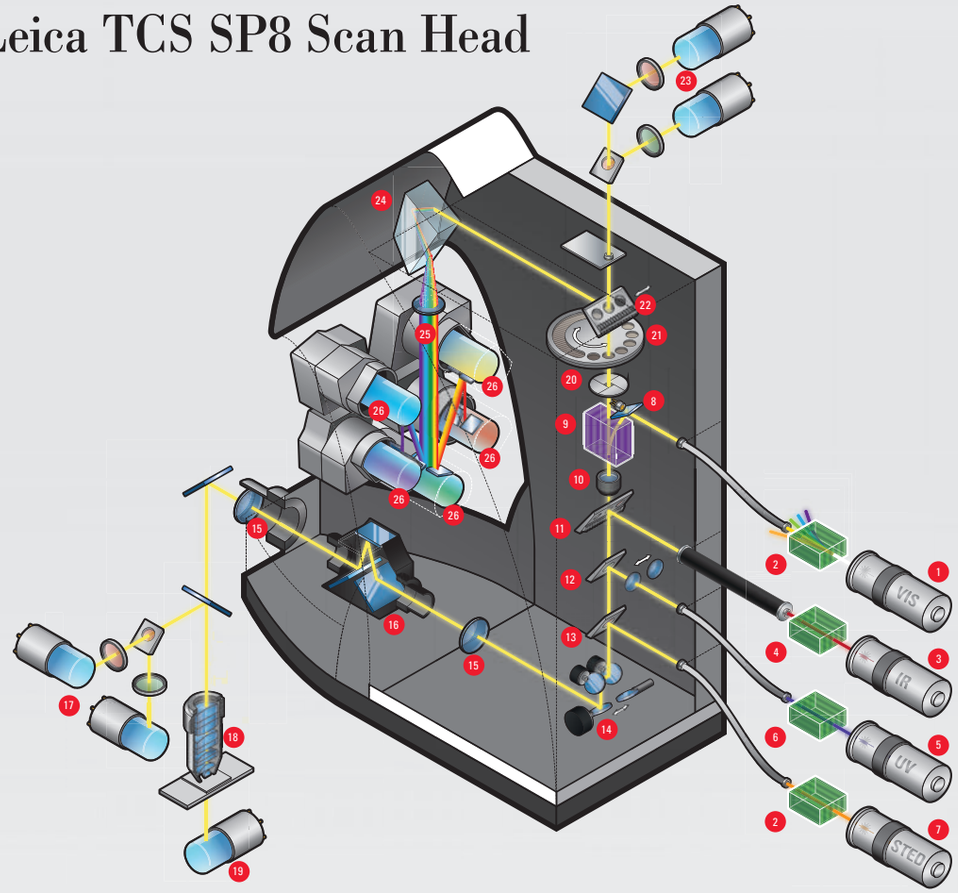
Leica TCS SP8 Scan Head

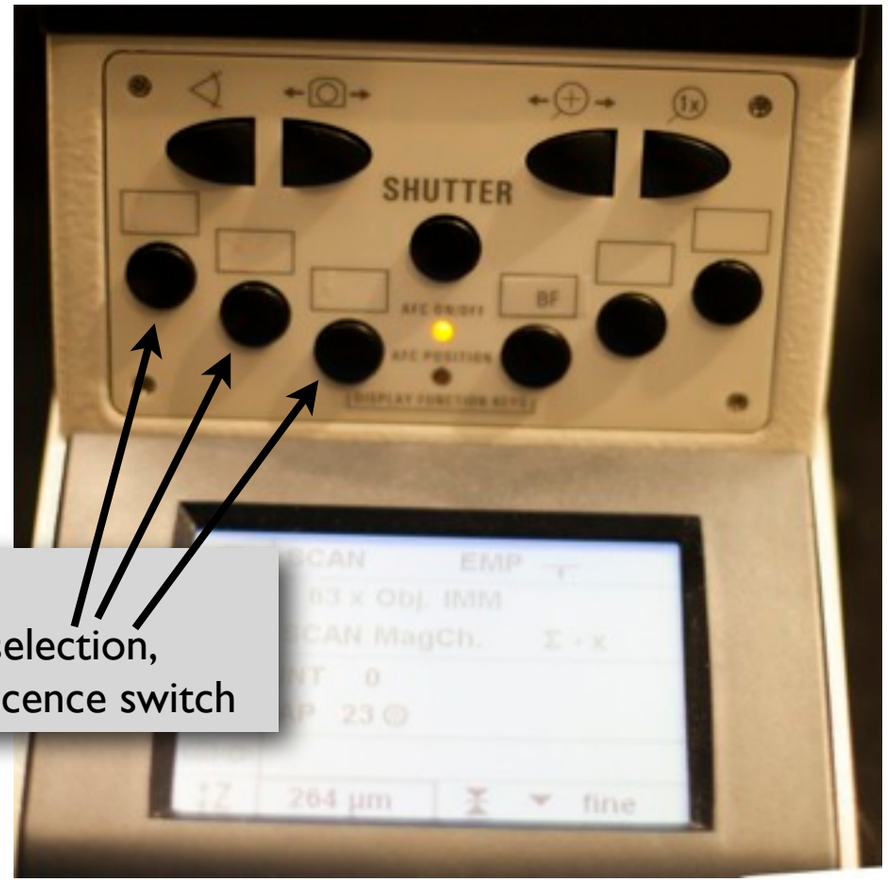


1 Visible line lasers or white light laser
2 Acousto-optical tunable filter (AOTF)
3 Infrared (IR) lasers *
4 Electro-optical modulation (EOM)
5 Ultraviolet (UV) lasers *
6 AOTF or direct modulation (DMOD)
7 STED laser *
8 Monitoring diode for Setlight
9 Acousto-optical beam splitter (AOBS), other options av
10 FRAP Booster *
11 IR laser incoupling
12 UV laser incoupling with CS2 UV optics
13 STED laser incoupling
14 FOV Scanner with Tandem Scanner option
15 Scan optics with alternative UVIS, HIVIS or VISIR coating
16 Scan field rotation (Abbe-König rotator) *
17 Reflected light detection (RLD) in non-descanned position *
18 Objective lens (different options available)
19 Transmitted light detection (TLD) in non-descanned position *
20 Square confocal pinhole
21 Fluorifier disc *
22 Outcoupling with X1 port *
23 External detection *
24 Prism-based dispersion
25 SP Detectior with spectrophotometer arrangement
26 Up to five photo-multipliers (PMT) or up to four hybrid photo-detectors (HyD)*

*optional

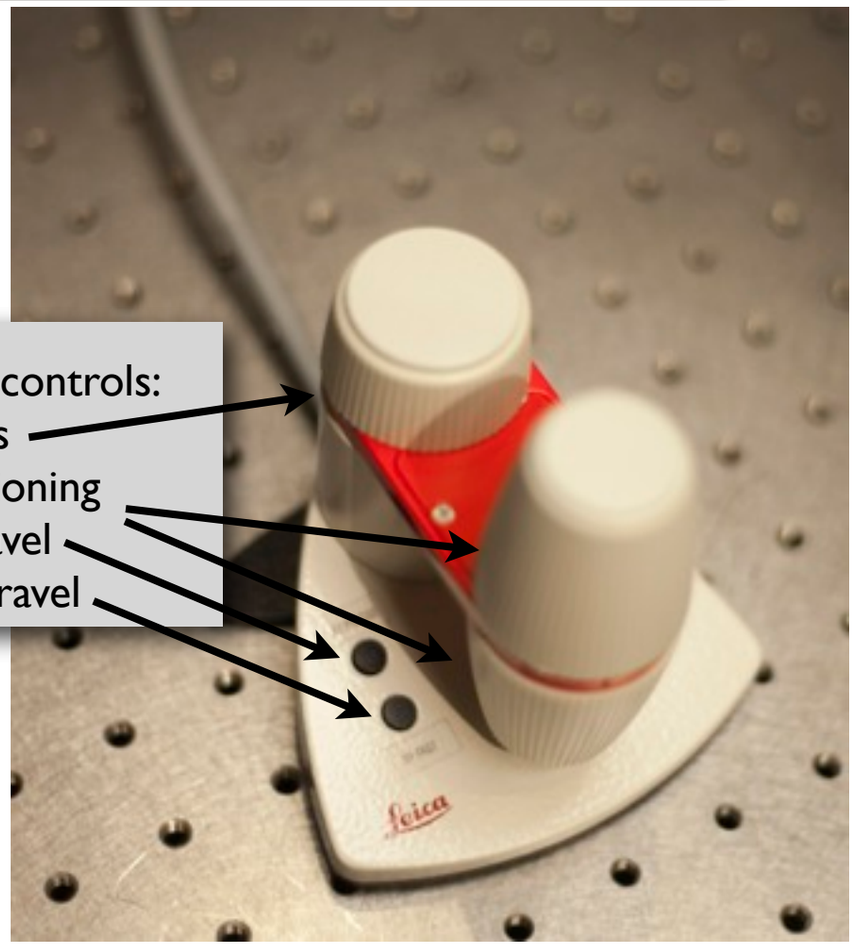
System operation

Scope left side:
TL/IL switches on/off transmitted light,
INT adjusts intensity



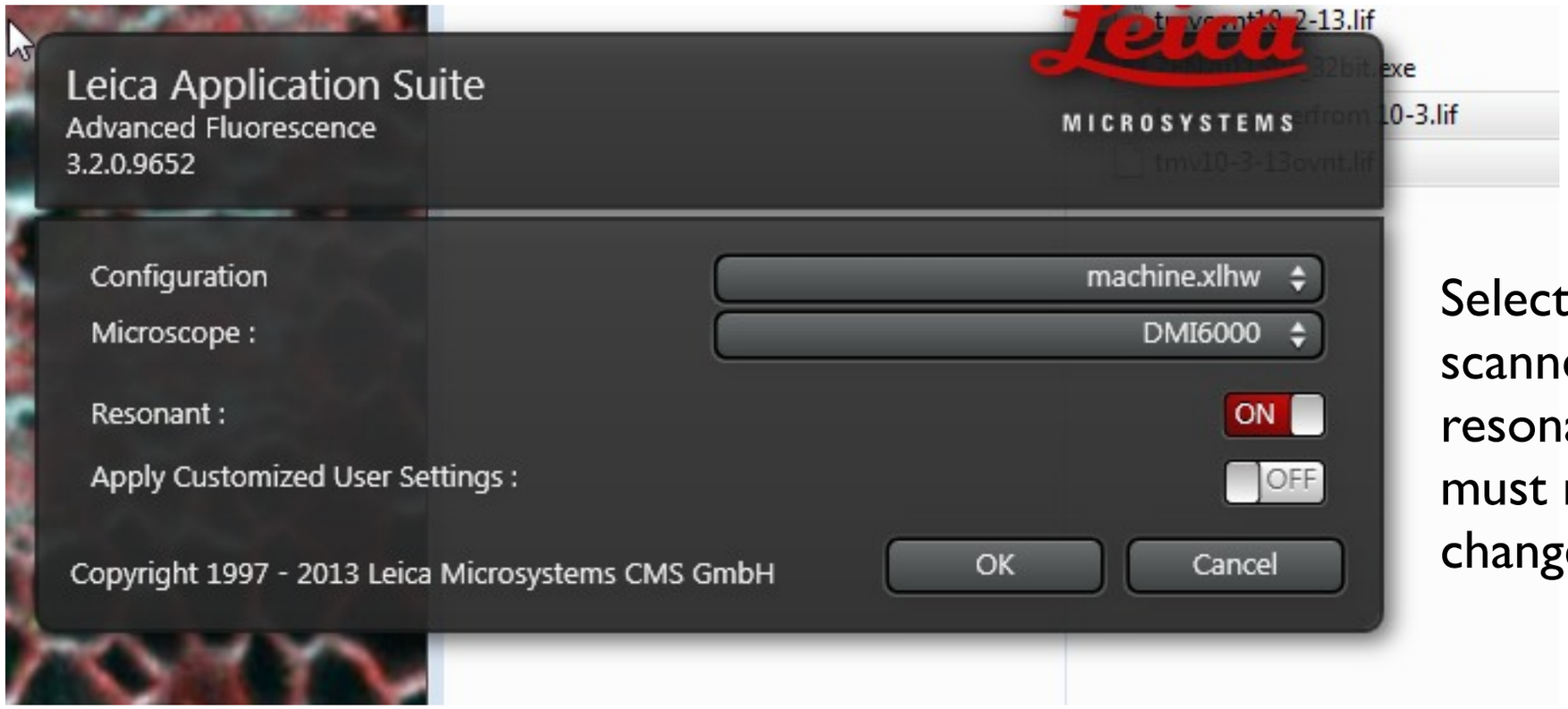
Scope front:
Epifluorescence filter selection,
Shutter controls epifluorescence switch

System switches:
Turn buttons on left-to-right and key on last, off in reverse order



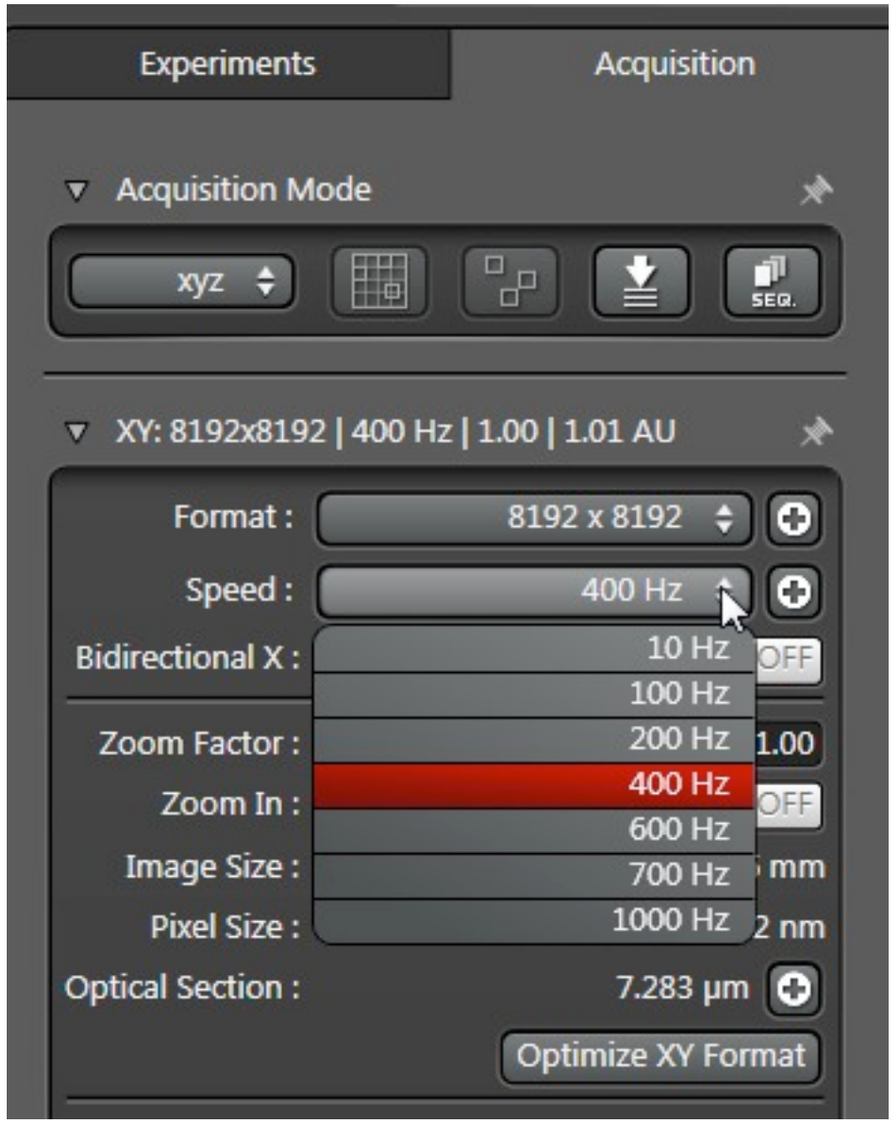
Manual stage controls:
Focus
X-Y positioning
Fine travel
Coarse travel

Software boot up

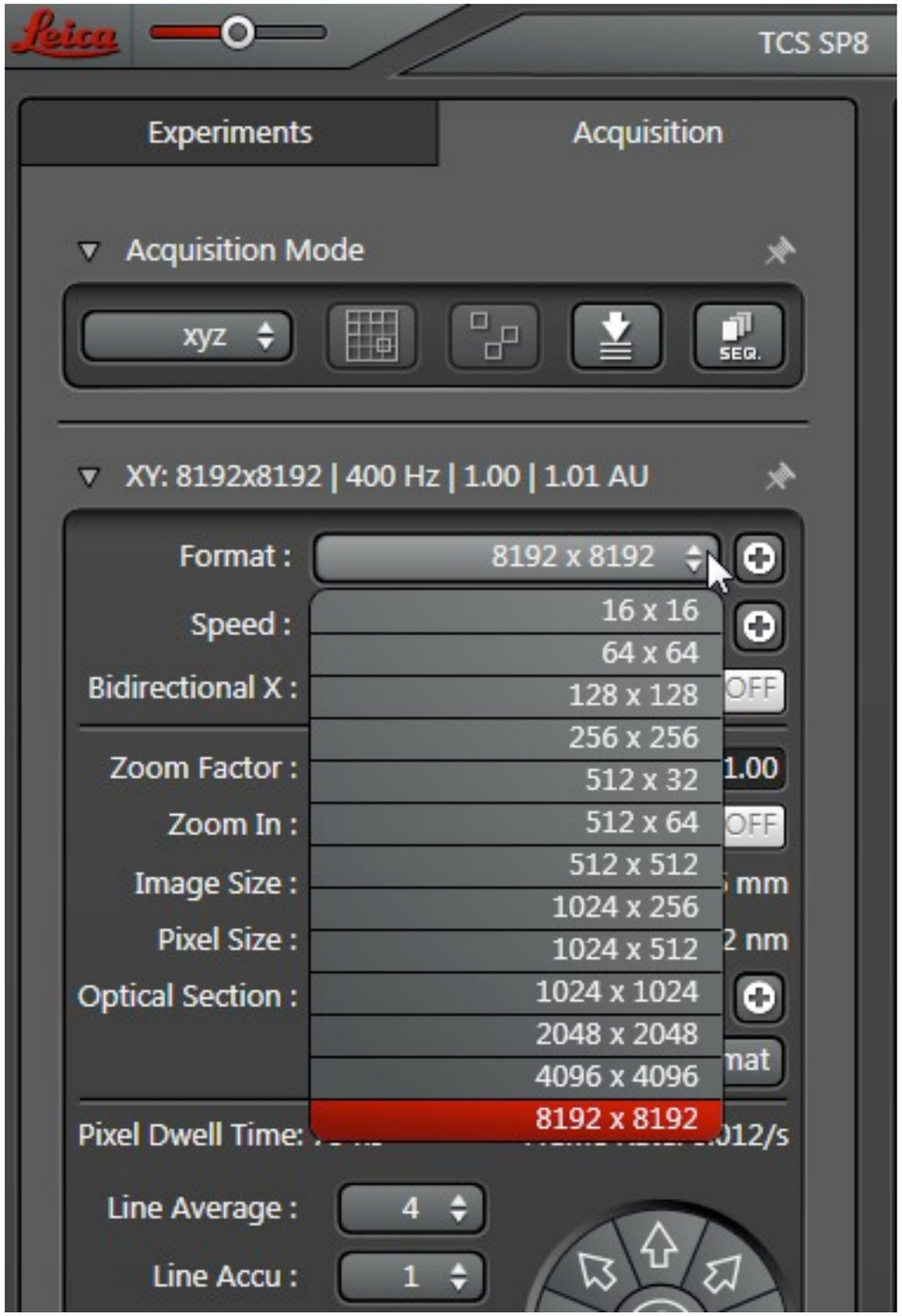


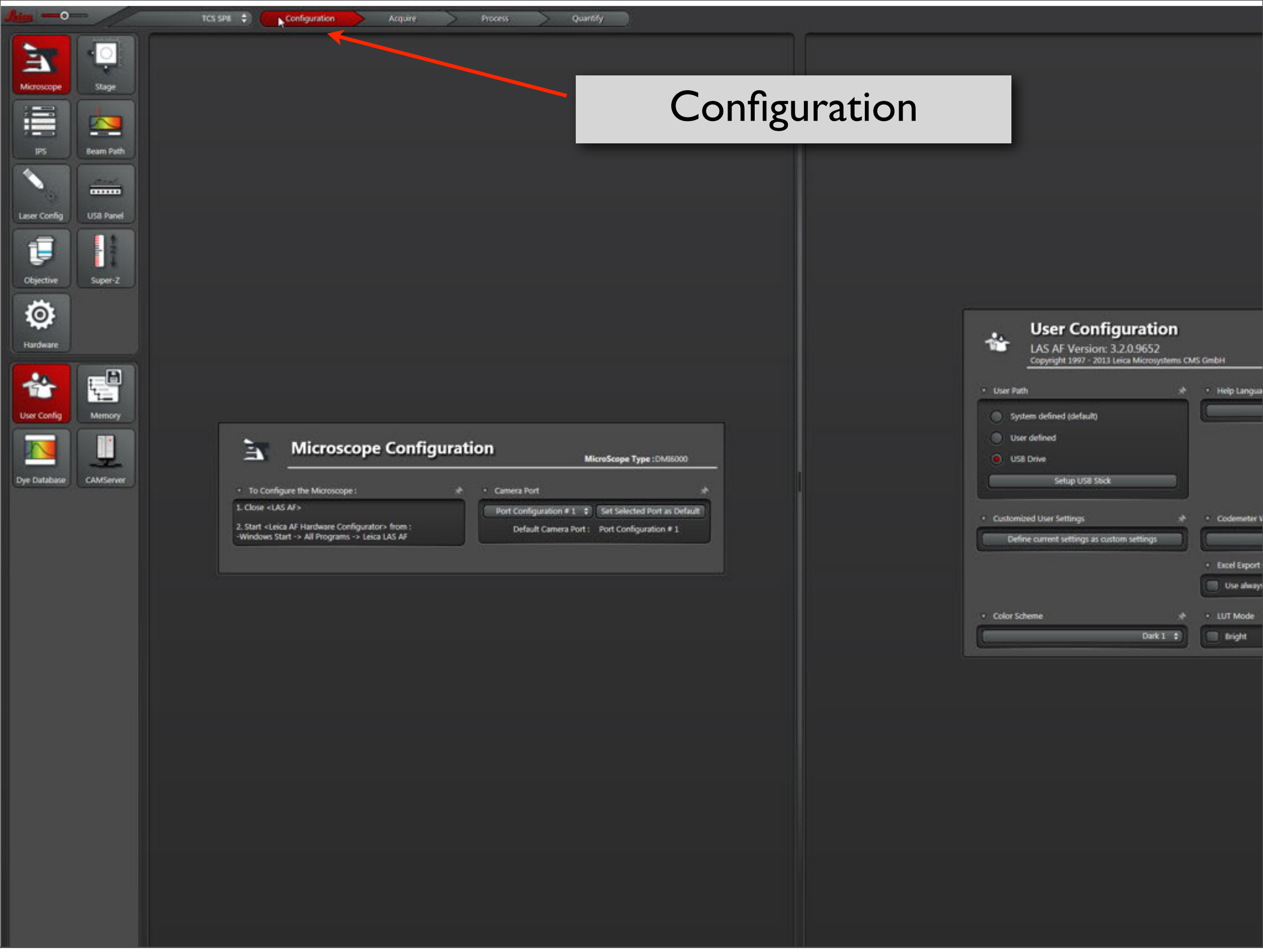
Select FOV or resonant scanner at boot-up: resonant off to get FOV, must re-start software to change

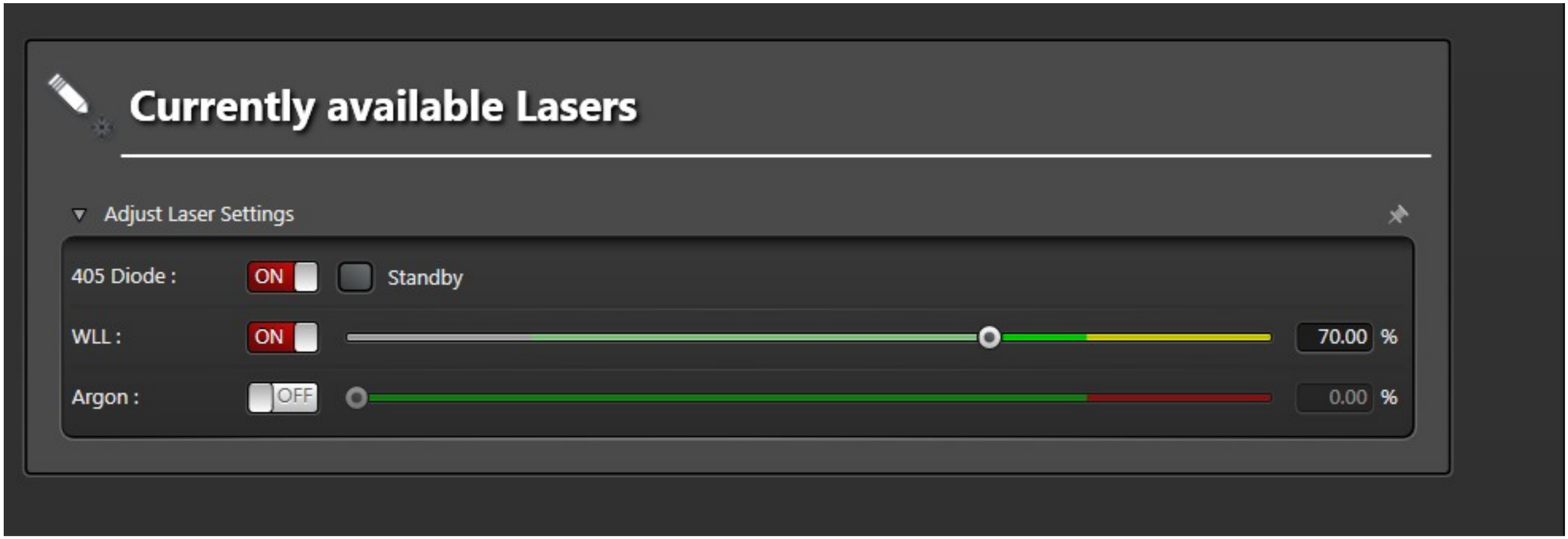
FOV speed selection



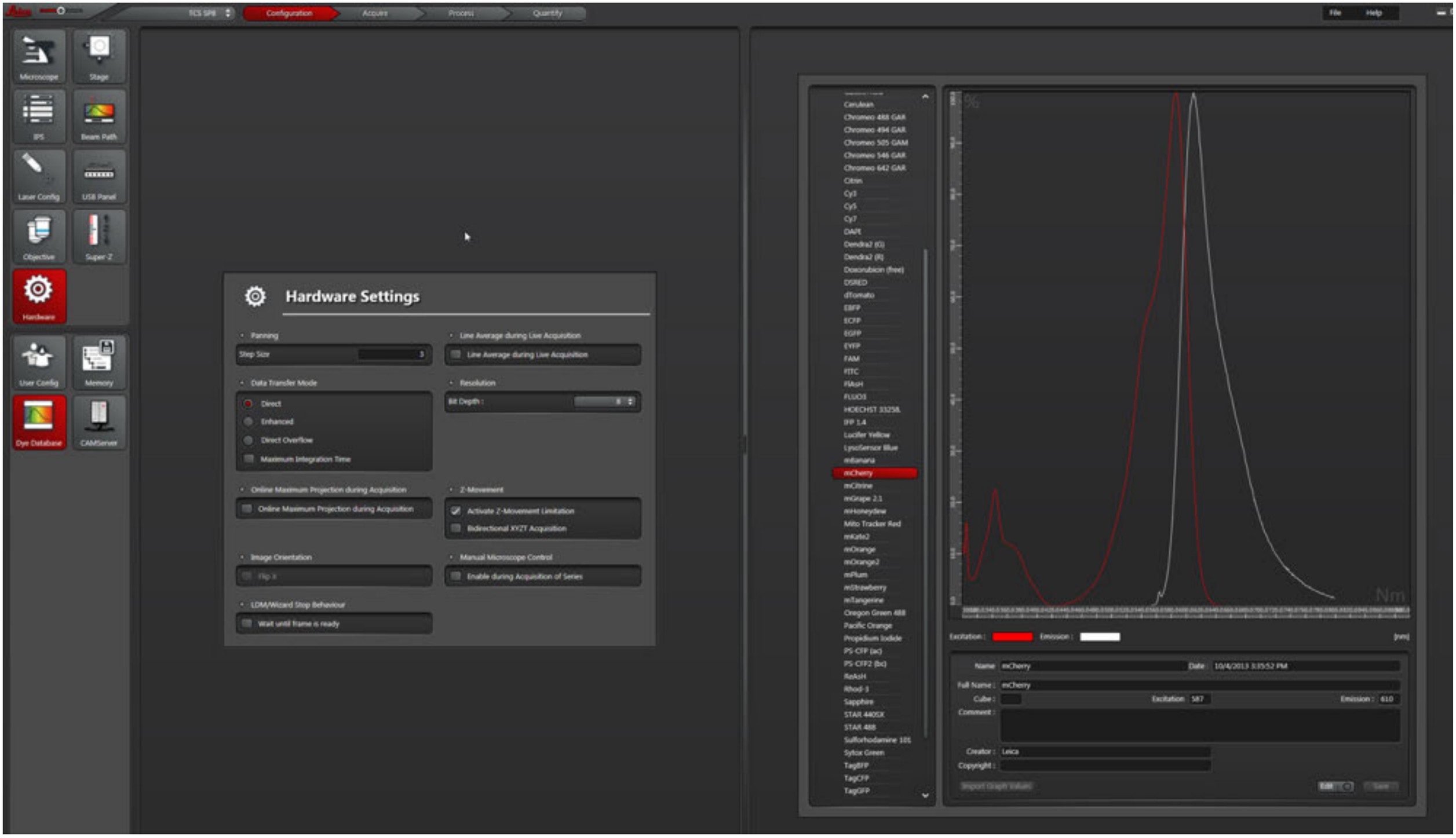
FOV size selection




TCS SP8
Configuration
Acquire
Process
Quantify
Microscope
Stage
IPS
Beam Path
Laser Config
USB Panel
Objective
Super-Z
Hardware
User Config
Memory
Dye Database
CAMServer
Configuration
Microscope Configuration
MicroScope Type : DMI6000
To Configure the Microscope :
1. Close <LAS AF>
2. Start <Leica AF Hardware Configurator> from :
-Windows Start -> All Programs -> Leica LAS AF
Camera Port
Port Configuration # 1
Set Selected Port as Default
Default Camera Port : Port Configuration # 1
User Configuration
LAS AF Version: 3.2.0.9652
Copyright 1997 - 2013 Leica Microsystems CMS GmbH
User Path
System defined (default)
User defined
USB Drive
Setup USB Stick
Customized User Settings
Define current settings as custom settings
Color Scheme
Dark 1
Excel Export
LUT Mode
Bright



WLL operated at 70% by default



Dye database

Objective Configuration

Selected Objective :HC PL APO UVIS CS2 63x/1.20 WATER

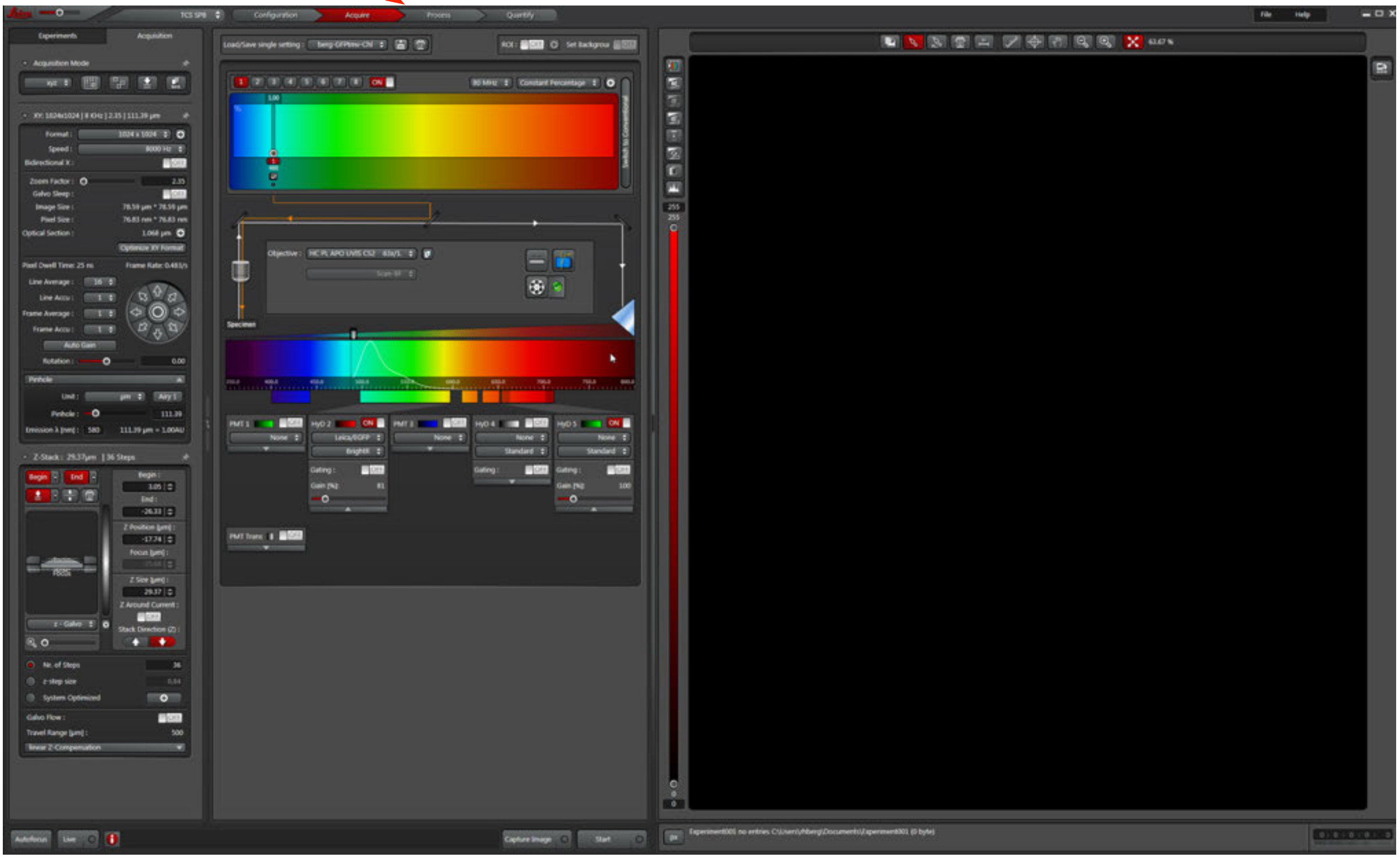
▼ Objectives

	HCX PL APO CS 10x/0.40 DRY
	HC PL APO CS2 40x/1.10 WATER
	Empty 1x/0.00
●	HC PL APO UVIS CS2 63x/1.20 WATER
	HC PL APO CS2 63x/1.40 OIL
	HCX PL APO CS 100x/1.44 OIL

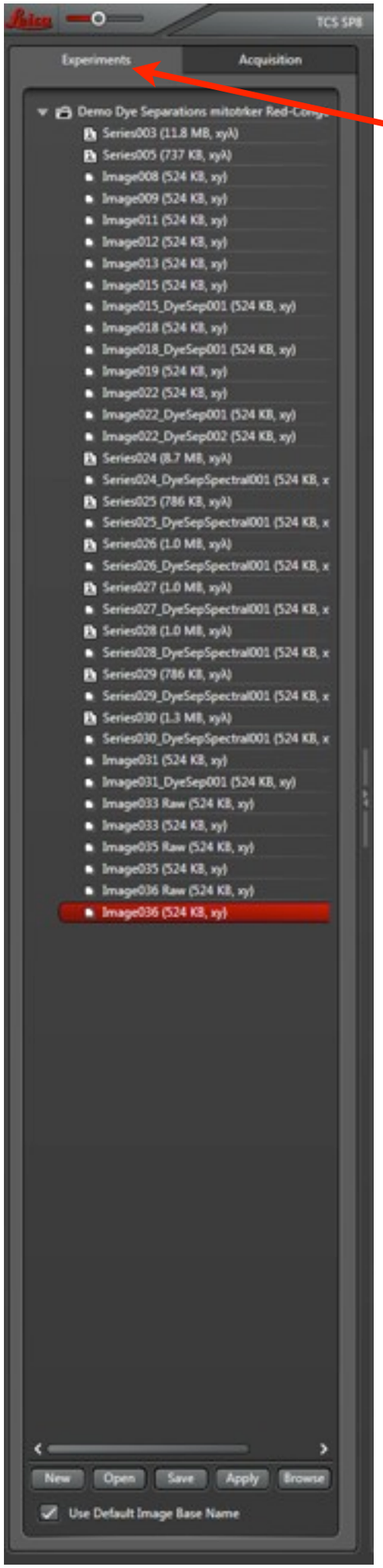
▼ Objective Attributes

Type :	HC PL APO UVIS (	Resolution XY(488nm) :	162.67	Phase Ring :	
Magnification :	63	Resolution Z(488nm)	290.28	IC Prisms :	D1;D1-P;D
Numerical Aperture :	1.2	Free Working Distance :	220	Technique :	
Immersion :	WATER	Focus Depth :	0	Cond. Prism DIC :	K10;K7
Coverglass :	0,14-0,19	Focus Offset :	0	Order Number :	11506355

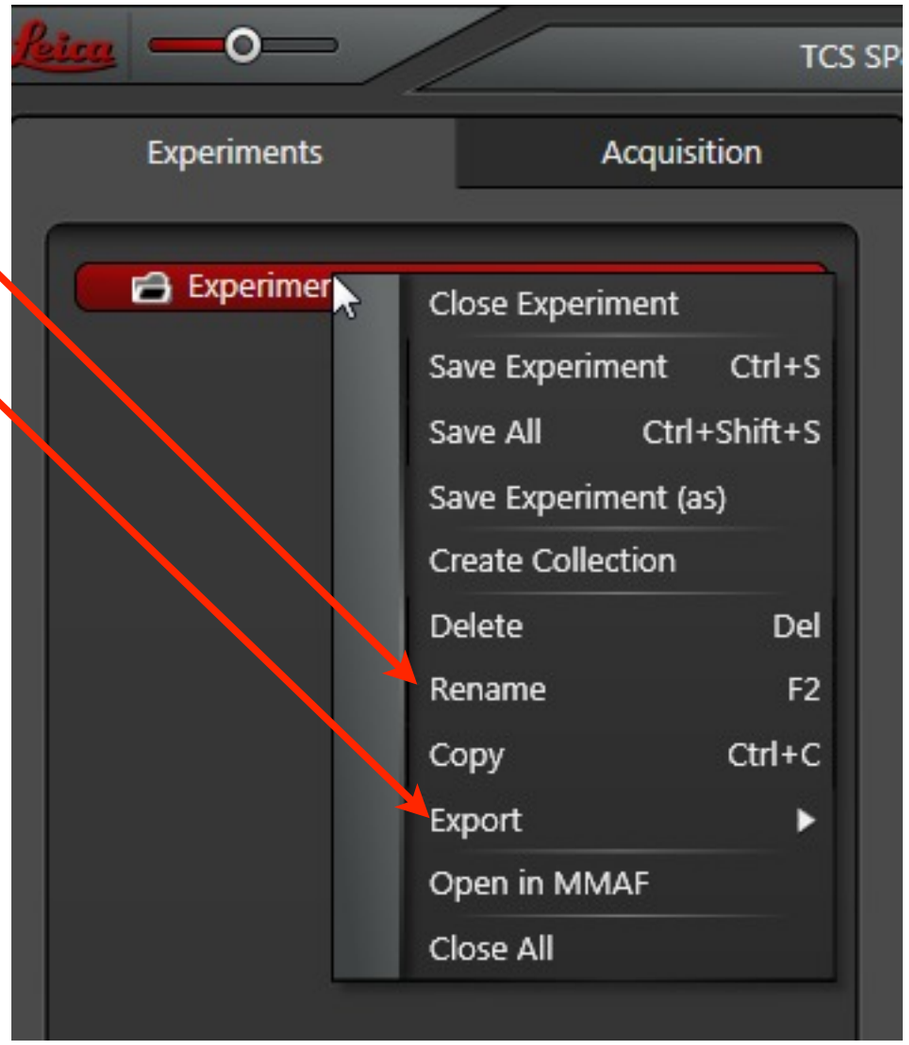
Acquire


Configuration
Acquire
Process
Quantify
Experiments
Acquisition
Acquisition Mode
Format :
Speed :
Zoom Factor :
Image Size :
Pixel Size :
Optical Section :
Optimize XY Format
Line Average :
Frame Average :
Auto Gain
Rotation :
Pinhole
Z-Stack : 29.37µm | 36 Steps
Begin
End
Z Position [µm] :
Focus [µm] :
Z Size [µm] :
Z Around Current :
Stack Direction (Z) :
No. of Steps
z-step size
System Optimized
Galvo Flow :
Travel Range [µm] :
Linear Z-Compensation
Objective :
Specimen
PMT 1
HyD 2
PMT 3
HyD 4
HyD 5
Gating :
Gain [%] :
PMT Trans
Autofocus
Live
Capture Image
Start

Experiment window



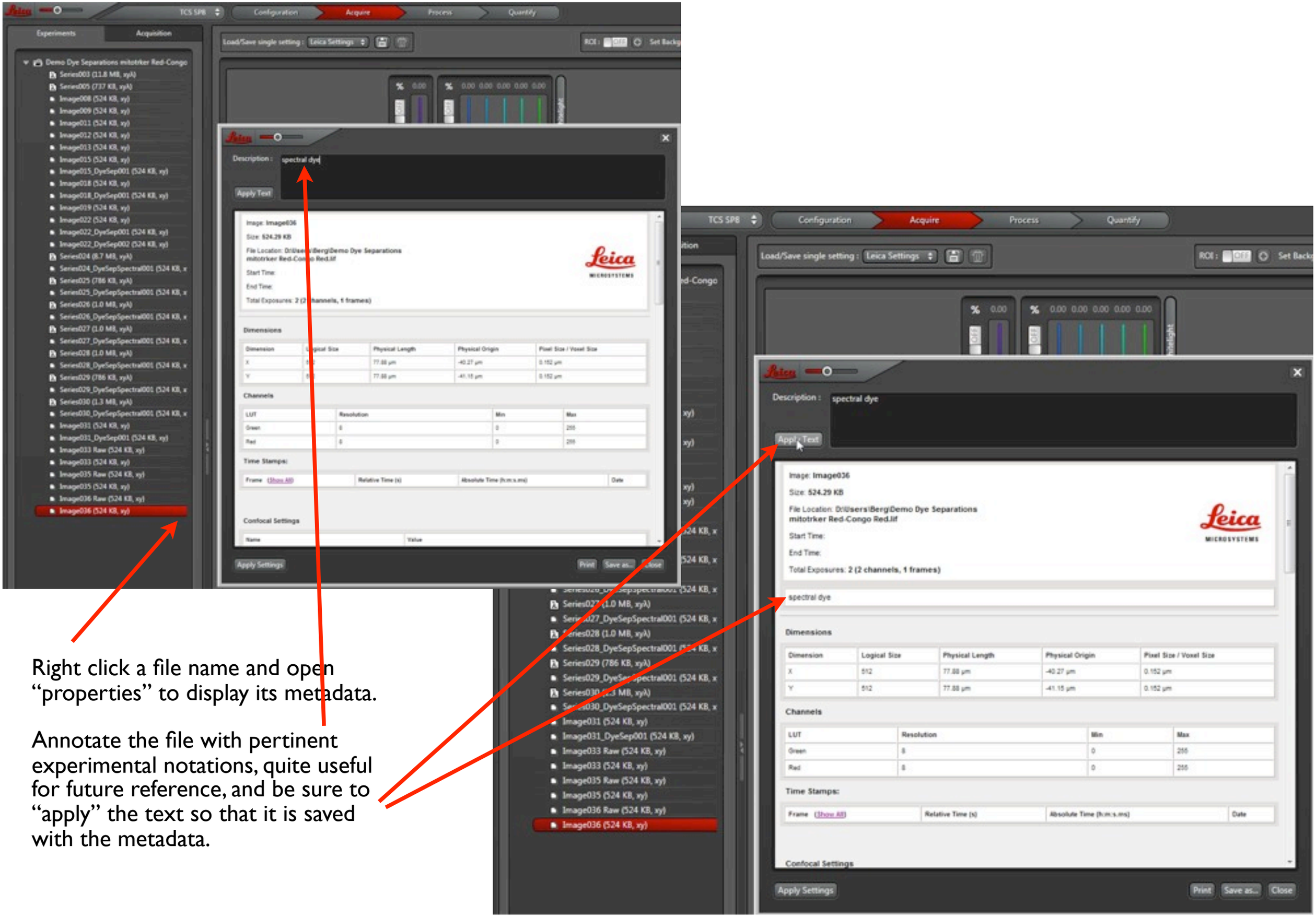
- Images are grouped into experiments, e.g., different samples
- An experiment is a single "lif" file
- FIJI (Image J) can open the individual images (see pdf on your disk), as can Imaris
- right-click experiment or file names to rename them, which is helpful when deciding which file to open
- images can be directly exported in other formats (e.g., tiff)



If system crashes restart the software and any unsaved data will be reloaded from the temporary cache, this should be saved before the cache is altered

Never alter the original data and keep it available in case it is needed by reviewers, editors, or lawyers

Experiment window



Right click a file name and open "properties" to display its metadata.

Annotate the file with pertinent experimental notations, quite useful for future reference, and be sure to "apply" the text so that it is saved with the metadata.

Opening “lif” files with imageJ/FIJI

Opening LIF files with FIJI

Bio-Formats Import Options

File>open gives this menu:

Stack viewing

View stack with: Hyperstack

Stack order: XYCZT

Dataset organization

- [ ] Group files with similar names
- [x] Open files individually
- [ ] Swap dimensions
- [ ] Open all series
- [ ] Concatenate series when compatible
- [ ] Stitch tiles

Color options

Color mode: Grayscale

- [x] Autoscale

Metadata viewing

- [x] Display metadata
- [ ] Display OME-XML metadata
- [ ] Display ROIs

Memory management

- [ ] Use virtual stack
- [ ] Specify range for each series
- [ ] Crop on import

Split into separate windows

- [ ] Split channels
- [ ] Split focal planes
- [ ] Split timepoints

Information

View stack with - The type of image viewer to use when displaying the dataset.

Possible choices are:

- **Metadata only** - Display no pixels, only metadata.
- **Standard ImageJ** - This option is deprecated (i.e. intended for use by old macros only). Please use *Hyperstack* instead.
- **Hyperstack** - Display the pixels in ImageJ's built-in 5D viewer.
- **Data Browser** - Display the pixels in LOCI's multidimensional Data Browser viewer. The Data Browser has some additional features on top of the normal ImageJ hyperstack.
- **Image5D** - Display the pixels in Joachim Walter's Image5D viewer. Requires the Image5D plugin.
- **View5D** - Display the pixels in Rainer Heintzmann's View5D viewer. Requires

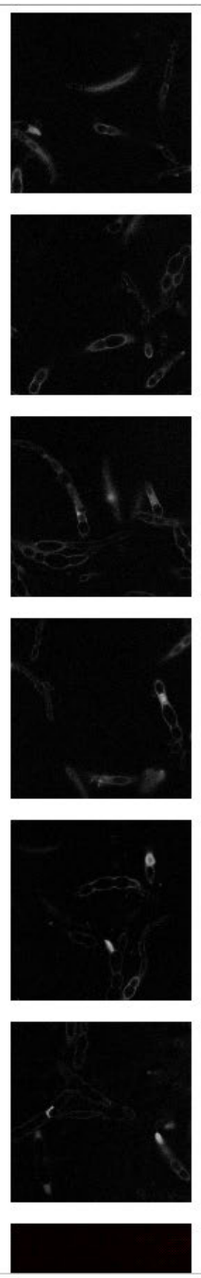
Novices: use these default settings and click OK

Cancel OK

Bio-Formats Series Options

Opening the LIF file displays the individual image files:
Tick the one you want to open and click OK
(large files will take longer to open)

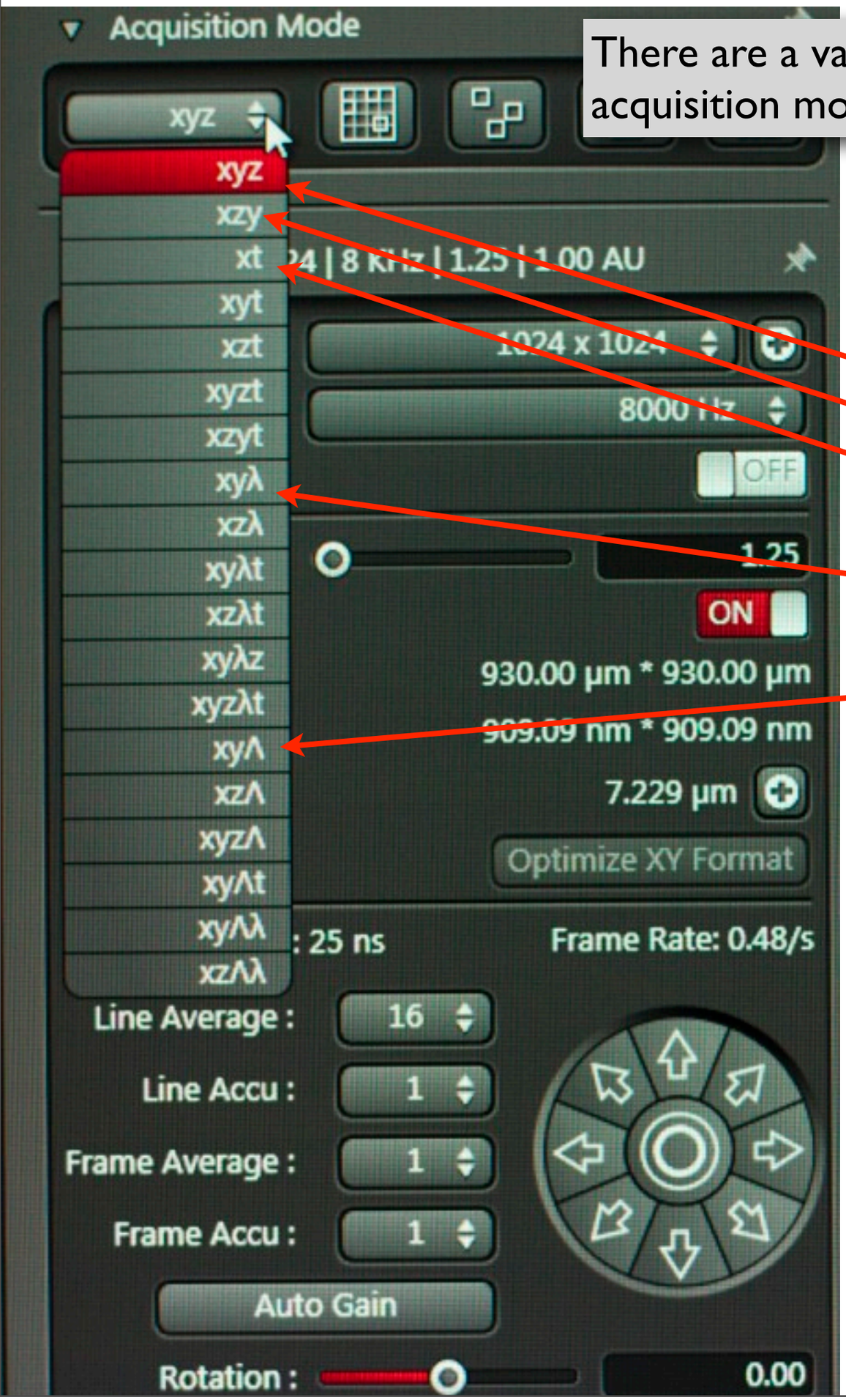
- [x] Series 1: Mark and Find 001/Position1001: 1024 x 1024; 1200 planes (2C x 25Z x 24T)
- [ ] Series 2: Mark and Find 001/Position2002: 1024 x 1024; 1200 planes (2C x 25Z x 24T)
- [ ] Series 3: Mark and Find 001/Position3003: 1024 x 1024; 1200 planes (2C x 25Z x 24T)
- [ ] Series 4: Mark and Find 001/Position4004: 1024 x 1024; 1200 planes (2C x 25Z x 24T)
- [ ] Series 5: Mark and Find 001/Position5005: 1024 x 1024; 1200 planes (2C x 25Z x 24T)
- [ ] Series 6: Mark and Find 001/Position6006: 1024 x 1024; 1200 planes (2C x 25Z x 24T)



Cancel OK Select All Deselect All

Acquire screen

- Image parameters
- Averaging, rotation
- Pinhole (single)
- Z stacks



- Single stack
- Slice vertically
- Point over time (fast)
- ..t different time lapse modes
- lambda emission and excitation scans

- here for resonant scanner: 1024x1024 is max size, 8k is the only speed

- change pixel size by zooming (in USB control panel as well) and/or formatting number of pixels--"optimize XY format" will change latter in FOV scanner

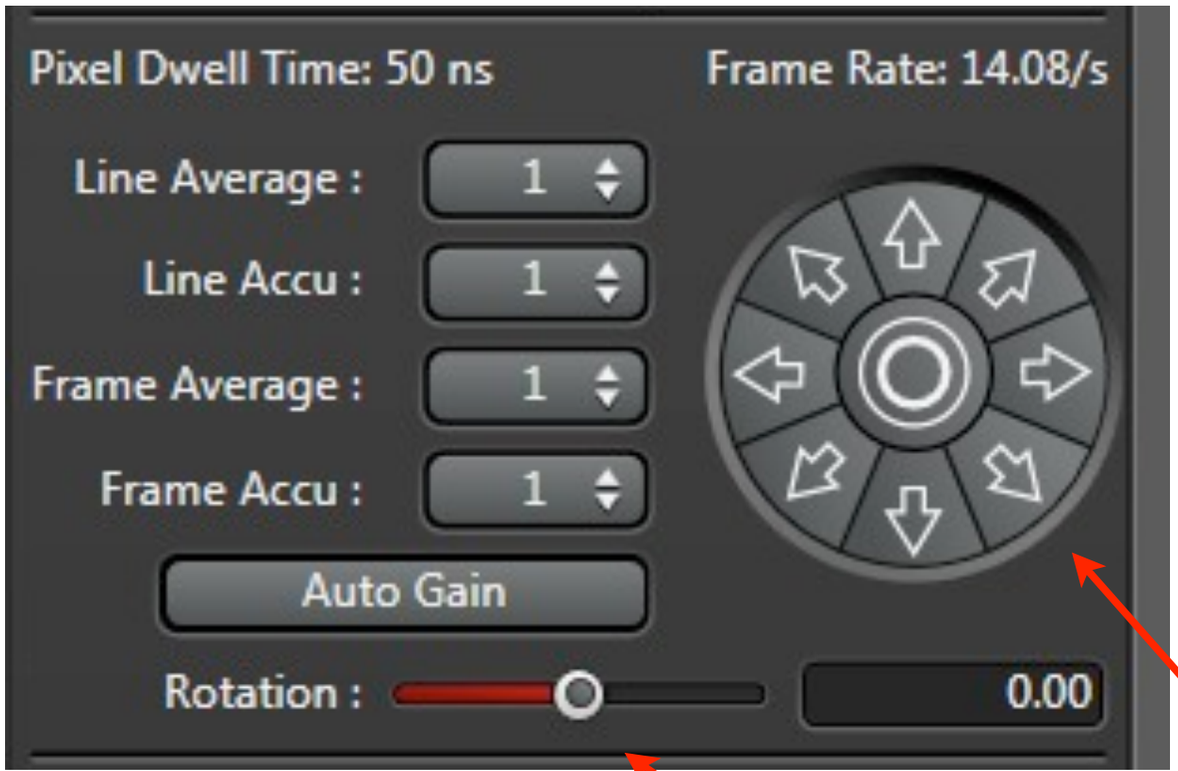
- digital image rotation is possible

- single pinhole; can change from 1 AU default by using slider

Averaging

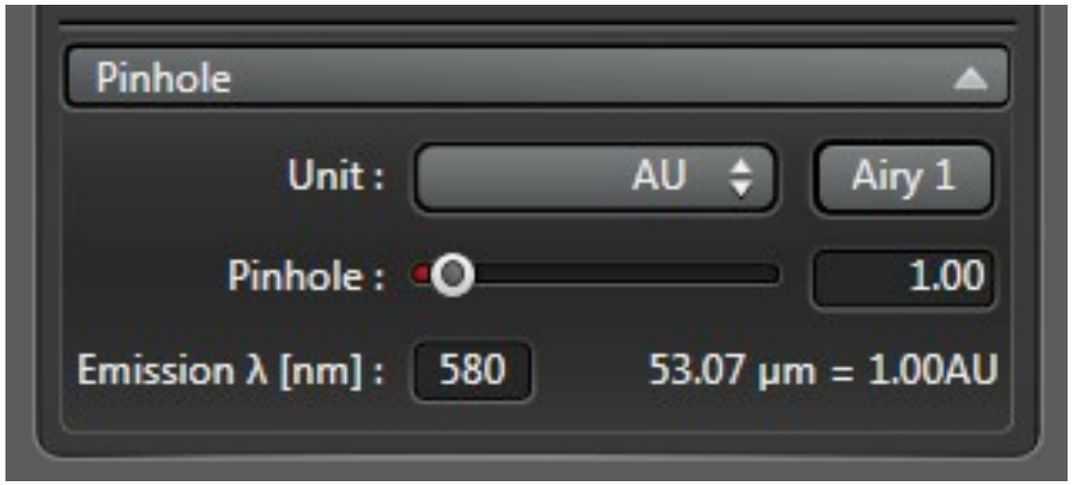
•Confocal images are usually noisy, seen as randomly distributed single pixel signals resulting from spurious detector signals, much more prevalent in PMTs than HyDs

•Commonly reduced by averaging several images of the field of view—single random noise pixels are averaged to a low value



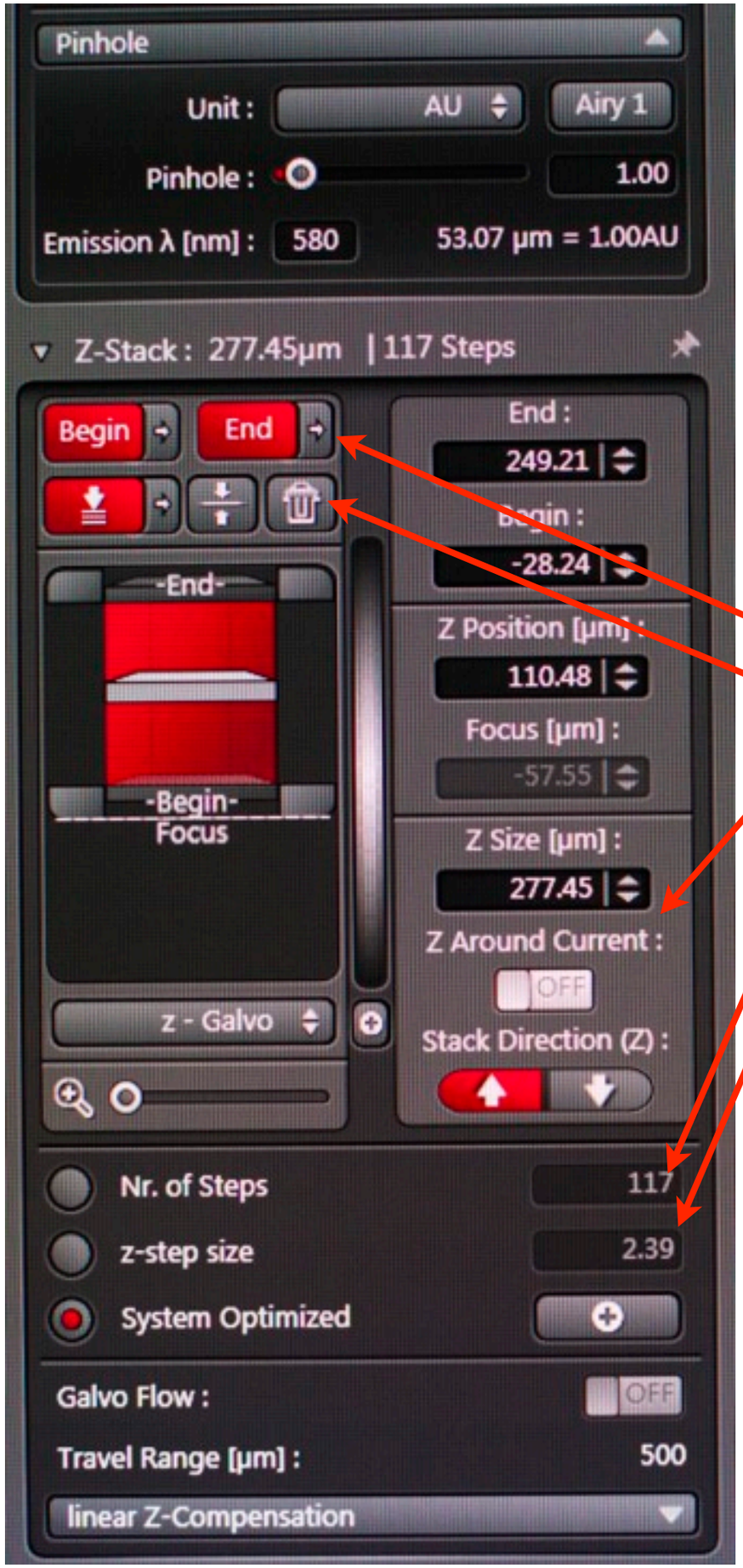
•line or frame are alternate controls
•here 16 line averaging in resonant scanner—each line is scanned 16 times before moving to the next
•accumulation is the alternate (adds signal rather than averaging as in line average)

•specimen can be moved using arrows here (gives fine-tuned control)
•specimen can be rotated (here or on USB panel), which rotates a Konig rotator

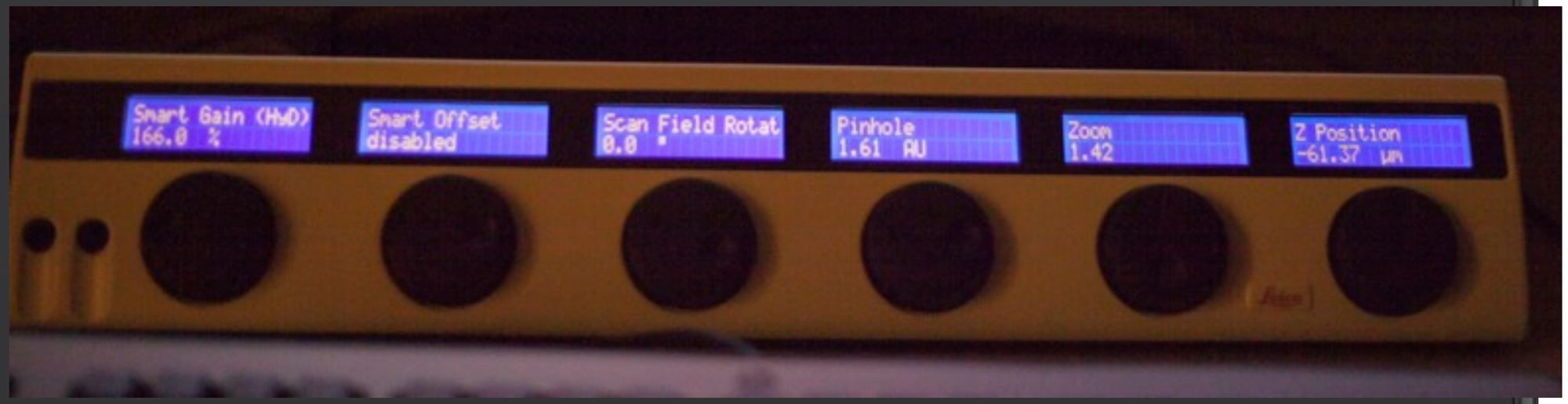


Default pinhole is one Airy Unit

- can specify whether to display AU or physical size
- slider or USB panel control of AU size



- Z stack menu, must use USB Z control for marking begin/end
- click arrow to move to begin, middle or end positions
- reset to zero by clicking trash can
- can make a stack around a current position
- can change direction of stacking
- number of slices
- slice spacing



Laser Config | USB Panel | Objective | Super-Z | Hardware

User Config | Memory | Dye Database | CAMServer

USB Control Panel

Panelbox Settings

Smart Gain | Smart Offset | Scan Field Rotation | Pinhole | Zoom | Z Position

others (250V per tu | Medium | Medium | Medium | 10µm per turn

Gain HyD 2
Gain HyD 4
Gain HyD 5
Gain PMT 1
Gain PMT 3
Gain PMT NDD1
Gain PMT NDD2
Gain PMT NDD3
Gain PMT NDD4
Gain PMT Trans
motCORR™
Offset PMT 1
Offset PMT 3
Offset PMT NDD1
Offset PMT NDD2
Offset PMT NDD3
Offset PMT NDD4
Offset PMT Trans
Panning (horiz.)
Panning (vert.)
Phase
PhaseSMD
Pinhole
Scan Field Rotation
Smart Gain
Smart Intensity
Smart Offset
Smart Wavelength

Display Settings

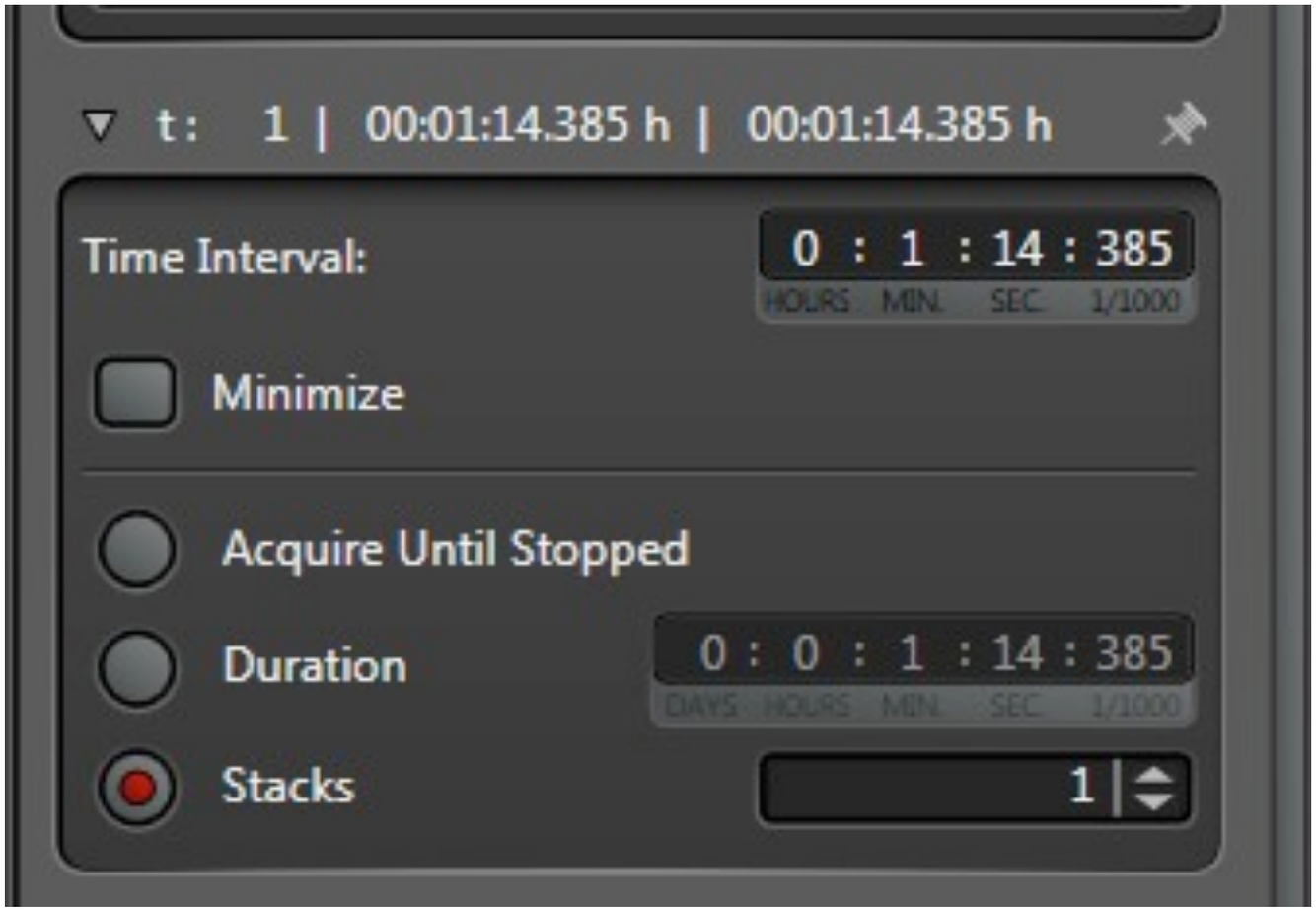
Contrast : 40.00 %

Intensity : 40.00 %

Load/Save control panel setting | Leica Settings

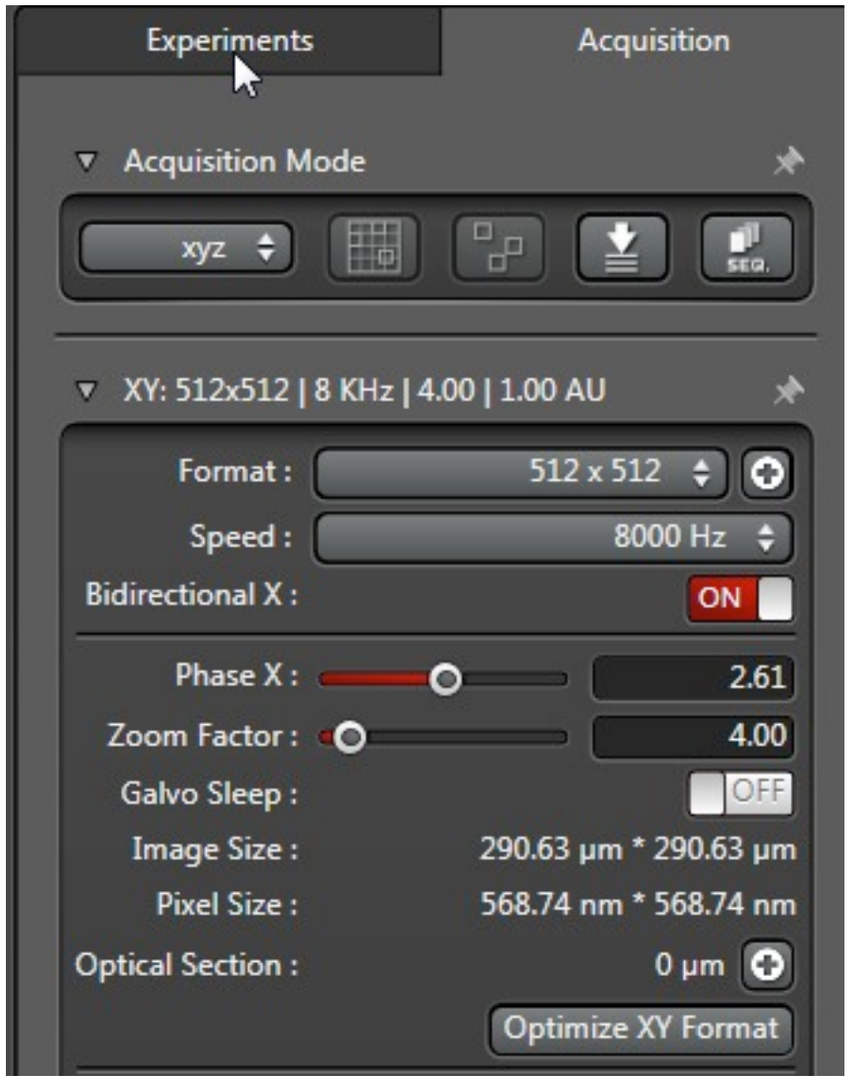
USB control panel is programmable (configuration or acquire window)

Compensation for signal drop-off within tissue



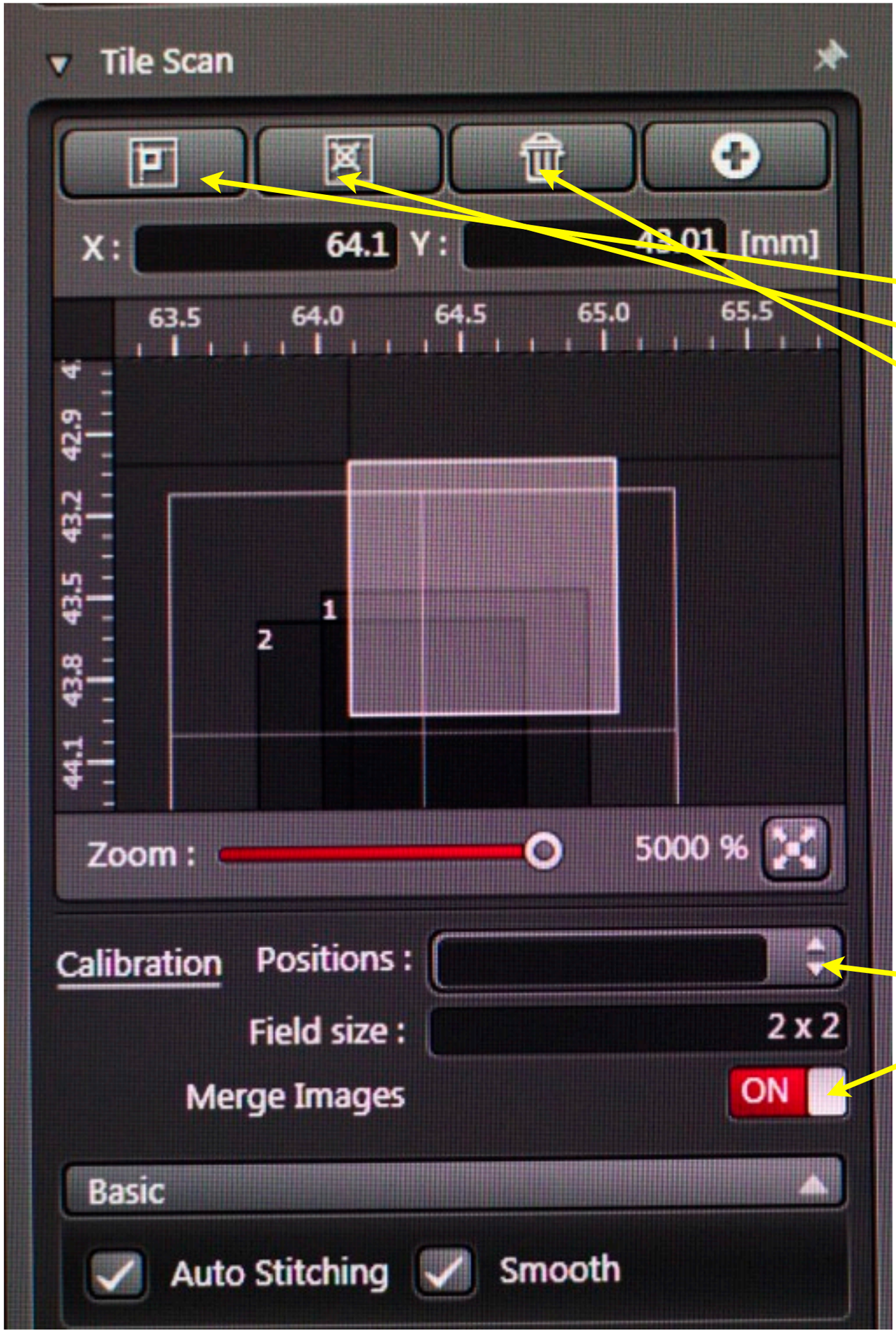
Time-lapse set-up

- minimize: no delay between stacks; when not ticked the time interval can be set to be longer than the time needed to acquire a stack (dark interval must be added to the acquisition time to give the interval)
- ...until stopped: flexible--can stop when appropriate
- duration: a set total time duration
- stacks: a set number of stacks



For high speed acquisition rates:

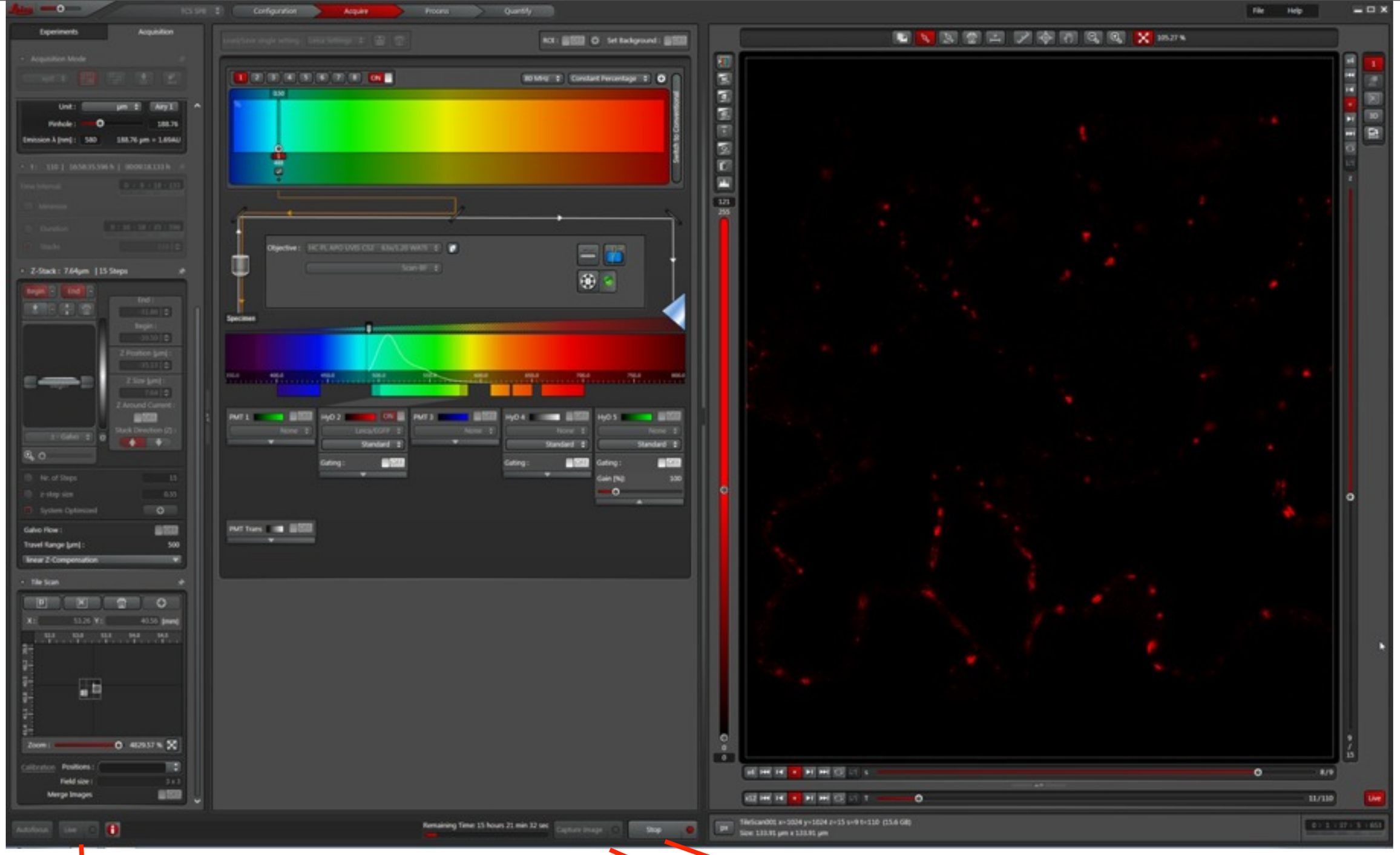
Use bidirectional scan and calibrate "phase" so that forward and reverse scan are synchronized. Also minimize the number of acquired pixels.



- mark position
- delete position
- delete all positions

- tiling/montage menu
- easiest is to mark positions for opposing corners--software fills in the number of images
- easiest way to identify corners is by using the scope's epifluorescence
- zoom is to show the montage layout optimally

- moves to indicated position
- merges montage after acquisition is completed



Single averaged scan

Single full feature scan (alters: start/stop)

Live fast scan without averaging

Acquire

- Laser line selection (here: white light laser) and power level

- objective, access to parameters

- Sliding mirror selection of detection bandpass

- PMT and Hyd detectors, five

Lasers

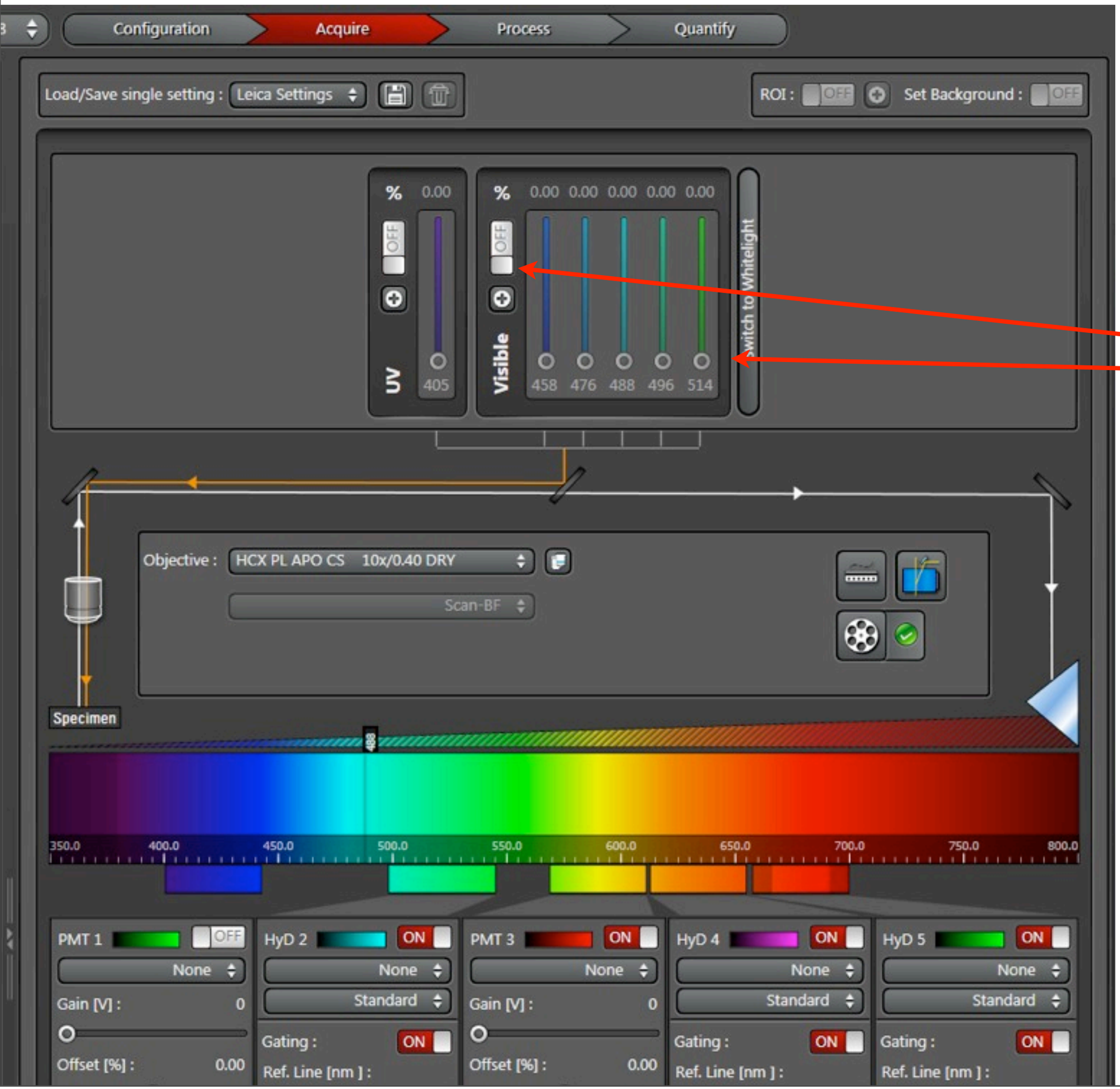
• The goal is to use a laser line that is optimal for the fluorophore (emits at a wavelength close to the excitation maximum for the fluorophore).

• Due to phototoxic effects, laser light reaching the specimen must be kept to a minimum. When lasers are powered on, they emit at their full output energy. This output is then sent through an AOTF modulator that gives the user control over the amount of that laser light reaching the sample. This can be measured by a power meter reading at the back focal plane of the objective. The slider gives a % transmission value.

• Kasha's rule: fluorophore emission will the be same regardless of how the molecule is excited.

Goal is to keep the slider as much as possible to the bottom.

The 405 and WLL are lower power than the argon laser--use the argon at a very low power setting (it is useful for FRAP).



Control of 405 and Ar lasers

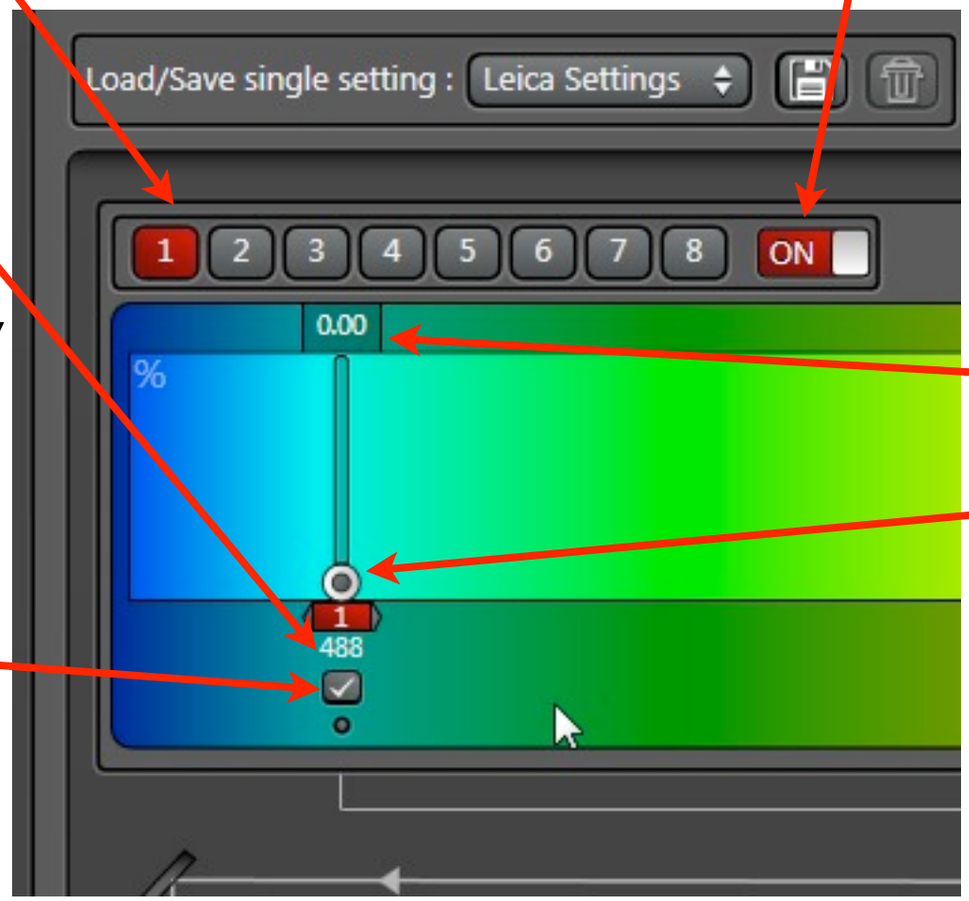
- activate line ("on")
- slider = modulation of intensity

Using WLL excitation

(after laser is turned on in configuration window)

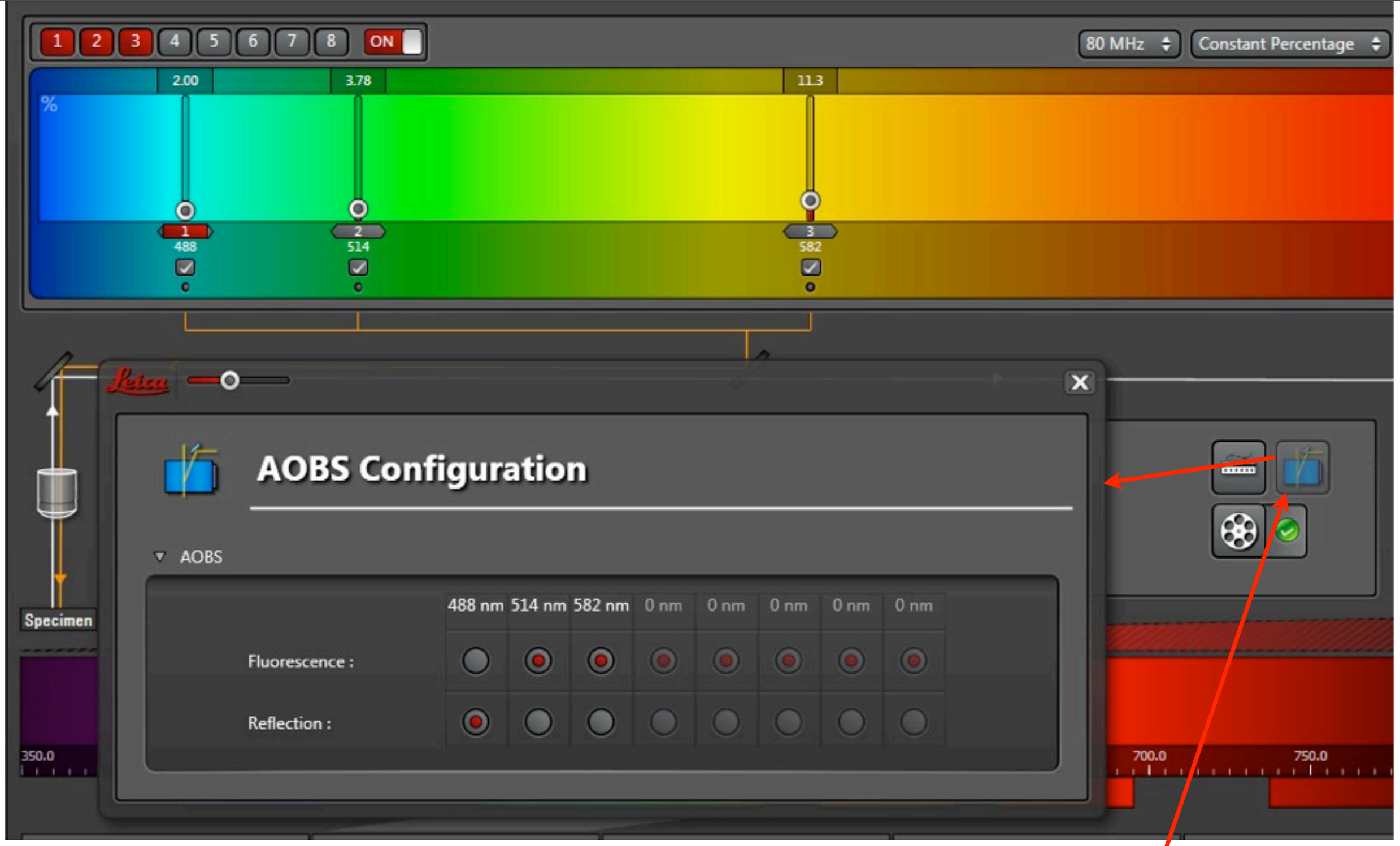
Select up to eight lines in region between 470-670 nm, to select nm excitation:
1--double click number and type in line number or click presets or,
2--slide the number laterally to the desired region

Activate WLL in AOTF



Activate the line

Set intensity:
1--double click and type desired percentage or,
2--slide this to desired percentage



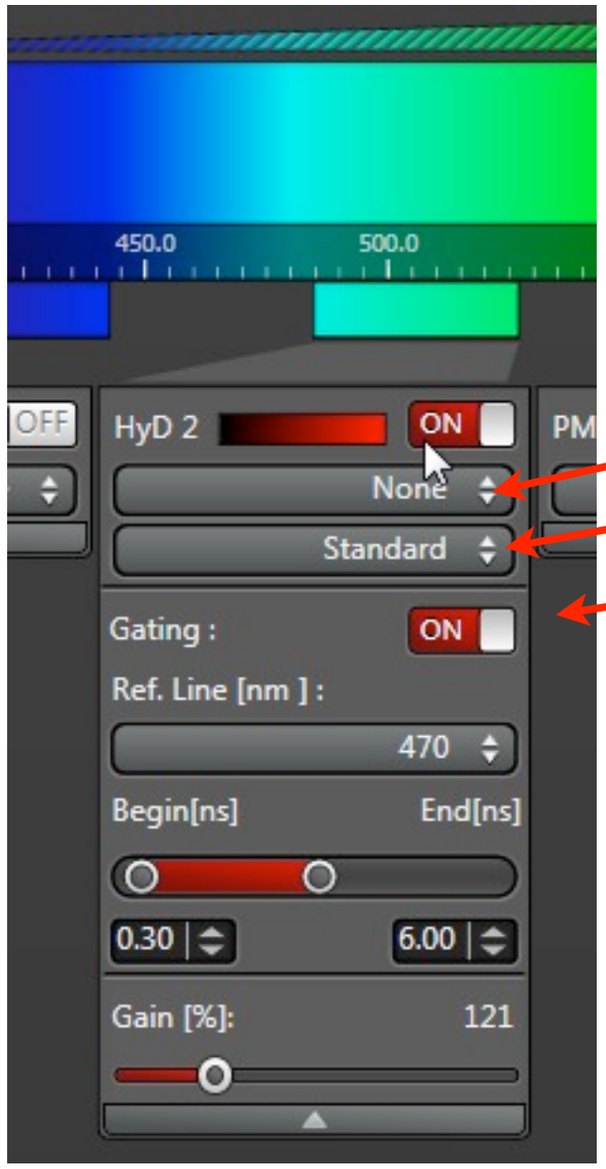
"Beam path" in "configuration" or in "acquisition":

shows AOBS settings (fluorescence blocks indicated line, reflection passes it)

Adjusting the detector window

With associated detector "on"

- click on each end of the window to adjust it up or down
- alternatively, double click window and type in desired values
- if needed adjust non-used windows to minimize them and/ or slide them out of the way



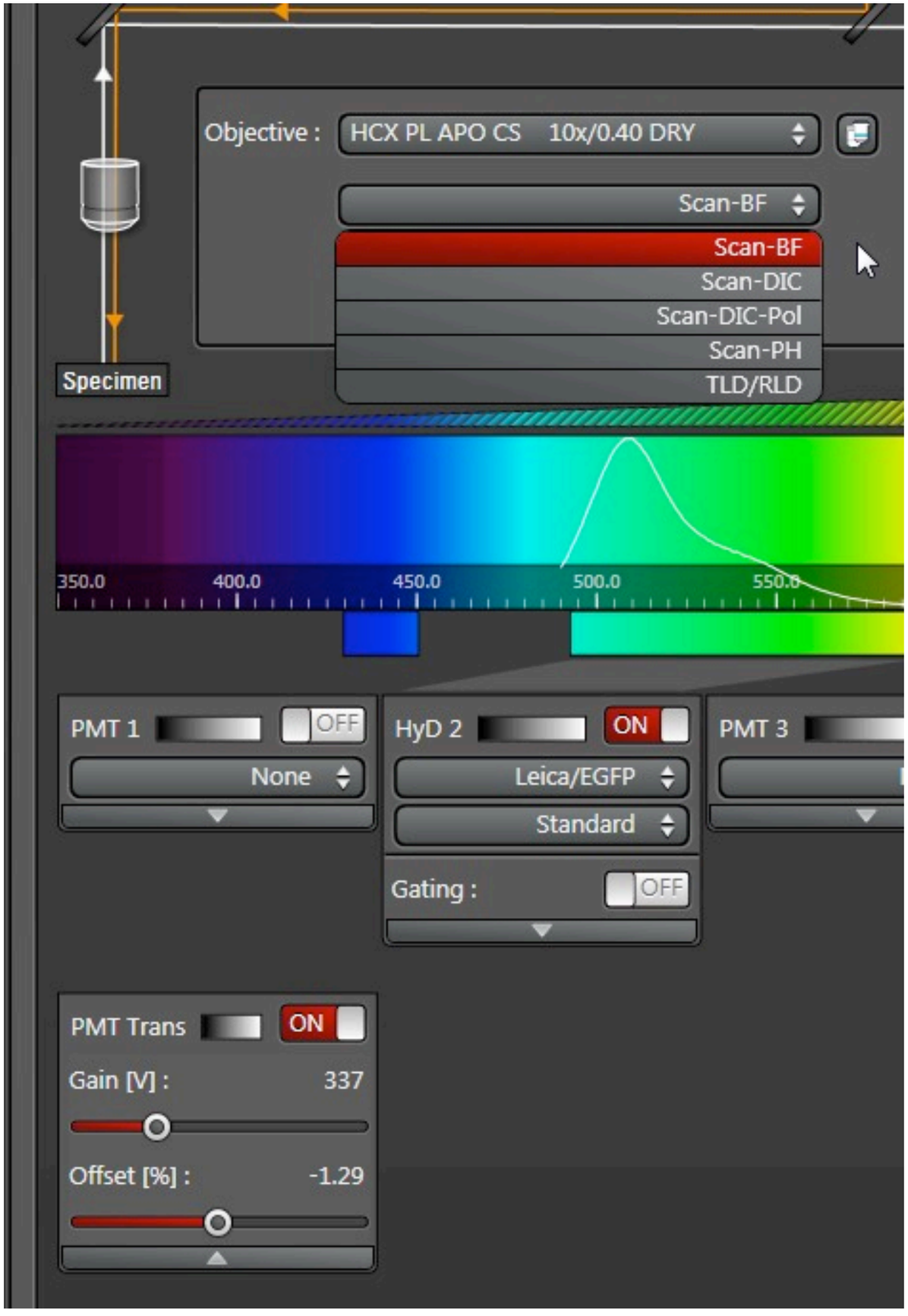
- database emission curve display option
- detection mode (standard, Bright R, photon counting)*
- gating mode (only for WLL)

*

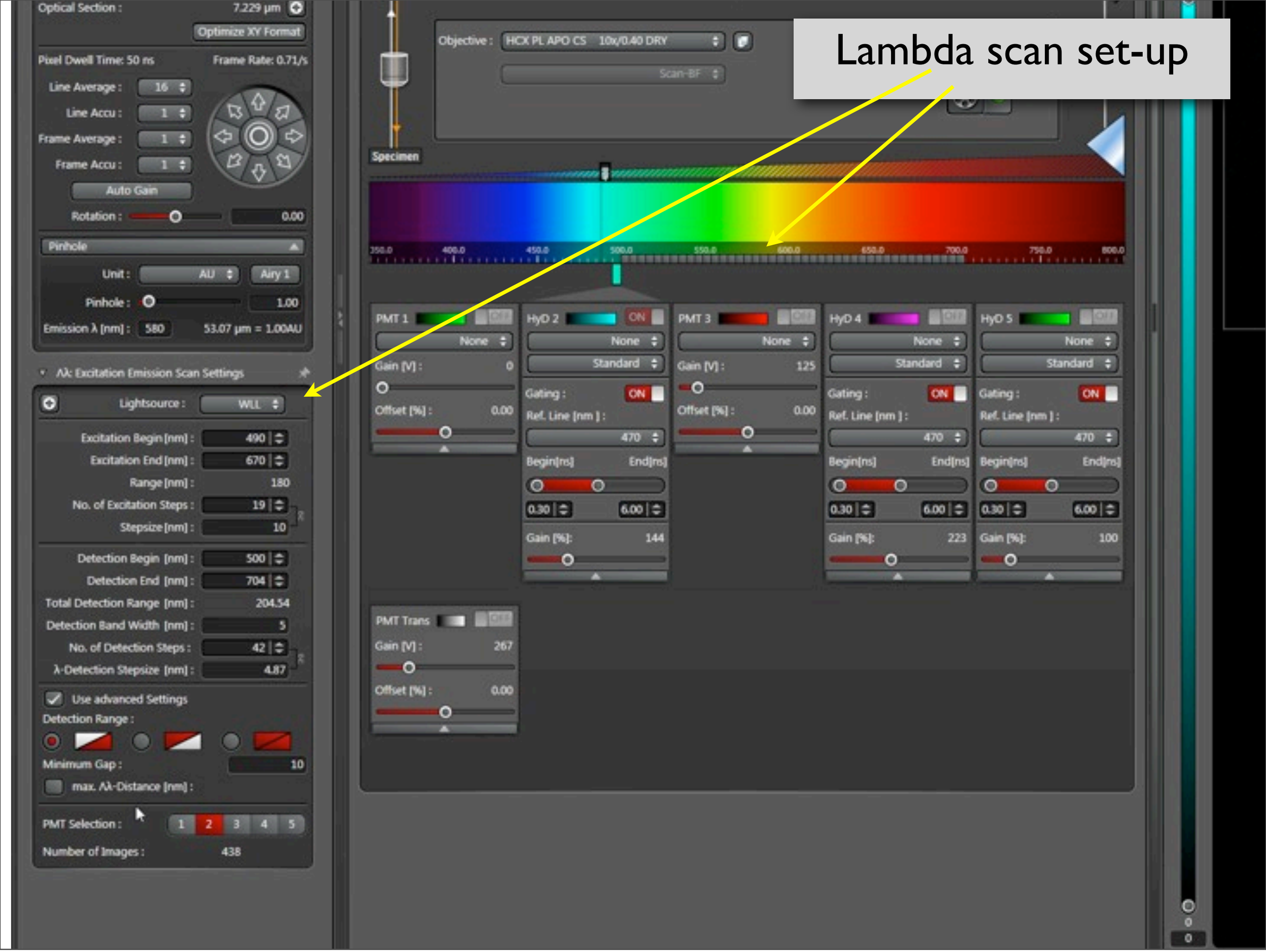
- standard: gain control linear response
- BrightR: non-linear gain control response--gamma adjust
- photon counting: each emission photon reaching the detector results in an intensity-count (1:1) in the image (statistically accessible)

- BF: brightfield
- DIC: true DIC (differential interference contrast, more tomorrow)
- DIC-Pol: polarized light

When transmitted light (PMT) detector is “on” a variety of imaging modes are available



Lambda scan set-up



105.27 %

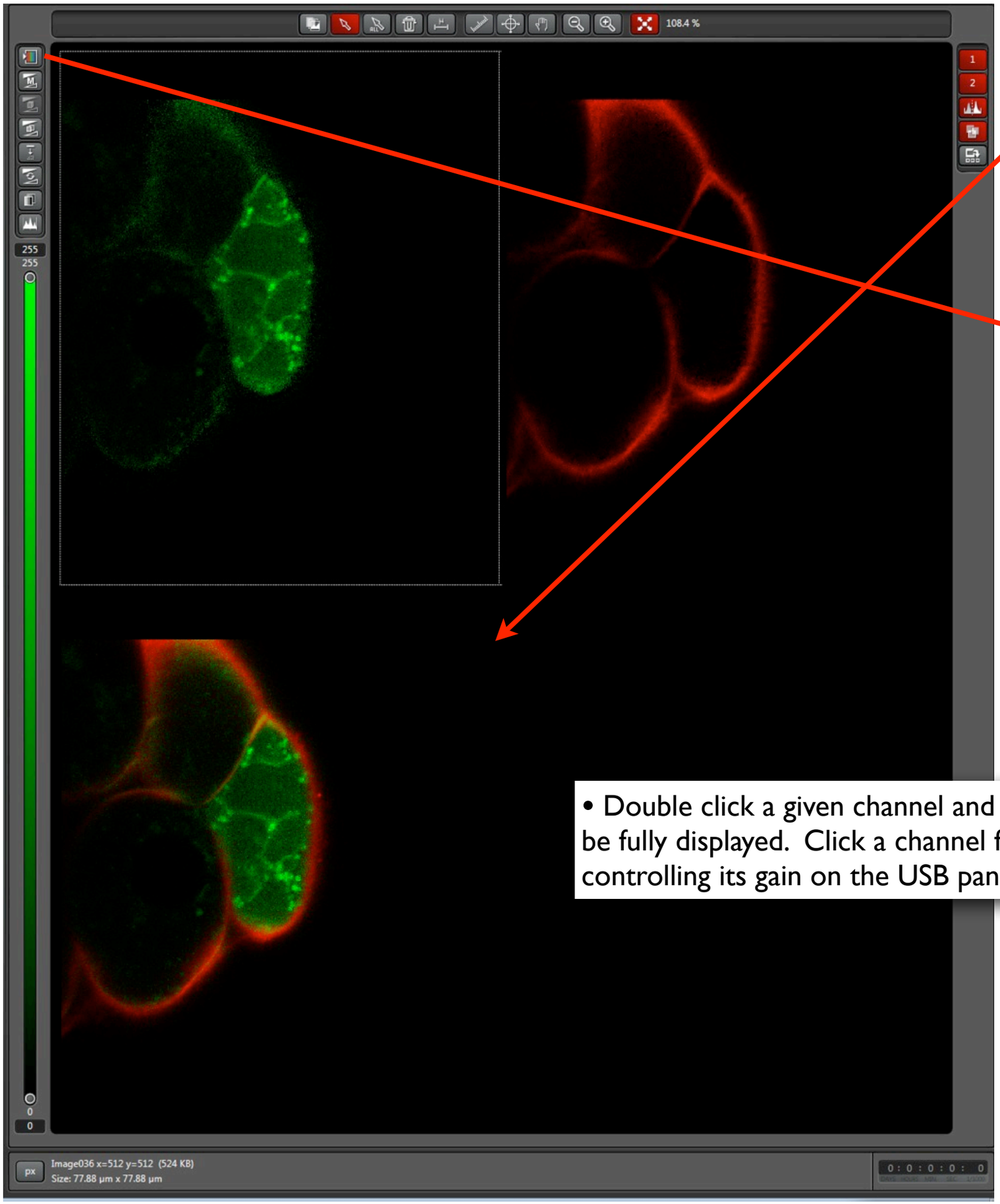
121

255

Switch to Conventional

100

- Slide down to stretch a dim signal to higher intensity
- Scroll through a Z stack



Channel overlay

- Each channel is given a number and also a merged channel is selectable

LUT

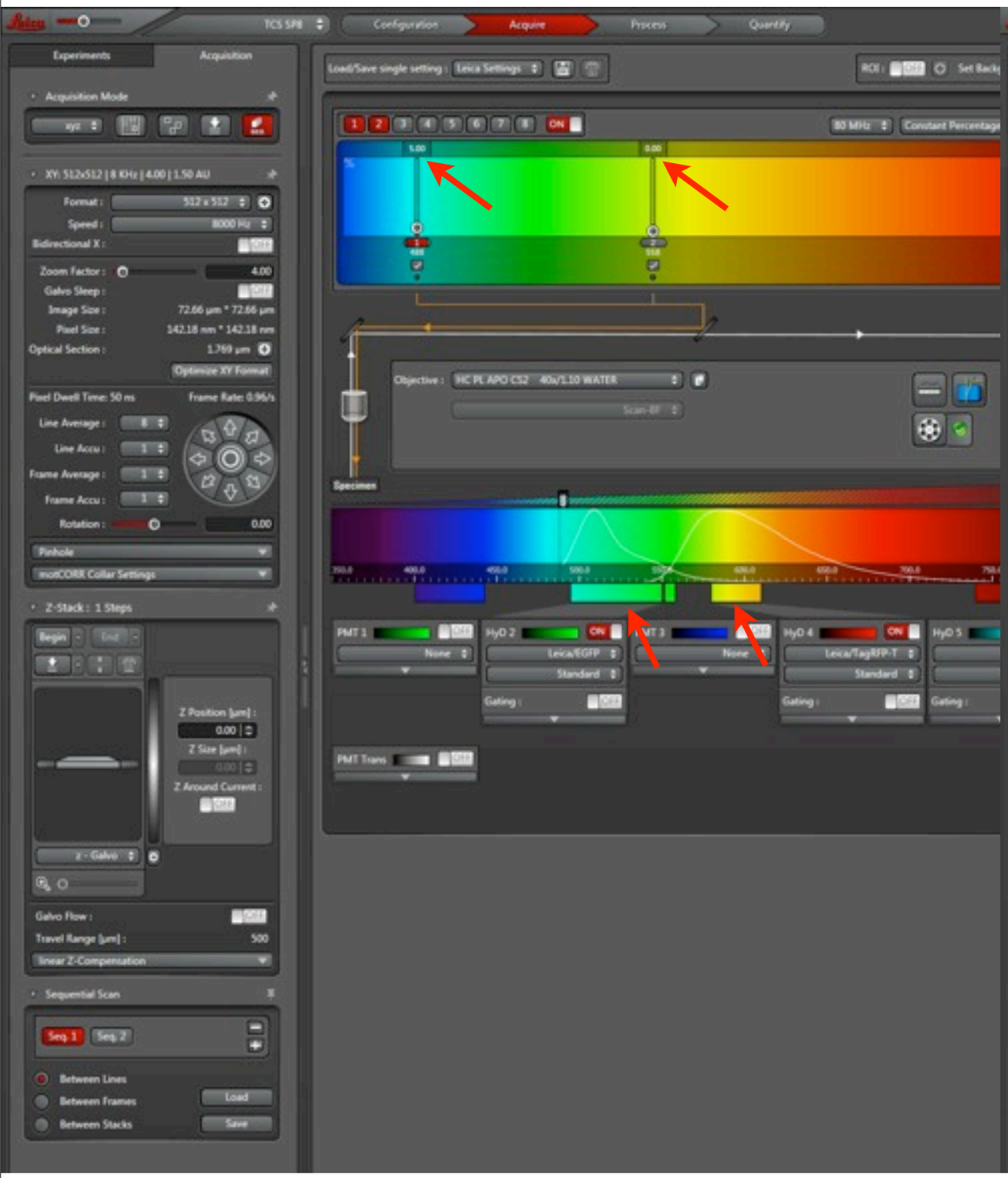
(look-up table)

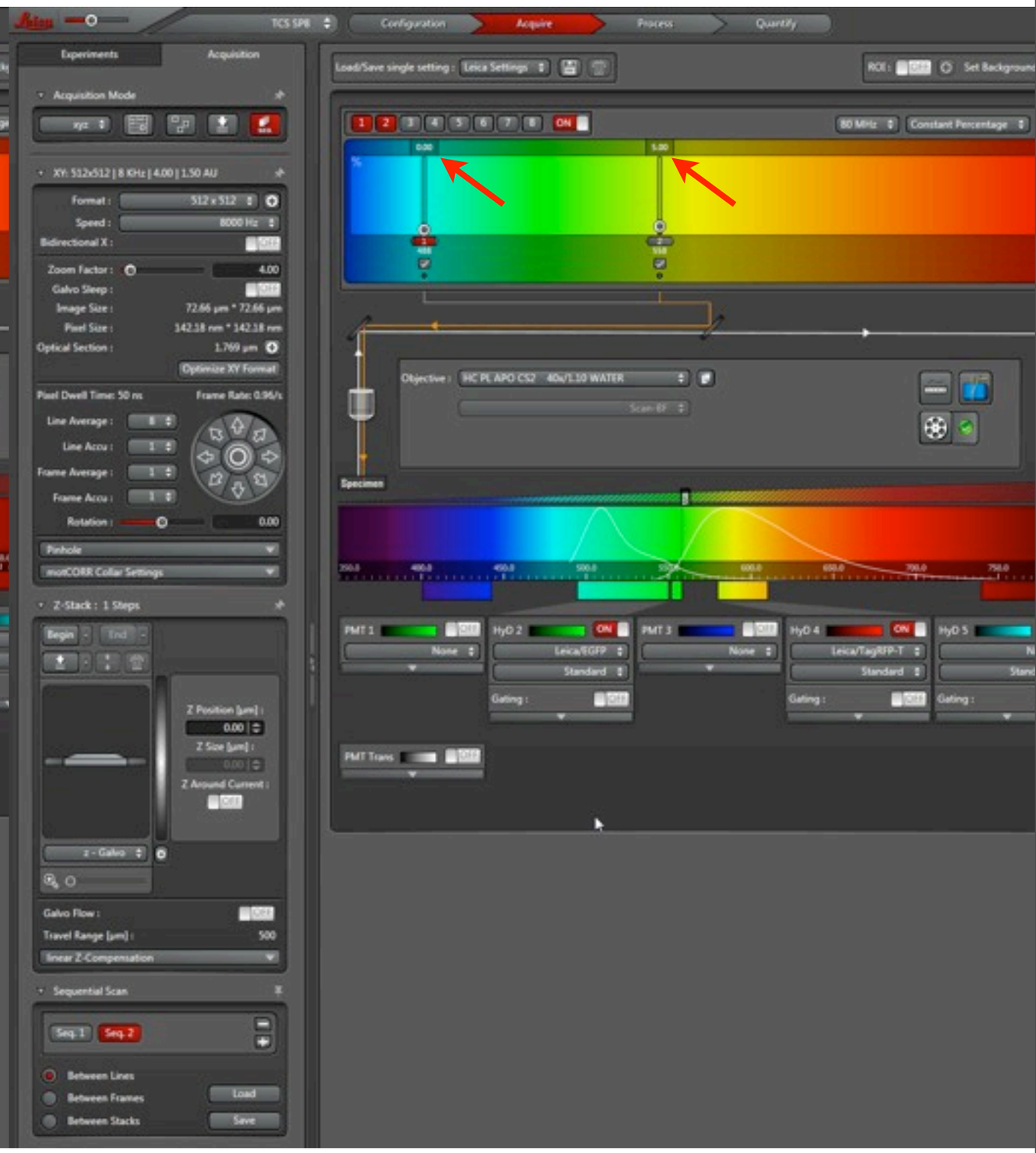
- LUT three sequential click steps: pseudocolor, dynamic range, white. Dynamic range: green is black value, blue is saturated value.

- Double click a given channel and it will be fully displayed. Click a channel for controlling its gain on the USB panel.

Channel display

Minimizing bleed-through by sequential acquisition



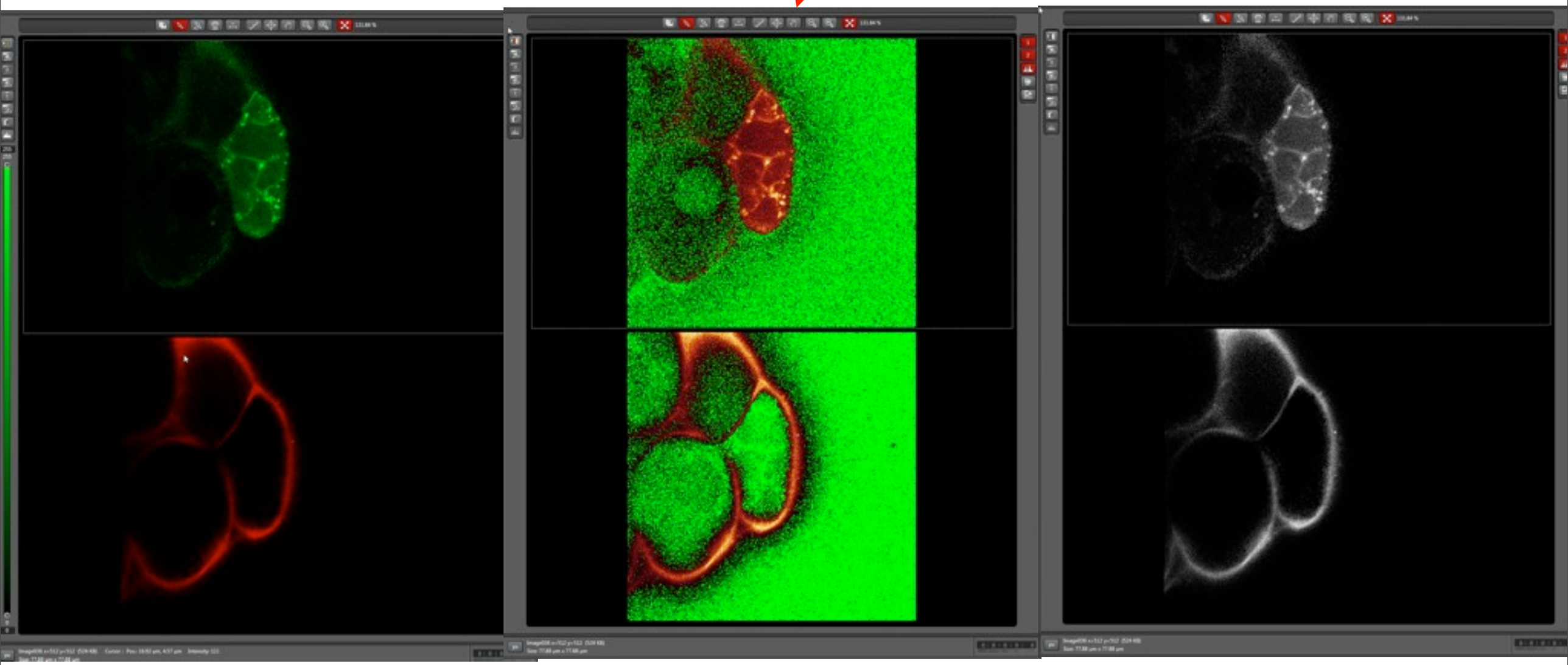


Scan one

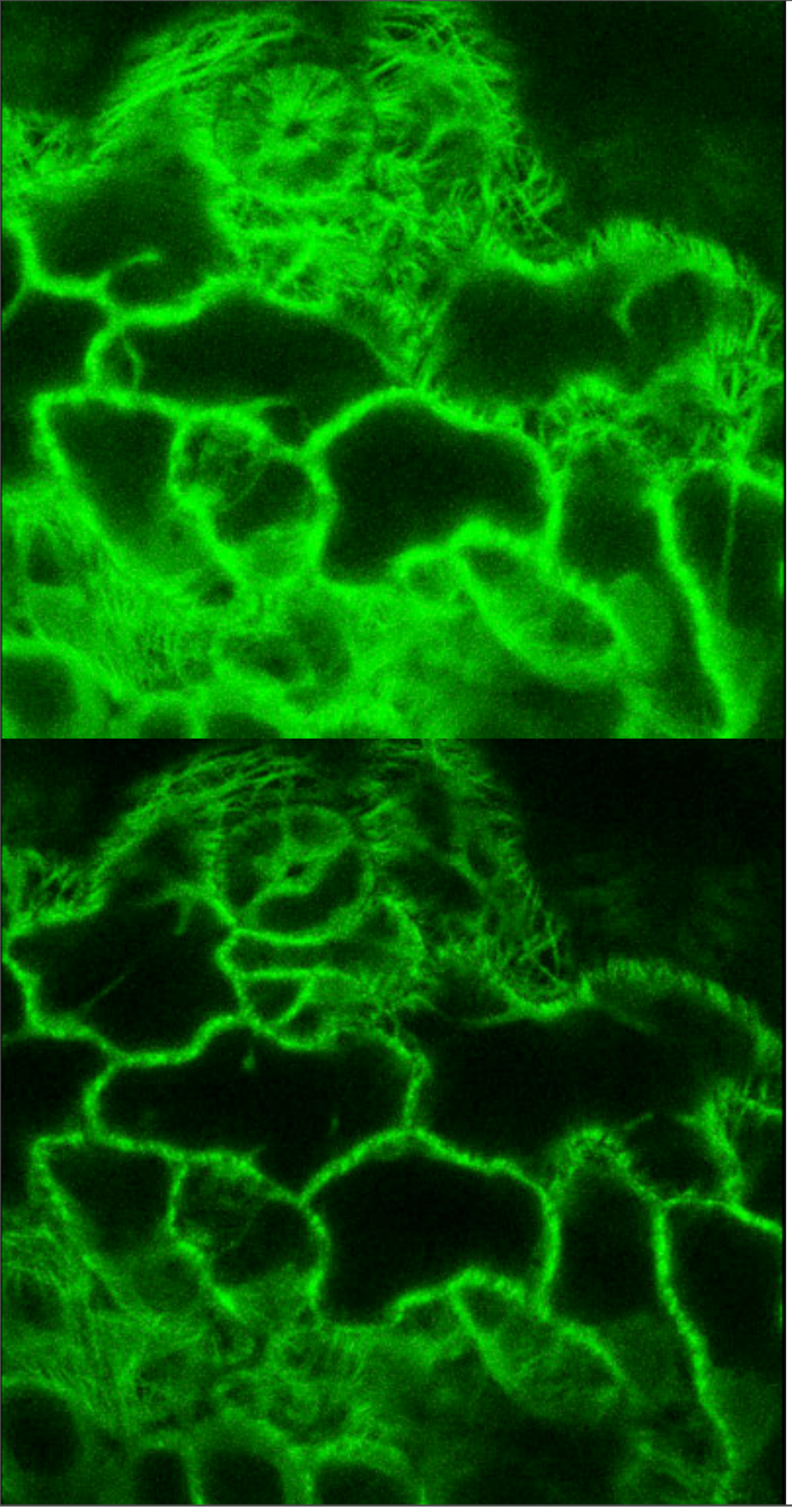
Scan two

Basic technique is to minimize bleed-through by adjusting S:N so that signal only occurs in the correct channel, set by using singly-stained controls. Then use the same acquisition S:N settings for the experiment sequence scan. In the SP-8 the detection sliders must not be different for the two scans. By scanning sequentially a given scan is therefore optimized for its fluorophore and lack of bleed into the other channel.

Adjusting Signal:Noise in the SP-8—click LUT button one time to get the dynamic range LUT



Green: pixels with an intensity value of zero=background should have this value
Blue: pixels with the maximum value (saturated)= avoid, lacks structure information
Warm colors: higher end of the intensity scale= ideal is to increase signal without getting into the blue range



Uncorrected

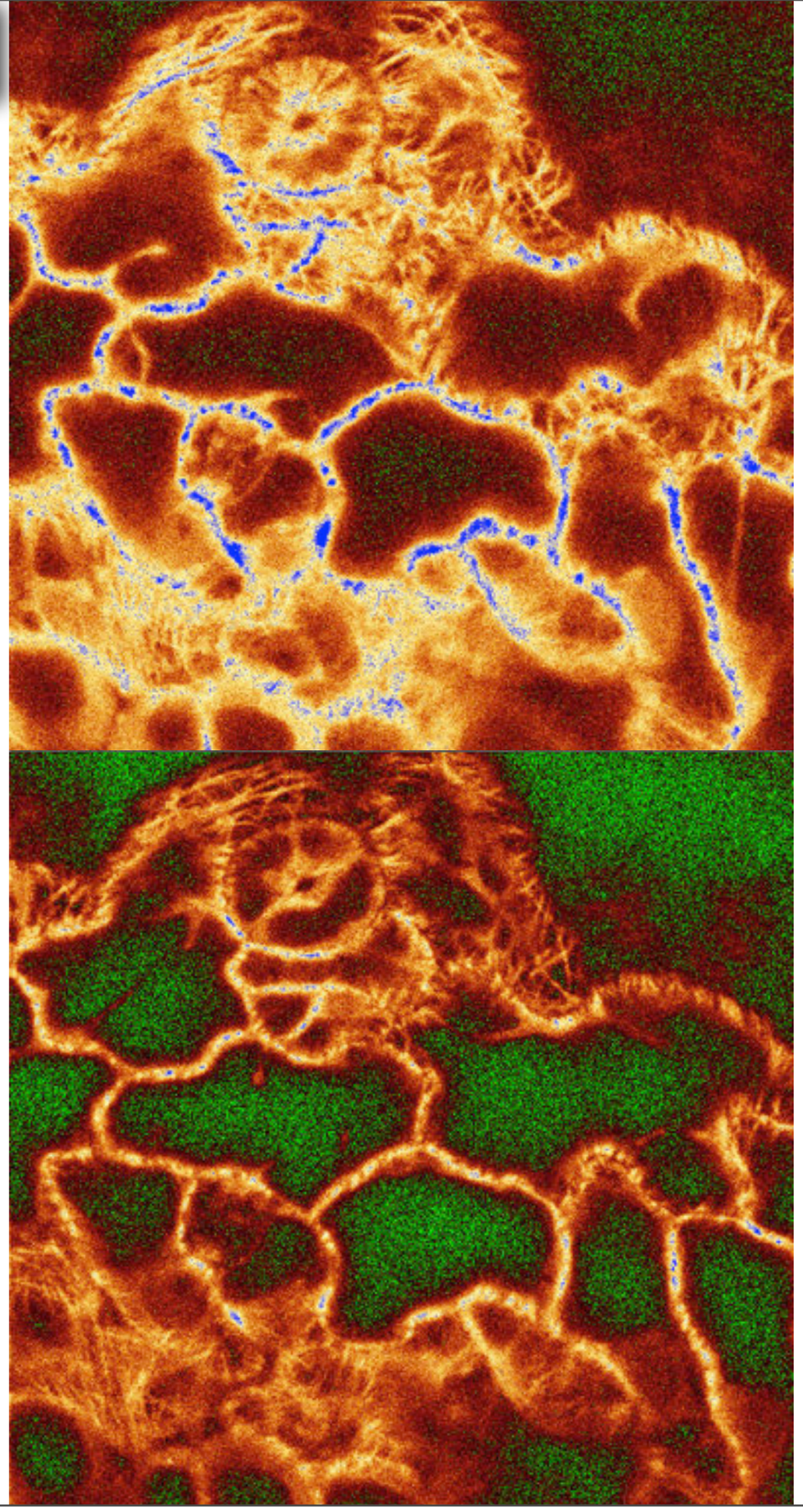
Green, black level, is adjusted by "offset" in USB panel.

Intensity not high enough?

1--increase gain and averaging
2--increase pinhole at expense of z axis resolution
3--increase laser power

Reduce intensity?

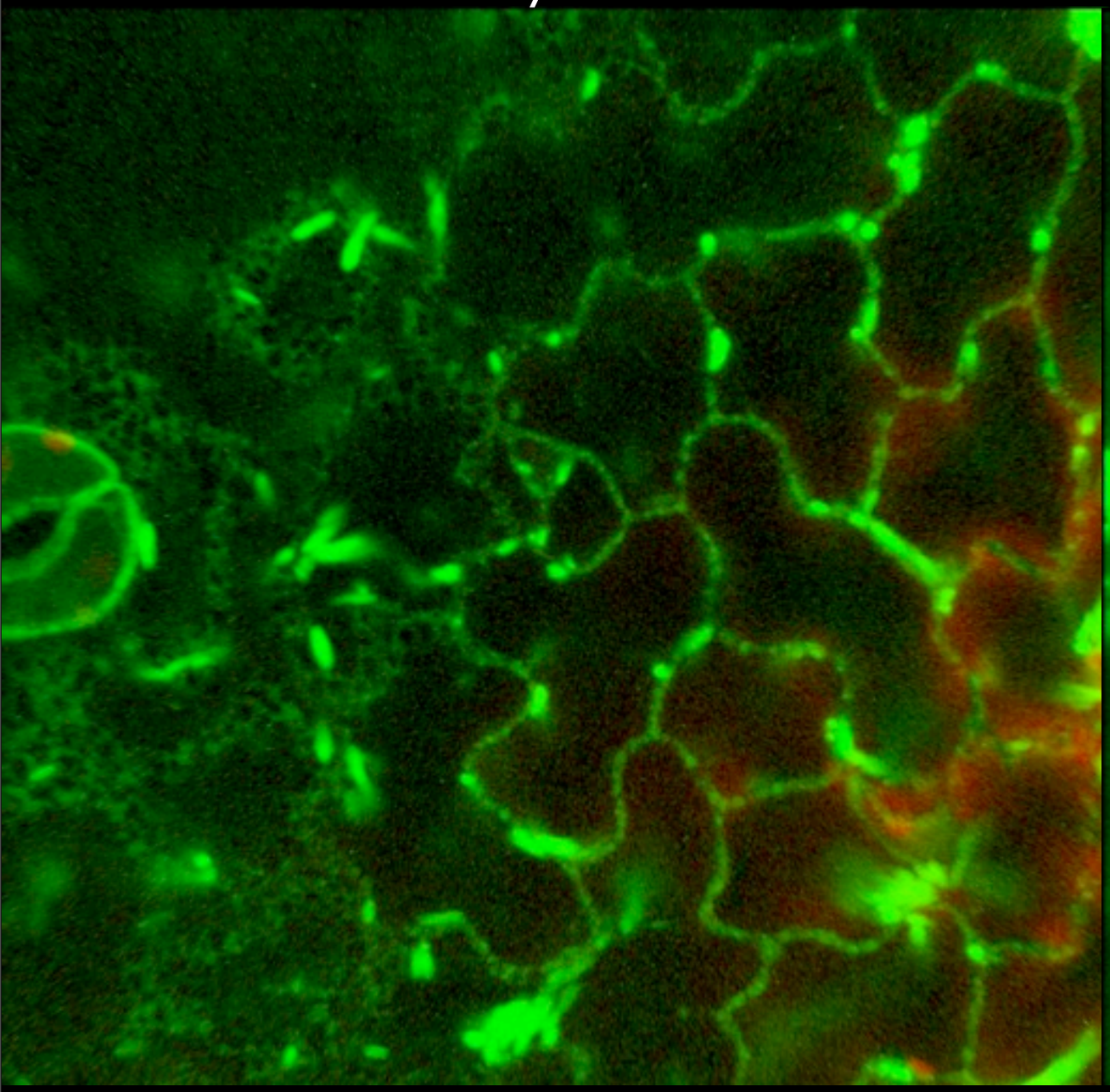
1--pinhole= 1 AU
2--reduce laser power
3--reduce gain



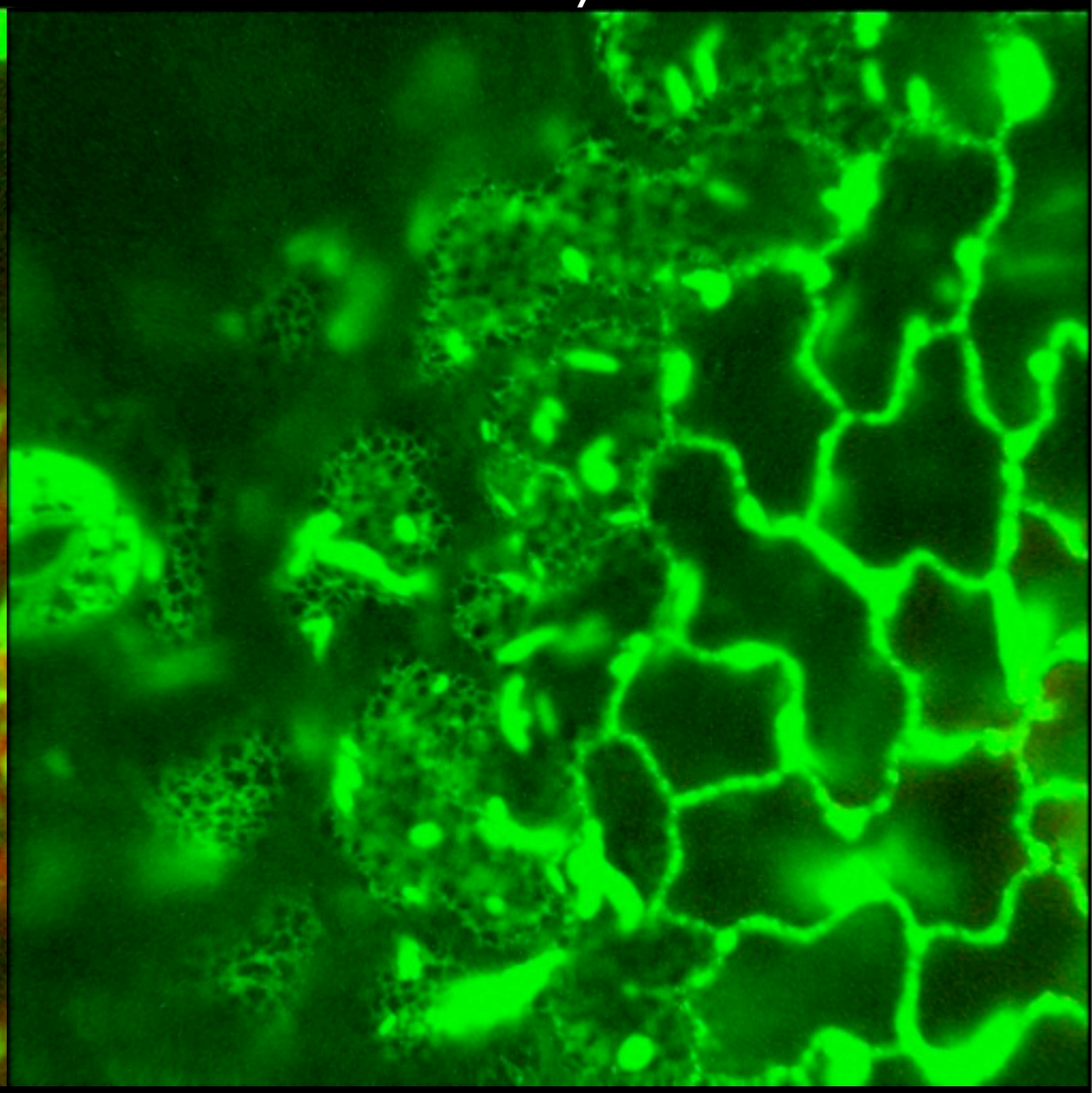
Corrected

Effect of changing pinhole size

One Airy Unit



Three Airy Units



- Increases the optical section thickness = samples more of the emitted photons, more signal
- More signal: opportunity to reduce laser power and therefore reduce photodamage
- Here: better sampling of the cortical ER, which is a curved sheet that is best seen with the increased depth provided by the bigger pinhole
- Thicker section means less Z axis resolution

Pixel size: satisfying the Nyquist criterion

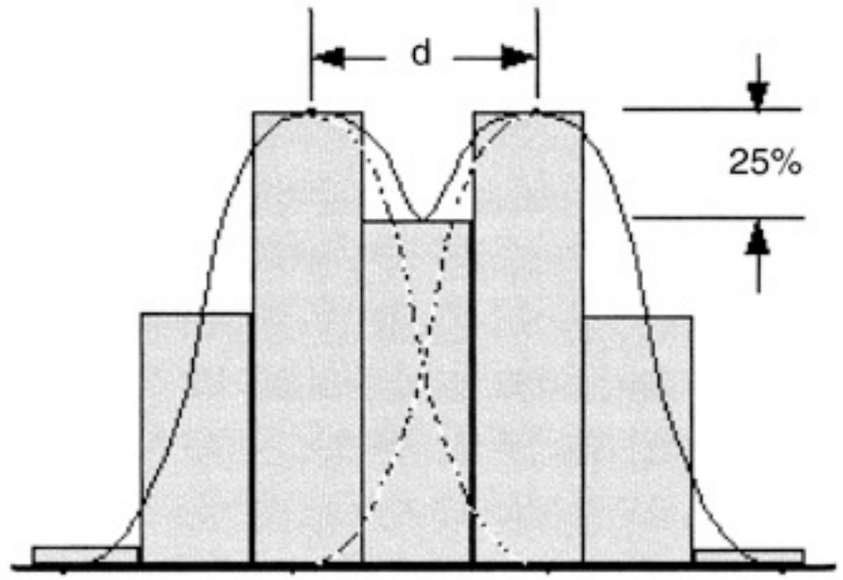
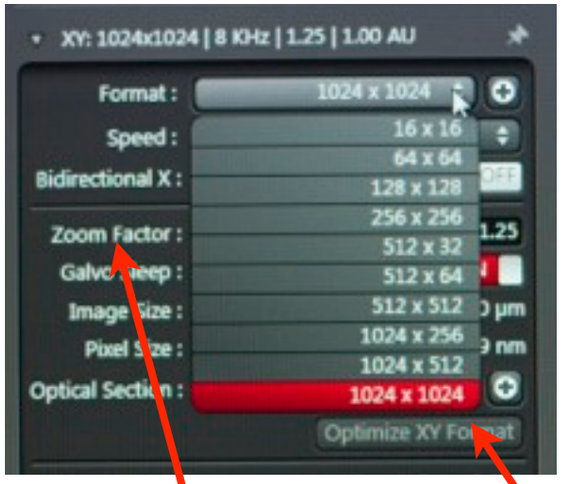


FIGURE 4.11. Nyquist sampling of an image of two points separated by the Rayleigh resolution.

The optical (Rayleigh) resolution must be sub-sampled by pixels in order to define the signal location accurately

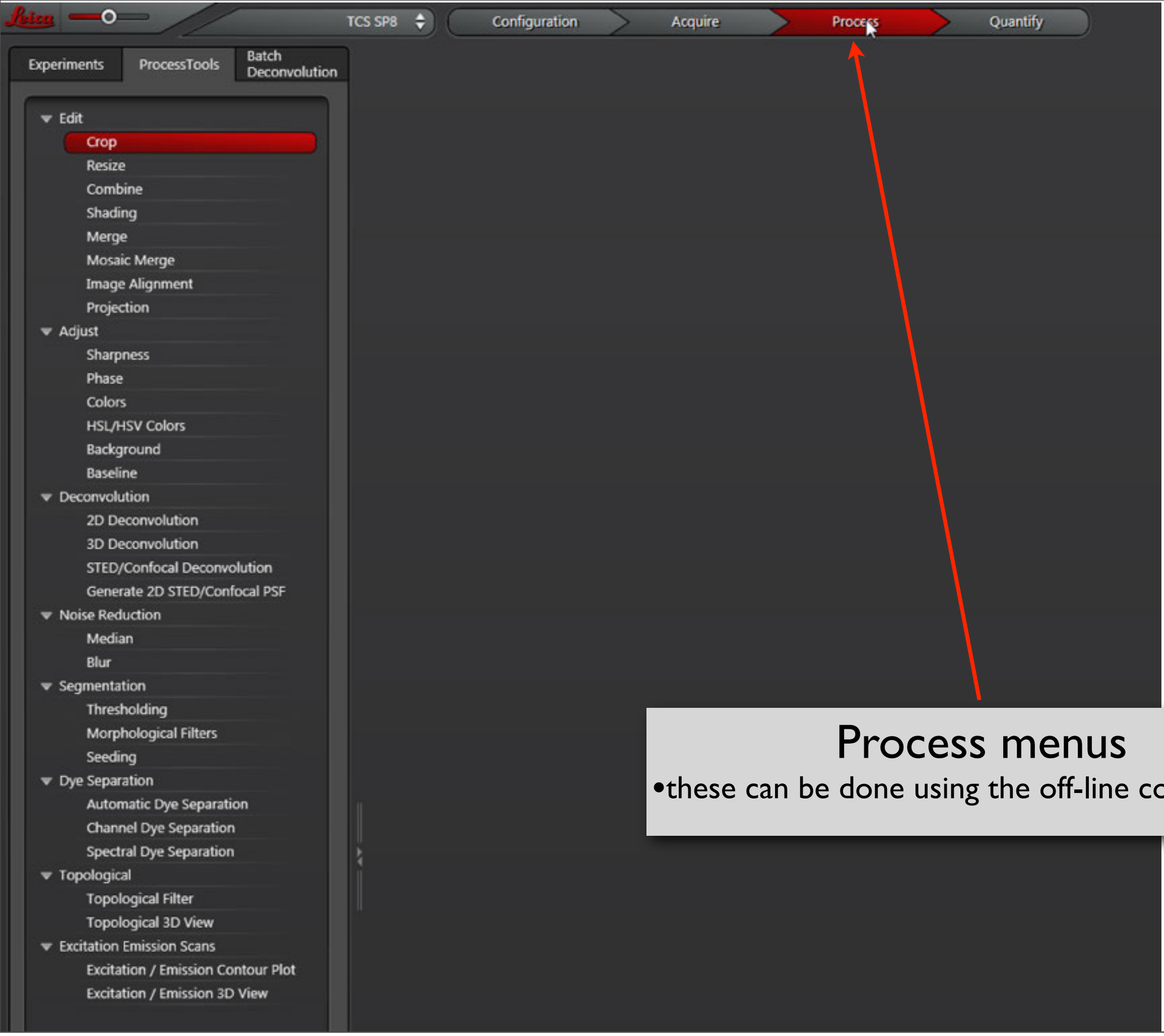
- ~2.3 X is typical
- E.g., if optical resolution is ~250 nm, then pixel size should be 250/2.3, or ~ 108 nm
- Pixels smaller than this make for oversampling, which subjects the specimen to excessive photodamage
- Pixel sizes considerably smaller than this do make for a better looking image, if this can be afforded

Pixel size adjustment on the SP-8



- Select larger image pixel dimensions: more pixels in the image=higher pixel resolution
- "Zoom" here or on the USB panel keeps the same number of pixels, in a smaller specimen region
- "Optimize XY format" will adjust them according to the Nyquist

Pixel size is here



Process menus

- these can be done using the off-line computer