Usermanual for

Leica SP8 confocal

MIC
Molecular Imaging Center

Contact information: hege.dale@uib.no & endy.spriet@uib.no

Table of content

Important information

Before you start working:

- Booking is compulsory through the online MIC booking system.
- Report any problems or issues to responsible personnel (Hege or Endy).
- If the heating system is on it means a user needs it later in the day, so do not turn it off.

After you have finished working:

- Check whether the system is booket later right after you and make sure the user shows up! If there is a gap, turn off the system (lasers, mercury lamp, turn off the software but leave computer and hardware running).
- If you are the last user fo the day, turn off the complete system incl. pc.
- Fill out the logbook and comment on the performance of the system or failure description.

Start up procedure

- Switch on the 3 green buttons
- Turn the key to ON position
- Turn on the fluorescence lamp,
  note the lamp hours in the log book.

- Log on & start the LasX-software
  (the initialization takes a few minutes)

- Configuration = machine.xlhv
  Microscope = DMI8
  Activate Resonant scanner and
  AFC (auto focus ctrl) if needed.

- Remember to activate the lasers (WWL and Blue diode) under Configuration tab and open the Laser Config. icon.

- ***OBS! Never let the detector band passes overlap with any active laser lines, this is also valid for INACTIVE detectors.***

Shut down procedure

- Turn off the lasers under Configuration tab / Laser Config. Icon
- Close the LasX software
- Check the booking page to see if someone is coming after you.
- ***If yes**:* Leave system on, log out and fill in the log book (make sure the next user shows up!)
- ***If no:*** Continue below.
- Shut down the computer

- Turn off the laser emission key and the green buttons.
- Switch off the fluorescence lamp and note the hours in the log book.
- Fill in the log book.
- **Clean the objective lens & put on the plastic hood.**

Saving data to the Biomic server:

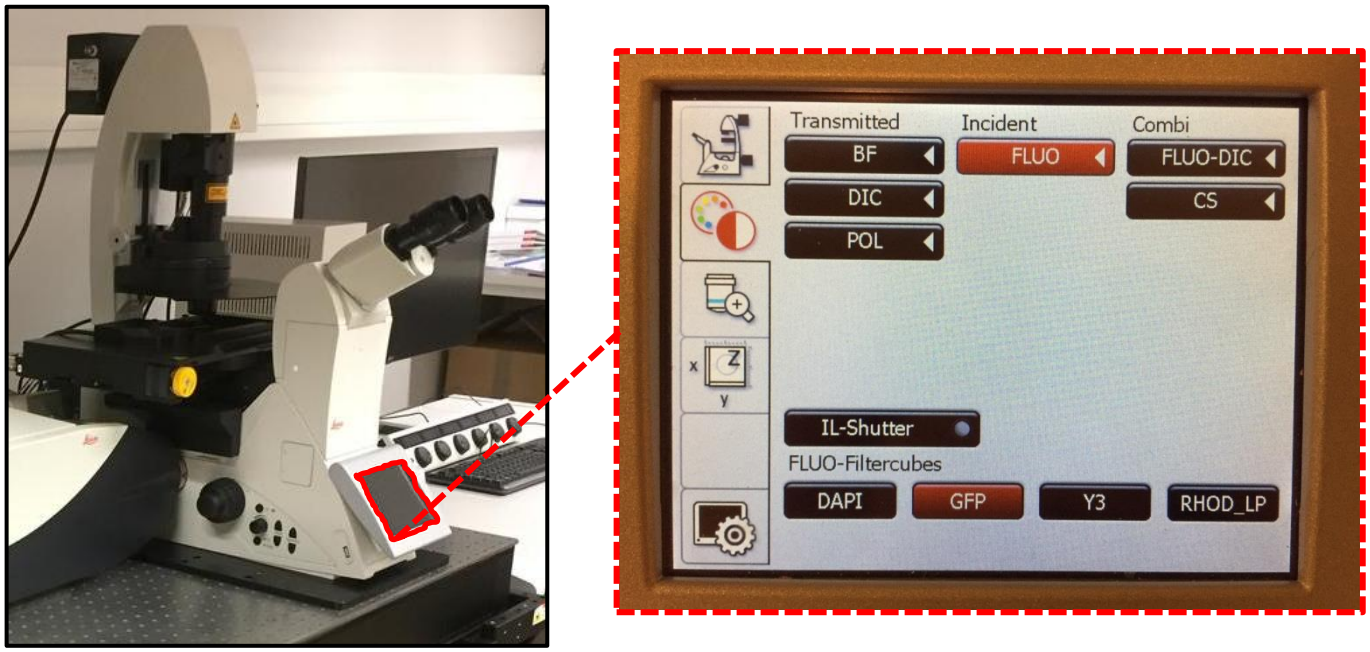
Mapping: \\klient.uib.no\felles\mofa\biomic

Log on: Username: **uib**your UiB username

Password: Your UiB password

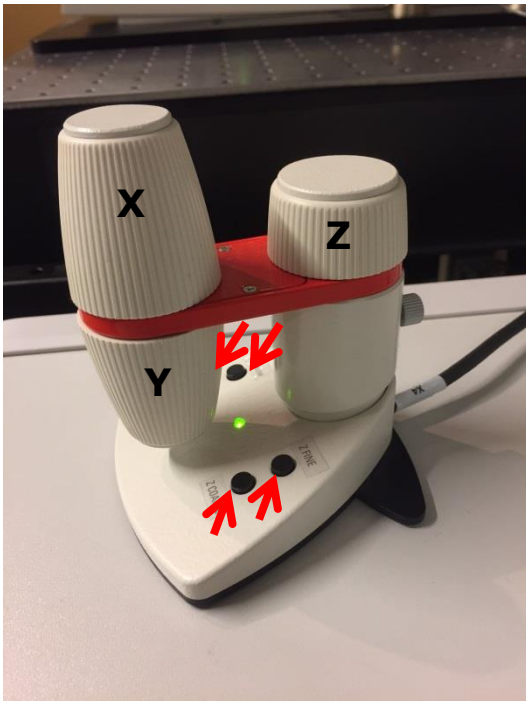
(You need access privileges to log onto the Biomic server, ask Torstein Ravnskog ravnskog@uib.no for this)

Operating the DMI 8 microscope stand



- There are few buttons you need to operate on this new microscope stand as everything can be operateded through the self-explanatory touch-screen.

- The x y and z controls are most useful with the joystick. Change sensitiviy from corse to fine when needed (arrows).



Objectives available

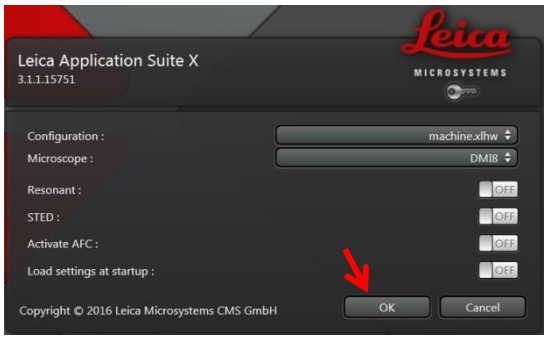
Magnification	NA	Type	Immersion media	Working distance (mm)	Suitable sample
20x	0.75	HC PL APO CS2	IMM (water, glycerol, oil)	0.68	
25x	0.96	HC Fluotar VISIR	water	2.4	
40x	1.1	HC PL APOnotCORR CS2	water	0.65	
63x	1.3	HV PL APO CORR CS2	glycerol	0.3	
100x	1.4	HC PL APO STED WHITE	oil	0.13	

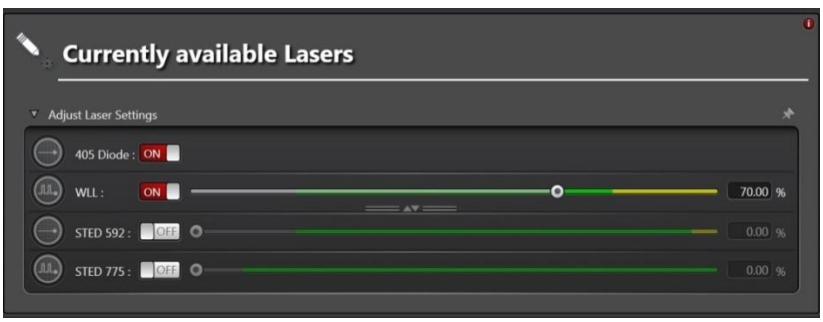
Lasers available on the system

The system is equipped with a 405nm laser and a white laser which emits wavelength in the range of 470nm – 670nm, suitable for all fluorochromes on the market in this range.

Starting the LAS X software

- Start LAS X (it's slow!)
- Leave configuration and microscope to «machine» and «DMI8».
- Turn on resonant scanner if planning to do live-cell imaging
- Activate AFC if planning long timelapse experiments.
- Once the software has initialized you can choose to maximize the icons with the slider at the top left.
- Turn on the lasers under «configuration-laser config».
- The 405 nm diode is used for blue fluorochromes (like dapi and hoechst).
- The White Laser (WL) will excite between 470nm-670nm. Leave the power output on 70%.





Select the appropiate objective

- Select the objective you want to use either inside software or on microscope ctrl panel (do not fysically turn on microscope due to movement restriction).

- Objective available:

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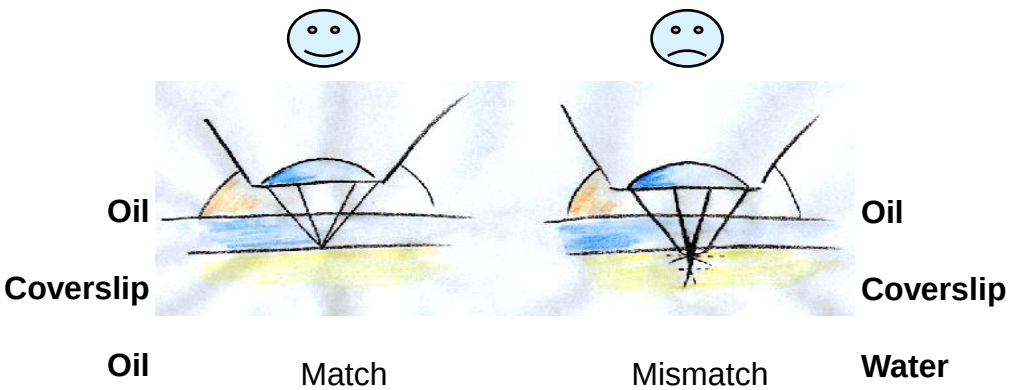
20x	0.75	HC PL APO CS2	IMM (water, glycerol, oil)	0.68	
25x	0.96	HC Fluotar VISIR	water	2.4	
40x	1.1	HC PL APOnotCORR CS2	water	0.65	
63x	1.3	HV PL APO CORR CS2	glycerol	0.3	
100x	1.4	HC PL APO STED WHITE	oil	0.13	

- For more details, go to configuration – objective.

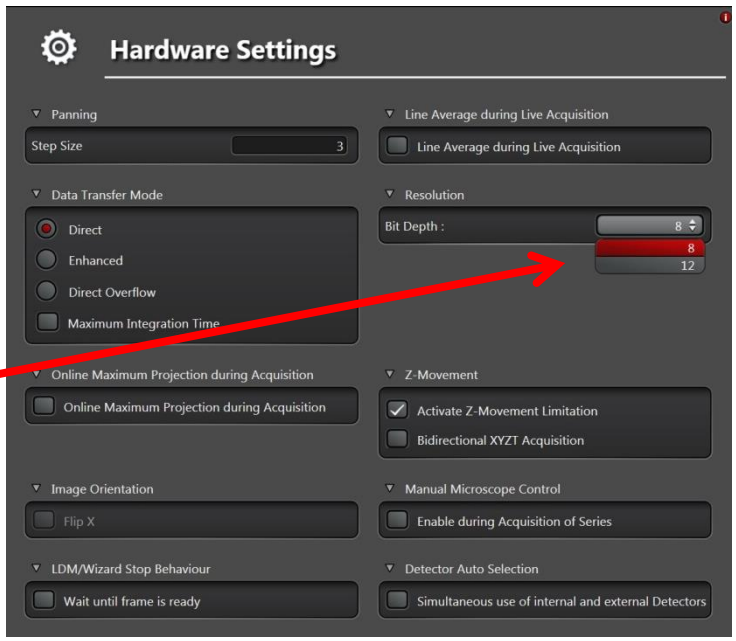
- Make sure you match the immersion media with the mounting media. Mismatch will lead to loss of intensity and resolution.



Customize control panel

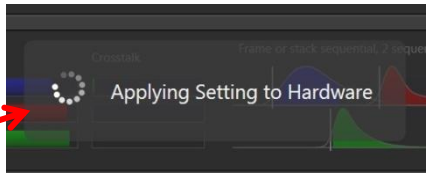
- For easier and smoother working, customize the control panel to you needs.
- Both item and sensitivity can be changed from the drop down menys.

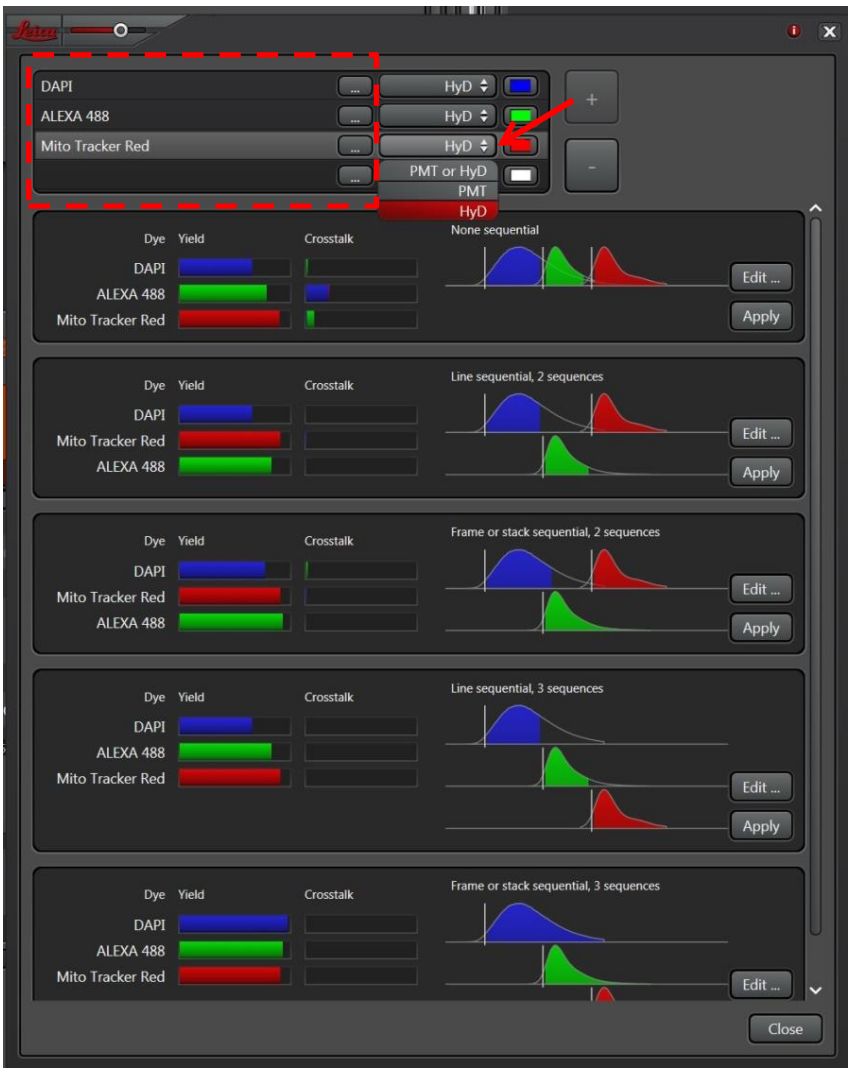
Bit depth and quantification



Setting up a sequencial scan

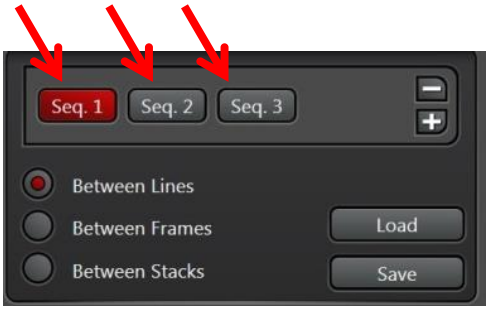
- Open the **dye assistant**.
- Choose your fluorochromes from the drop down list.
- Define which type of detector you need (read more about detectors on page…)
- For most optimal imaging, choose the **line sequencial** setting.
- Be patient for the hardware to initialize!





Setting up a sequencial scan

- The SEQ will indicate that you are scanning sequentially.
- Click between the different sequences (without scanning) and check that emission bands are not moving between sequences.



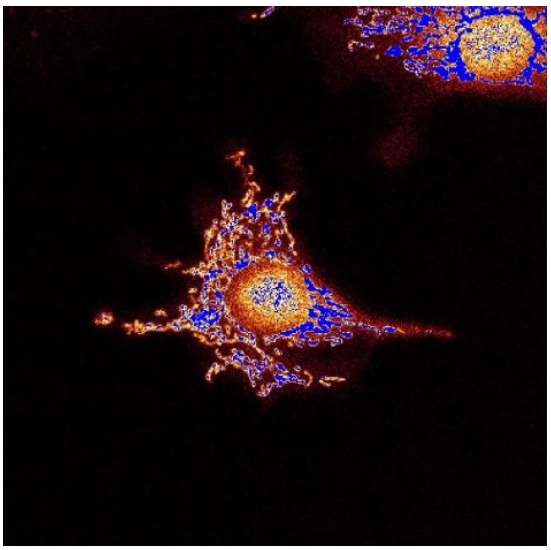
Lasers should always be active but lower the laser % to zero for the lasers that should not apply for the spesific seq. scan.

Image optmization

- We recommend to set scanning speed to 600 and turn on bidirectional X scanning.
- The «Live» button on the lower left corner is used for a preview scanning of the sample.
- The «Start» button on the lower right is used to acquire the image/stack.
- «Capture Image» will acquire just the preview image, not the whole stack or all sequences.

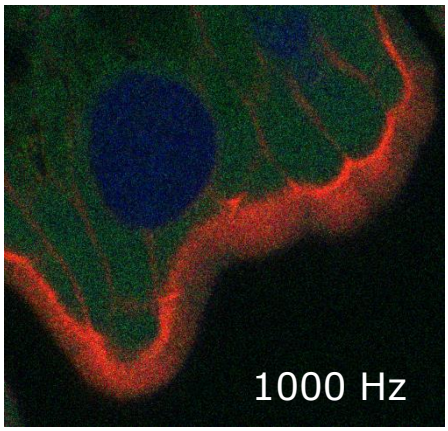
- Use the Quick LUT on a regular basis to make sure you stay away from over- and under-saturated pixels.

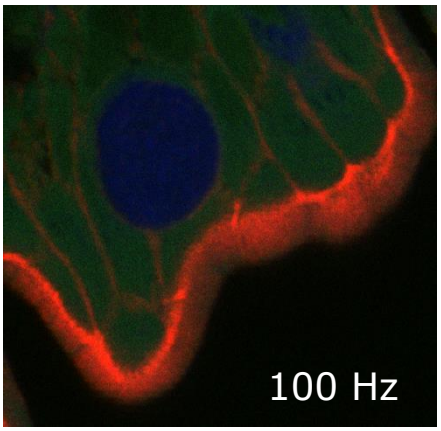
Blue pixels = saturated
Green pixels = undersatrated
Black-**orange**-**yellow**-**white** = signal between 1 and 255 (in a 8 bit image)



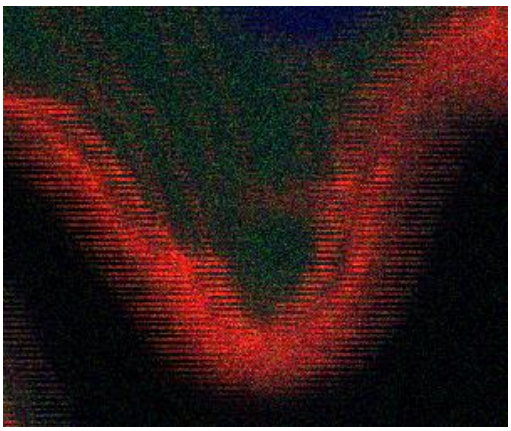
Scan Speed

- Scan speed is measured in Hz (lines per second). It refers to the frequency of two galvanometers which drive scanning mirrors, shading laser light across the specimen.
- You can select between 10-1800 Hz on the regular scanner while the resonant scanner is fixed to 8000 Hz (with min zoom of 1.25).
- The slower the speed, the higher singnal-to-noice ratio (SNR).

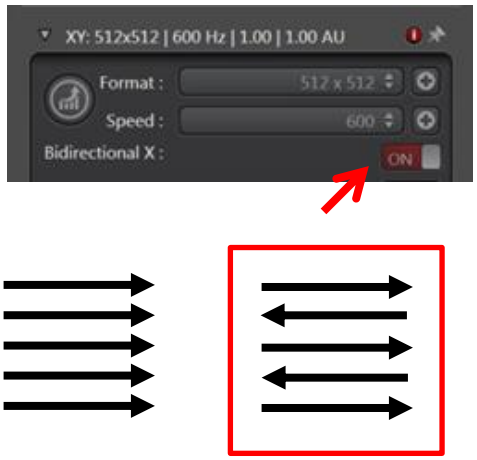




- Bidirectional scanning will double the speed as pixels are recorded in both directions!
- If you encounter mismatch in the phase, you can correct this with the control panel (phase correction).

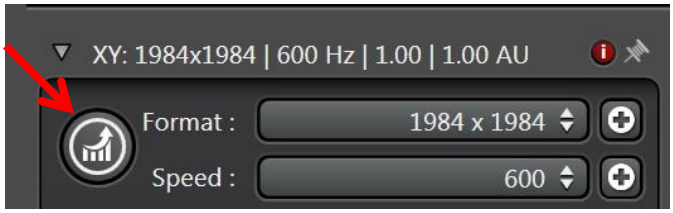


What mismatch in phase looks like.

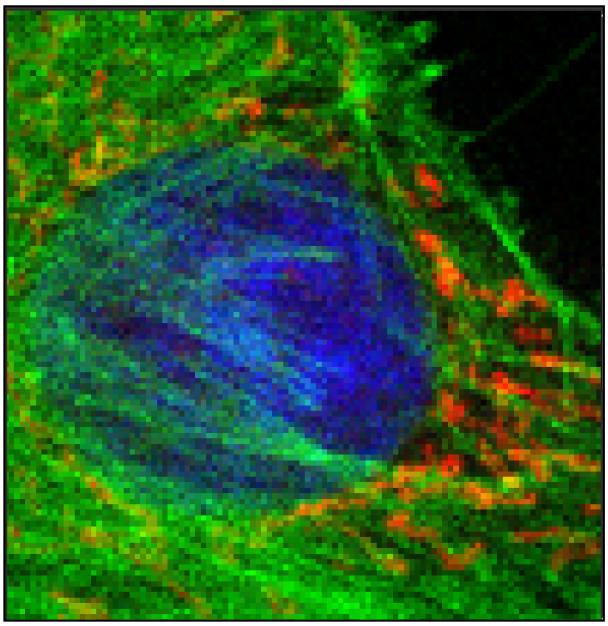


Scan Format

- For preview scanning, 512x512 pixels is reasonable (default).
- For acquiring the optimal resolution, use the **optimal format** button. This will take into account the zoom factor, speed and light wave. Always optimize with the same seq. scan active.

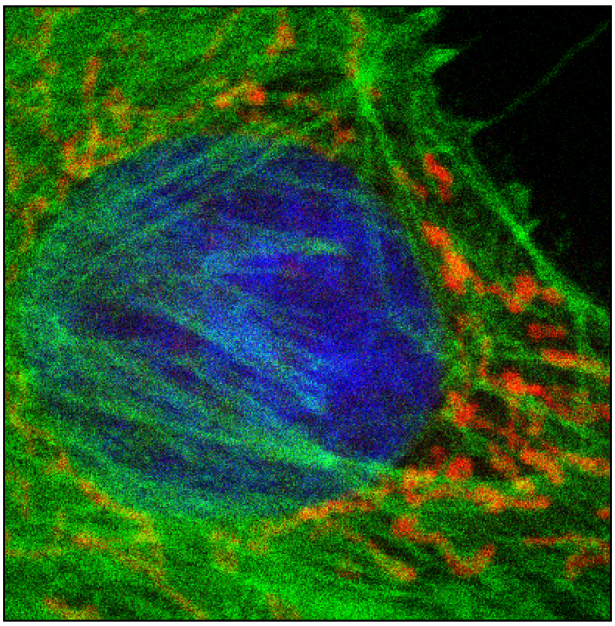


- Pixel size will be set to the best resolution for the objective, zoom used and wavelength used (differences occur if you use between lines or between frames).
- If scanning between frames, optimize when you are on the green channel.



512x512 (pixel size 227nm)

undersampling

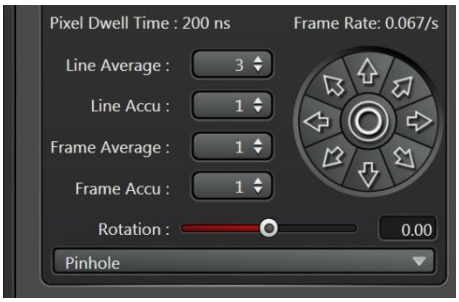


1984x1984 (pixel size 59nm)

correct sampling

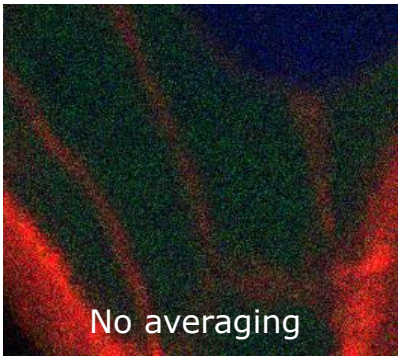
Averaging

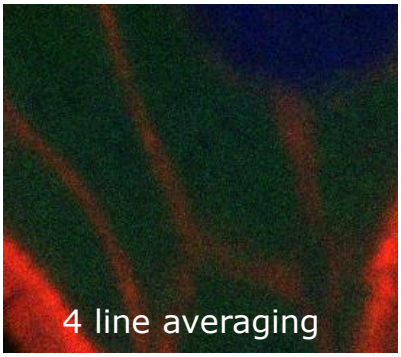
- To improve your image quality, you will need to apply averaging.

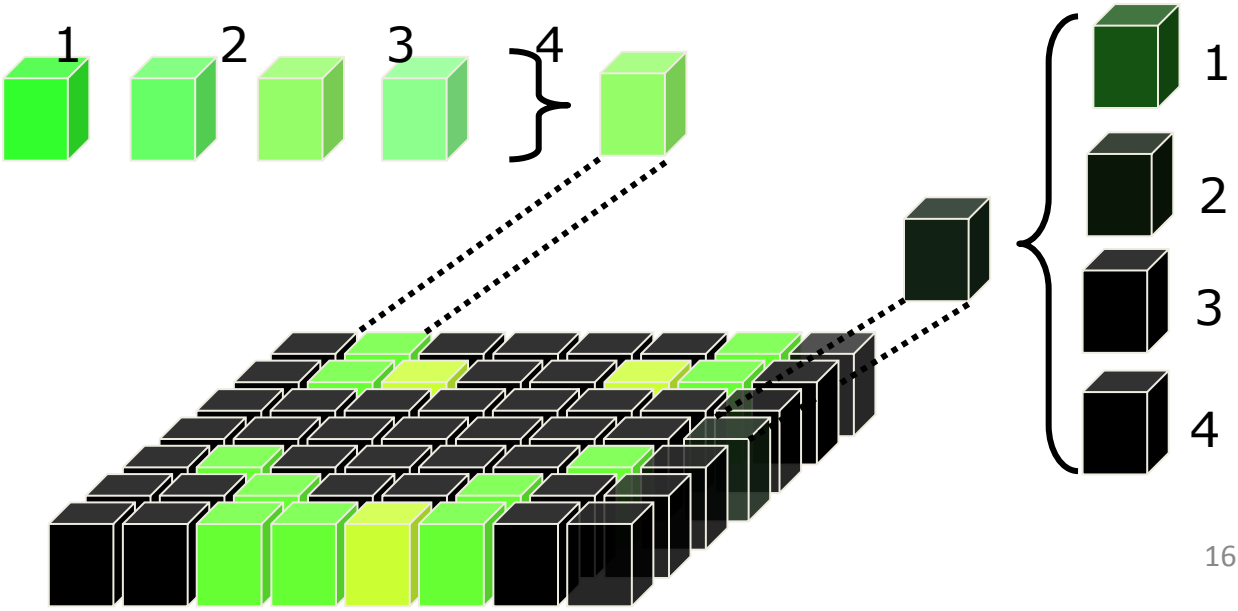


- You can choose between line averaging, frame averaging or a mixture of both.

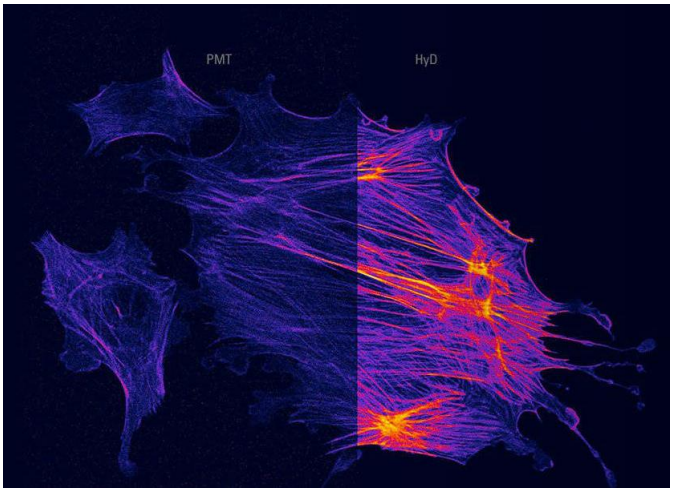
- Averaging takes the sum of pixels from the specified number of scan and uses arithmetic mean as the final value of the image. Averaging preserved those persistent pixel values that are mostly specific signal while divide away along times those fluctuated values that are mostly noise.





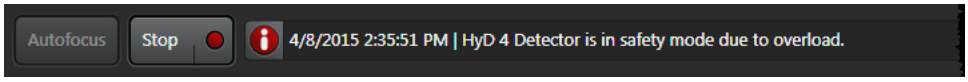


Photomultiplier tube (PMT) versus Hybrid detector (HyD)

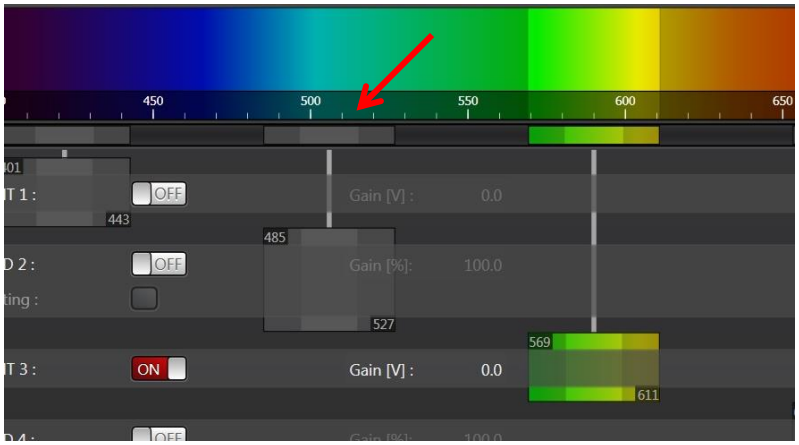


- Use PMT when you have very bright fluorescence.
- PMT will handle imaging with bright and faint signals.
- Use PMT when imaging reflection.

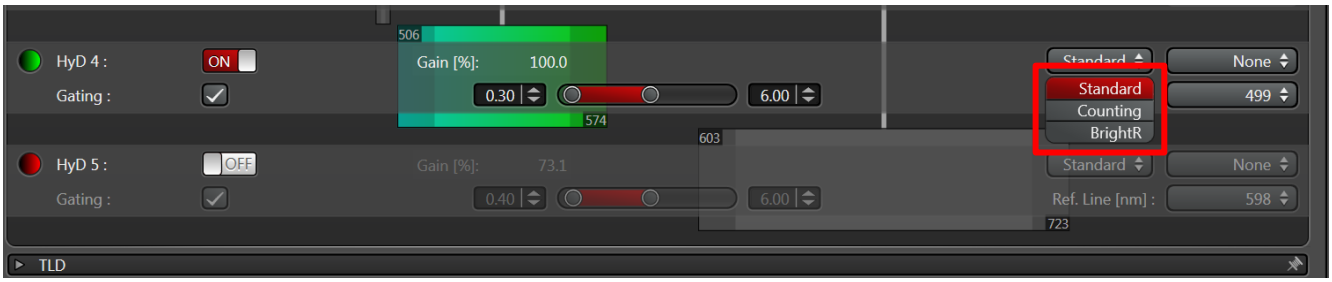
- HyD are extremely ligh- sensitive and high exposure will negatively effect their life. There is a shut down mechanism.
- Images will have less background noise.



- Never turn on a laser over an inactive or active HyD!



HyD modes: standard and counting



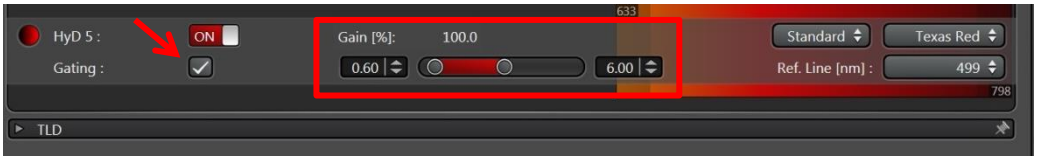
- The **standard** mode is used for regular image acquisition. Leave the gain on 100% and adjust laser%.

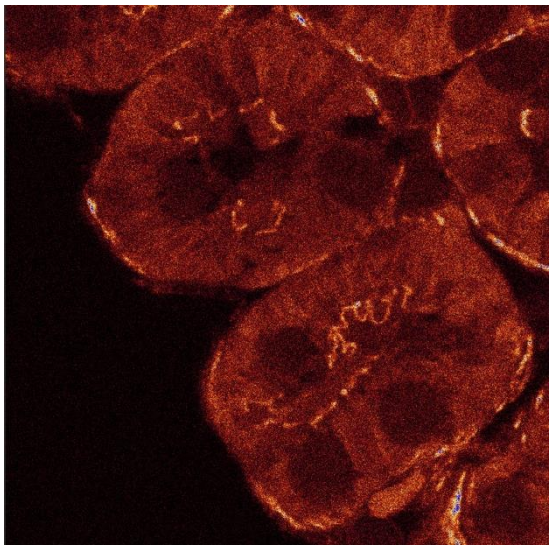
- **Counting** mode will display the image based on the number of photons detected per pixel over a constant integration time.
- Counting mode is used for ratio imaging and image correlation
- Pixels will fill up with photons like a basket. The higher the bit depth during this process, the larger the basket. A 10 grey-scale value will correspond to 10 photons.

- **BrightR** mode makes it possible to display very bright areas and weakly fluorescent structures within the specimen in an image. By amplification it brings out weak light signal in images with very bright and very dark areas.

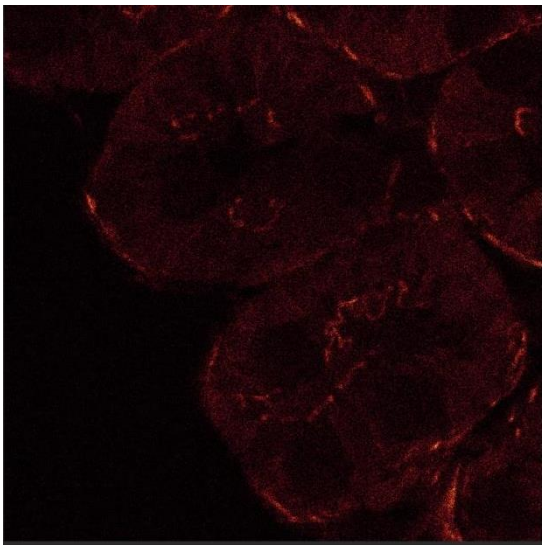
Hybrid detector (HyD) and Gating

By using gating you will define the start and end of image acuisition in relation to the laser pulse by using the slider. In this way you can exclude the background generated by excitation light.

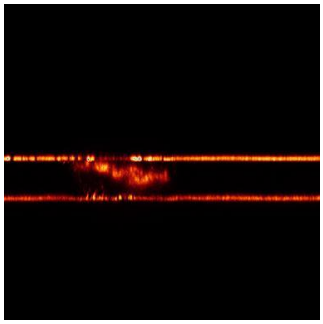


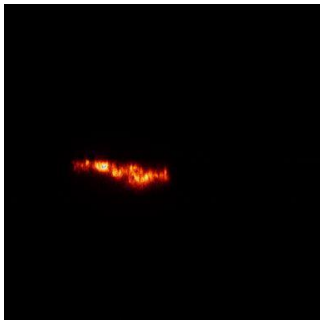


No gating



gating

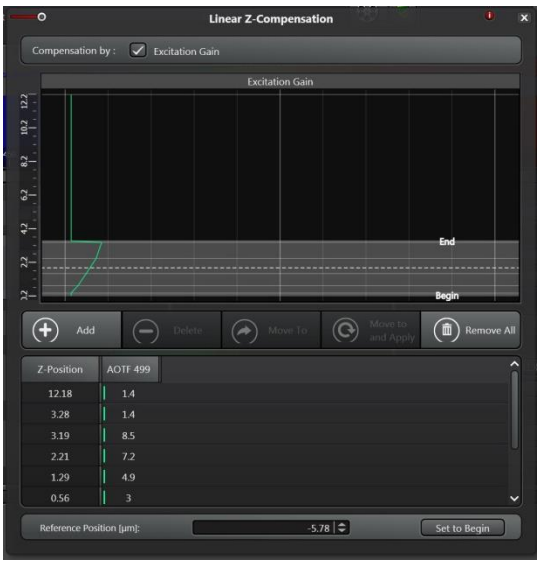


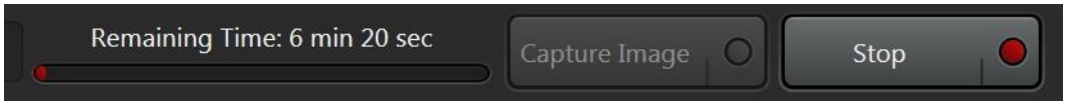


Gating will effectlively remove reflection from the coverslp (example shows xzy scan).

Acquiring a z-stack

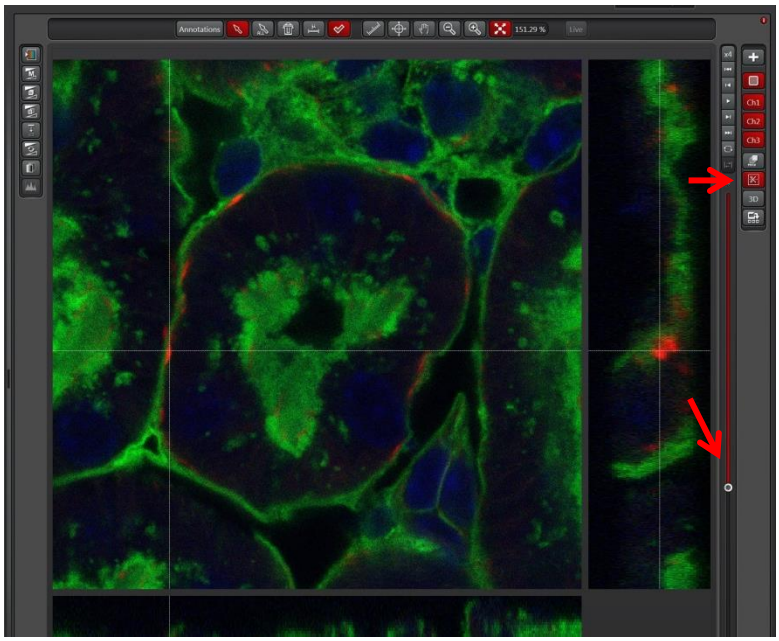
- Open the z-stack window (if it's not there, you do no not have the correct mode. Make sure you are in xyz or xyzt).
- Go to live mode and move to the top of the sample (or region of interest) using the z-position knob.
- Click on **begin** indicating your starting point. Move through the sample and click on **end** to define the bottom position.
- Define the **z-step size** of your choise. (0.6µm will acquire images back-on-bakc. 0.3µm should give you 50% overlap which is needed for 3D reconstruction). You can also leave the **system** to **optimize**.
- If you notice loss of signal in z, this can be **compensated** with linear AOTF control (there is also the option of voltage compensation if using PMT detectors). Always try to start the z-stack the furthest away from the objective.

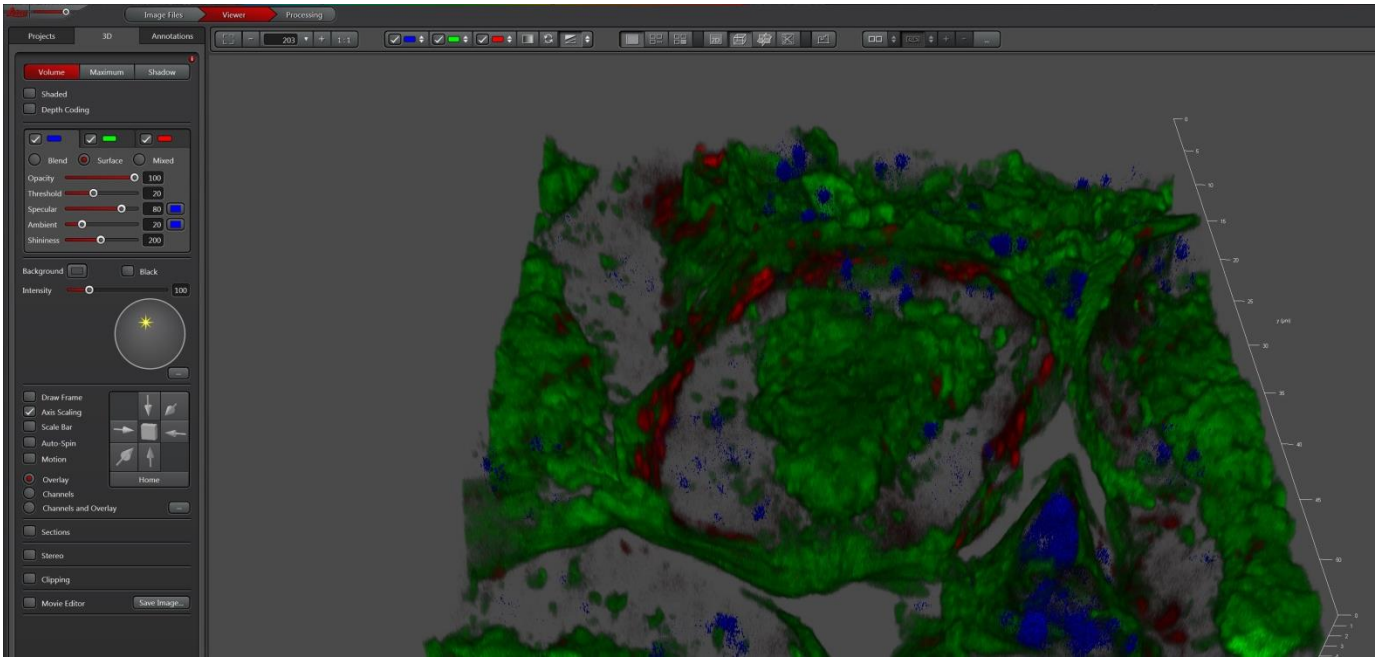




Visualization and handelig z-stack

- A stack can be visualized by moving the z slider.
- Orthogonal sectioning can be useful to visualize details in all dimensions in a 2D image.
- The 3D viewer is very useful. Here you can rotate the sample, create surface rendering, 3D orthogonal slicer and generate movies.

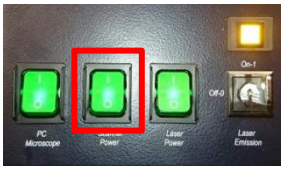




Live cell imaging – incubator and CO_2 control

Doing a successfull live cell imaging takes some planning ahead.

- Turn on the microscope (middle green button) the day before and leave it on for at least 6h to ensure stability in z.

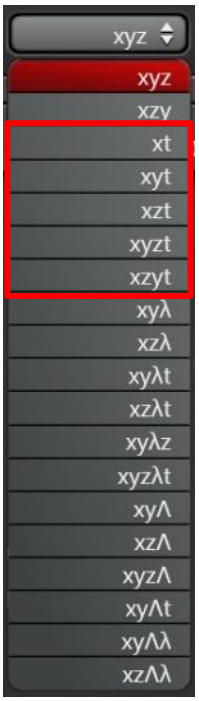


- Carefully mount the heating stage in place (remember to remove it after use and place the standard stage back on). Be careful not to loose any of the tiny screws. The stage is also very sensitive to pressure, so take care!

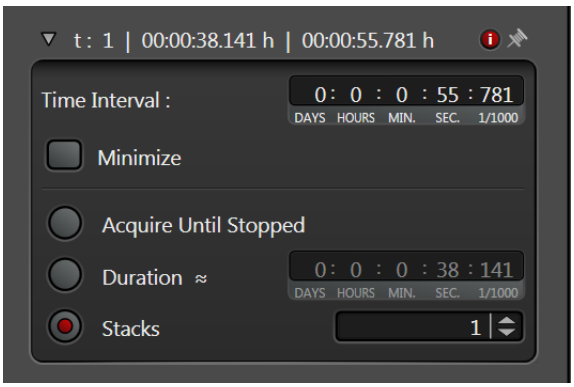
- Turn on the TokaiHit controller 30 min before imaging (main switch + objective heater and CO_2 controller).

- Carefully place the objective heater around the objective you will use. This will prevent the immersion media to cool down and change RI.

Live cell imaging – setup

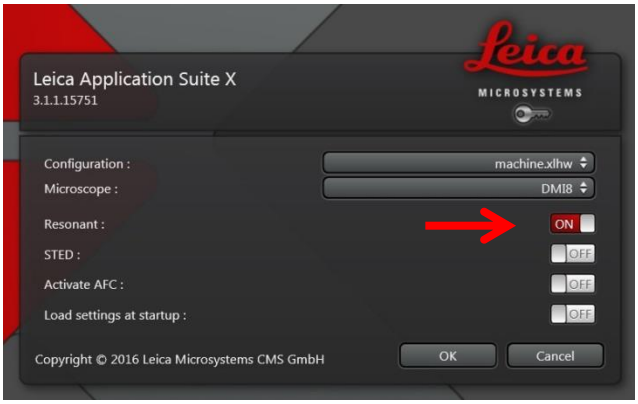


- Select a mode including a timelapse (t).
- The timelapse window will appear and interval and duration can be set.
- If you check «minimize» the system will acquire images non-stop.
- You can define interval and set the duration.
- Use «acquire until stopped» if you are sitting by the system and waiting for a specific for a special event to happen.

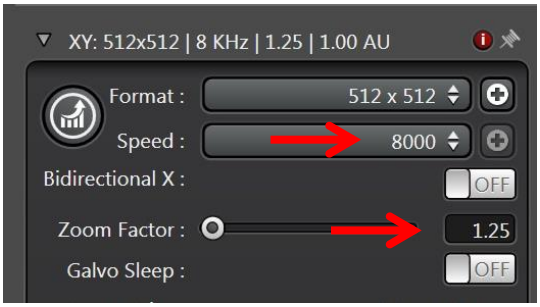


Live cell imaging – when speed is a factor

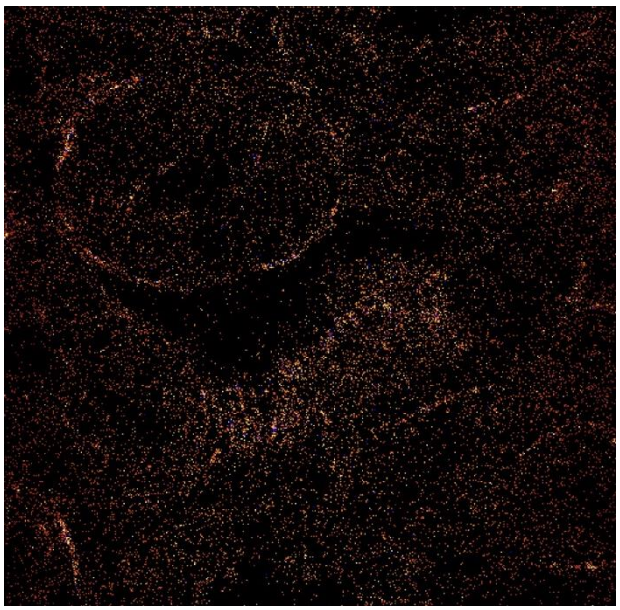
- If you need high speed heck «resonant» when starting up the system.

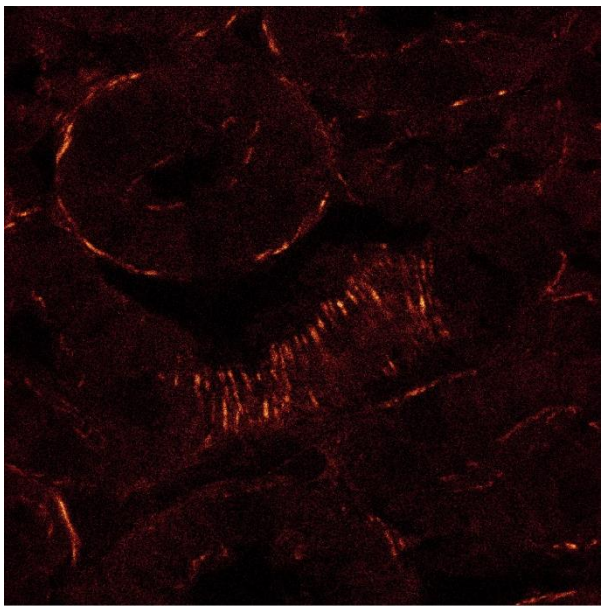


- Scan speed will be fixed at 8000Hz and the minimum zoom will be 1.25



- It is normal for the image to look pixelated and strange in live mode. Once you add averaging, the image quality will improve.



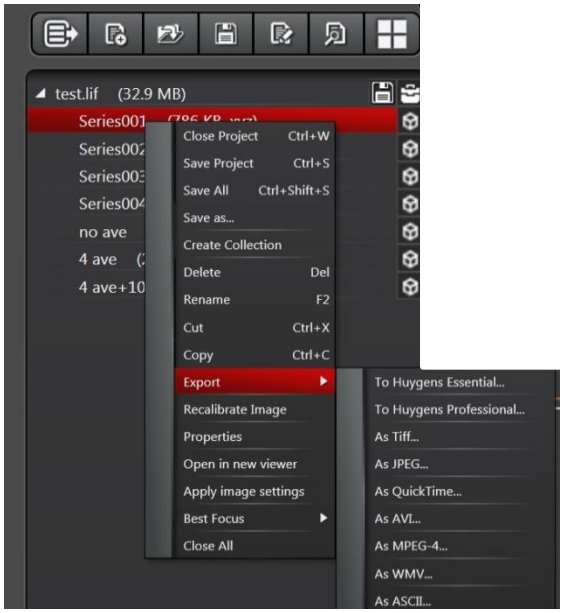


NB! The regular scanner can also be used for live cell imaging.

Saving and exporting data

- Always save the **raw data** as «**lif**».

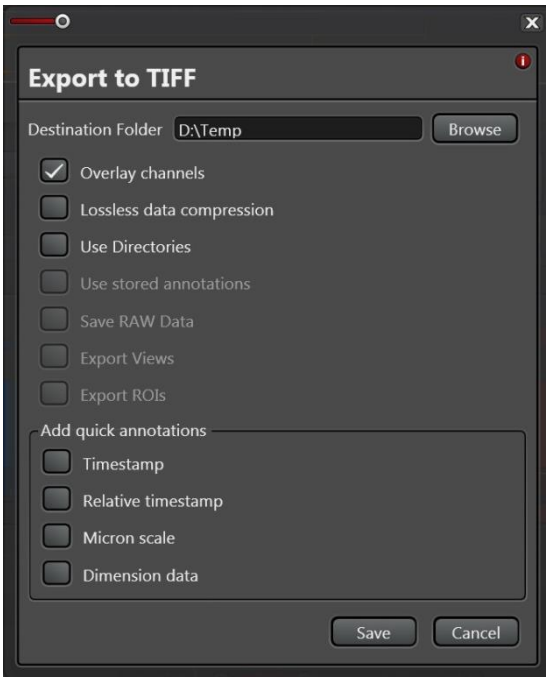
- To export your images, select either the whole folder (test.lif in this example) or a single image and right mouse click. We recommend exporting to **tiff**.



- Choose a destination folder (on the server!) and leave all boxes empty to export each channel separate with the colors.

- «save raw data» will export each channel separate but all in black and white.

- «overlay images» will export only overlay.



- You can also install the free **LAS AF Lite** software (only pc compatible!) which will handle both images acquired from SP5 and SP8.

LAS-AF-Lite_2.6.0_7266_Setup.exe

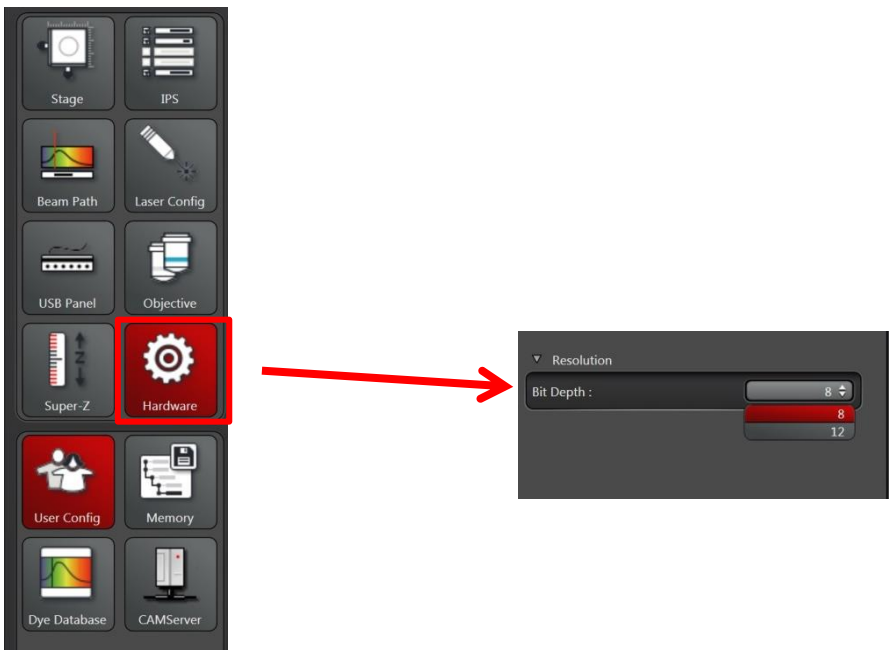
Bit depth – visualization or quantification

- The bit depth tells you how many grey levels there are in the image (or in each channel). Your eyes will not differantiate between 8 or 12 bit.

  8 bit = 256 different grey values
  12 bit = 4096 different grey values

- We use 8 bit for regular imaging and 12 bit for quantification analysis.
- The size of the image (megabite) increase with the bit.

- When you start up the LAS X software, the bit size will be 8 bit as default.




TCS SP8
Configuration
Acquire
Process
Quantify
Analysis
Open projects
Acquisition
Acquisition Mode
xyz
XY: 512x512 | 600 Hz | 1.00 | 1.00 AU
Format : 512 x 512
Speed : 600
Bidirectional X : ON
Phase X : -31.04
Zoom Factor : 1.00
Zoom in : OFF
Image Size : 116.25 µm * 116.25 µm
Pixel Size : 227.5 nm * 227.5 nm
Optical Section : 0.896 µm
Pixel Dwell Time : 800 ns
Frame Rate: 0.767/s
Line Average : 1
Line Accu : 1
Frame Average : 1
Frame Accu : 1
Rotation : 0.00
Pinhole
Z-Stack :
Sequential Scan
Seq. 1
Seq. 2
Seq. 3
Between Lines
Between Frames
Between Stacks
Load
Save
Load | Save | Roi
Load/Save single setting : Leica Settings
ROI : OFF
Set Background : OFF
Bleachpoint : OFF
ON
Constant Percentage
Switch to Conventional
Objective : HC PL APO CS2 100x/1.40 OIL
Fluo Turret : Scan-BF
Specimen
Internal
PMT 1 : OFF
HyD 2 : OFF
Gating :
PMT 3 : OFF
HyD 4 : OFF
Gating :
HyD 5 : ON
Gain [%]: 100.0
Standard
None
Mito Tracker F
xyz
xyz
xzy
xt
xyt
xzt
xyzt
xzyt
xyλ
xzλ
xyλt
xzλt
xyλz
xyzλt
xyΛ
xzΛ
xyzΛ
xyΛt
xyΛλ
xzΛλ