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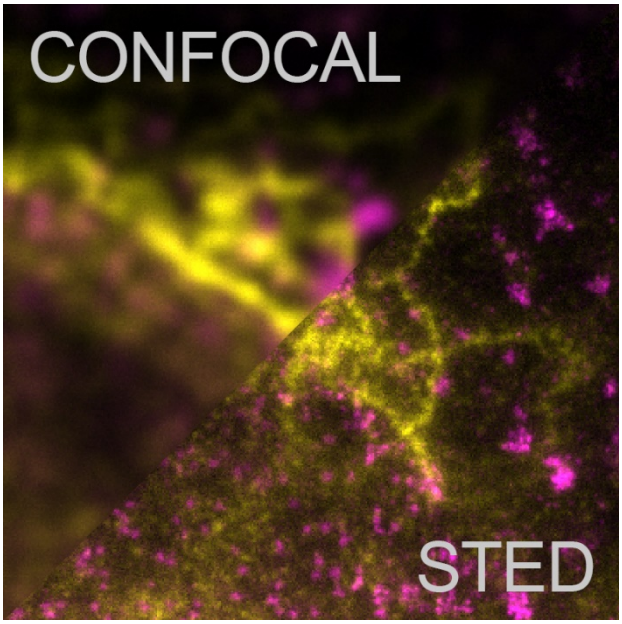
BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

STEDYCON

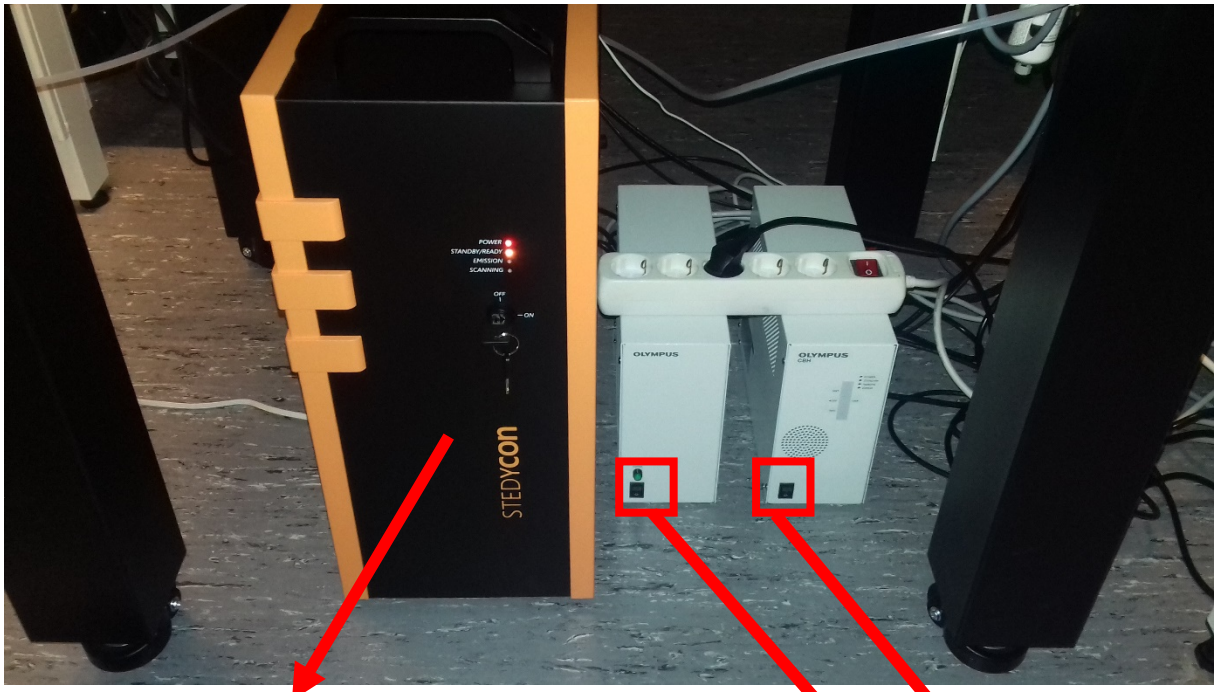
STED super resolution microscope

Manual/Quick guide



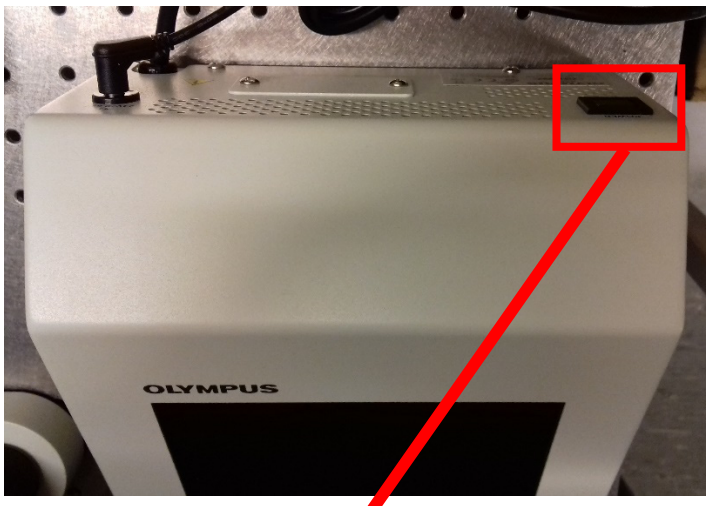
Matyas Molnar, Biovis 2020

Starting the microscope

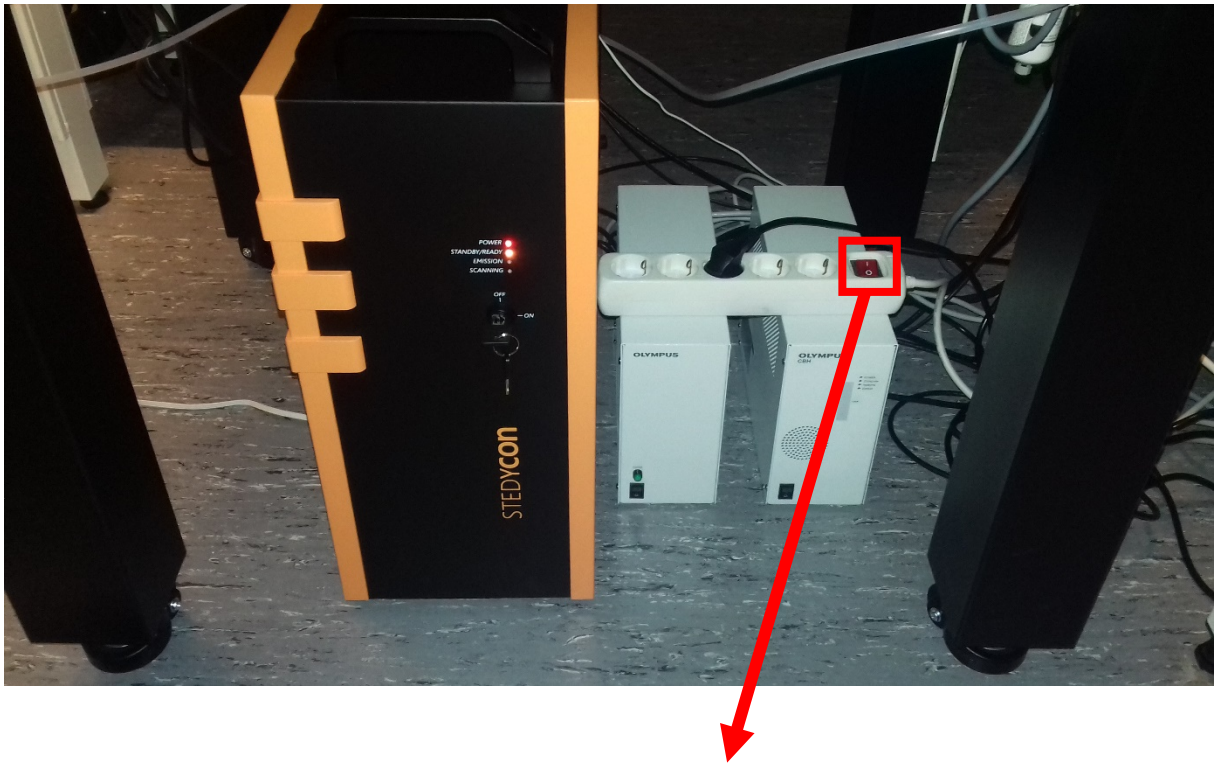


The STEDYCON box is always on standby. When yellow light is visible next to the ”Standby” text, follow the startup steps.

Turn ON the two **control boxes** for the Olympus microscope and wait 1 minute

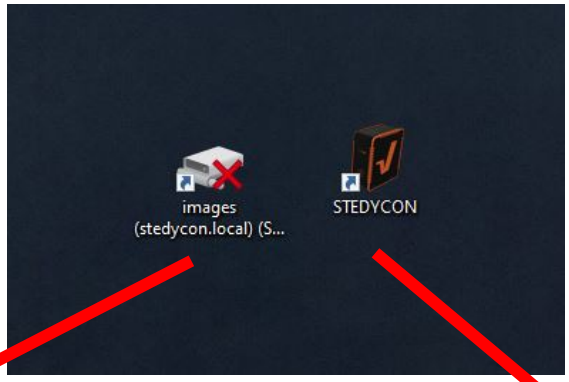


Turn ON the **TFT control** and wait till fully booted



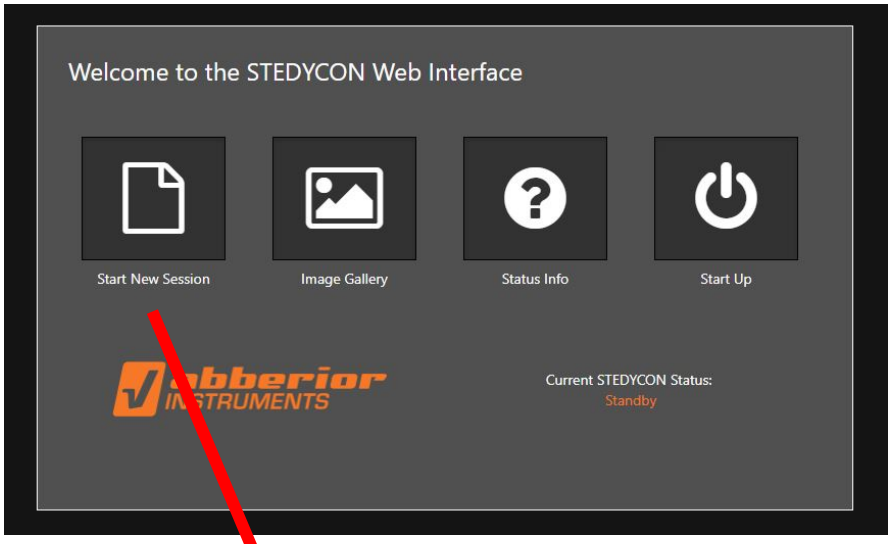
Turn ON the power for the LED excitation light source

Turn ON the **computer**, when windows booted, wait 1 minute



Location where images are saved (into the Stedycon control box, not computer)

Start the browser based STEDYCON control software



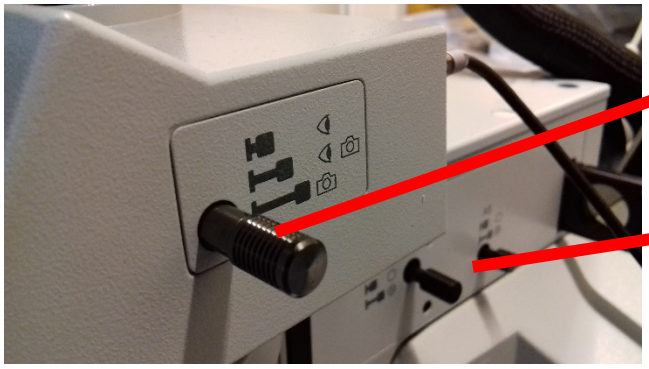
Start new session

Focusing the sample with the microscope

Finding the focus of the sample is extremely tricky with this microscope, as the 100x/1.45 objective has a very small working distance. There is a high risk for breaking the sample and damaging the objective. All effort for focusing should be done with extra care.

Please note that if some of the settings/knob/filter/lamp is not in the right position (or not working properly), no light is delivered to the correct light path thus the signal can be never found. Focusing this way (with no signal) has a high risk for breaking the sample and damaging the objective.

During focusing, the objective moves up and down, the stage doesn't move vertically. Before focusing, test which direction brings the objective closer to the sample, and which direction brings it away from it.

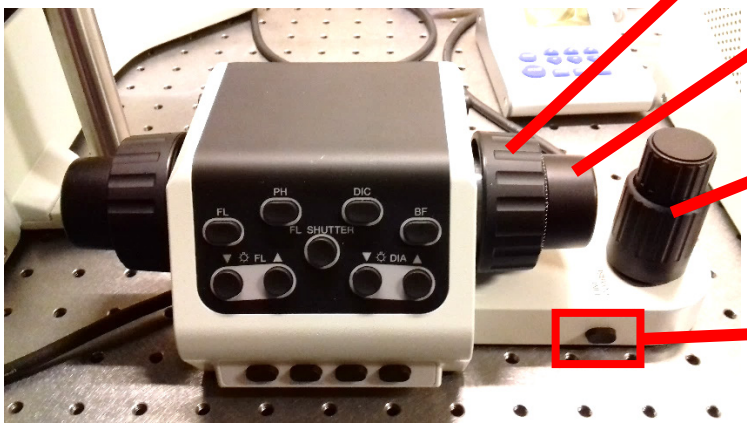


Push in this knob completely to deliver light to the eyepiece

Aperture knobs are pushed in always (apertures are fully open)

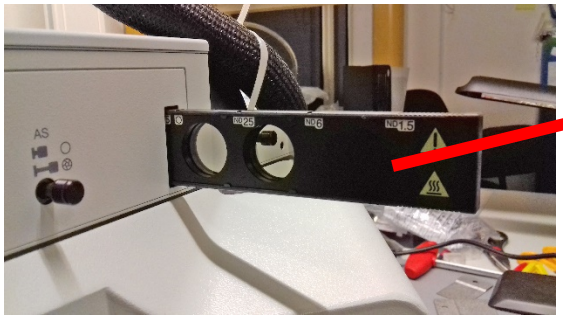
Coarse focusing (objective moves up/down)

Fine focusing (objective moves up/down)



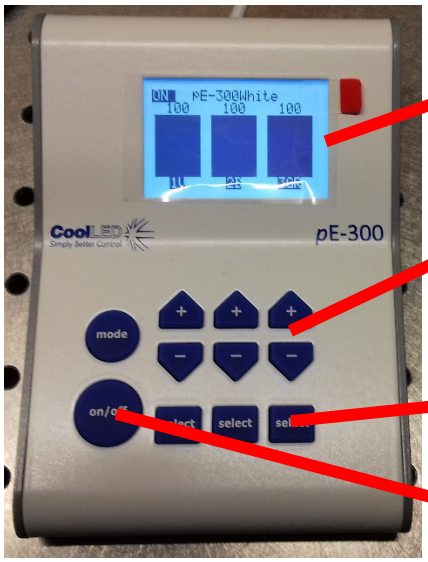
XY movements for the stage (test and make sure which knob is X and which one is Y)

Coarse/fine switch for the stage XY movements



ND filter rod is pushed completely **to the right side**

Focusing the sample with fluorescent channels



Settings for the different LED colors (Violet, Blue, Green): ON/OFF and intensity 0-100.

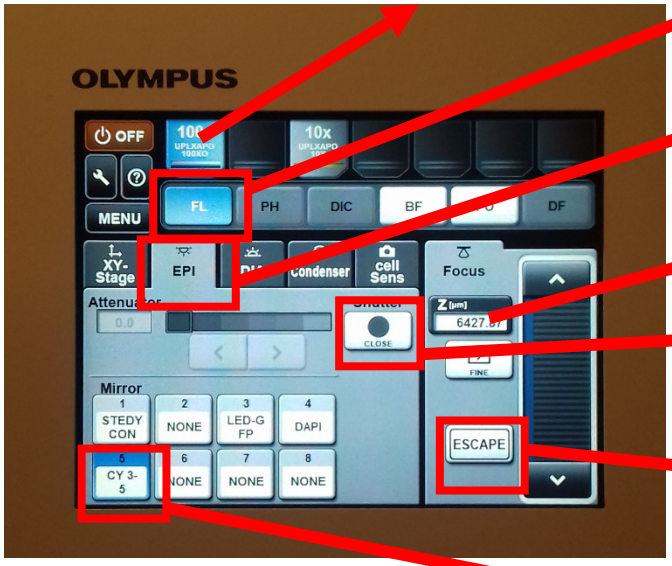
Change the **intensity** of the LEDs 0-100. Maximum is usually fine for cell samples. **Before looking into the eyepiece, check the light's power by showing a paper or your palm to the eyepiece! Don't blind yourself!**

Select the different LED colors (V; B; GR) for changing the intensity or turn ON/OFF

Turn ON/OFF the selected LEDs

Select the 100x/1.4 objective

Select the FL mode for fluorescent imaging



Open the EPI tab, for fluorescent imaging

Z (focus) position, see "hint" below

Open the fluorescent shutter

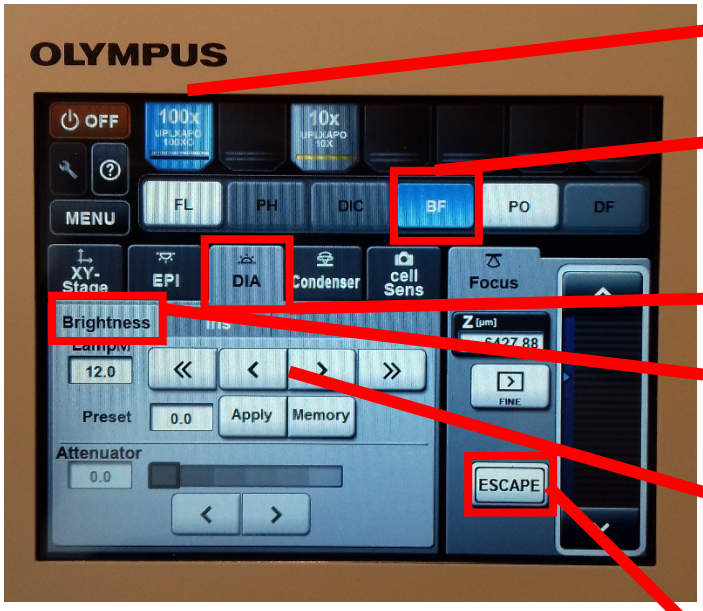
In case you need to move the objective UP/DOWN with big steps (e.g. to put oil on the sample)

Select your fluorescent channel (LED-GFP / DAPI / CY 3-5)

Now there should be light and fluorescent signal through the eyepiece. If nothing is visible, remember that there might be still no light to the eyepiece due to missed settings, malfunction, bad sample, or being in the wrong sample location (no cells in the field of view) etc... Don't start to move the objective UP/DOWN desperately, be careful.

A good hint: For a normal, properly prepared microscope slide where cells are embedded in mounting media (with #1.5 cover slip), **the approximately Z (µm) position is: 6420**. You can move to that approximately position without any sample. After this, slide your sample carefully under the objective and focus UP/DOWN with fine movements till you see your sample in focus. You should be pretty close at Z: 6420, with some luck something should be already visible there. **If you use the sample pins** (to push down the sample so it can't move), **the Z position can be approx.: 6420 - 6450**. Don't push the pins down with force, only lightly. To open: rotate the pins away from the sample.

Focusing the sample with brightfield illumination



Select the 100x/1.4 objective

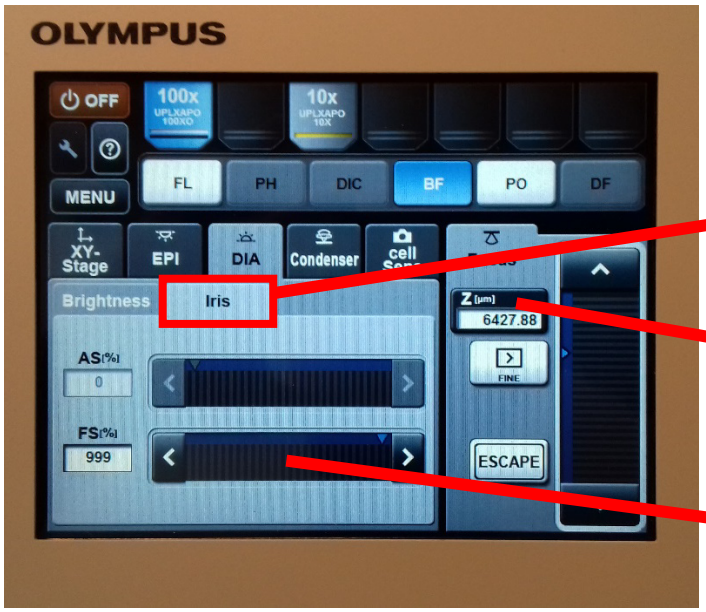
Select the **BF mode** for brightfield imaging

Open the DIA tab for brightfield imaging

Open the Brightness tab to change the lamp's intensity

Change the lamp's intensity UP/DOWN with small or big increments. Just put it to maximum.

In case you need to move the objective UP/DOWN with big steps (e.g. to put oil on the sample)



Open the Iris tab to change the lamp's iris

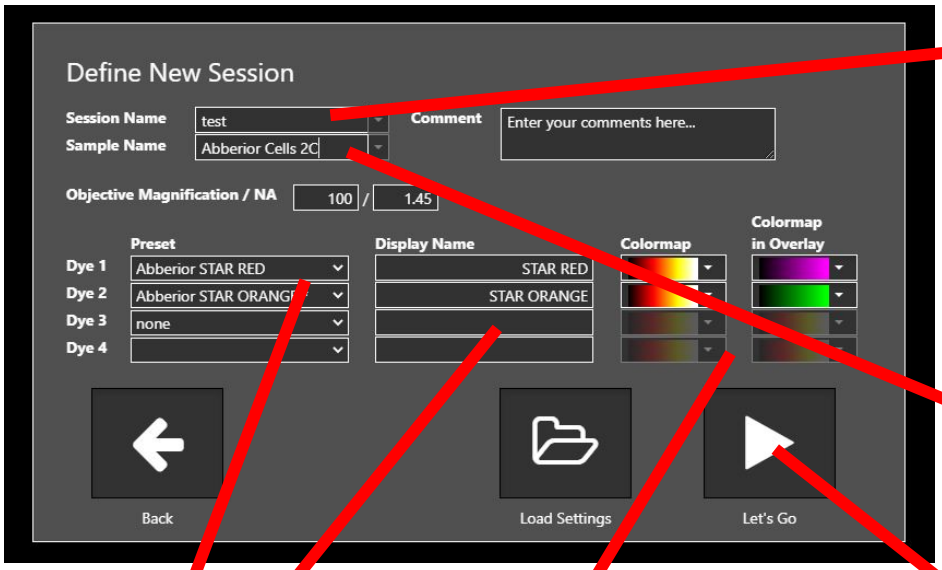
Z (focus) position, see "hint" below

Change the size of the iris, at the beginning try to open it to the maximum size to see some light.

Now there should be light and fluorescent signal through the eyepiece. If nothing is visible, remember that there might be still no light to the eyepiece due to missed settings, malfunction, bad sample, or being in the wrong sample location (no cells in the field of view), etc... Don't start to move the objective UP/DOWN desperately, be careful.

A good hint: For a normal, properly prepared microscope slide where cells are embedded in mounting media (with #1.5 cover slip), **the approximately Z (µm) position is: 6420**. You can move to that approximately position without any sample. After this, slide your sample carefully under the objective and focus UP/DOWN with fine movements till you see your sample in focus. You should be pretty close at Z: 6420, with some luck something should be already visible there. **If you use the sample pins** (to push down the sample so it can't move), **the Z position is approx.: 6420 - 6450**. Don't push the pins down with force, only lightly. To open: rotate the pins away from the sample.

Starting the session



Give a name to the session. That will be the folder's name where all the images are saved.

Add a name for the sample

Select the dyes from the preset, and name the dyes

Select colors for the channels

Start the session with the defined settings

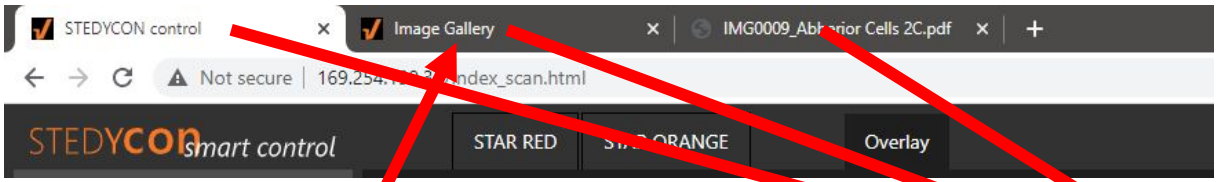
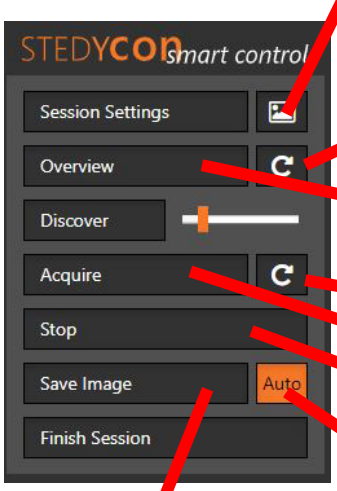


Image gallery, saved images during the session



Scan fast confocal **overview** images - full field of view - **continuously**

Browser based control software, look out for the **tabs**

Scan one fast confocal **overview** image - full field of view

Acquire confocal and/or STED images

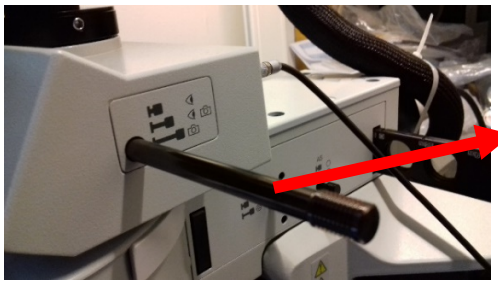
Acquire confocal and/or STED images **continuously**

Stop any scan without saving

Force to **save an image** (e.g. unfinished scans)

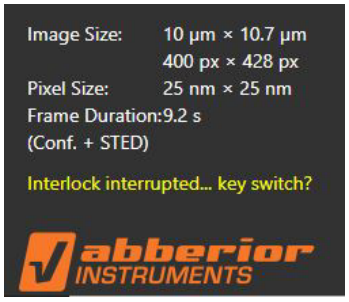
Auto-save button, leave it ON: Every single and final (if continuous/loop button is used) overview and acquisition is saved (as an .OBF raw file) if finished properly.

Imaging with the microscope (using the software)

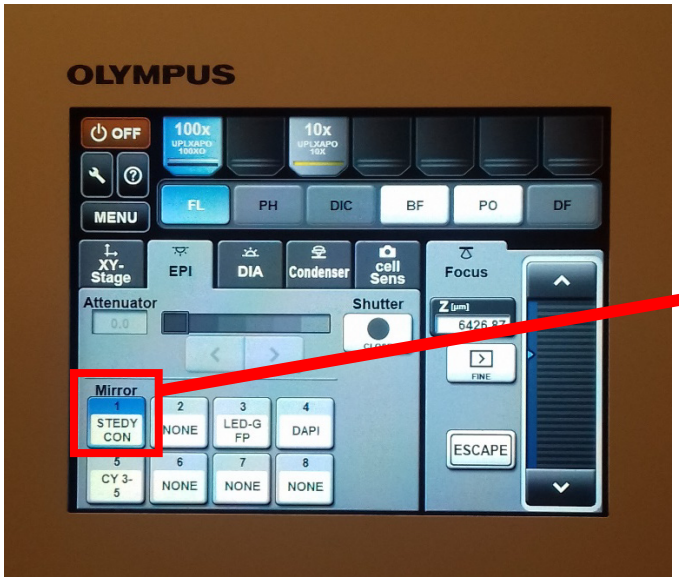


The **knob** should be **pulled out completely** to deliver light to the STEDYCON.

Double check this with the green "Interlock OK"; OR "connected" sign on the bottom left corner of the software (laser interlock is OK). If the knob is pushed in for eyepiece visualization, a yellow "Interlock interrupted" sign must be visible.

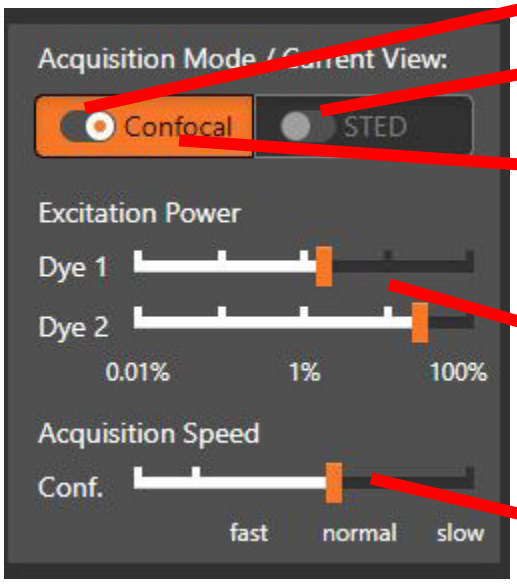


Don't go further and don't start any laser scanning if you see a problem with the laser interlock, as it can be dangerous! Never look into the eyepiece during laser scanning!



In the EPI tab of the TFT control, **select position 1: STEDYCON**

Confocal



Confocal mode is activated

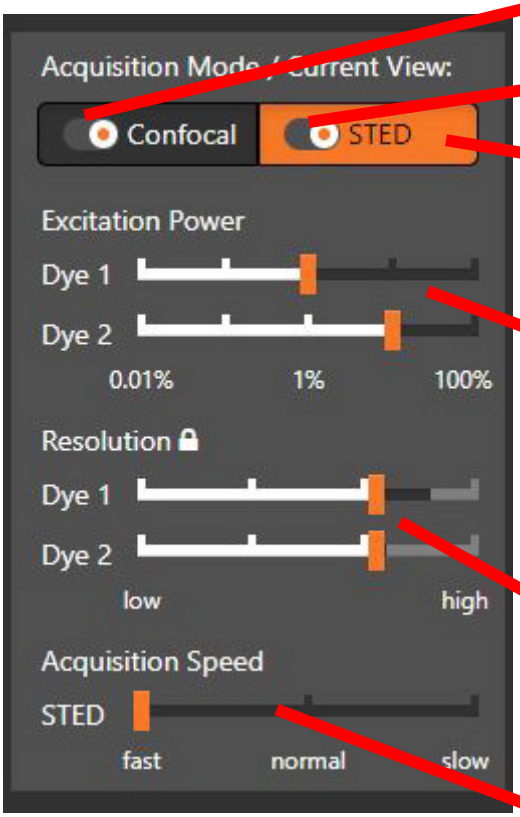
STED mode is not activated

Confocal mode is visible in the image window and confocal settings are visible in this tab (see below)

Laser excitation powers for the confocal dyes (two active dyes at the moment)

Confocal scanning speed (balance between imaging time and quality)

STED



Confocal mode is activated

STED mode is activated

STED mode is visible in the image window and STED settings are visible in this tab (see below)

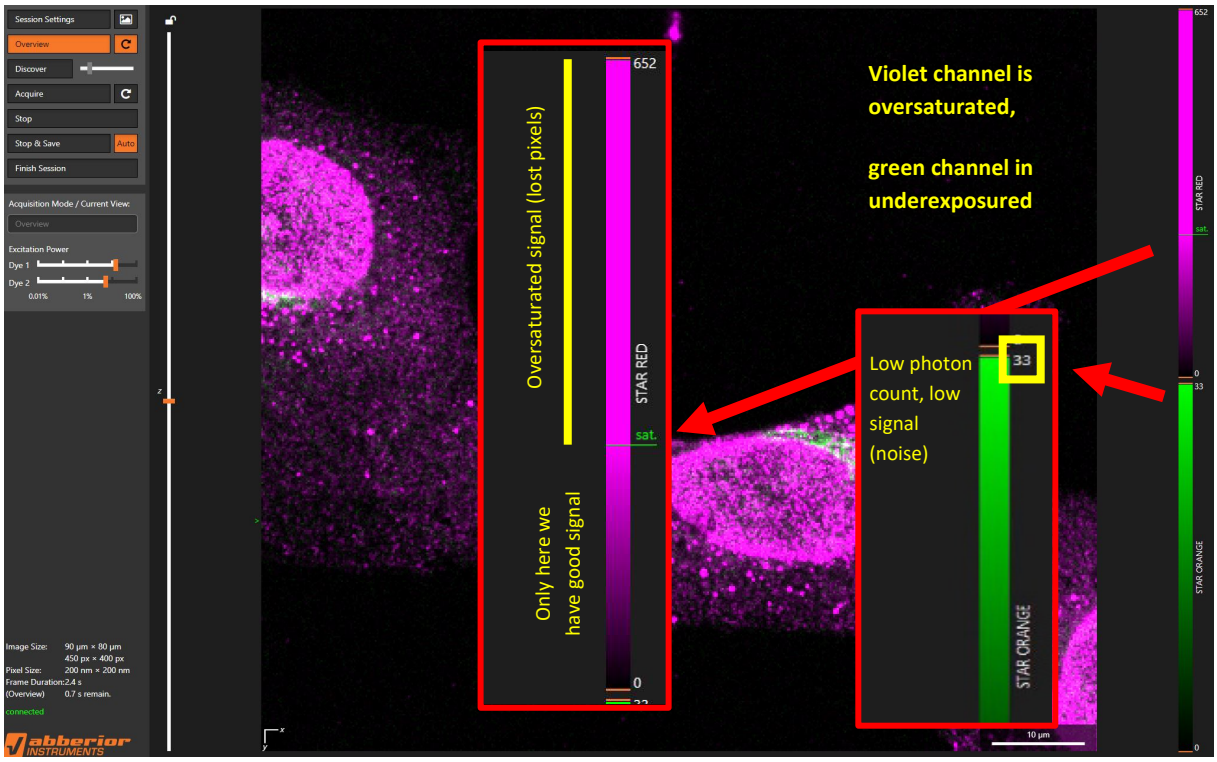
Laser excitation powers for the STED dyes (two active dyes at the moment)

STED resolution, intensity of the STED depletion laser. Higher intensity means higher STED resolution, check **"pixel size"** on the bottom left corner

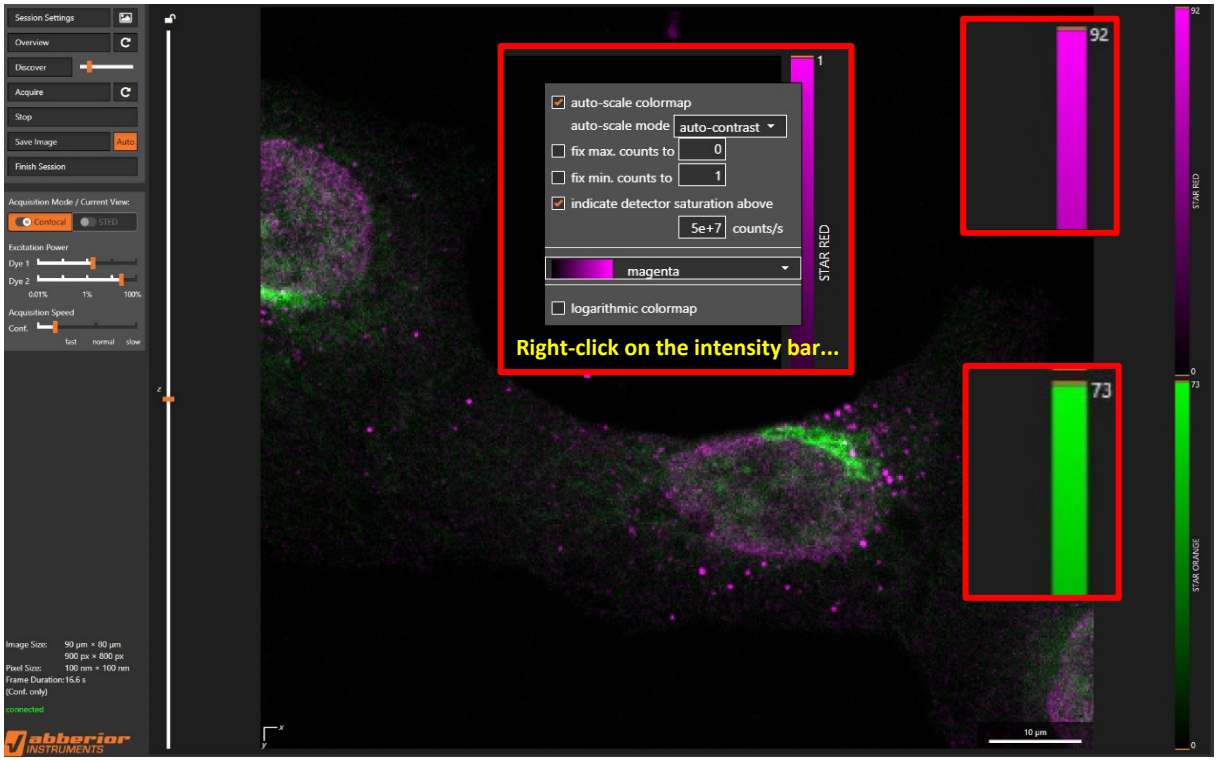
STED scanning speed (balance between imaging time and quality)

Overview scan and image canvas – checking the correct intensity of the dyes

To get good intensity and avoid underexposure/oversaturation, have at least around 70 photon counts per channel but don't increase the laser intensity to a high level where the green saturation bar is visible (detector is saturated). Photon counts are visible on the top of the channel's intensity bar.

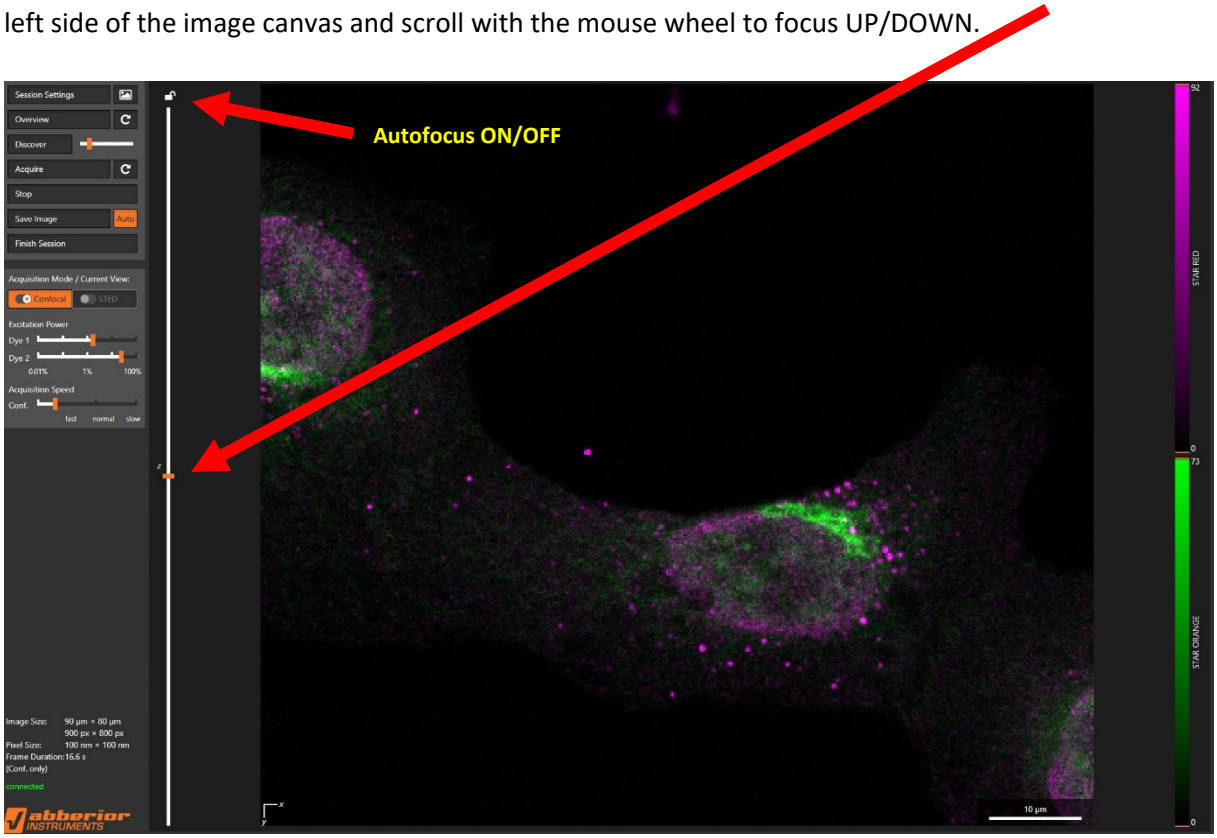


The image below shows good intensity, no overexposure and photon counts are over 70 for all the channels. The excitation laser's intensity can be still increased to avoid noise till: (1) sample is not bleaching during the normal (slower) imaging and; (2) detector is not saturated (overexposure).



Focusing

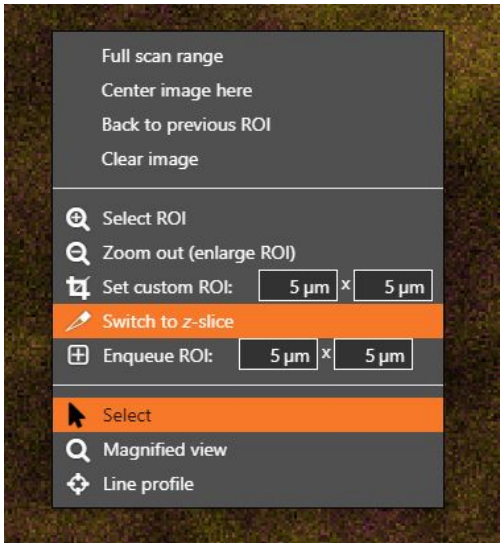
Focusing can be done either with the microscope's focusing knobs (coarse/fine; be careful); or using the piezo for very fine movements. To use the piezo, move the mouse cursor to the **"z level"** on the left side of the image canvas and scroll with the mouse wheel to focus UP/DOWN.



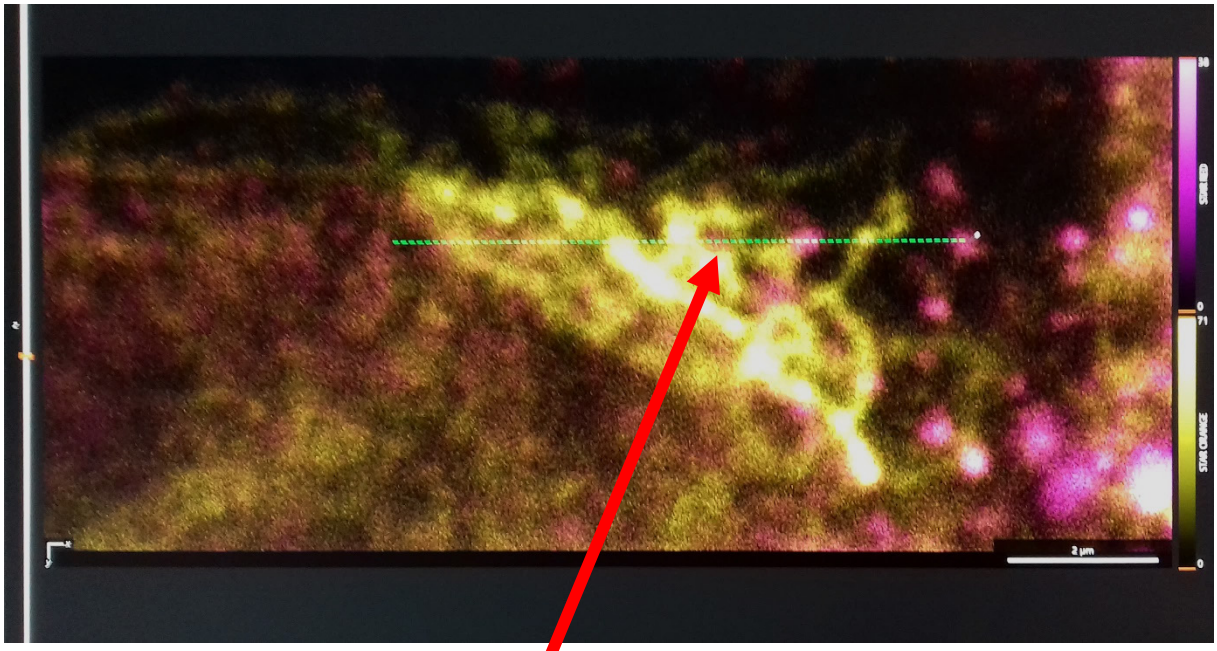
Autofocus is not ON, focus drift can happen during longer scans

Autofocus is ON, focus is locked on the last focal plane modified by the piezo

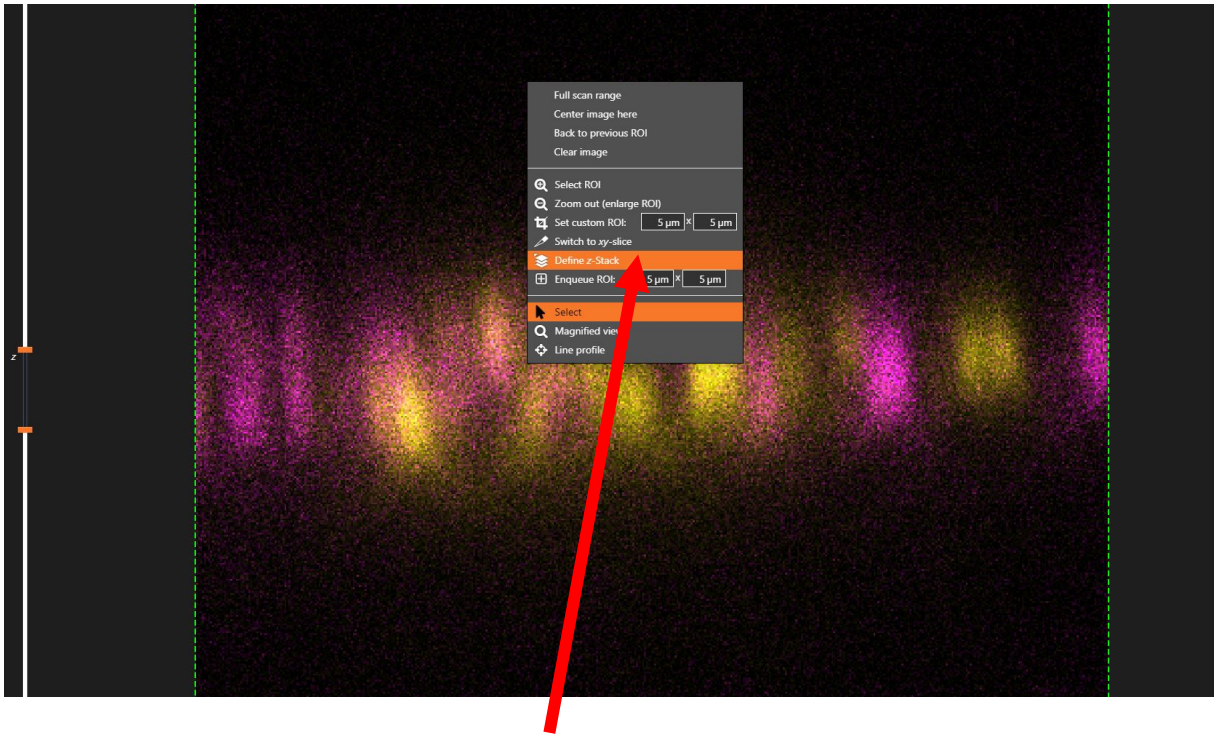
Z-stacking (3D imaging)



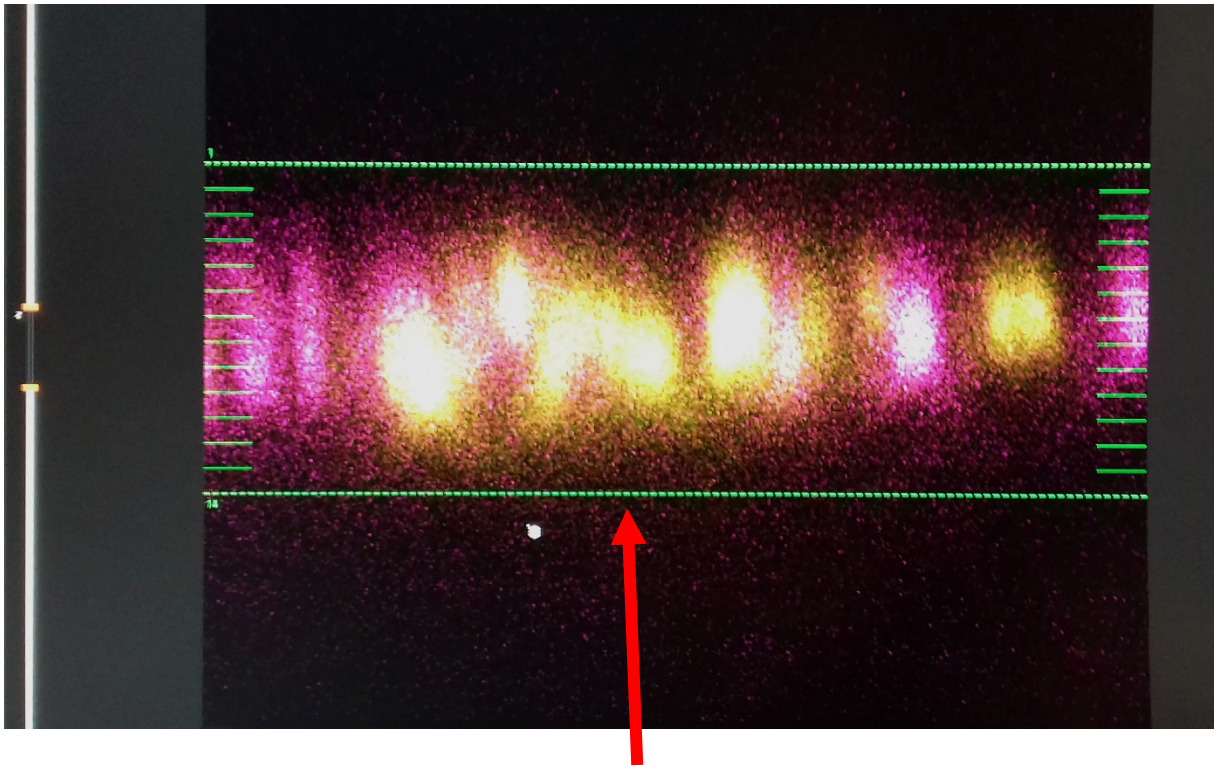
Right-click on the overview where Z-stacking is needed, and select the **"Switch to z-slice"** option.



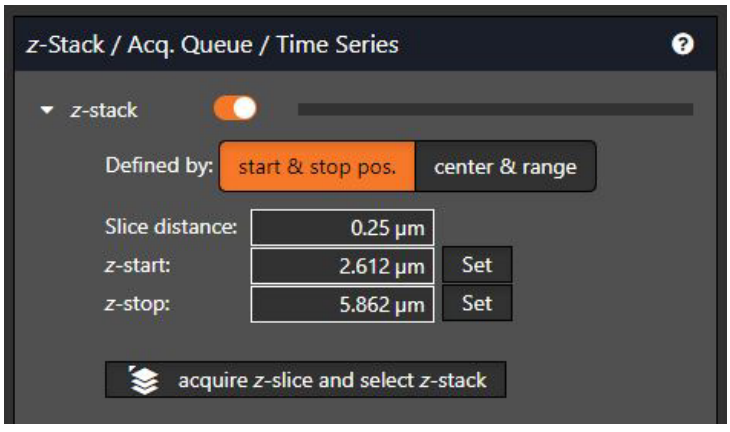
With left-click and holding the left mouse button, draw a horizontal or vertical line for the desired position (XZ or YZ). After this, hit the **”Acquire button”.**



After the XZ or YZ scan is done, **right-click on the image** and select the **”Define z-stack”** option.

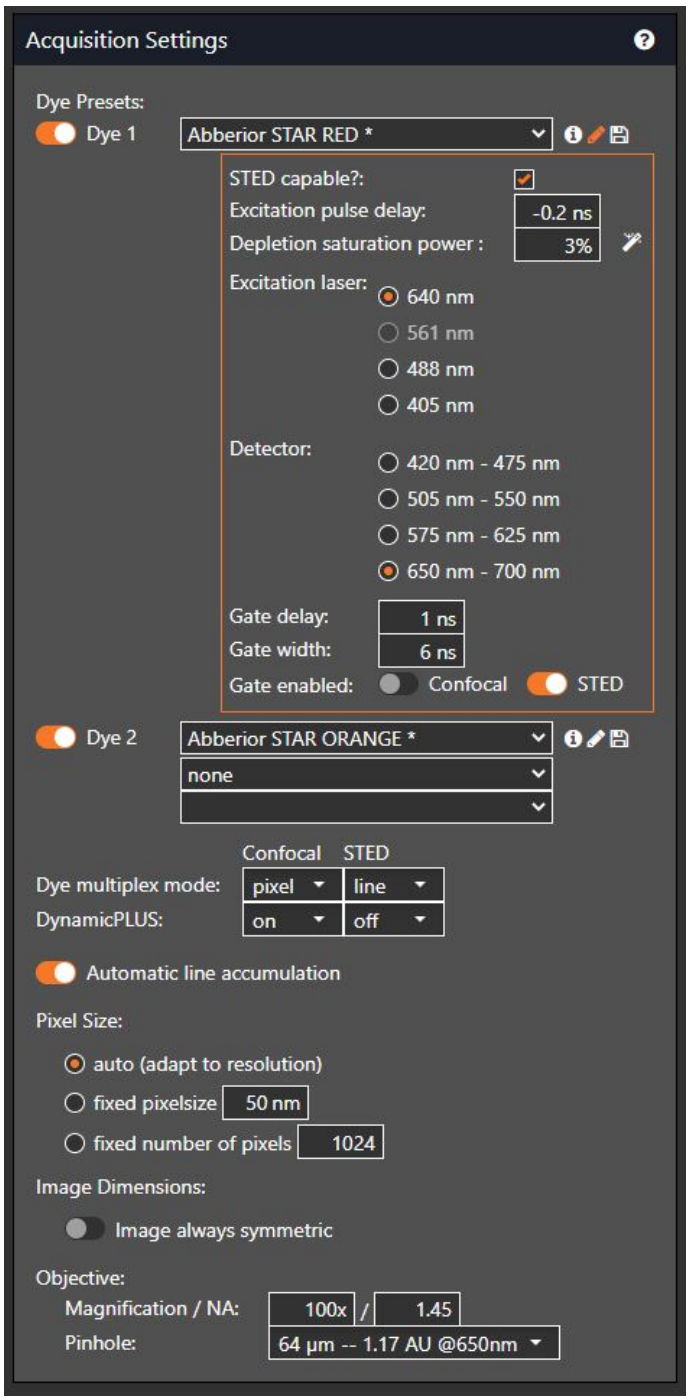


With left-click and holding the left mouse button, mark vertically the start (click left mouse button) and stop (release left mouse button) position for the Z-stack. The number of stacks will be visible on the screen. After this, hit the **"Acquire button"** and the 3D scanning starts in confocal AND/OR in STED mode.



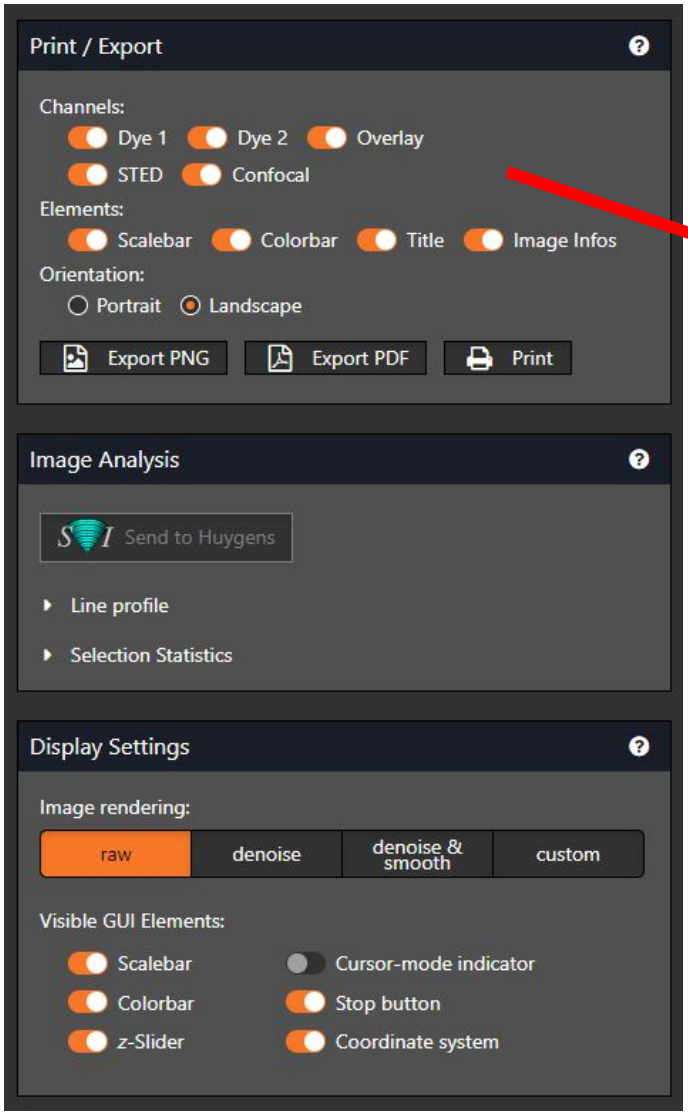
For changing the slice number or distance, and for other settings, check the "**Advanced menu/Z-stack"** on the right side of the software

Advanced menu



The settings for the specific dyes (laser excitation, detection range, time gating, pulse delay, etc) can be changed here or new dye can be added manually.

Don't play here unless you know what you are doing and always save the modified dyes separately (don't overwrite the default settings for dyes).



If autosave option is ON (default), every single and final (if continuous/loop button is used) overview and acquisition is saved (as an .OBF raw file) if finished properly.

Here we have more option for exporting in different file formats. PNG is a lossless format (just like .TIFF) and uses compression. The other option is .PDF.

Select what you want to include for the export (channels, overlay, confocal/STED, info, etc) and hit the ”Export PNG” or the ”Export PDF” button.

Recommended dyes, mounting media and coverslip for STED imaging

6.8 Dyes and Embedding Media for STED Microscopy

6.8.1 Recommended Dyes for STED @ 775 nm

Table 1. List of recommended STED dyes for STED @ 775 nm.

Exc. 488 nm	Exc. 561/594 nm	Exc. 640 nm
Abberior STAR 520 SXP	Abberior STAR 600	Abberior STAR 635P
Abberior STAR 470 SXP	Abberior STAR 580	Abberior STAR 635
		Abberior STAR RED
Dyomics 520XL	Alexa Fluor 594	
Dyomics 480XL		Atto 647N*
	Atto 590*	Atto 655*
Atto 490LS*	Atto 594*	Atto 633*
Chromeo 494		Alexa Fluor 647°
		SiR°

*Abberior STAR dyes may be purchased from Abberior GmbH, Göttingen, Germany; Alexa Fluor dyes may be purchased from Life Technologies Inc., Carlsbad, CA, USA; ATTO dyes may be purchased from ATTO-TEC GmbH, Siegen, Germany. Dyomics dyes may be purchased from Dyomics GmbH, Jena, Germany; Chromeo dyes may be purchased from Active Motif, Carlsbad, CA, USA. *Dyes are hydrophobic which might lead to pronounced background. °Dyes are sensitive to photobleaching – optimization of the imaging conditions might be necessary.*

Further dyes are shown on the web page of the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany: http://nanobiophotonics.mpibpc.mpg.de/old/dyes/

6.8.2 Embedding Media for STED Microscopy

Following embedding media are recommended for 2D STED microscopy:

- Abberior Mount Solid (Abberior GmbH, Göttingen, Germany)
- Abberior Mount Solid Antifade (Abberior GmbH, Göttingen, Germany)
- Mowiol/DABCO
- Prolong Antifade Gold (Life Technologies Inc., Carlsbad, CA, USA)
- Prolong Antifade Diamond (Life Technologies Inc., Carlsbad, CA, USA)

Following embedding media are recommended for 2D and 3D STED microscopy:

- Abberior Mount Liquid (Abberior GmbH, Göttingen, Germany)
- Abberior Mount Liquid Antifade (Abberior GmbH, Göttingen, Germany)
- TDE (Abberior GmbH, Göttingen, Germany)

Please do not use Vectashield, Vectashield HardSet or (other) embedding media containing *p*-phenylenediamine as an antifading reagent.

Please do not use DAPI, Propidium Iodide or Ethidium Bromide (in high amounts) for labeling or in embedding media. These dyes may be excited by the STED laser and thereby might lead to high background and blurred images.

6.8.3 Recommended Live-Cell Imaging Media for STED microscopy

Several media can be used for imaging of living cells. In general imaging media for STED microscopy have to be non-absorbing in the excitation channels and non-fluorescent in the different fluorescence channels.

For mammalian cells frequently HDMEM (Dulbecco's Modified Eagle's Medium, buffered with HEPES, Invitrogen, USA) or DMEMgfp-2 (Evrogen, Moscow, Russia) is used.

For yeast cells, many Synthetic Complete media (without yeast extract and peptone) may be used.

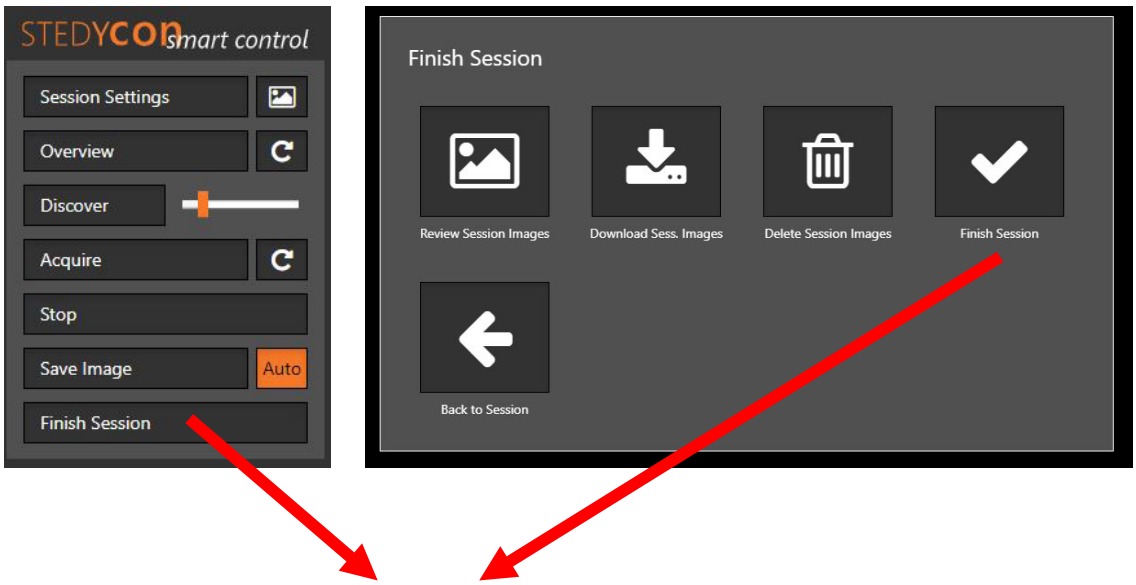
>>> Coverslip thickness is #1.5 (170 µm) <<<

Shutting down the microscope

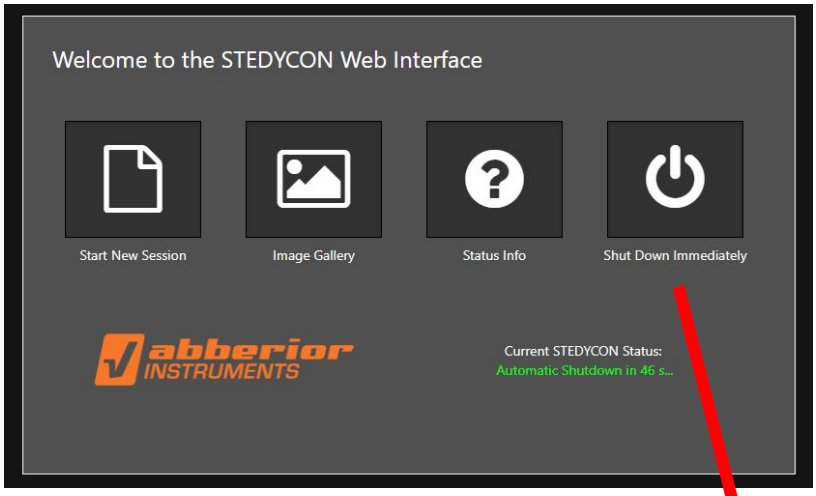
Remove your sample and CLEAN. Wipe off all oil residue with ethanol and tissue.

Save and export all your files in your desired format and location.

- The raw .OBF files (autosave) saved to an internal computer of the STEDYCON, not to the small PC. If you need those files later, you need a full start of the STEDYCON to reach them.

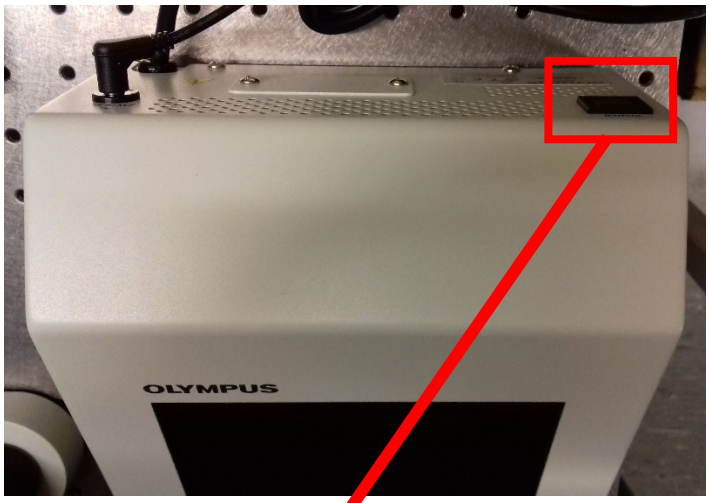


Click on **"Finish session"**

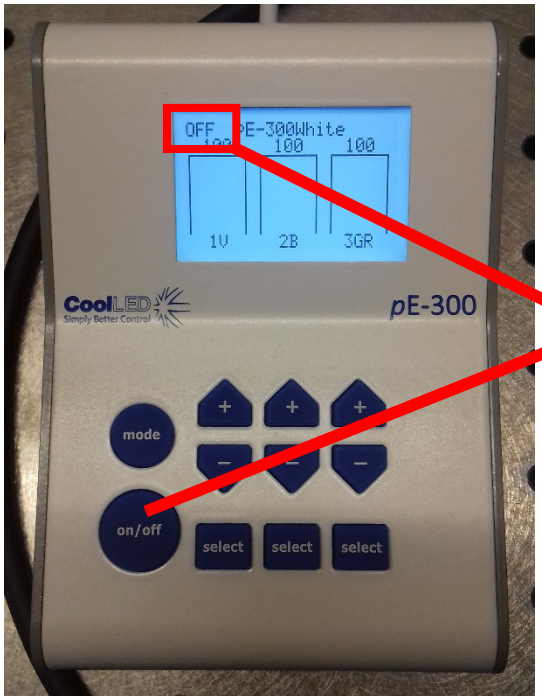


Click on **"Shut Down Immediately"** and wait till the STEDYCON is fully shut down to Standby, check the yellow indicator lamp next to the "Standby" text.

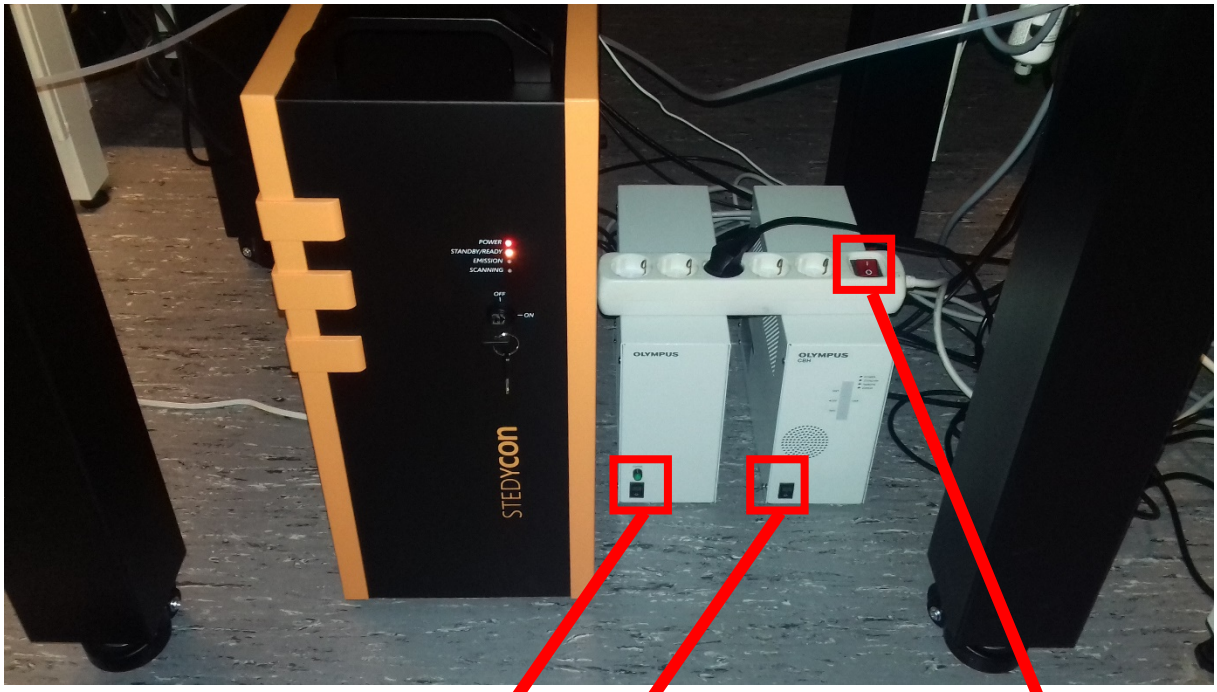
Shut down the computer (through Windows 10)



Turn OFF the **TFT control** and wait till it's fully shut down



Turn off all the LEDs (V, B, GR) and check on the TFT for the **"OFF"** text. Wait 2 minutes so the LEDs can cool off.



Turn OFF the two **control boxes** for the Olympus microscope

Turn OFF the **power cord** for the LED box