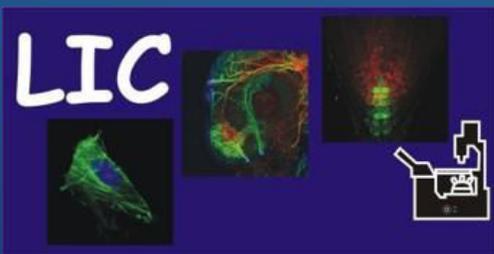


Video Tutorial - Basic

Leica Sp8-I-STED- used for STED

room 00.024

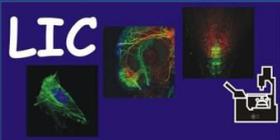
Life Imaging Center 2020





Start Software

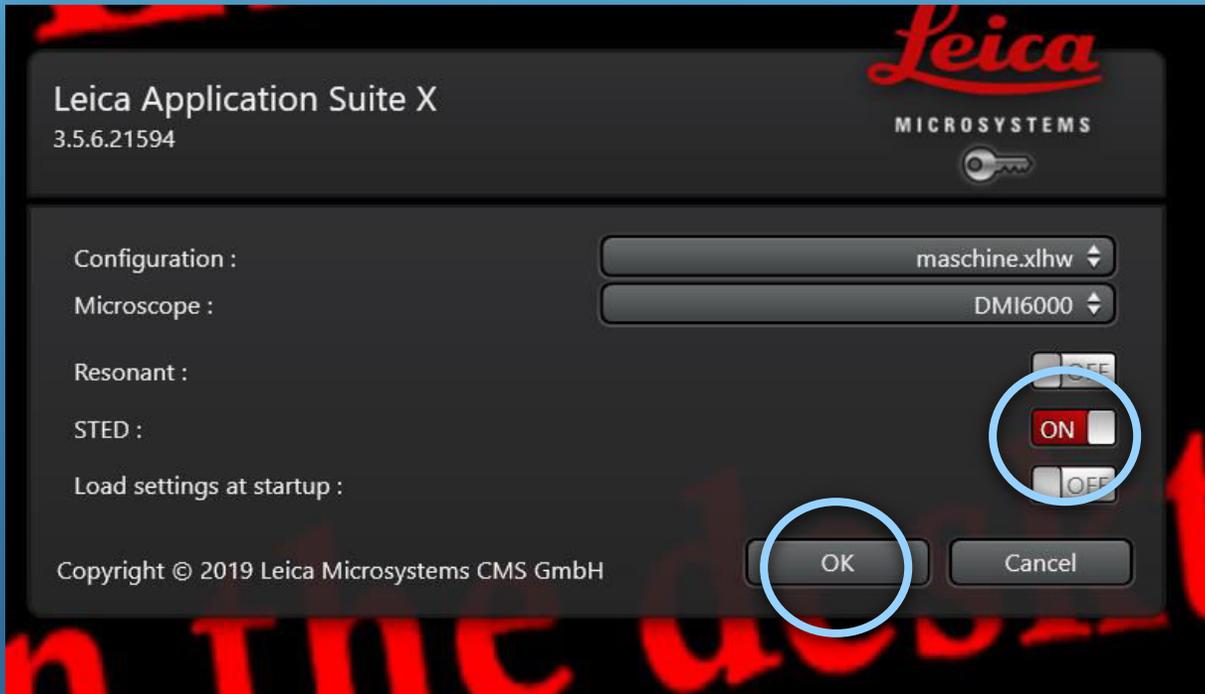
Click on LASX Icon to start **LASX** Software



Life Imaging Center

SP8-I-STED





Start Software

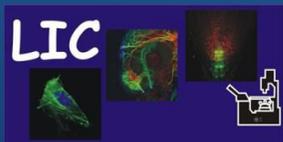
Configuration: **maschine**

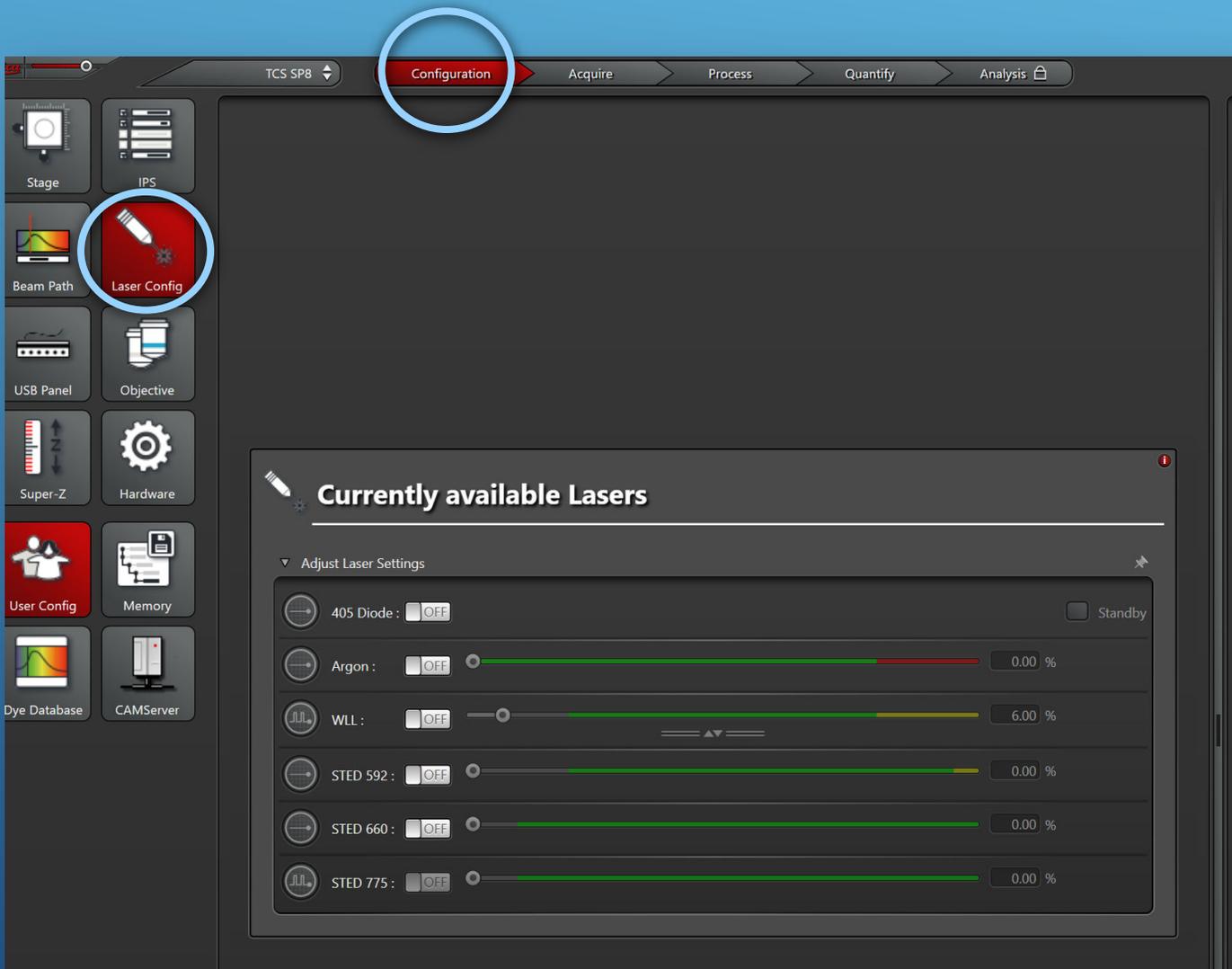
Microscope: **DMI6000**

For **STED** use

| | |
|---------------|-----------|
| Resonant | OFF |
| STED | ON |
| Load settings | OFF |

Press **Ok**

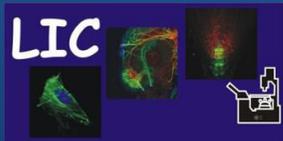




Turning on Lasers

Click on the **Configuration** tab at the top of the application

Click on the "**Lasers Config**" button within configuration



TCS SP8 Configuration Acquire Process Quantify Analysis

Stage IPS Beam Path Laser Config USB Panel Objective Super-Z Hardware User Config Memory Dye Database CAMServer

Always switch laser in this sequence **ON**

Currently available Lasers

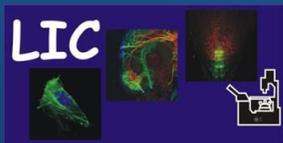
Adjust Laser Settings

| | | |
|-----------|-----|---------|
| 405 Diode | ON | Standby |
| Argon | OFF | 0.00 % |
| WLL | ON | 70.00 % |
| STED 592 | ON | 51.90 % |
| STED 660 | OFF | 0.00 % |
| STED 775 | ON | 51.46 % |

Turning ON Lasers

Turn **ON** only needed laser

- !!! very **important sequence** when switching **on** and **off** laser lines !!!
- For STED always 592nm (for alignment)



TCS SP8 Configuration Acquire Process Quantify Analysis

Stage IPS Beam Path Laser Config USB Panel Objective Super-Z Hardware User Config Memory Dye Database CAMServer

Always switch laser in this sequence **OFF**

Currently available Lasers

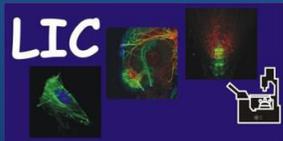
Adjust Laser Settings

| | | |
|-----------|-----|---------|
| 405 Diode | ON | Standby |
| Argon | OFF | 0.00 % |
| WLL | ON | 70.00 % |
| STED 592 | ON | 51.90 % |
| STED 660 | OFF | 0.00 % |
| STED 775 | ON | 51.46 % |

Turning Off Laser

...after your session

always switch laser in this sequence
OFF



TCS SP8 Configuration Acquire Process Quantify Analysis

Stage IPS Beam Path Laser Config USB Panel Objective Super-Z Hardware User Config Memory Dye Database CAMServer

The **White Laser (WL)** will excite between 470nm-670nm

- Leave the power output on 70%

Currently available Lasers

Adjust Laser Settings

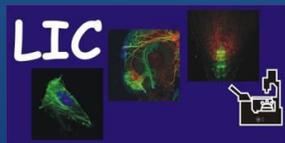
| Laser | Status | Power (%) |
|-----------|--------|-----------|
| 405 Diode | ON | Standby |
| Argon | OFF | 0.00 |
| WLL | ON | 70.00 |
| STED 592 | ON | 51.90 |
| STED 660 | OFF | 0.00 |
| STED 775 | ON | 51.46 |

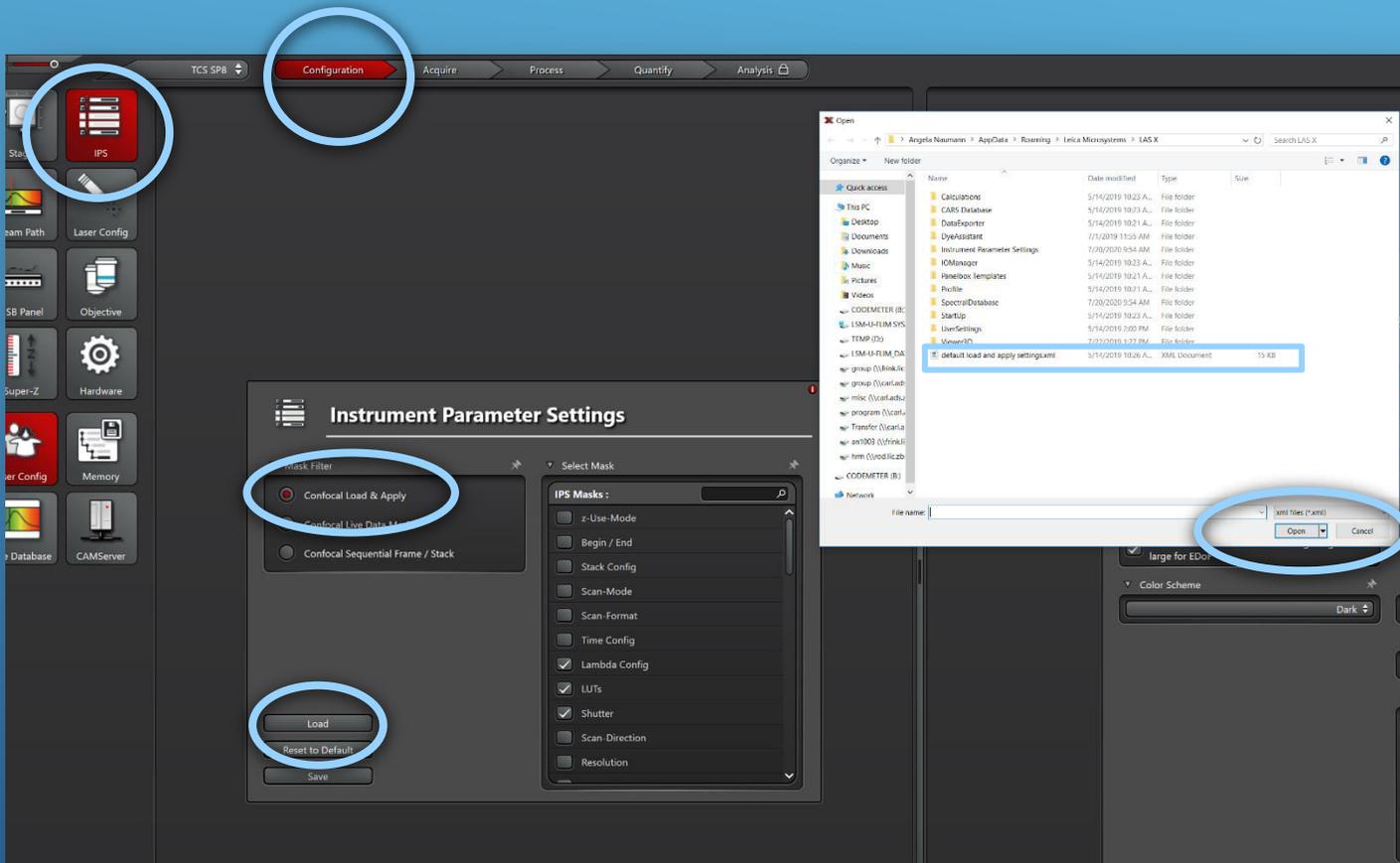
Laser Power

Turn up the power

WL 70%

STED for warming up to 50%





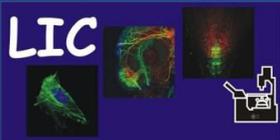
Instrument Parameter:

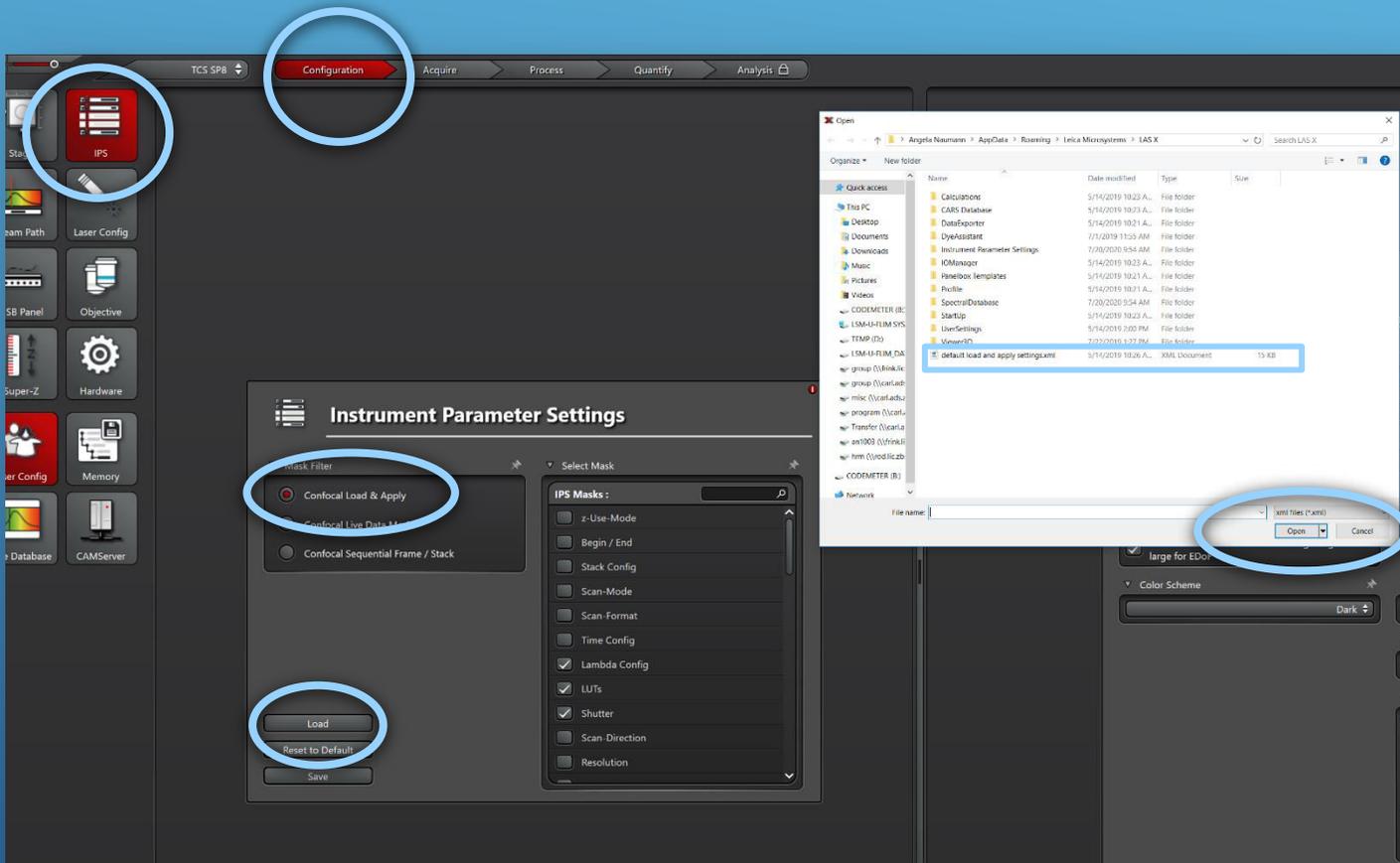
Open **Configuration**
Open **IPS**

Select „Confocal Load and Apply“
Press **Load**

Window will open

Select „Default Load and Apply“ file
Press **Open**

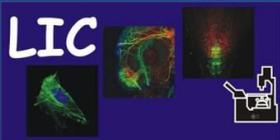


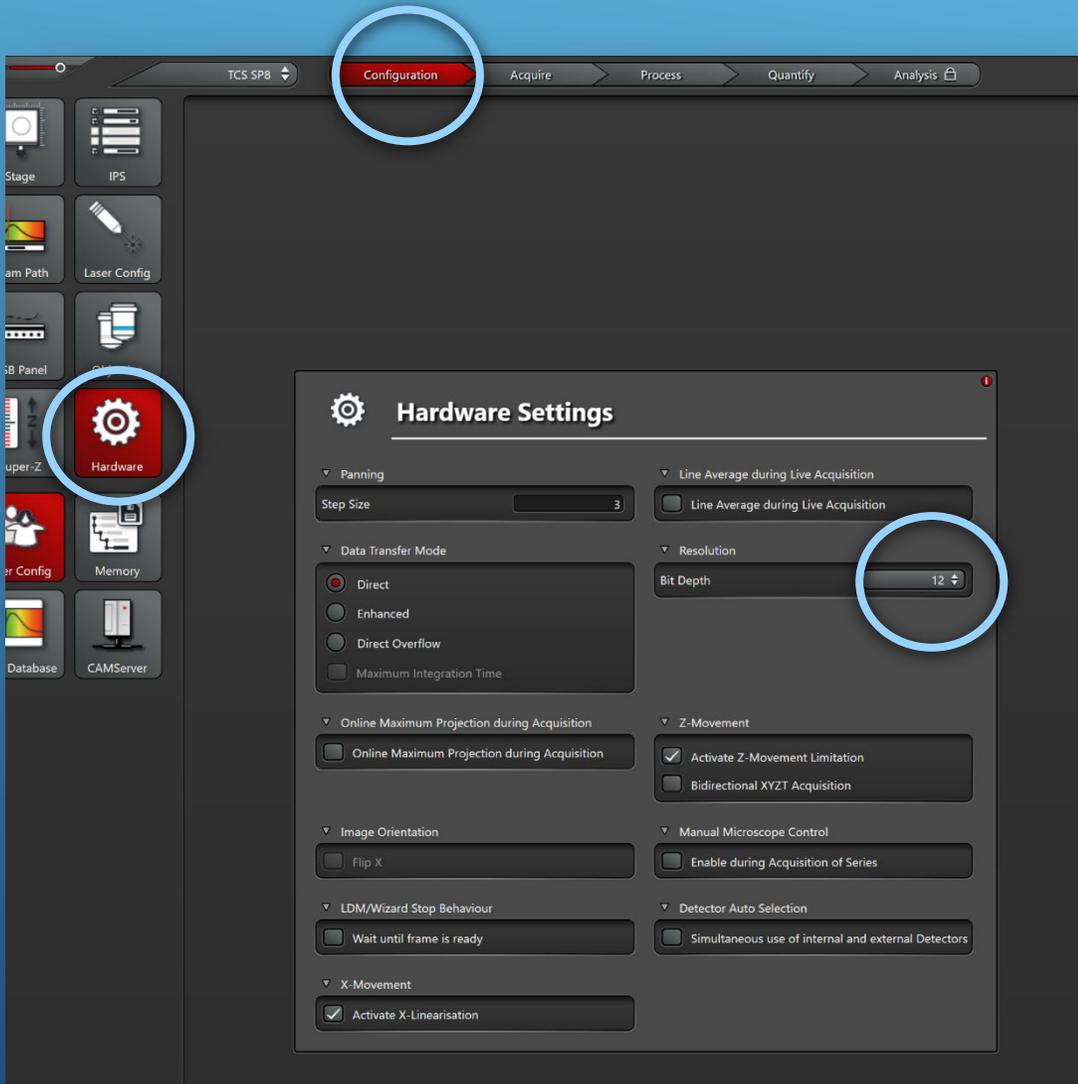


Instrument Parameter:

Instrument Parameter defines specific settings, which should be transferred later from an already recorded image using “Apply settings” to the hardware:

ie. Resolution - scan format - channel set up and so on



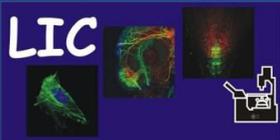


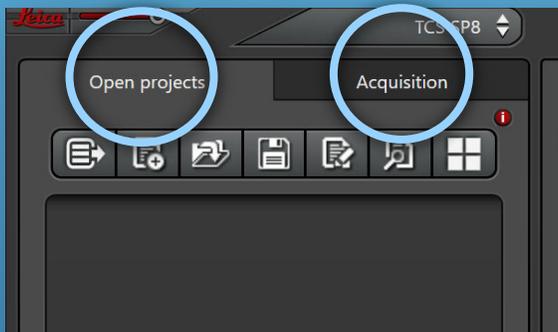
Change Bit Depth

- 8 bit** 256 Grey Shades
- 12 bit** 4095 Grey Shades
- 16 bit** 65536 Grey Shades

i.e. grayscale intensity stored as an 8-bit integer is giving **256** possible different shades of gray from black to white

12 and 16bit are recommended

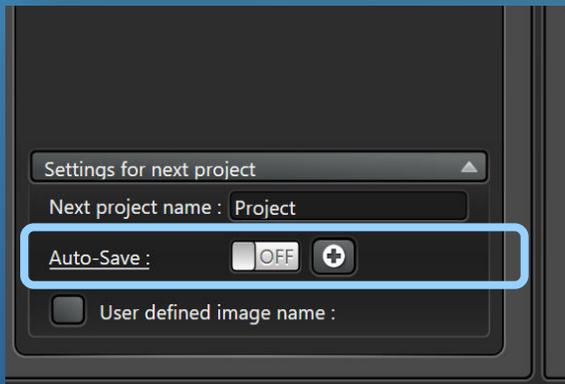




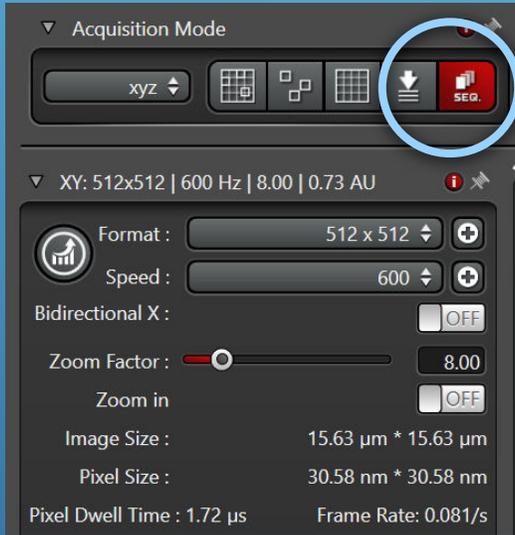
Projects/Acquisition pane

The **Acquisition tab** – which controls the microscope

The **Open Projects tab** - which is the data management area.

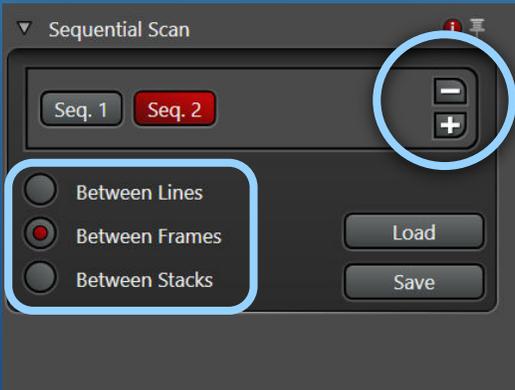


- As image data is captured (using *Capture image* or *Start - Button* in the *Acquisition tab*) it is **temporary** stored under this tab until the user tells the software to save the project
- Please **do not turn** on the **Auto-save function** (bottom of the Project tab)



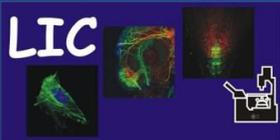
Acquisition pane – Sequential Scan

The SEQ will indicate that you are scanning sequentially



Plus + and Minus – you can duplicate or remove sequences

- For **Live** Imaging use **Between Lines**
- For **Fixed** sample use **Between Frames**
- For **Fixed** sample and **Z-stack** use **Between Stacks**



Objective for STED

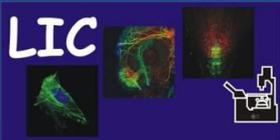
Select the appropriate STED objective inside the software :

- **HC PL APO CS2 93x/1.3 GLY**



Make sure you match the immersion media with the mounting media or use the objective correction ring to adjust in xzy mode.

Mismatch will lead to loss of intensity and resolution and STED effect

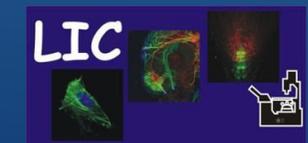


Light Path - Laser & Detector

Activate **Seq** scan tool

To control whether STED works, we will first set up a confocal and in addition a STED channel,

- Later you work only with a STED channel.



Light Path - Laser & Detector

Set up now **Seq 1** as confocal channel

Use for **excitation** of the fluorophores the **WWL** (470 up to 670) and setup specific wavelength - here 488

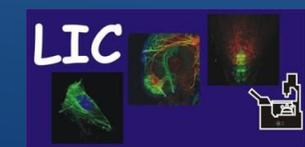
Activate **HYD** and adjust detection range according to the emission of your fluorophore

Adjust - using **ZOOM** and **FORMAT** -the **pixelsize** to 20-30 nm range – here 24nm

Open **STED panel**, but leave laser **OFF**

The screenshot displays the SP8-I-STED software interface. Key elements include:

- Top Panel:** A color-coded wavelength scale from 405 nm to 800 nm. A red box highlights the 'WWL' (White Light Laser) section, and a blue box highlights the '488' nm mark.
- Left Panel:** System parameters such as 'Format: 1024x1024', 'Speed: 700', and 'Zoom Factor: 5.00'. A blue box highlights the 'Format' and 'Zoom' controls.
- Center Panel:** Objective and Fluo Turret settings. A blue box highlights the 'Objective' and 'Fluo Turret' dropdowns.
- Bottom Panel:** A spectral plot showing emission intensity across wavelengths. A blue box highlights the plot area. Below it, detector settings for 'HyD 2' are shown, with 'Gain [%]' set to 100.0.
- Bottom Left Panel:** 'STED Delay Control' and 'Sequential Scan' settings. A blue box highlights the 'Seq. 1' selection in the 'Sequential Scan' section.

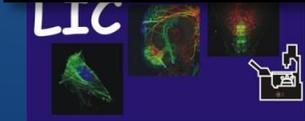
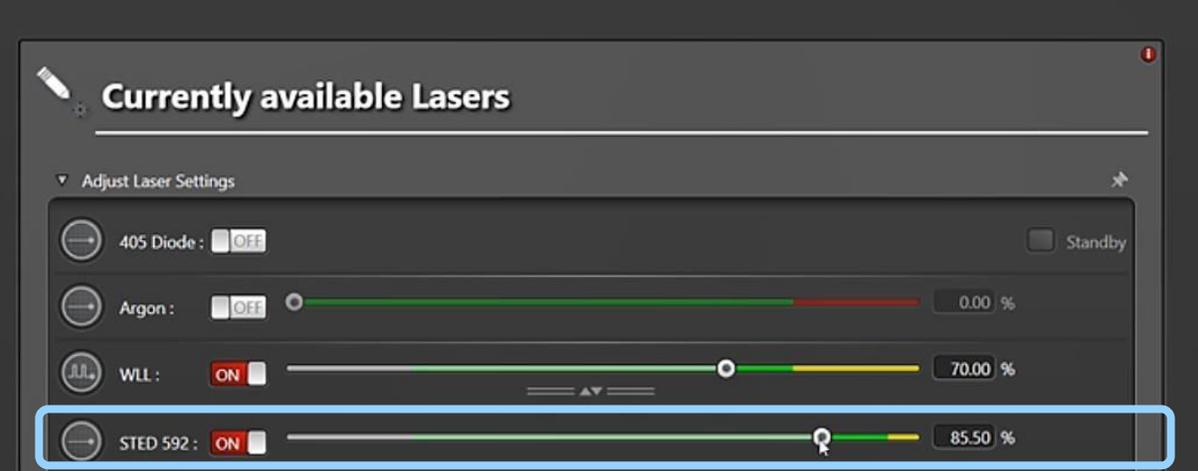


Light Path - Laser & Detector

To set up the STED channel - **duplicate Seq 1**

Go to **Configuration tab** and **laser window** and increase the during the experiment used STED laser power to ~ **85%** - here the 592nm

Go back to **Acquisition tab** and configure **Seq 2** as STED Channel



Light Path - Laser & Detector

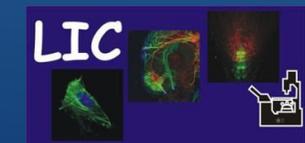
STED % switch **ON**

- Select the **appropriate depletion laser** for your fluorophore.

Leave STED 3D % - **OFF**

Note that no inactive detector may be positioned below this laser-line.

The screenshot displays the SP8-I-STED software interface. At the top, a color-coded light path diagram shows the laser line (488 nm) and the detection path. The STED 3D % switch is set to OFF, and the STED % switch is set to ON. The STED Dichroic Slider is set to SD 592. The Fluor Turret is set to Scan-BF. The objective is HC PL APO CS2 93x/1.30 GLYC. The specimen is shown in the center. The internal view shows the laser line at 488 nm and the detection path. The detector settings are shown below, with HyD 2 selected and its gain set to 150.0. The STED Delay Control is set to 0 ps. The Sequential Scan section shows Seq. 1 and Seq. 2 selected.



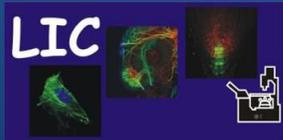
Light Path - Laser & Detector

Altering here **% Power** changes STED effect, i.e. the resolution in XY, see visualization of the PSF.

However remember, bleaching also increases with higher STED **%** power.

The screenshot displays the SP8-I-STEM software interface, which is used for controlling a STED microscope. The interface is divided into several sections:

- Top Left:** Emission wavelength settings (580 nm) and motCORR Collar Settings (0.0% to 100.0%).
- Top Center:** A color-coded bar representing the light path or detector response, with a scale from 405 to 775 nm. A blue circle highlights the 100% power setting.
- Top Right:** STED 3D and STED Z controls, with a blue circle highlighting the 100% power setting.
- Middle:** Objective (HC PL APO CS2 93x/1.30 GLYC) and STED Dichroic Slider (SD 592) settings.
- Bottom Left:** Z-Stack settings (2.31 μm, 10 Steps) and a sketch of the estimated effective PSF, which is circled in blue.
- Bottom Center:** A detailed light path diagram showing the specimen, internal components, and detector channels (PMT 1-5, HyD 2-4).
- Bottom Right:** Detector channel settings, including Gain [V], Offset [%], and Ref. Line [nm].



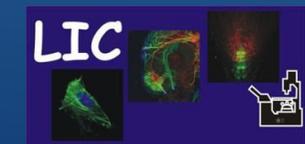
Light Path - Laser & Detector

To achieve even better effects, activate **Gating**

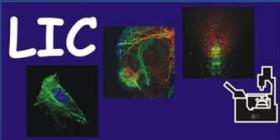
With **Gating** you define the start and end of your image acquisition in relation to your laser pulse (ref. line):

- exclude background of laser reflection
- increase STED resolution
- decrease intensity (need higher laser %)

The screenshot displays the SP8-I-STED software interface. At the top, a color bar shows the wavelength range from 405 nm to 775 nm. Below this, the 'Specimen' section shows the objective (HC PL APO CS2 93x/1.30 GLYC) and the STED Dichroic Slider (SD 592). The 'Internal' section shows a graph of the light path with a red line indicating the laser pulse and a green line indicating the image acquisition. The 'HyD 2' detector is highlighted with a blue box, showing its gain (150.0) and gating settings (0.30 and 12.00). The 'PMT 1' through 'PMT 5' sections show their respective gain and offset settings. The 'TLD' section is also visible at the bottom.



Thanks for viewing



Life Imaging Center

SP8-I-STED

