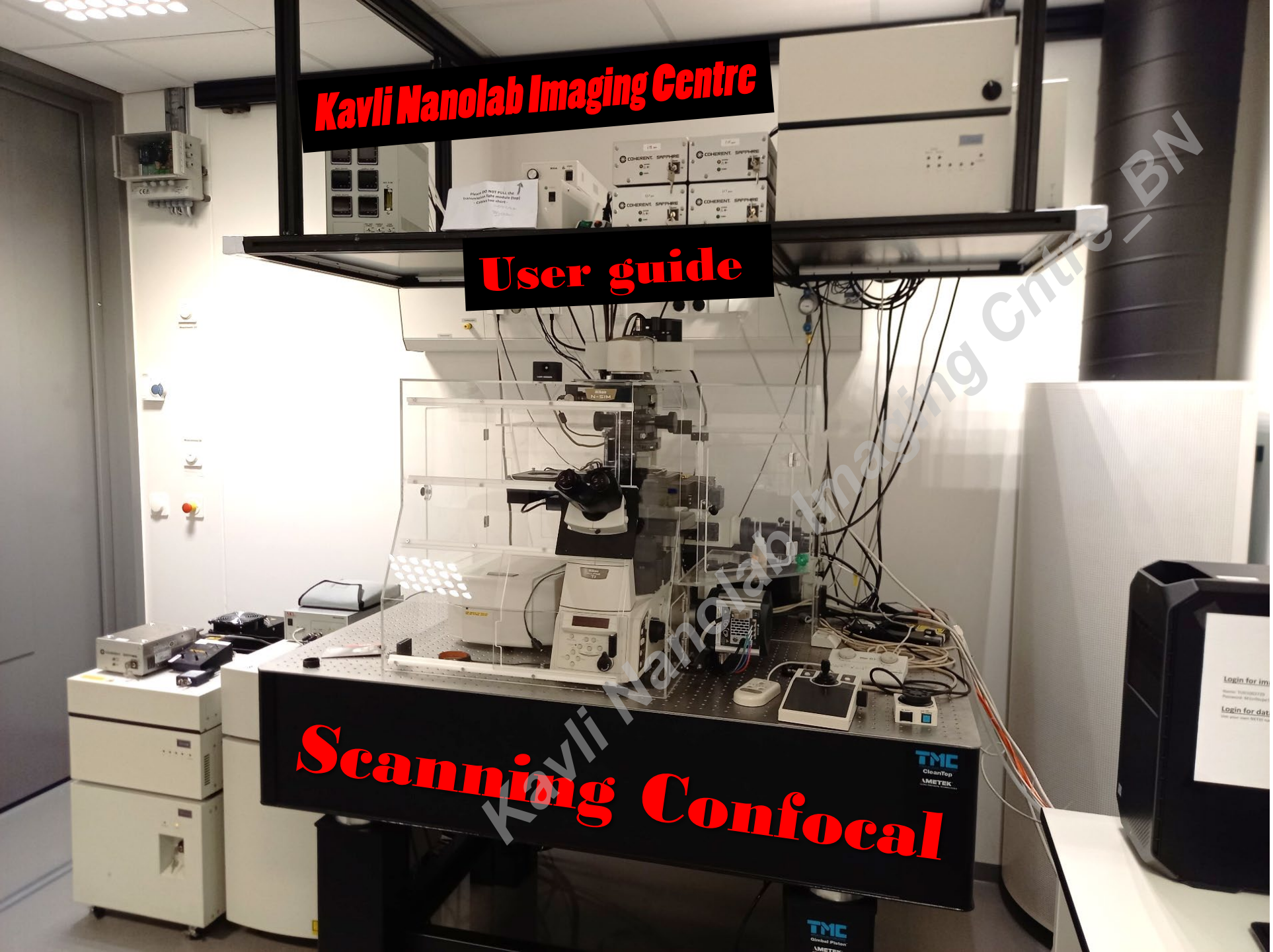
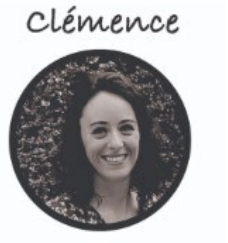


Co-author:
Clémence Taisne (AJ lab)



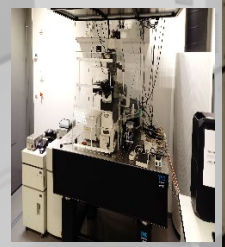
Start-up:

#4
Turn On Main switch (lamps/stage/heating)

#3
Switch your Individual lasers

#1
Switch A1R control

#2
Turn On Laser controller



!
Make sure all cage doors are closed

#5
Turn on PC and log into Nis Elements

Clémence Taisne

Focus on your sample- eyepiece- general:

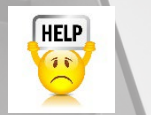
#1 Choose the eyepiece path in the software (Epi/Dia)* *Page#17*

#2 Starting from objective height Z=500µm
Raise the objective until you see your sample

If you do not see increase in brightness- Stop! You might damage the objective.

Check*:
Try both BF/Flu
Do you see light on your sample?
Is the transmitted path open?
Is your sample mounted properly and matched objective WD (penetration depth)?

*Better ask for help than damage the Objective! Don't force it!



Confocal Vs. wide field : Pinhole

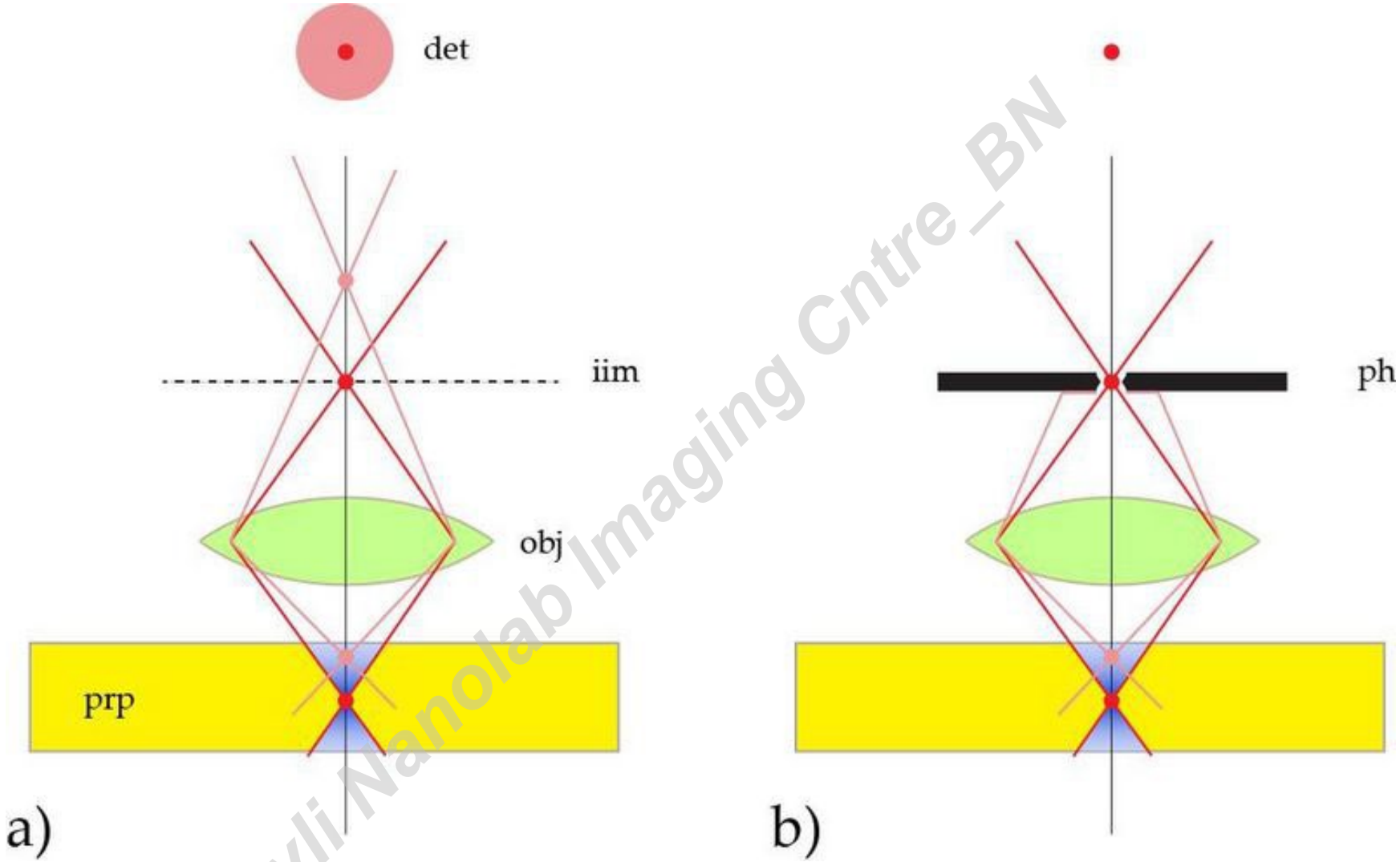
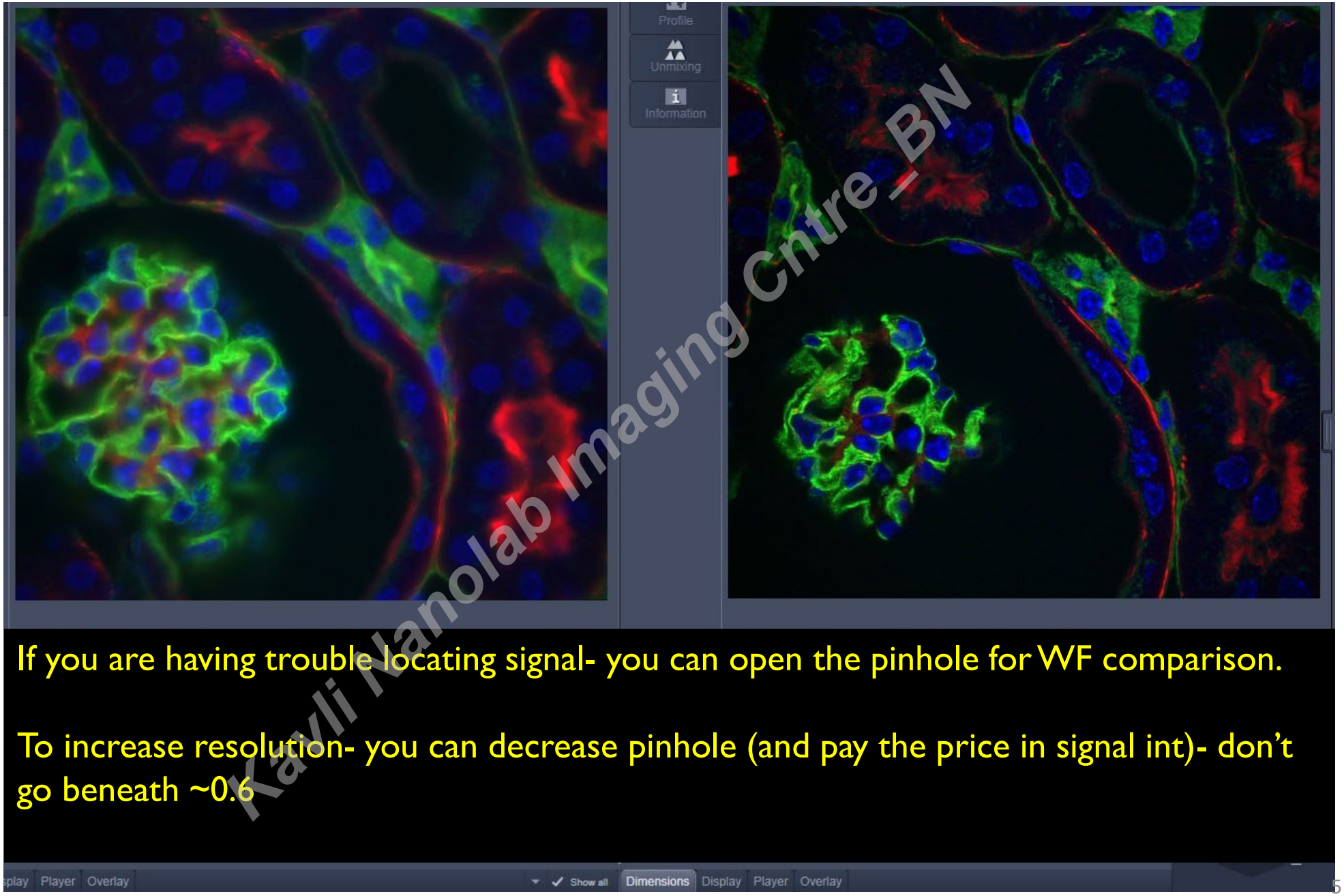


Figure 1: The optical knife. a) a compound microscope generates an intermediate image (iim) by an objective lens (obj), containing both focal (red) and extrafocal (pink) signal emanated by the sample (prp). When imaging a single spot, the detector (det) will record both a spot-shaped feature from the focal plane and extended blurry discs from other regions. b) by introducing a pinhole aperture (ph) in the intermediate image plane, nearly all extrafocal signal is cut off and solely the emission from the focal plane can reach the detector. The spatial filter generates an optical section.

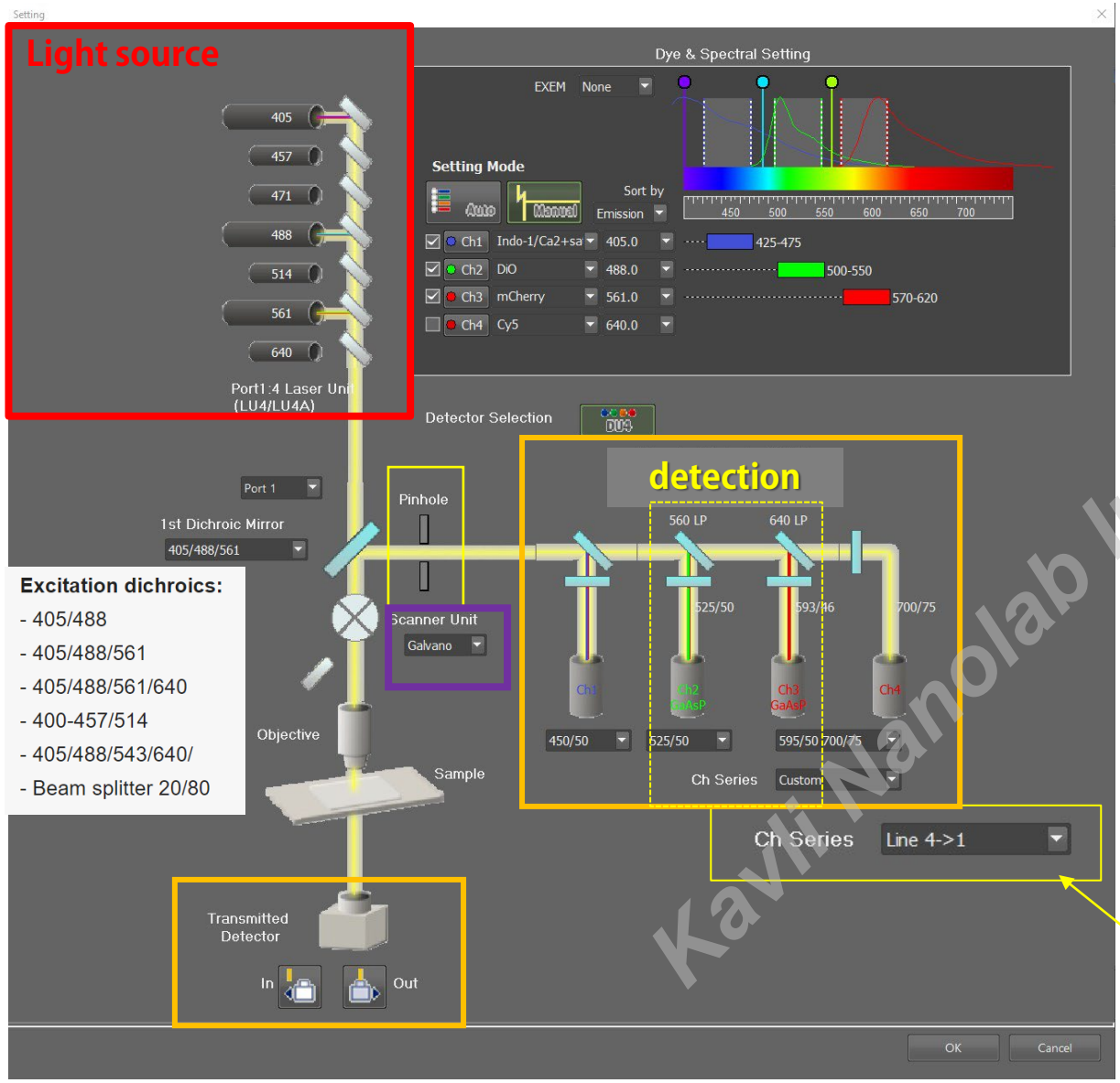
https://www.leica-microsystems.com

Confocal Vs. wide field : Pinhole



http://www.microscopist.co.uk/essential-techniques/confocal-microscopy/

Light path



Excitation dichroics:

- 405/488
- 405/488/561
- 405/488/561/640
- 400-457/514
- 405/488/543/640/
- Beam splitter 20/80

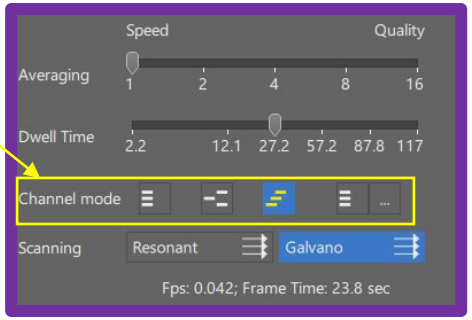
Fluorescence filters:

Detector	Dichroic beam-splitter	Fluorescence Emission filter
conventional PMT detector	495 LP 515LP	450/50 482/35
GaAsP detector	560 LP 640LP	525/50 540/30
GaAsP detector	640LP	595/50
conventional PMT detector	-	700/75

Choose the detector according to signal (wavelength/energy)

Work in low gains

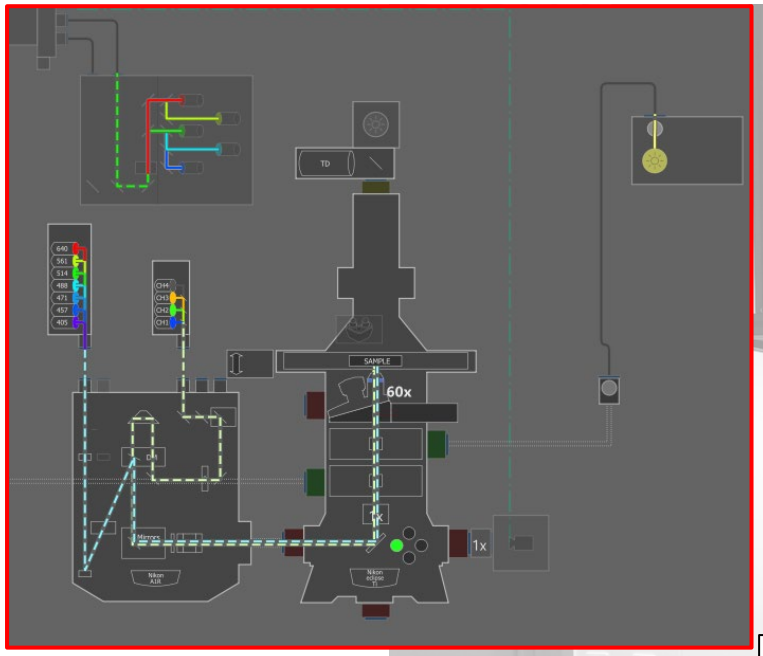
Illuminate red shifted first



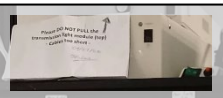
To enhance SNR in resonant fast scan, you can add line average

To enhance SNR in Galvano scan, increase pixel dwell time

Confocal Scanning Microscope :



motorized XY stage ctrl



Confocal scan-descan:

Piezo Z stage

Stage control (XY and Z)

Light Path: In the Nikon A1 systems, the light follows a scan-descan path. The light leaves the lasers, enters the scan head, moves through the optical train to the sample, and excites it. The fluorescence is then collected in the objective and returned to the scan head. Since fluorescence is red-shifted, it can be re-directed via a dichroic mirror to exit out the rear optical output ports. This process (where the light is directed with the scan mirrors in both directions) is known as descanning. After that, the light moves to the A1 controller where it interacts with the PMTs and the signal is converted to an image in the computer.

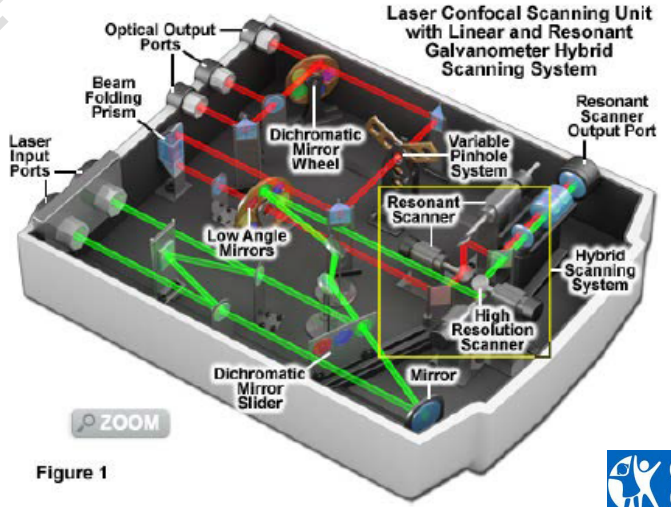
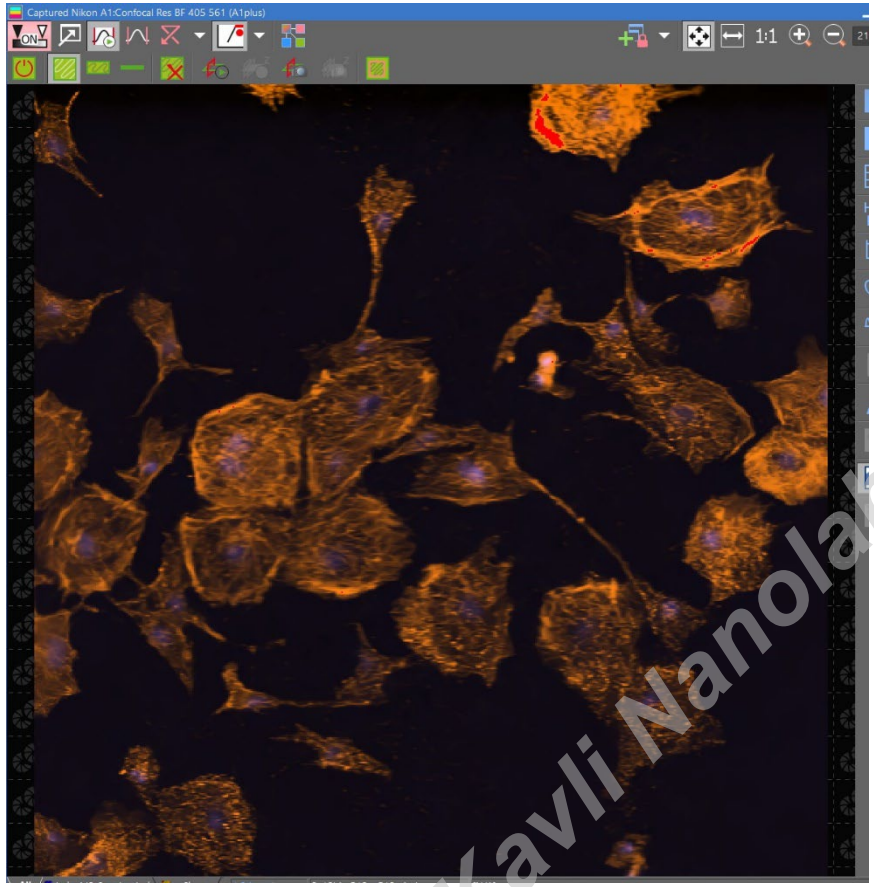


Figure 1

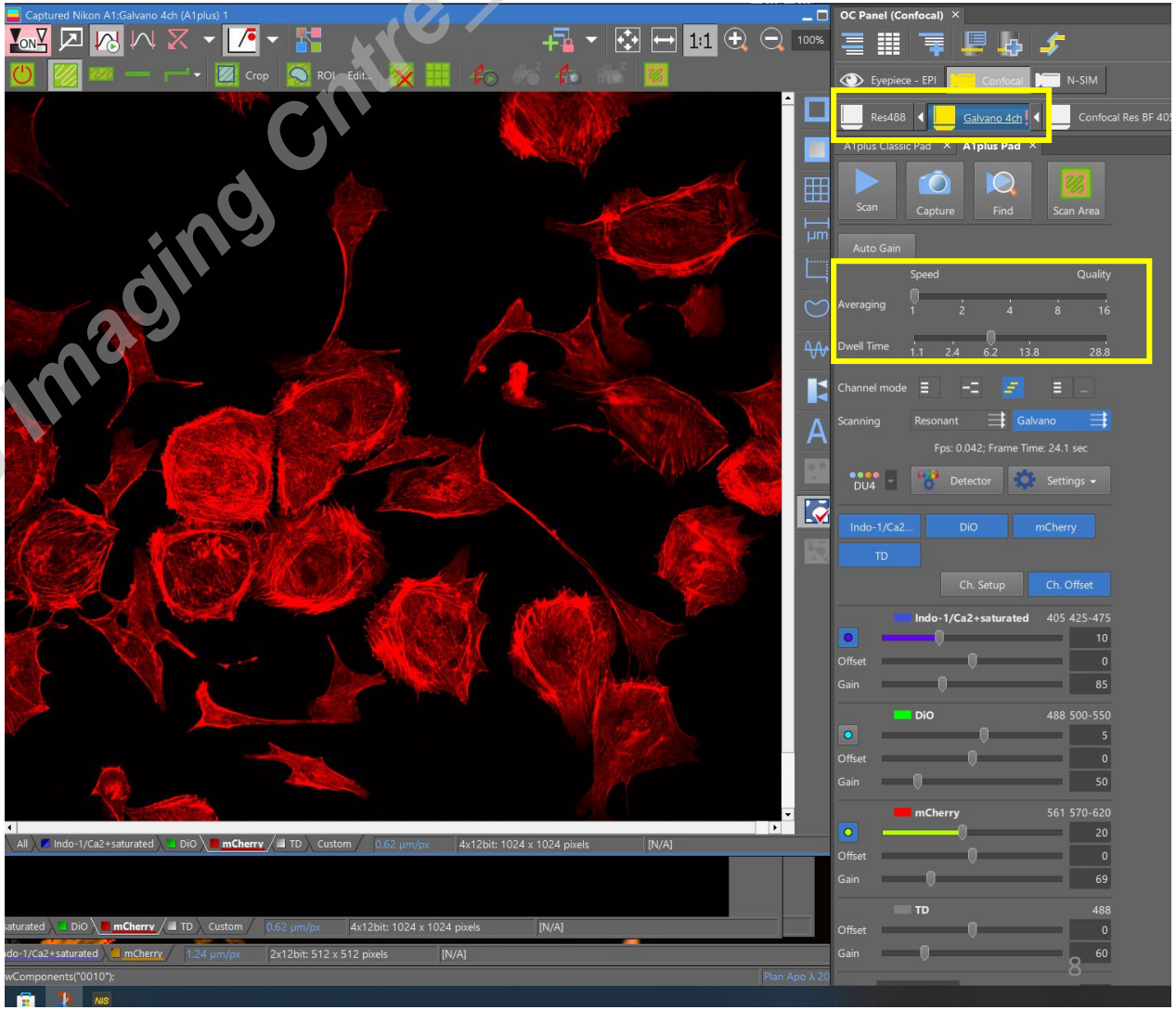
Clémence Taisne

Scanner Options

Resonant scanner (8KHz), no Av

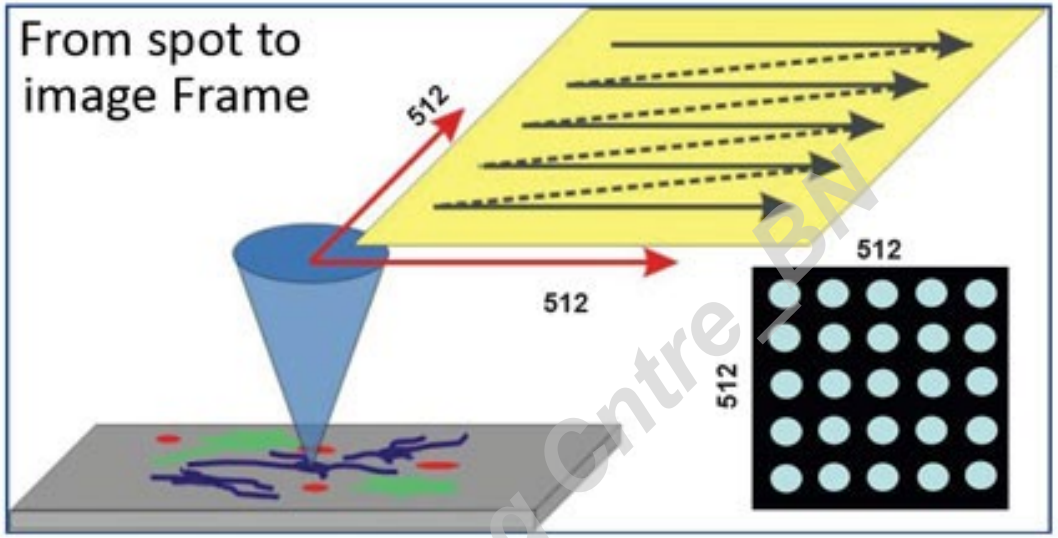


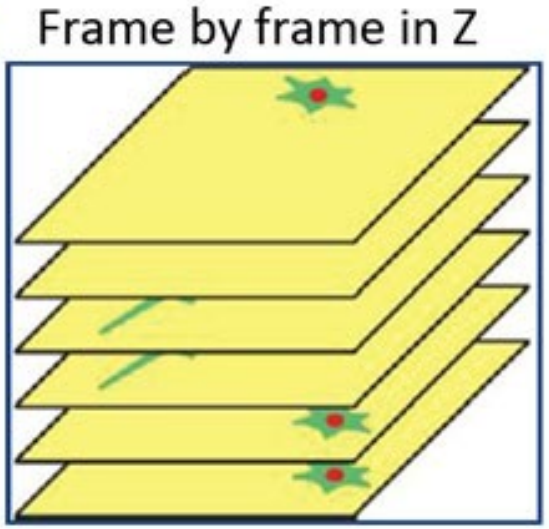
Galvano scanner (adjustable), no Av



Scanning confocal-

Optimizing dwell time





http://www.microscopist.co.uk/essential-techniques/confocal-microscopy/

Acquires *one pixel at a time*

Frame time = pixel dwell time × pixel number

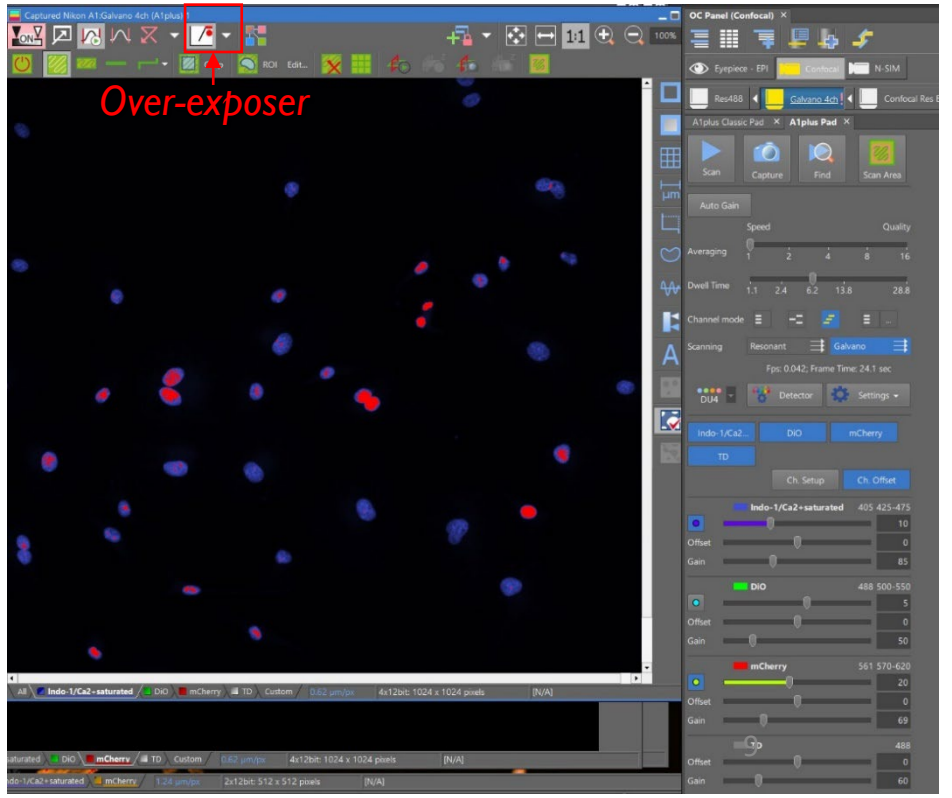
To decrease dwell time, must *increase emission rate*

to maintain signal level

⇒ Must *increase illumination intensity*

Where's the limit?

- Phototoxicity?
- Saturation



Prof. Zvi Kam, Weizmann Institute of Science

Sampling:

Pixel size/pixel number/scan area

* *There is no advantage in over-sampling (high pixel number) when no additional information can be retrieved*

- *However, under-sampling will result in loss of data*

- *Nyquist Sampling:*
  the sampling interval must be at least twice the highest spatial interval. If the smallest resolvable feature is 5µm, then each detector pixel must sample intervals that are ≤ 2.5 µm.

The optimal spatial sampling can be retrieved from the software

Nyquist rate and PSF calculator

Microscope type: Confocal

Numerical aperture: 1.3

Excitation wavelength: 488 nm

Emission wavelength: 520 nm

Number of excitation photons: 1

Lens immersion refractive index: Oil 1.515

☐ Calculate a Point Spread Function

Calculate

Nyquist rate and PSF calculator

Results

This is the parameter list used in this calculation:

Parameter	Value
Microscope type	Confocal
Numerical aperture	1.3
Excitation wavelength	488
Emission wavelength	520
Number of excitation photons	1
Lens immersion refractive index	1.515

The optical axis lays along z. Your Nyquist sampling is:

x: 46 nm
y: 46 nm
z: 165 nm

— Set your zooms and scanning steps so that each pixel covers a x-y area of 46 nm × 46 nm (or smaller)
— Calibrate and set your z-stepper so that it takes steps of 165 nm when acquiring a 3D stack (or smaller)

For Confocal Microscope images recorded with pinholes of around 1 Airy disk diameter, lateral sampling distances may be up to 1.6 times of the recommended Nyquist values without significantly compromising image quality. When small pinholes are used (< 0.5 Airy disk), up to 1.3 times larger; with very large pinholes (> 4 Airy disks), up to 2 times.

https://svi.nl/nyquistV2/

Focus on your sample- Perfect Focus system:

near-infrared 870-nanometer LED and CCD line sensor record and correct for thermal/vibration drifts

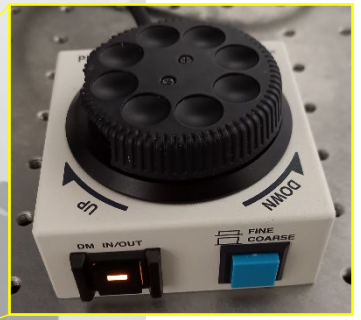
#1 Starting from objective height Z=500µm

Focus on your sample using BF/EpiFlu manually

#2 Enable PFS*

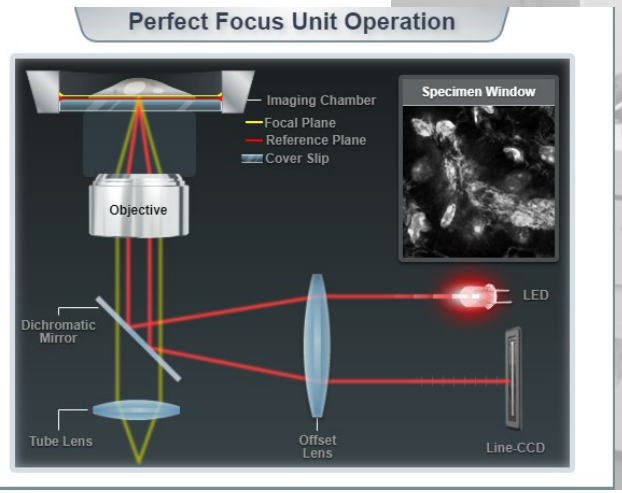
PFS will blink until finding surface- then beep

***needs to be activated close to the surface**



In/Out

Fast/Slow



#3 Z is now disabled- continue with the PFS controller

Move the wheel until your sample is in good focus.

The PFS offset can be used in the software

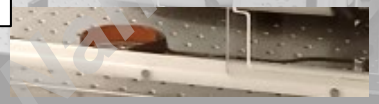
https://www.microscopyu.com/applications/live-cell-imaging/correcting-focus-drift-in-live-cell-microscopy

Environmental Control:

3. Turn on the controller, set to the right values.
!Note- Only use "Stage Heater" and not "Sample Temp"

2. Place water inside the stage heating chamber (around the sensor).
**make sure the sensor is touching sample*
***Don't forget to empty the water when done!!*

1. wrap heater band around your objective



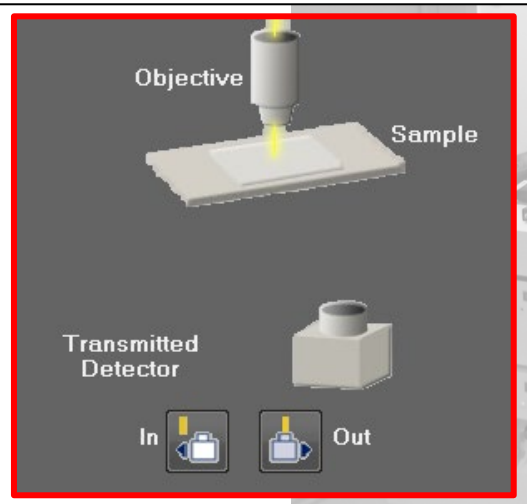
4. For mammalian cells- open the CO_2 valve

Epi-Flu and BF (Hardware):

Halogen lamp/brightfield

Mercury Lamp-
Epi flu:
405/488/561

*If you have trouble seeing BF- check in the software that the TD is in position "Out"



White light is controlled best via software*

Relevant filters are chosen by the software

Mercury Lamp controller

ND : Neutral Density Filters
The higher is the number, the darker is the filter (for strong signal).
The lower is the number, the lighter is the filter (for weak signal)

Low energy light- keep ND @ 1

Start Nikon Acquisition (NIS-Elements):

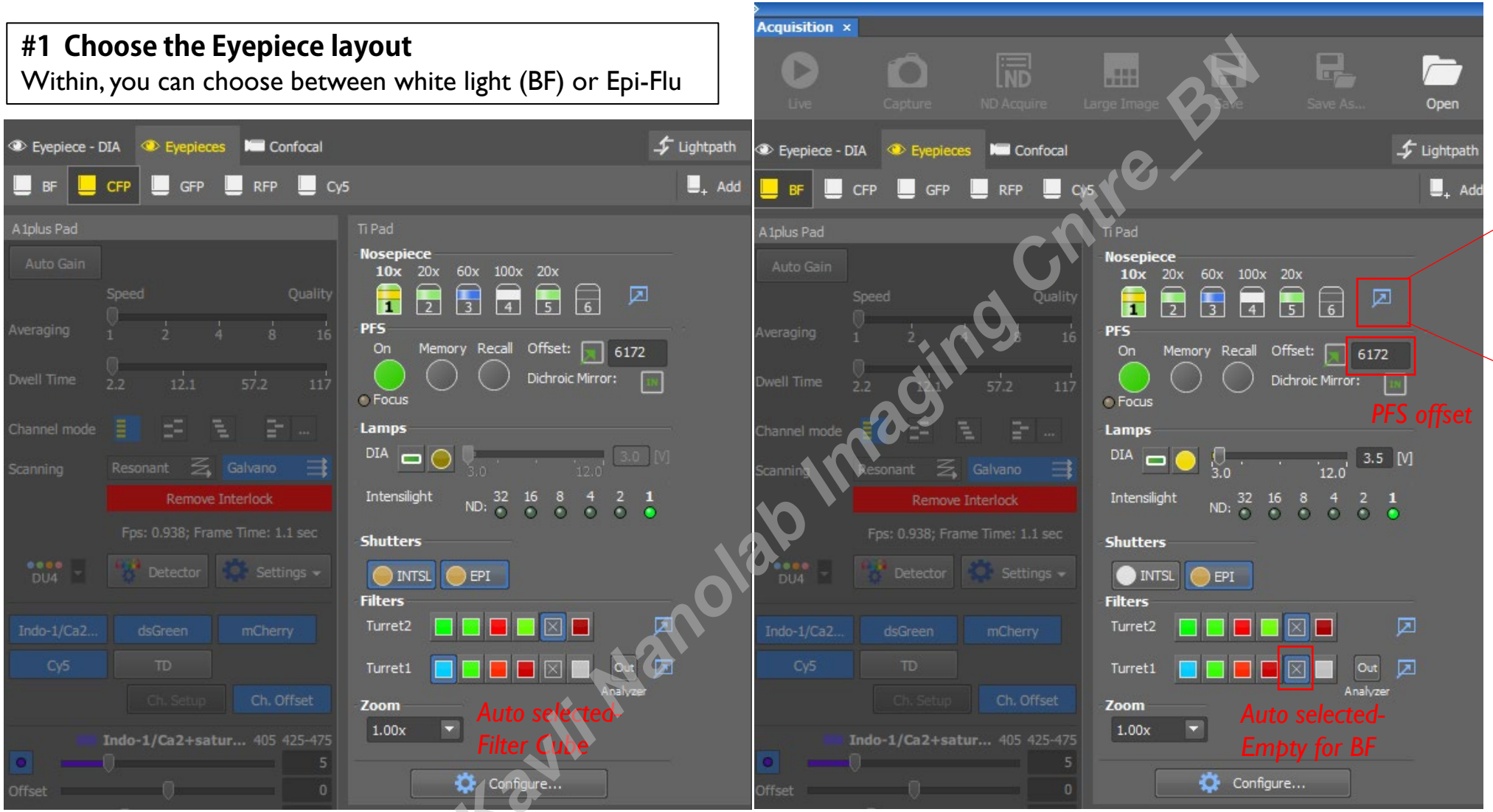
#1 log in to the PC with your username and password

#2 NIS-Elements should be opened without further dialog, showing your last setup

#3 Save on local folder (C/Data/Username) and when done copy to network folder

Epi-Flu and BF (Software):

#1 Choose the Eyepiece layout
Within, you can choose between white light (BF) or Epi-Flu



You can find here info for each Obj

#2 Troubleshooting
Check the selected filter matches; that the relevant shutter is open

Shift to confocal:

Choose the Confocal tab layout. Remove interlock
Within, you can easily shift between your defined pre-sets:

#1 quick Resonant scan:

Fixed scan speed of 8KHz
Bidirectional scan

Recommended to work on 1 laser line only to protect sample

Limited scan resolution (512)- reduce scan size for optimal resolution

Add averaging- cleans noise
Add denoise.ai – smoothens the image

Open pinhole to 3AU for thicker sectioning
Lower laser power, increase detector gain

#2 High resolution Galvano scan:

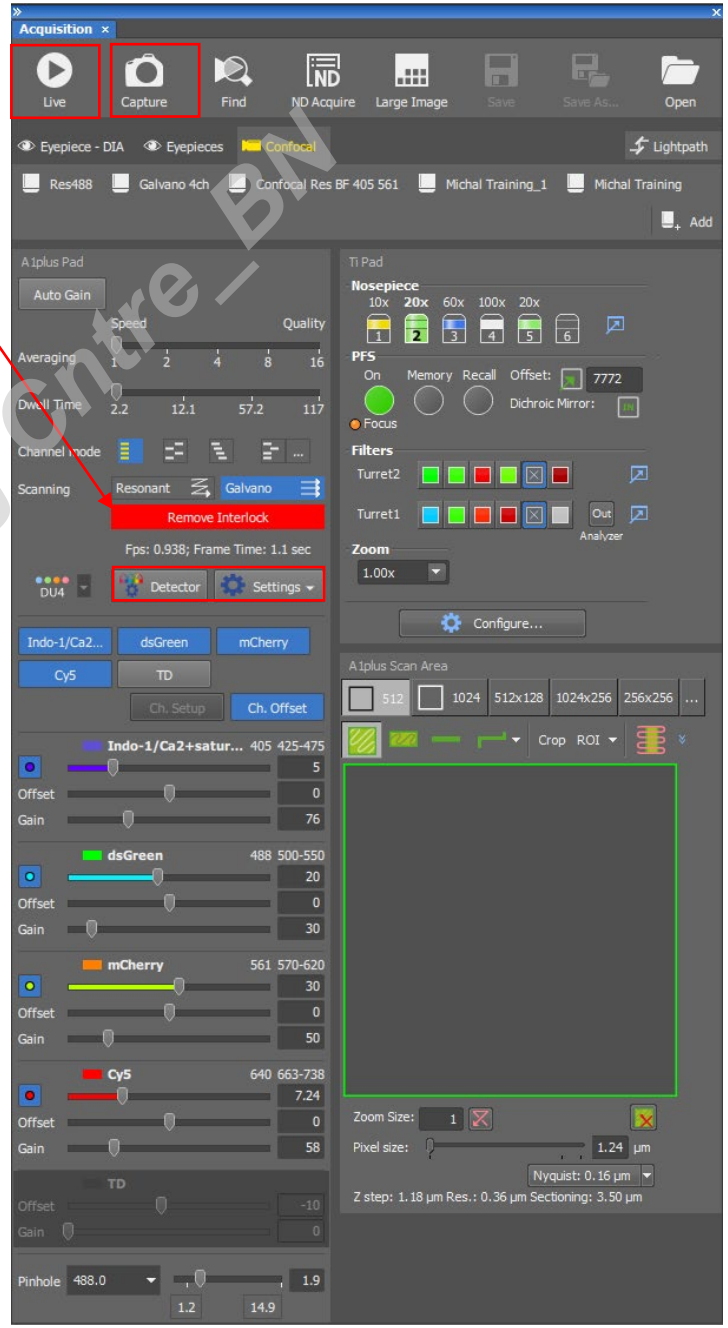
Adjustable scan speed/Dwell time
*Does not operate well in fast mode

Adjustable scan resolution- check for optimal sampling

Close pinhole for higher Z resolution

Try to work in minimal gain and higher laser

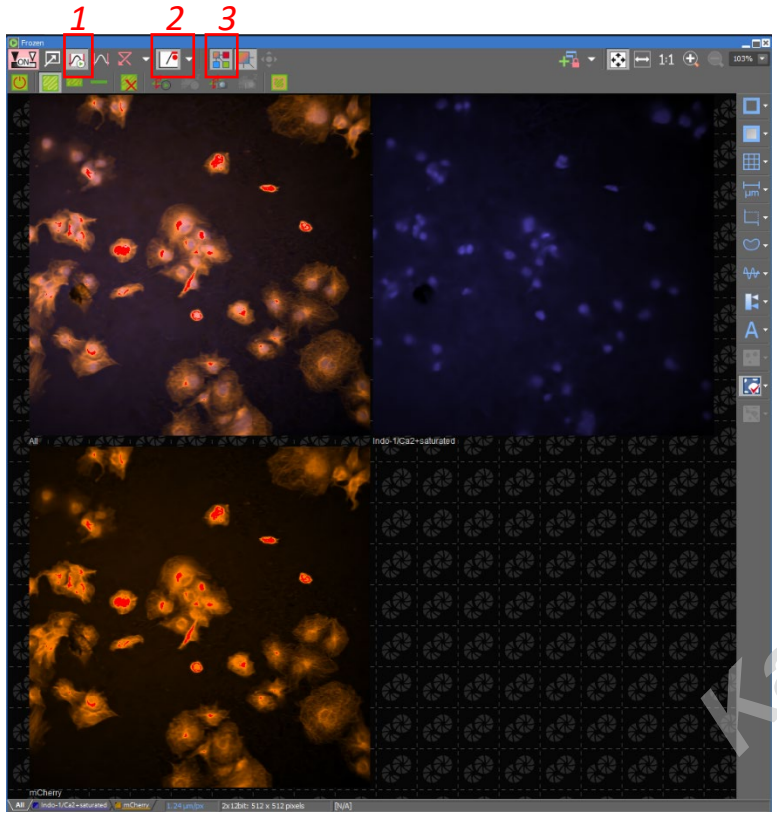
Work only on these two



Confocal basics:

#4 View:

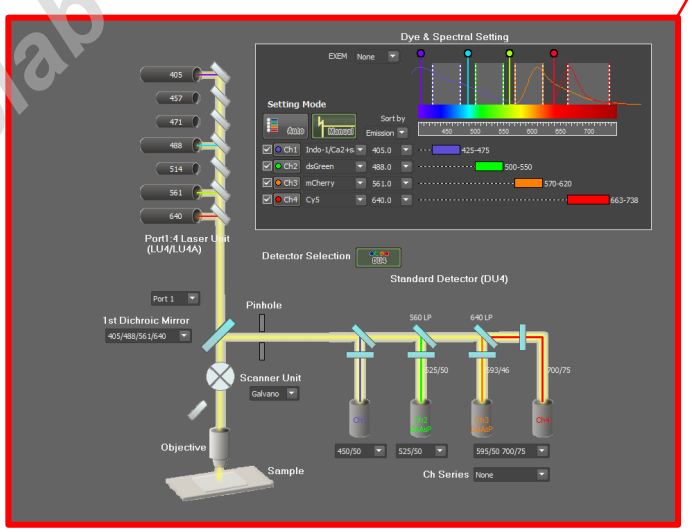
1: Auto threshold
2: Oversaturated pixels
3: Multi-channel view

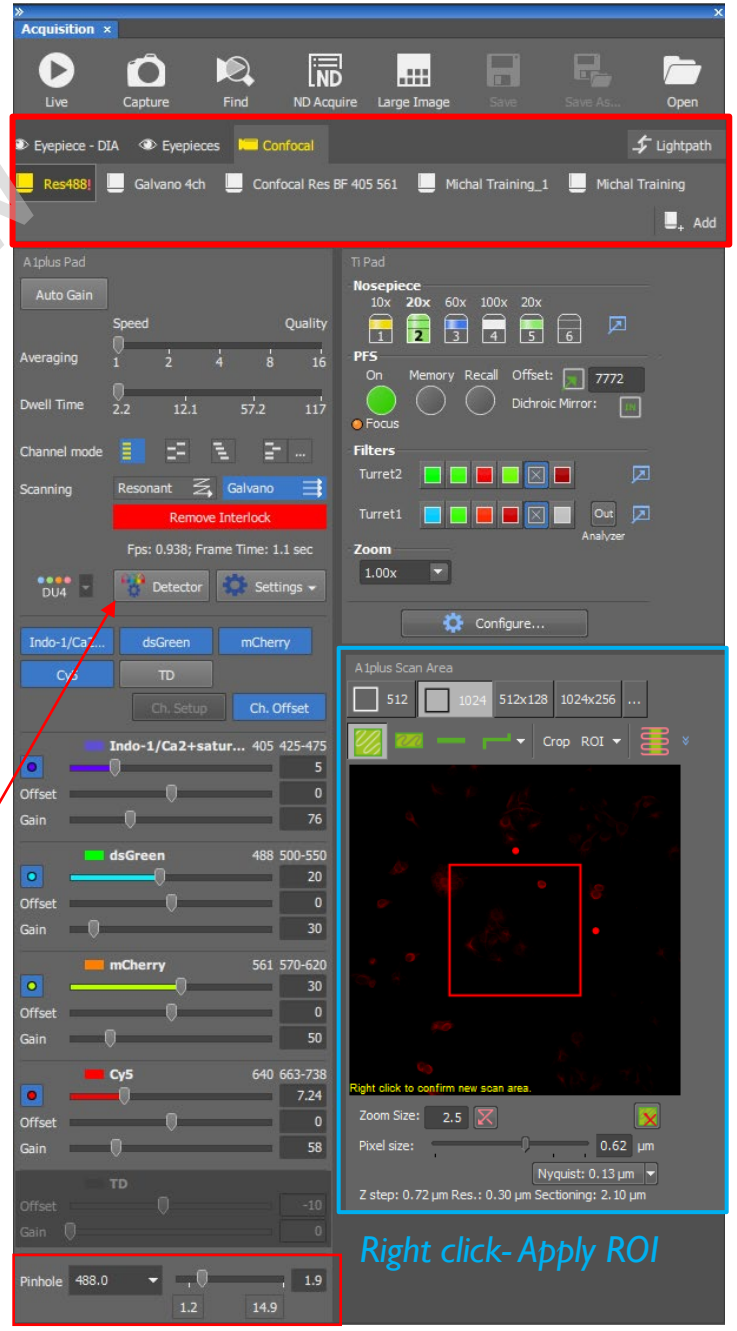


Right click- Apply Camera settings- to keep your parameters

#3 Optimize if needed:

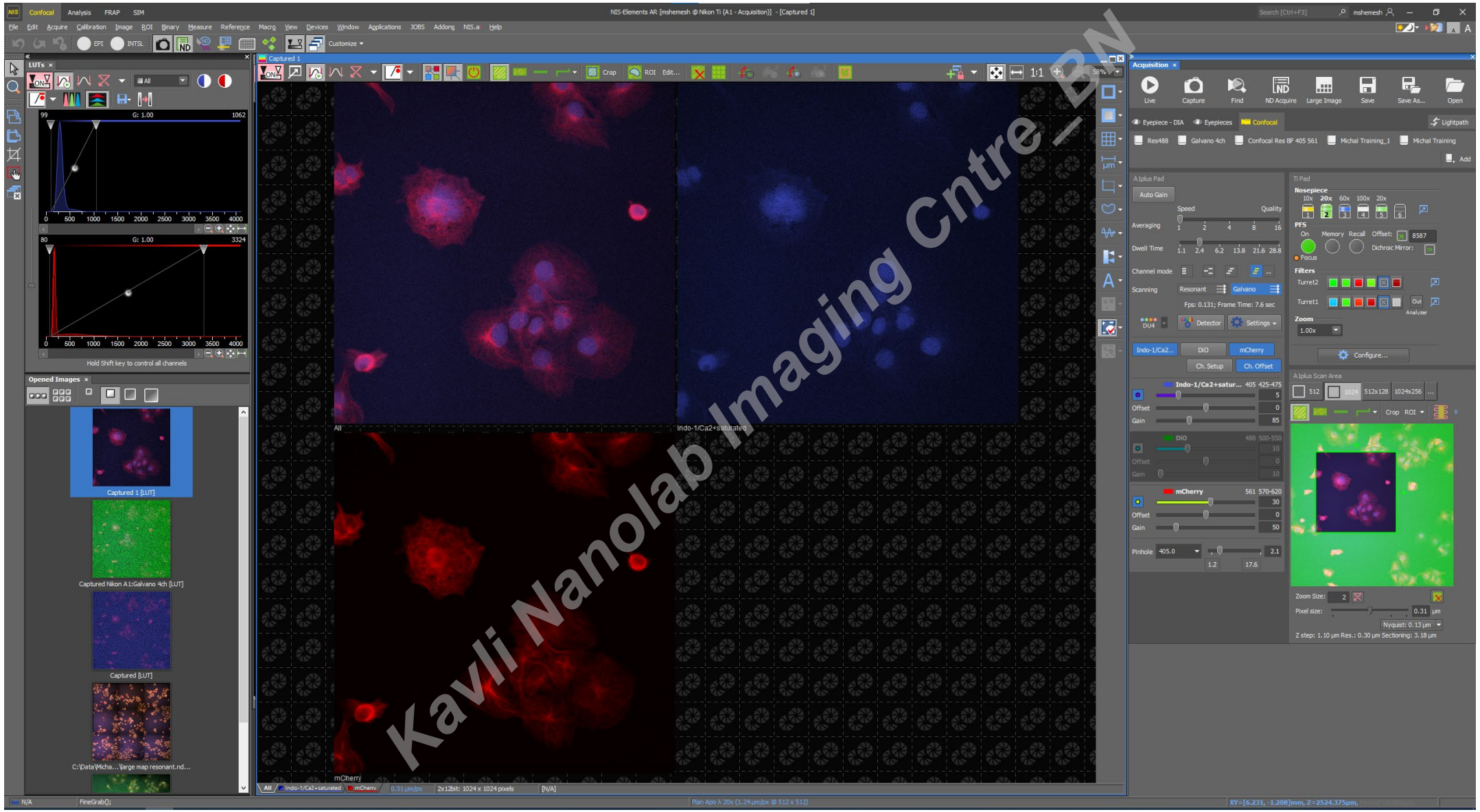
Change channels/order/intensity
Pixel number, size& dwell time





Right click- Apply ROI

Example settings galvano 2Ch:



Confocal Set experiment- Large Image

Try to work in Resonant

Keep PFS on

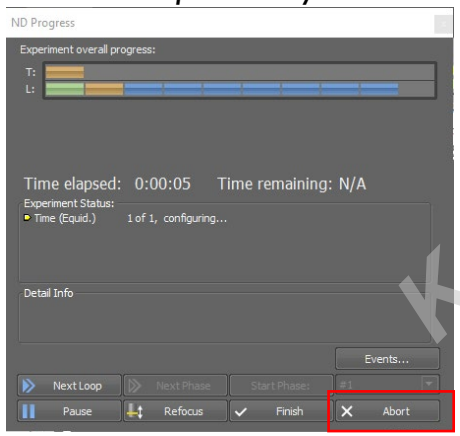
Make sure you save data properly

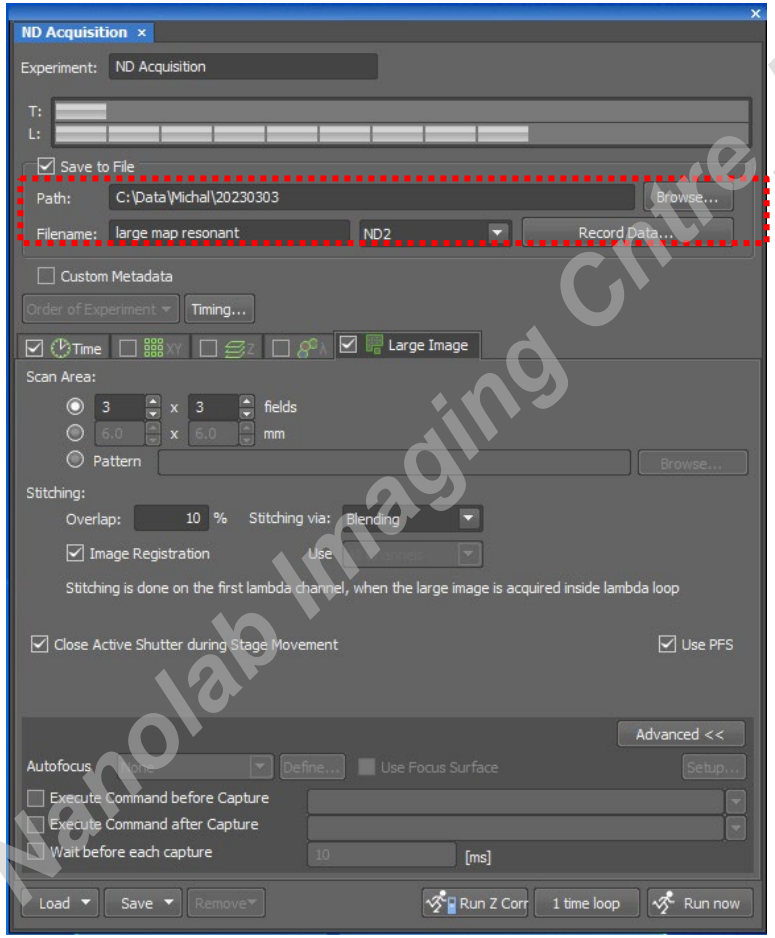
Set one time loop (software requirement)

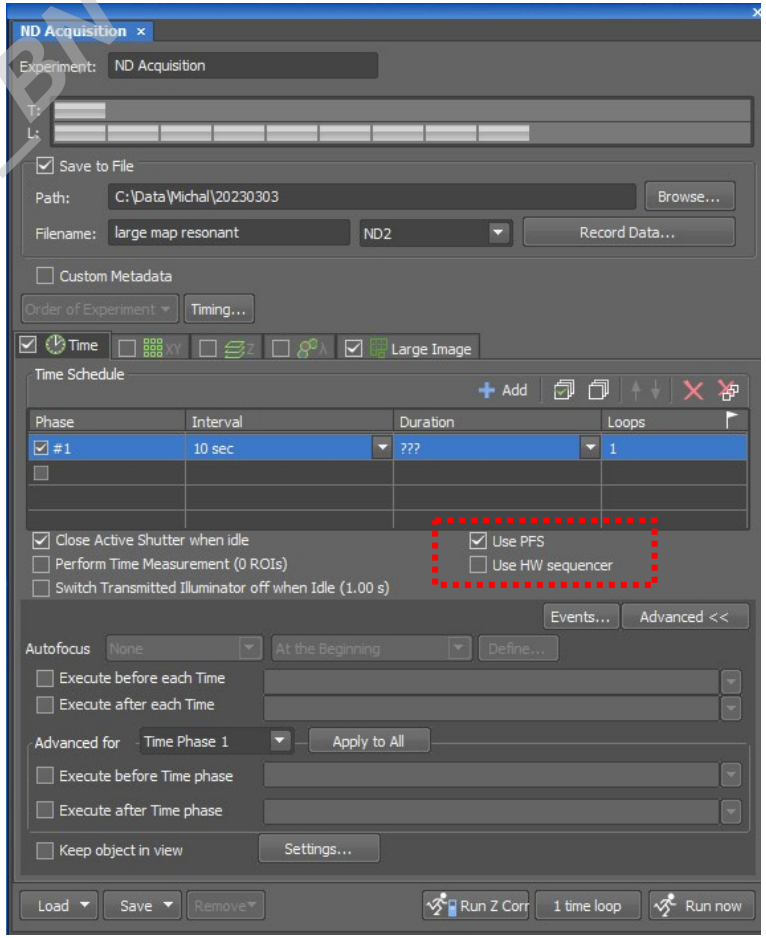
Only set Fields and not area

Keep 10% overlap

You can quit at any time!

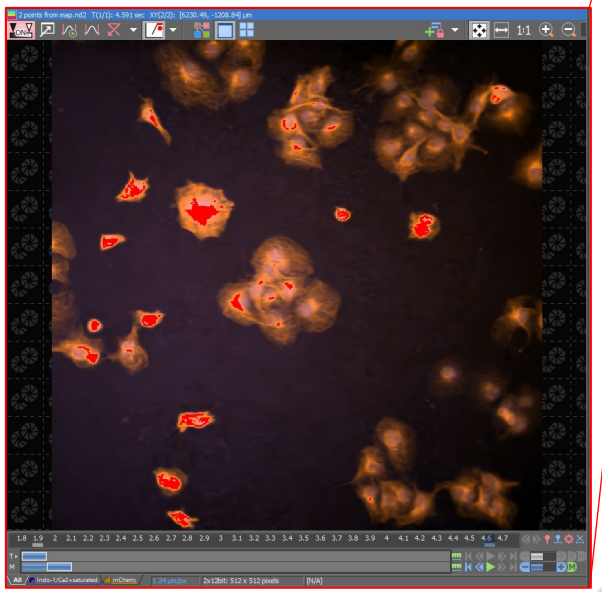


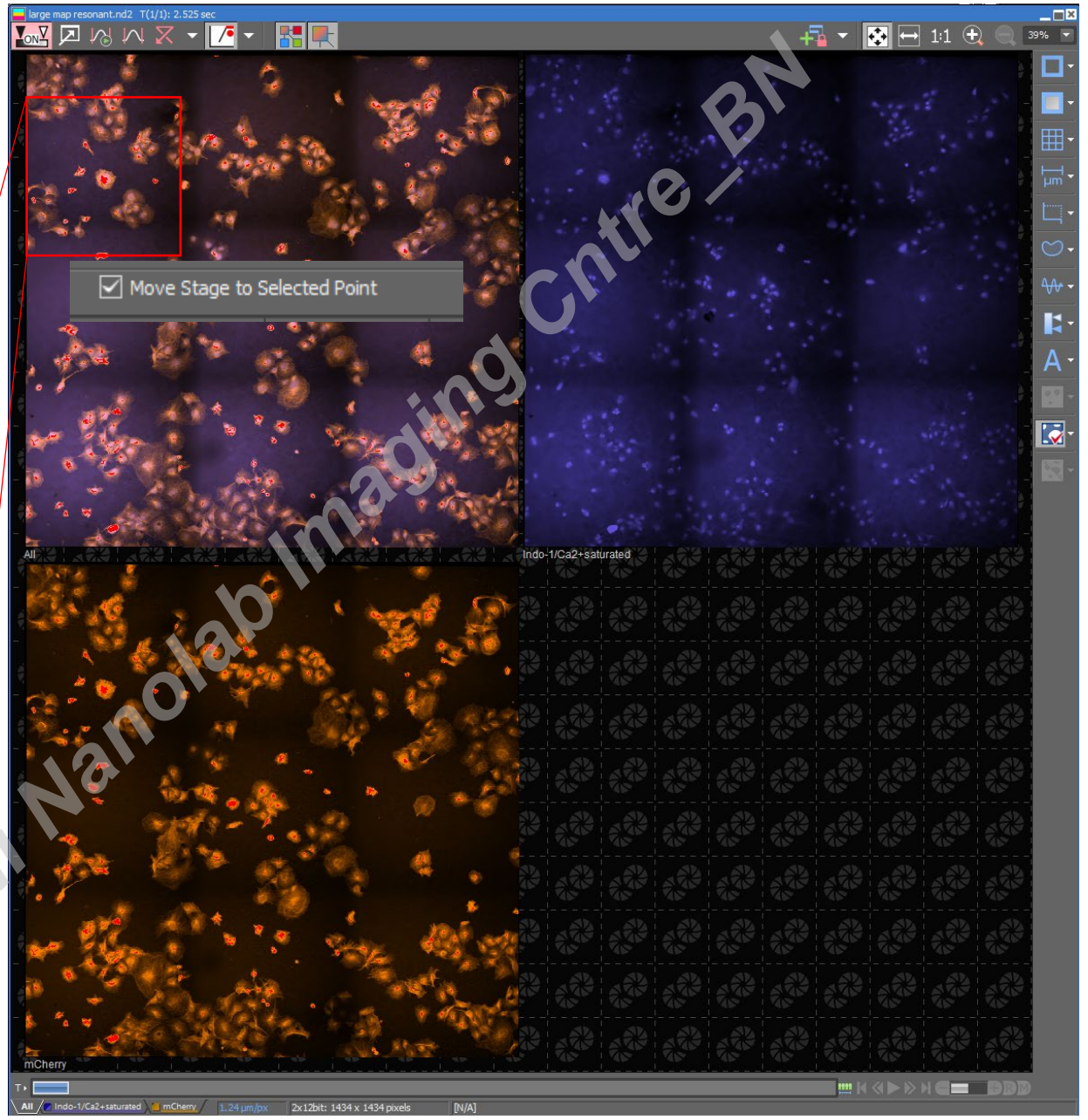




Confocal Set experiment- Large image as map for point list:

You get only the merged image (no individual areas) as one file.
Right click and "move this point to center/Move stage to selected point/Add to point list" will allow you to use this map for point list



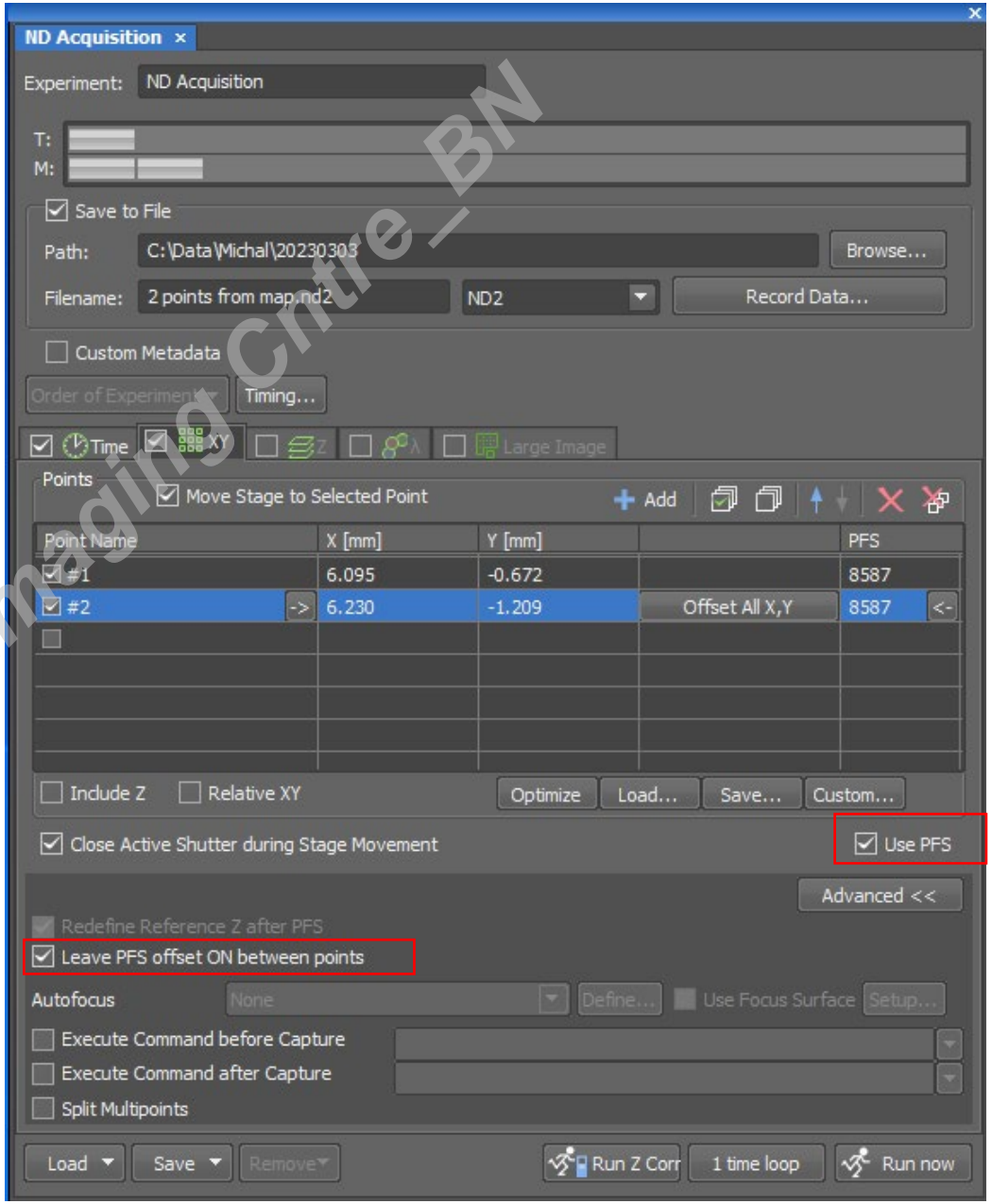


Point list with PFS:

If you want to keep PFS (for example, time experiments), you can choose either to:

leave PFS ON between points (short distances only!)

or OFF (in this case PFS will shut down on stage movement and re-engage when reaching new point in list



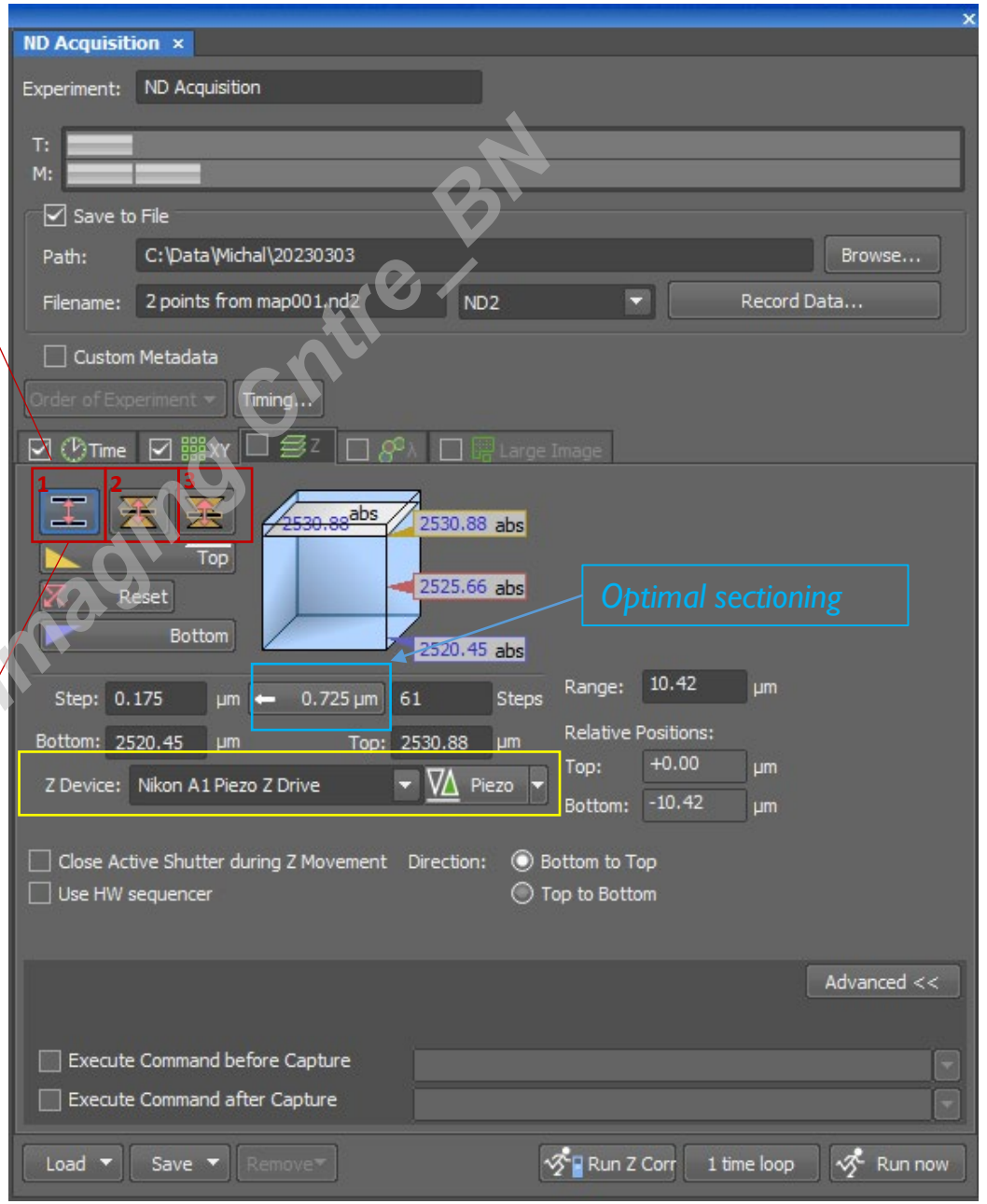
Z stack- basic:

1: Absolute- use resonant pre-set to quickly go through the top and bottom boundaries of your sample

2: Relative- once you have a mid-section in good focus, and you know sample size, you can directly set the same thickness above/below

3: Relative- you can set different thickness below/above

You can choose which device to work with. Piezo gives you better accuracy, Ti (objective) works faster

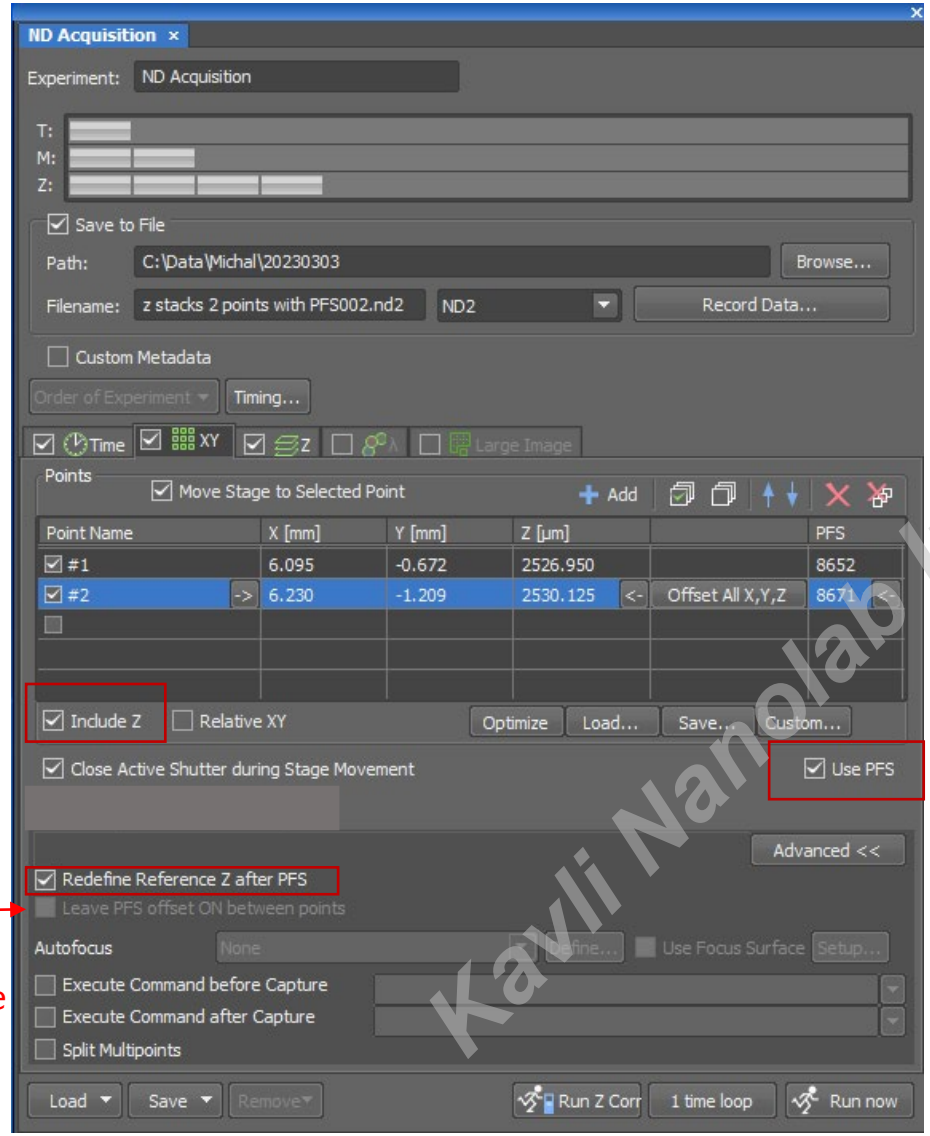


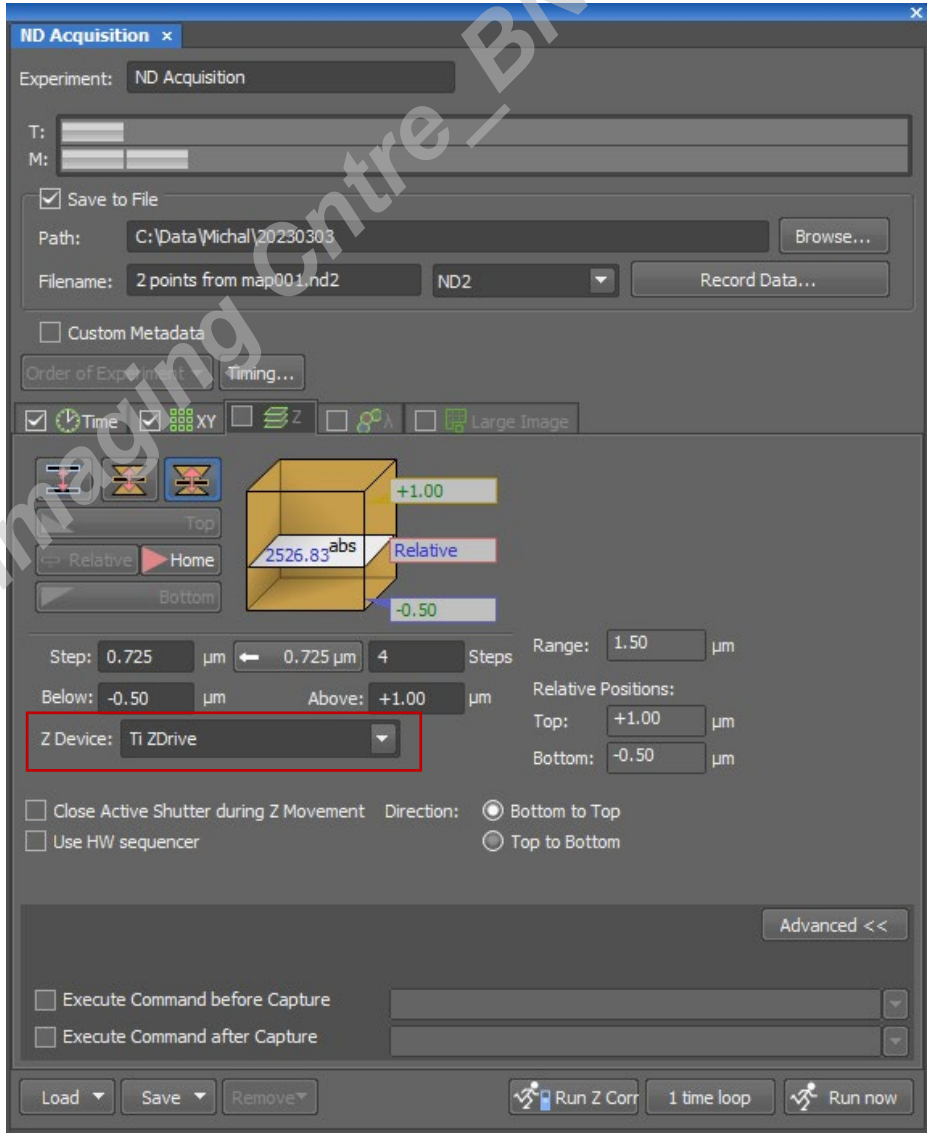
Z stack- point list-PFS:

Keep the PFS ON, it will close when starting a z-stack and reactivate when reaching new position (you should hear beeping in every new position). This is especially important with time experiments

Don't!

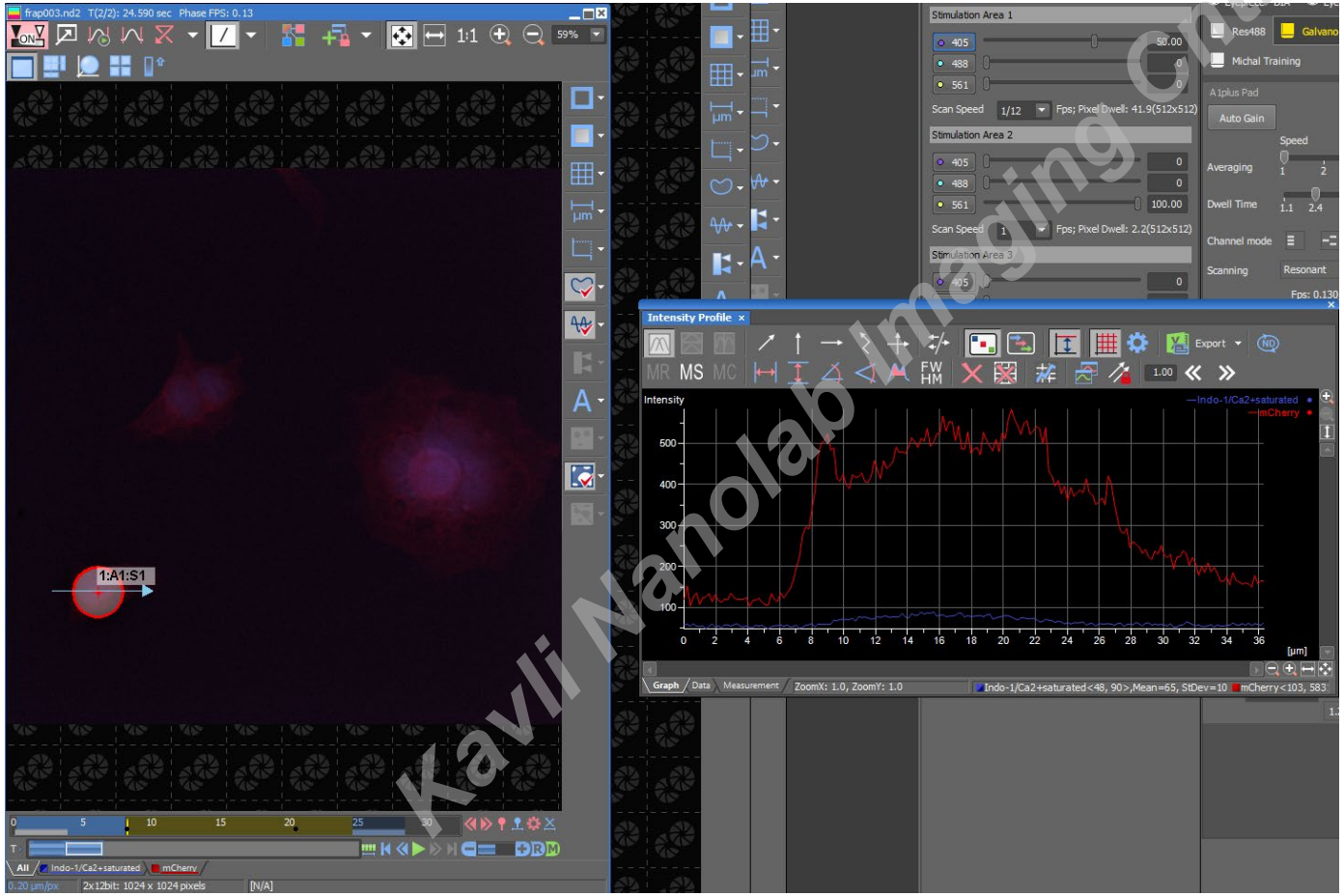
PFS doesn't handle large distances

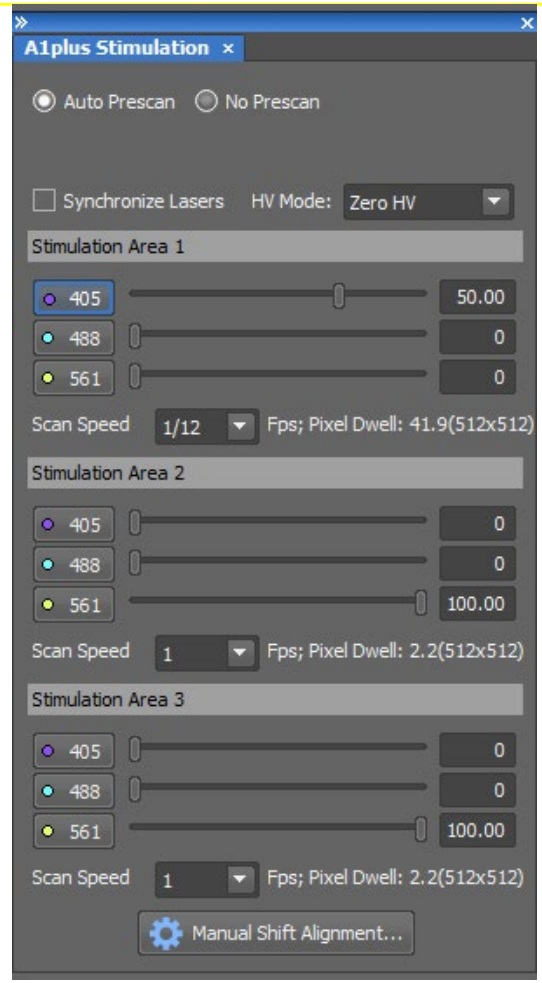




FRAP

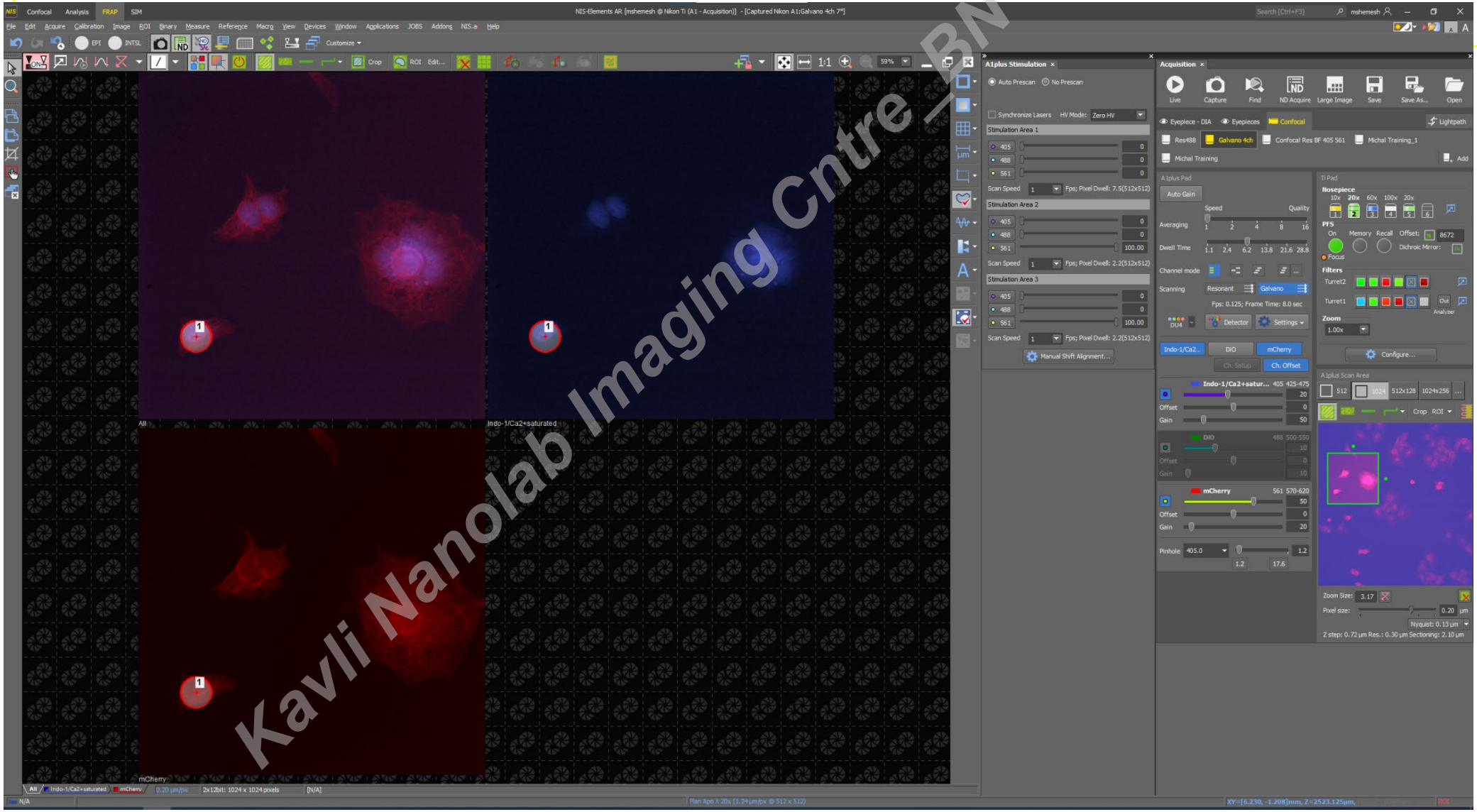
1, go to FRAP tab, work with ND stimulation (experiment setup) and A1 plus stimulation (lasers)





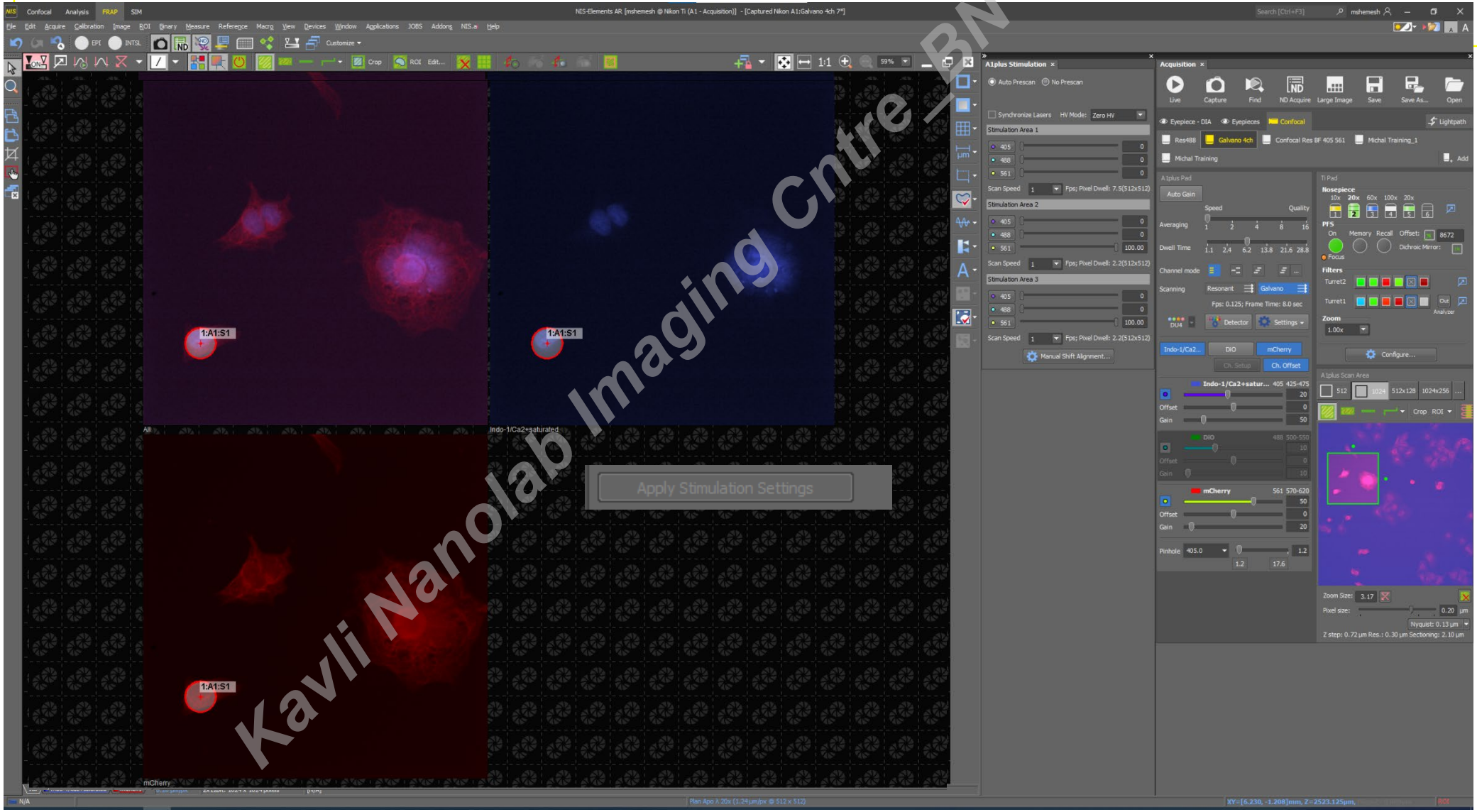
FRAP

2, Draw a circular ROI and assign it as **stimulation ROI (A1:S1)**



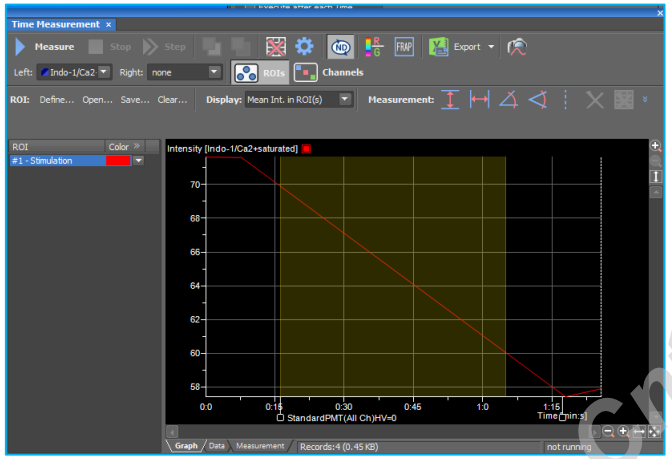
FRAP

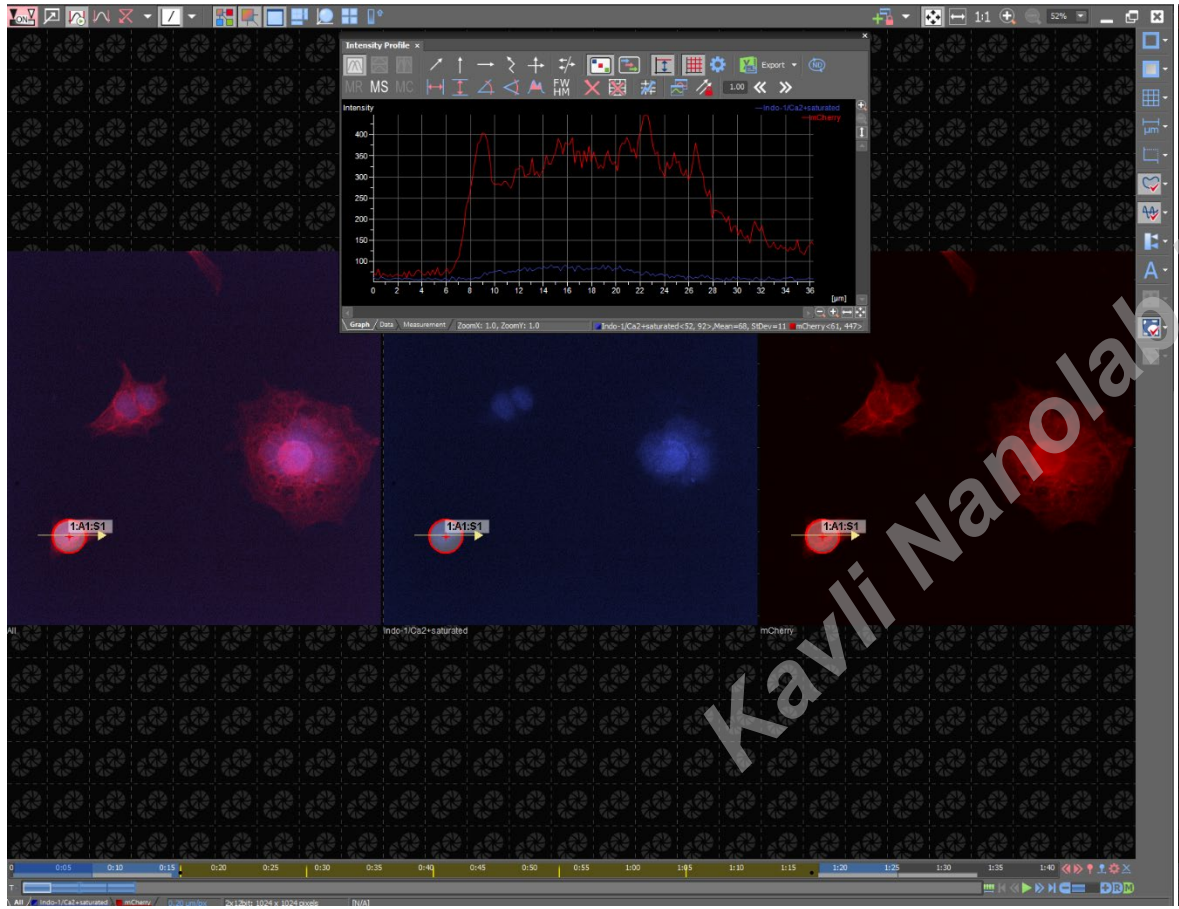
2, Draw a circular ROI and assign it as **stimulation ROI (A1:S1)**

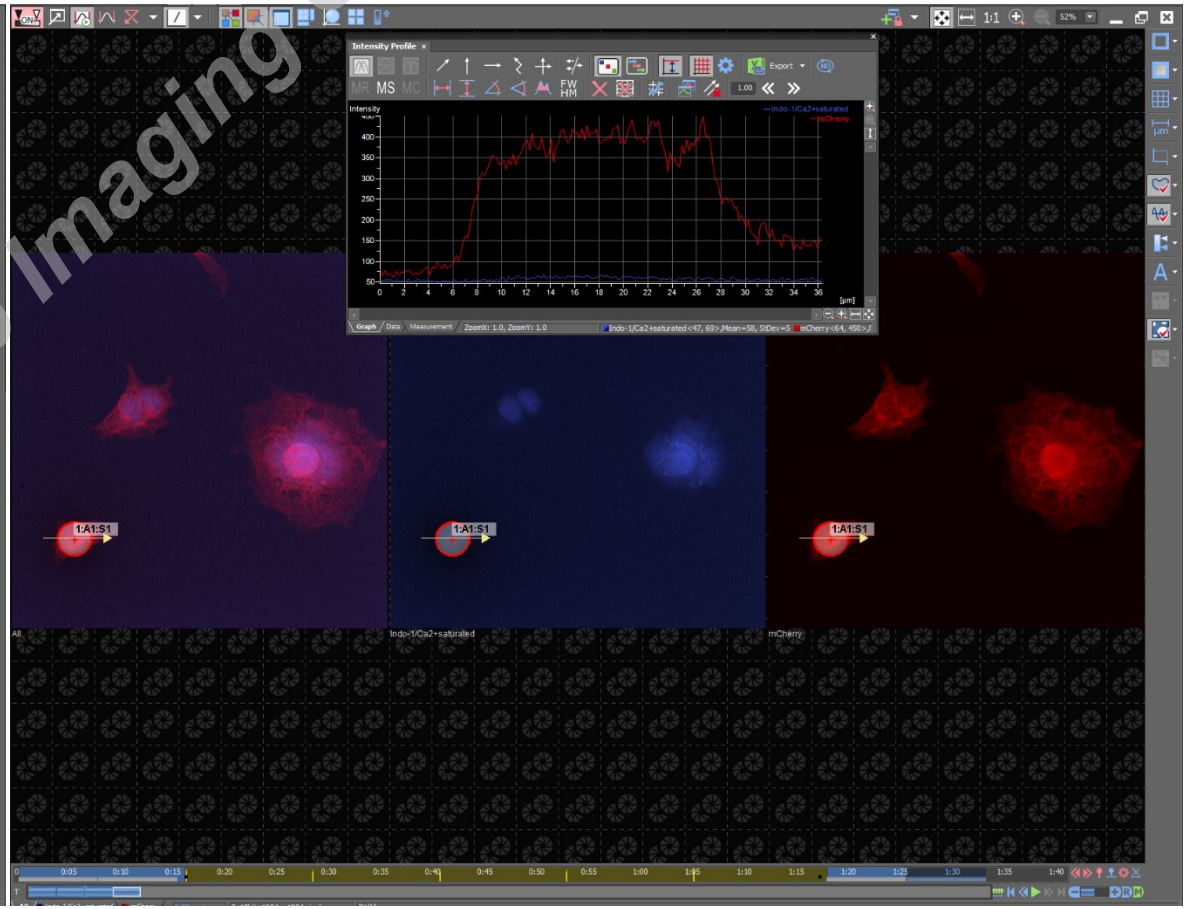


FRAP- check

You can use *time measurements*
Or compare line intensities
Before/after







FRAP- ND experiment

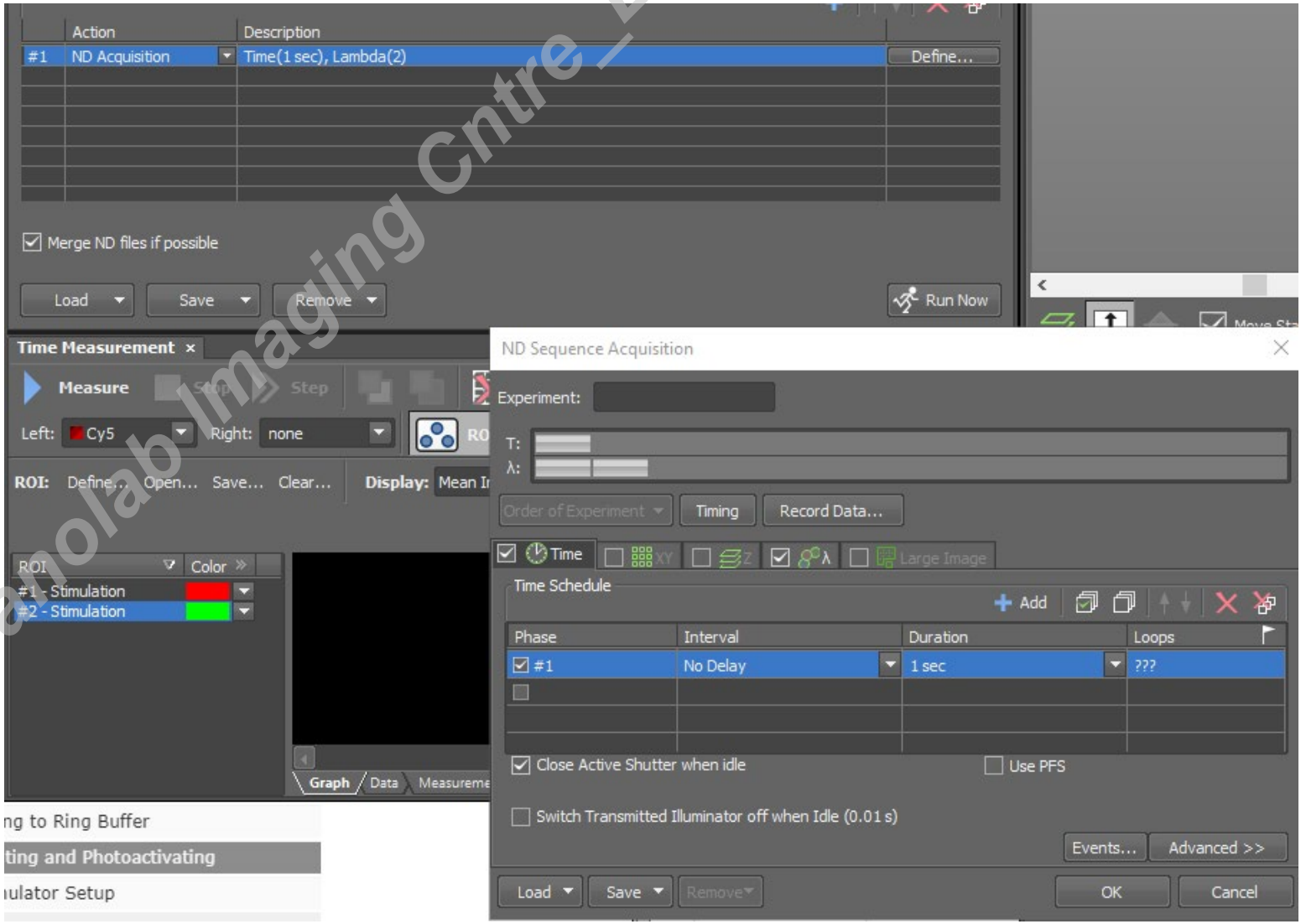
In your time lapse experiments, you can define an action of acquisition and/ or stimulation

Acquisition can be set before/after bleaching, but not simultaneous (same lasers)

Bleaching parameters should be the same as in your stimulation check

ROI should be defined correctly: you can set a measurement ROI, Background and Reference ROIs.

Your **lasers** should be activated in acquisition before used for bleaching



Shut down:

#3
Turn Off Main switch
(lamps/stage/heating)

#1
Go to Esc position
Remove your sample
Clean the Objective!!
Shift back to 10X

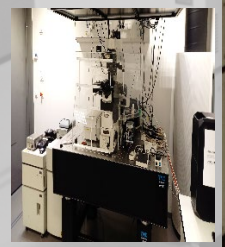
#2
Save your data- copy to Network folder*
log out from your windows account

*** All local data will be deleted monthly- copy immediately*

#4
Switch Off your
Individual lasers

#6
Switch Off
A1R control

#5
Switch Off Laser
controller



Make sure all cage
doors are closed