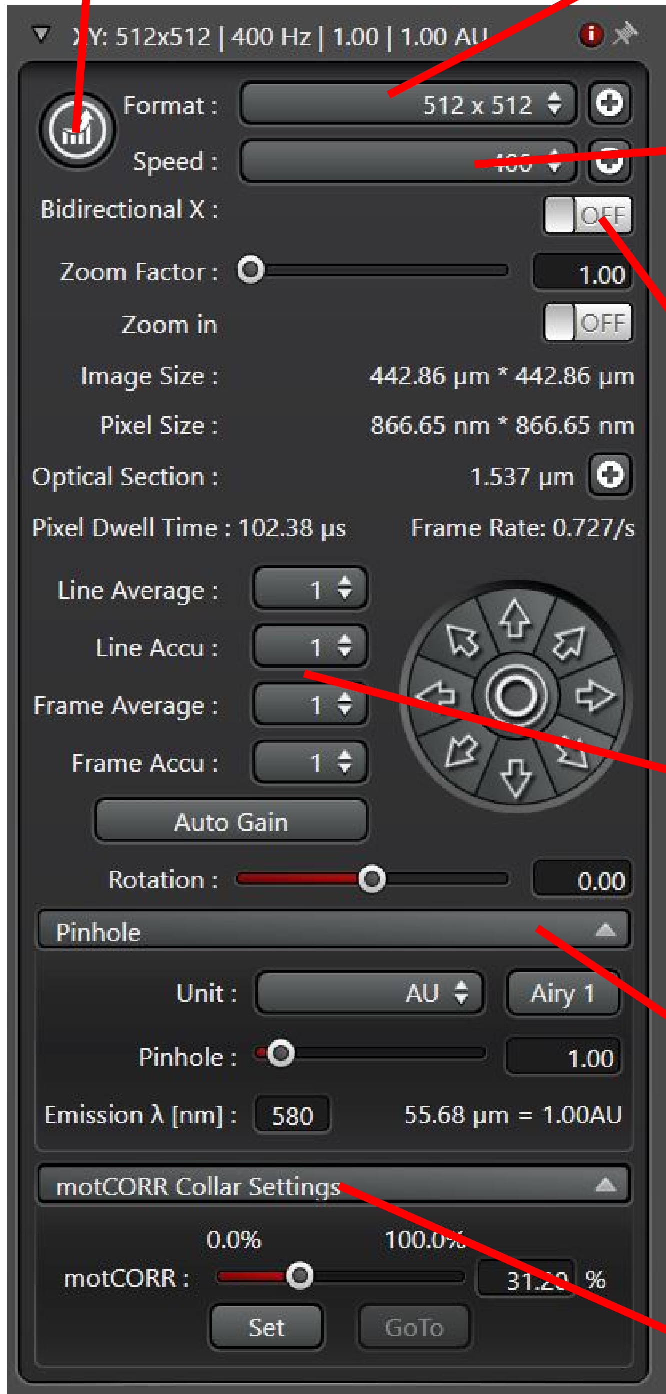
Right channel is not selected (not clicked), we change the detector gain for the other channel

Underexposure (green color); the histogram is clipped on the left side. To avoid clipping and to remove the green, use “offset”

Overexposure (blue color); the histogram is clipped on the right side. To avoid clipping and to remove the blue, lower the “gain” or “laser power”

Acquisition menu

Optimize resolution (pixel size)



Set resolution (pixel size) manually. If you are unsure, use the **optimize** button. For 3D imaging use small, like 512x512 to save time/avoid bleaching.

Scanning speed (Hz): go up with the speed to decrease, go down to increase the quality. No golden rule, it is sample dependent, if unsure try the sample with different speeds, and use the best for the real acquisition. Again COMPROMISE between time and quality, decide what is more important.

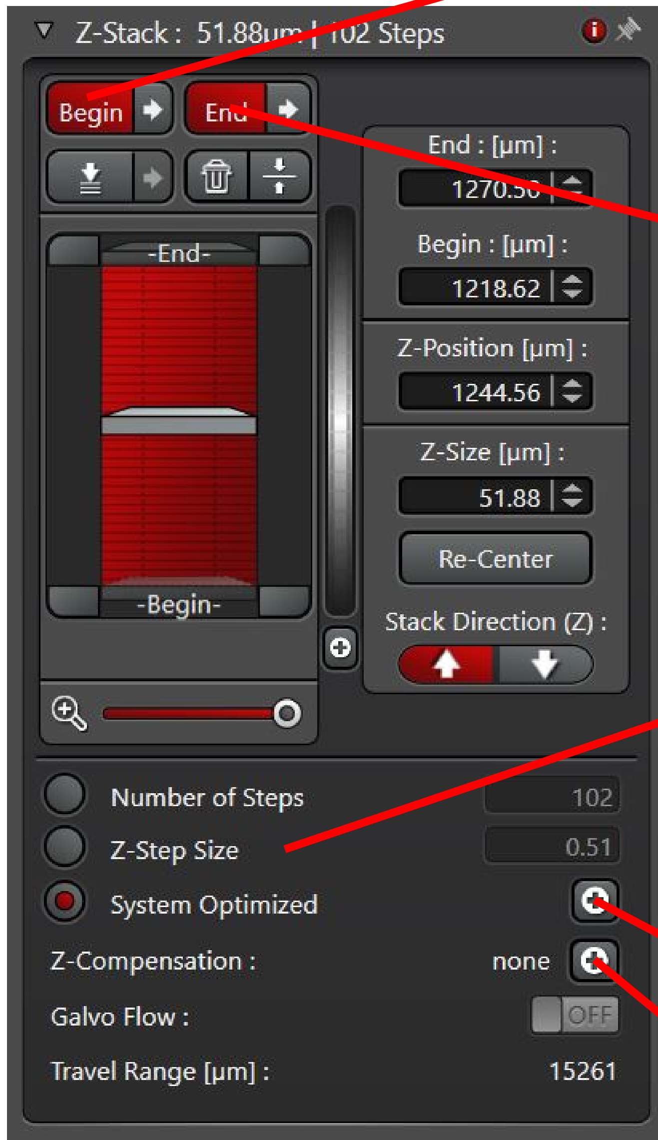
Bidirecional scanning: Before starting a scan always test if X phase is correct. If unsure, don't use it, it can ruin you imaging.

Averaging: noise (random pixels) are removed with averaging process. Select the number of image to be used for averaging (again time vs. quality!) and choose between line and frame averaging.

Pinhole: changes the thickness of the optical section. Click **1 AU button** to get confocal imaging. There is no pinhole adjustment for the multiphoton setup.

Motorized correction ring: when using water objective, the motCORR function compensate for deviations in the coverslip thickness. Follow the Leica manual (to open: (i) button) to set up the correction.

Z-stacking (3D imaging)



1. Go to **Live** mode, use the focus knob to find the end of the sample and push **Begin** to select the starting position of the sample where the first 2D stack should be made.
2. Focus to the other end of the sample and hit **End** to select the position where the last stack should be made.
3. Select the number of 2D stacks. Two ways of doing it, either one can select **Z-step Size** and type in the interval between two stacks or select the **Number of steps** and type in the desired slice number. If unsure, go for the **System Optimized** button to not lose any information between 2
4. Advanced optimization setup
5. Setup for linear laser or detector gain compensation when signal is getting lost in depth

The image window

Over- /Underexposure LUT

Auto contrast

Add **scalebar** and **annotations**

Zoom

View all channels separately

View histogram

Hide/view channels

3D viewer (opens up in new module)

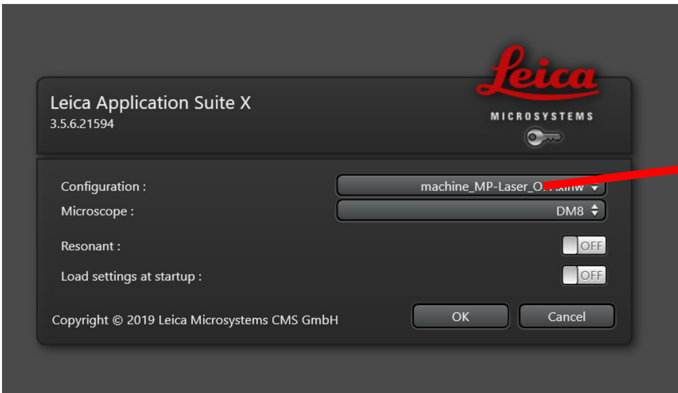
Right click on the image to **Snapshot a** view (for MIP or orthogonal view for example)

Show gallery (for Z-stacks, time-stacks, etc)

Saving, exporting

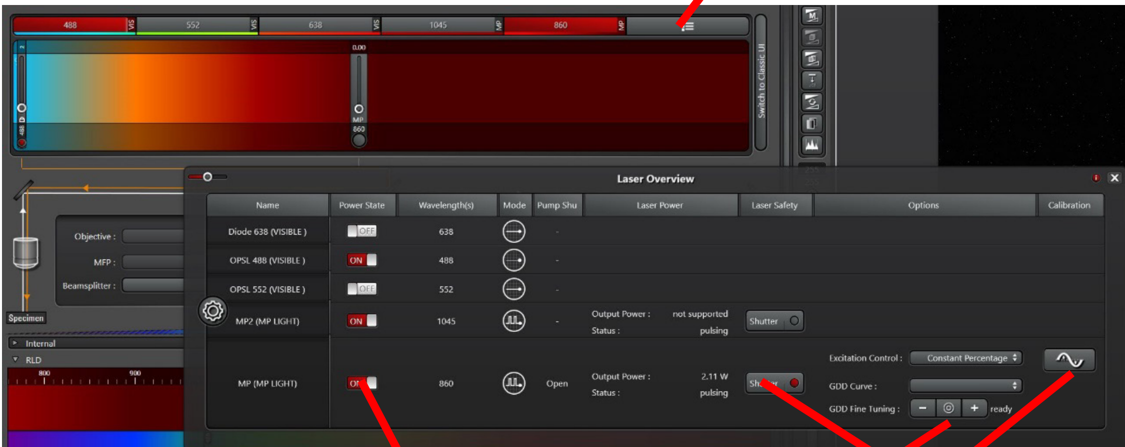
In LAS X projects can be created and saved. In a project, many images, datasets, snapshots can be saved and later re-used. **Saved** data will end up in .lif files that can be opened in LAS X, ImageJ, and Imaris. If image file is needed, right click on a dataset and select **Export**.

Multiphoton imaging



1. For multiphoton imaging, start the LAS X software in **MP-Laser_ON configuration**

2. Open **Laser config**



3. Turn ON the MP laser(s)

4. After the laser(s) is pulsing, to open the Laser shutter **press and hold** the **Shutter** button

For imaging with the MP laser, the GVD can be fine-tuned here

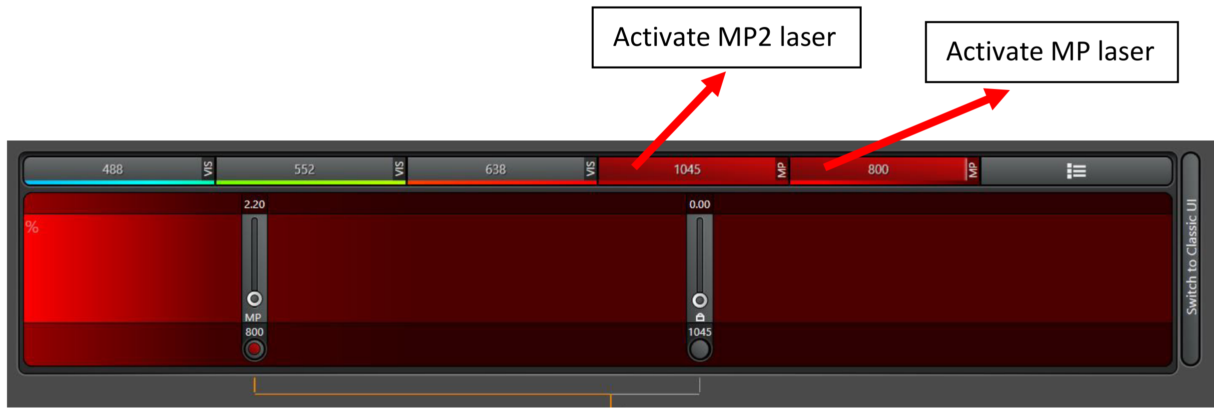
The MP laser's power calibration can be started here to obtain constant power (e. g. for absorption spectroscopy)

There are two infrared femtosecond laser line is available for this microscope due to the nature of the used parametric oscillation:

- fundamental pump and idler beam (MP: tunable 680-1300 nm);
- signal beam (MP2: fixed 1045 nm).

Activate MP2 laser

Activate MP laser



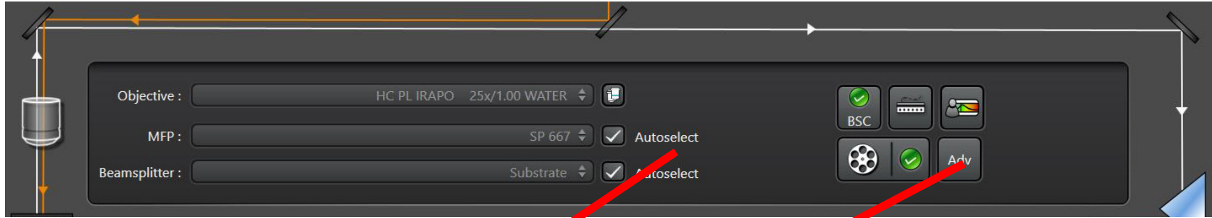
Tune the laser's wavelength by grabbing ONLY the top part here and move it left/right.

Be very careful, not to touch this region! The laser intensity can be dragged easily to a high value and the sample can be destroyed immediately.

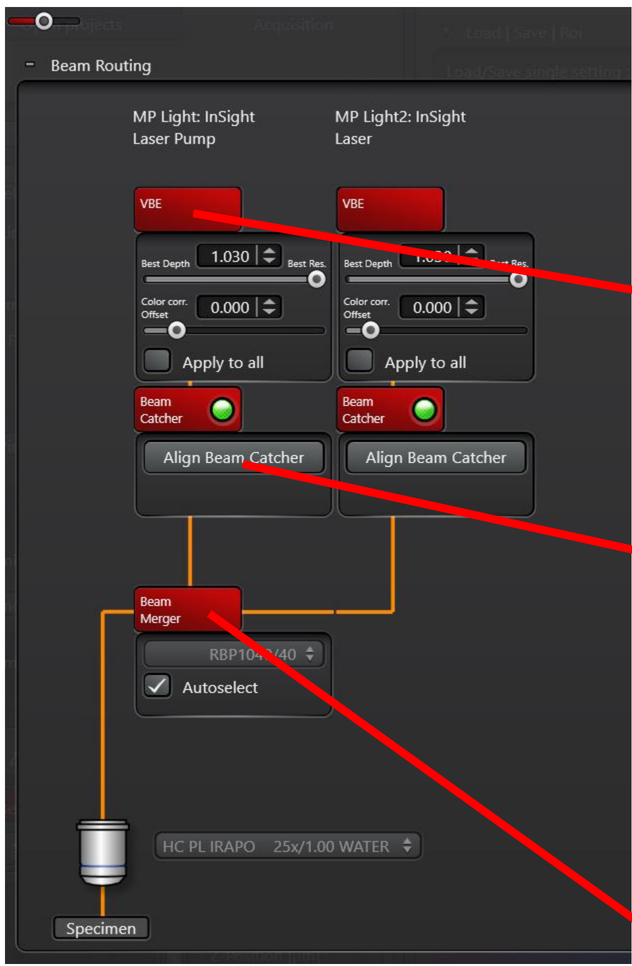
To change intensity, left click the center of the button and use the mouse wheel to change small increments.

Open the final shutter for the laser

Remember all the safety points and be careful when working with the infrared laser!



If unsure, leave them on autoselect



Set up the **Variable beam expander.** Using the beam expander, the diameter of the laser beam can be optimally adapted to the entrance pupil of the objective. It' a balance between depth and resolution. Default value is 1,030.

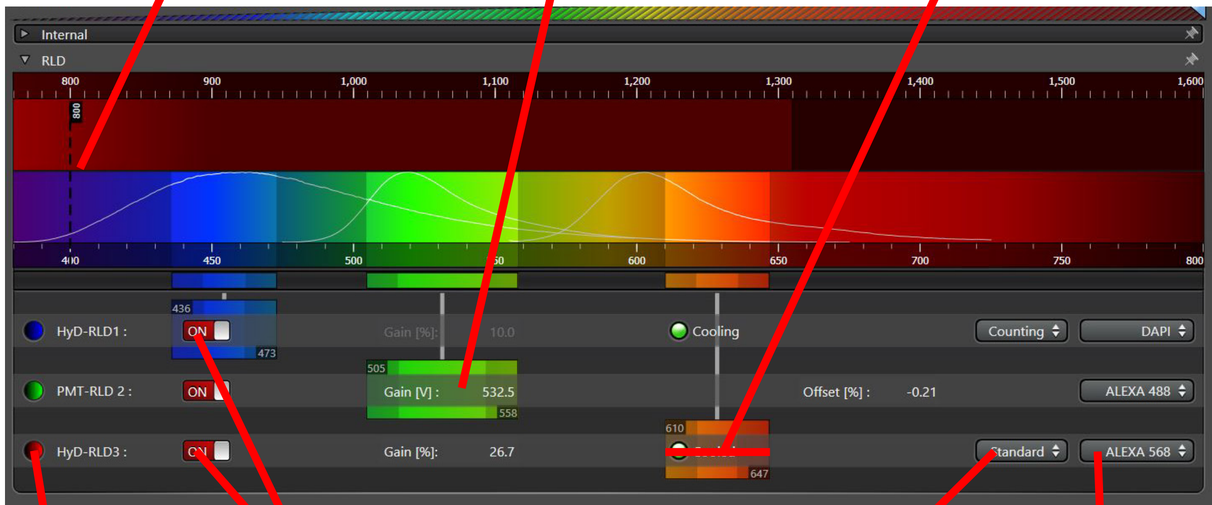
Shifts of the laser beam in position and orientation can occur. Use the **Beam catcher** to optimize the MP beam's position for the desired laser line.

If both MP laser lines are used together, select the proper beam merger. If unsure, check autoselect.

MP laser wavelength and doubled frequency line in the emission spectrum, to help setting up SHG detection

Gain [V] for the detectors

Spectral range for the detectors



Detection mode for the HyD (Hybrid) detectors.

Draw emission curve for fluorophores to help setting up the detection range

Change virtual color for a channel

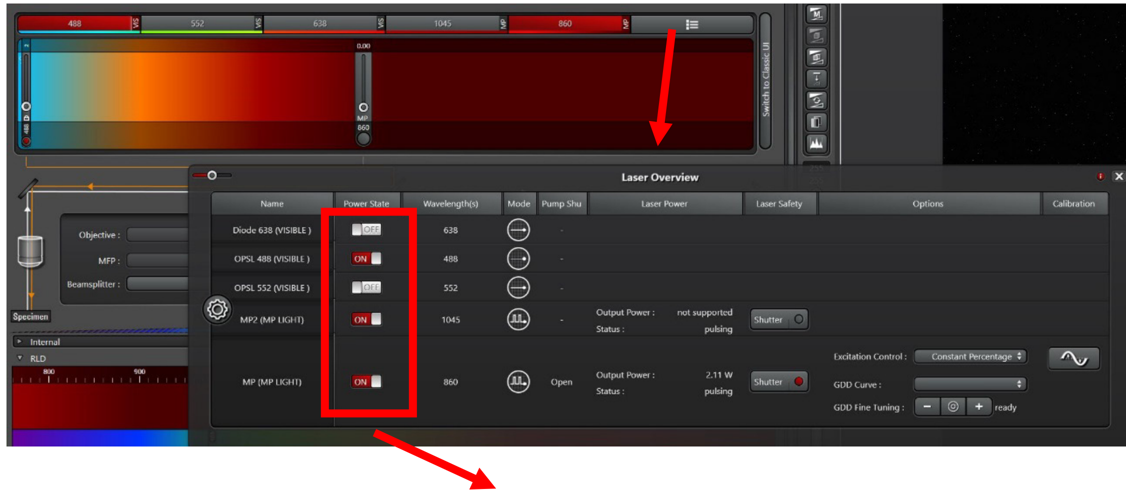
The HyD (Hybrid) detectors are very light sensitive and can be destroyed easily. Before opening the curtains and turning ON lights, **turn OFF all HyD detectors! If high pitched noise is sounding the HyD detectors are overloaded, turn OFF all the HyDs immediately.**

Commonly used fluorophores for multiphoton imaging with their two-photon absorption range

Fluorochrome	Absorption	Emission
Alexa Fluor 350	720-800	440
Alexa Fluor 488	720-800	515
Alexa Fluor 546	720-840	569
Alexa Fluor 568	720-840	596
Alexa Fluor 594	720-850	610
Alexa Fluor 633	720-900	647
AMCA	780-800	444
bis-MSB	680-750	420
Bodipy	900-950	512
Calcium Crimson	900	615
Calcium green	780-850	531
Cascade Blue	750-800	420
Coumarin 307	780-800	530
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
Dansyl Hydrazine	700-750	440
DAPI, Hoechst	700-820	455, 478
DiA	800-860	580
DID	780-820	670
DiO	780-830	510
eCFP	800-900	476
eGFP	820-950	509
eYFP	860-950	532
Fluorescein	780 - 820	519
Indo-1 free	690-720	490
Indo-1 Ca2+	690-720	400
Lucifer Yellow	860-890	533
Mito Tracker red	750-840	600
Nile Red	810	640
Oregon Green	780-860	526
Propidium Iodide (PI)	820-850	617
Rhodamin B	800-840	600
Rhodamine 123	780-860	550
Sytox Green	740-760 or 880-940	524
TRITC	800-840	572

Shutting down the microscope

1. **Clean** after yourself and **swing out** the stage arm from the way of the objective
2. In LAS X software **save** everything, **close** all laser shutters and **turn OFF all lasers**



To turn OFF the lasers, **use ONLY the ON-OFF sliders** in the **Laser Overview window** (in the **Acquire tab**) or in the **Laser Config window** (in the **Configuration tab**).
For the multiphoton laser, **DON'T use "laser shutdown", "full shutdown", "complete shutdown"** and similar buttons, that would turn OFF the laser completely and the next user wouldn't be able to use the system. For the multiphoton laser, only the emission is needed to be turned OFF, and that's done by the ON-OFF sliders shown above.

After all the sliders turned OFF, verify that the emission for the multiphoton laser is OFF by:

- Checking that the indicator lamp is not glowing on the top of the blue laser box (on the back side of the microscope table)
- Checking the text on the laser chiller box (between the microscope table and the window). The emission is OFF if:
  - The text says "THERMAL RECOVERY", and the power is 0.0W
  - The text says "READY" and the power is 0.0W

3. **Close** down the LAS X software
4. **Shut down** the computer, **wait** till fully OFF

----- After Computer is OFF, the following steps are not strict, the order can be changed -----

5. **Turn OFF** the confocal box behind the PC desk in the following order:
   a. Laser Emission key
   b. Laser power
   c. Scanner power
6. **Turn OFF** the microscope stand controller, white box next to the curtain
7. **Turn OFF** the TFT screen with joystick on the PC desk
8. **Turn OFF** the fluorescence lamp – **if turned OFF, can't be turned ON for the next 30 min**

➢ The chiller for the Ti:Sapphire laser AND for the NDD detectors stay ON

During vacation, BioVis might turn OFF the chillers for:
1. Ti:Sapphire laser (Full shutdown through LAS X software)
2. NDD detector