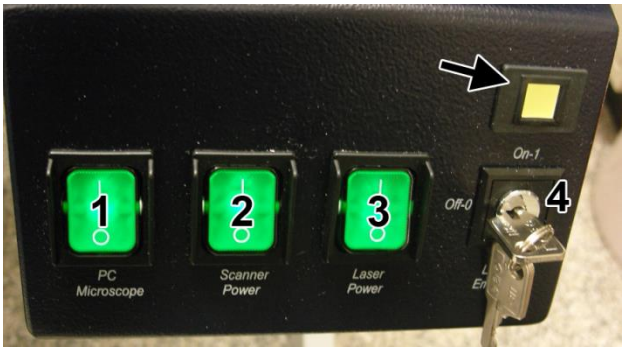
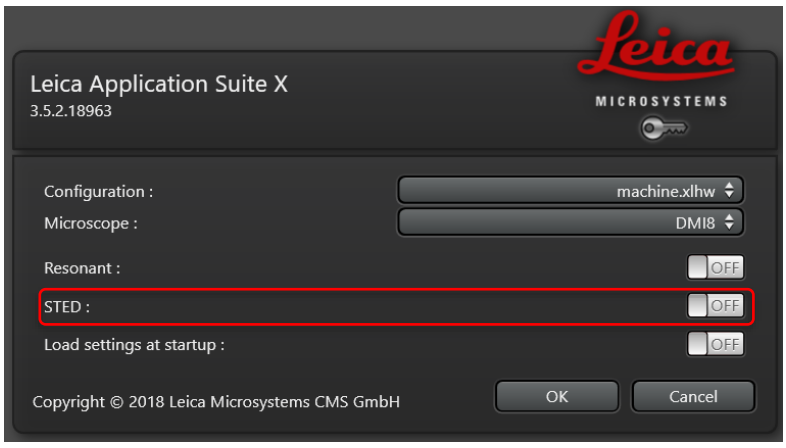
Leica SP8 STED 3x WLL Confocal Laser Scanning Microscopy User Guide

1. Switch on the **PC Micrscopy (1) wait 10 sec → Scanner Power (2) wait 10 sec → Laser Power (3) wait 10 sec**. Then turn the **Laser Emission key (4)** on; the yellow light will come on (arrow).
2. Turn on the **Leica Arc lamp power supply (5).**
3. **For STED Super resolution imaging, turn the depletion lasers; turn the keys for 592nm and 640nm lasers > The SHG Ready LED light should be on green. Turn the key for 775 nm.**



4. Log on to LAS-X user account and start the **LAS X** program.

5. Select "**machine.xlhw**" for Configuration and "**DMI8**" for Microscope. **For STED super resolution**, click **STED ON**. Click OK. (*Option: if you will use resonant scanner, click Resonant to ON*).



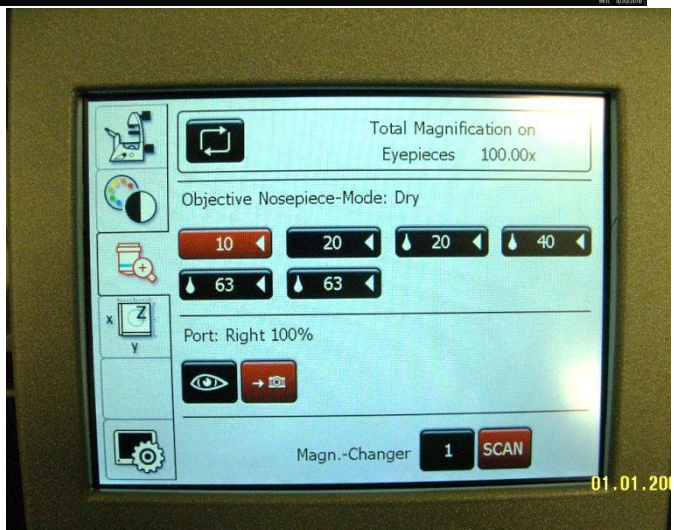
6. When another message regarding initializing the xy stage, click No.

7. Once initialization finishes, the image acquisition interface will open.

<u>Operating the DMi8 microscope.</u>
The SP8 STED DMi8 has the following objectives;
10x HC PLAPO 0.40 NA, WD 2.2 mm
20x HC PLAPO 0.75 NA, WD 0.62 mm
63x HC PLAPO, 1.40 NA, Oil immersion, WD 0.14 mm
100x HC PLAPO, 1.4 NA, Oil STED White, WD 0.13 mm

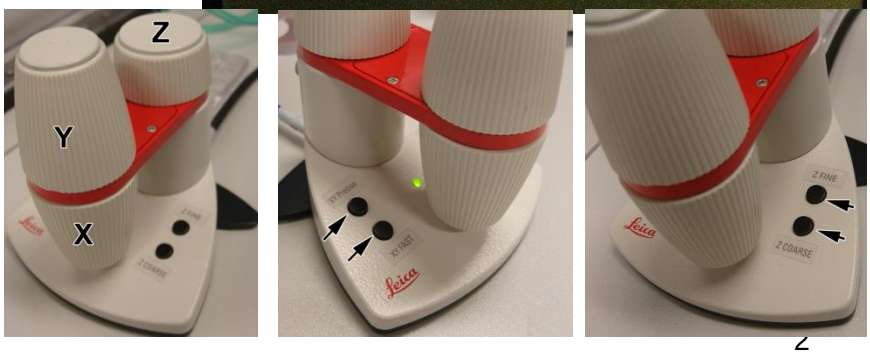
Objectives can be selected on the touchscreen panel.
Upon selecting objectives that don't need immersion medium, i.e. dry, it will turn immediately to the selected objective.

However, if you select an objective that requires an immersion medium, the button will flash for confirmation. Touching once more will take effect.



The XY stage movement and the Z focusing are controlled by a remote controller on the desk.

The speed of XY stage movement can be selected by **XY Precise** or **XY Fast** buttons on the left bottom.



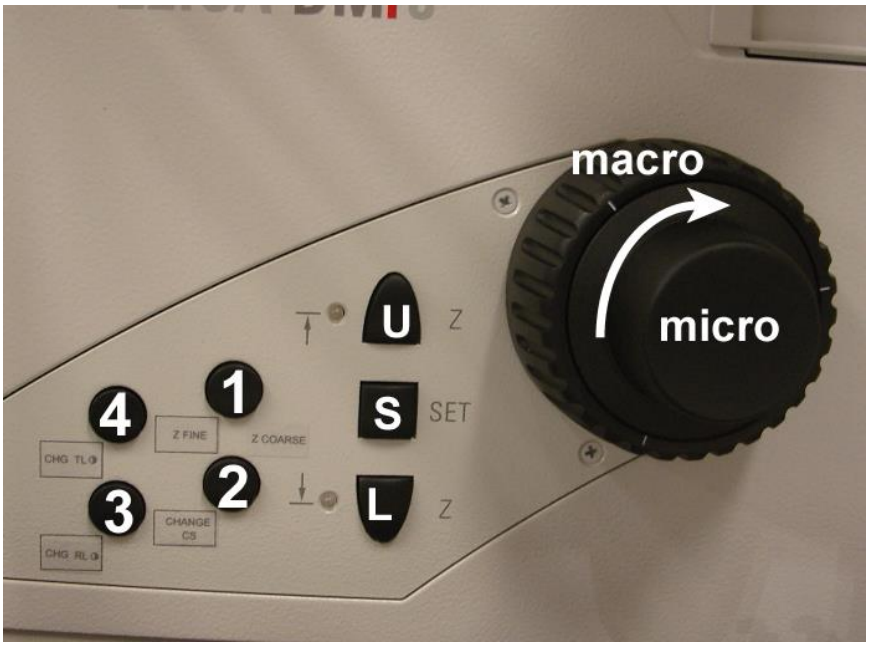
For Z focusing, turning clockwise will objective closer to the sample. You can toggle between Coarse and Fine focus movement by pushing the buttons on the right bottom.

On the left side of the microscope, you can control focus by turning macro and micro focus knob (arrow direction moves the objective upward).

1) Shutter button – turns on/off the light to the eyepieces.
2) Intensity control knob – controls the intensity of either fluorescence or brightfield light to the eyepieces
3) This button toggles between brightfield (TL) and Fluorescence mode.

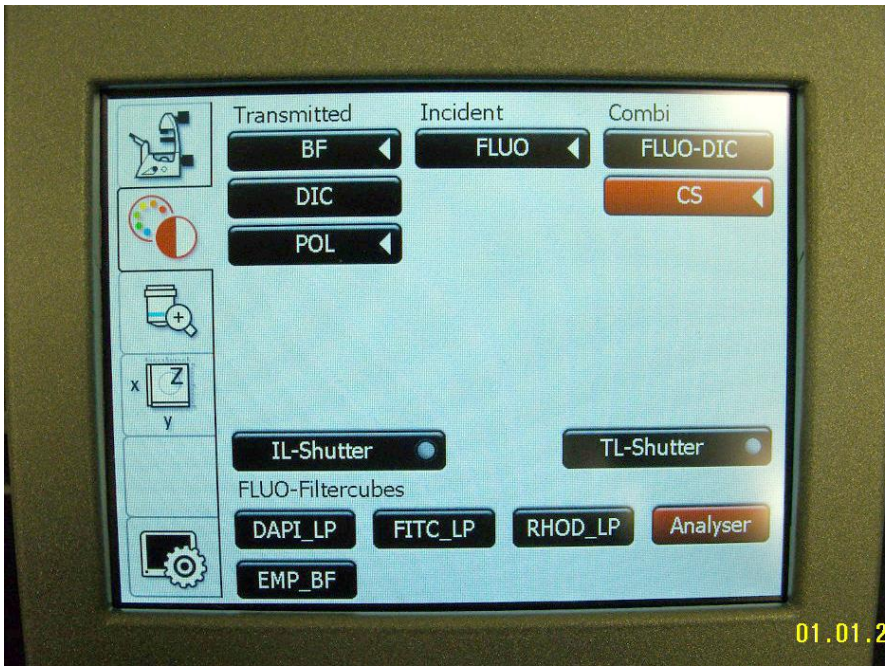
On the right side of the microscope, you also control the focus with the focus knob.

1) **Z Fine/Coarse toggle** button for focusing knob.
2) **CS** (confocal scanning) button – direct the light path to the confocal laser scanner for imaging.
3) **CHG RL** button – switch to fluorescence viewing mode.
4) **CHG TL** button – switch to brightfield (TL) viewing mode.

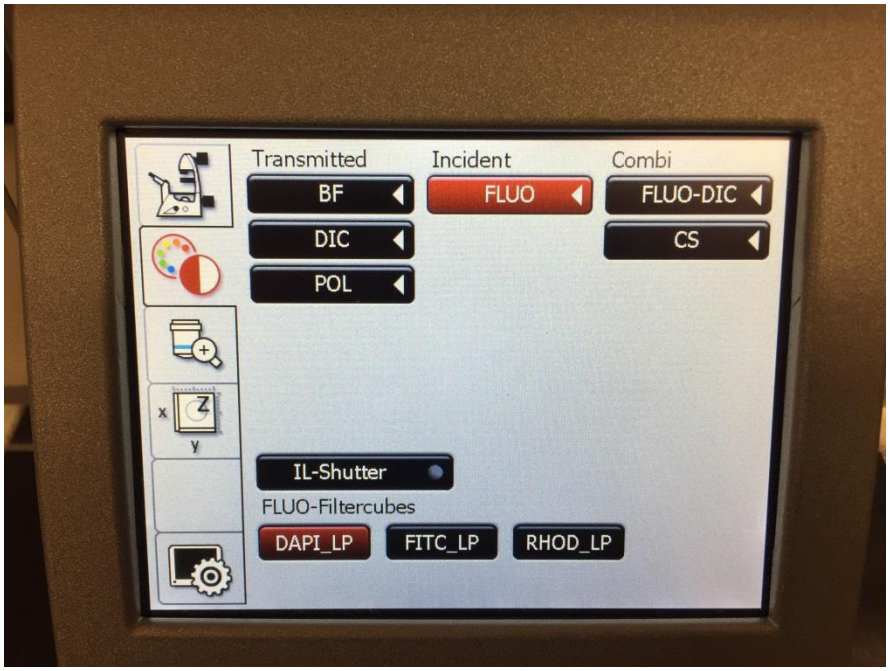


U,S, and L buttons allows to set the upper and lower limit of the objective movement.
Push **T** and **S** together to set the upper limit (the top green light will turn on). Push **T** and **S** again to clear the limit (the light will be off).
Do the same for the lower limit by pressing **L** and **S** buttons together.

This panel will be used most often to change the viewing mode, i.e. viewing with the eyepiece in brightfield or fluorescence or change to confocal scanning for image acquisition.
For viewing with brightfield, touch BF.

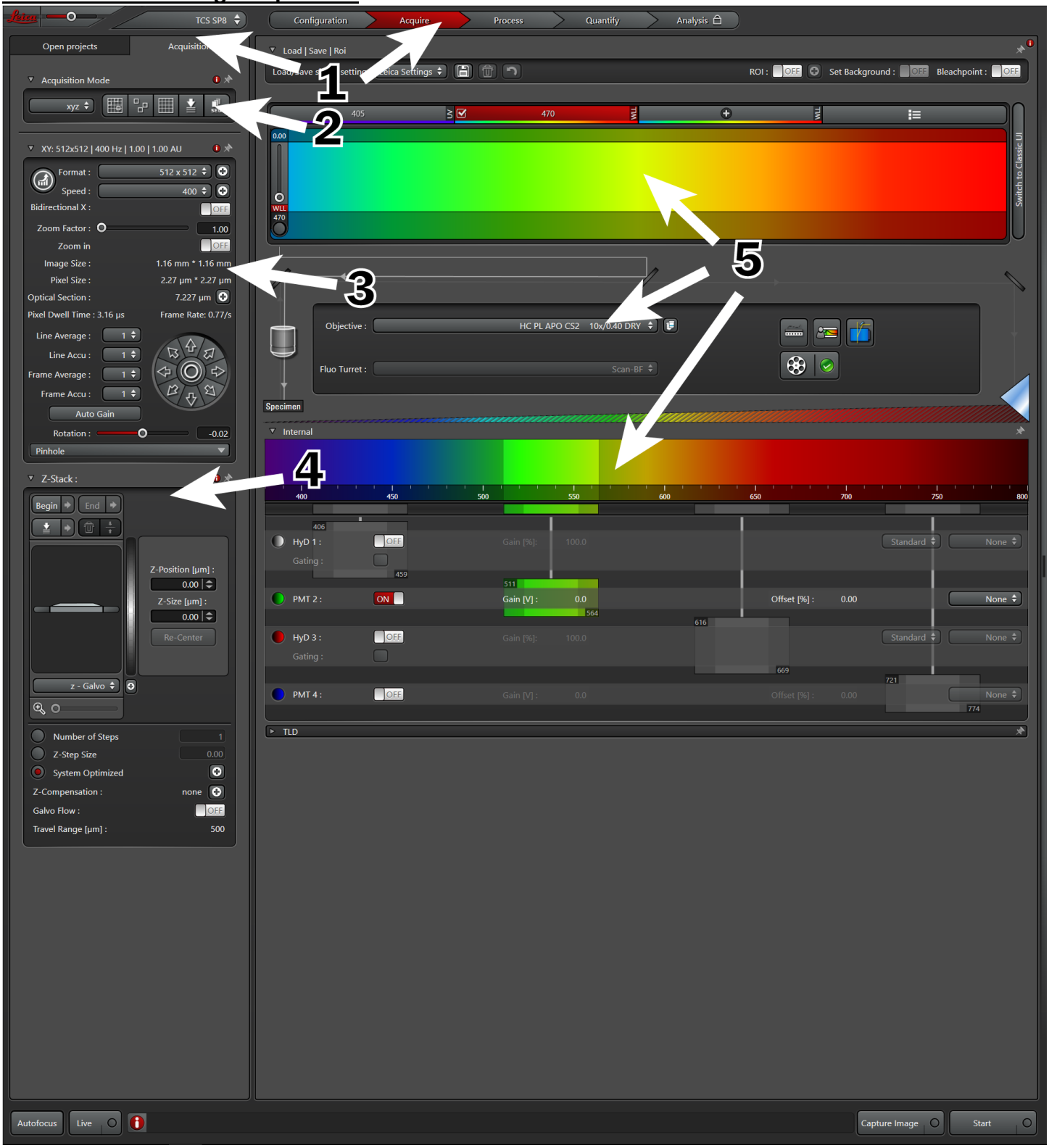


For viewing with fluorescence light, touch FLUO, and IL-Shutter to open the shutter for fluorescence illumination. You can view the sample with eyepieces between DAPI_LP (blue), FITC_LP (green), and RHOD_LP (red) filters.

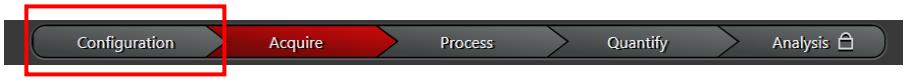


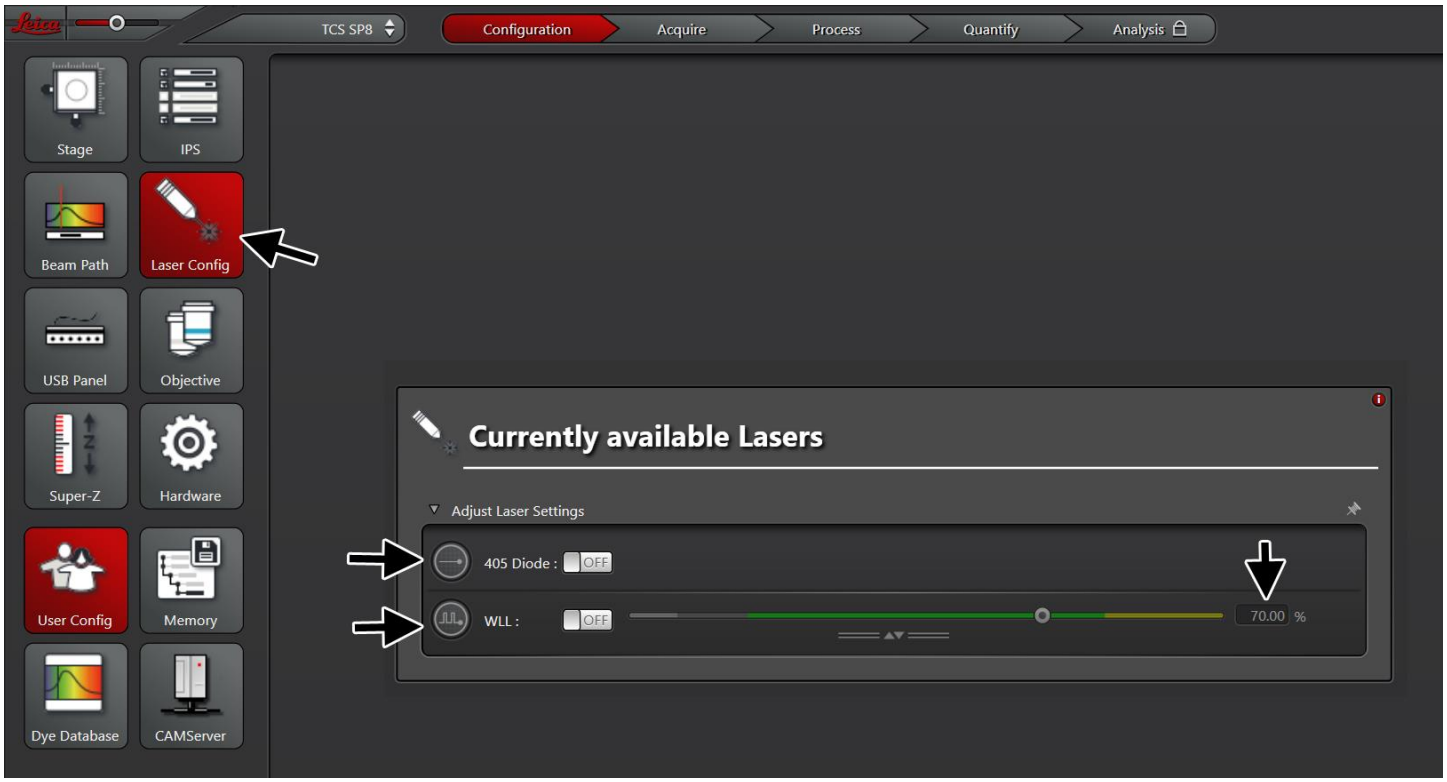
When you find the region of interest for imaging, touch CS to change the lightpath to the confocal scanning.

Basic multicolor Image acquisition

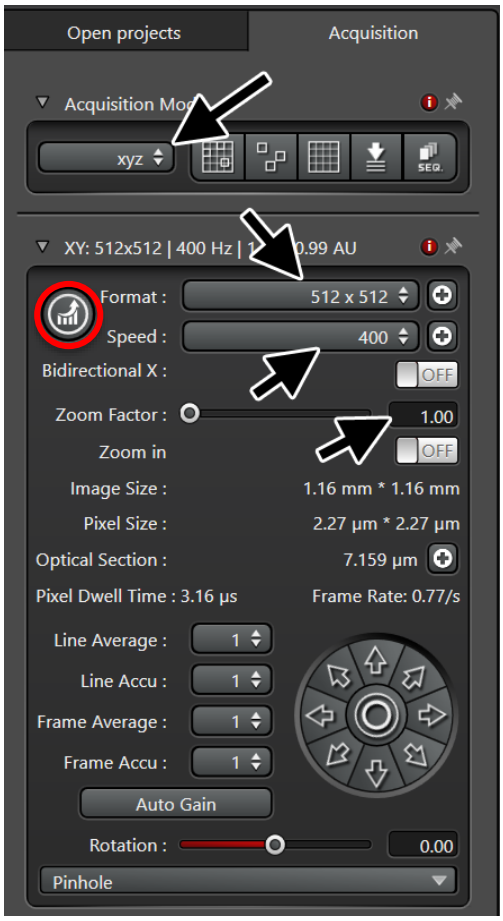


The default starting tab upon starting is **(1) Acquire** tab, where you set the acquisition parameters, such as **(2)** imaging mode (i.e. xyz, xyt, multipoint, sequential), **(3)** image format (size, speed, optical zoom, line/frame averaging, pinhole), **(4)** z stack parameters, and **(5)** selection and control of lasers and detectors.
Before you set up an imaging condition, click **Configuration** tab.





On the **Configuration** window, click **Laser Config** button. On **the Currently available Lasers panel**, turn on **WLL** (white light laser) and **405 Diode** (if your sample has DAPI or 405 nm excitation dye). **Keep the WLL laser power at 70% (default).**



Go back to **Acquire** tab. Select **xyz** as **Acquisition Mode** (you can choose any mode from the drop-down list).

Format: the default Image size is 512x512 pixel. They can be changed from the drop-down menu. 512x512 (default) format is a good image size with a good acquisition speed. 1024x1024 increases resolution but will take longer to acquire an image.

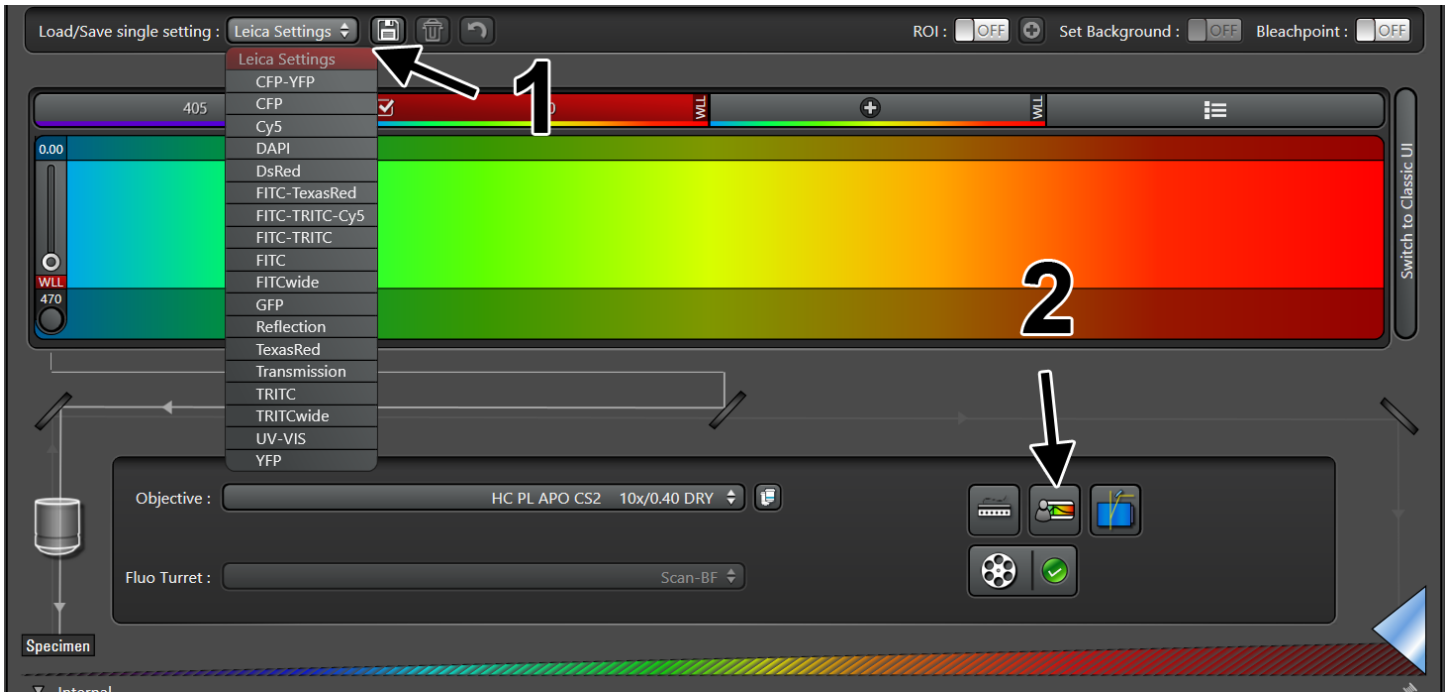
Optimize XY Format (red circle): The image format size is automatically adjusted according to the zoom factor to set the pixel size in the correct relation to the optimum resolution (Nyquist sampling).

Speed: the default scanning speed is 400 Hz. You can go faster with 600 or 700 Hz, minimizing bleaching the samples. However the image quality will drop.

Zoom Factor: 1 is the original magnification of the current objective *(note: some faster scan speeds automatically increase the zoom).* Increasing zoom factor will multiply the magnification of the objective (i.e. 20x objective with 2x zoom factor will be like using 40x objective).

Line average: This may remove some background noise by averaging pixel values from multiple (determined from the drop-down menu) line scans. **Frame average**: this does the same effect as line average but it does by multiple whole frame scans.

Line (Frame) accumulation: This will increase the intensity by adding multiple scans.
For multicolor imaging, you can choose from the default **Leica Settings** (1) or you can define with **Dye Assistant** (2).



Click the **Dye Assistant**  button. On the pop-up panel, select dye and detector type one by one (Arrows).

Click  to choose the dye from the drop-down menu.

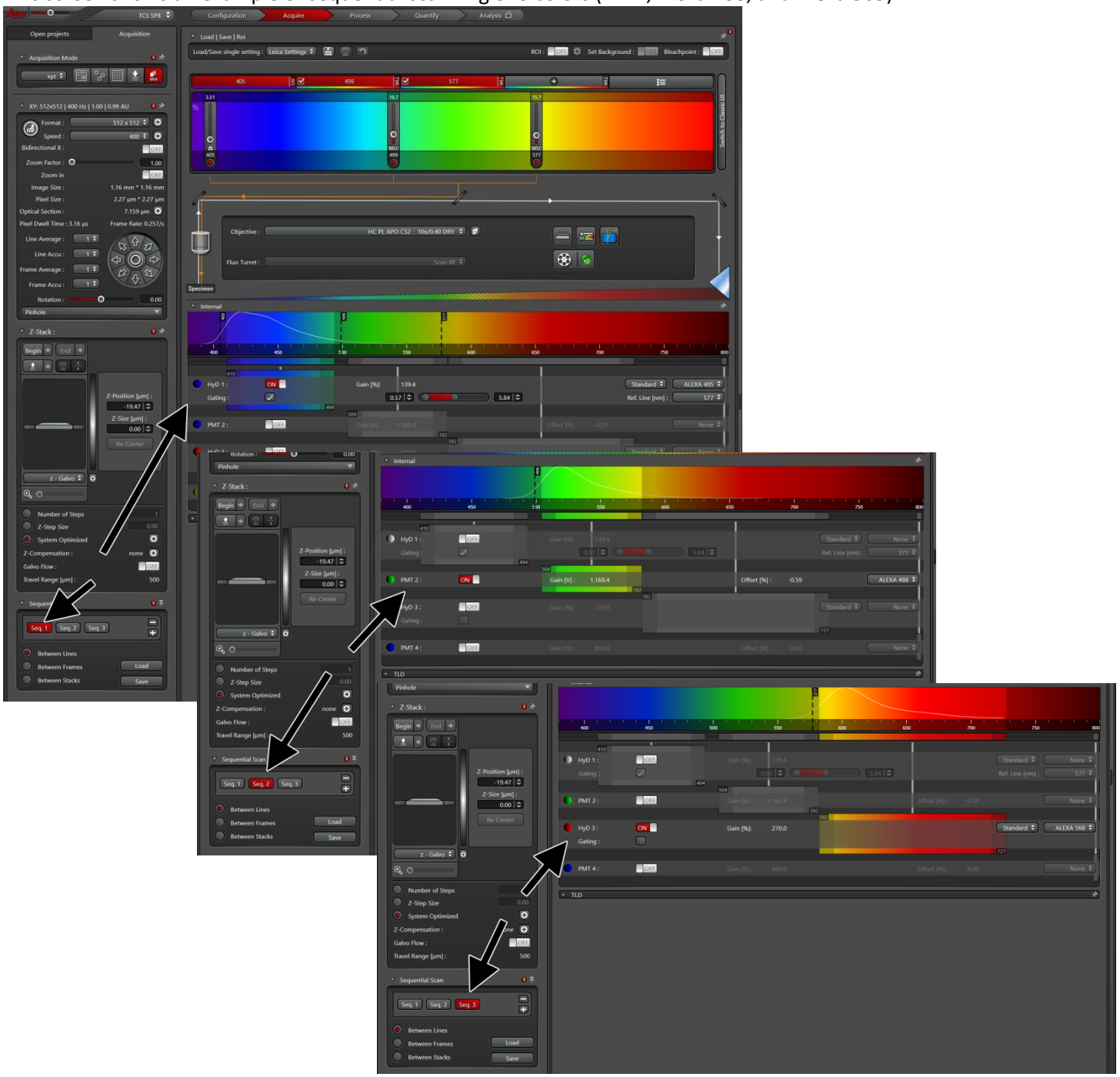
Click  to choose the detector type (If you are not sure about what type of detector you will use, select **PMT or HyD**.)
*****Use PMT when imaging reflection of laser (reflection image mode)*****

Once you select more than two dyes, it will show imaging options, such as “None sequential”, “Line sequential. 2 sequences” etc.

For the fluorophores close in emission wavelength (such as GFP and RFP), choose either “Line sequential” or “Frame or stack sequential”

For the fluorophores well separated in emission (such as Alexa 488 and Cy5), choose “None sequential”.

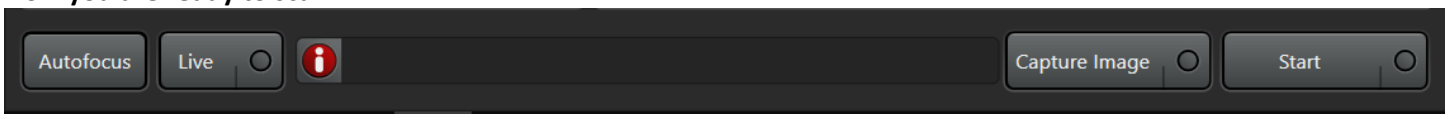
This screen shows an example of sequential scanning of 3 colors (DAPI, Alexa 488, and Alexa 565).



You need to increase laser power by sliding the laser power bars or by double click to type the number.

You can shut off the specific laser lines by clicking the red dot (it will be gray out). Keep the laser power around 5~10%.

Now you are ready to scan.



Click 

button to start scanning with lasers. You can see the image of continuously scanned sample in 3 colors. The button will change into 

. Click Stop 

button to stop scanning.

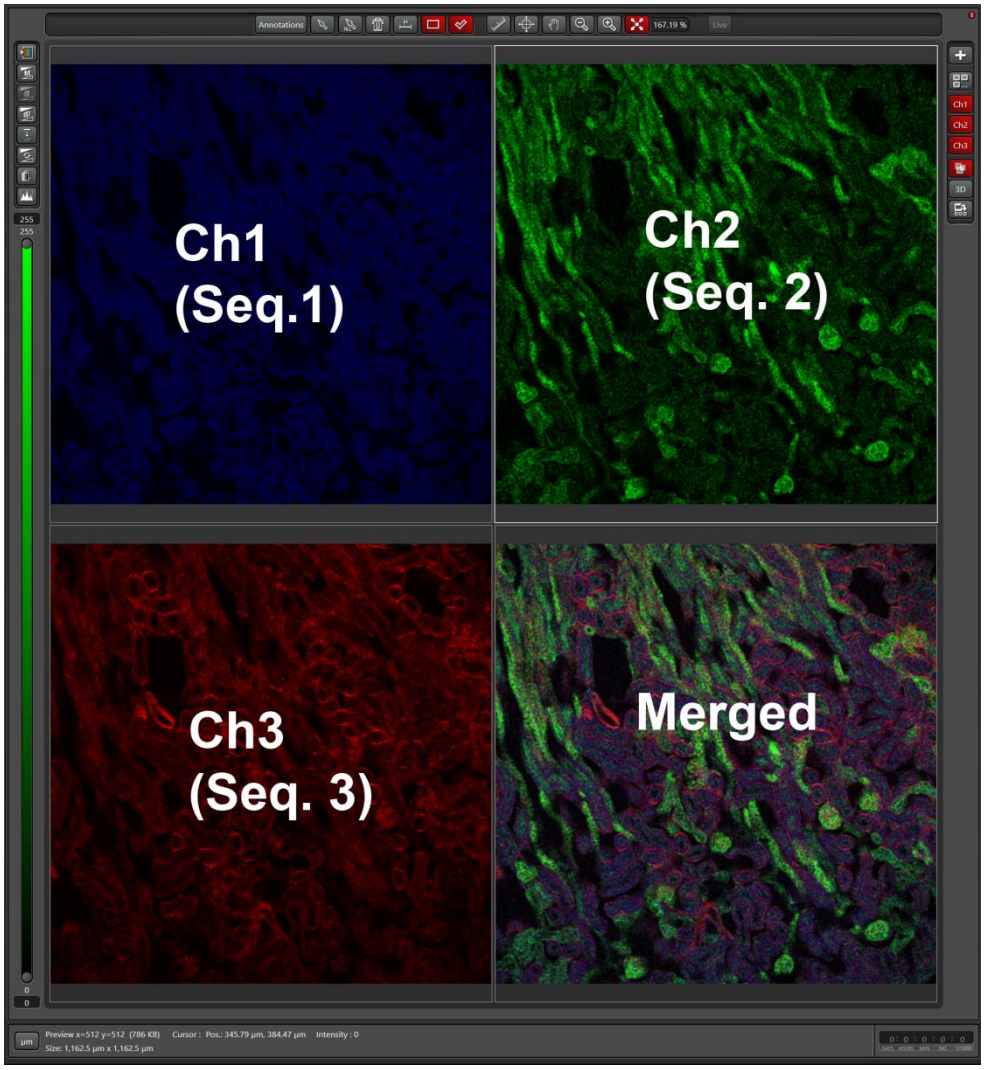
You can change how they look on the live window with some buttons on the window.

With the left side button, you can control the image color modes, histogram.

By clicking Quick LUT  button, you can change the display in color, LUT, or gray scale. In LUT mode, you can visualize whether the pixels have saturation (showing in blue) or complete dark (showing in green).

This button  will change the view between multichannel and single panel.

This  merge button create a view or panel of merged channels.



By clicking Ch1, Ch2, Ch3 buttons, you can turn on off the display of the channel.

This  will create another viewing area for other images you acquired, for instance, to compare the images.

Some parameters of the image such as brightness, contrast, and zooming, can be adjusted quickly with the knobs on the desk.

Smart Gain: controls voltage of the PMT, thereby the brightness of the image. The higher the number, the brighter the image. Recommended PMT gain between 600 and 800 and HyD gain between 50 and 100.

Smart Offset: this controls the contrast by changing mostly the background of the image.

Scan Field Rotat: it rotation the scanning direction, which rotate the view of scanned image without actual rotation of the sample.

Pinhole: The default is 1.00AU. Keep this number at 1.00, unless you need dimmer/thinner section (less than 1.00) or need brighter and thicker section (larger than 1.00).

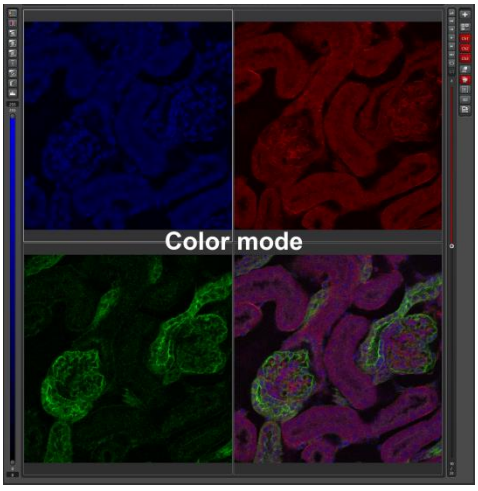
Zoom: it allow you to zoom up the image by scanning smaller area with the same pixel numbers, thereby increasing the resolution. Keep the zoom factor less than 4.

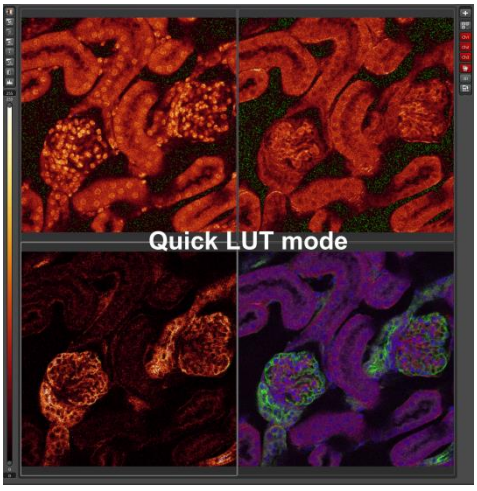
Z Position: This knob changes the focus for focusing and setting z stack series. Depending on the selection (arrow) on the **Z stack panel**, it will move the stage top holder fast (**z-Galvo**) or move the objective (**z-Wide**). For z series imaging of typical cell/tissue section samples, **z-Galvo (default)** is recommended.

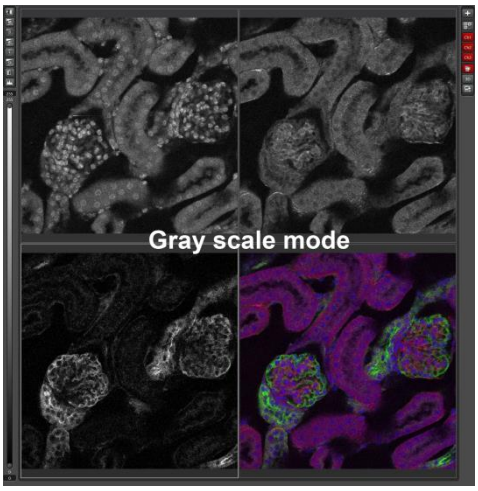
Adjusting image acquisition condition.

While scanning, you need to adjust acquisition setting to get as good images as possible. Mostly it requires adjustment of laser powers, detector sensitivity, background reduction, and spectral window for each fluorophore emission.

Using the Quick LUT  is helpful to set appropriate imaging parameters. In the Quick LUT mode, the saturated pixels appear in blue and the black background pixels appear in green. **Try to get the few saturated pixels with black background by changing the laser power, the Smart Gain, and the Smart Offset.**





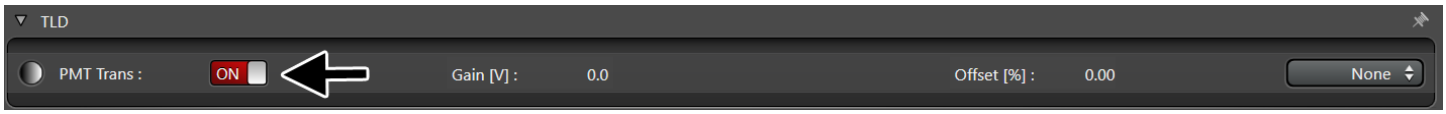


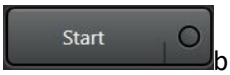
Click on the channel that you want to change, depending on the type of the detector, the **Smart Gain** and/**or Smart Offset** will be activated (HyD detector allow **only Smart Gain**, and **Smart Offset** will be disabled).

Increasing the **Smart Gain** number will increase brightness of the channel. Decreasing the **Smart Offset** number will reduce the background without affecting the brightness of the specimen. If necessary, increase the laser power.

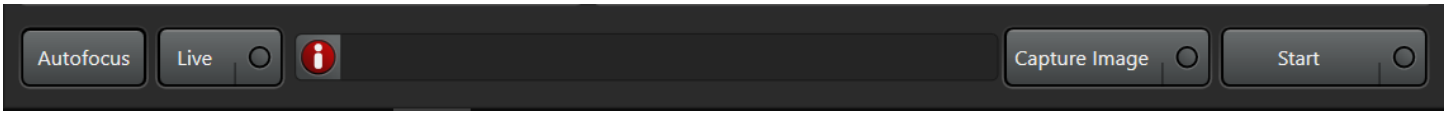
For **HyD** detector, use **Smart Gain** to increase the brightness. If you sample shows high background, you can't use **Smart Offset** to decrease background because the **Smart Offset** is disabled. Instead, try to use **Gating** and keep the lower number **to 0.30 (default)**. Gating can exclude background or glass reflection by excitation laser.

If you need to add the bright field image (non-confocal) to the acquisition, click TLD to activate transmission light detector.



Once you are satisfied with the imaging condition, stop scan. Then, click 

button to acquire images.

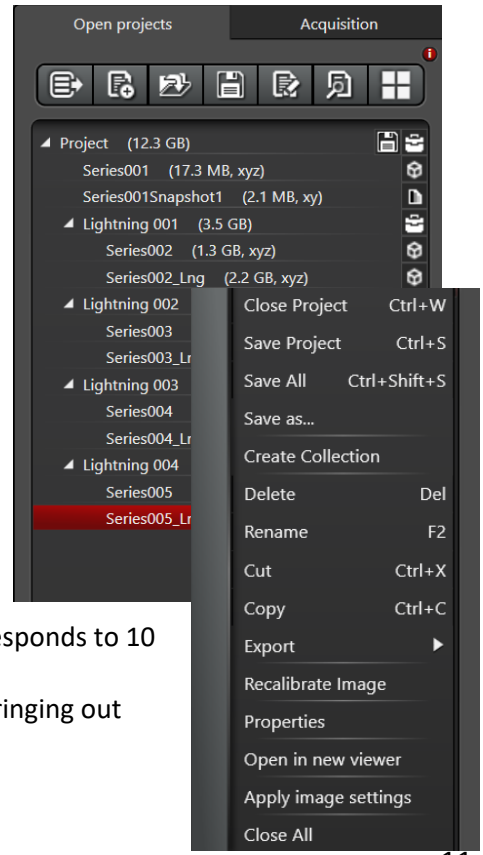


When the image acquisition is done, the image will be temporarily saved as Project in the **Open projects** tab.

If you want to change the default name (image, series, etc) of the images, right-click on the mouse to open the context menu.

Click  button to save the project that includes all the images you obtained. Choose Data (:E) drive, open your lab folder, and create your own folder to save the project. You can change the name of the project.

The project will be saved as **.lif file, which can opened in Image J or Fiji.



<u>*HyD detector has 3 mode</u>

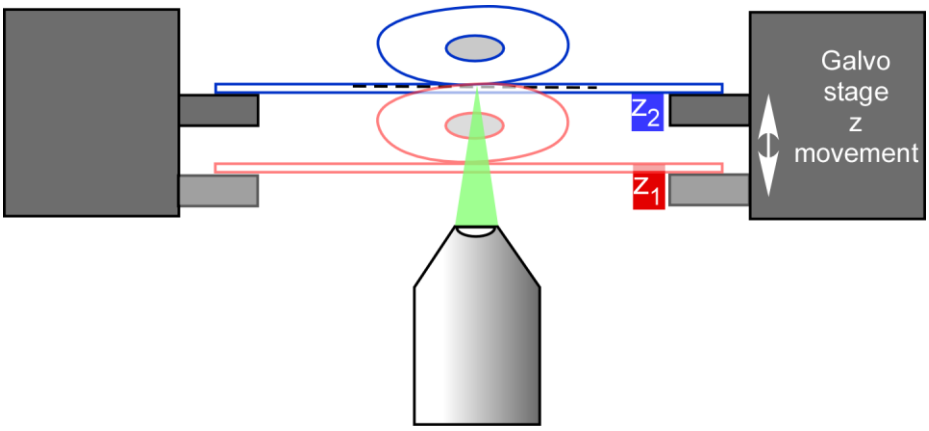
1. Standard - for regular image acquisition.
2. Counting – for ratio imaging and image correlation. Pixels function as a basket. The higher bit depth, the larger the basket. A 10 gray-scale value corresponds to 10 photons.
3. BrightR – for display very bright and weak fluorescent areas efficiently by bringing out the weak signal.

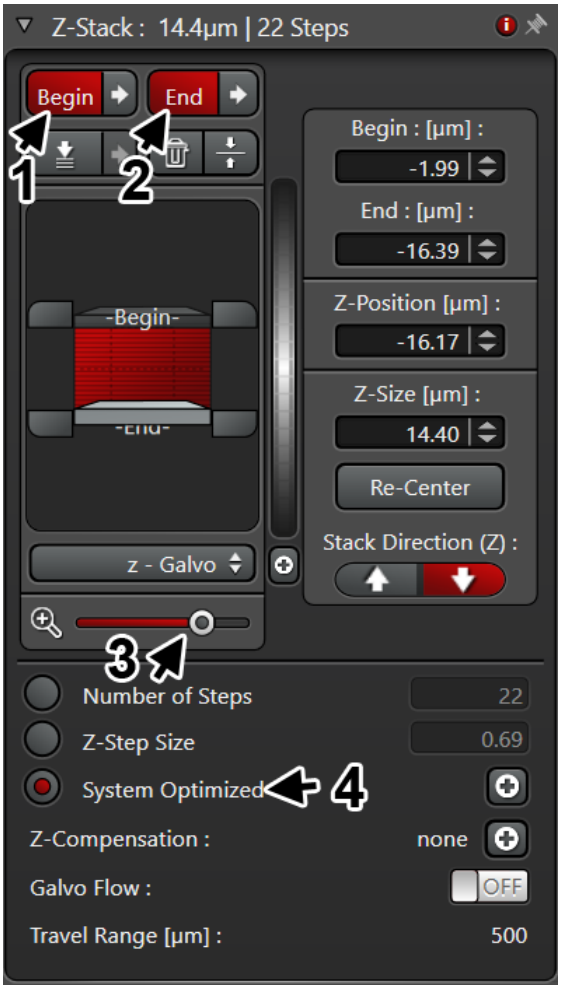
Z stack image acquisition.

- For imaging z stack from top to bottom of your samples (cells or tissues), you need to set the range for these focal plane as Begin and End. You can set either the top or the bottom of the sample as either Begin or End.

- Turn **Z Position knob (6)** clockwise, which lowers the Galvo stage (Z1), to focus the top of the sample (deeper focus). Click **Begin (1)** button.

- Turn Z position knob counterwise, which raises the stage (Z2), to focus the bottom of the sample. Click **End (2)** button.





- You can visualize the range better by slide the **magnifiation slider (3).**

- **Stop scanning.** The program will calculate **the Number of steps** and the **Z-step size**, based on **System Optimized (4)(default). This is recommended for optimal 3D rendering and quantification.**
  If you want different z-step size, click on Z-step size button, and enter the number, which is in µm scale. The Number of Steps will change accordingly.

- If you notice signal loss in deeper z, try to use **Z-compensation**.

- If all the settings are good, click 

button to acquire z-stacks. When it finishes, the z-stacks will be stored as **Series*** ** under project.

Visualization of z stack images

The individual slice image of a Z stack can be viewed by **play buttons (1).**

The **Max button (2)** will project (combine) all the slices of the stack into a 2D image.

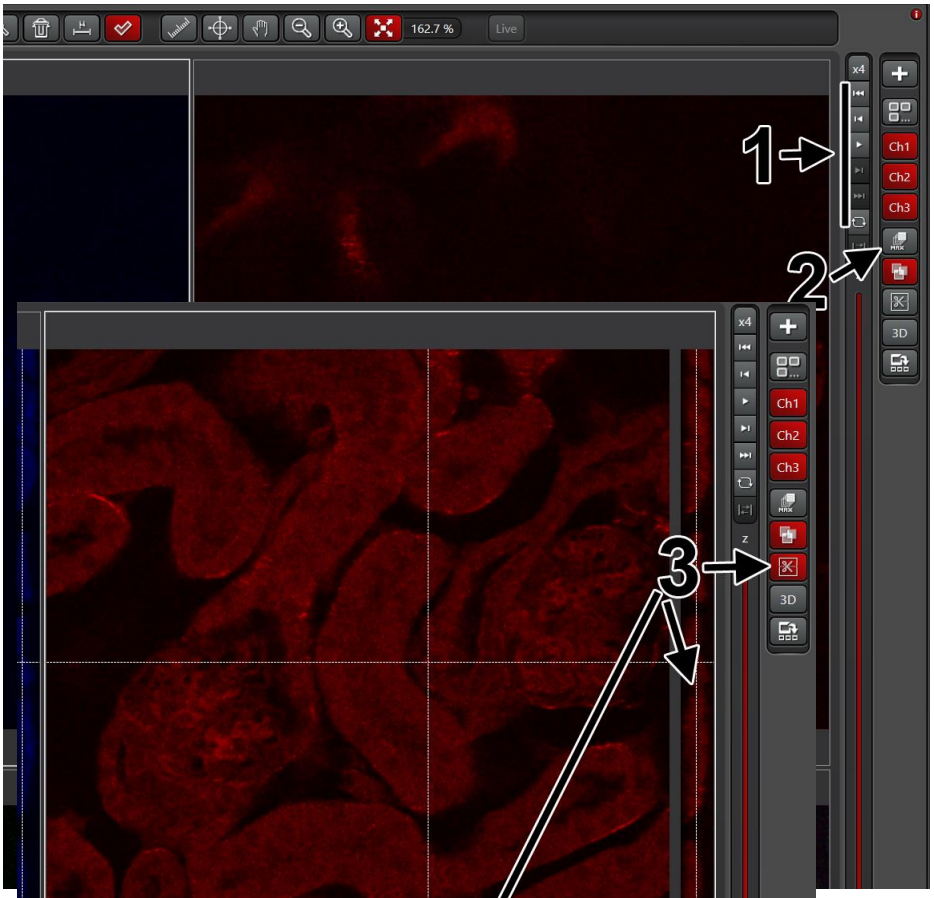
The cross sectional view along the x and y axes can be visualized by **Orthogonal sectioning view button (3).**

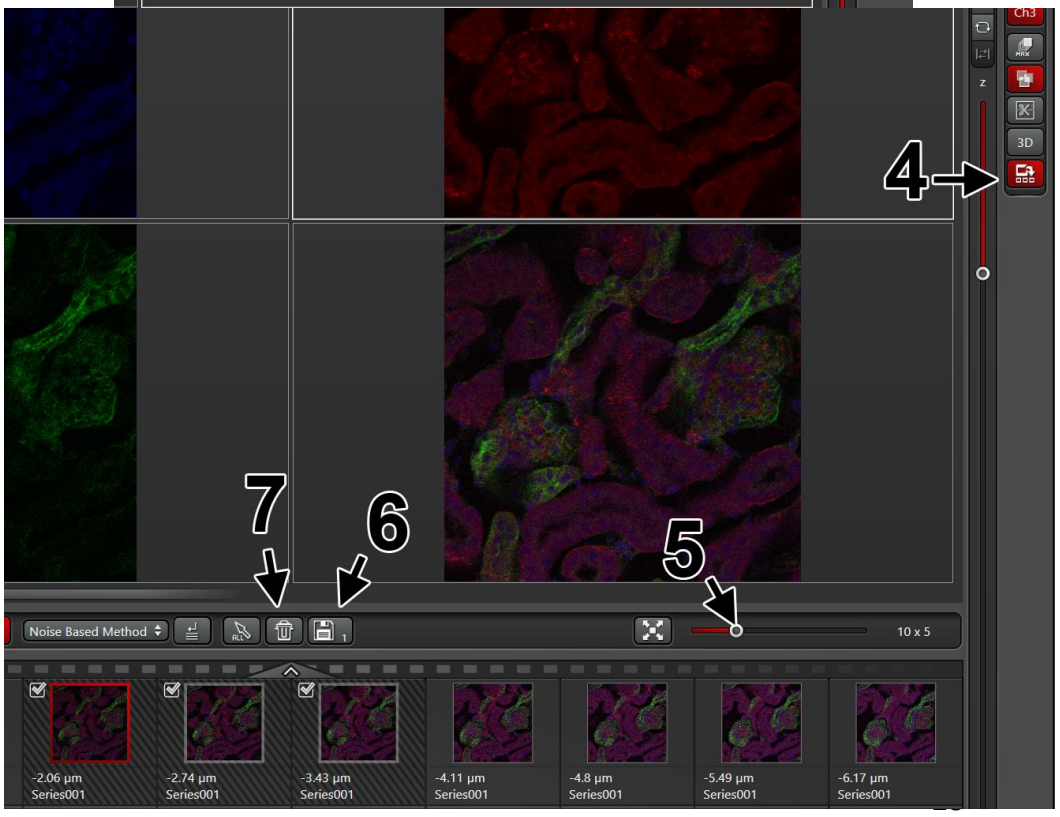
All the individual slices can be visualized as thumbnail views by **Show Gallery button (4).**

The size of the thumbnail images can be changed **(5).**

You can select specific slices by checking the checkbox and save them separately **(6).**

You can deselect the current selections **(7)**

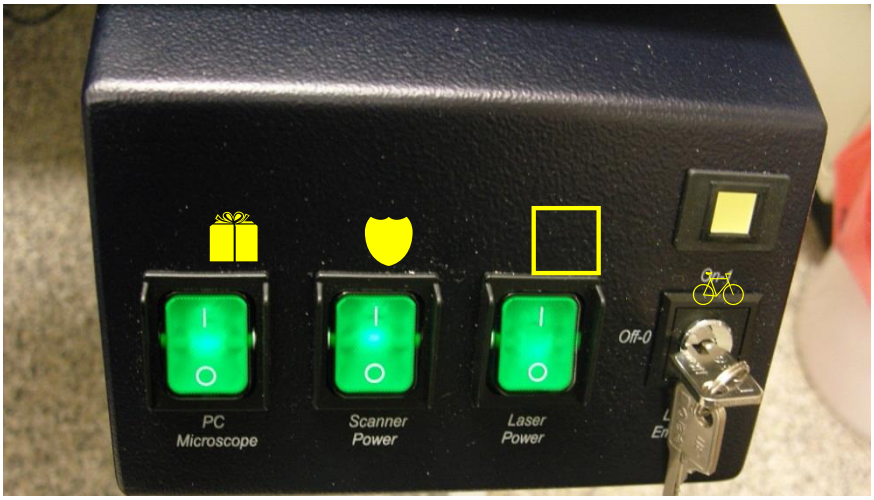




Shutting down the system

1. Make sure you saved all the image data before you exit the program.
2. Exit the program and shut down the Windows.
3. (If you used the incubation chamber, turn off the incubation systems (the Cube and the Brick) and close the gas tanks.)
4. Turn off the Xenon arc lamp.

5. Turn the laser key off 🚲.
6. Turn the switches in order of □ ♥ 🎁.



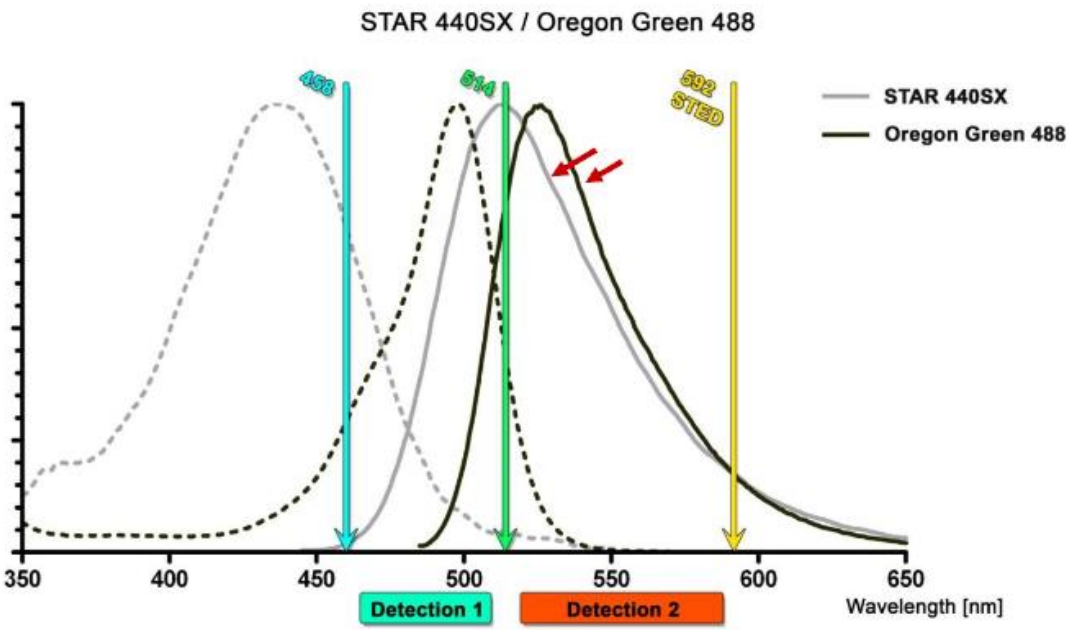
STED Super-resolution Imaging

Things to consider when using STED method.

- Excitation of fluorophores should be between 470 and 560 nm
- Fluorophores should not be excited by STED lasers (i.e. 592nm, 660nm and 775 nm)(no excitation at depletion wavelength)
- Fluorophore's emission should some overlap with STED laser wavelength.
- Dyes should have high photo stability.
- Dye pairs for 2C STED should have little cross excitation (combination of dyes with large and normal Stokes shift) and have good STED efficiency at the same wavelength.

Some working green dyes for STED

Dye	Excitation Laser line	Vendor	Remark
Standard dyes			
Alexa 488	488 or 514	Invitrogen	
Atto 488	488 or 514	ATTO-TEC	
Chromeo 488	488 or 514	Active Motif	
Chromeo 505	488 or 514	Active Motif	Recommended for 2C
DyLight 488	488 or 514	Pierce Technology	
FITC	488 or 514	Invitrogen	
FluoProbes 488	488 or 514	Interchim	
Oregon Green 488	488 or 514	Invitrogen	Recommended for 2C
Oregon Green 514	514	Invitrogen	
Large Stokes Shift dyes			
Abberior STAR 440SX	458	Abberior	Recommended for 2C together with Chromeo 505 or Oregon Green 488
ATTO 425	458	ATTO-TEC	Dye separation required for 2C; resolution not optimal
BD Horizon V500	458	Becton Dickinson	Streptavidin; recommended for 2C with Chromeo 505 or Oregon Green 488
Cascade Yellow	458	Invitrogen	Resolution not optimal
NBD-X	458	Anaspec	No conjugated Ab available; dye separation required for 2C
Pacific Orange	458	Invitrogen	Dye separation required for 2C
Fluorescent Proteins			
Citrin	514		Recommended
eGFP	488		Results highly dependent on specimen
EmGFP (Emerald Green)	488		
eYFP	514		Recommended
Venus	514		Recommended



For efficient STED, the emission spectra of both dyes need to have significan emission at the STED wavelength.

Dye combination for 2 color STED

- **With single 592 nm depletion**
  - BD Horizon V500 and Oregon Green 488 (or Chromeo 505)
  - Abberior STAR 440SX and Oregon Green 488 (or Chromeo 505)
  - Pacific Orange and Alexa 488.

- **With single 660 nm depletion**
  - Alexa Fluor 532 and TMR or Alexa Fluor 568
  - Oregon Green 488 and TMR or Alexa Fluor 568

- **With two 592 and 770 nm depletion**
  - Chromeo 494 and ATTO 647N or Abberior STAR 635 (best choice, no dye separation needed.)
  - Alexa 532 and ATTO 647N or Abberior STAR 635 (no dye separation needed)
  - Mega 520 and ATTO 655 or ATTO 665 (dye separation might not necessary)

STED Tips and Tricks

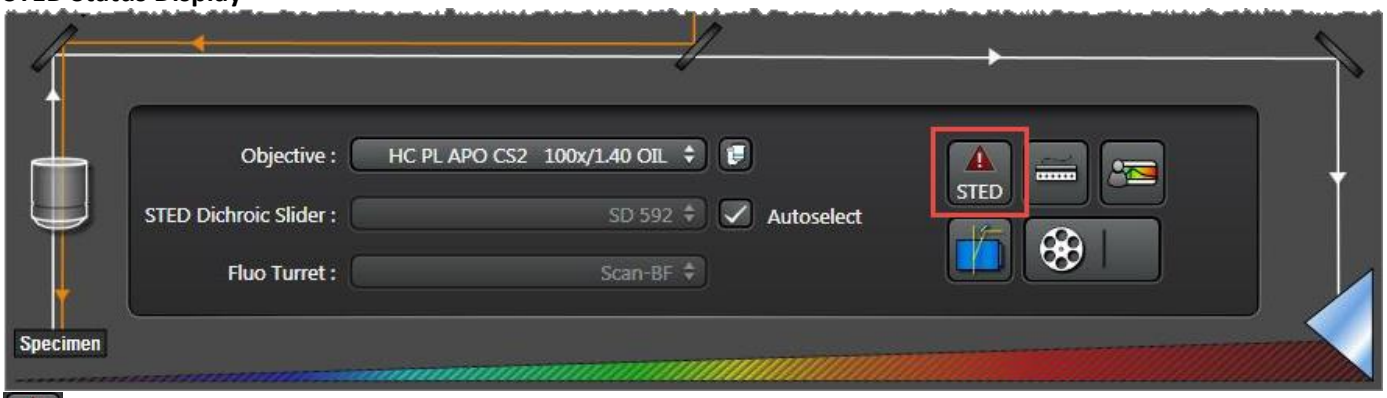
- **Sub-optimal excitation of dyes**: if background noise is problematic, consider not excitating the dye at peak wavelength. This may help to avoid cross-talk between dyes.
- **STED depletion laser power**: Although the resolving power increase as STED laser power increases, 100% laser power will bleach your fluorophores. Instead try using it at 30% power and see what you get. It may be good enough for your sample.
- **Gated STED**: use the HyD detector with gating. By gating out the initial signal, it can dramatically reduce background.
- **Frame accumulation**: This can be useful in STED when the fluorescence after STED depletion can be very low.
- **Deconvolution**: Once image obtained, it is recommended to perform deconvolution to improve the image resolution.
- **Mounting media**:
  - Prolong antifade kit (without DAPI) or Prolong Gold – allow at least 24 to 48 hrs to cure as the refractive index increases with curing time. The regions of interest need to be located within 30 μm from the coverslip.

- Thiodiethanol (TDE, Sigma #88559) – viscous water based medium for deep imaging of tissue sections. The TDE solution is used sequentially (50%,70% 15-30 min at each step), then to 97%+antifade as final mounting medium. The coverslip must be sealed with nail polish.
- Vectashield is **NOT** recommended.

STED Laser Configuration

- Pulsed STED (775 nm) laser
  - White Light Laser (Trigger master) + 775 nm STED (Trigger slave): the trigger master emits the pulses and the trigger slave is synchronized with the trigger master uisng a time delay.
  - Following the switch ON/OFF sequence of the lasers is important when working with these laser combination
    - Switching ON – First, swith ON the trigger master and then trigger slave. Otherwise the trigger slave will not be activated and the system will issue a report.
    - Switching OFF – First, switch OFF the trigger slave and then the trigger master.
- After finsihg a STED experiment, switch the lasers back OFF by clicking the respective ON/OFF button.
- "Align beams" function should be carried out after warming up the system (30~60 min after the system startup) the first time, and subsequently every 2~3 hours.

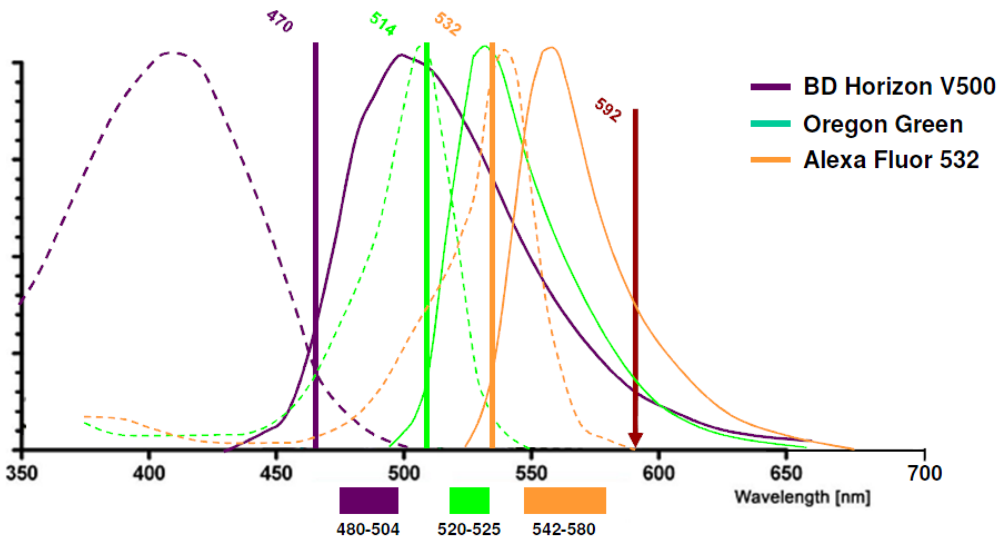
STED Status Display



This warning suggest that certain settings are not suitable for STED acquisition. By clicking the button, a dialog opens the specific error message.

All STED settings are correct.

Selected spectra for 3 color gated STED



Dual Color acquisition of gated STED

STED: 592nm, 660nm, 775 nm					
Dye 1			**Dye 2**		
Name	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**
BD Horizon V500	458/470	475-513	Oregon Green488/ Chromeo 505	514/520	523-580
Oregon Green 488	470	480-520	Alexa Fluor 532	545	555-580
Alexa Fluor 532	514	520-565	TMR/TRITC/ Alexa Fluor 568	580	590-650
Alexa Fluor 514/ Oregon Green 488	505	515-565	TMR/TRITC/ Alexa Fluor 568	580	590-650
ATTO 594/ Alexa Fluor 594	532/590	600-630	STAR635P	635/650	655-750
TMR/TRITC	532/550	560-630	Alexa Fluor 647	635/650	655-750

Triple Color Acquisition of gated STED

STED: 592nm, 660nm, 775 nm								
Dye 1			**Dye 2**			**Dye 3**		
Name	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**
STAR 440SX	470	475-505	Oregon Green488	510	515-530	Alexa Fluor 532	540	550-585
Oregon Green 488	470	475-525	Alexa Fluor 532	532	538-550	TMR/TRITC	580	590-650
Alexa Fluor 514	480	490-535	Alexa Fluor 546	540	545-580	Alexa Fluor 594	590	600-650
TRITC	550	560-590	ATTO 594	600	610-640	STAR 635P	660	665-750
Alexa Fluor 594	580	600-615	Alexa Fluor 633	620	625-655	Alexa Fluor 660	660	665-750
Alexa 488	488	500-545	TRITC/TMR	550	560-635	STAR 635P	640	750

- Secondary Antibody: use high concentration, i.e. 1/100.

- Do not use:
    - Other fluorescent proteins not excitable by any laser lines from the WLL laser (470nm~580)
    - DAPI (replace with TO-PRO 3, YOYO-3, PicoGreen)
    - QDOT or other fluorophores excited by the 405 nm laser.
    - Nailpolish sealants that can quench fluorescence or produce autofluorescence (i.e. color nailpolish)

- USE only #1.5 Coverslip and mounting medium without DAPI.