

Kavli Nanolab Imaging Centre

User guide

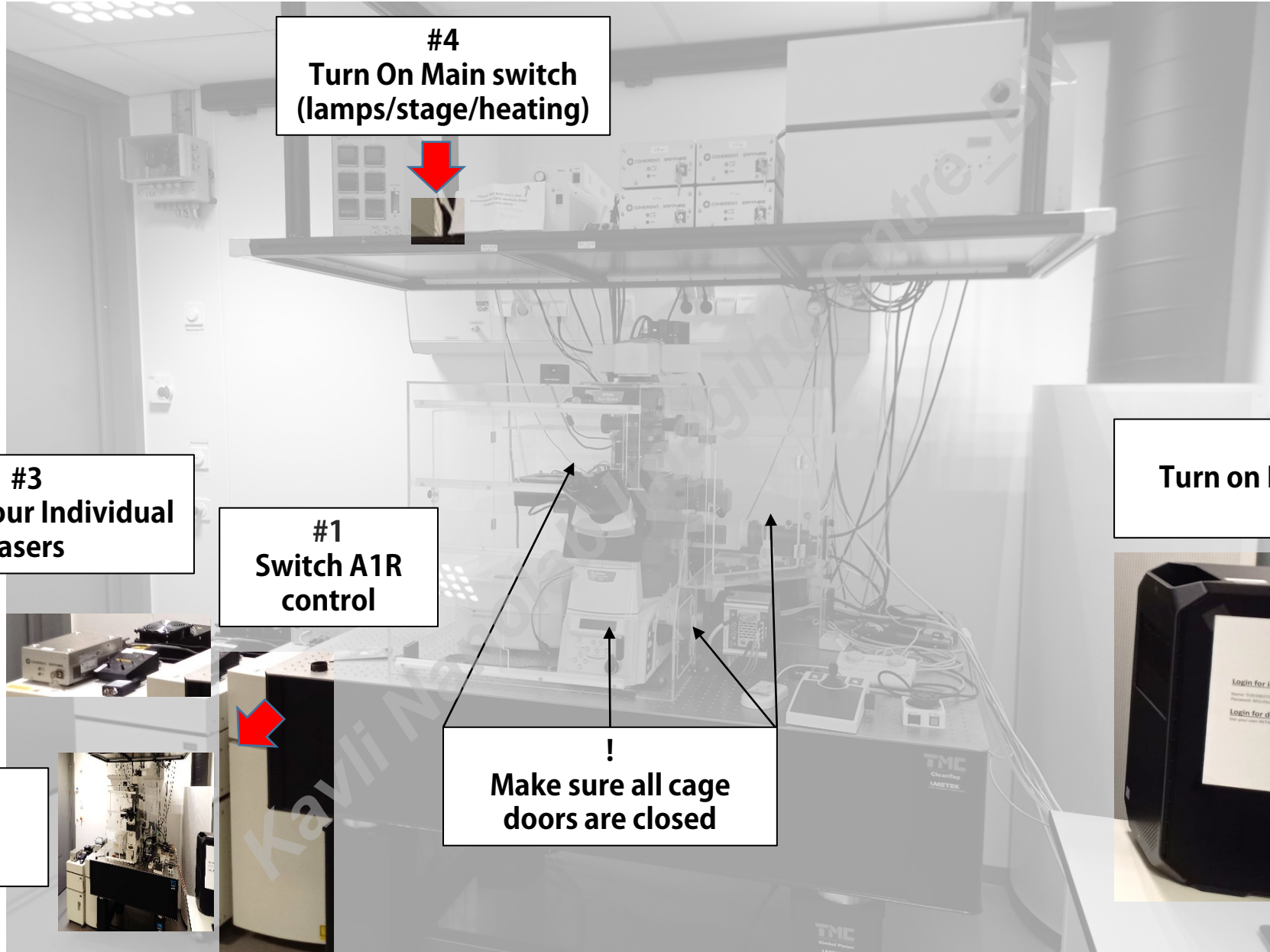
Scanning Confocal

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Clémence



Start-up:



Focus on your sample- eyepiece- general:

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

#2 Starting from objective height $Z=500\mu\text{m}$

Raise the objective until you see your sample

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

Check*:

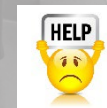
Try both BF/Flu

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

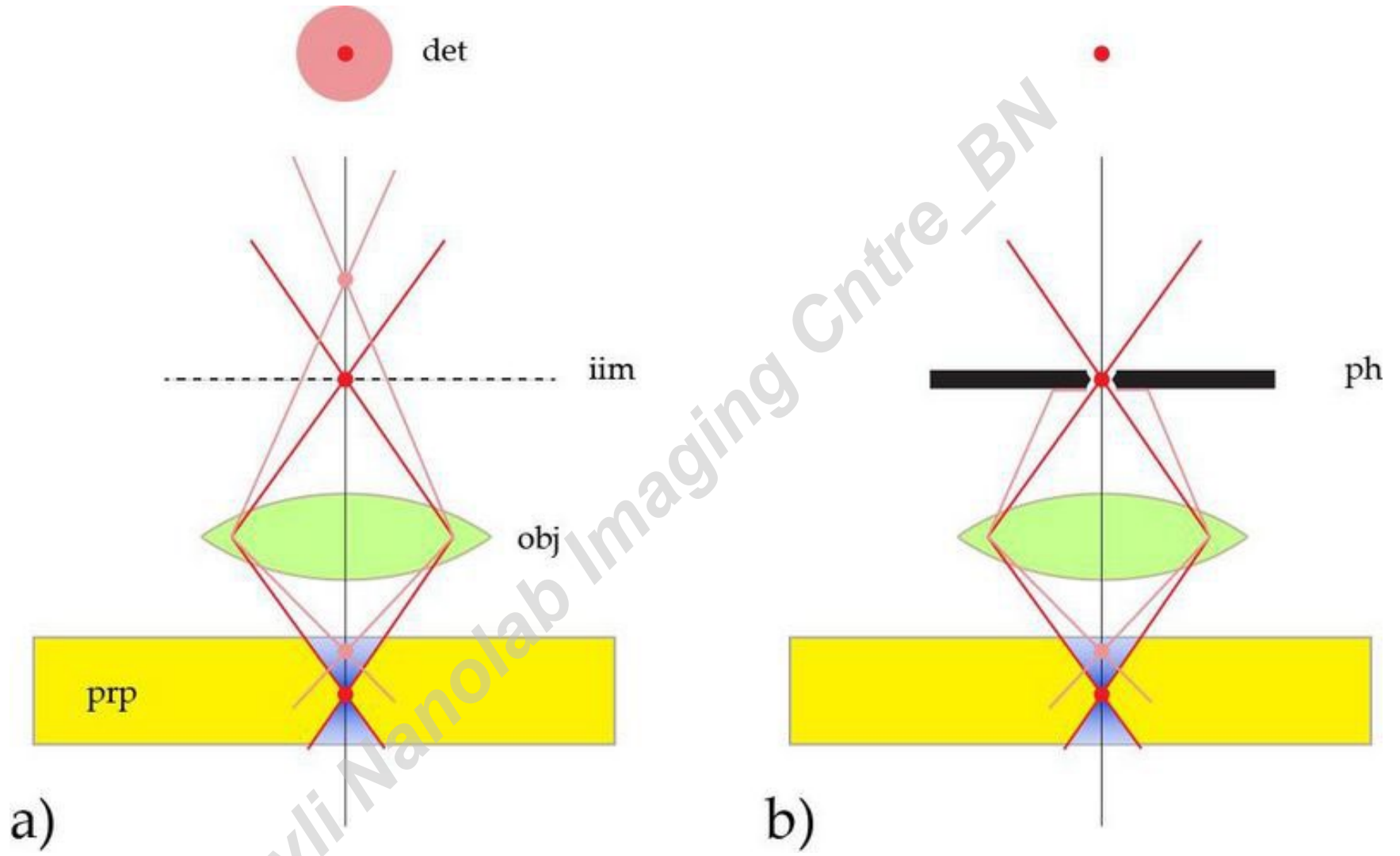
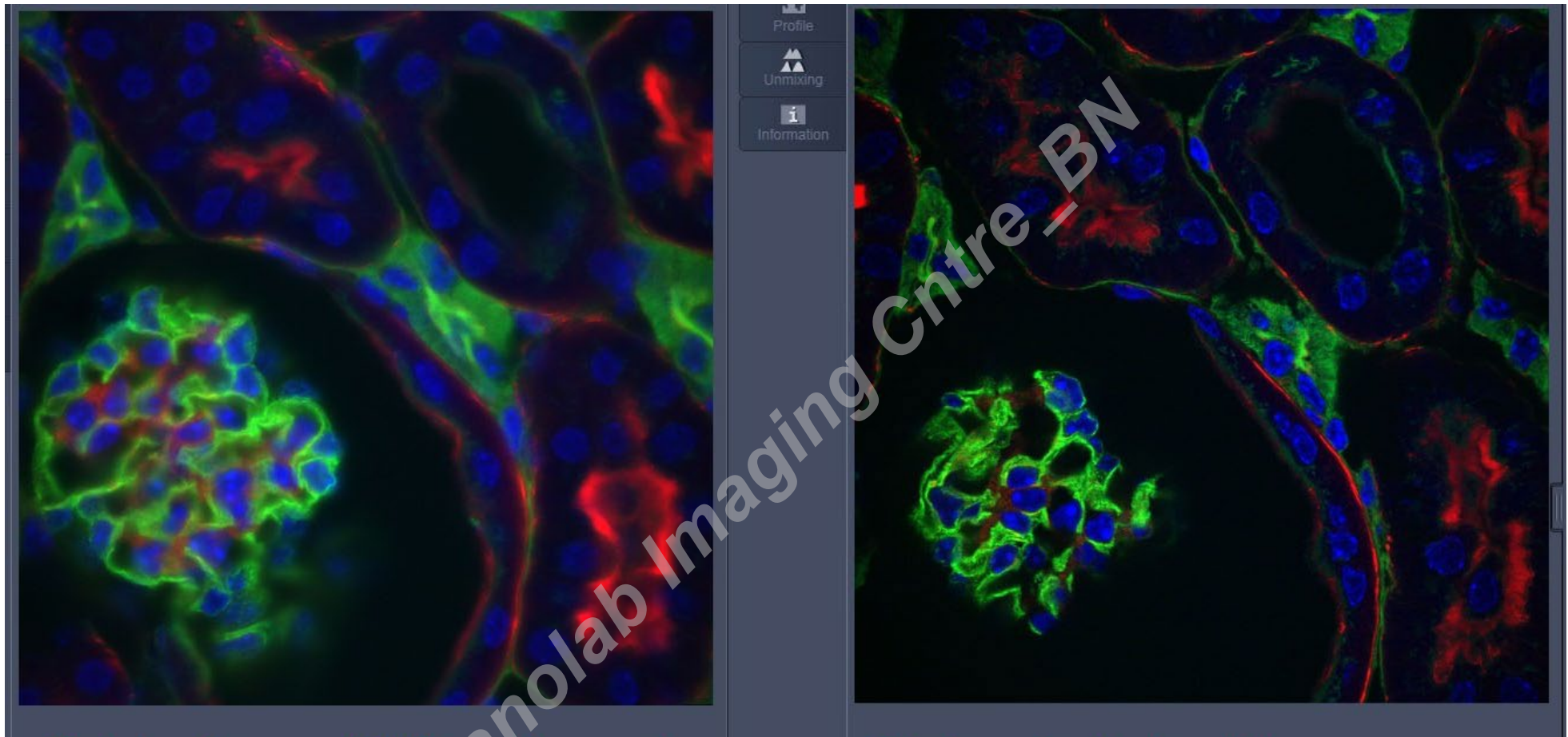


Figure 1: The optical knife. a) a compound microscope generates an intermediate image (iim) by an objective lens (obj), containing both focal (red) and extrafocal (pink) signal emanated by the sample (prp). When imaging a single spot, the detector (det) will record both a spot-shaped feature from the 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.

Confocal Vs. wide field : Pinhole



If you are having trouble locating signal- you can open the pinhole for WF comparison.

To increase resolution- you can decrease pinhole (and pay the price in signal int)- don't go beneath ~ 0.6

Light path

Light source

Port1:4 Laser Unit (LU4/ILU4A)

Excitation dichroics:

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

Dye & Spectral Setting

EXEM None

Setting Mode

Sort by Emission

Ch1 Indo-1/Ca2+sa 405.0 425-475

Ch2 DiO 488.0 500-550

Ch3 mCherry 561.0 570-620

Ch4 Cy5 640.0

detection

560 LP 640 LP

525/50 593/46 700/75

Ch1 450/50 Ch2 525/50 Ch3 595/50 Ch4 700/75

Ch Series Custom

Ch Series Line 4->1

Transmitted Detector

In Out

Fluorescence filters:

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

Speed Quality

Averaging 1 2 4 8 16

Dwell Time 2.2 12.1 27.2 57.2 87.8 117

Channel mode

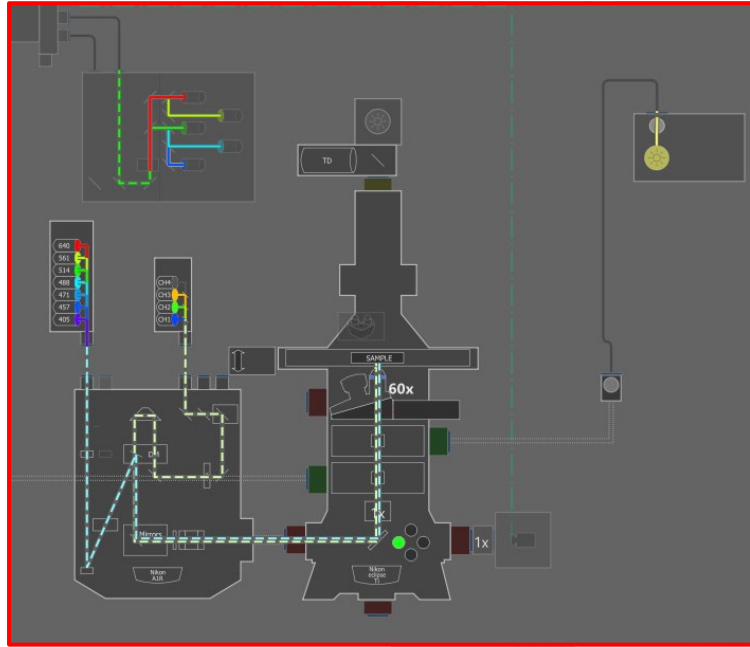
Scanning Resonant Galvano

Fps: 0.042; Frame Time: 23.8 sec

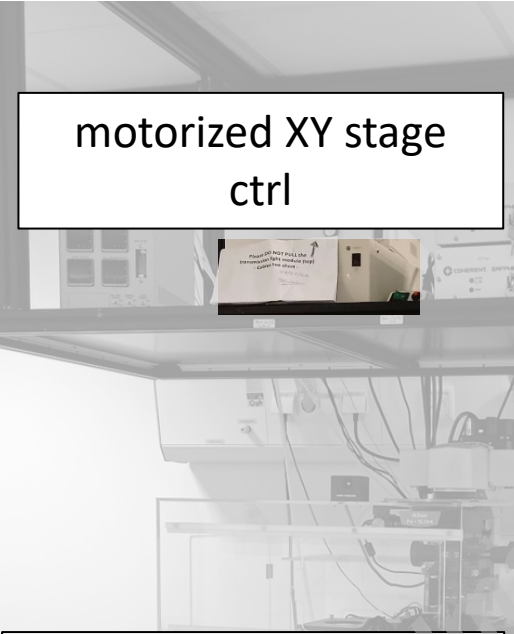
To enhance SNR in resonant fast scan, you can add line average

To enhance SNR in Galvano scan, increase pixel dwell time

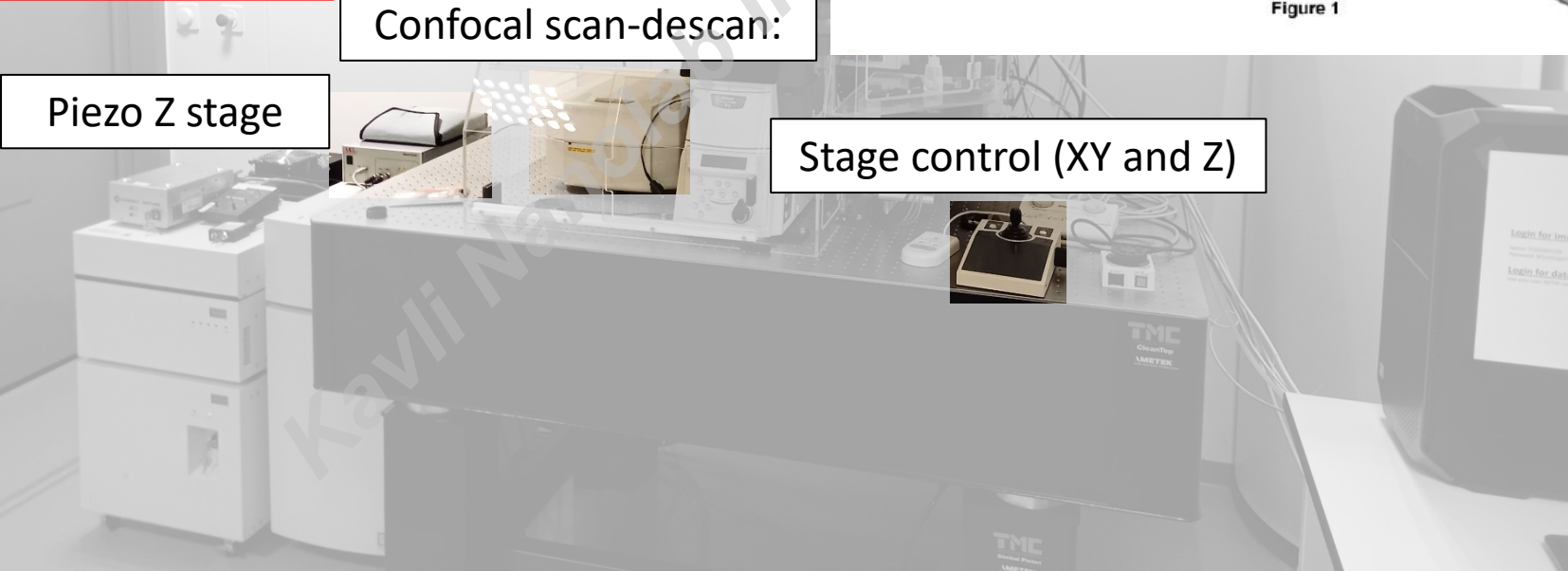
Confocal Scanning Microscope :



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.

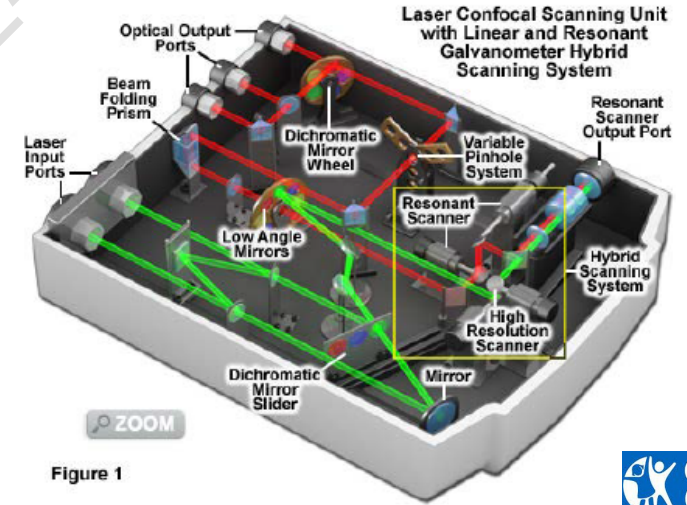
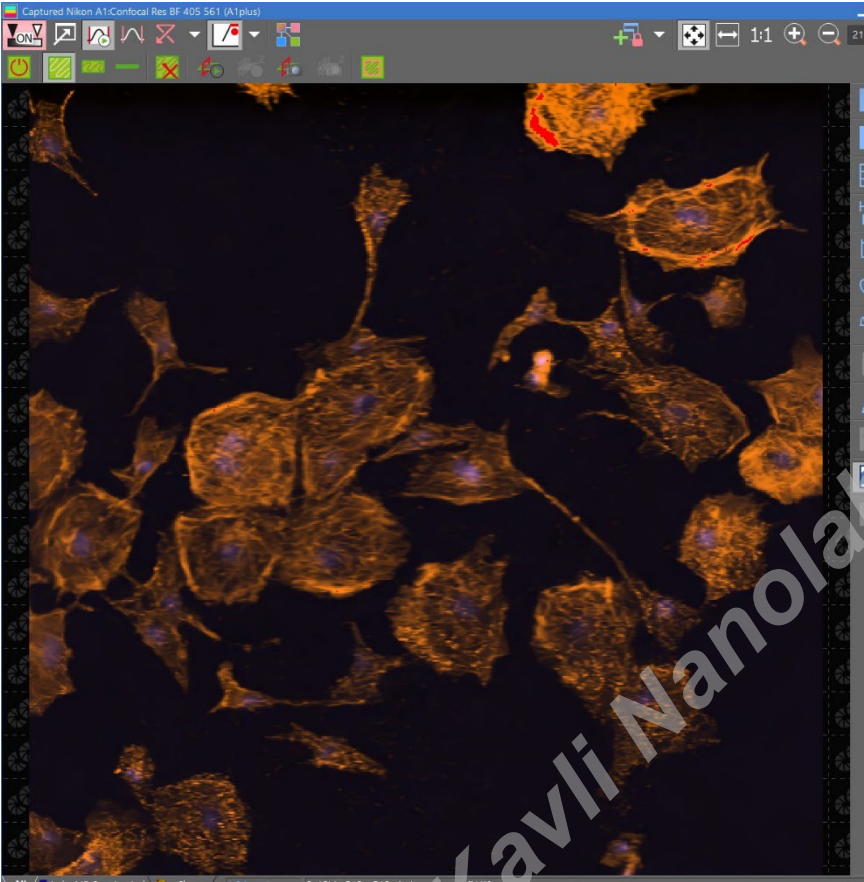


Figure 1

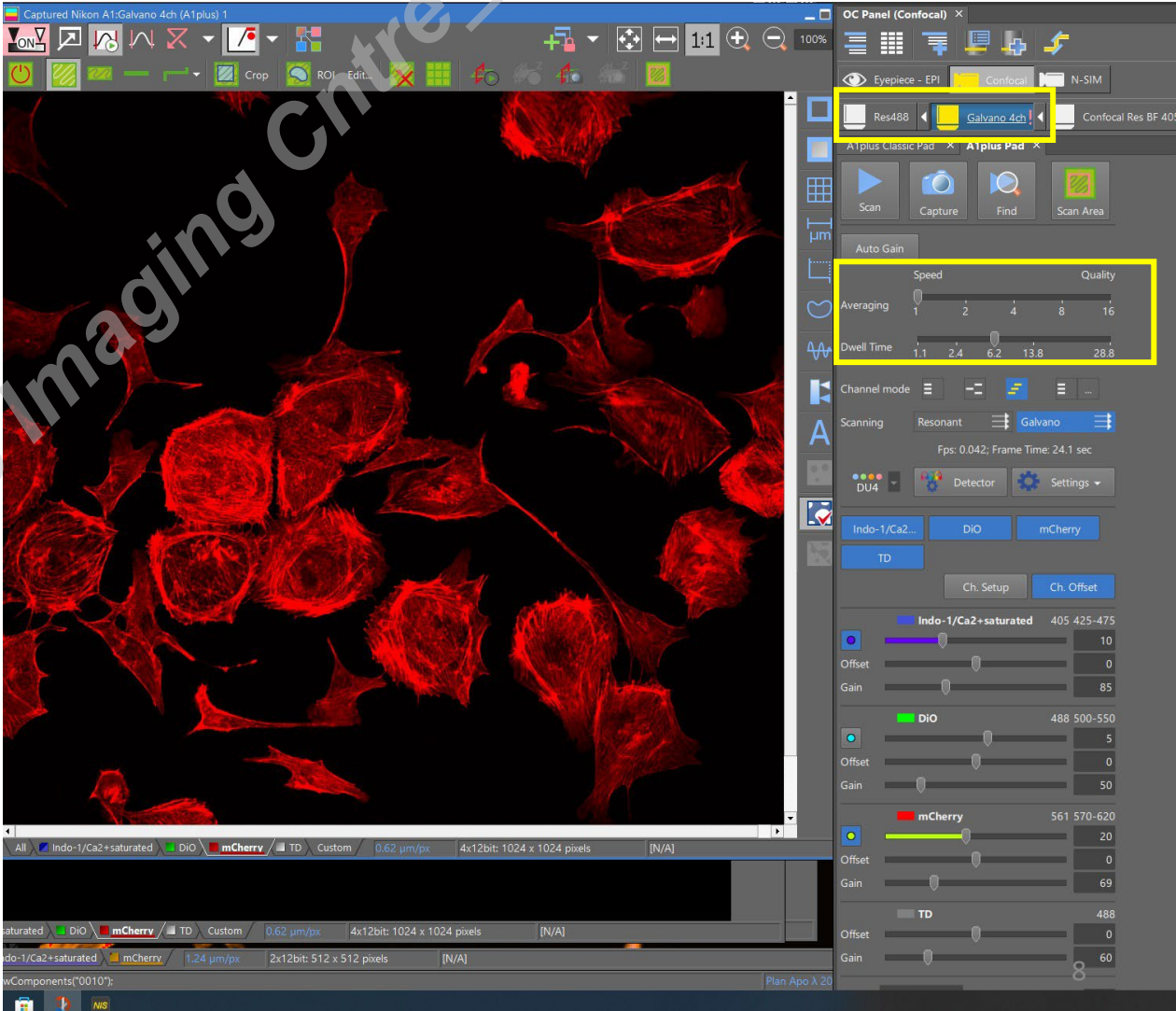


Scanner Options

Resonant scanner (8KHz), no Av

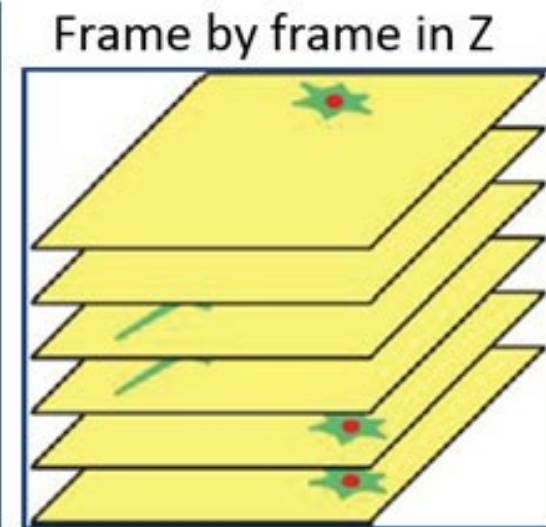
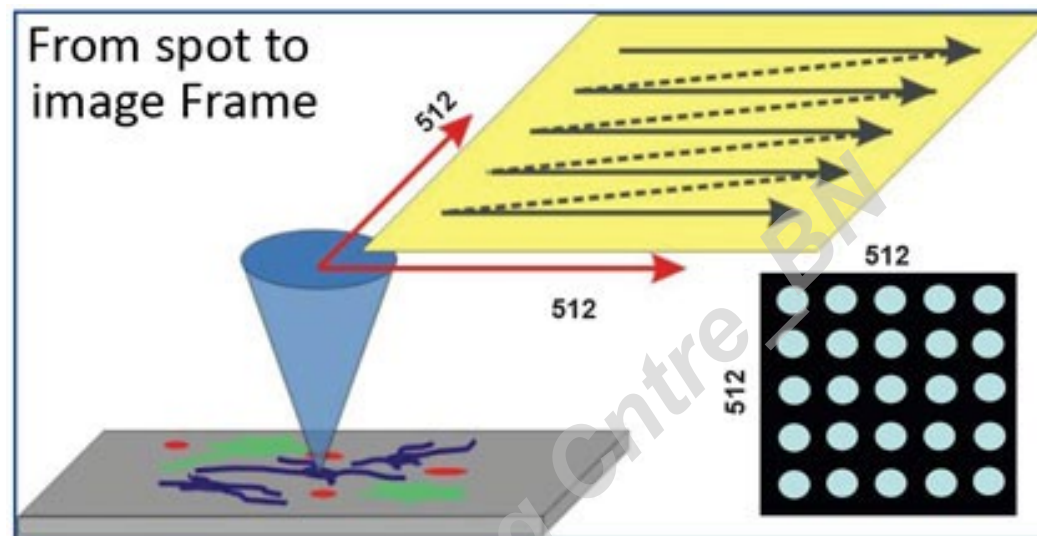


Galvano scanner (adjustable), no Av



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 \times pixel number

To decrease dwell time, must *increase emission rate*

to maintain signal level

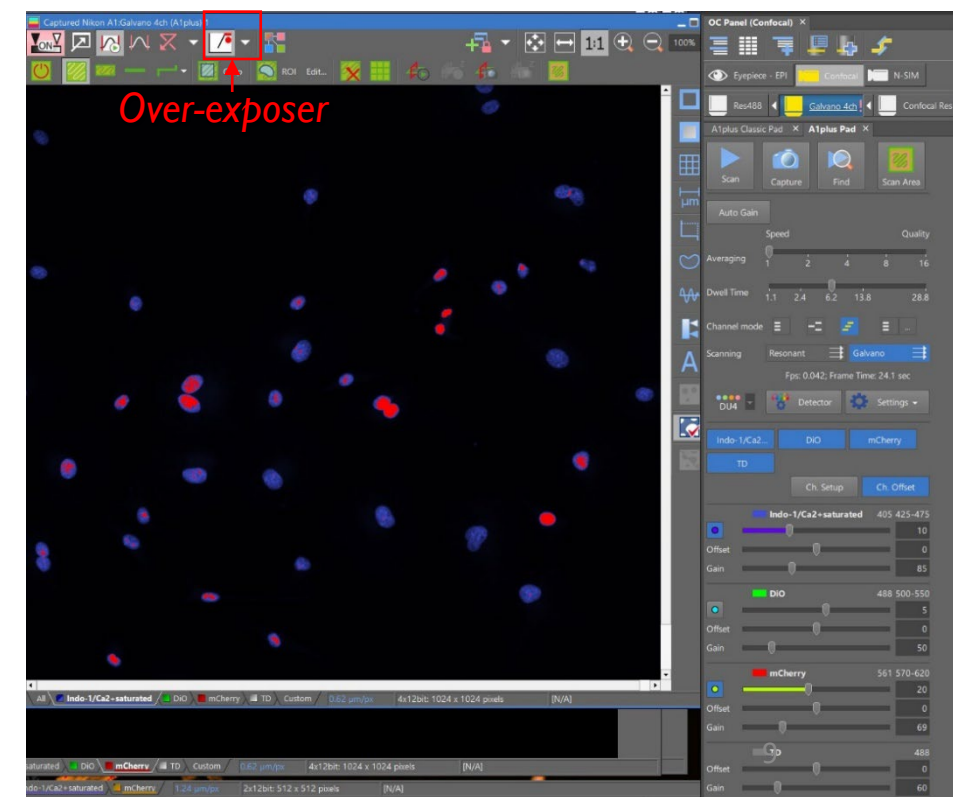
\Rightarrow Must *increase illumination intensity*

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 $5\mu\text{m}$, then each detector pixel must sample intervals that are $\leq 2.5\mu\text{m}$.

The optimal spatial sampling can be retrieved from the software



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

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.

Focus on your sample- Perfect Focus system:

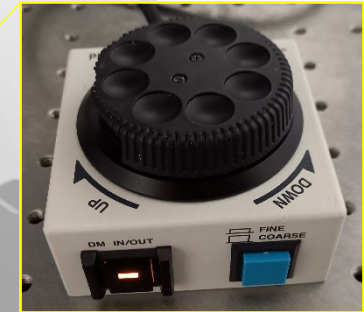
near-infrared 870-nanometer LED and CCD line sensor record and correct for thermal/vibration drifts

#1 Starting from objective height $Z=500\mu\text{m}$

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

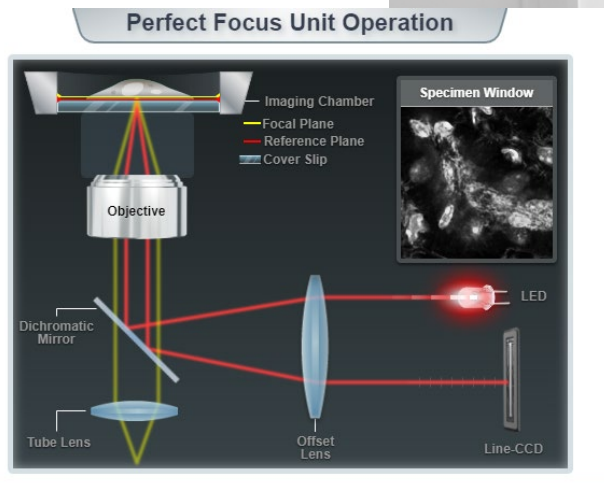


In/Out

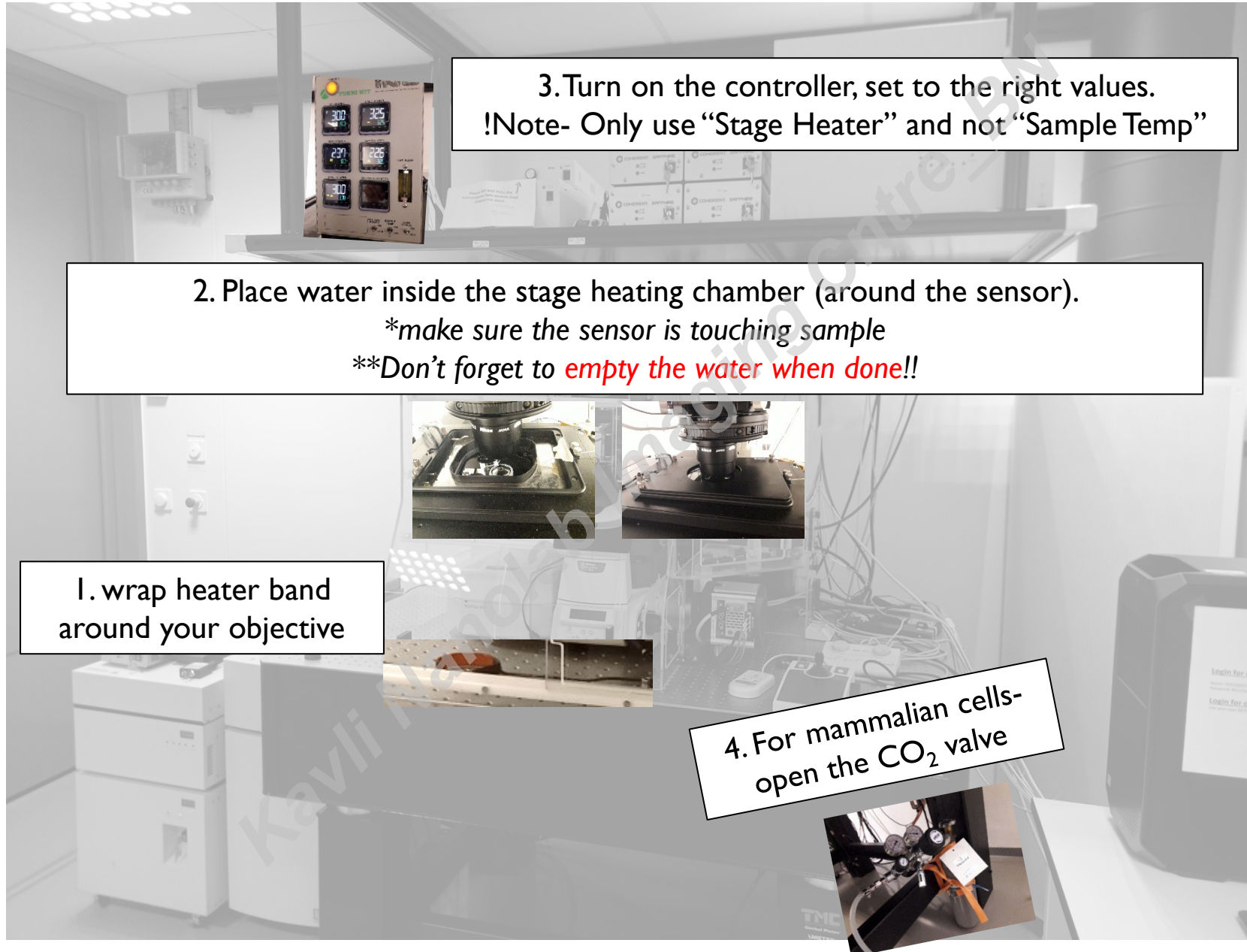
Fast/Slow

#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



Environmental Control:



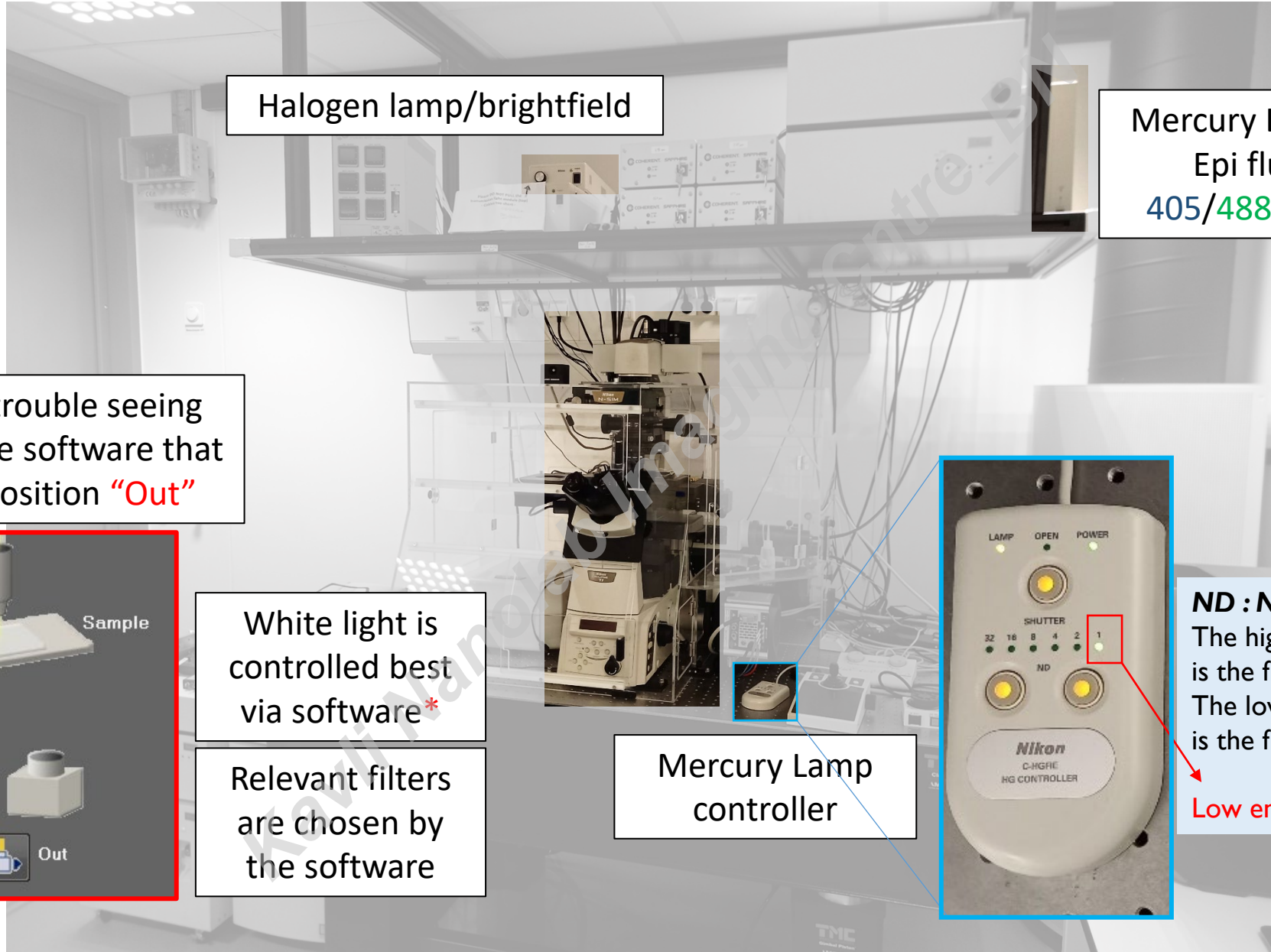
3. Turn on the controller, set to the right values.
!Note- Only use "Stage Heater" and not "Sample Temp"

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 CO₂ valve

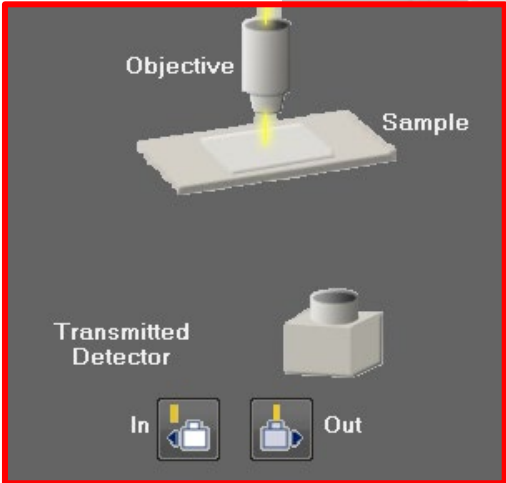
Epi-Flu and BF (Hardware):



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

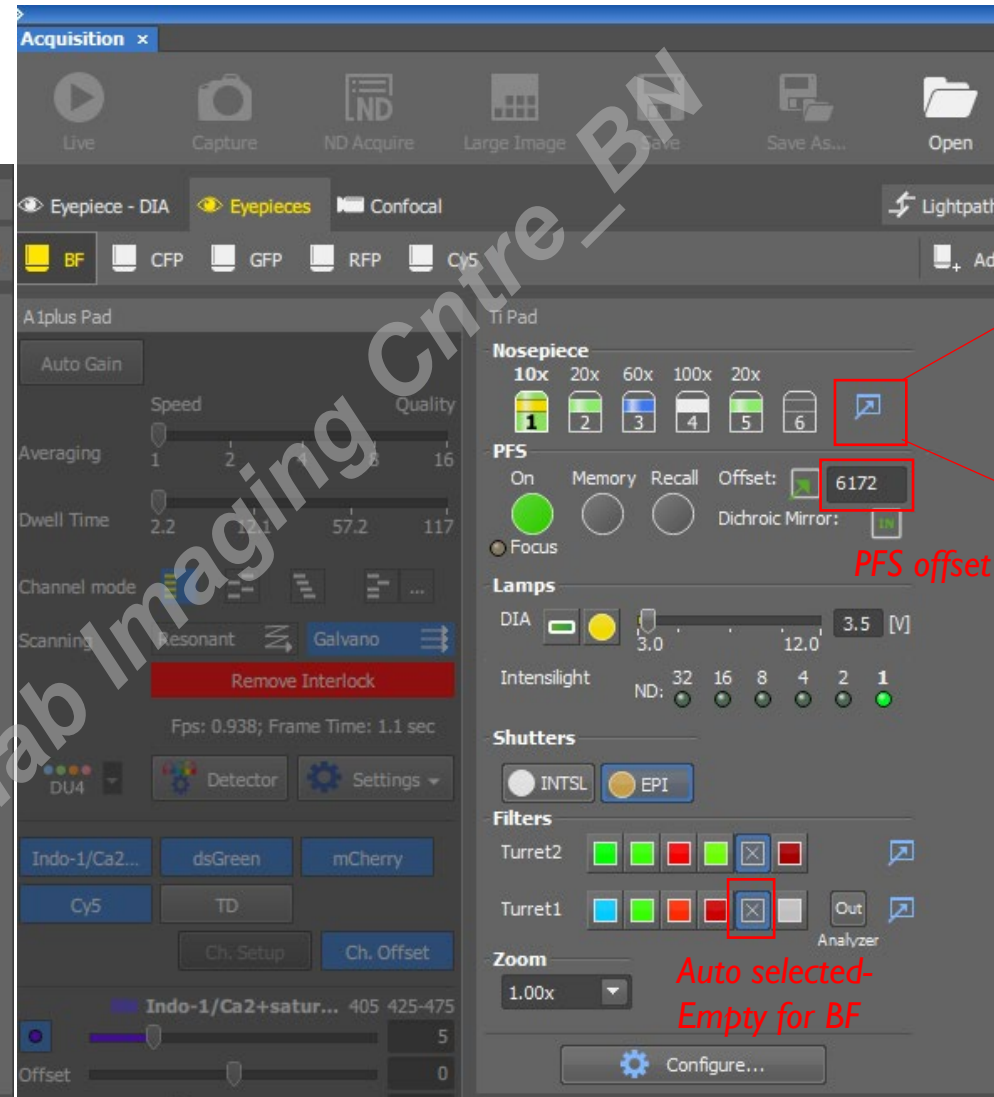
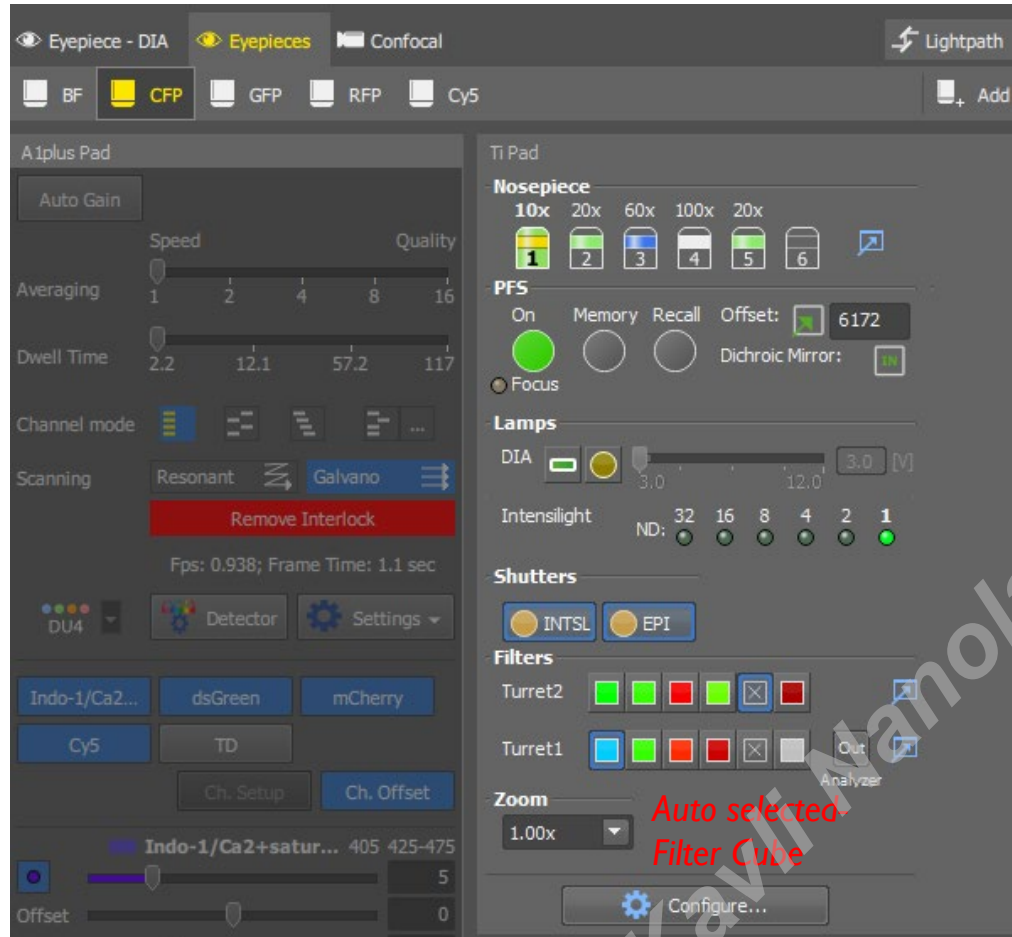


#3 Save on local folder (C/Data/Username) and when done copy to network folder

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

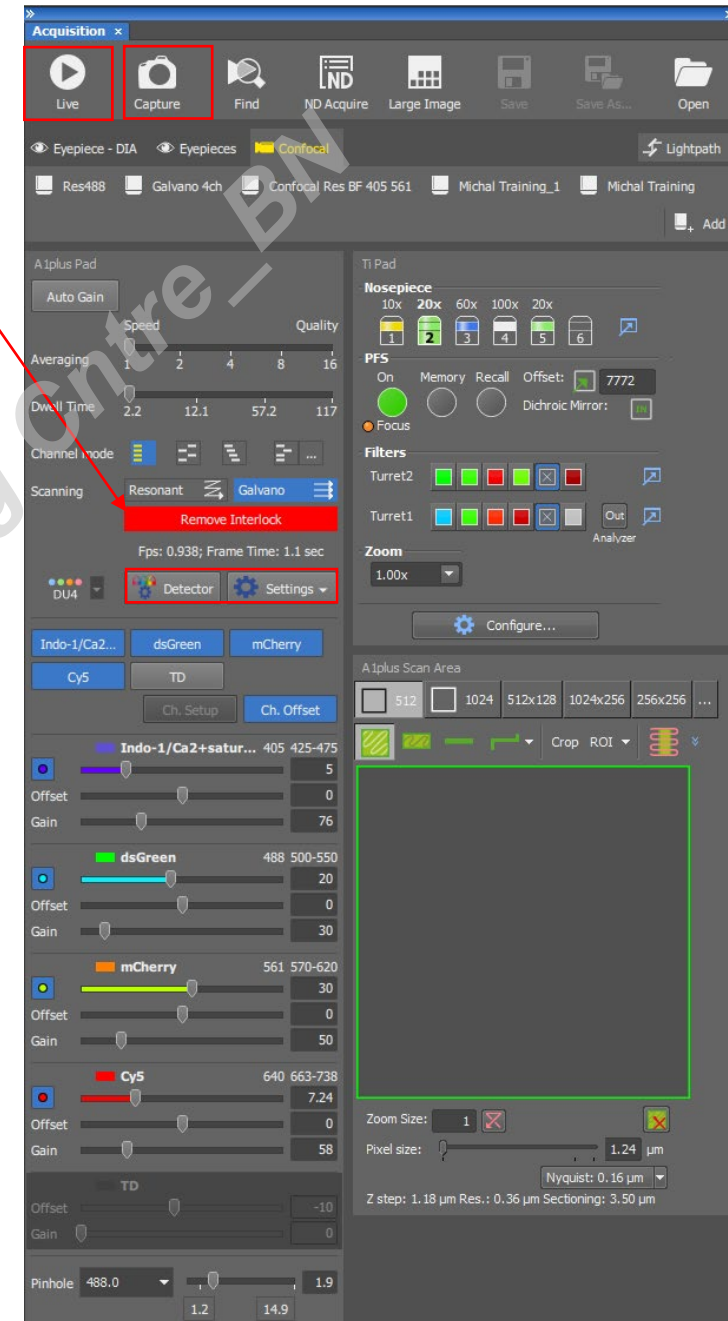
#2 High resolution Galvano scan:

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

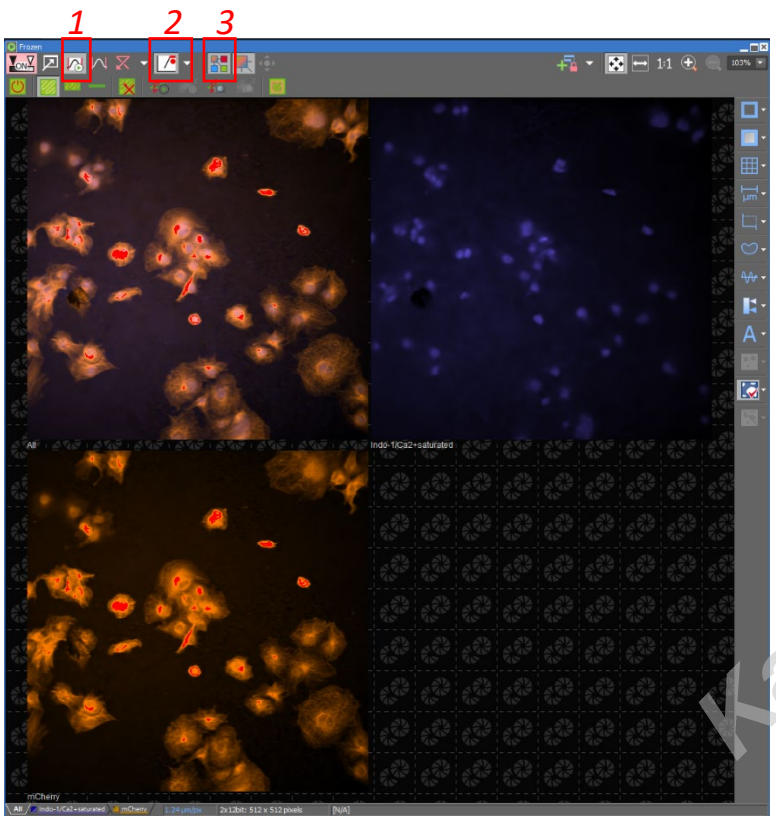
Work only on these two



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

Channel	Wavelength	Offset	Gain
Indo-1/Ca2+	405.0	0	76
dsGreen	488.0	0	30
mCherry	561.0	0	50
Cy5	640.0	0	58

Right click-Apply ROI

Example settings galvano 2Ch:

The screenshot displays the NIS-Elements AR software interface for a microscope acquisition. The main window shows a multi-panel view of a cell sample with red and blue channels. The interface includes several key components:

- Top Menu:** File, Edit, Acquire, Calibration, Image, ROI, Binary, Measure, Reference, Macro, View, Devices, Window, Applications, JOBS, Address, NIS, Help.
- Acquisition Panel (Right):**
 - Live/Capture/Find:** Buttons for real-time viewing and image capture.
 - Lightpath:** Selection of light paths (e.g., Confocal).
 - Speed/Quality:** Sliders for acquisition speed and image quality.
 - Averaging:** Selection of averaging levels (1, 2, 4, 8, 16).
 - Dwell Time:** Selection of dwell time values (1.1, 2.4, 6.2, 13.8, 21.6, 28.8).
 - Channel mode:** Selection of channel modes (e.g., Galvano).
 - Filters:** Selection of filters for Turret1 and Turret2.
 - Zoom:** Selection of zoom level (1.00x).
 - Channel Settings:** Individual settings for channels like Indo-1/Ca2+, DIO, and mCherry, including gain, offset, and pinhole.
- Left Panel:**
 - LUTs:** Look Up Tables for color mapping.
 - Opened Images:** List of captured images (e.g., Captured 1 [LUT], Captured Nikon A1:Galvano 4ch [LUT]).
- Bottom Status Bar:** Displays current acquisition parameters such as Plan Apo A, 20x (1.29 um/obj), @ 512 x 512, and XY coordinates.

Confocal Set experiment- Large Image

Try to work in Resonant

Keep PFS on

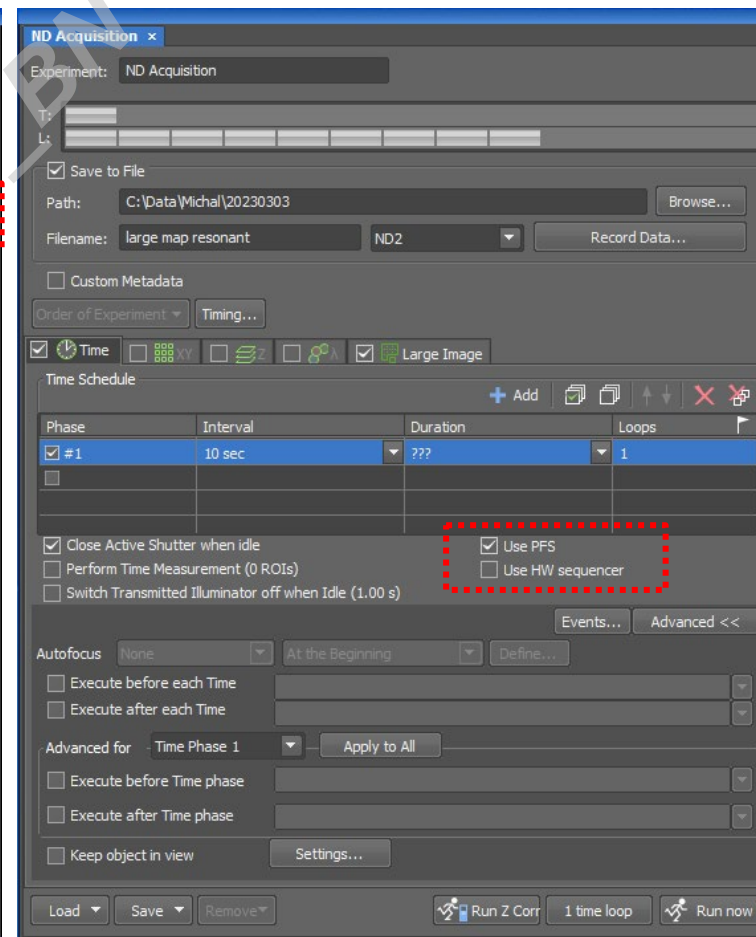
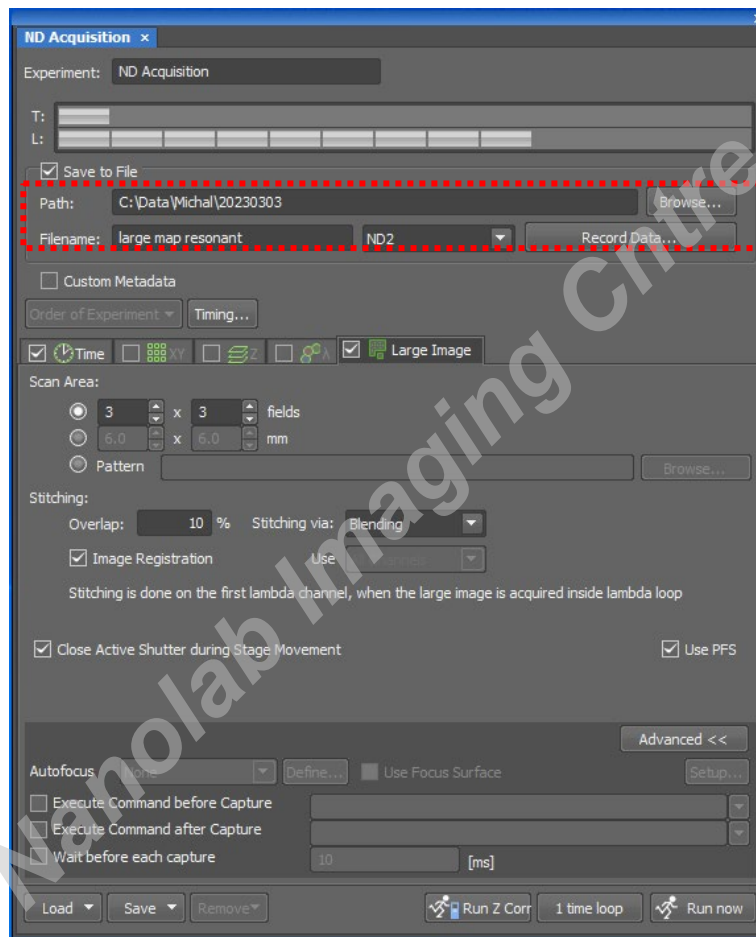
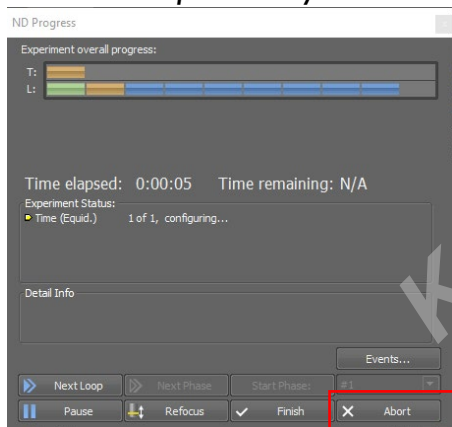
Make sure you save data properly

Set one time loop (software requirement)

Only set Fields and not area

Keep 10% overlap

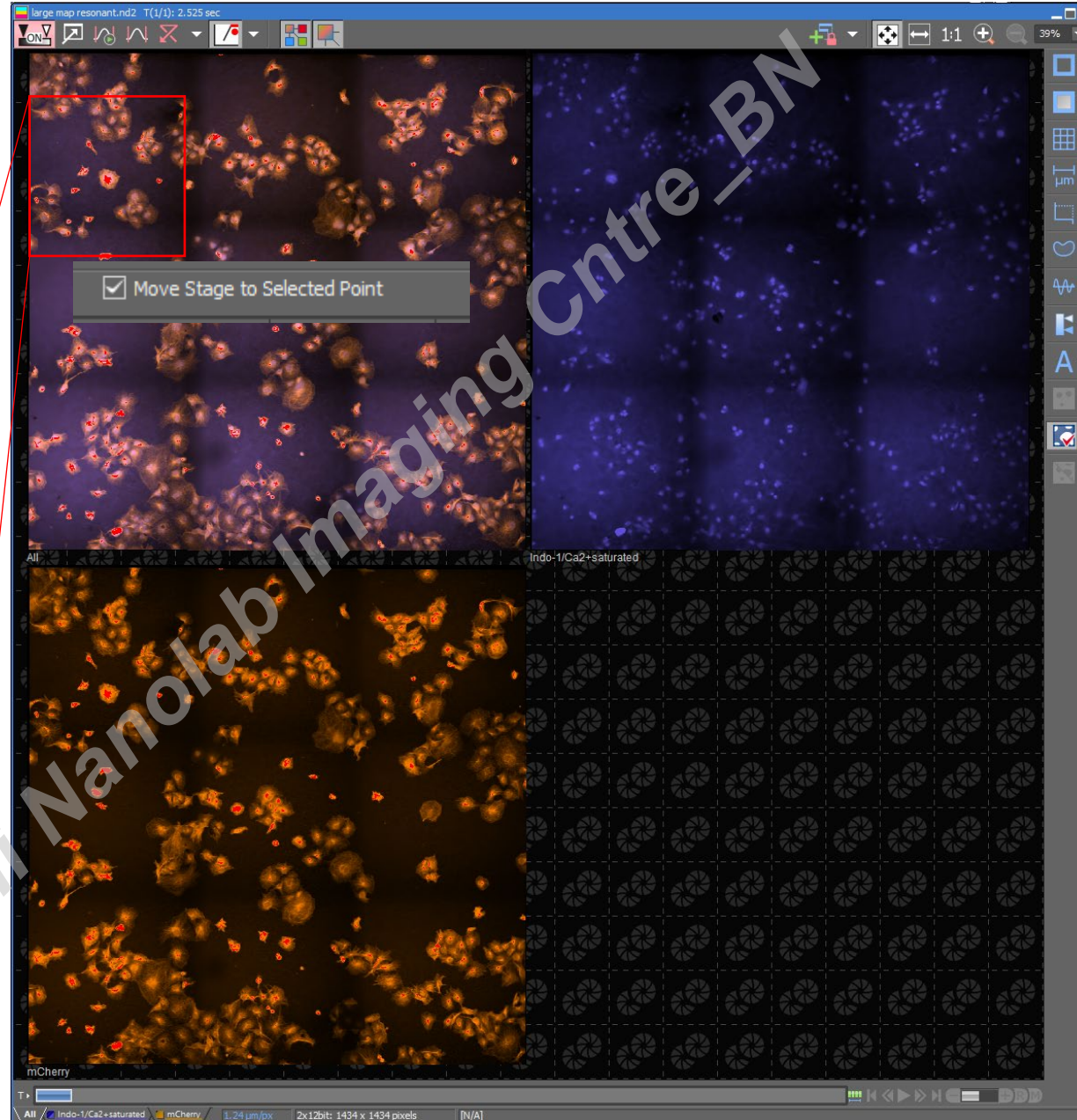
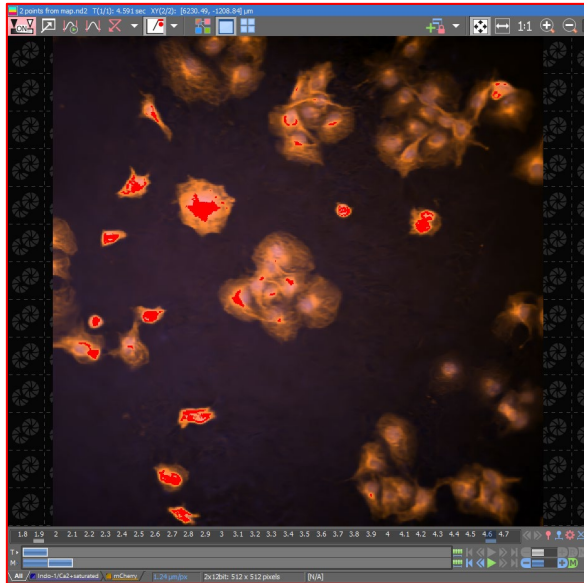
You can quit at any time!



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

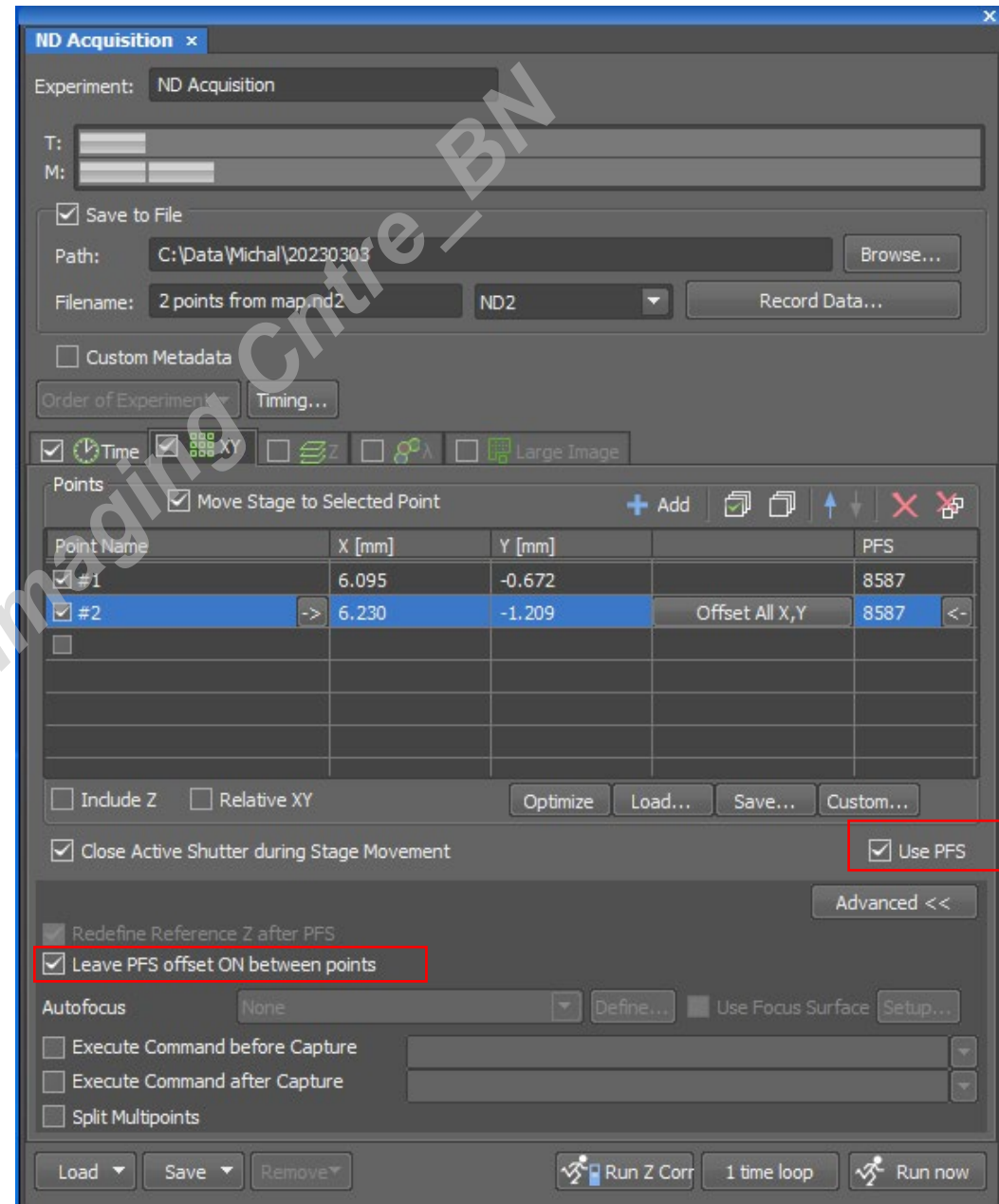


Point list with PFS:

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)



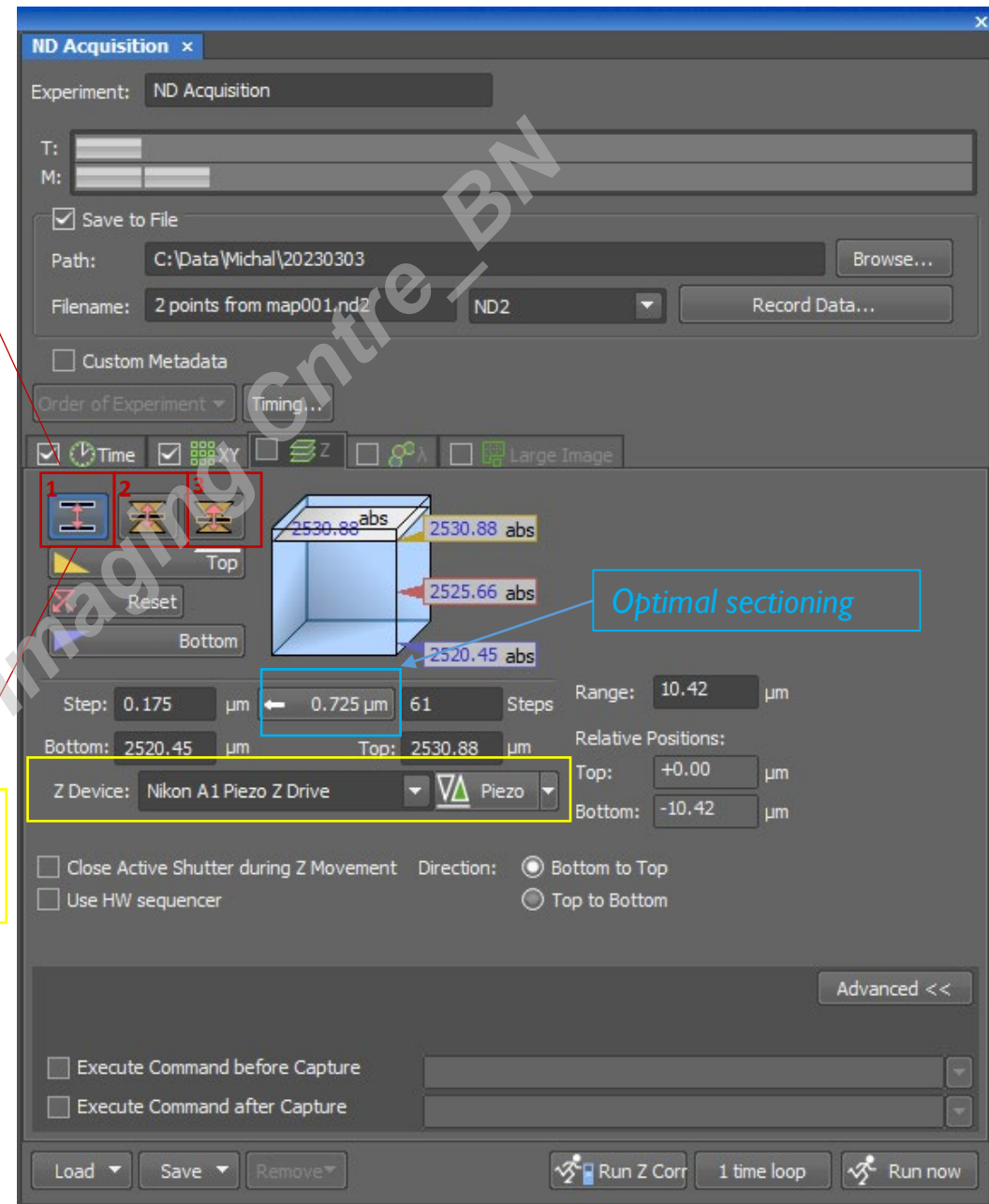
Z stack- basic:

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

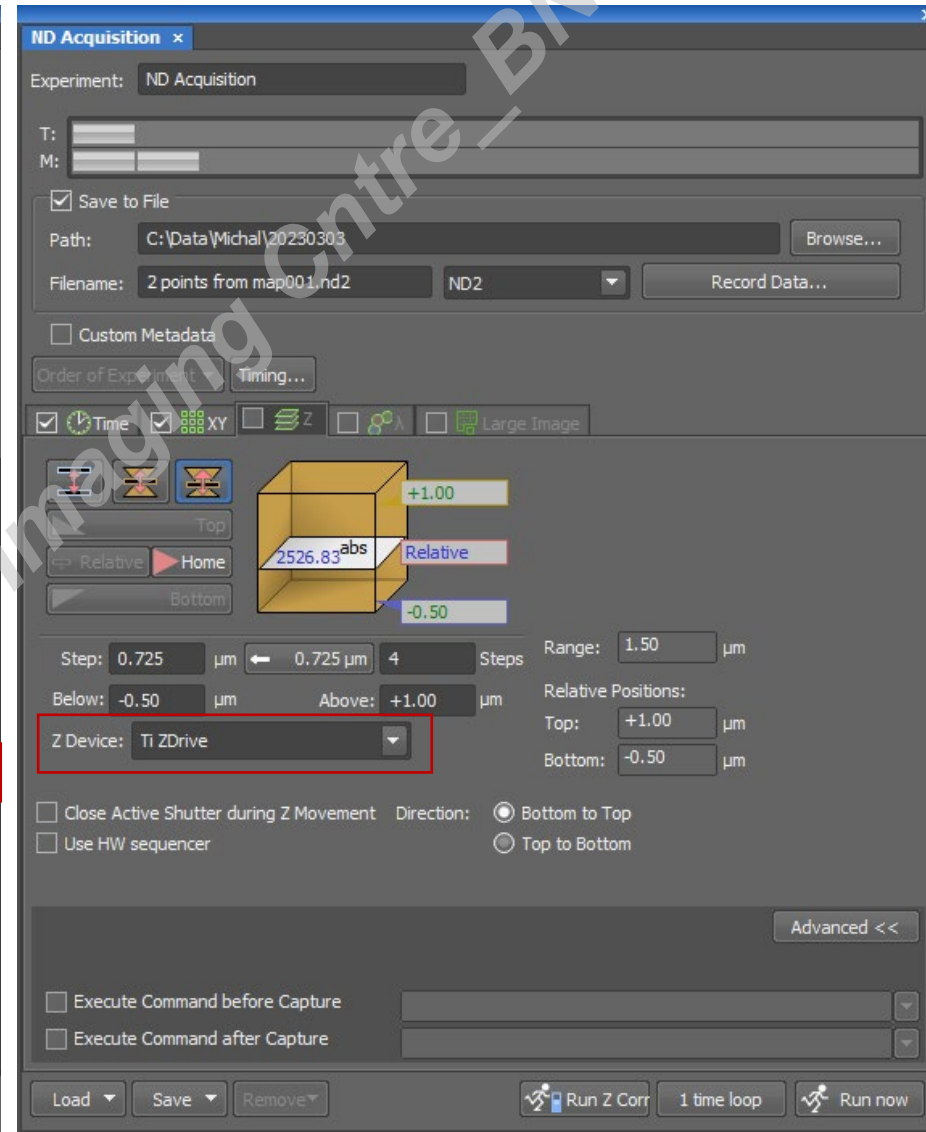
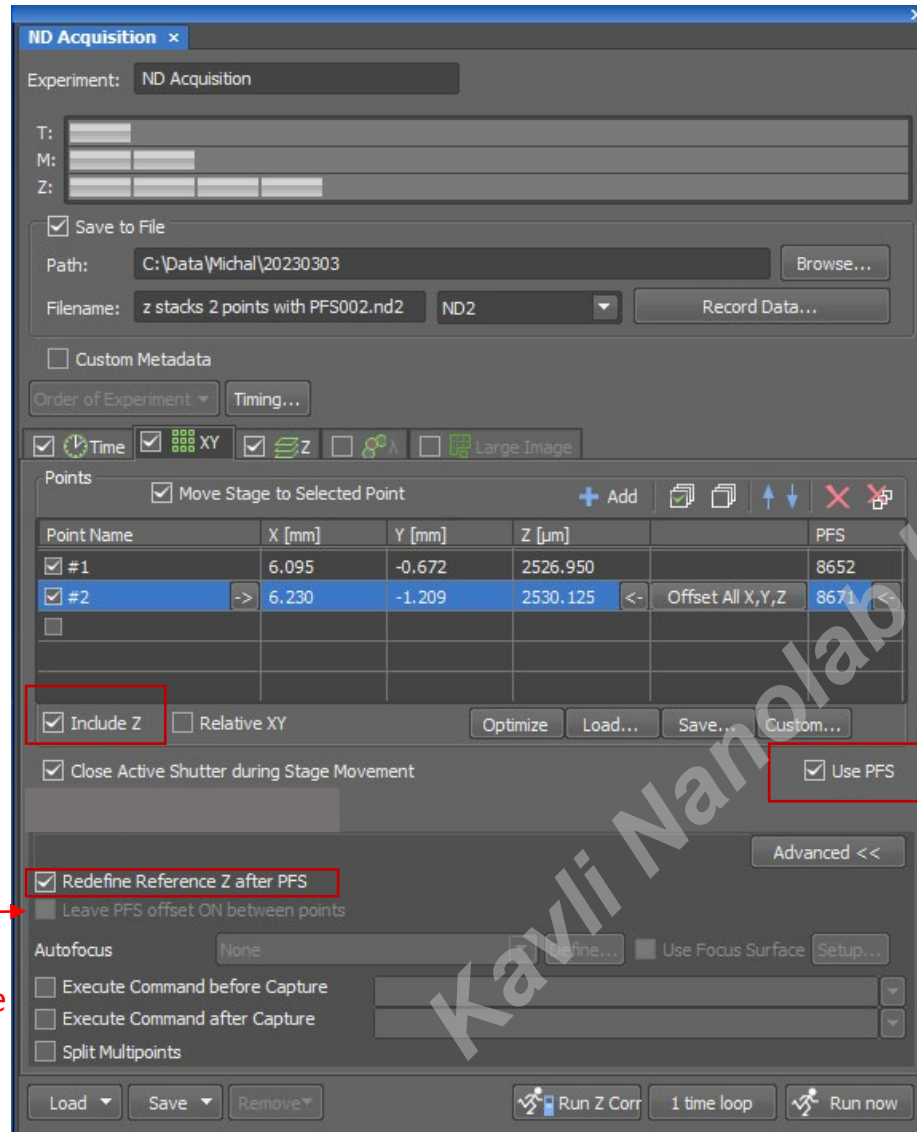
3: Relative- you can set different thickness below/above

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

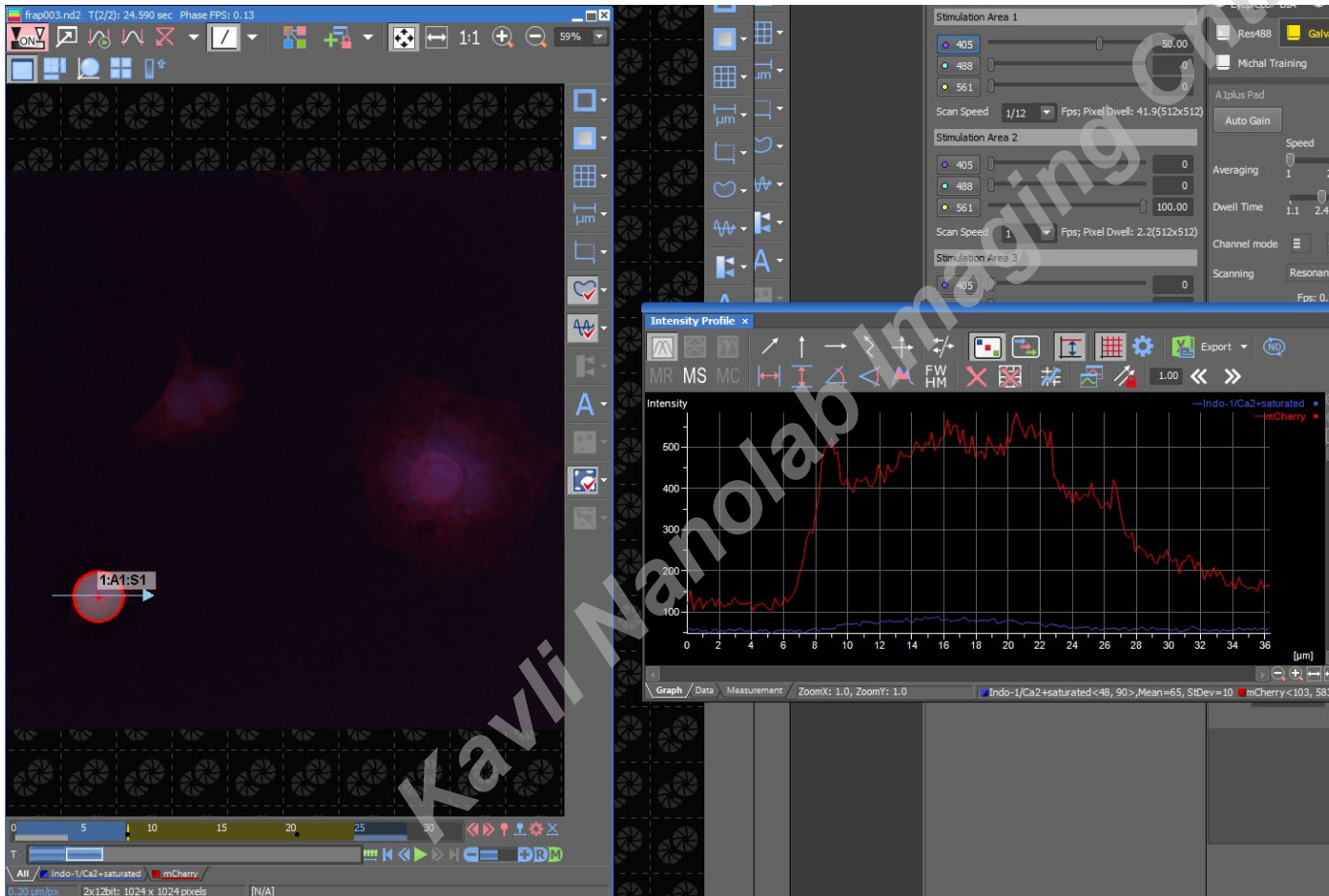


Don't!

PFS doesn't handle large distances

FRAP

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



The 'A1plus Stimulation' control panel is shown on the right. It features a 'Prescan' section with 'Auto Prescan' selected and 'No Prescan' unselected. Below this, there are checkboxes for 'Synchronize Lasers' and 'HV Mode' set to 'Zero HV'. The panel is divided into three sections for 'Stimulation Area 1', 'Stimulation Area 2', and 'Stimulation Area 3'. Each section contains three intensity sliders for wavelengths 405 nm (blue), 488 nm (cyan), and 561 nm (yellow). The 'Scan Speed' and 'Fps; Pixel Dwell' parameters are also adjustable for each area. A 'Manual Shift Alignment...' button is located at the bottom.

2, Draw a circular ROI and assign it as **stimulation ROI (AI:SI)**

The screenshot displays the Nikon NIS-Elements AR software interface during a FRAP (Fluorescence Recovery After Photobleaching) acquisition. The main window shows a microscope view with a grid overlay. A circular ROI (Region of Interest) is drawn on the image, labeled '1'. The ROI is assigned as a stimulation ROI (AI:SI). The software interface includes several panels:

- Acquisition Panel:** Shows acquisition parameters such as Eye-piece, DIA, Eye-pieces, Confocal, Res488, Galvano 4ch, Confocal Res BF 405 561, and Michal Training_1. It also displays the Aplus Pad, Auto Gain, Speed, Quality, Averaging, Dwell Time, Channel mode, Scanning, Resonant, Galvano, Fps, Frame Time, Detector, and Settings.
- Stimulation Area Panel:** Shows three stimulation areas (Stimulation Area 1, 2, and 3) with parameters for Scan Speed, Fps, Pixel Dwell, and Manual Shift Alignment.
- Filters Panel:** Shows filters such as Turret2, Turret1, and Zoom.
- Channel Panel:** Shows channel settings for Indo-1/Ca2, DIO, mCherry, and other channels, including Offset, Gain, and Pinhole.
- ROI Panel:** Shows the ROI settings, including the ROI name (1), ROI type (Circular), and ROI size (512x512 pixels).

The software interface also displays the following information:

- File: Edit Acquire Calibration Image ROI Binary Measure Reference Macro View Devices Window Applications JOBS Addons NIS.a Help
- Search [Ctrl+F3] mshemesh
- Plan Apo A 20x (1.24 µm/px @ 512 x 512)
- XY=[6.238, -1.208]mm, Z=-2523.125µm

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

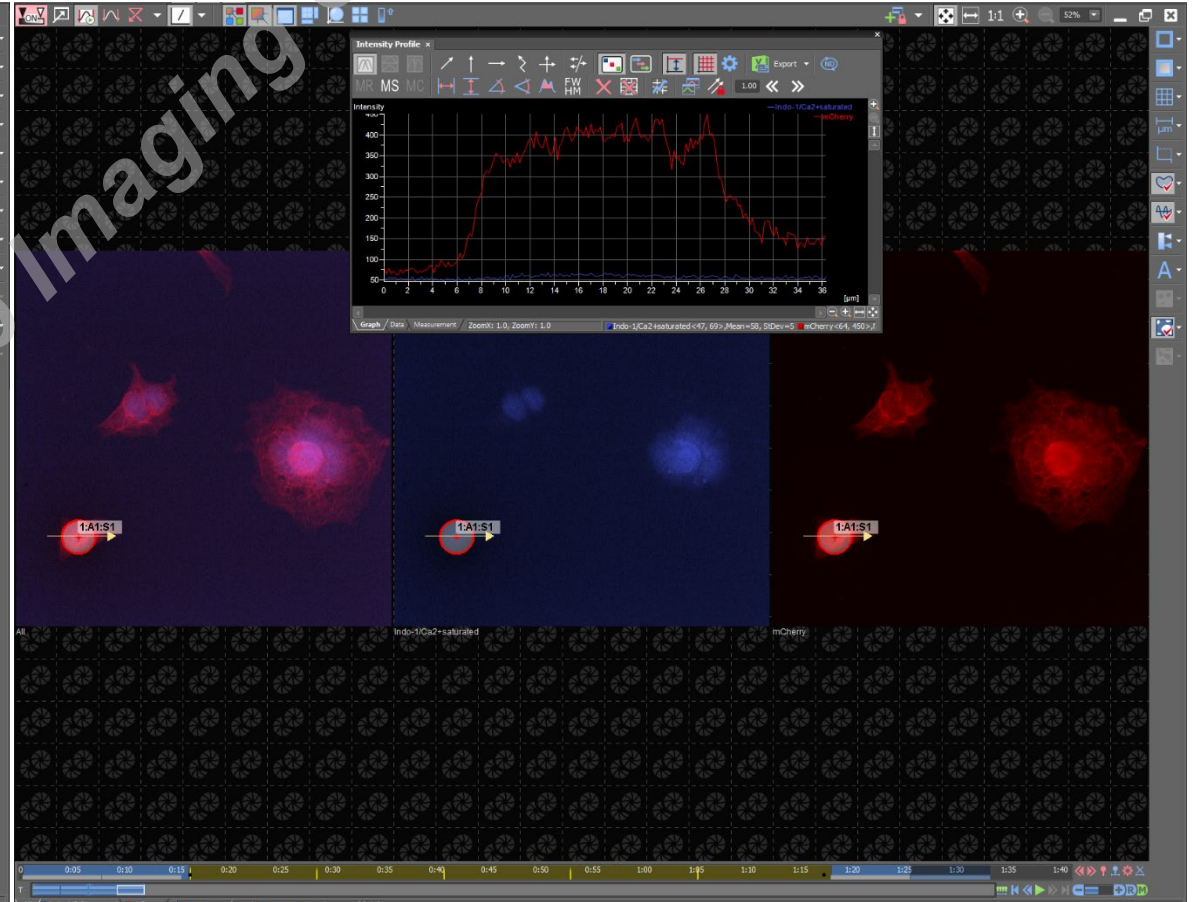
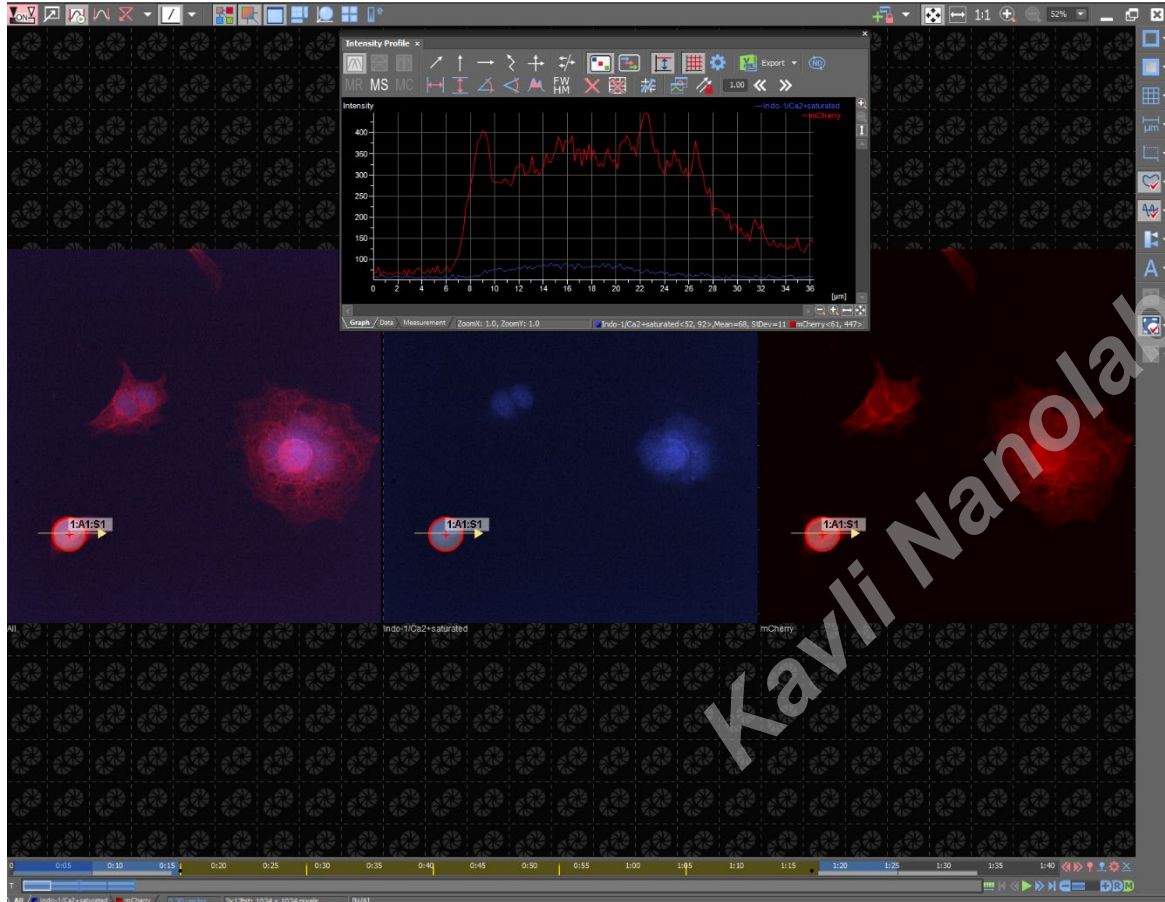
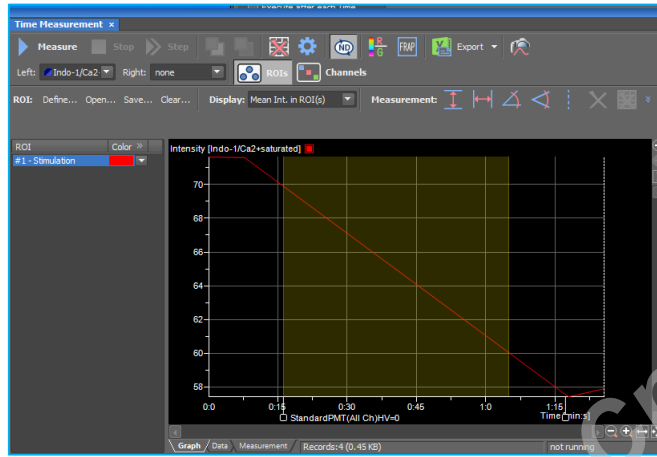
The screenshot displays the Nikon NIS-Elements AR software interface for FRAP acquisition. The main window shows a 3x3 grid of microscopy images. The top-left image shows a cell with a red circular ROI labeled "1:A1:SI". The top-middle image shows a blue channel with a similar ROI. The bottom-left image shows a red channel with the ROI. A central button labeled "Apply Stimulation Settings" is visible. The right-hand side of the interface contains several control panels:

- A1plus Stimulation**: Includes options for "Auto Prescan" and "No Prescan", "Synchronize Lasers", and "HV Mode: Zero HV". It lists three "Stimulation Area" configurations with parameters for "405", "488", and "561" lasers, "Scan Speed", and "Fps; Pixel Dwell".
- Acquisition**: Features a "Live" button, "Capture", "Find", "ND Acquire", "Large Image", "Save", "Save As...", and "Open" options. It shows "Eye-piece: DIA", "Eyepieces: Confocal", and "Res:488 Galvano-4ch".
- Channel mode**: Shows "Resonant" and "Galvano" modes, "Fps: 0.125; Frame Time: 8.0 sec", and "Detector" settings.
- Filters**: Includes "Turret2" and "Turret1" settings.
- Zoom**: Set to "1.00x".
- A1plus Scan Area**: Shows "512" scan area and "Crop ROI" options.
- Channel Settings**: Includes "Indo-1/Ca2", "DIO", "mCherry", "Offset", and "Gain" sliders for each channel.
- Pinhole**: Set to "405.0" and "1.2".
- Zoom Size**: Set to "3.17".
- Pixel size**: Set to "0.20 μm".
- Z step**: Set to "0.72 μm Res.: 0.30 μm Sectioning: 2.10 μm".

A large watermark "Kavli Nanolab Imaging Centre" is overlaid diagonally across the center of the image.

FRAP- check

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



FRAP- ND experiment

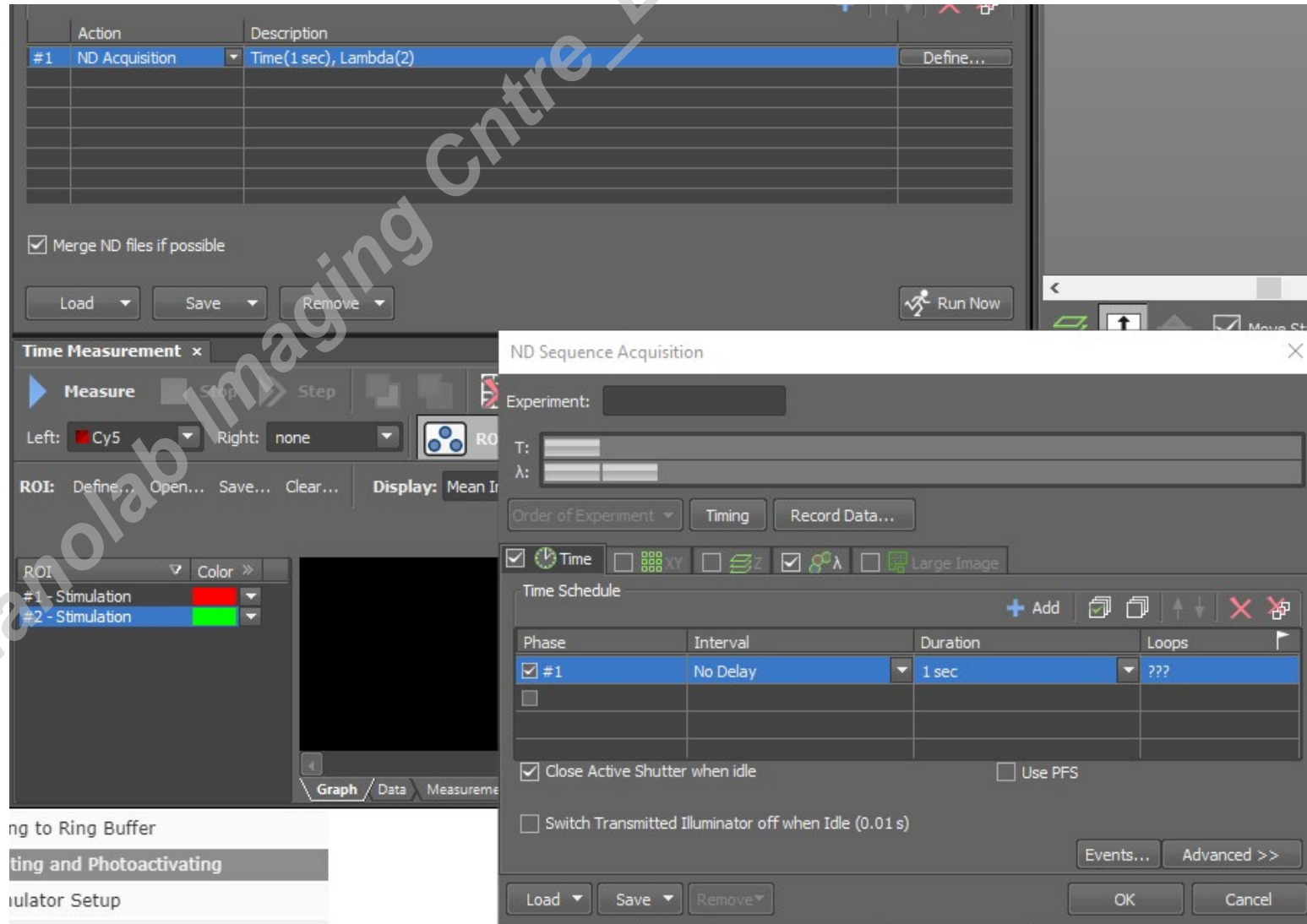
In your time lapse experiments, you can define an action of acquisition and/ or stimulation

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

#4
Switch Off your Individual lasers

#6
Switch Off A1R control

#5
Switch Off Laser controller

Make sure all cage doors are closed

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