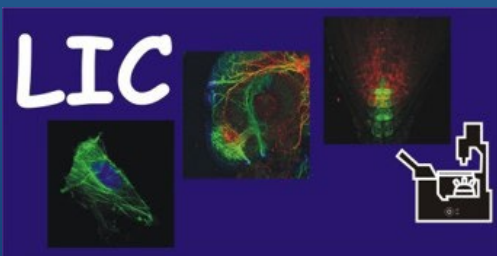


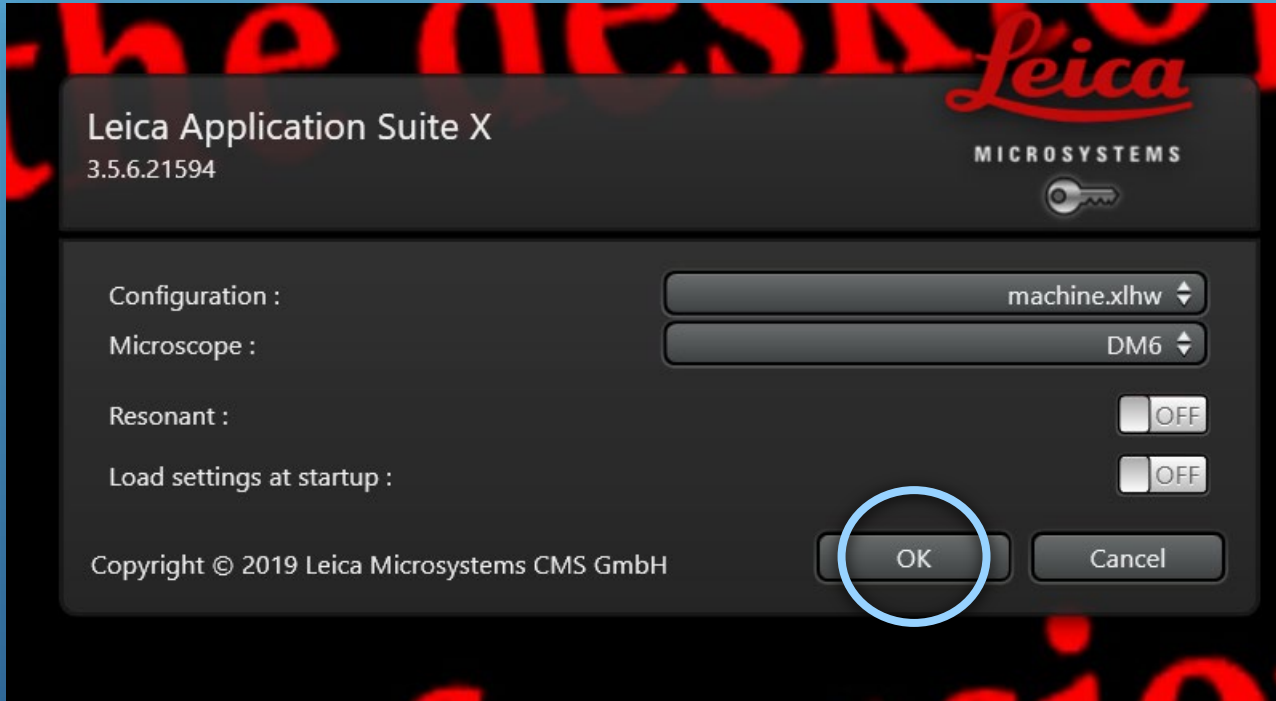
# Video Tutorial - Basic

**Leica Sp8-U-FLIM** (confocal use)

room 00.017

Life Imaging Center 2020

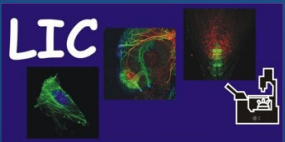


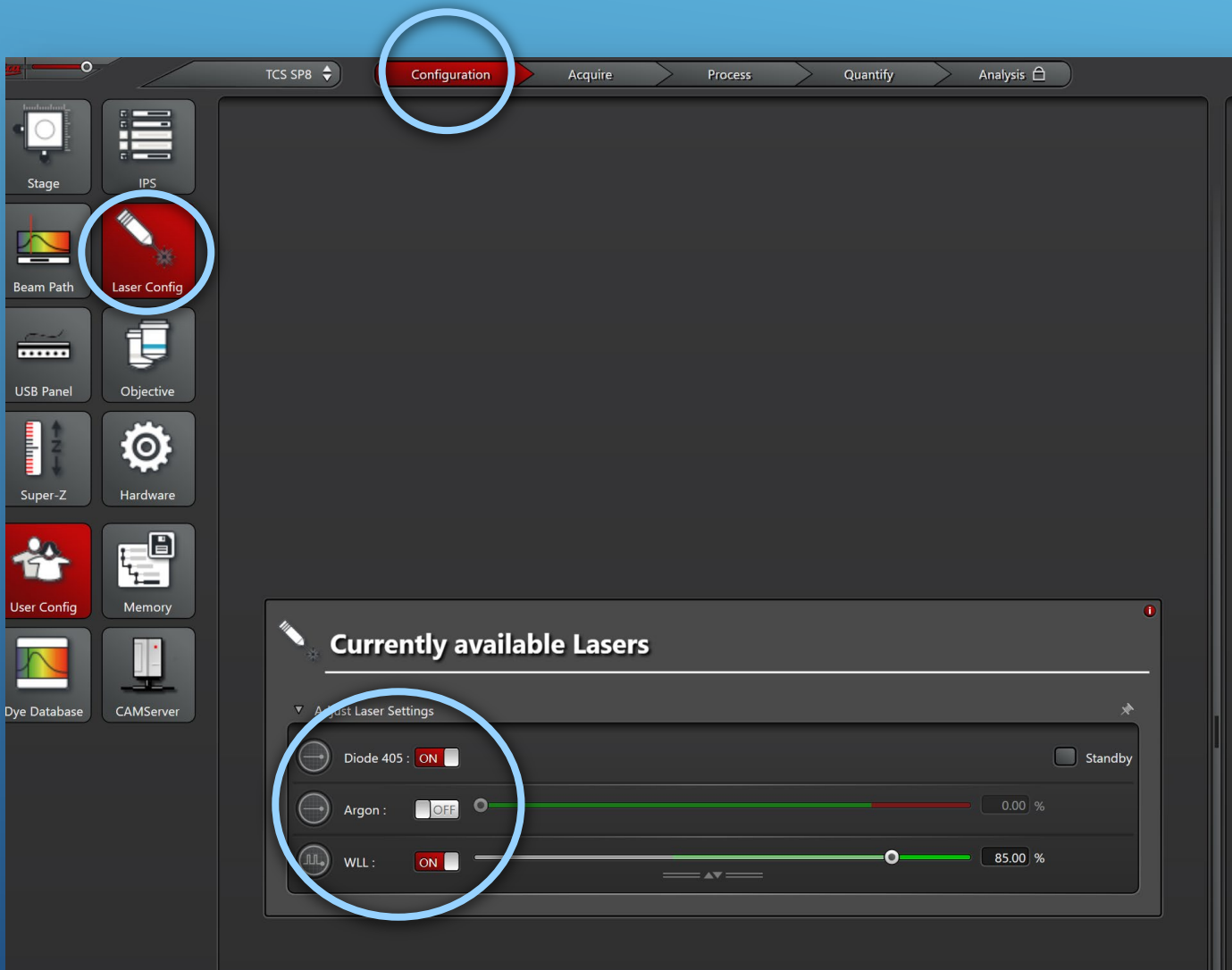


## Start Software

Start **LASX** Software

Press **Ok**



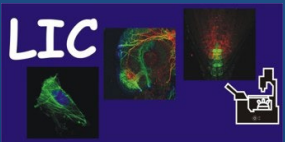


## Start Laser

Open **Configuration**  
Open **Laser Configuration**

Switch Specific Laser **ON**

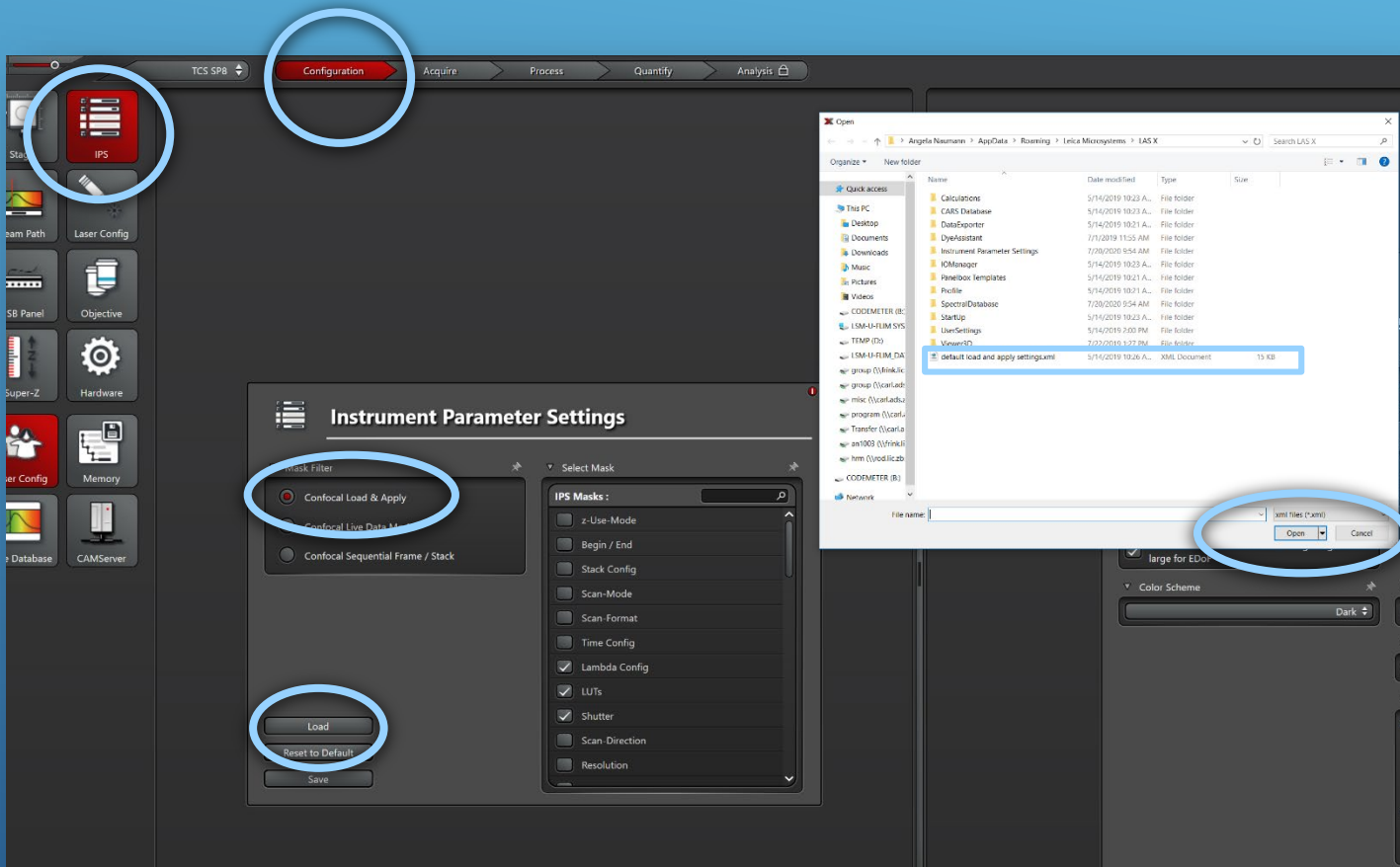
The **White Laser (WL)** will excite between 470nm-670nm.  
Leave the poweroutput on 85%



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SP8-U-FLIM

universität freiburg



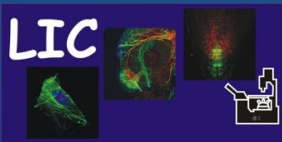
## Instrument Parameter:

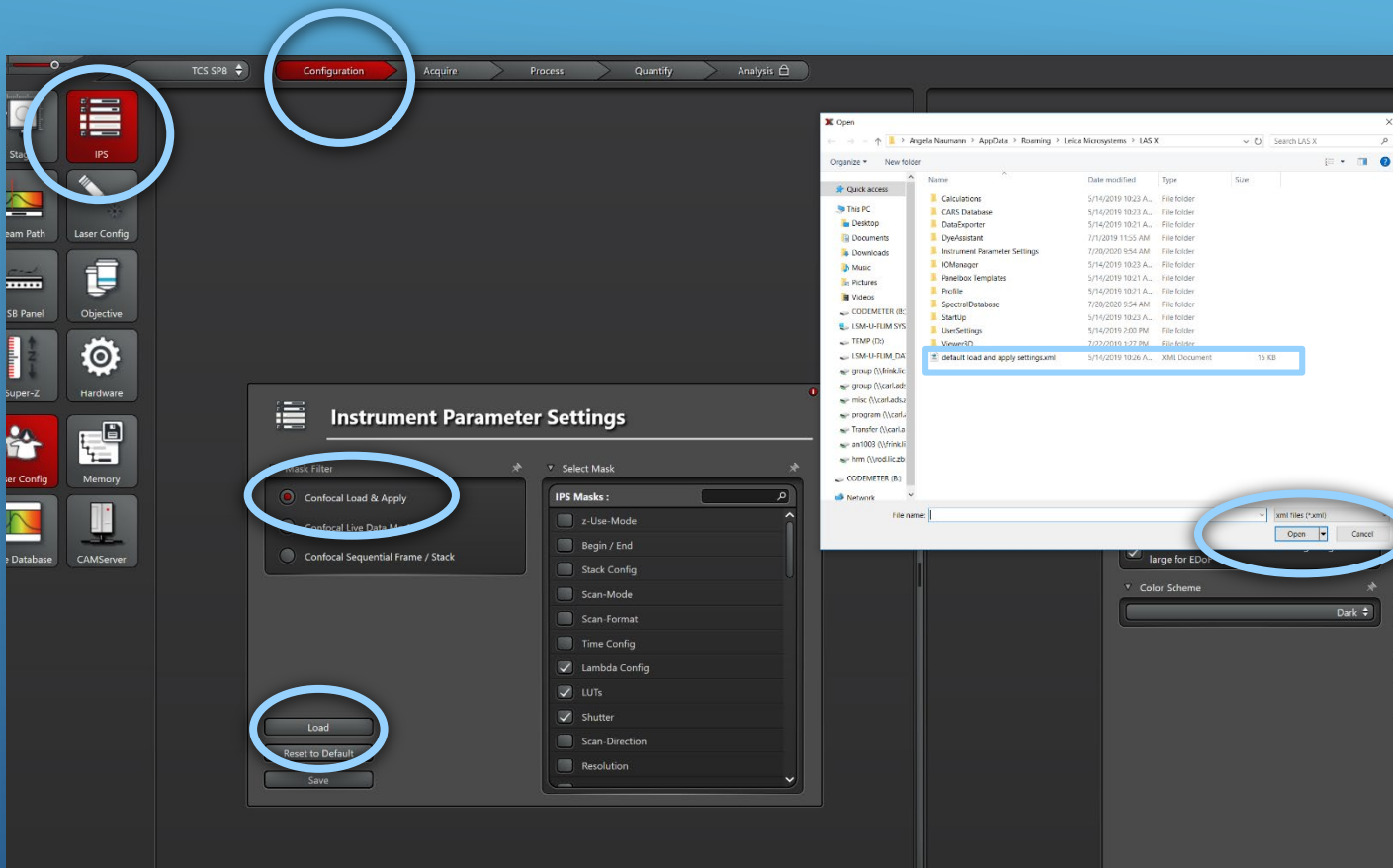
Open **Configuration**  
Open **IPS**

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

Window will open

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

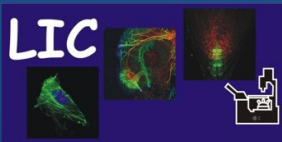


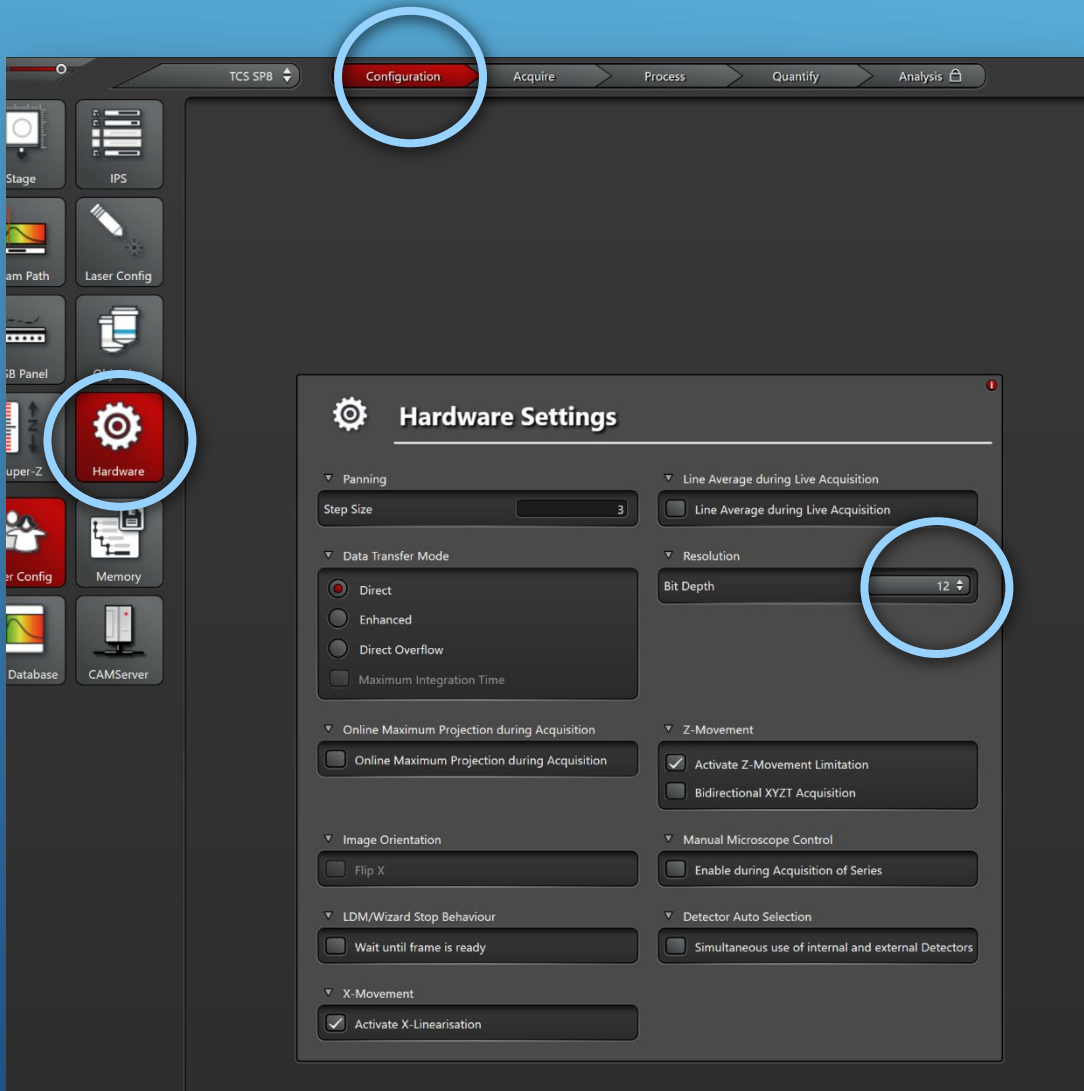


## Instrument Parameter:

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



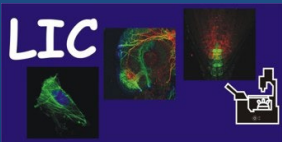


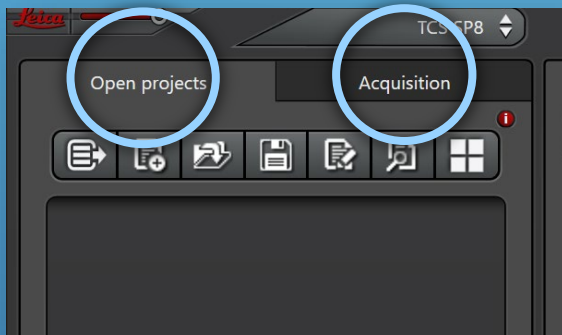
## Change Bit Depth

- 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**

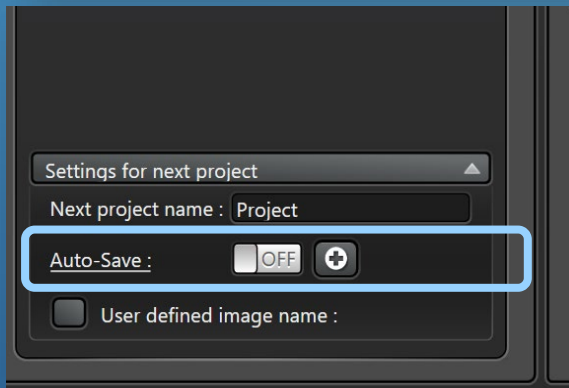




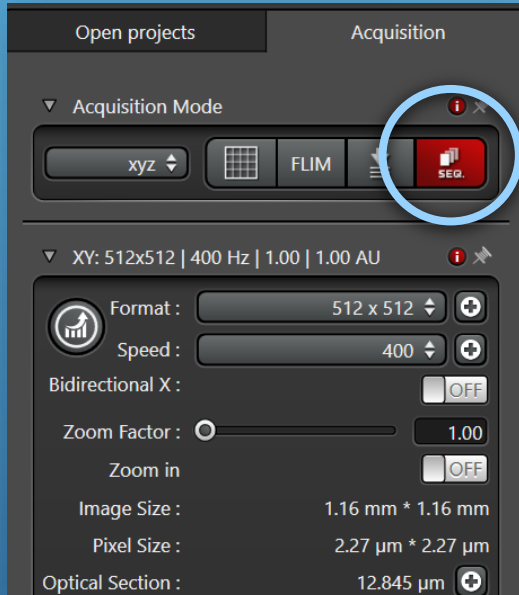
## Projects/Acquisition pane

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

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

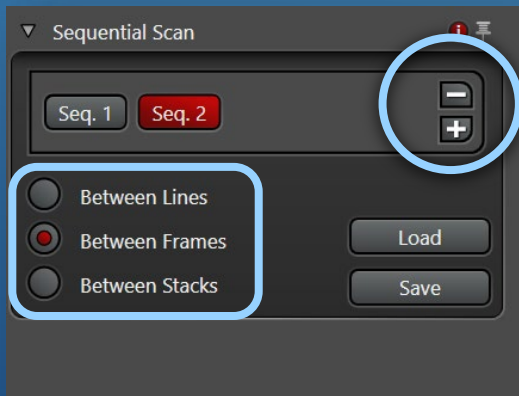


- As image data is captured (using *Capture image* or *Start* - Button in the *Acquisition tab*) it is **temporary** stored under this tab until the user tells the software to save the project
- Please **do not turn** on the **Auto-save function** (bottom of the Project tab)



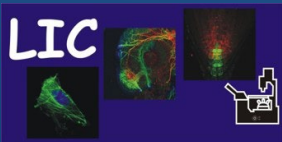
## Acquisition pane – Sequential Scan

The SEQ will indicate that you are scanning sequentially



Plus + and Minus - 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**



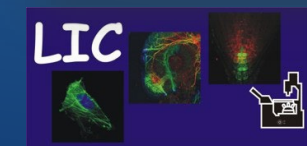


## Light Path - Laser & Detector

Access to **UV** (405) **VIS** (458, 476, 488, 496, 514)  
Wight-Light Laser – **WWL** (470 up to 670)

You can open the **Dye-Assistant**.

- Choose fluorochromes from the drop down list and detector type



# Light Path - Laser & Detector

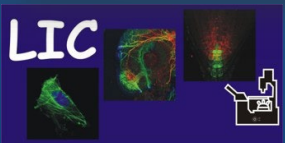
## Photomultiplier – PMT

- Used with bright signals ( like Dapi)
- Access to Gain and Offset

## Hybrid Detector – HYD

- Used with weak signals
- Extremely light sensitive
- No access to Offset
- Standard Gain is 100%

**Never turn laser (on) over an inactive or active detector**



## Hybrid Detector - Modi

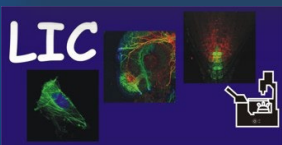
**Standard** mode used for default image acquisition. Start with gain on 100% and adjust the laser power

**Counting** mode will display the image based on the number of photons detected per pixel over a constant integration time

- used for ratio imaging and image correlation

**BrightR** mode makes it possible to display very bright and weakly fluorescent structures within an image.

- used for ratio imaging and image correlation

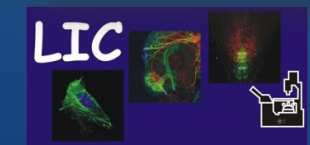


# Hybrid Detector - Gating

**Gating** defines **Start and End** of image acquisition in relation to the laser pulse by using the slider

- Could be used to exclude the background generated sometimes by excitation light

The screenshot displays the Leica SP8-U-FLIM software interface. At the top, there are menu options like 'Load | Save | Roi' and 'Load/Save single setting: Leica Settings'. Below this, a color calibration bar shows various wavelengths (405, 458, 476, 488, 496, 514, 470, 648) and their corresponding intensity levels. A central diagram shows the specimen and the internal detector setup. Below the diagram, there are controls for the objective (HC PL FLUOTAR 10x/0.30 DRY) and the fluo turret (Scan-BF). The 'Internal' section shows a graph of the laser pulse and the detector response. The 'HyD 5' section is highlighted with a red box, showing the 'Gating' checkbox checked and the 'Gain [%]' set to 100.0. The 'Gating' slider is set to 0.60 and 6.00. Other sections include 'HyD 3' and 'HyD SMD 4', both with 'Gating' checkboxes unchecked. The 'TLD' section is also visible at the bottom.



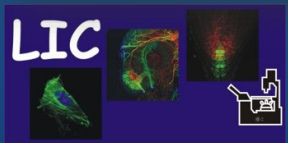
# Light Path - Laser & Detector

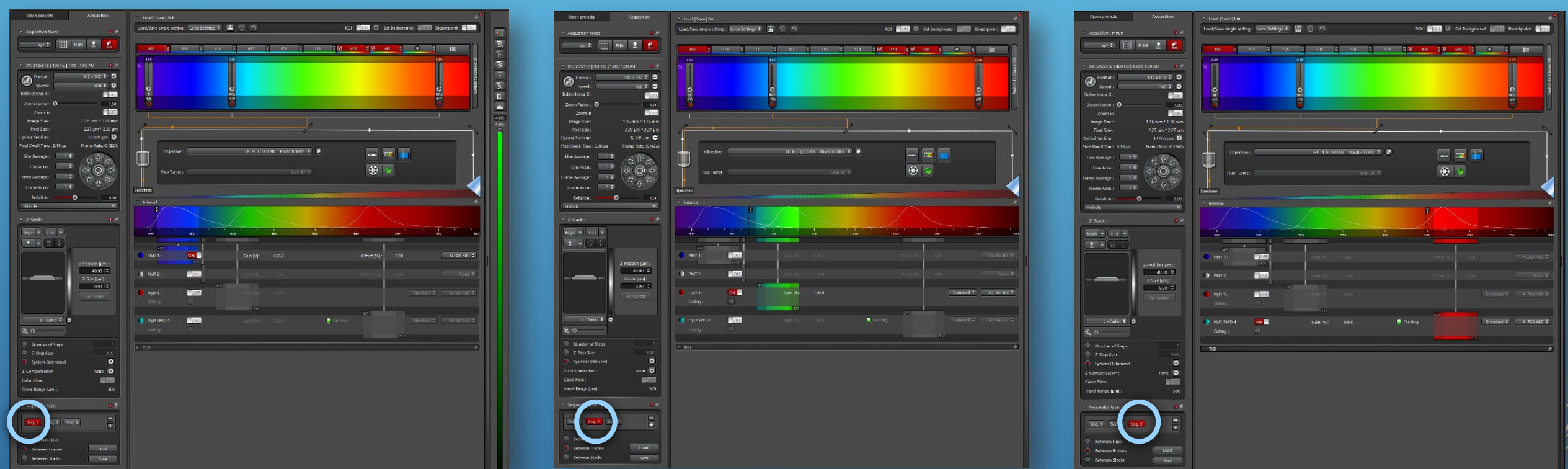
## Simultan - Scanning

- Example: here simultan scan with three laser lines:
  - Risk of bleed through
  - But fast

However we recommend : sequential scan

The screenshot displays the control interface for a microscope system. At the top, there are menu options like 'Load | Save | Roi' and 'Load/Save single setting: Leica Settings'. Below this, a horizontal bar shows various laser lines with their wavelengths (405, 458, 476, 488, 496, 514, 470, 648) and intensity levels (1.07, 2.13). A blue box highlights the 405, 470, and 648 nm lines. Below this is a schematic of the light path, showing the objective (HC PL FLUOTAR 10x/0.30 DRY) and the specimen. The 'Internal' section shows a spectral plot with three peaks corresponding to the laser lines. The 'Detector' section shows settings for four detectors: PMT 1 (ON, Gain 638.2, Offset 0.00, ALEXA 405), PMT 2 (OFF, Gain 0.0, Offset 0.00, None), HyD 3 (ON, Gain 100.0, Standard, ALEXA 488), and HyD SMD 4 (ON, Gain 100.0, Standard, ALEXA 647). A blue box highlights the PMT 1 and HyD 3 settings.

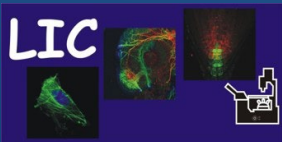


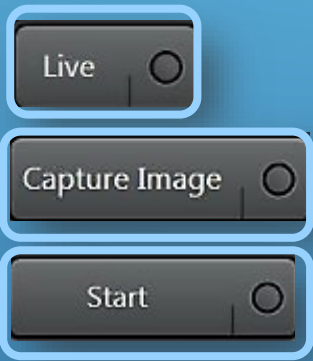


# Light Path - Laser & Detector

## Sequential - Scanning

- Example: here sequential scan with three laser lines:
  - Reduced risk of bleed through
  - Slower than simultan scanning

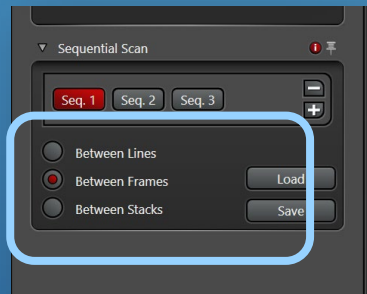




«**Live**» button [lower left corner] is used for a preview scanning

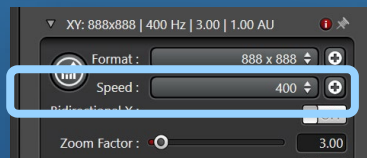
«**Capture Image**» will acquire just the active channel as image, no z-stack

«**Start**» button used to scan all channel, z-stack – start whole experiment



**Switch Seq.** between each

- **Line** - used for live sample,
- **Frame** or **Stack** used for fixed sample

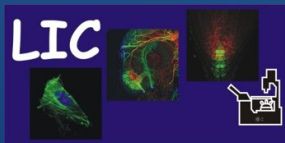


**Scan speed** can be adjusted individually

Scan speed is measured in Hz (lines per second) between 10 -1800Hz

- Lower speed – higher signal to noise ratio (SNR), risk of bleaching
- Faster speed – lower signal to noise ratio (SNR), low risk of bleaching

**We recommend as default speed: 600**



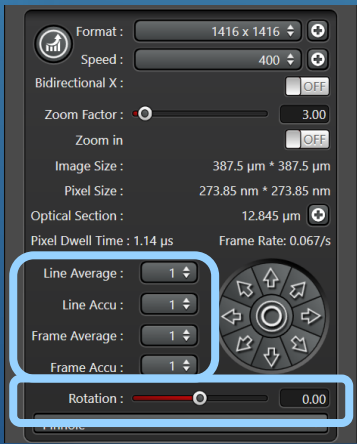


## Bi-directional

Bidirectional scanning will double the speed as pixels are recorded in both directions!



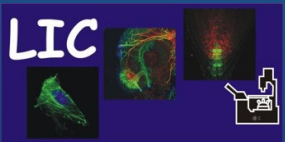
If you encounter mismatch in the phase, you can correct this with the control panel (phasecorrection).



**Average Function** (arithmetic mean as final pixel value)  
Used to improve image quality

- **Line** - recommended - especially for live sample
- **Frame** - used for weak and strong bleaching dyes

**Rotation** used to orientate the sample







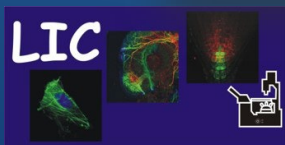
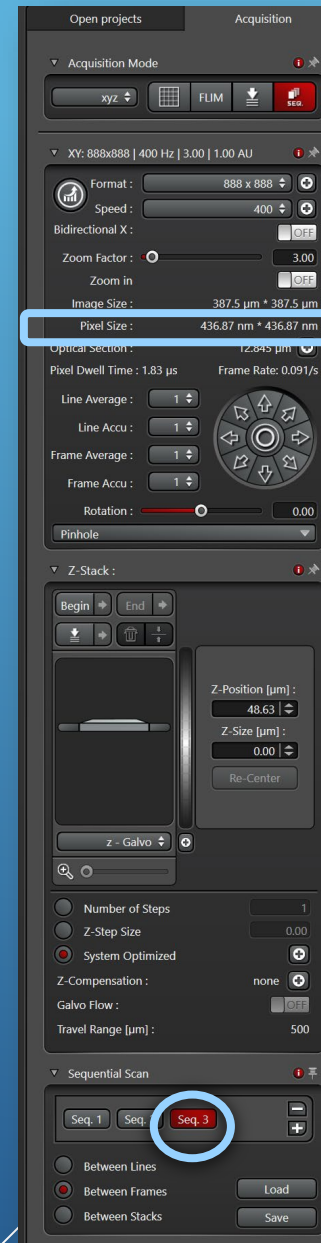
## Scan Format

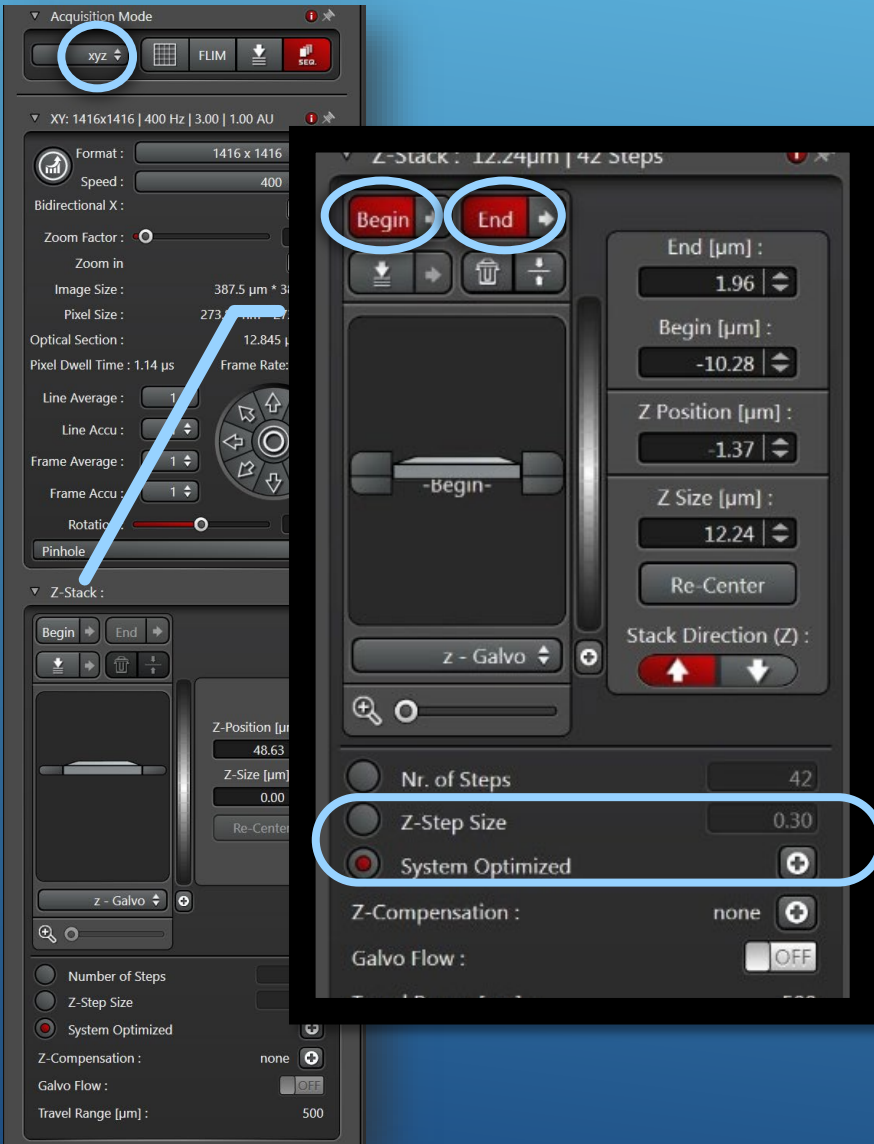
### Preview

- For preview scanning use 512x512 pixel as default

### Acquisition

- For optimal resolution, use the **«optimal»** button.
- This will take into account the NA of the objective and the active channel wavelength and **set up the recommend pixelsize**
- Therefore always optimize with the same Seq. active to avoid differences in your experiment





## Acquisition Z-stack

Make sure that you are in XYZ - Mode

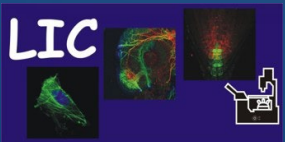
### Define begin and end

Press «**Live**» and move to the **top** of sample region of interest using the right z-position knob of the Leica panel tool in front of you – **click « begin »**

Move through the sample to the **bottom** of the region of interest– **click « end »**

### Define the z-step size

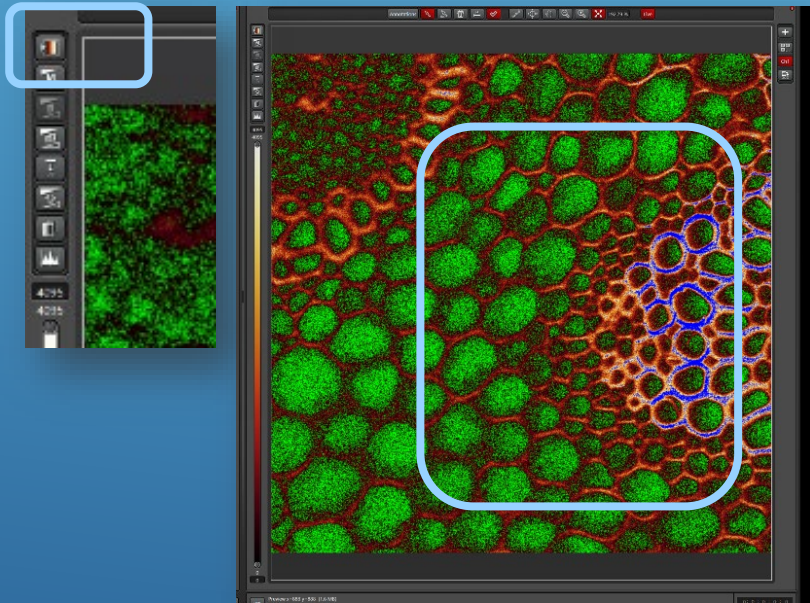
- manually (Z-step size) or
- choose « **optimized** », which calculates the best resolution of the system and set the size to 50% overlap. This is needed for high resolution and 3D reconstruction. **Recommended**



## Additional Tips - Range indicator

- «**Range indicator**» button
- top left at the image container
- used to avoid **over-and under-saturated pixel**

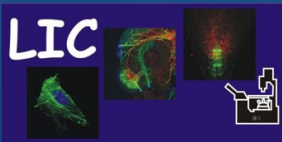
- « **Blue pixels** » saturated
- « **Green pixels** » undersaturated
- « **Black-red-yellow-white** » = good signal



# Thanks for viewing

## Additional Information

- Video Tutorial: Navigator Leica Sp8-U-FLIM



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SP8-U-FLIM

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