KING'S College LONDON

Wohl Cellular Imaging Centre

Nikon A1R Confocal Microscope

A1R - STEP BY STEP INSTRUCTIONS CONTENTS PAGE

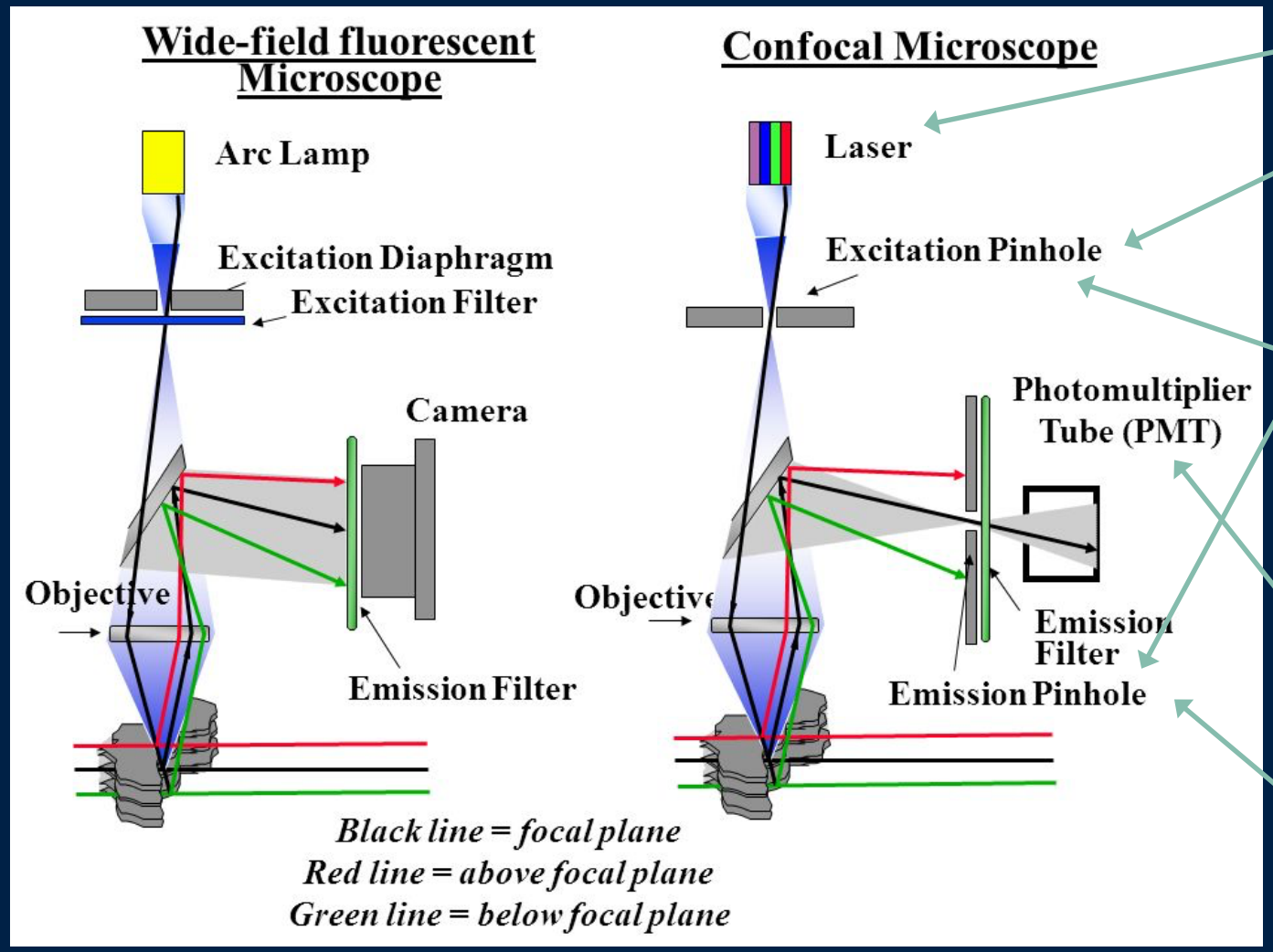
Before Using The Facility…

To use the facility the individual must undergo training…

- Tour of the facilities
- T & C agreements
- First training session (2 hours)
- Second training session with user's samples (2 hours)
- Additional training sessions may be required
- Additional lens installation training (optional)
- Access to booking system: http://ppms.eu/kcl-wohl

Basic Principles Of A Confocal Microscope

Confocal microscopy improves image resolution by using a more focused excitation light source and allow users to observe more selective emitted light. Confocal microscopy bridges the gap between widefield and electron microscopy.



Lasers produce intense monochromatic light which excites a small target zone instead of flooding a large area like a Arc lamp.

The excitation pinhole force lasers into a spot shaped light source.

The excitation and emission pinhole focuses on the same exact spot on the specimen, thus making them confocal.

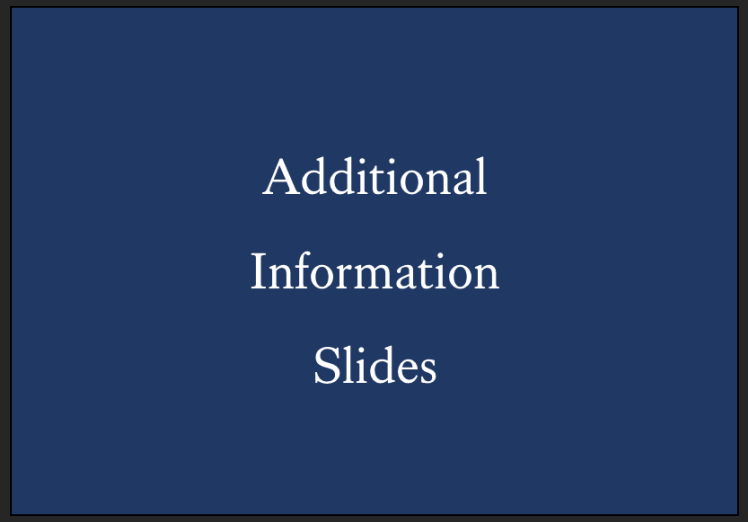
If you enlarge the pinhole to much, the microscope will behave like a wild-field system.

The photomultiplier tube (PMT) is the detector and amplifier for emitted signals. The charge of the PMT affects the amplification (gain). PMT is more sensitive for blue light (15%) than red (4%).

The emission pinhole act as a spatial filter, restricting any light not originated from the focal point.

STEP BY STEP INSTRUCTIONS

The rest of this document will take you from focusing on your sample to optimising your image to what to do at the end of your session.



The blue slides contains additional information you might find helpful.

STEP BY STEP INSTRUCTIONS

STEP 1

System ON

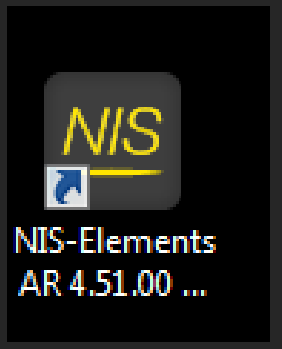
System On

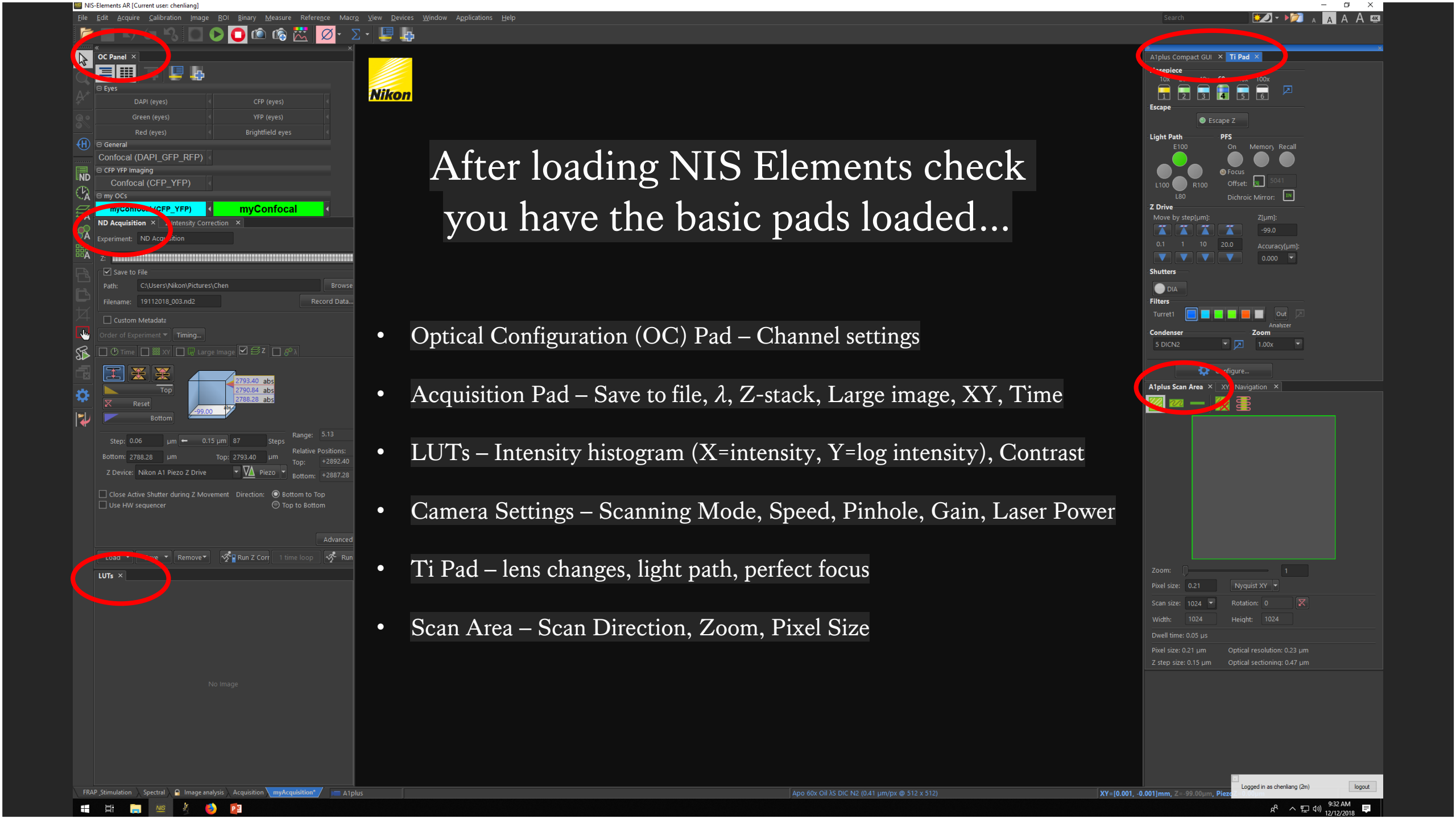
If the incubator is on do not turn the incubator off.

1. Switch on the A1R confocal system by following the numbered switches.

2. **Make sure the stage is empty before turning on the Microscope switch at the right, far back.**

3. **ALWAYS login to NIS-Elements Software before loading any sample, this checks if all systems are connected.**



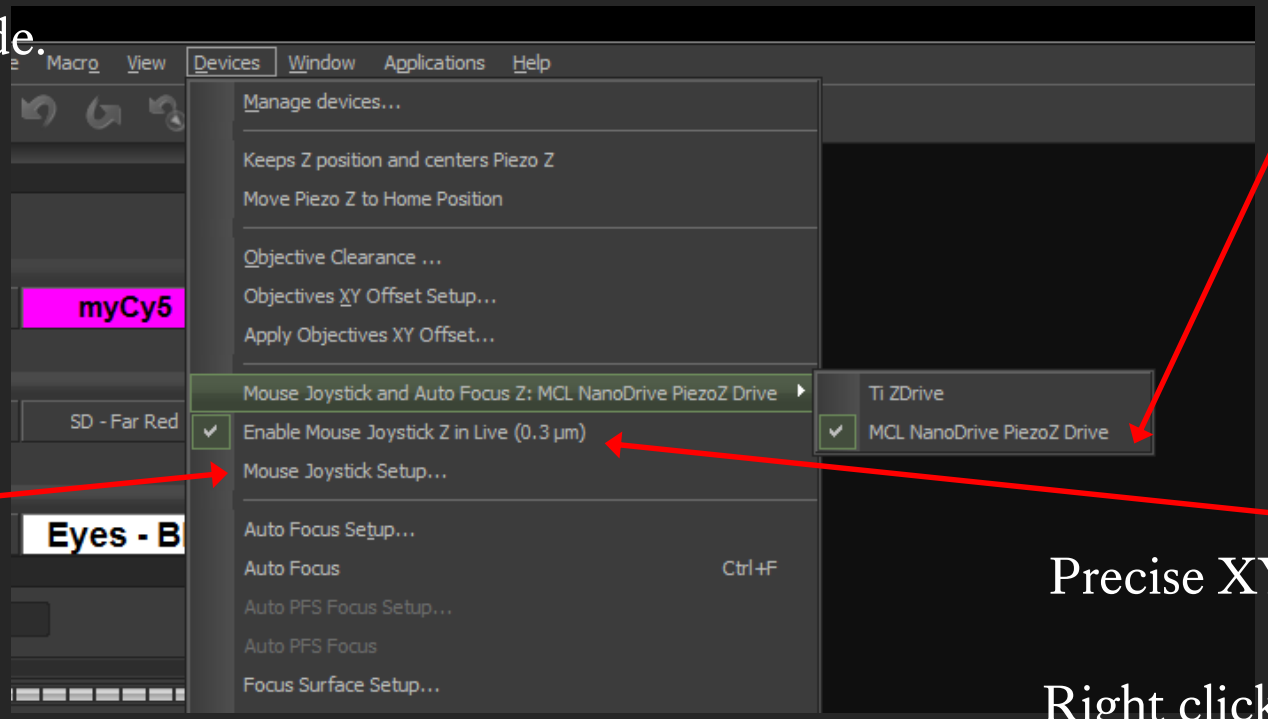

After loading NIS Elements check you have the basic pads loaded…
Optical Configuration (OC) Pad – Channel settings
Acquisition Pad – Save to file, λ, Z-stack, Large image, XY, Time
LUTs – Intensity histogram (X=intensity, Y=log intensity), Contrast
Camera Settings – Scanning Mode, Speed, Pinhole, Gain, Laser Power
Ti Pad – lens changes, light path, perfect focus
Scan Area – Scan Direction, Zoom, Pixel Size

Use Mouse Wheel To Focus And Move In Live Mode

This will be set up for you in your 2nd training session.

Mouse wheel is used to zoom in when not in live mode.

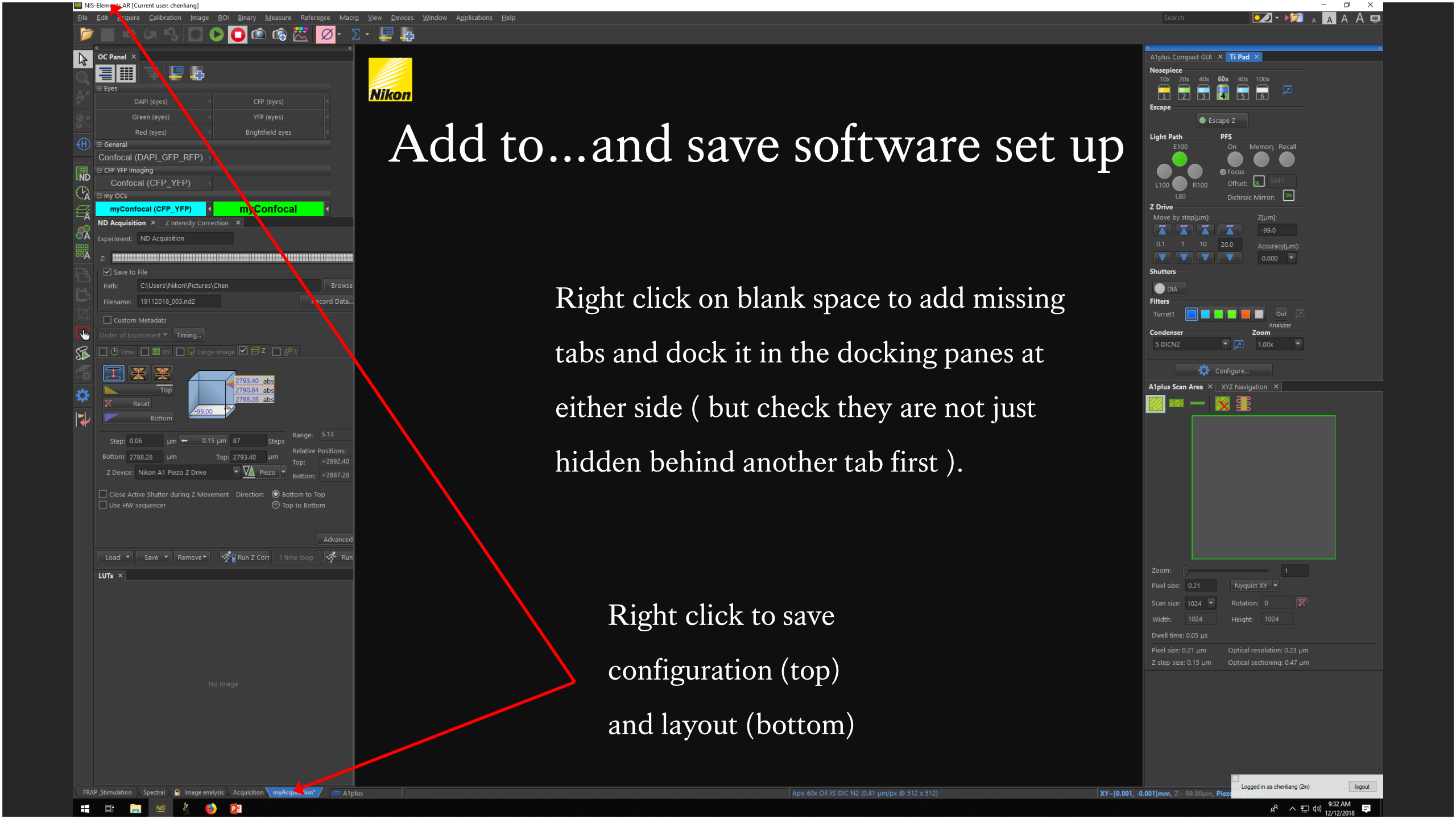
Select Piezo Drive to increase precision.



Options for extra fine Z control.

Precise XY movement in live mode.

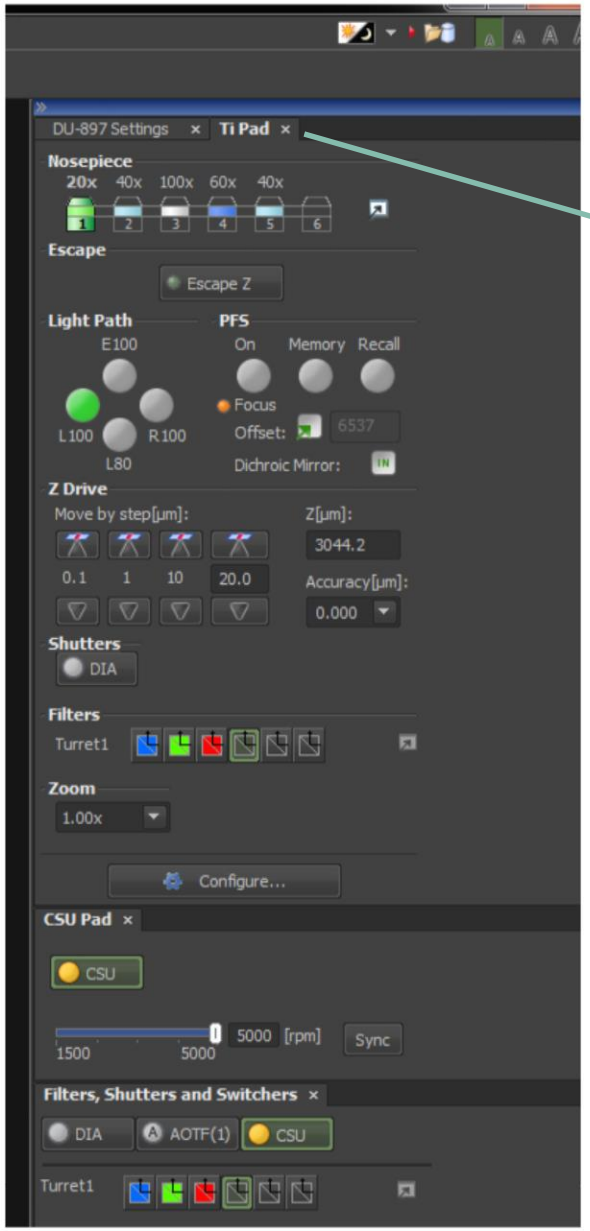
Right click for move object to centre option.


Add to…and save software set up
Right click on blank space to add missing tabs and dock it in the docking panes at either side (but check they are not just hidden behind another tab first).
Right click to save configuration (top) and layout (bottom)

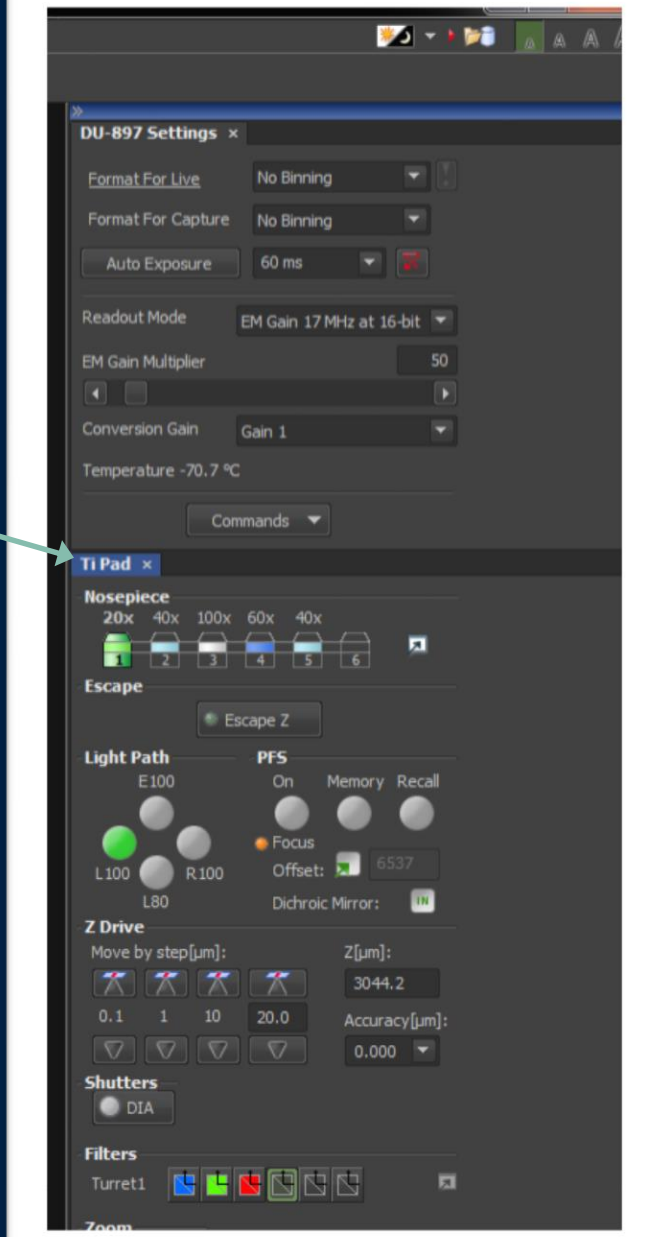
Microscope and software set up and layout

Additional Information Slides...

Software Hidden Panels



Mouse left click hold, drag and drop to rearrange docking pane.



Software Layout

Live Stop

Capture: This is not automatically saved

Camera Settings

Eye View

Shared Default DO NOT CHANGE

Set up mouse wheel control (focus in live mode) (zoom when not in live mode)

LOWER the OBJECTIVES

Lens Options

Camera view.

Exposure/Gain/Laser/averaging settings can be set up and saved for each channel in Camera settings for my OCs

Perfect Focus

Zoom

Acquisition pad - for setting up Z-stack, Large image, multi-channel, time and XY positioning.

SAVE AS: Get into a good habit of setting up file path at the start of session. Any images taken using the "Run now" button will be automatically saved.

If using the dial to zoom 1.5X, UPDATE the software, they do not automatically sync and can cause errors during large image stitching.

Direction of Scan

Field of view dependant on laser scanning zoom (not the same as the dial zoom mentioned above!)

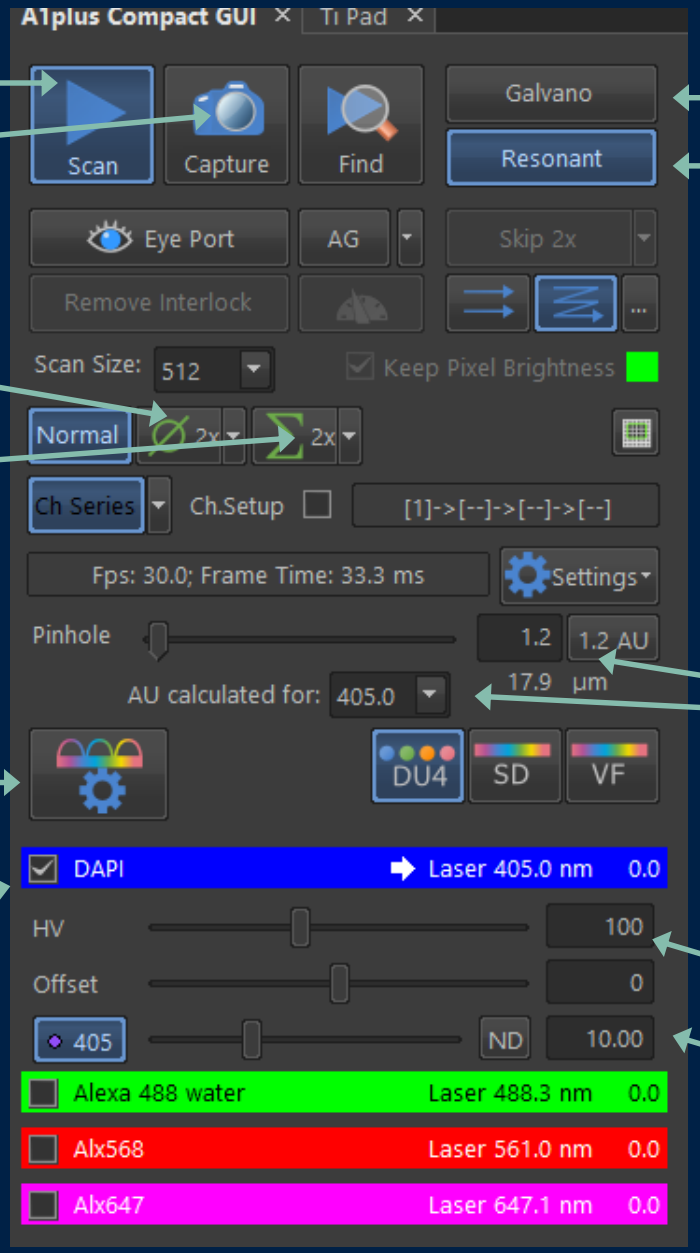
LUTs

Show intensity in a histogram format

Contrast manipulation

Saturation indicator options

Software Layout: Camera Settings



Live view

Capture

Precise scanning mode

Fast scanning mode

Averaging

Integrate

Settings – Pathways, wavelength, filters…

Laser scanning channels

Pinhole for a particular channel, can use recommended size or type in preferred size.

Gain (signal amplification)

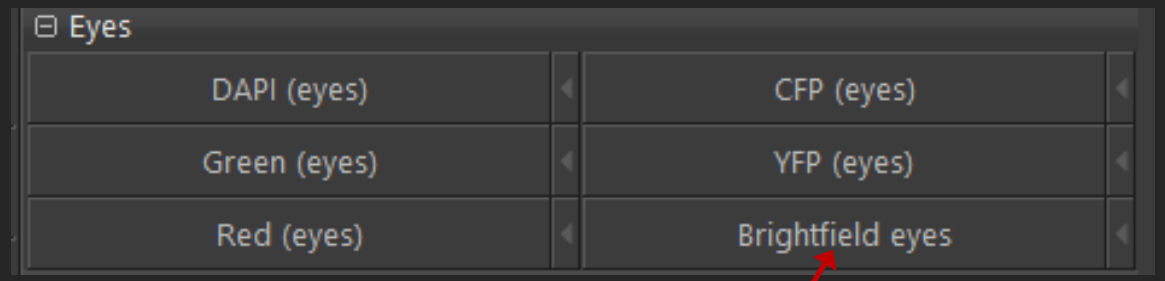
Laser Power

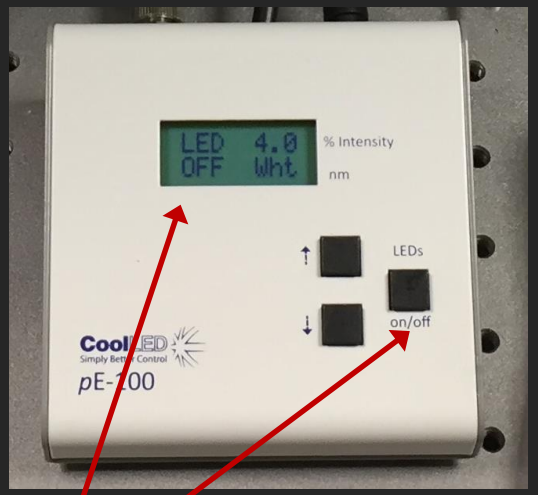
STEP BY STEP INSTRUCTIONS

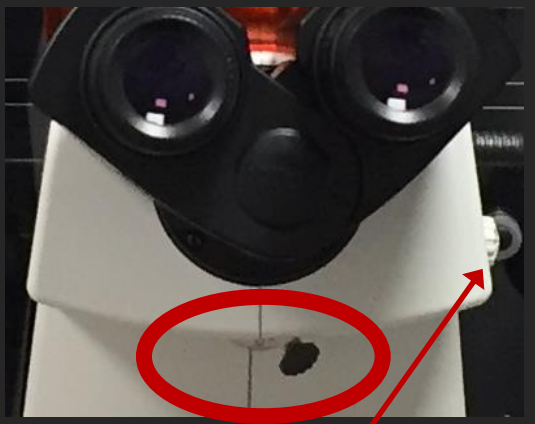
STEP 2

Lens And Focus

Checking Lens for Damage and Cleanliness







Make sure you are on brightfield eyes and the illumination is on. Check the lens for any damage by putting the toggle beneath the eyepiece to the **LEFT** and zoom on the lens using the button here.

ALWAYS put the toggle back to the RIGHT

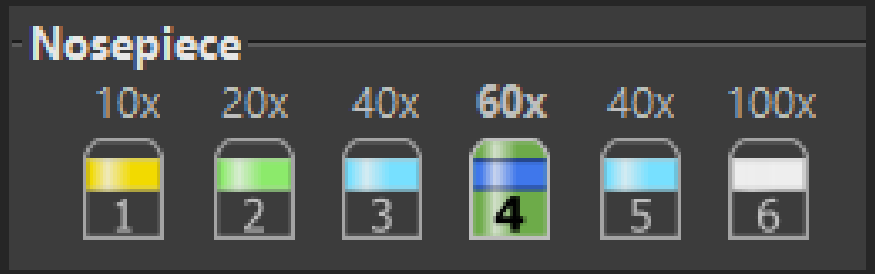
after you're done and before you start to focus.

Cleaning OIL Lens

1. Wipe away any excess oil with dry lens tissue (normally after your session)
2. Wrap lens tissue around your finger and soak up some Isopropanol and clean lens from centre outwards (REPEAT 3 TIMES)
3. Clean once more with dry lens tissue

- During your session if you are switching between oil/dry lenses, just wipe away excess oil on the lens and your slide, lower your objectives down, before switching.

Changing Lens



ALWAYS

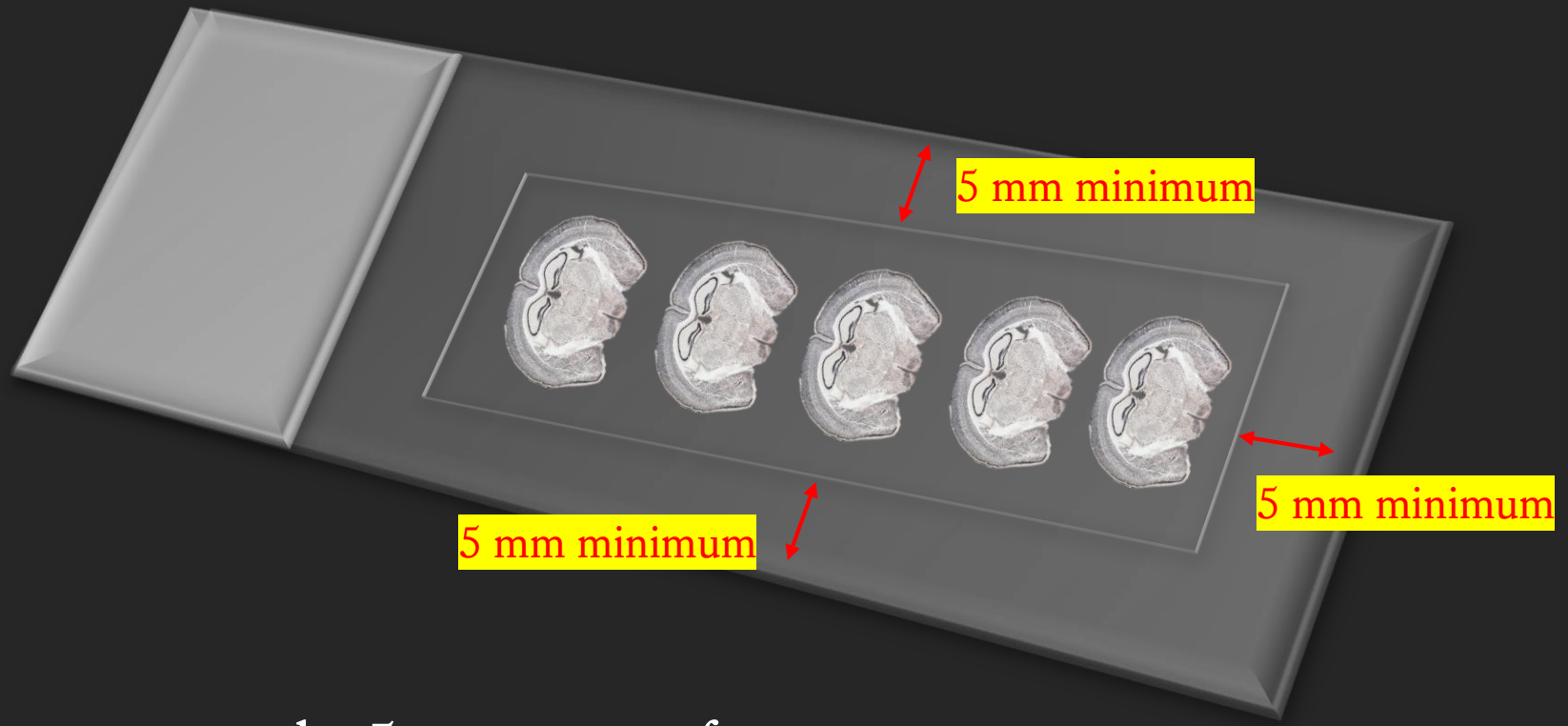
Lower the lenses as far as they can go before inserting the stage and/or clicking on another lens.

To lower lens…

- Rotate wheel clock-wise
- Bottom = this number no longer reduces
- Be careful - zero doesn't mean it's at the bottom

Lower the lenses between changing <u>slides</u>.

Load your sample into the stage



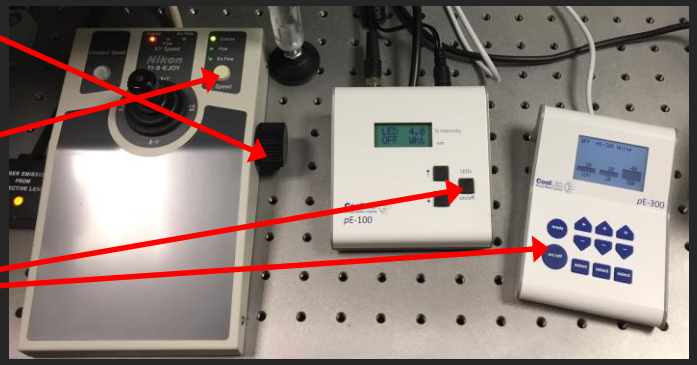
Make sure to mount your sample 5mm away from the edge of the slide! You cannot image too close to the edge of the slide, it can damage the lens!

Focus

ALWAYS put the toggle back to the RIGHT after you're done and before you start to focus.

DRY LENS ONLY (20X Air and 40X Air)

- Use Wheel and joystick on the control pad to adjust and focus.
- Make sure you are on the specimen.
- Focus indicator comes when it detects a reflective surface. (Do not rely on numbers as the lowest setting is not always set to ZERO, to set the stage XZ to 0µm press here.)
- When the green light comes on STOP moving up immediately (lower it back down again if needed, otherwise the focal plane might be passed).
- Switch coarse XZ to fine (Z button), coarse XY to fine (twist the joystick)
- Select one of the "EYE" options in the software.
- Turn on and adjust the brightfield or Epi-fluorescence illumination intensity.
- Find focus using eye piece.



OIL LENS

- Focus indicator does NOT work for oil/water lens.
- Wheel upwards on coarse ONLY until lens comes in contact with oil.
- Switch coarse XZ to fine, coarse XY to fine (twist the joystick)
- Find focus using eye piece.
- Find focus on camera.

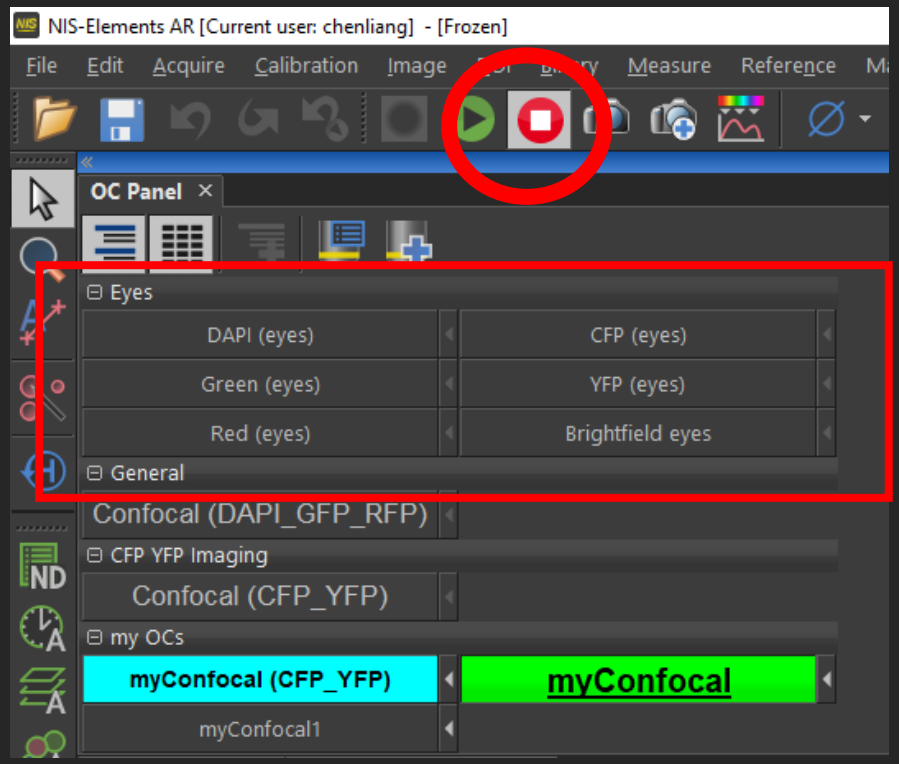
STEP BY STEP INSTRUCTIONS

STEP 3

Change from Eyes to Camera View

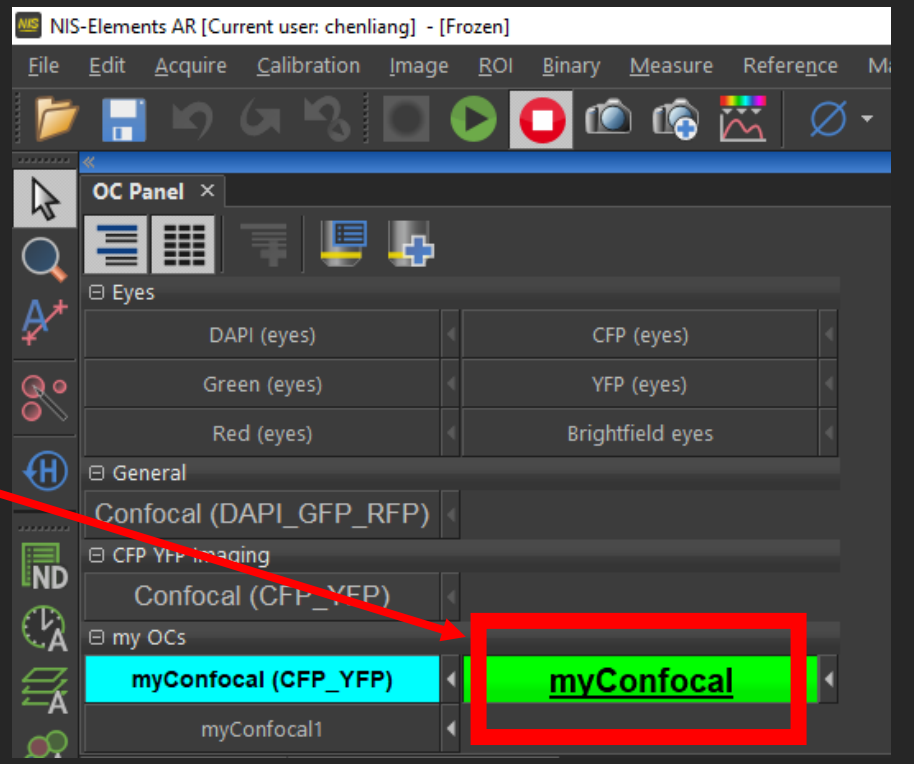
From this point forward we will be adjusting things on the computer screen so we need to work in camera mode instead of looking down the eye piece.

Remain on STOP setting.

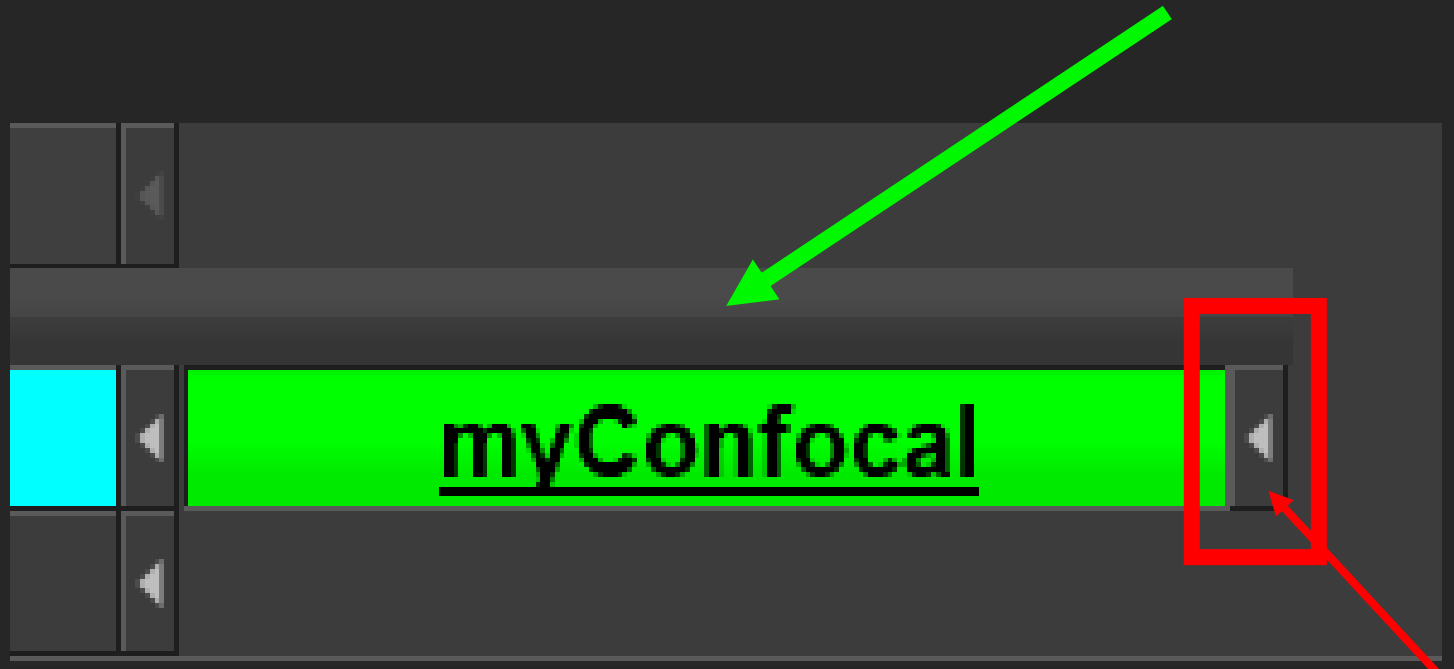


Click on ‘myConfocal’

This moves from eyes to camera.



Click on the green area “myConfocal”



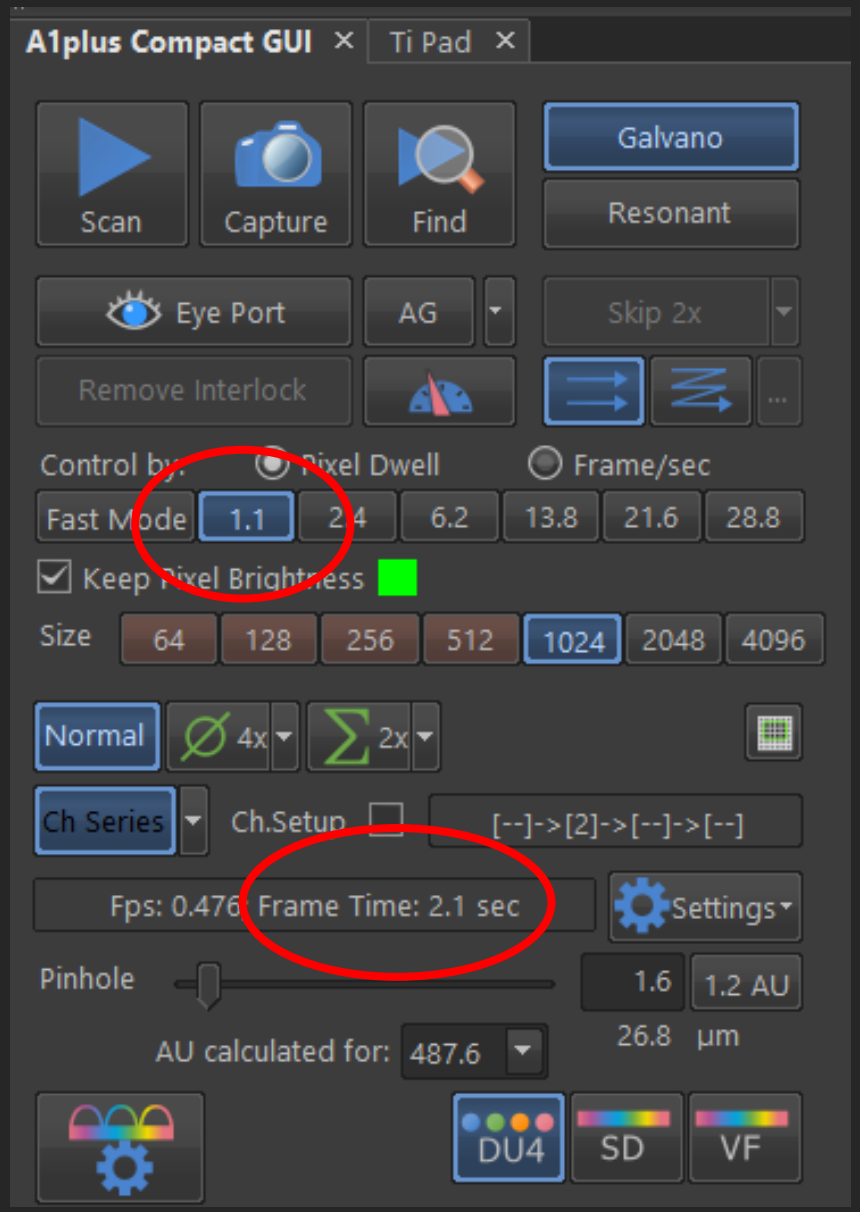
DO NOT CLICK ON THIS AT THE MOMENT

STEP BY STEP INSTRUCTIONS

STEP 4

Choose A Laser Scanning Mode

Galvano:
Precision point laser scanning

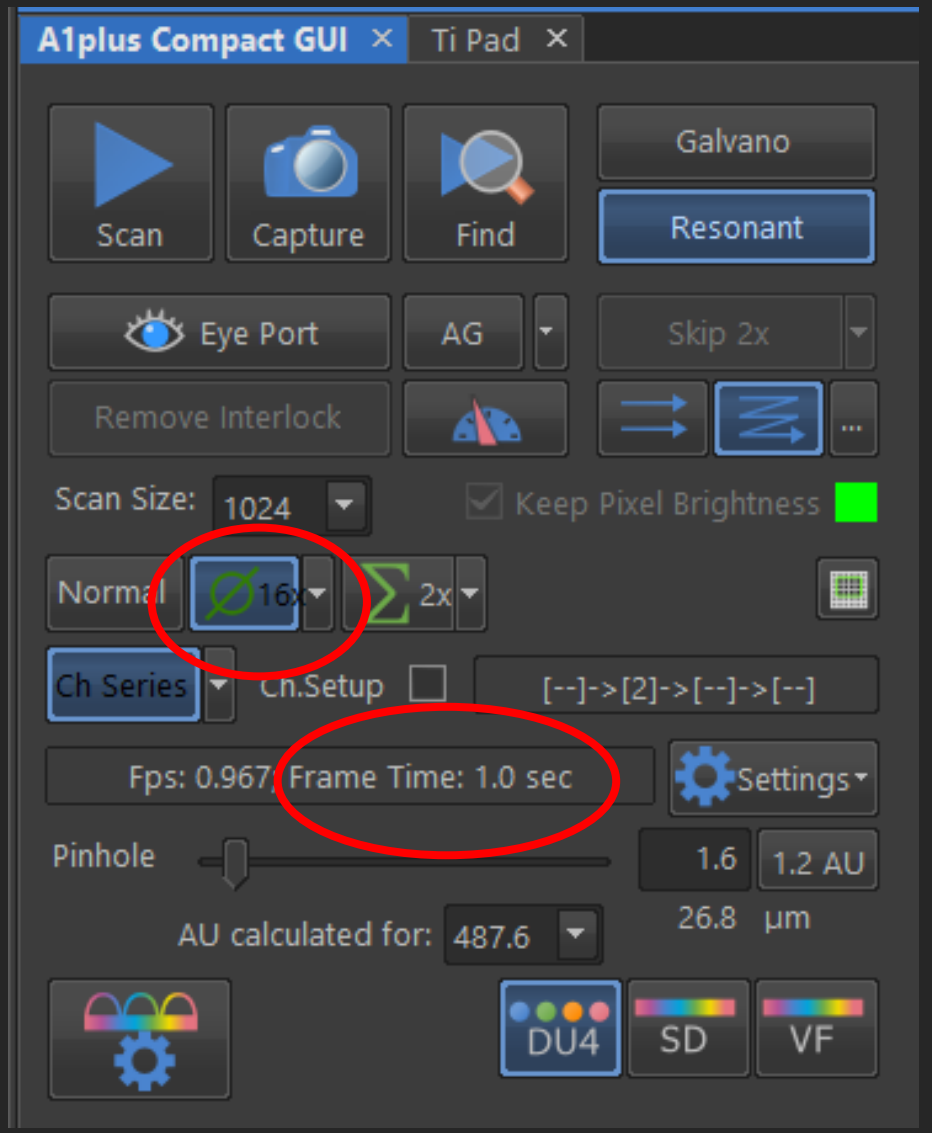


Even when Galvano option scans your sample once and Resonant has 16 times averaging (scans your sample 16 times) Resonant mode still only take half the time to capture.

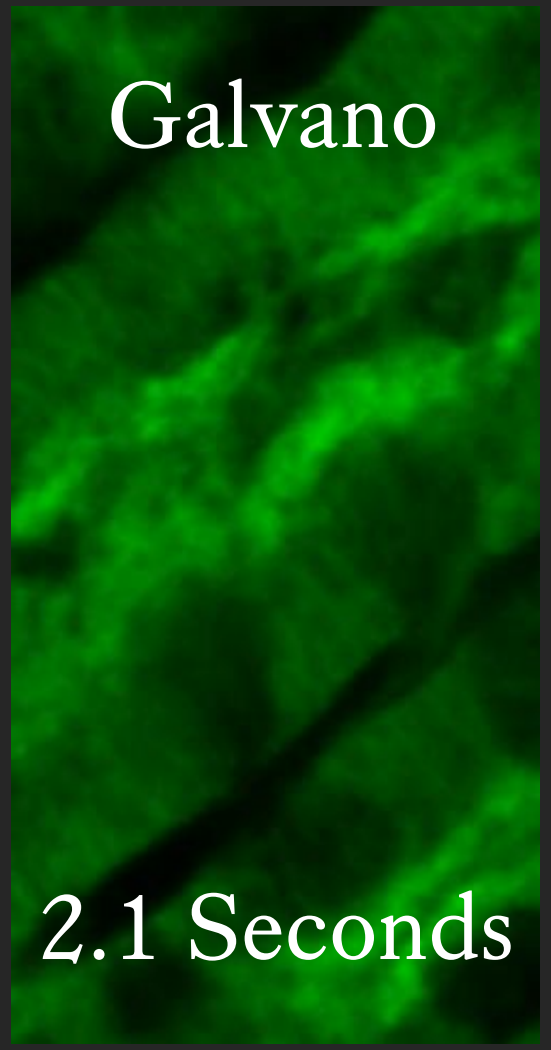
↓

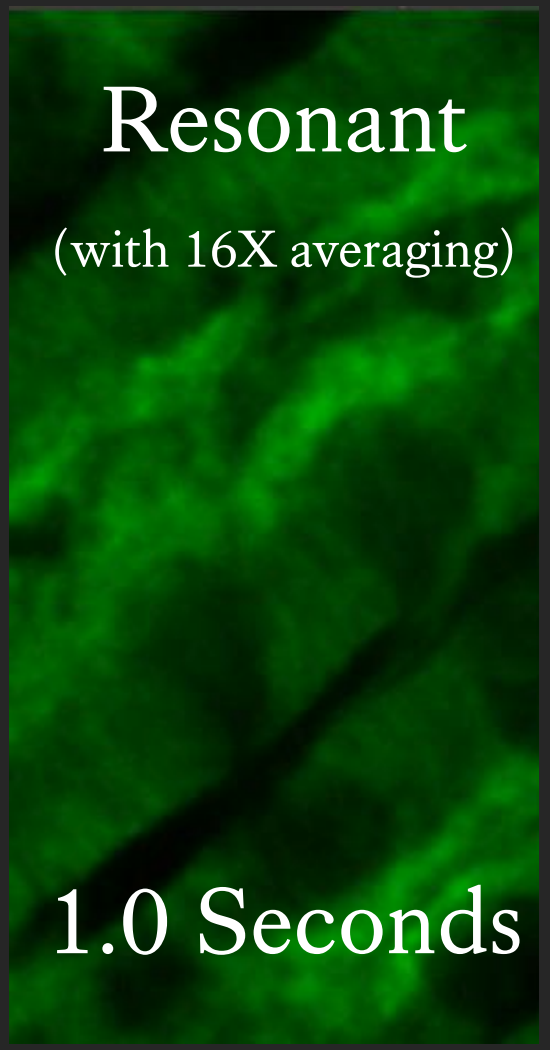
Lets compare background noise and image quality…

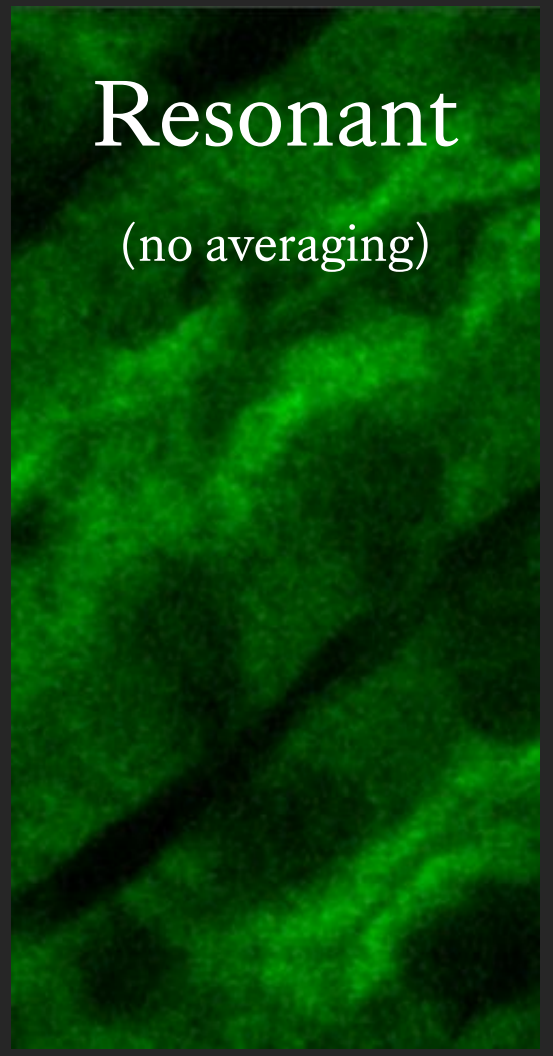
Resonant:
faster laser scanning
(recommended for live imaging, less bleaching)



Galvano vs Resonant (with 16X averaging)







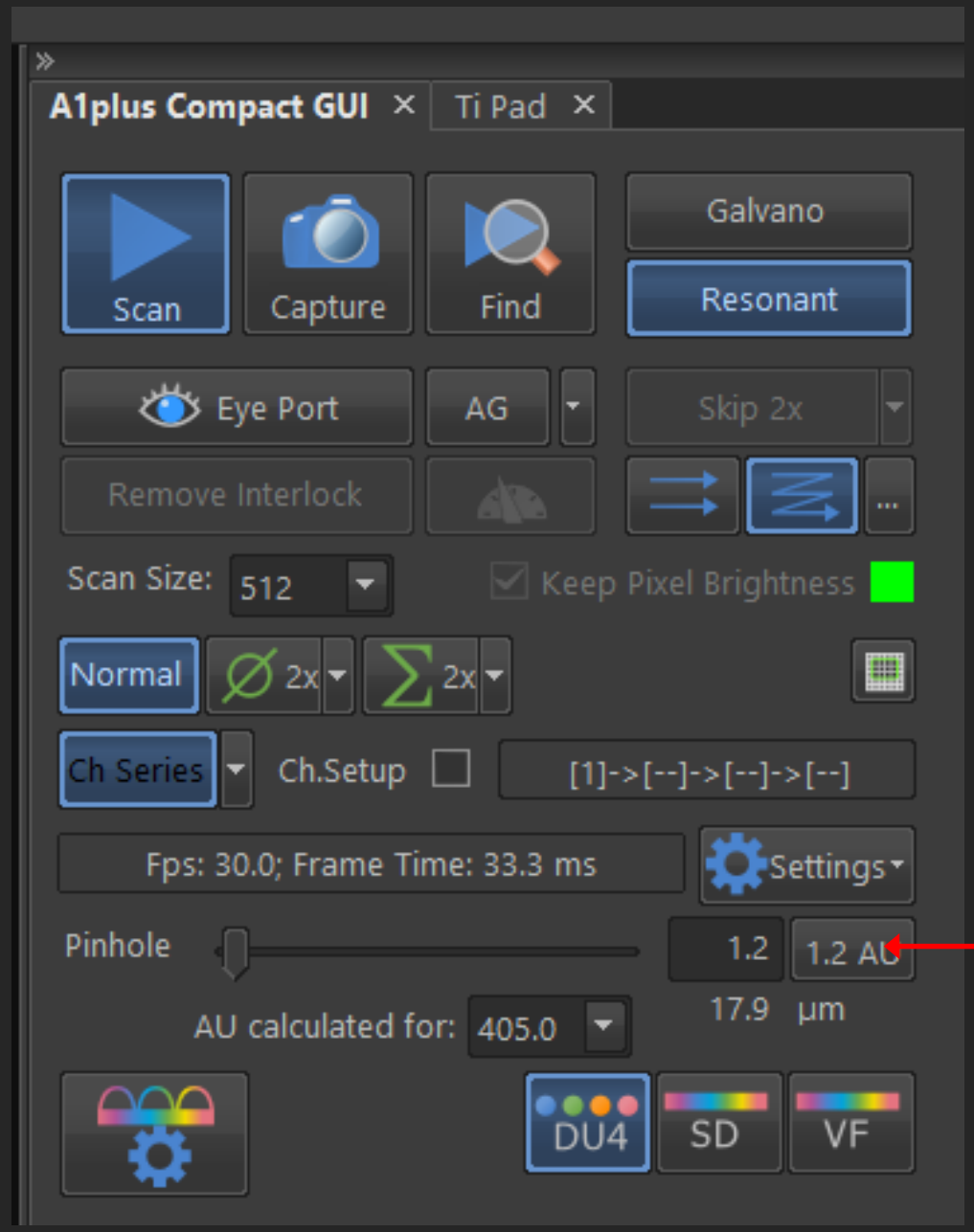
When switching from Resonant to Galvano, the software resets these settings every time, so please do the following:

1. Select Pixel Dwell
2. 1024
3. 1.1

STEP BY STEP INSTRUCTIONS

STEP 5

Setting Up Initial Live View

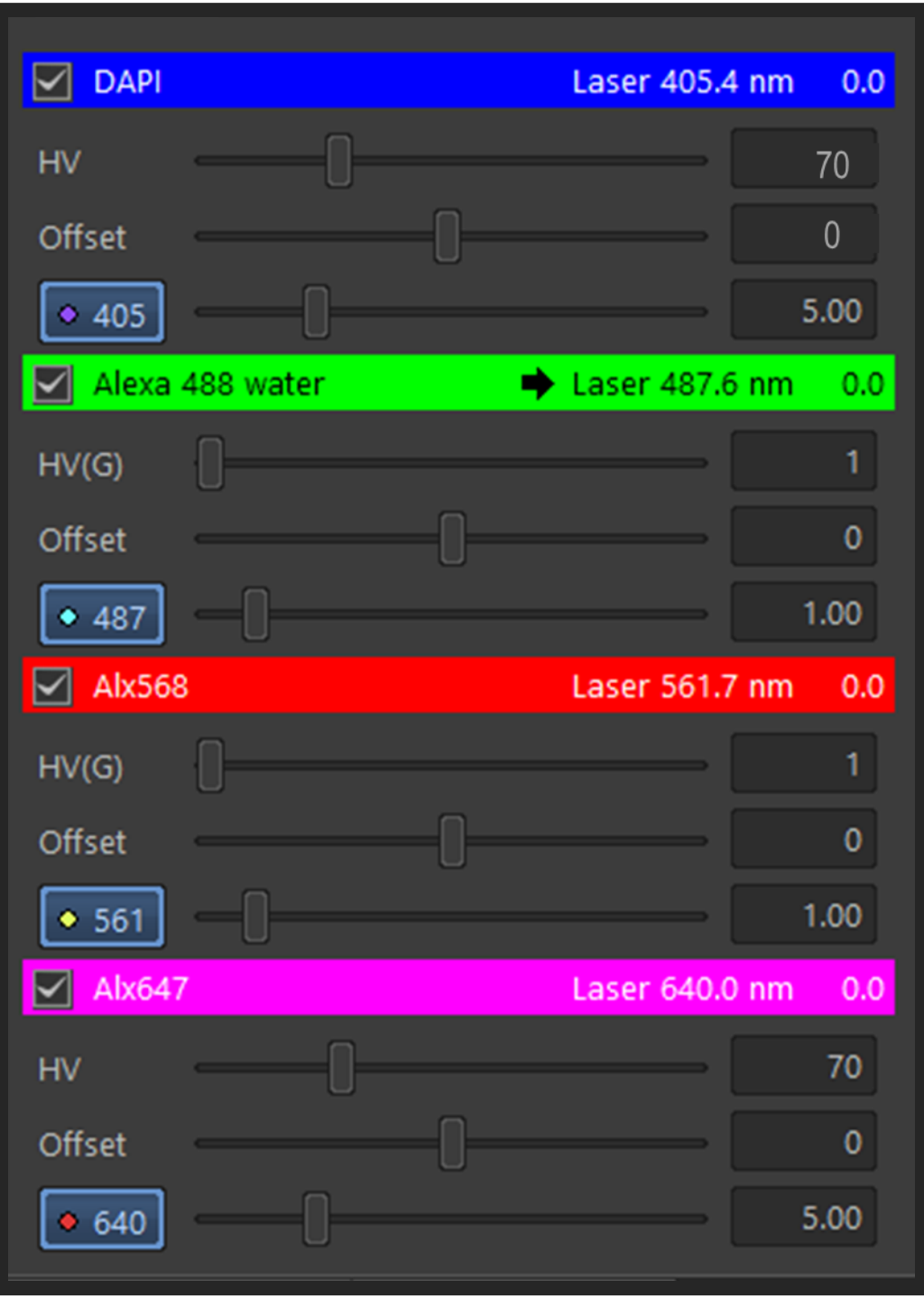


Set your Pinhole size to 1.2AU

Click

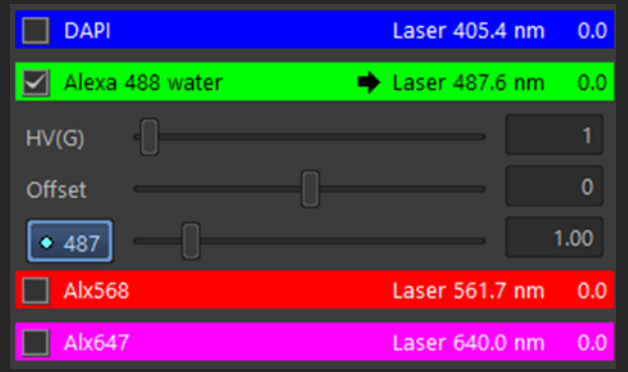
To set pinhole to

recommended starting point.



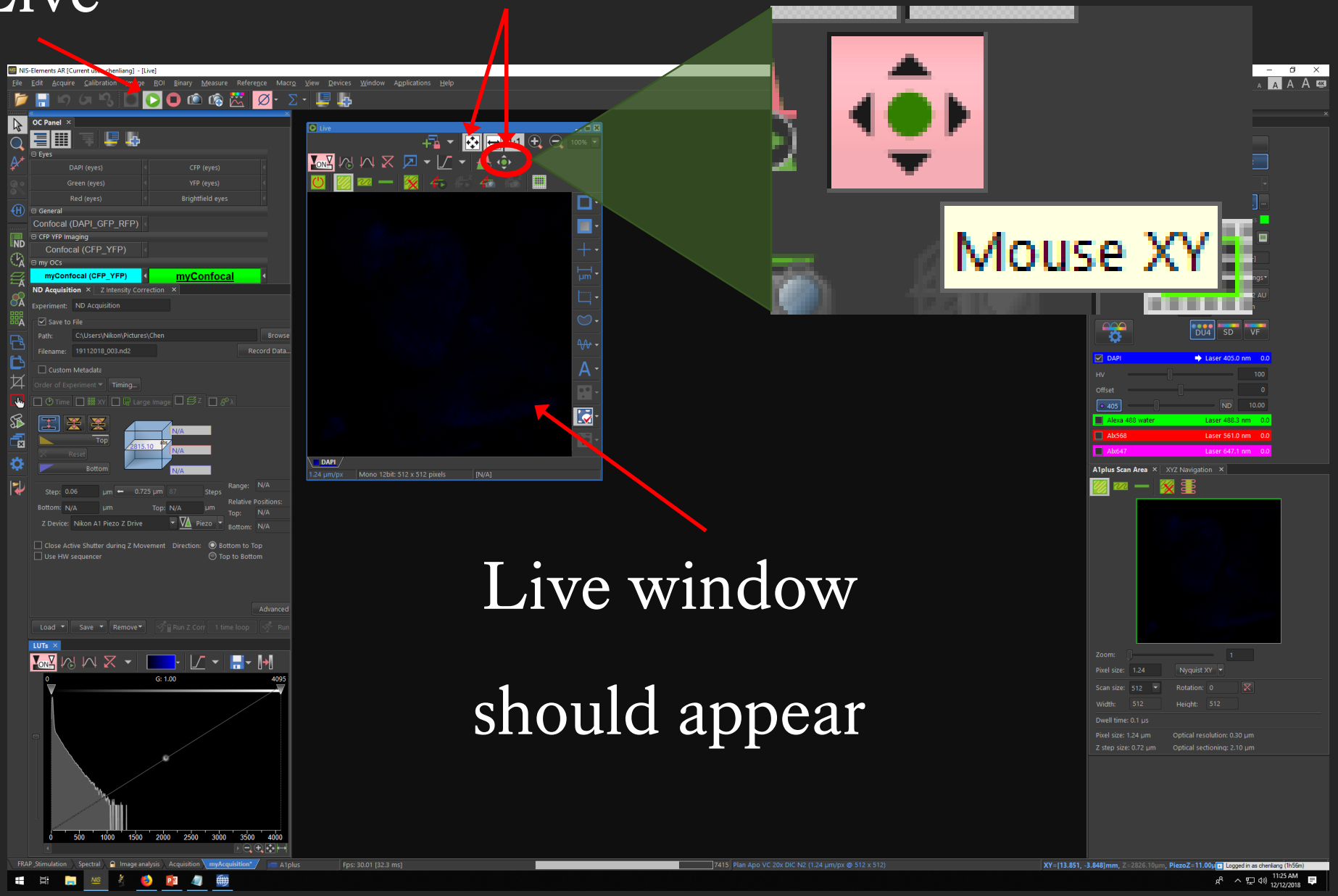
1) First input these initial setting numbers into your channel set up and update the **myConfocal** button. (This gives you a safe starting point)
2) Choose a single channel you want to see first.
3) Uncheck all the other channels but that one.

e.g. 488 channel



Click on Live

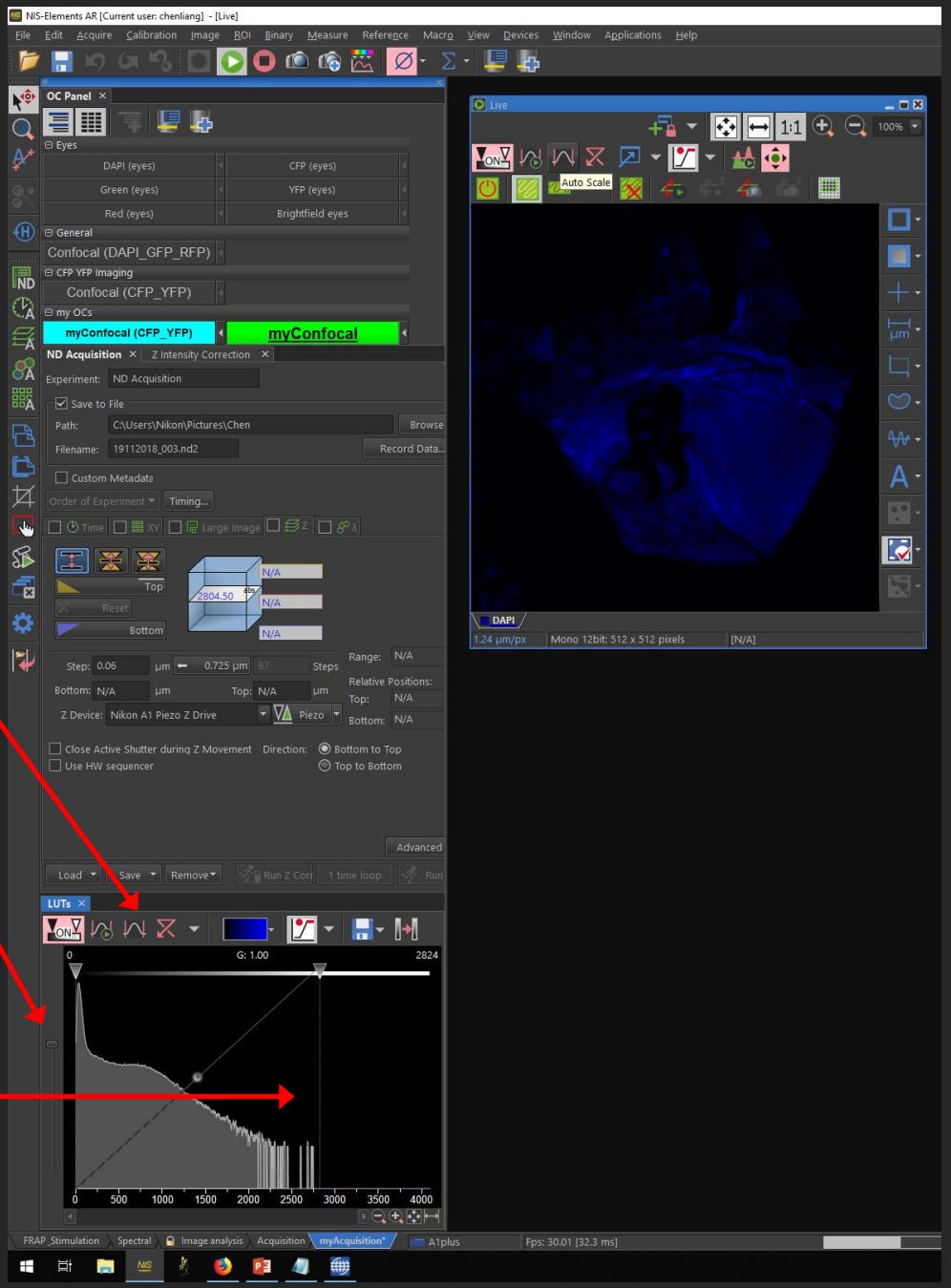
While on Live click on this and this to drag your field of view using your mouse



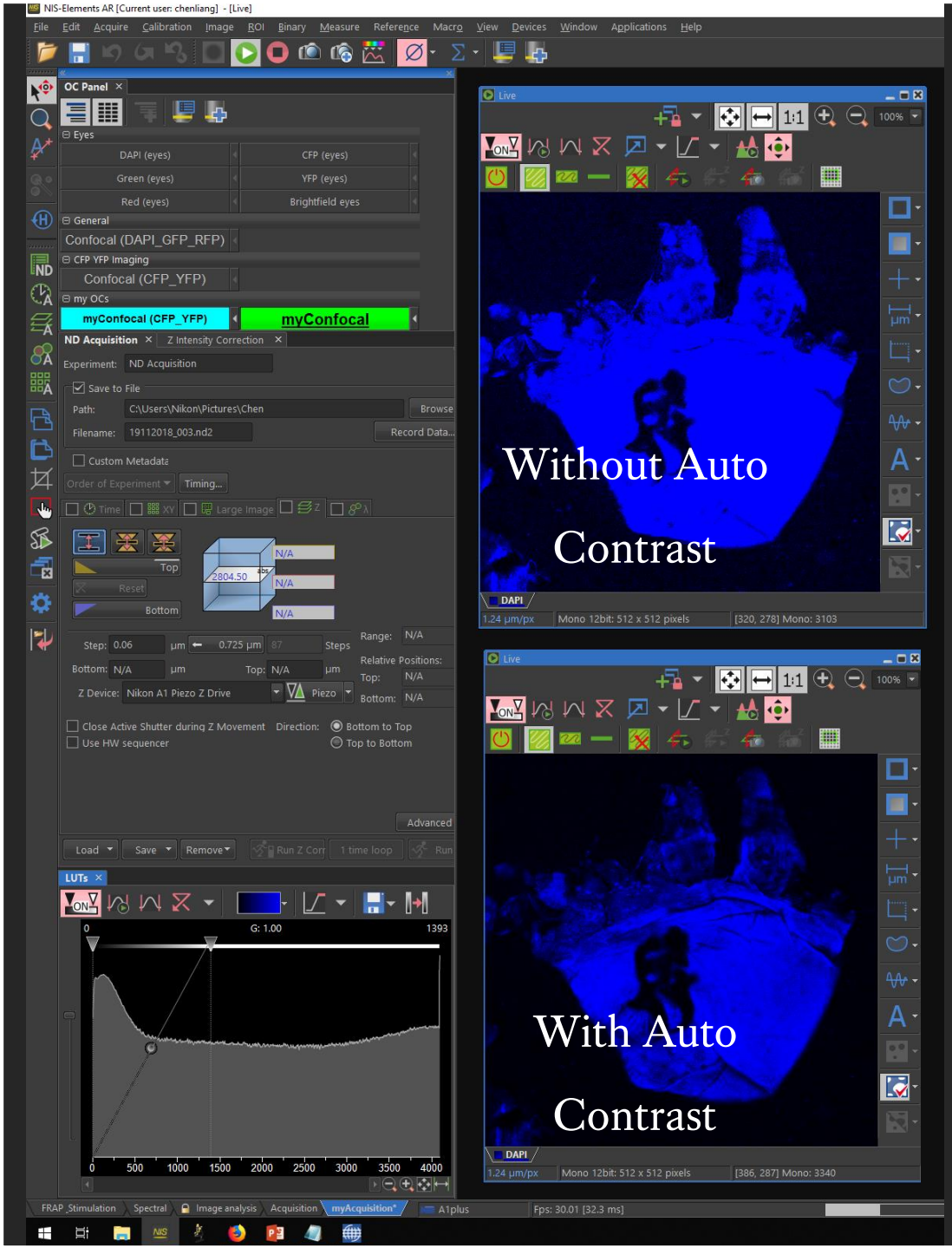
Live window should appear

To visualise your image…

1. Click on auto contrast
2. Slide this bar to the top
3. Drag the contrast line towards the left.



4. Starting to see something on the screen…
5. Now bring it into focus (hover mouse over image and use mouse wheel to change focus.)
6. Once in focus STOP scanning to preserve your sample.



While you are changing focus, your LUTs will change accordingly…

You may find you have too much or too little signal…

So now we move on to optimising your camera settings to get your signal just right…

LUTs

Additional Information Slides...

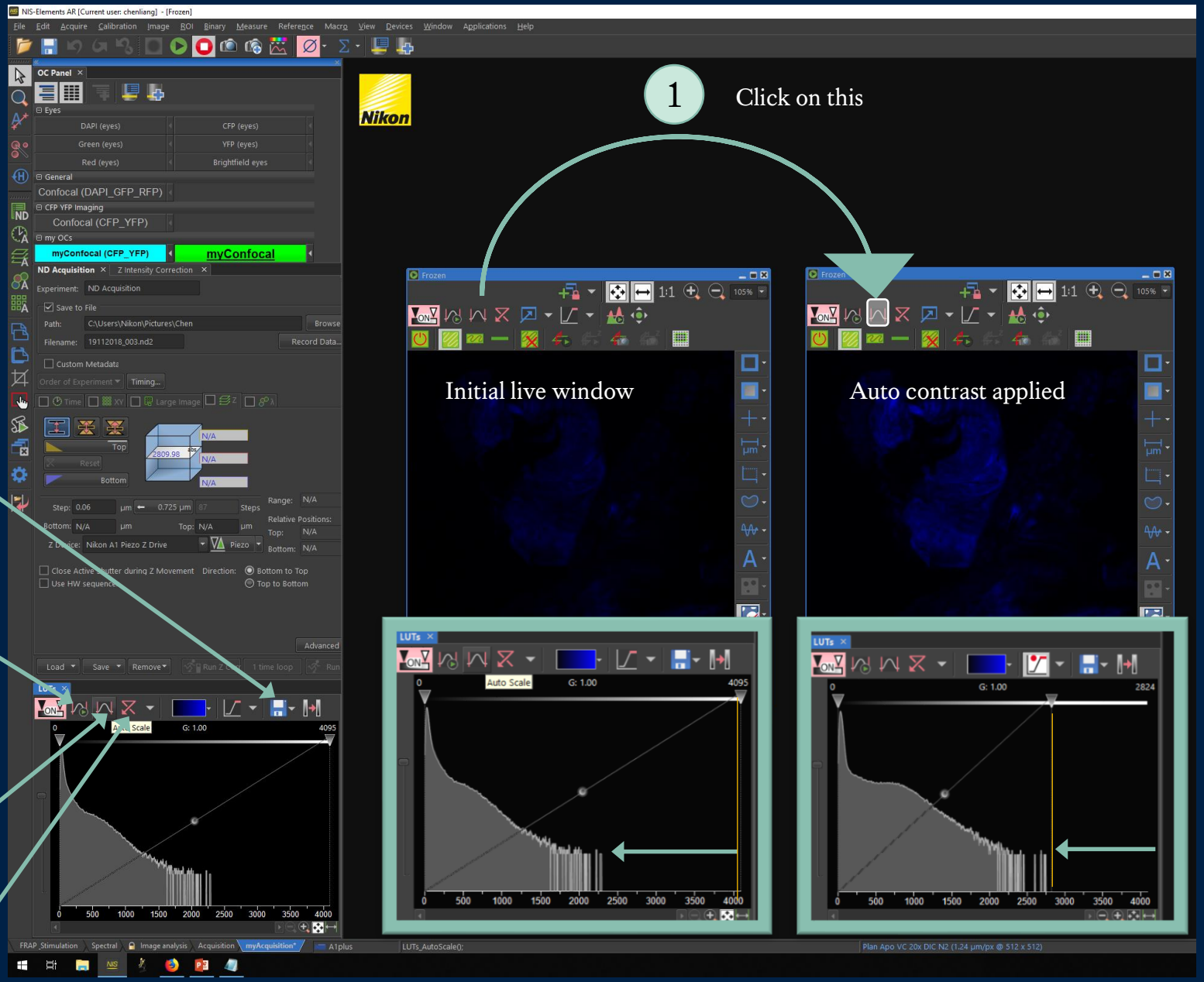
LUTs And Contrast

LUTs can be saved in a separate file and applied to other images.

Continuous auto contrast (there may be flickering while in live view)

Auto Contrast

Reset LUTs



LUTs explained.

- Changing LUTs to visualise your sample better does not change your raw data (signal intensity).
- This means when you open your image again in e.g. ImageJ, LUTs will not be applied.
- If you change LUTs before quantitative analysis, it is recommended to save the LUTs and apply it to all comparable images.

Why do we need to change the contrast?

The camera in this microscope captures shades from 0 to 4000, initially the LIVE window shows you all these different shades, but the signal from this sample only reach roughly 2500 therefore we only need to work within the 0 to 2500 range.

Auto contrast brings the contrast into a range for you to better visualise your sample.

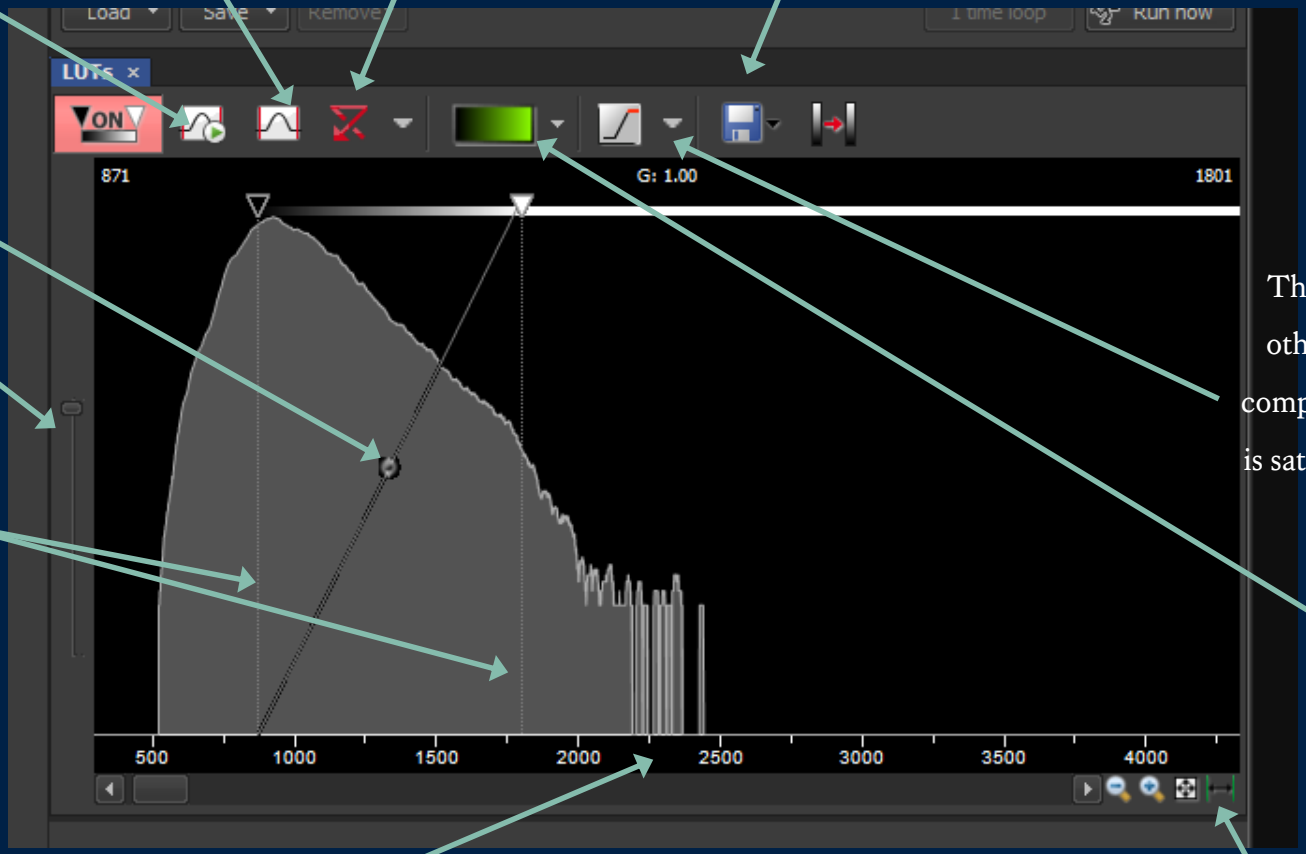
LUTs in more detail…

Continuous auto-contrast while in live view – can cause a flickering effect.

Auto-contrast

Delete all contrast adjustments

To keep analysis consistent, you can copy and paste LUTs across different captured images.



Makes dim targets brighter while keeping bright target the same.

Controls the Y axis log graph

Controls the X axis contrast, this doesn't change the captured raw data.

The spinning disk does not over saturate, but on other microscopes, this is for viewing saturation, complementary colour recommended. Once an area is saturated, it loses any intensity value information.

The camera is a black and white camera. You can assign any colour combination to your captured image.

Drag and change the threshold of this histogram for better visualisation.

X axis - relative to #-bit camera (e.g. 16-bit CCD camera gives 65,536 different intensity values, of which you should not go over 50,000). Y axis – log intensity scale

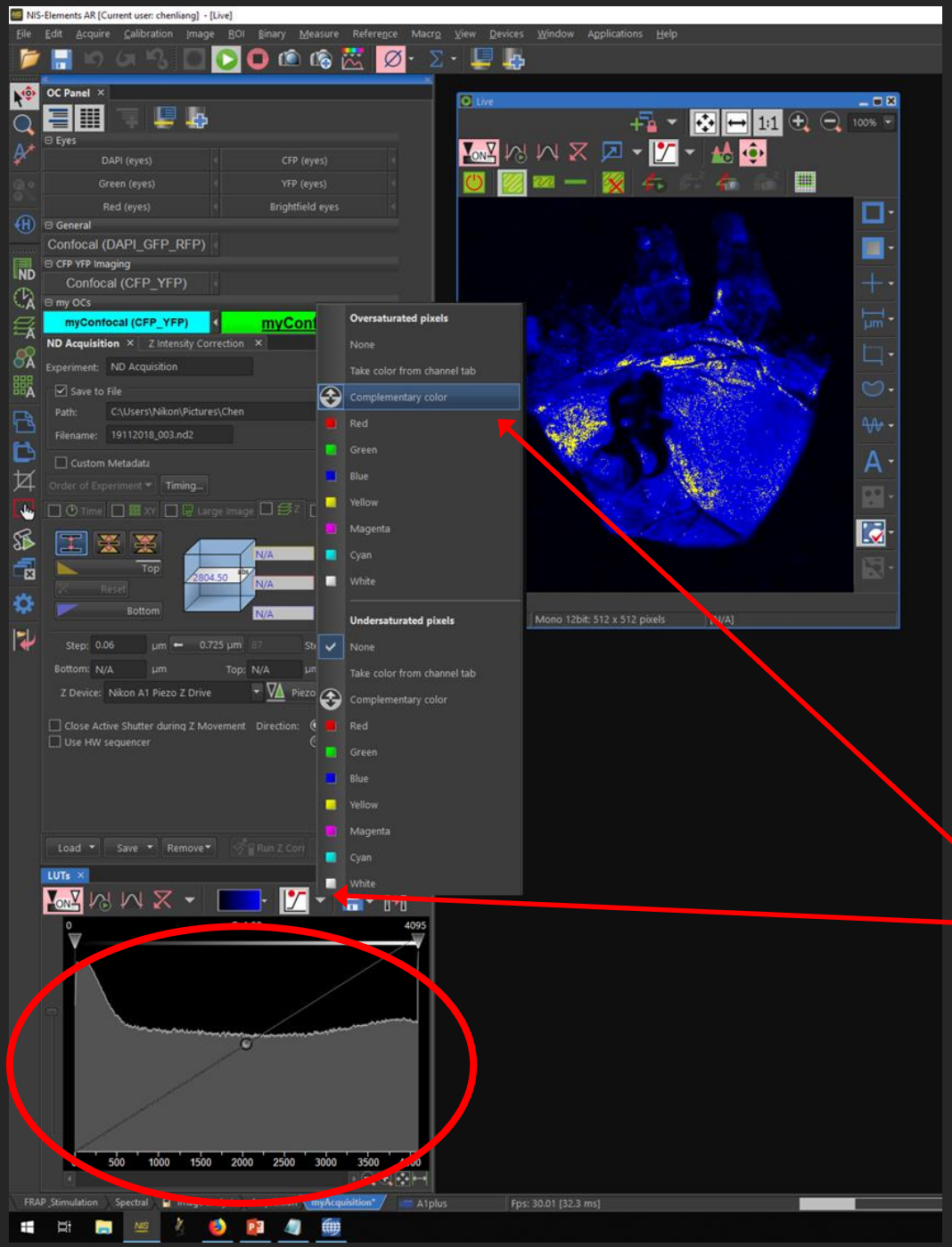
Fit the histogram to this space

STEP BY STEP INSTRUCTIONS

STEP 6

Optimising your camera settings

...when you have too much signal

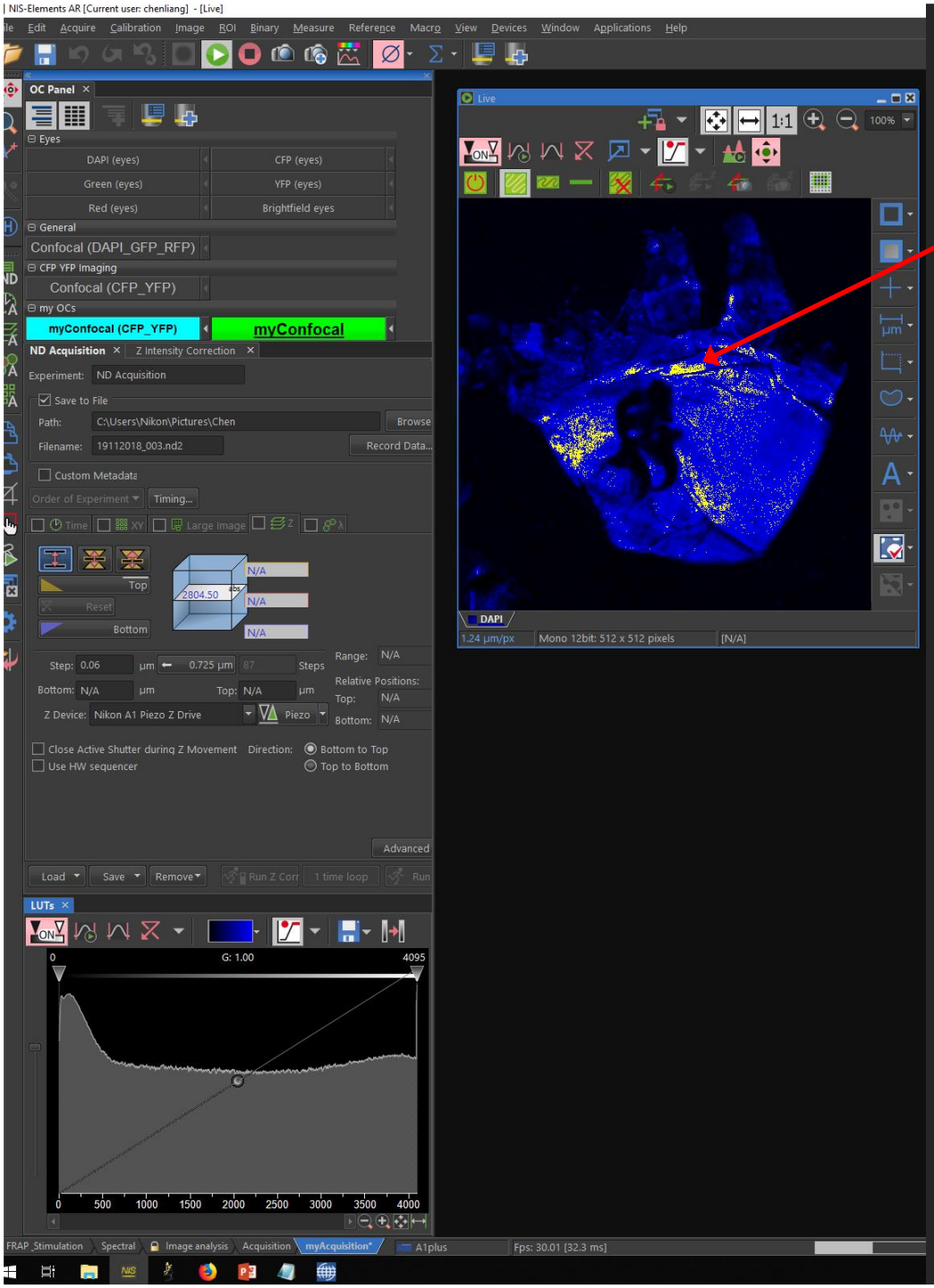


Do I need to optimise my settings?

How to tell if you have too much or not enough signal?

When your LUTs graph is filled up like this, you may be oversaturating your sample.

Turn on your oversaturating indicator by selecting complementary colour in the drop down.

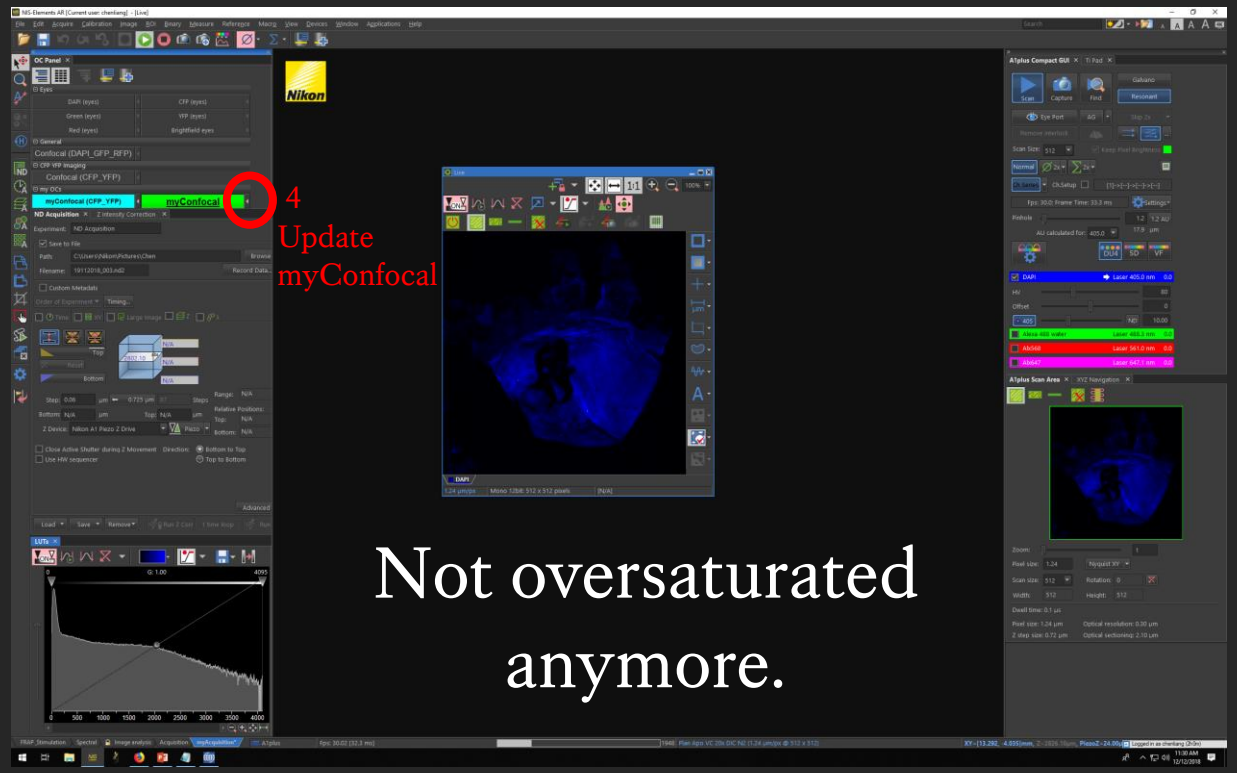


Oversaturation!

Oversaturation means the camera is picking up too much signal, and the camera can no longer determine the actual intensity of your signal, it just knows that sample is ‘bright’.

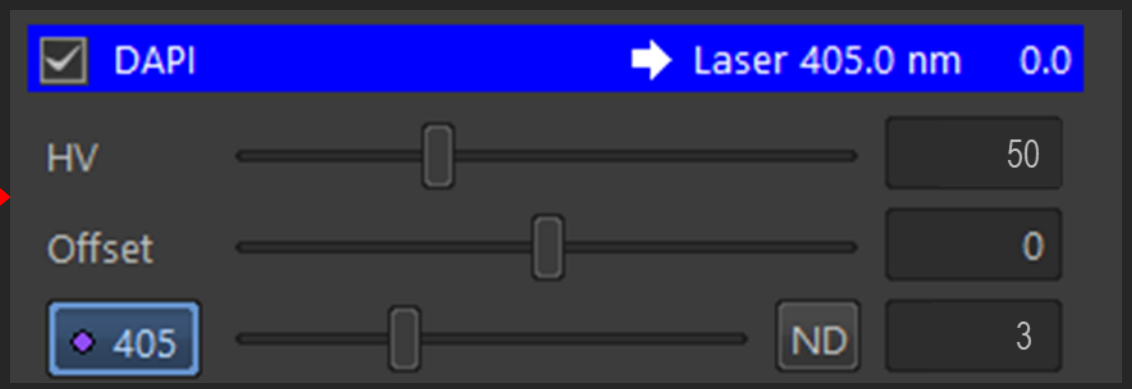
This can cause you problems during analysis, because you won’t have intensity information.

To fix oversaturation…



Not oversaturated anymore.

1. Reduce the Gain (HV) and laser power to reduce oversaturation.
2. Press ENTER to confirm change.
3. Go to live view again to check.
4. Update changes in myConfocal



STEP BY STEP INSTRUCTIONS

STEP 6

Optimising your camera settings

...when you don't have enough signal

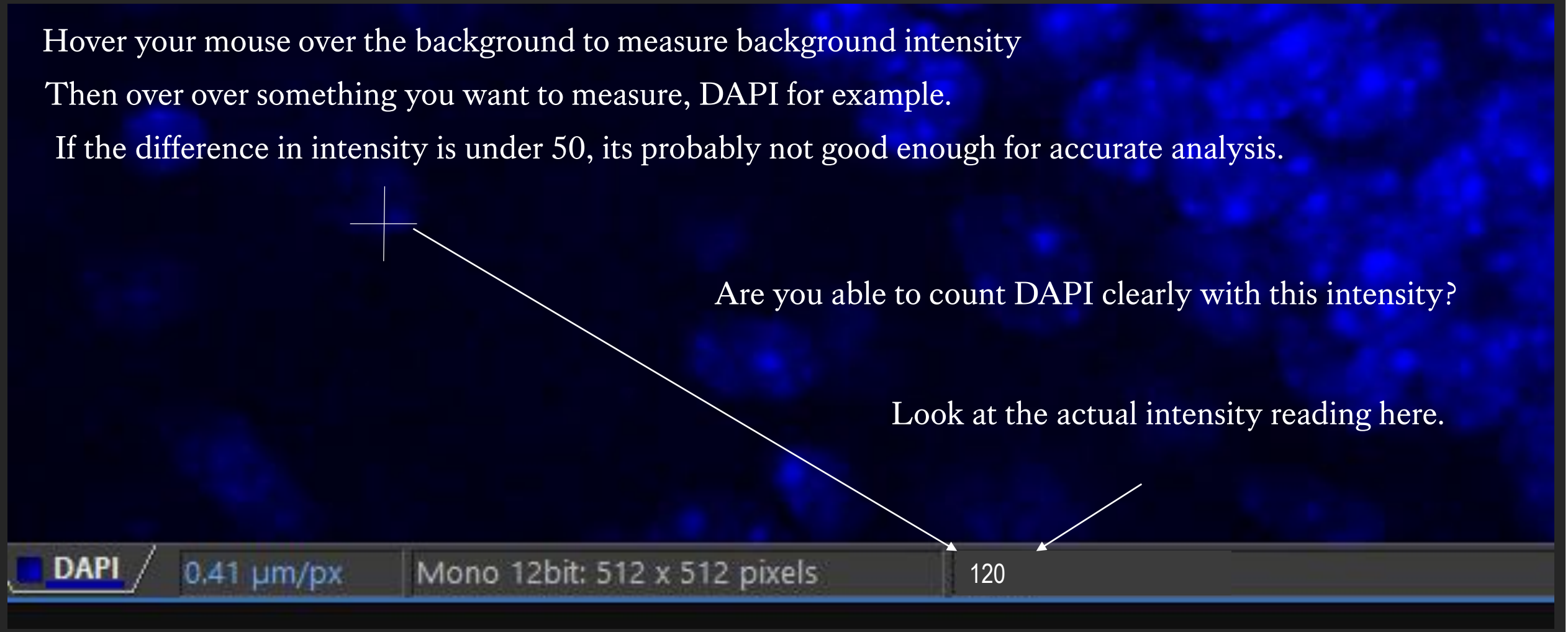
How much signal is enough signal?

That really depends on what you want to measure…

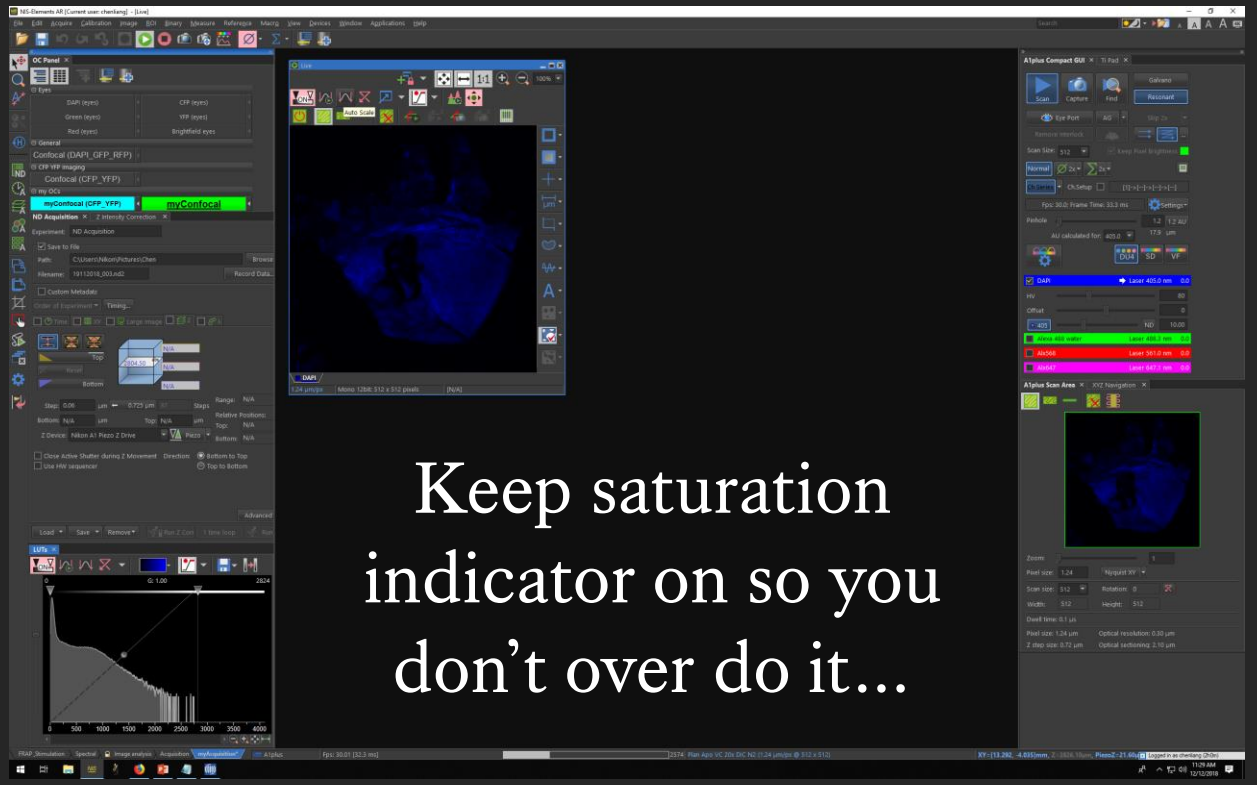
Hover your mouse over the background to measure background intensity

Then over over something you want to measure, DAPI for example.

If the difference in intensity is under 50, its probably not good enough for accurate analysis.

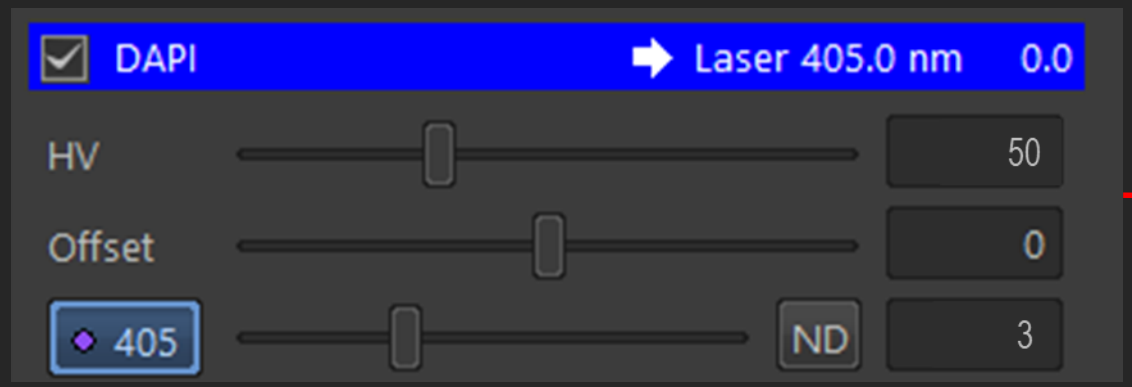


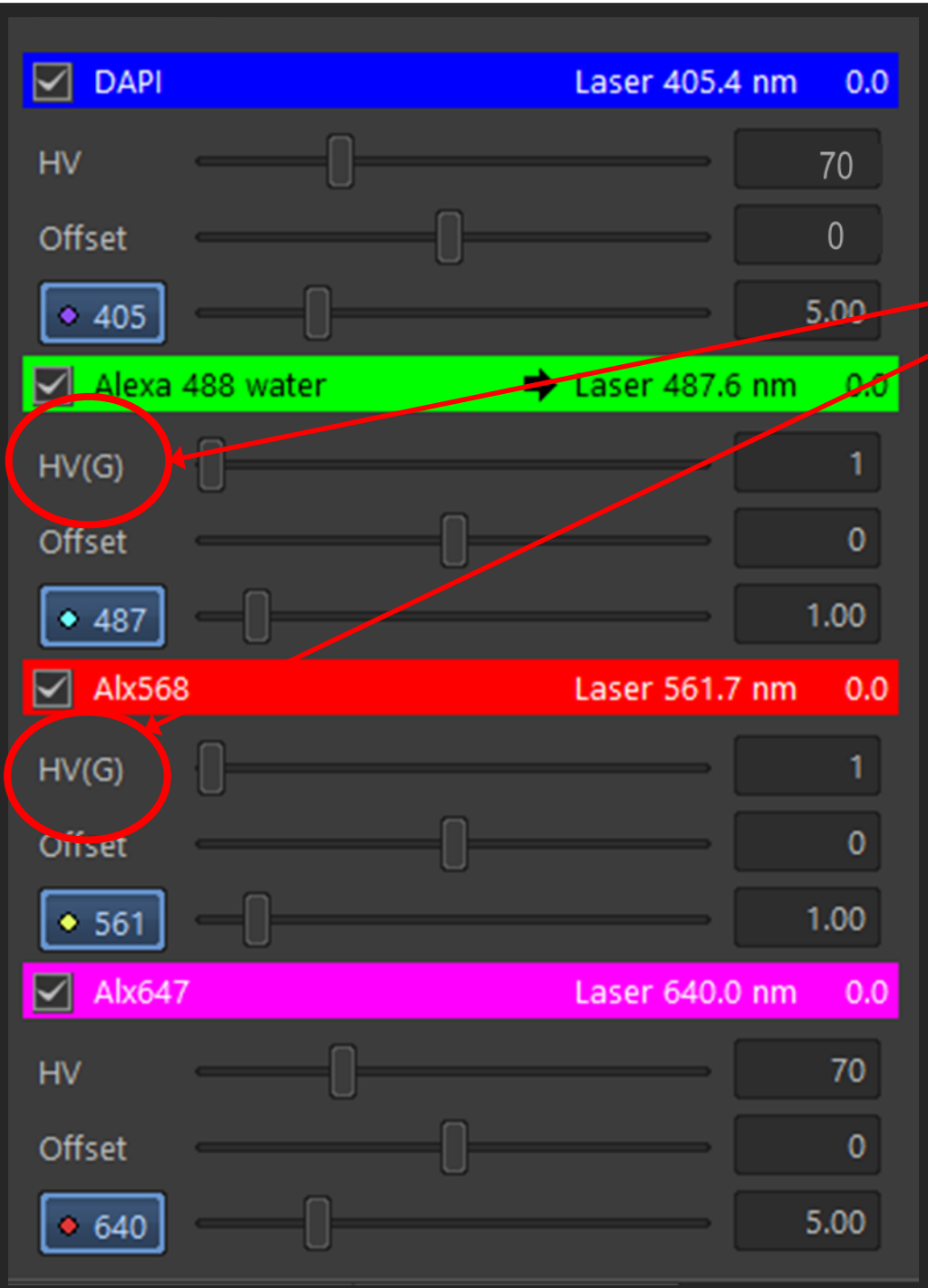
To increase signal…



Keep saturation indicator on so you don't over do it…

1. Increase Gain (HV) and laser power.
   (In SMALL increments for green and red channels)
2. Press ENTER to confirm change.
3. Go to live view again to check.
4. Update changes in myConfocal





How much to increase by?

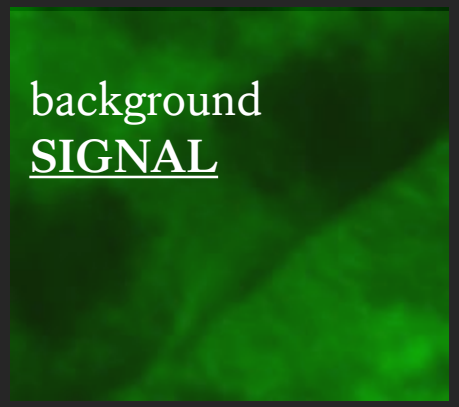
‘G’ means be gentle!

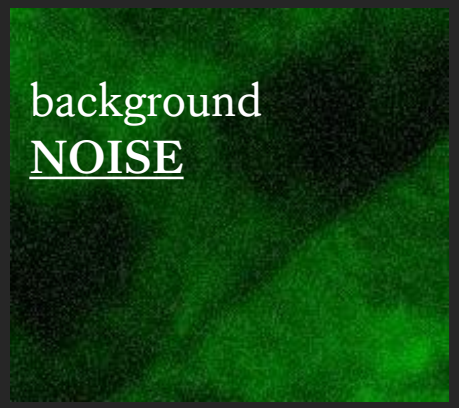
For green and red channels increase gain and laser power by no more than 5 each time.

DO NOT Increase HV above 50 !!!

For DAPI and far-red try changing by no more than 10 at a time.

STEP BY STEP INSTRUCTIONS





STEP 6

Optimising your camera settings

...when there is too much background SIGNAL

use OFFSET.

OFFSET…

helps to adjust the background voltage level to appear black on the computer screen, offset does this by **shifting** the entire amplitude of the signal without altering actual amplitude.

e.g. when offset -5 is applied to amplitude voltage 5 – 15 (figure 5a)

it will shift to voltage 0 – 10 (figure 5b)

but the actual difference between the voltage remains at 10 volts difference.

Gain can then be applied to amplify the amplitude of the voltage (figure 5c).

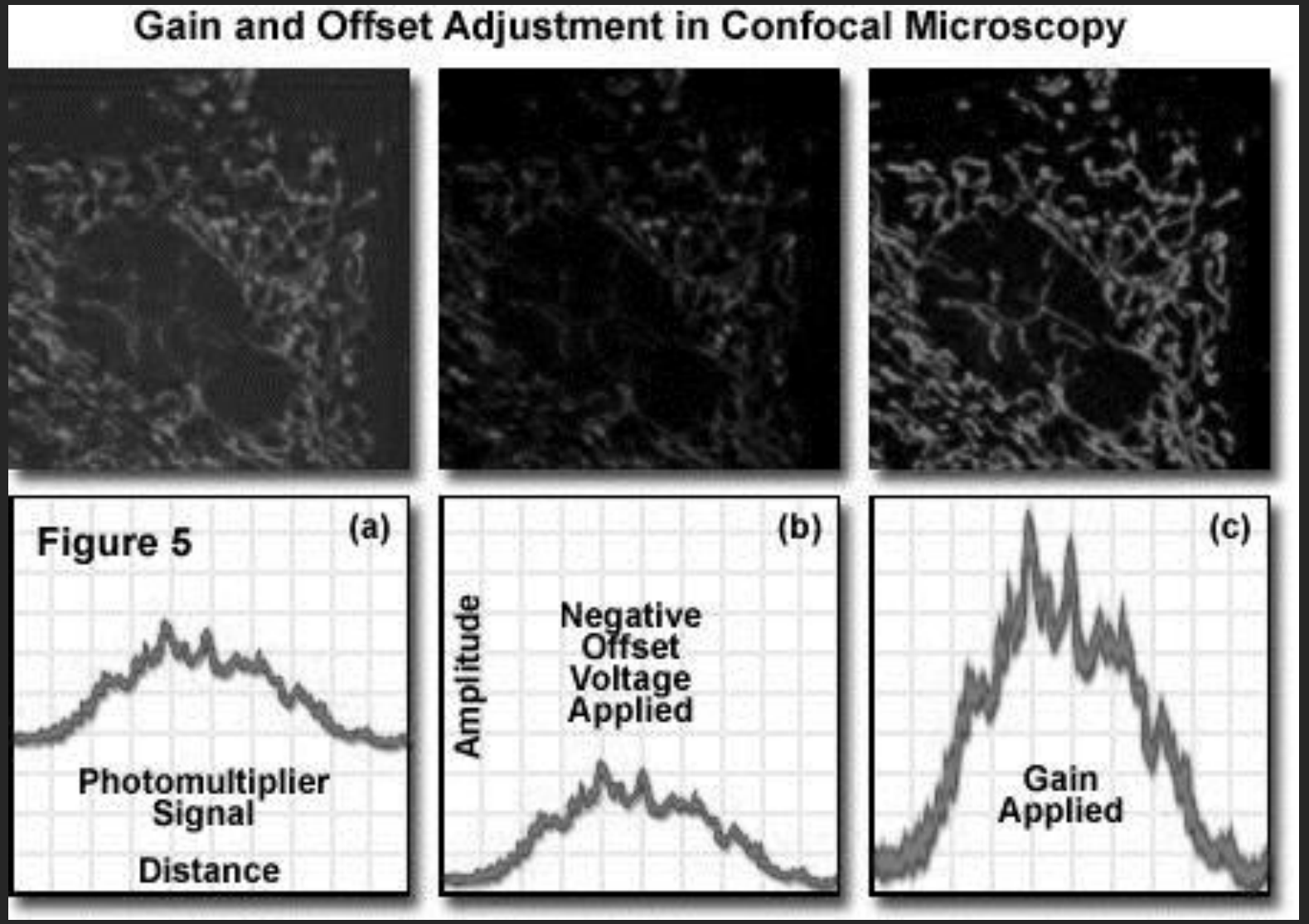
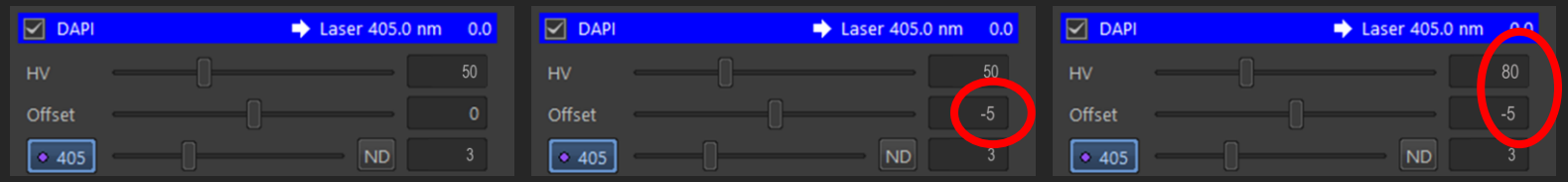
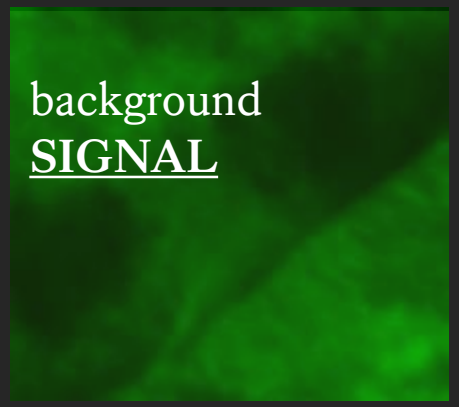
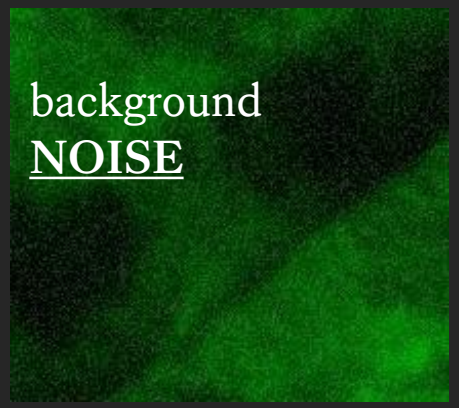


Image modified from: https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/confocal/pmtintro/



STEP BY STEP INSTRUCTIONS





STEP 6

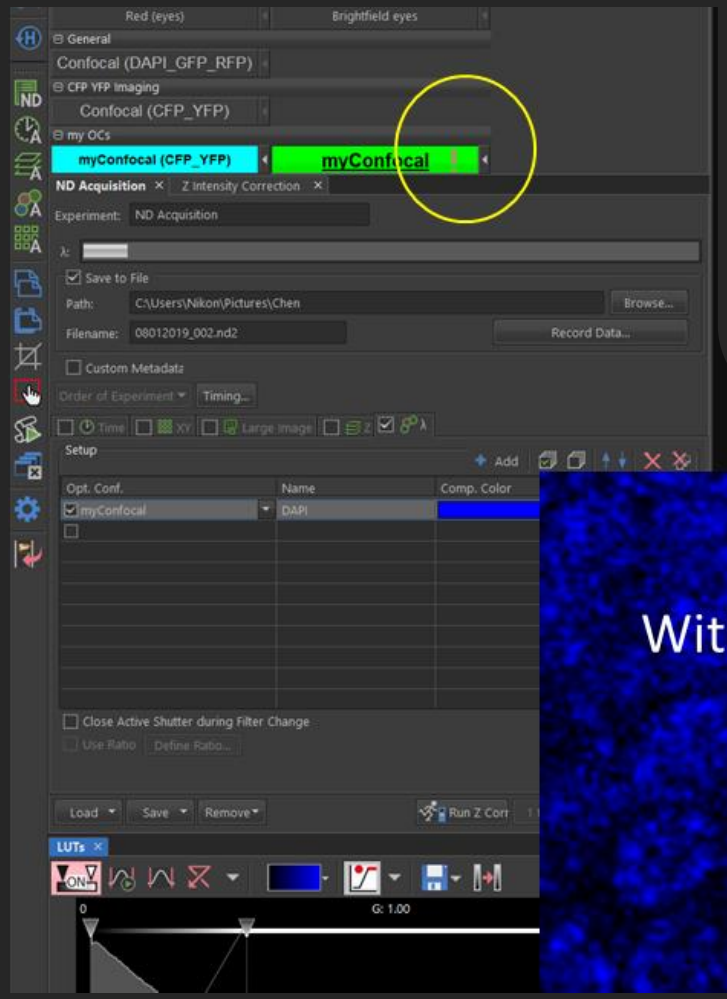
Optimising your camera settings

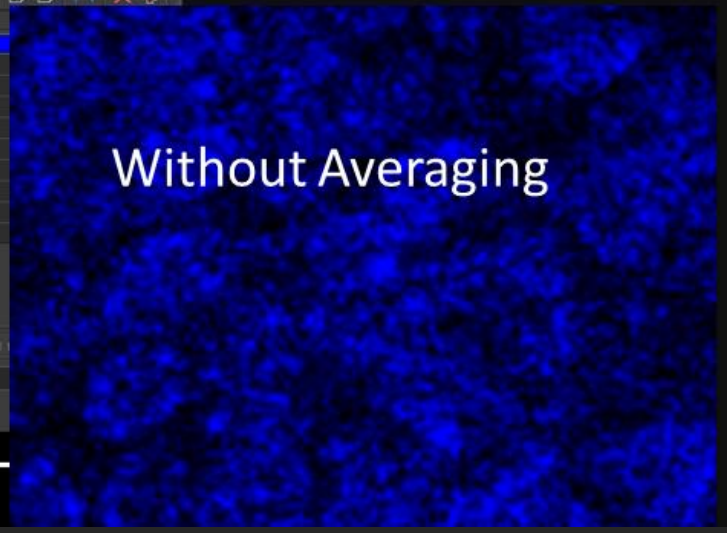
...when there is too much background **NOISE**

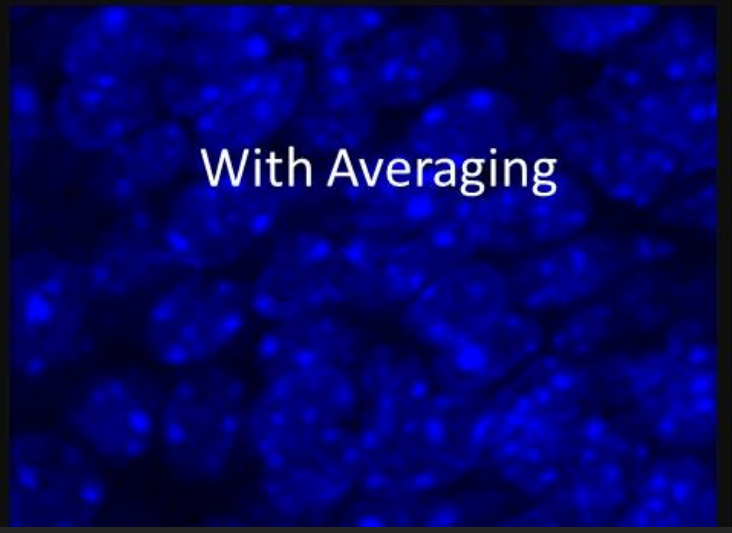
use **AVERAGING**.

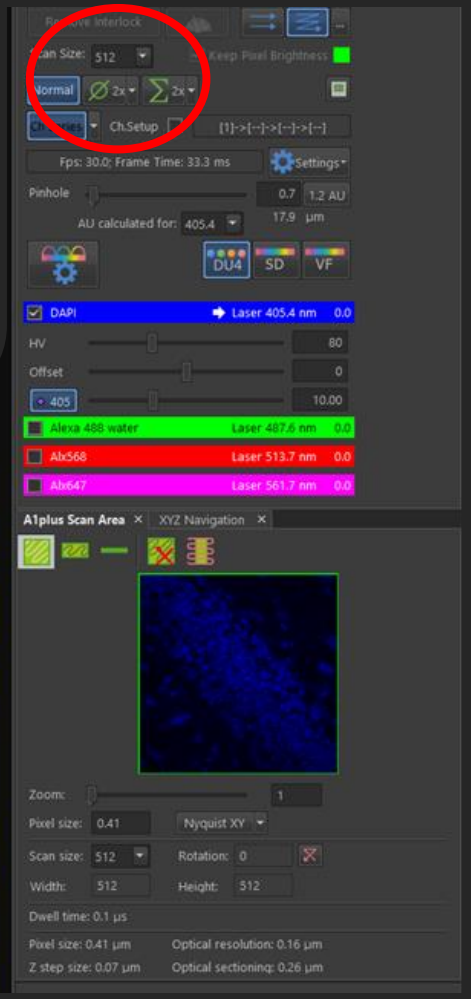
Averaging

- Takes multiple images and averages them out.
- Beware 8X averaging increase your imaging time by 8 folds.
- Remember to update myConfocal







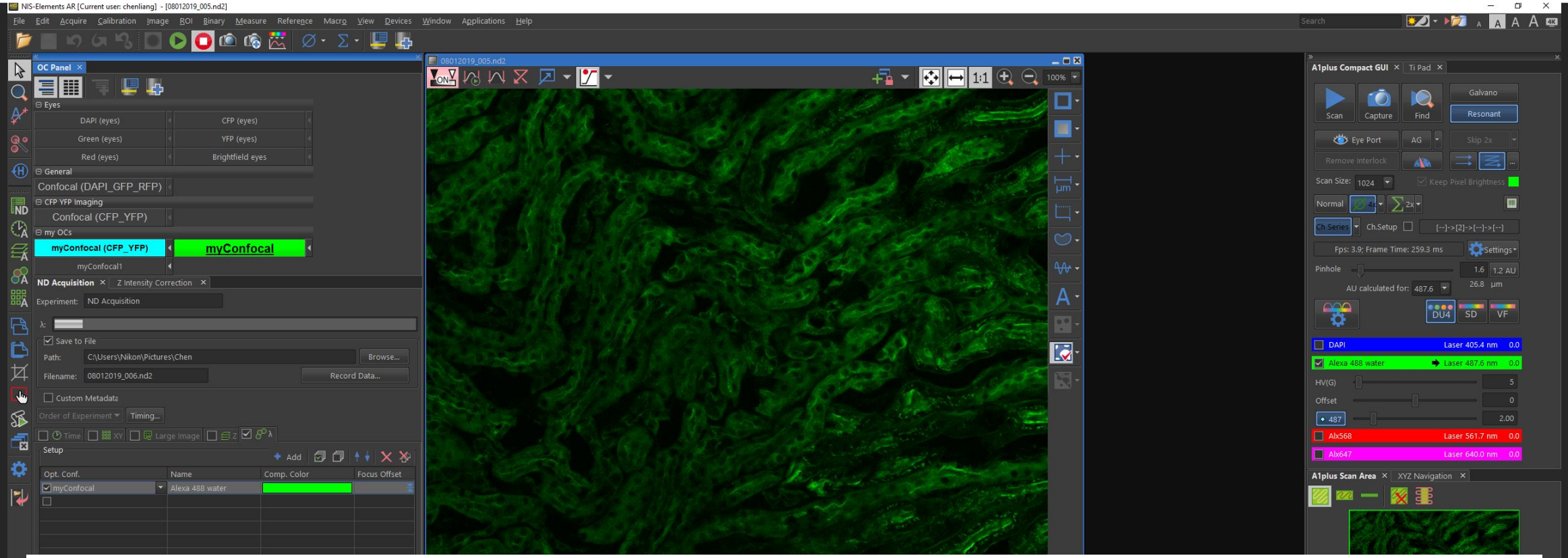


STEP BY STEP INSTRUCTIONS

STEP 6

Optimising your camera settings

...now do STEP 6 for every channel you want to use.



Optimise your camera settings for each channel you want to use during imaging.

1) While you are on STOP.
2) Check the box of another channel you want to use.
3) Uncheck any other selected channels
4) i.e. Check GFP channel then unselect DAPI in this case
5) Input the initial gain and laser power!!! If you haven't done so at the beginning of your session.
6) Be very GENTLE with your GREEN and RED channels.
7) Click on LIVE and continue to optimise by repeating STEP 4 for each channel you want to use.

A Helpful Table: Camera Settings – What Does It Do?

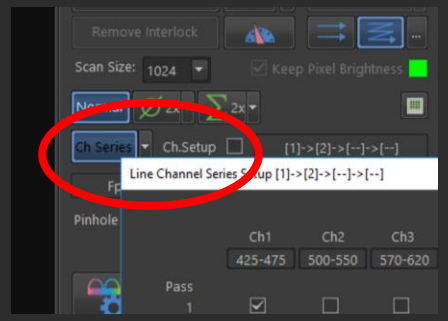
Camera Settings	Concept	Pro	Con	When To Change This...
Averaging	The camera takes multiple images and form an averaged image.	• Reduce noise	• Increase acquisition time drastically	• Increase when your image have lots of noise.
Binning	Combines the charges (signal) from adjacent pixels to form one “super” pixel.	• Faster read out • Increase signal to noise ratio	• Trades resolution for sensitivity	• Increase when there is very little signal from your sample, causing low intensity in your image.
Exposure	Longer exposure means the camera has longer to collect the emitted light. Ideally exposure time should be just below the saturation threshold.	• Detector receive more signal from your sample	• Phototoxicity • Bleaching • Fade • Acquisition time	• Increase when signal captured is not enough to give you the intensity level you need. • Decrease to preserve your sample.
Gain	Controls how much the signal is amplified before reaching the detection system. Increased gain makes photomultiplier tube plates more negatively charged = more amplification.	• Amplifies signal without causing bleaching or phototoxicity	• Gain increases noise	• Increase when signal is low and you have sensitive samples. • Decrease if you have too much noise.
Laser Power	The % power of a very photon-dense light source, focused in a very tight beam.	• Penetrates deeper into sample • Increase signal	• Bleaching • Heating • Harmful to sample	• Increase when you have thick samples or need more signal. • Decrease if you have sensitive sample, especially live samples.

STEP BY STEP INSTRUCTIONS

STEP 7

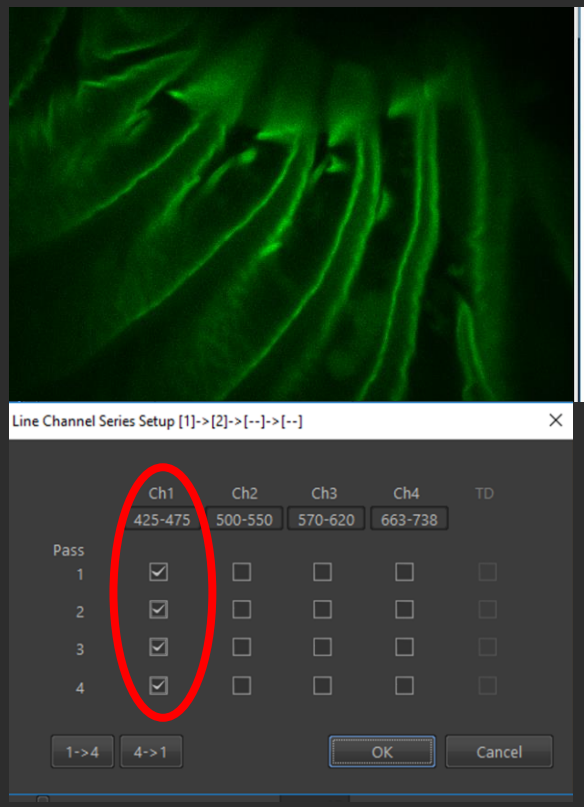
How to avoid bleed-through

Channel Series

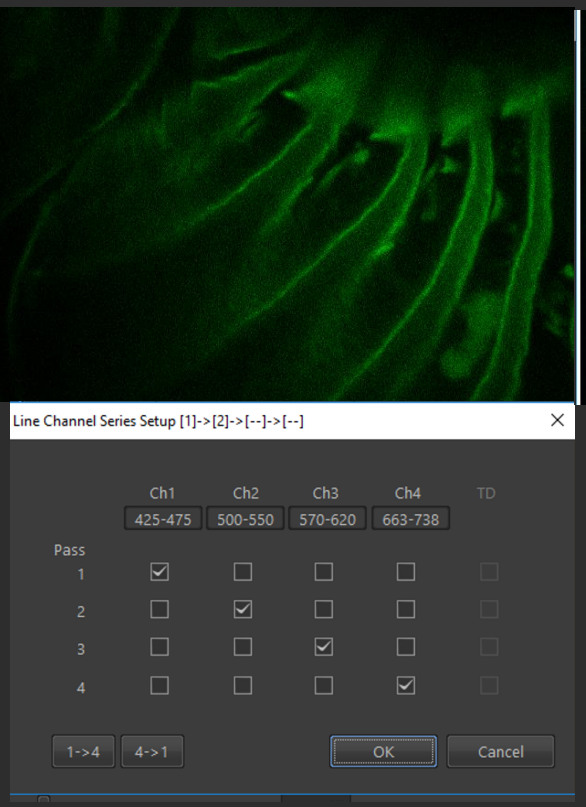


In your camera settings

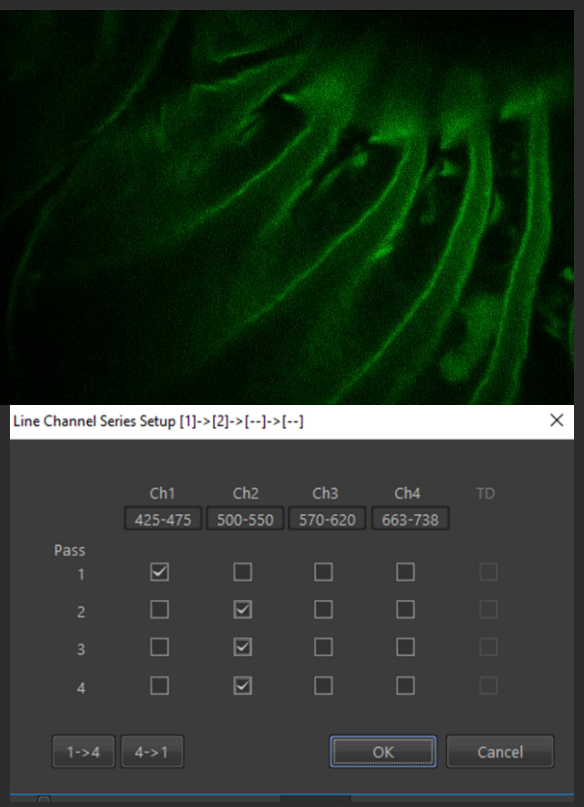
In this example DAPI signal is bleeding into the GFP channel.



This indicated you can scanning all channels at the same time, this is quick but can give false signal if bleed through occurs.



This indicated you are scanning each channel individually, this avoids bleed though but is a slower scanning method.



If DAPI is the only channel bleeding through, then you can scan DAPI alone and scan all other channels together to save time.

STEP BY STEP INSTRUCTIONS

STEP 8

Optimising your image resolution.

Up until now I've gone over how to change your camera settings to change the amount of signal intensity you can get. But the amount of signal and how much detail you can see within that signal are two different things.

Confocal microscopes can provide high resolution images, so here are some concepts to help you understand what actually changes image resolution and also what you need to change on the microscope and in this software to get the best possible image resolution.

But keep in mind, this is just a demonstration and when you come to do your own imaging, you'll have to consider what analysis you want to do and decide on how much image detail or resolution you actually need. Because the higher the resolution generally the more bleaching and the longer it takes to image.

To optimise your resolution you have 3 decisions to make...

1. Choosing a suitable lens and immersion medium

2. Set pinhole size (recommended: equal to or smaller than 1.2 AU)

3. Set sampling frequency (Zoom and Z stack step size)

Resolution

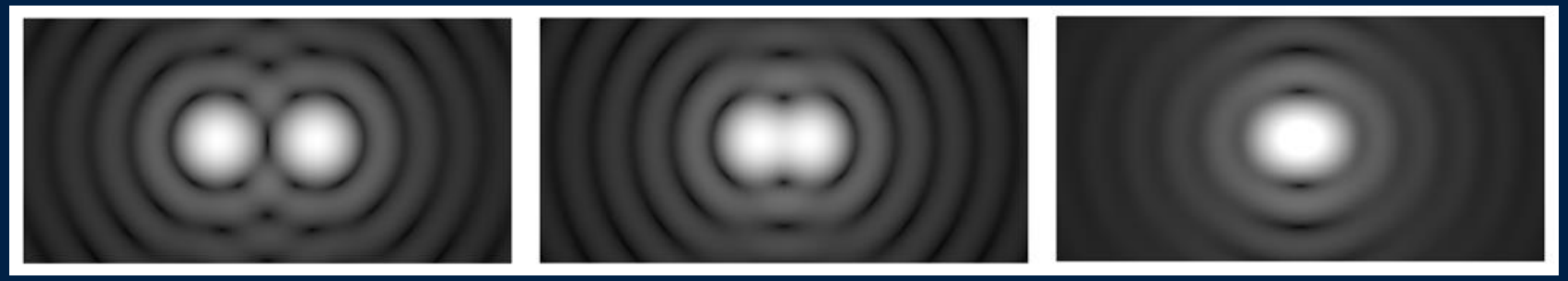
Additional Information Slides

Please read the following additional information slides to familiarised yourself with these microscope concepts.

Resolution is…

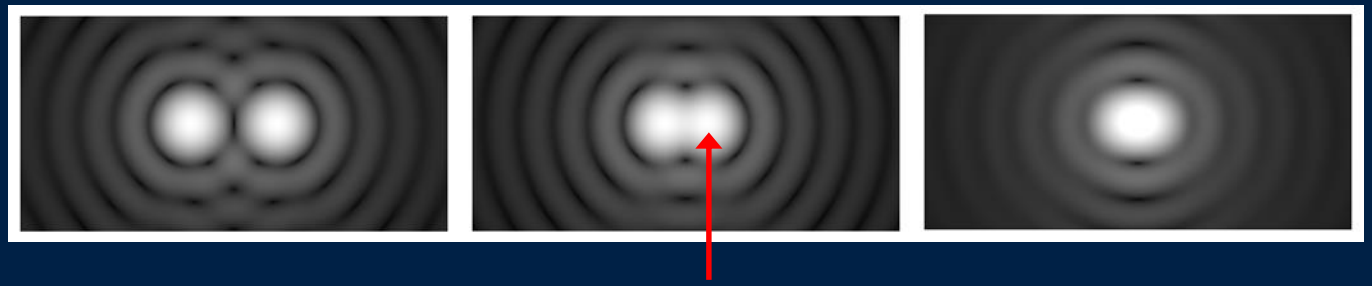
The ability to distinguish separate objects.

The highest resolution you can achieve is called the resolution limit, basically it's as close as 2 objects can get and still be visualised as 2 separate objects.



This limit exists because…

Light coming from a very small point spreads out in Airy Disks



When another object overlaps the first peak intensity ring, the drop in intensity between the two objects is enough for us to categorised them as 2 different objects.

$$\text{Lateral (XY) Resolution} = 0.61\,\lambda / \text{NA}$$

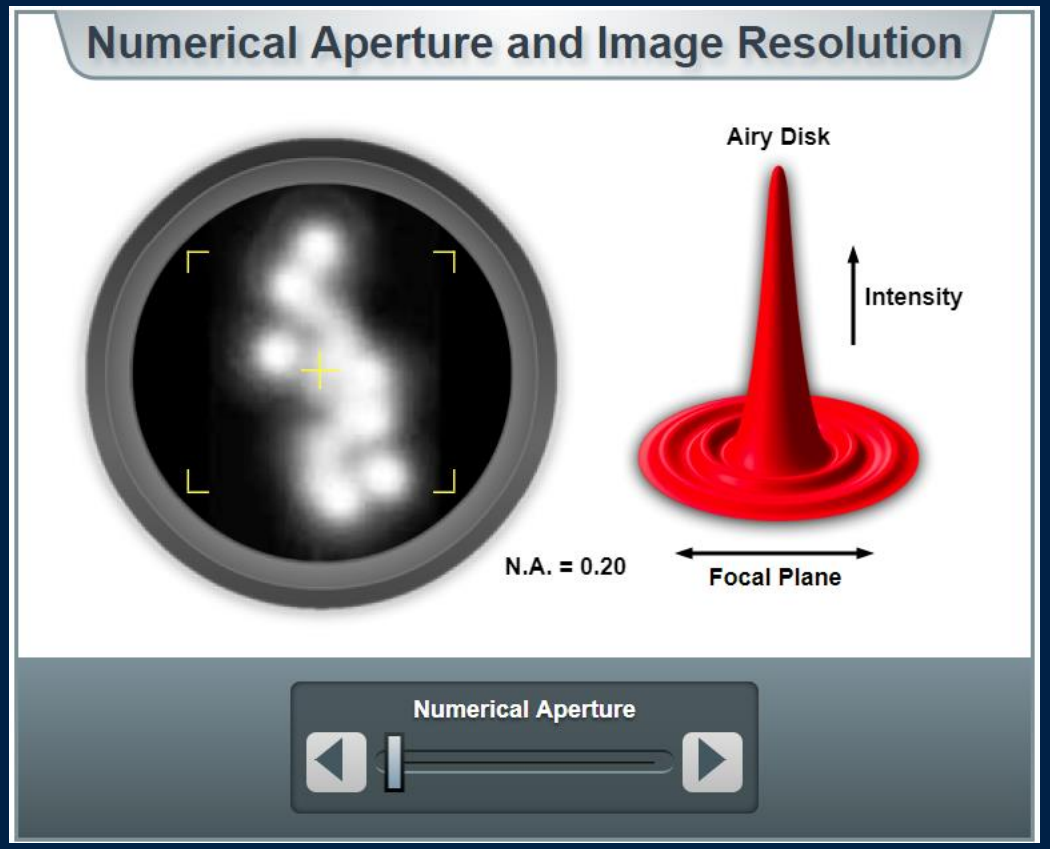
$$\text{Axial (XZ) Resolution} = 2\,n\,\lambda / \text{NA}^2$$

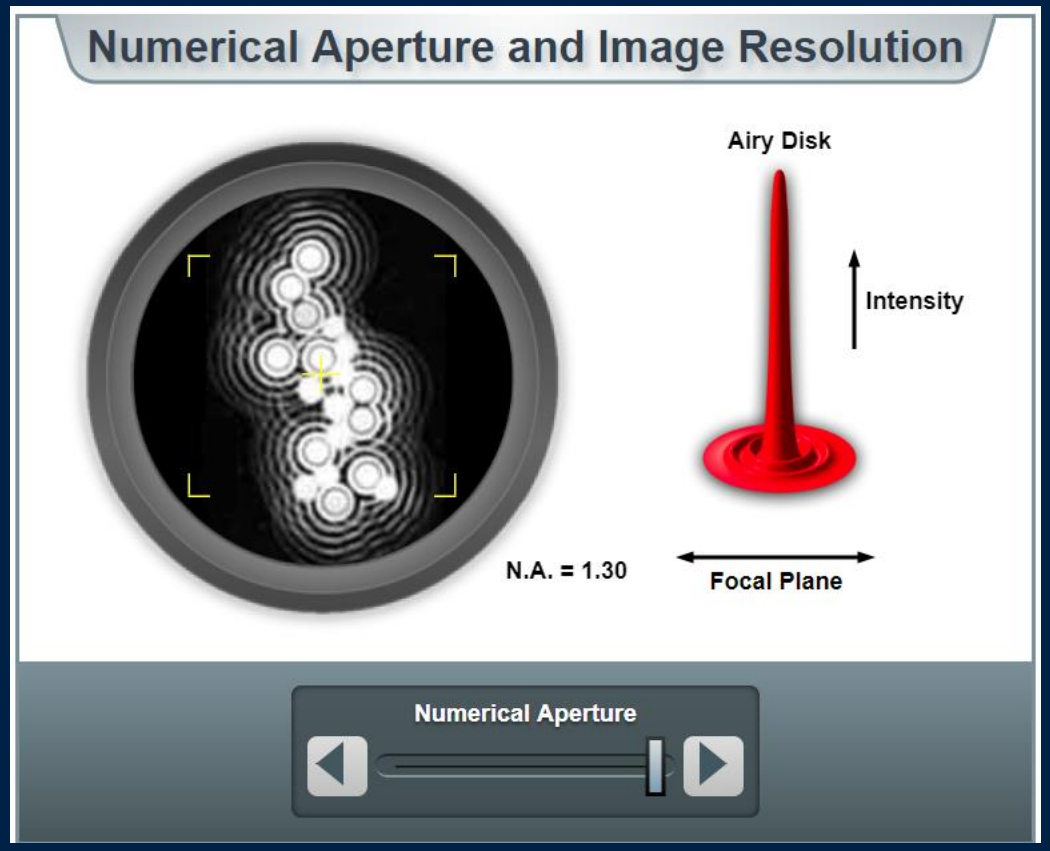
Rayleigh Criterion

Lateral (XY) Resolution = $0.61\ \lambda / NA$

Axial (XZ) Resolution = $2\ n\ \lambda / NA^2$

E.g.
0.61 x 480nm / 0.8 = 366nm
0.61 x 480nm / 1.4 = 209nm



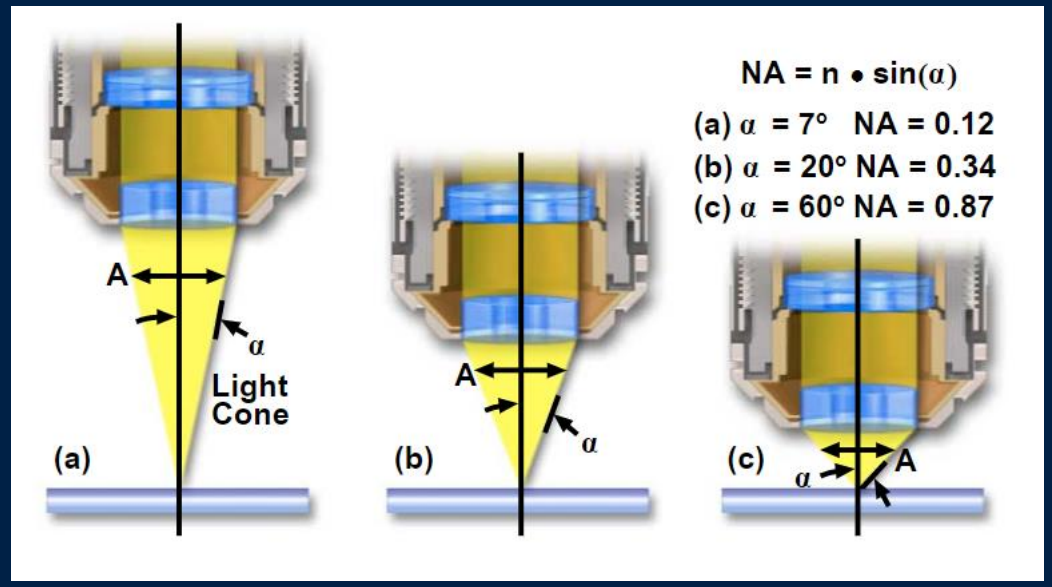


Changing the Numerical Aperture changes resolution!

Numerical aperture (lens dependent)

NA is a measure of a lens' ability to gather light and detail.

Different lenses have different Numerical apertures



The closer the lens is to your sample and the better matched your immersion medium means you'll have a higher NA and can gather more detail from your sample.

Higher NA = better resolution

Numerical Aperture (NA) = n × sin (α)

The immersion medium's refractive index:

Air = 1

Water = 1.33

Glycerine = 1.47

Immersion Oil = 1.51 = Glass (coverslip)

Angle between lens and sample.

Z Resolution = $2\ n\ \lambda\ /\ NA^2$ is affected further by refractive index.

Optimise your image resolution by…

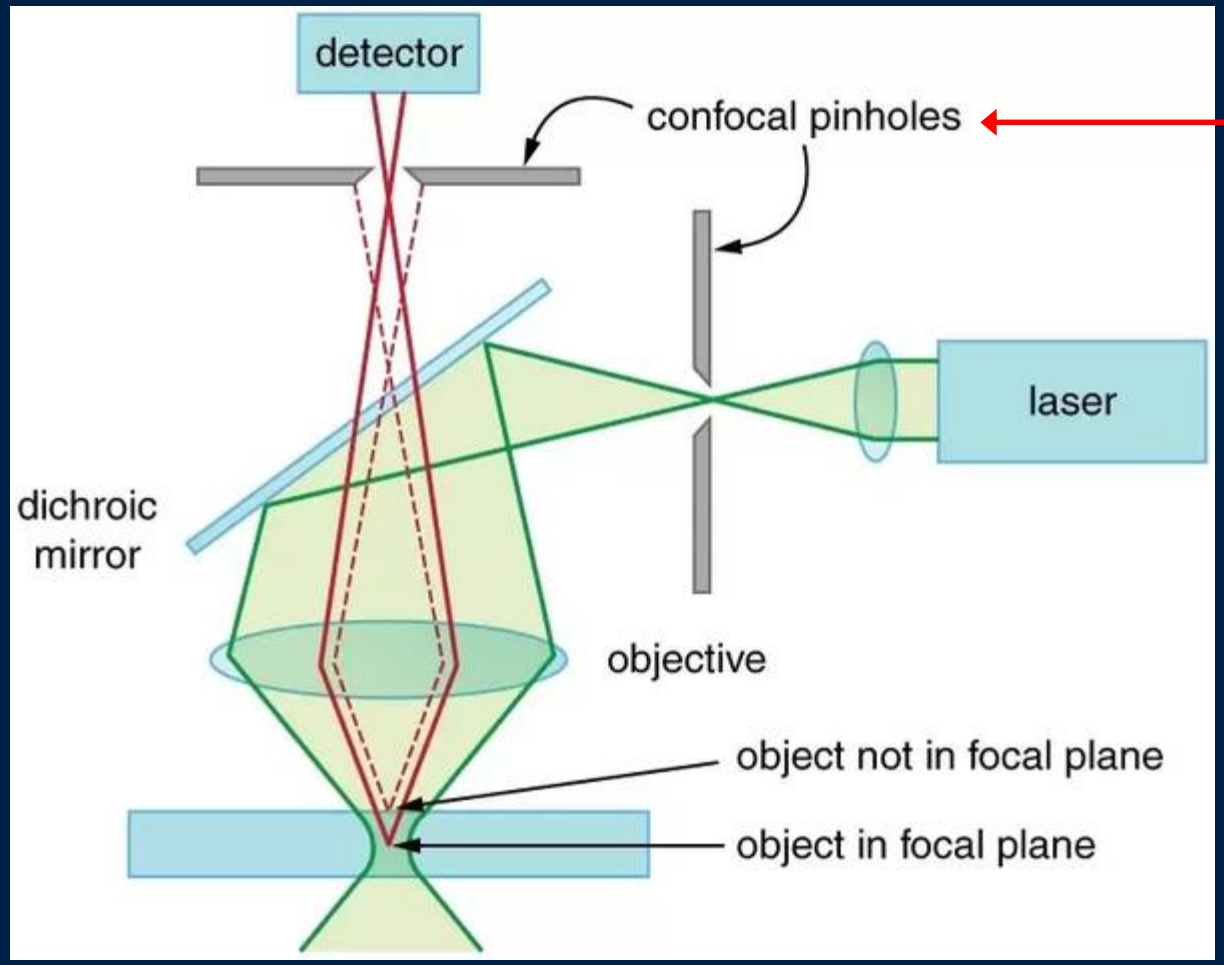
1. Choosing a suitable lens and imaging medium

2. Set pinhole size (recommended: equal to or smaller than 1.2 AU)

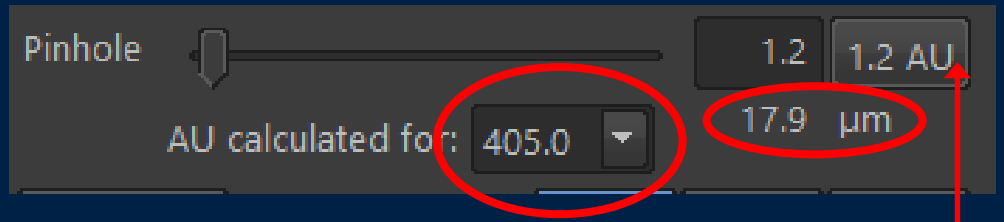
3. Set sampling frequency (Zoom and Z stack step size)

Airy disk and numerical aperture affect all microscope machines

so what makes confocal systems higher resolution than widefield?



Pinholes : minimises the detection of out of focus signal.



Recommended Pinhole size is 1.2AU

Be aware 1.2AU for different wavelength results in different pinhole sizes.

Optimise your image resolution by…

1. Choosing a suitable lens and imaging medium

2. Set pinhole size (recommended: equal to or smaller than 1.2 AU)

3. Set sampling frequency (Zoom and Z stack step size)

After optimizing the NA and pinhole size, the next thing to consider is how to actually take the image…

You can have the most powerful lens and smallest pinhole but if you don’t set up sampling frequency correctly, then you won’t capture enough detail to end up with a high resolution image.

Sampling frequency
(pixel and step size dependent)

- When your specimen emit fluorescent light, it is picked up by the camera and gets translated into digital pixels.

- When we decide on sampling frequency we are telling the microscope how many pixels we want to record within a fixed distance.

- For optimal resolution, that fixed distance would be the smallest resolution limit you can achieve (lens and pinhole).

- If you only sample once, that is unreliable, like if you ran an experiment without any repeats.

- So you need to sample more than once, within your resolution limit.

A standard sampling frequency can be worked out using the Nyquist Limit (N) equation.

$$N = 0.3 \lambda / NA$$

Most researchers uses the Nyquist limit equation to works out a sampling frequency of *2.2*, which means you need to sample *2.2* times within your resolution limit ($0.61 \lambda / NA$) to be sure the signal you are sampling is real.

So how do you apply this *2.2* times sampling frequency…

So how do you apply this 2.2 times sampling frequency…

Lateral (XY) Resolution

$= 0.61 \lambda / NA$

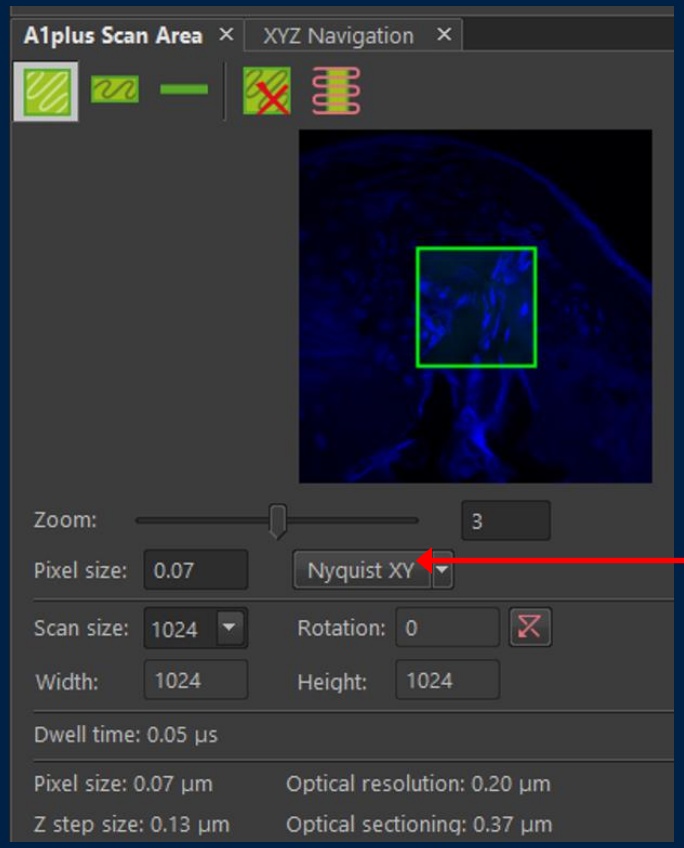
Scan area / Zoom

Axial (XZ) Resolution

$= 2 n \lambda / NA^2$

Z stack step size

Lateral (XY) sampling frequency can be changed using the ZOOM option.



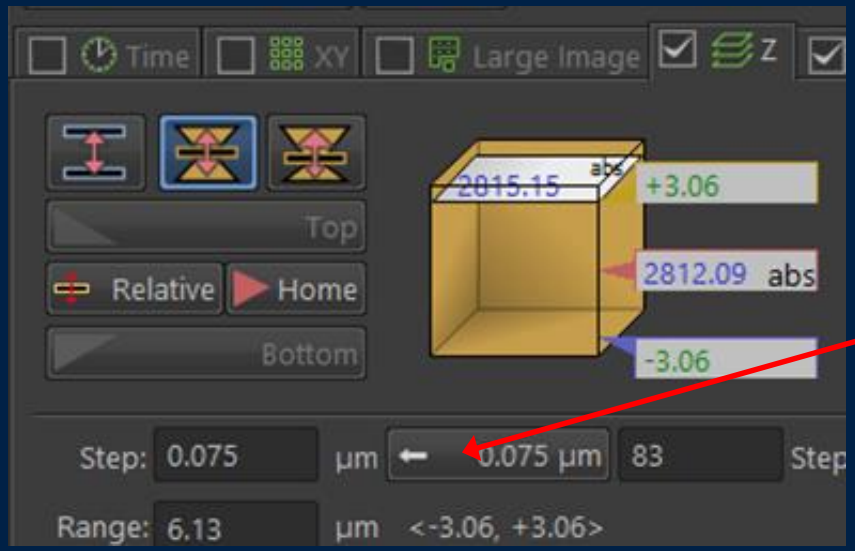
The software knows the lens and pinhole size you are using, so it calculates your resolution limit and Nyquist limit for you.

All you need to do is click on Nyquist XY for recommended pixel size. The correct Zoom will be automatically applied.

You are at your resolution limit, so even if you zoom in even further, you won't be able to distinguish any more detail from your specimen.

Axial (Z) sampling frequency can be changed using the Z stack step size option.

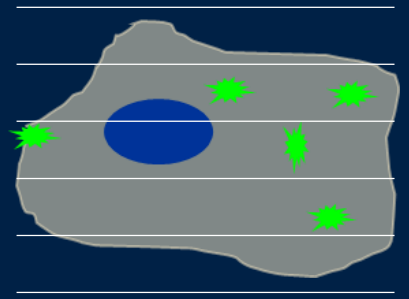
You can apply this during your acquisitions set up later…



The software again calculated your resolution limit for you. All you need to do is click here to apply the recommended step size during Z stacking.

You don’t have to use the recommended step size…

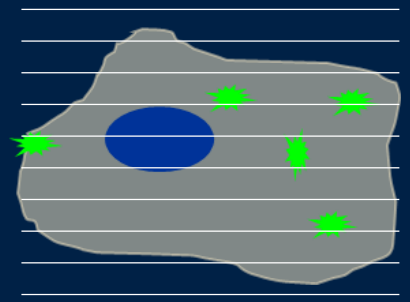
Under-sample



Make step size bigger

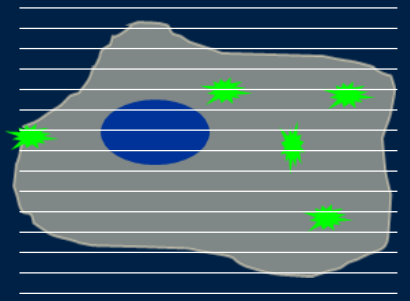
Step size e.g. 0.5µm

Recommended Step Size



Step size e.g. 0.3µm

Oversample



Decrease by 1/3

Step size e.g. 0.2µm

Faster acquisition
Less bleaching

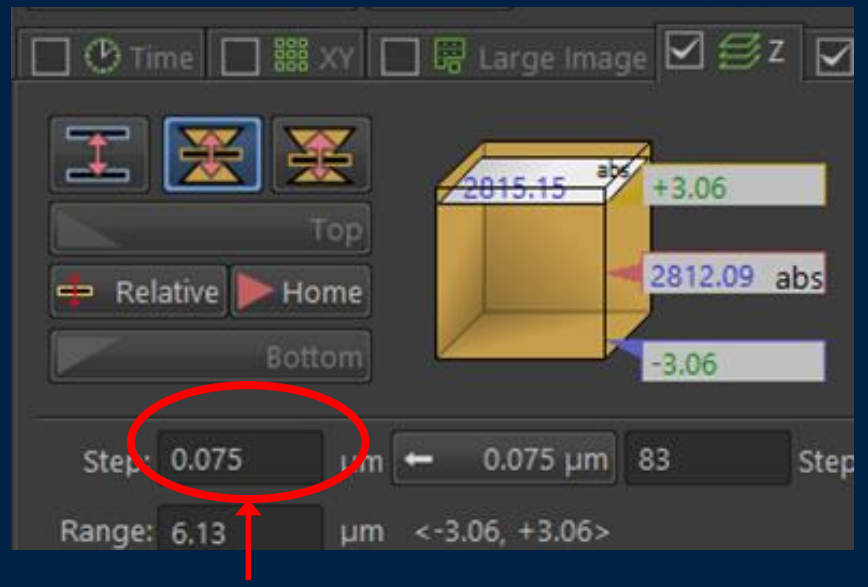
Slower acquisition
More bleaching

What happens when you under-sample

Actual Image

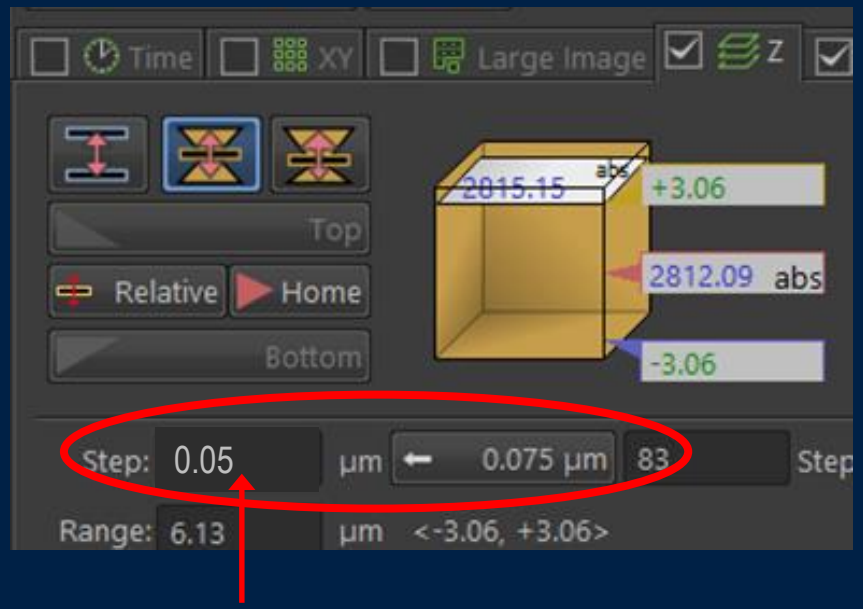
Under-sampled Image

Aliasing occurs when signal becomes indistinguishable and create distortions.

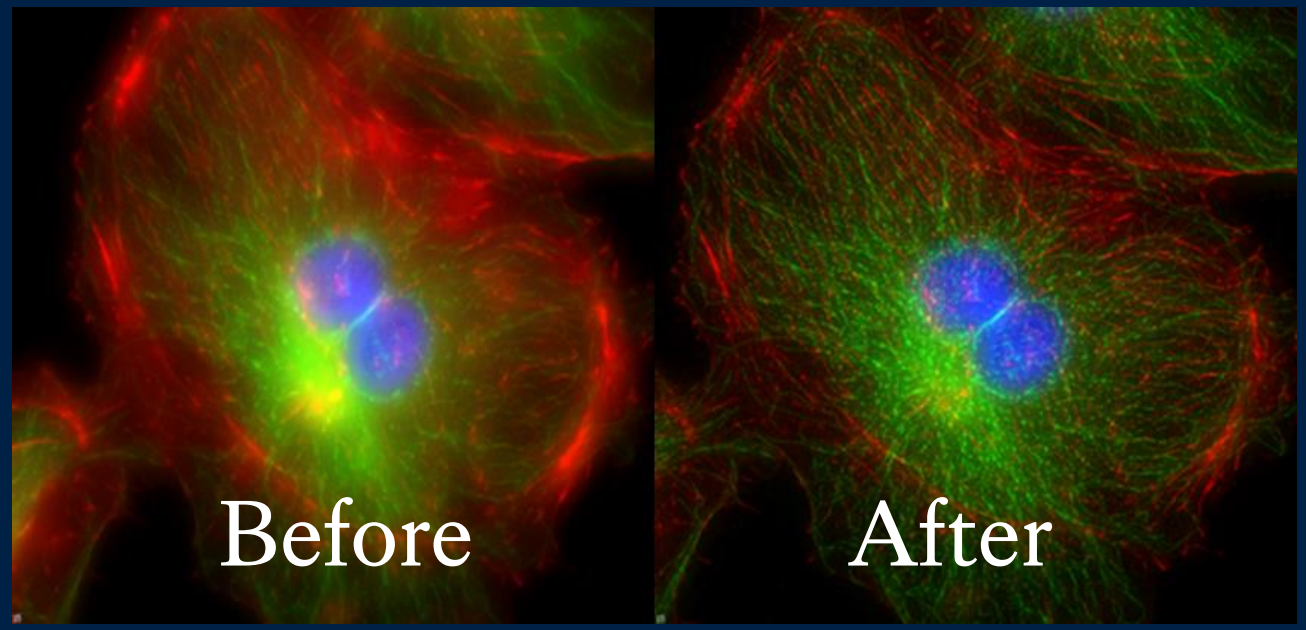


- Under-sampling can save time and minimise bleaching but you do loose information.
- To under-sample, type in a step size bigger than the recommended, E.g. in this case 0.1µm

What happens when you oversample



- You are capturing more information than you need when you oversample.
- But computer algorithms can use this information to 3D Deconvolve, digitally relocate signals for higher resolution images.
- To oversample, type in a step size smaller than the recommended, E.g. in this case 0.05µm



3D Deconvolution

Image copied from http://www.biology.wustl.edu/imaging-facility/specs-deltavision.php

Once everything is optimised, remember to save the camera settings in myConfocal

Moving on to Acquisition Settings…

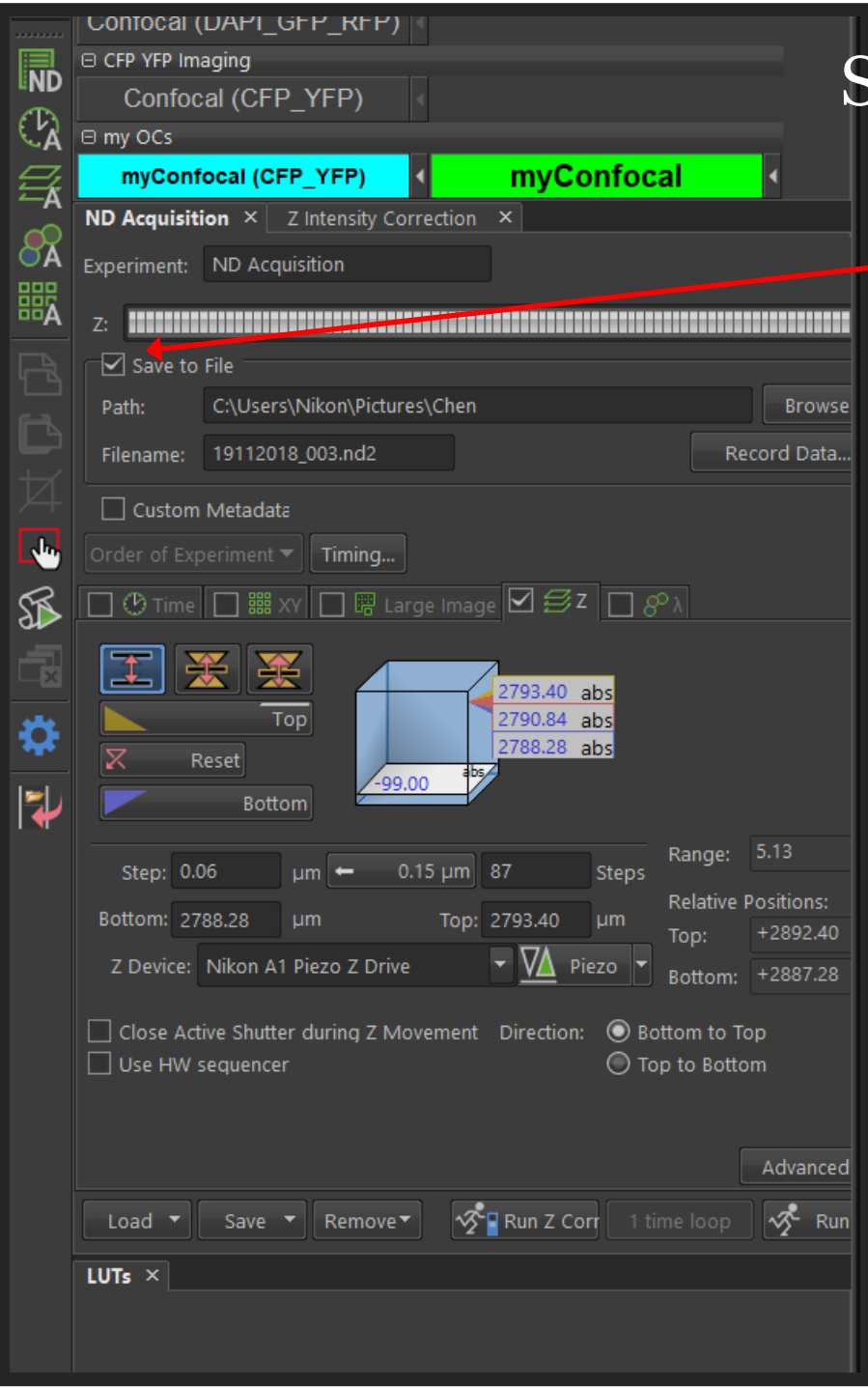
STEP BY STEP INSTRUCTIONS

STEP 9

Acquisition Settings

... Save to File

Set Up File Path (SAVE)



1) Check Save to file option

2) Go to Browse and select C:\Users\Nikon\Pictures

3) Create/find your folder, set up new folder for this session if needed.

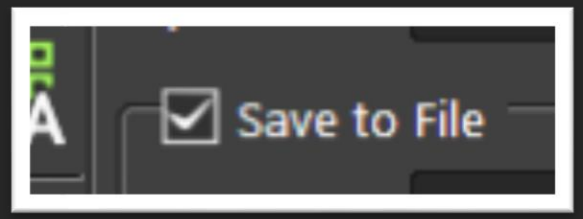
4) Recommended file name: Experiment_Name_Date_001

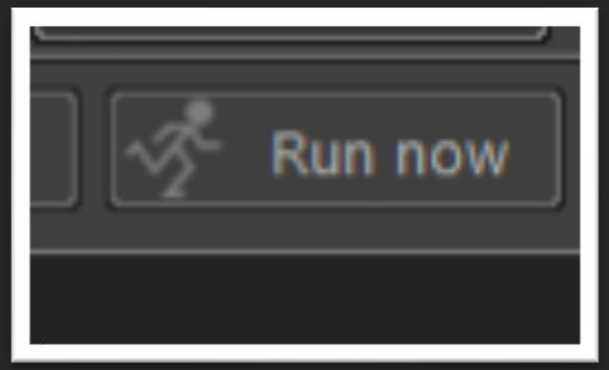
5) Finished, anytime you press ”Run now” a new file will be automatically saved in the nd2 format.

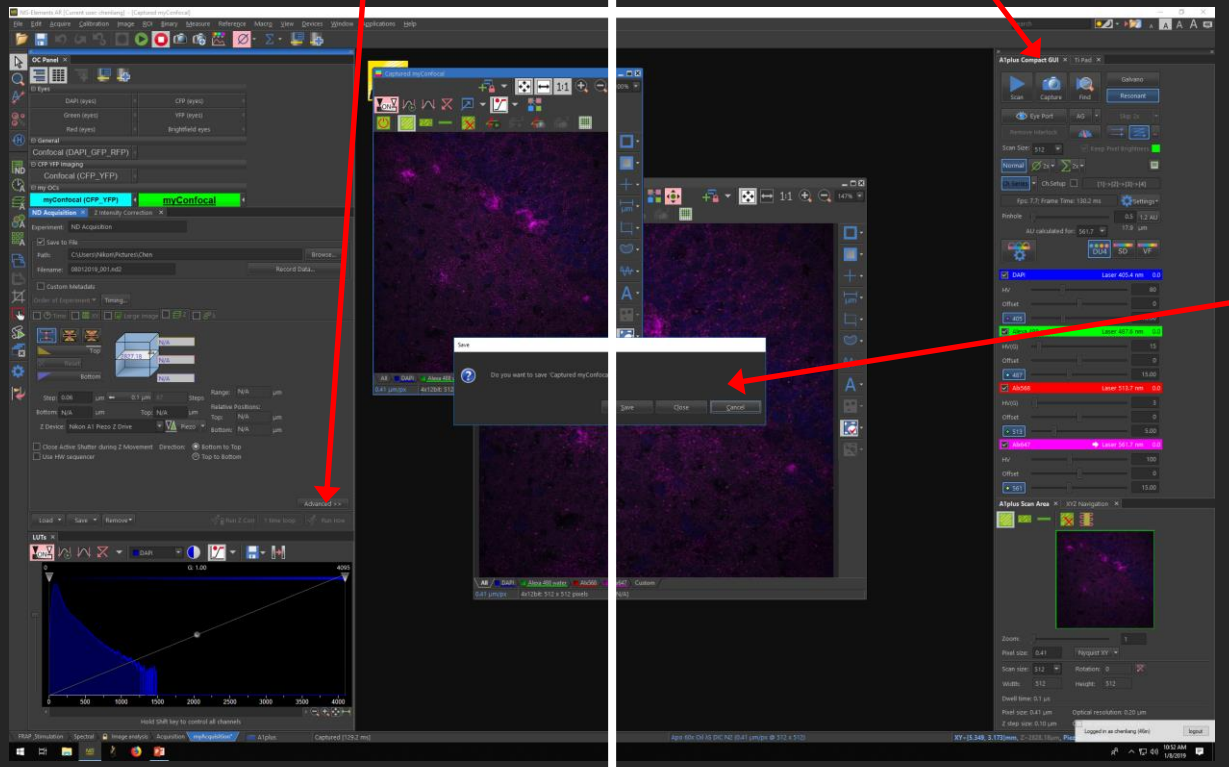
If you don’t put “_001” at the end of the first file name, the software will automatically name your second image ....._001, then _002, _003 for subsequent images.

Run VS Capture

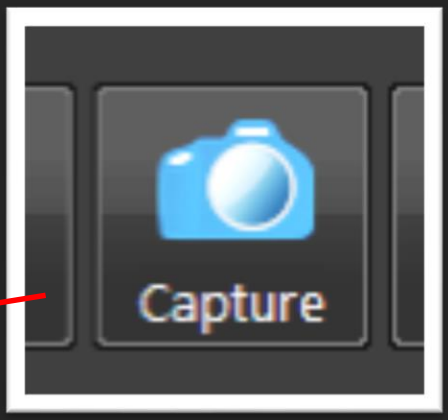
Automatically saved if ‘Save to File’ is ticked







Not automatically saved



Box will appear and you can save or discard image.

After ‘Run now’ if you make changes to your image (such as adding ROIs) then save this ‘new’ image by going to file and ‘SAVE AS’ so you don’t overwrite your raw data.

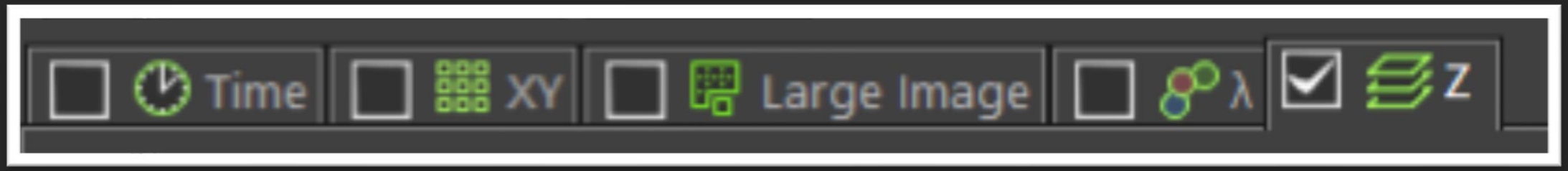
STEP BY STEP INSTRUCTIONS

STEP 9

Acquisition Settings

... Order or acquisition tabs

Fastest acquisition tab sequence

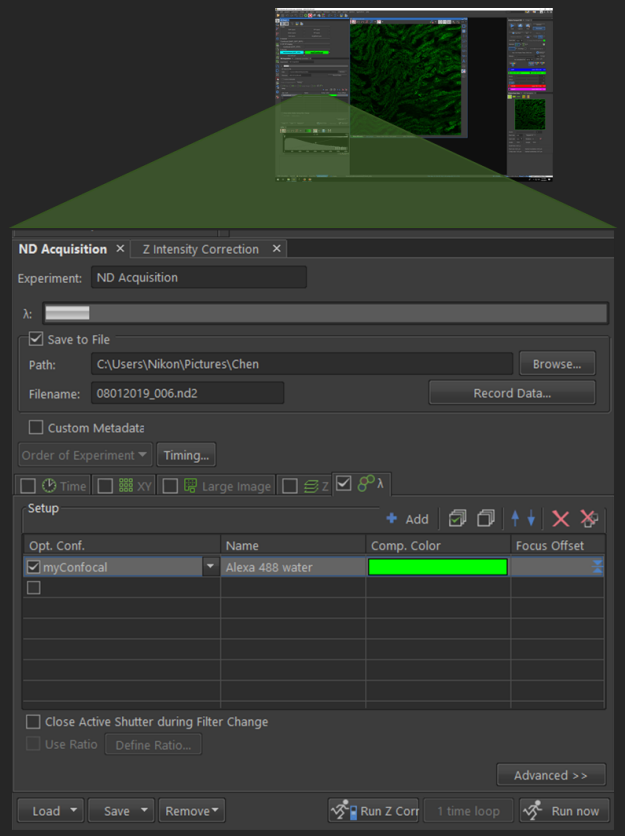


The Software will prioritise the tab on the RIGHT.

Do not put ‘Large Image’ tab on the right.

Tick the box for all acquisition functions you want to use.

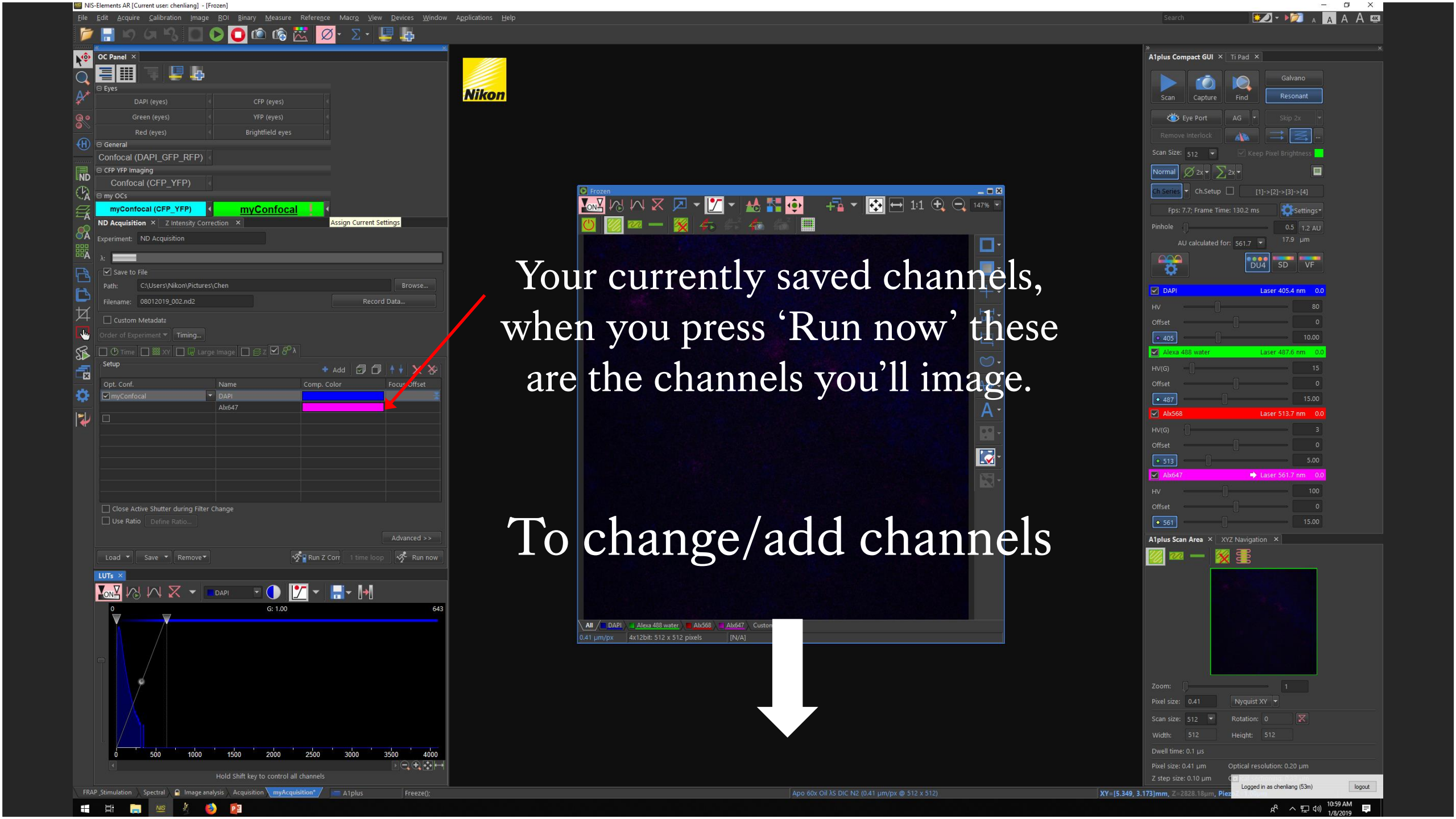
STEP BY STEP INSTRUCTIONS

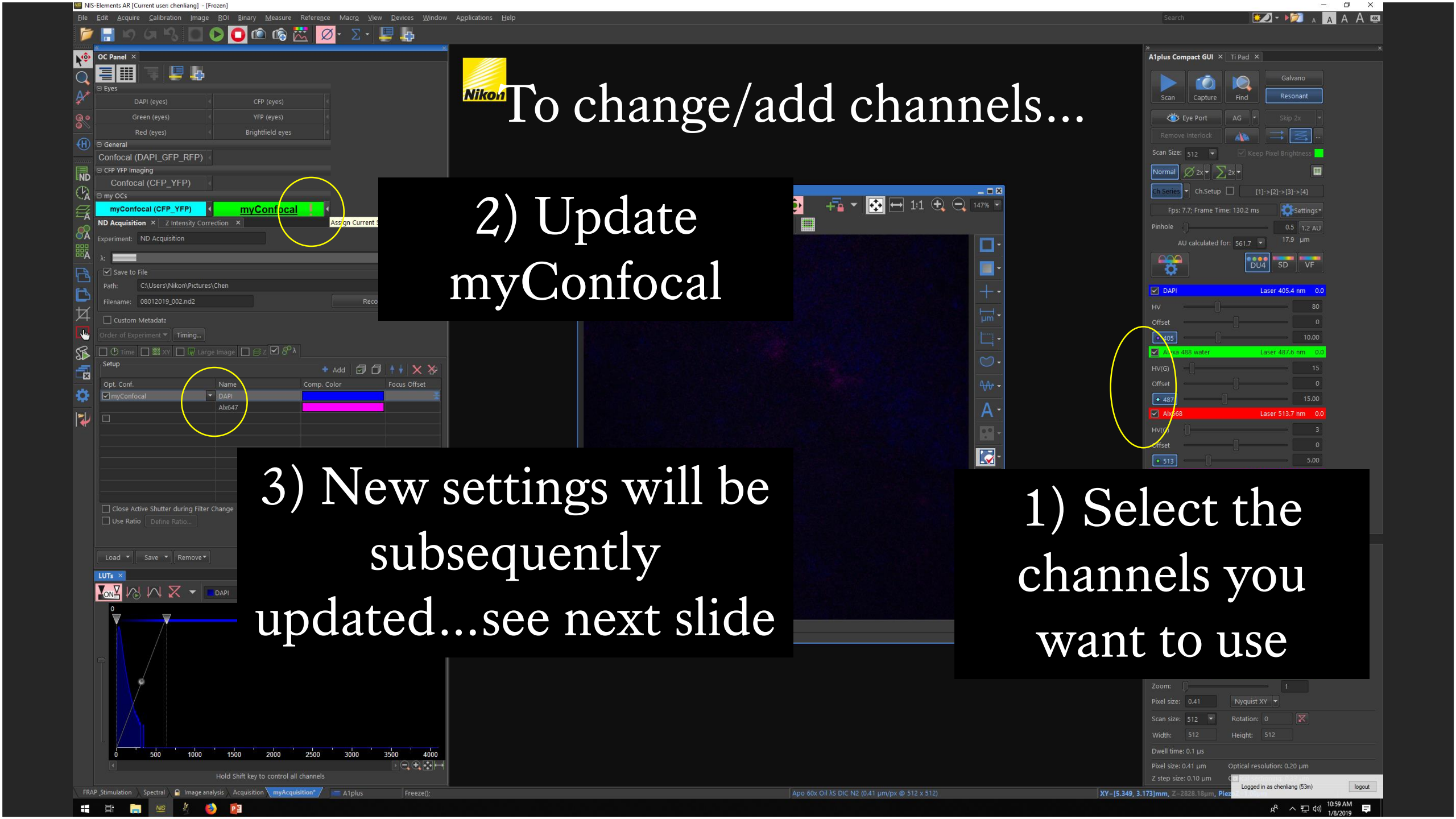


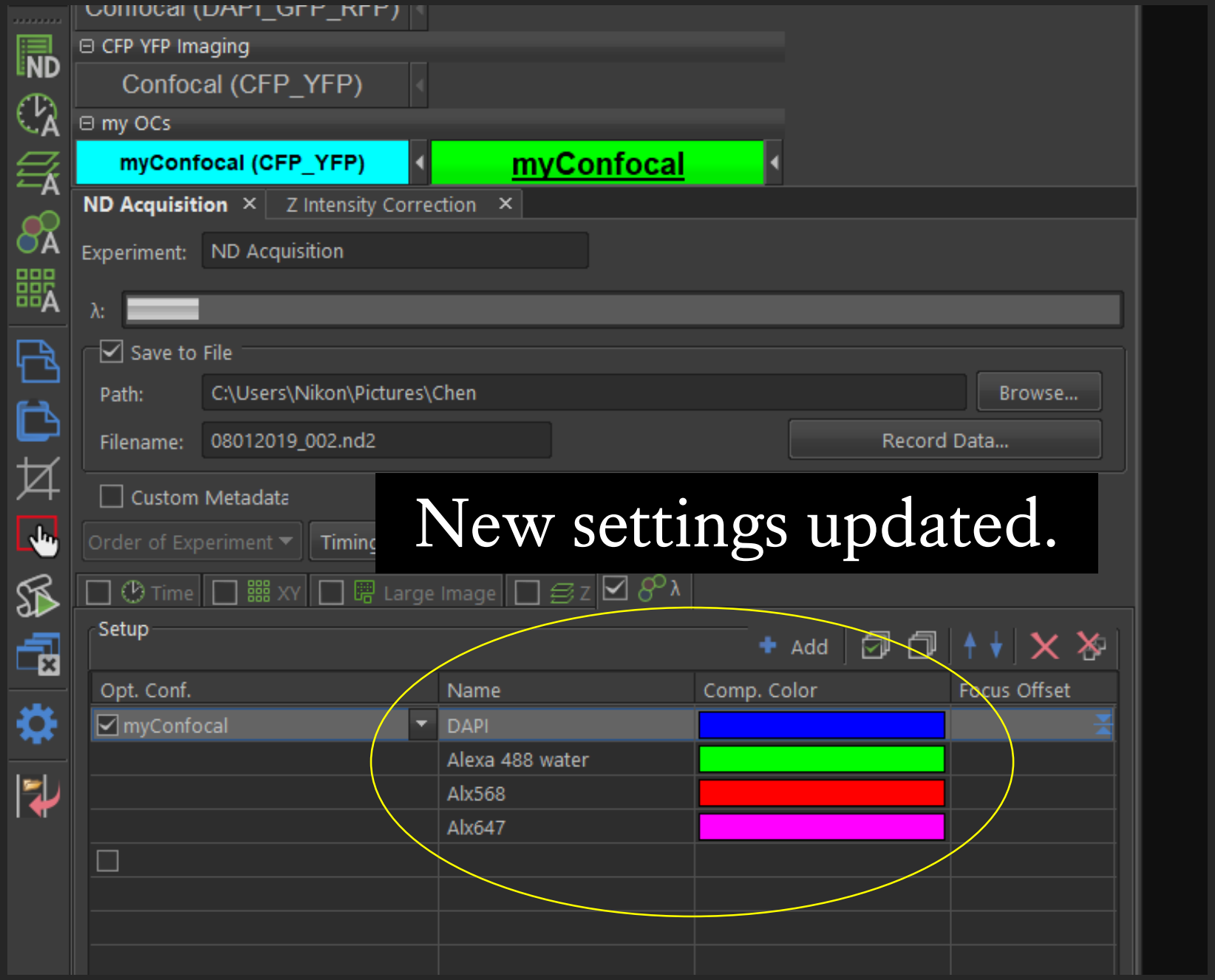
STEP 9

Acquisition Settings

... Lambda (laser channels)


Your currently saved channels, when you press ‘Run now’ these are the channels you’ll image.
To change/add channels

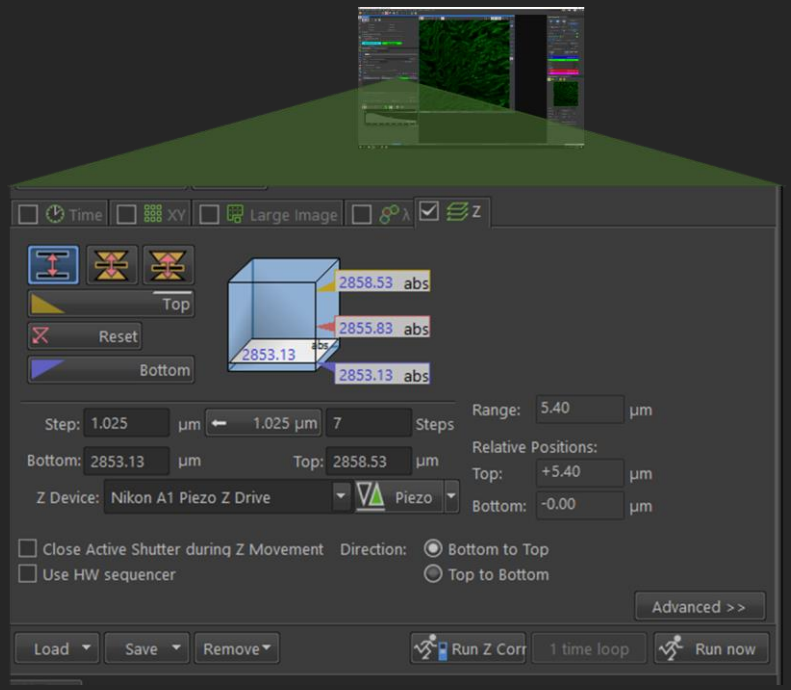

To change/add channels…
2) Update myConfocal
3) New settings will be subsequently updated…see next slide
1) Select the channels you want to use


CFP YFP Imaging
Confocal (CFP_YFP)
my OCs
myConfocal (CFP_YFP)
myConfocal
ND Acquisition
Z Intensity Correction
Experiment:
ND Acquisition
λ:
Save to File
Path:
C:\Users\Nikon\Pictures\Chen
Browse...
Filename:
08012019_002.nd2
Record Data...
Custom Metadata
Order of Experiment
Timing
Time
XY
Large Image
Z
λ
Setup
Add
Opt. Conf.
Name
Comp. Color
Focus Offset
myConfocal
DAPI
Alexa 488 water
Alx568
Alx647
New settings updated.

STEP BY STEP INSTRUCTIONS

STEP 9

Acquisition Settings



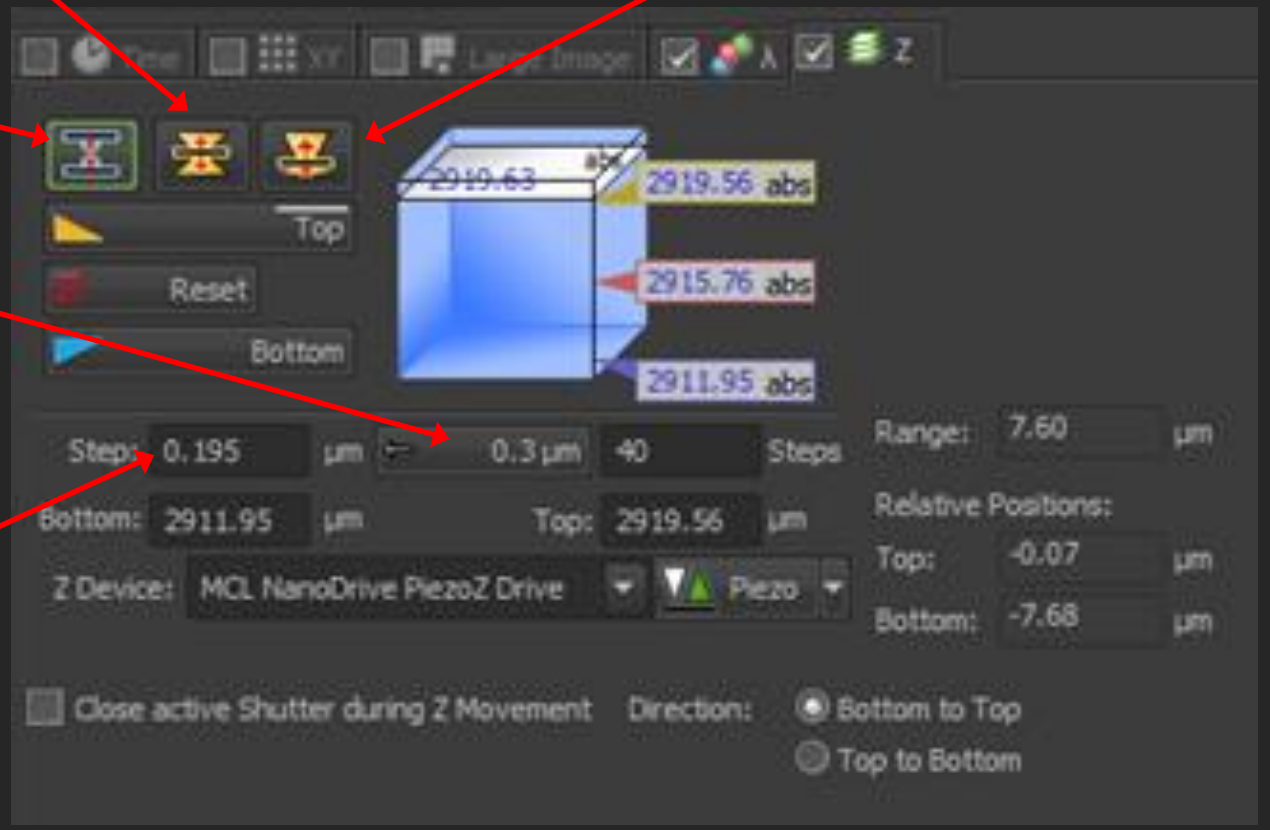
... Z stack

Z stack Basic Options

Set top and bottom: use mouse wheel to focus and define the exact range of your Z stack.

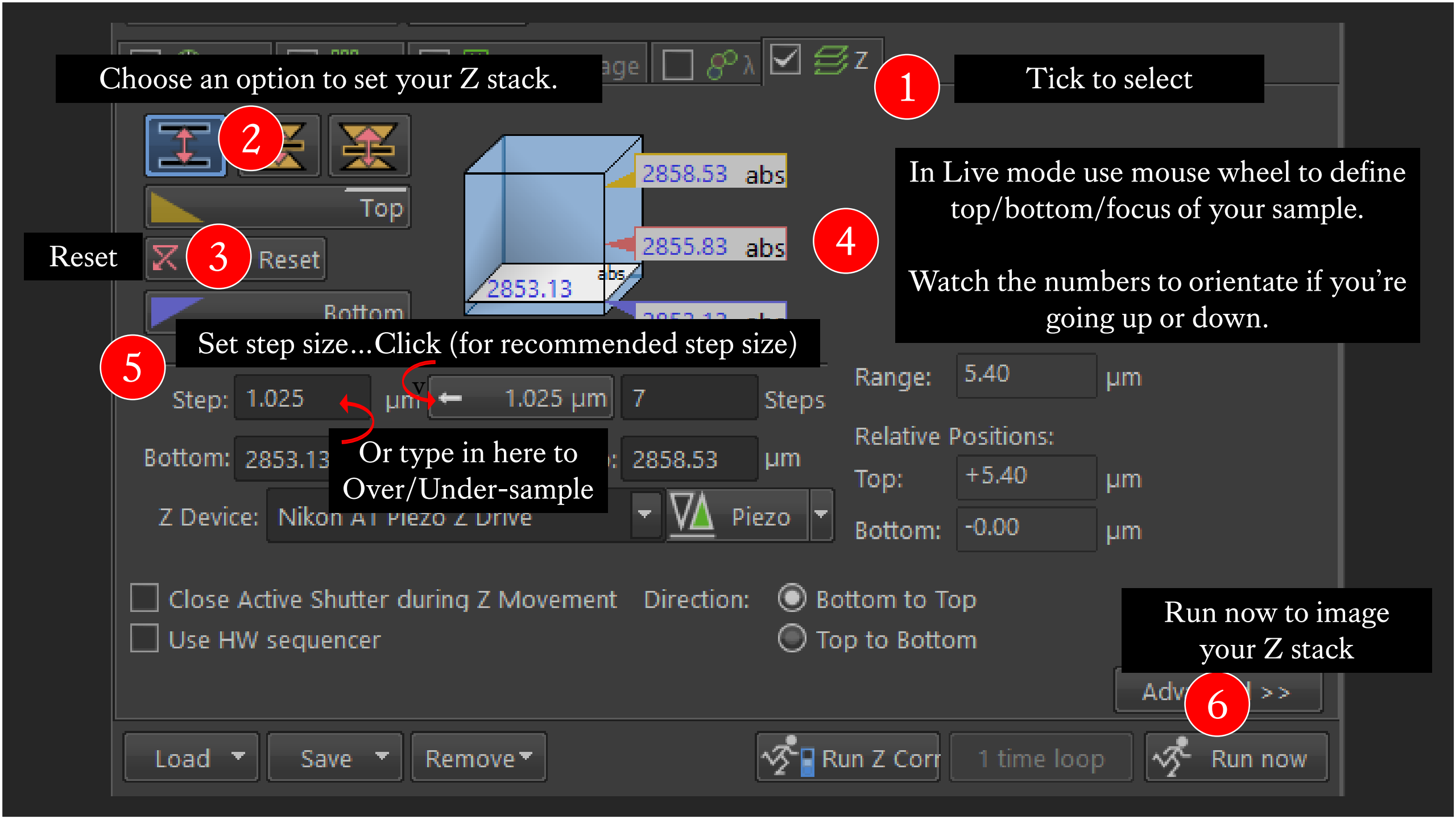
Set Middle: use mouse wheel to find the mid point of your Z focus and set equal distance above and below the focal plane. (Useful if your sample is symmetrical along the Z axis)

Asymmetrical: find focal plane and then set different distances above and below. (Useful for  like cells)

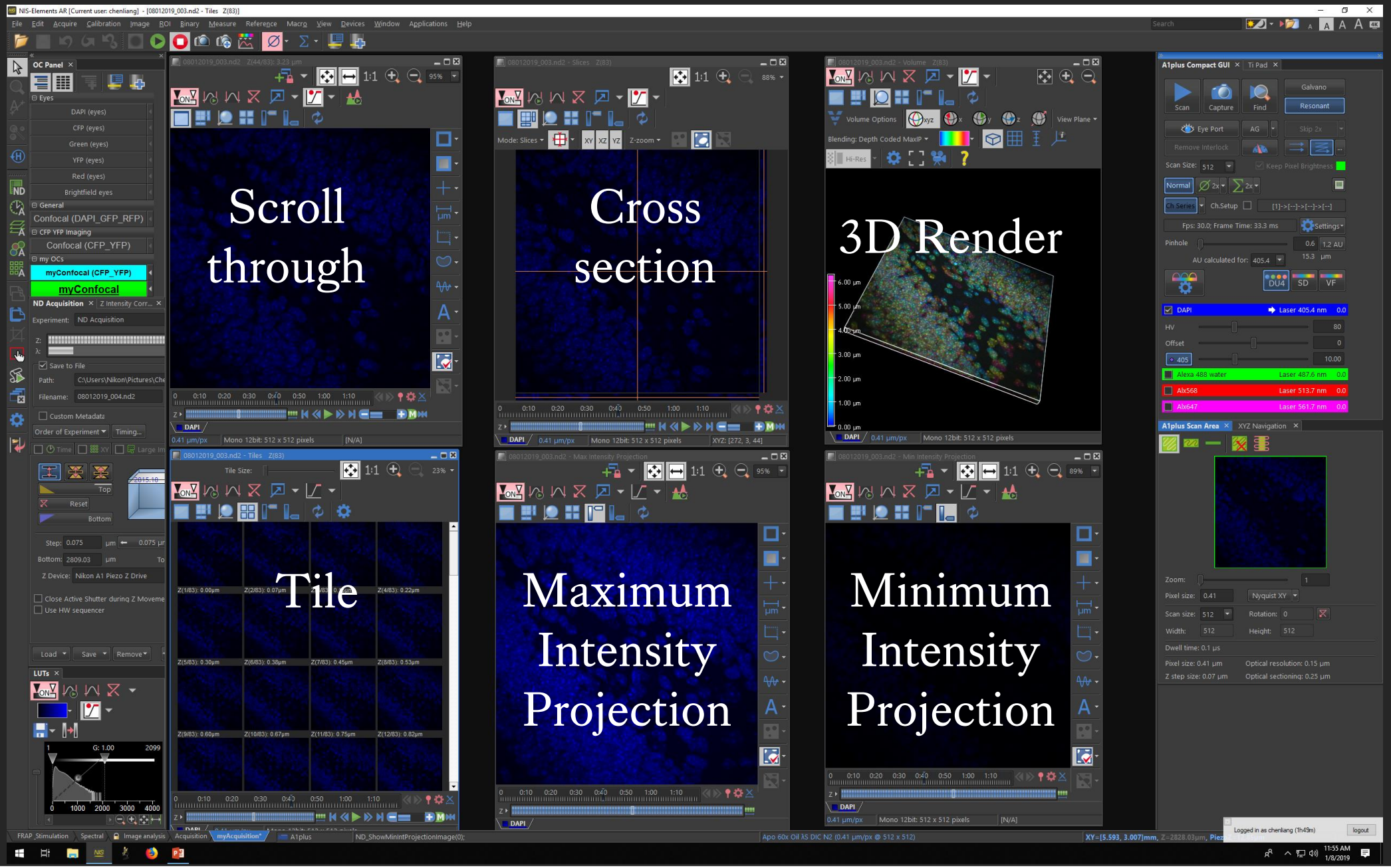


Step Size

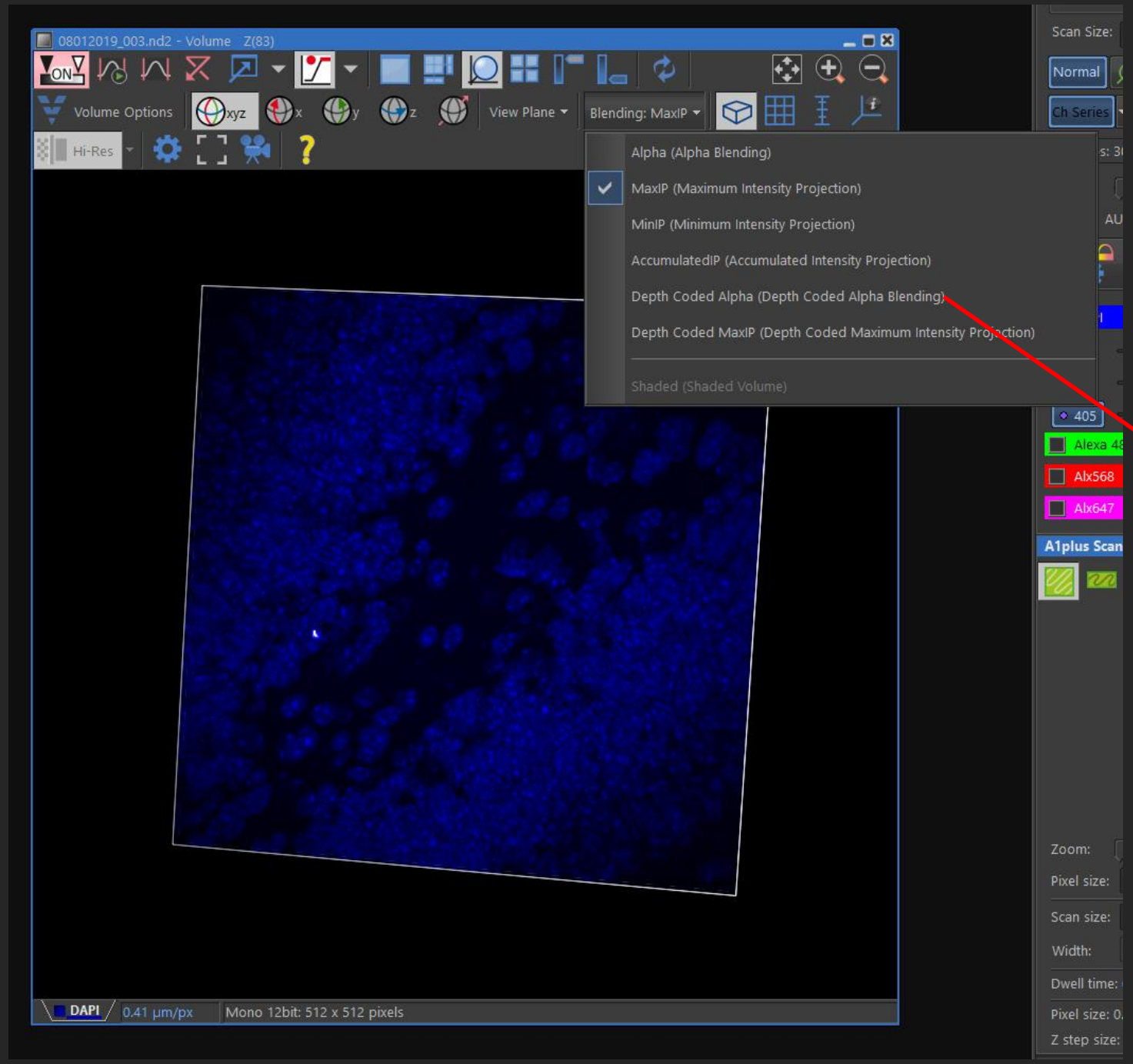
- You can set step size or number of steps.
- Use recommended step size to capture all the information.
- Fewer steps than the recommended is called under-sampling and you may loose information.
- More steps than the recommended is called over-sampling (typed in here) which is required for 3D deconvolution.

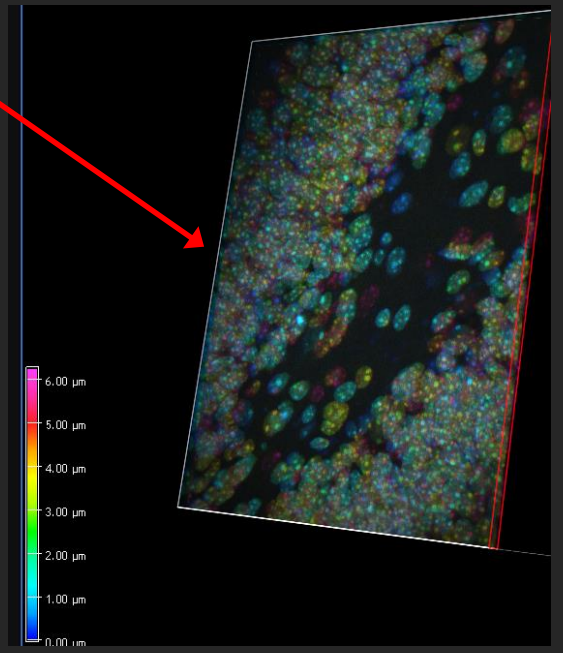

Choose an option to set your Z stack.
1
Tick to select
2
Top
Reset
3
Reset
Bottom
2858.53 abs
2855.83 abs
2853.13
4
In Live mode use mouse wheel to define top/bottom/focus of your sample.
Watch the numbers to orientate if you're going up or down.
5
Set step size…Click (for recommended step size)
Step: 1.025 µm
1.025 µm
7 Steps
Range: 5.40 µm
Relative Positions:
Top: +5.40 µm
Bottom: -0.00 µm
Bottom: 2853.13
2858.53 µm
Or type in here to Over/Under-sample
Z Device: Nikon A1 Piezo Z Drive
Piezo
Close Active Shutter during Z Movement
Use HW sequencer
Direction:
Bottom to Top
Top to Bottom
Run now to image your Z stack
6
Load
Save
Remove
Run Z Corr
1 time loop
Run now

Z stack View Modes



In 3D rendering you have different rendering modes.

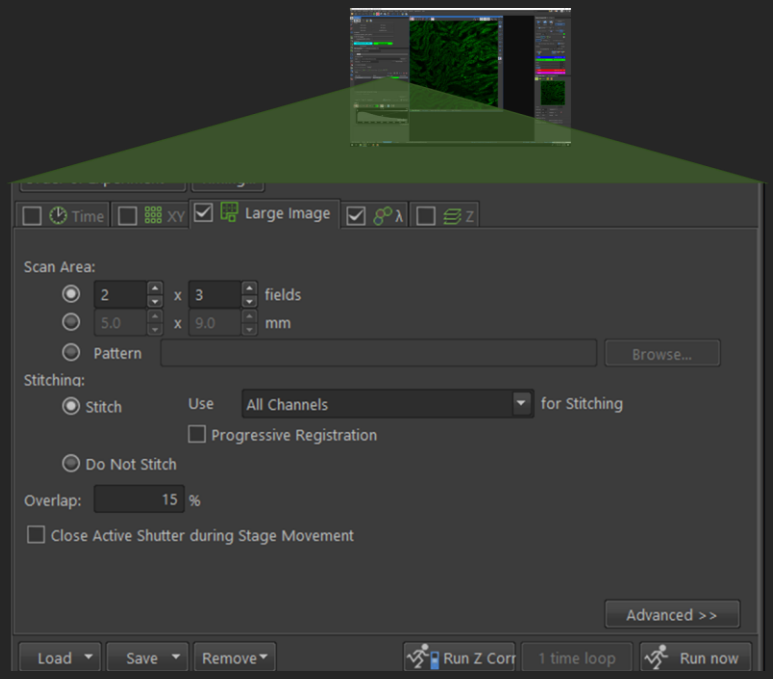




STEP BY STEP INSTRUCTIONS

STEP 9

Acquisition Settings



... Large Image

Time | XY | Large Image | λ | Z

1

Tick to select, Lambda must be ticked too if not using Z stack.

Set scan area

Scan Area:

3

2 x 3 fields

X axis Y axis

5.0 x 9.0 mm

Pattern

2

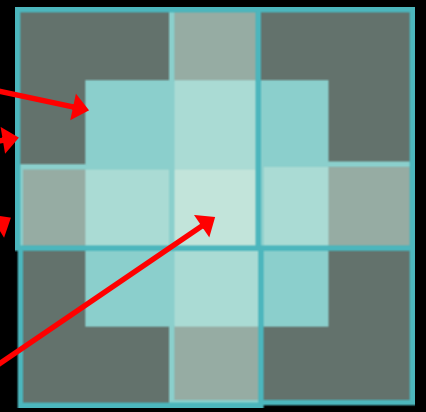
In Live mode, move to the centre of your field of view.

Live field of view

Large Image

Overlap

Live view centre



Stitching:

4

Stitch

Use overlapping image edge to stitch

Progressive Registration

Do Not Stitch

Overlap: 15 %

5

15% overlap minimum!

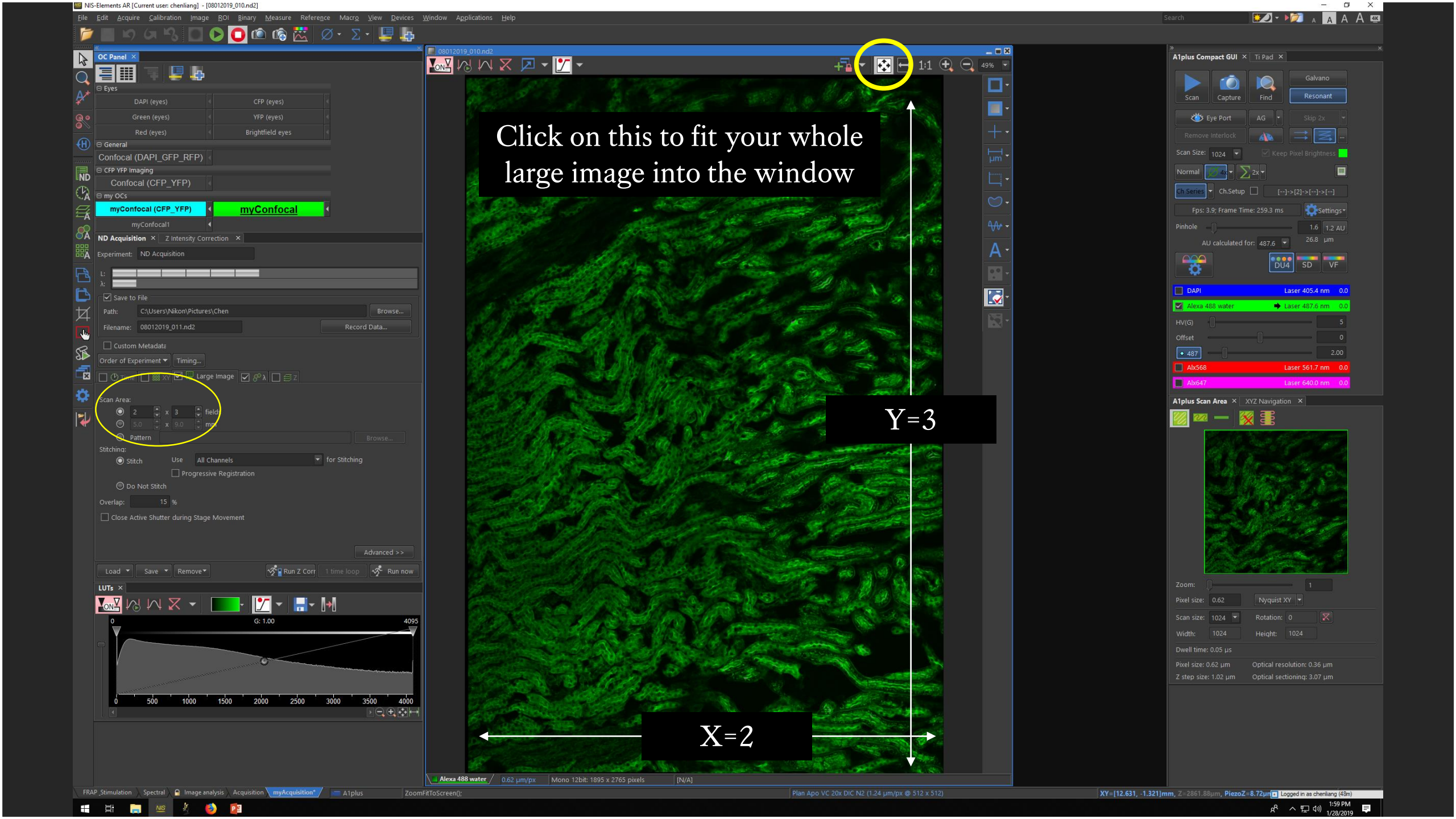
Close Active Shutter during Stage Movement

Run now to image your Large Image

Advance

6

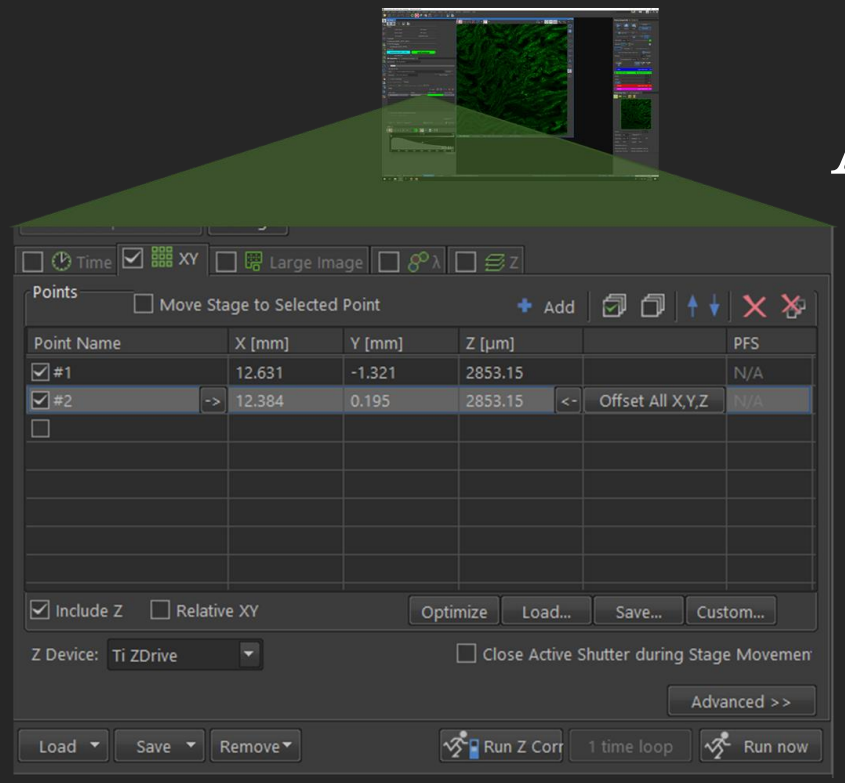
Load | Save | Remove | Run Z Corr | 1 time loop | Run now


Click on this to fit your whole large image into the window
Y=3
X=2
Scan Area:
Large Image
Stitching:
Stitch
Do Not Stitch
Overlap:
Close Active Shutter during Stage Movement
Advanced >>
Load
Save
Remove
Run Z Corr
1 time loop
Run now
LUTs
Eyes
DAPI (eyes)
CFP (eyes)
Green (eyes)
YFP (eyes)
Red (eyes)
Brightfield eyes
General
Confocal (DAPI_GFP_RFP)
CFP YFP Imaging
Confocal (CFP_YFP)
my OCs
myConfocal (CFP_YFP)
myConfocal
myConfocal1
ND Acquisition
Z Intensity Correction
Experiment:
Save to File
Path:
Filename:
Record Data...
Custom Metadata
Order of Experiment
Timing...
Browse...
Pattern
Stitching:
Use
All Channels
for Stitching
Progressive Registration
Overlap:
Close Active Shutter during Stage Movement
A1plus Compact GUI
Ti Pad
Scan
Capture
Find
Galvano
Resonant
Eye Port
AG
Skip 2x
Remove Interlock
Scan Size:
Keep Pixel Brightness
Normal
Ch Series
Ch.Setup
Fps: 3.9; Frame Time: 259.3 ms
Settings
Pinhole
AU calculated for:
DU4
SD
VF
DAPI
Laser 405.4 nm
Alexa 488 water
Laser 487.6 nm
HV(G)
Offset
Alx568
Laser 561.7 nm
Alx647
Laser 640.0 nm
A1plus Scan Area
XYZ Navigation
Zoom:
Pixel size:
Nyquist XY
Scan size:
Rotation:
Width:
Height:
Dwell time: 0.05 µs
Pixel size: 0.62 µm
Optical resolution: 0.36 µm
Z step size: 1.02 µm
Optical sectioning: 3.07 µm
Alexa 488 water
0.62 µm/px
Mono 12bit: 1895 x 2765 pixels
[N/A]
FRAP_Stimulation
Spectral
Image analysis
Acquisition
myAcquisition*
A1plus
ZoomFitToScreen();
Plan Apo VC 20x DIC N2 (1.24 µm/px @ 512 x 512)
Logged in as chenliang (48m)
1:59 PM
1/28/2019

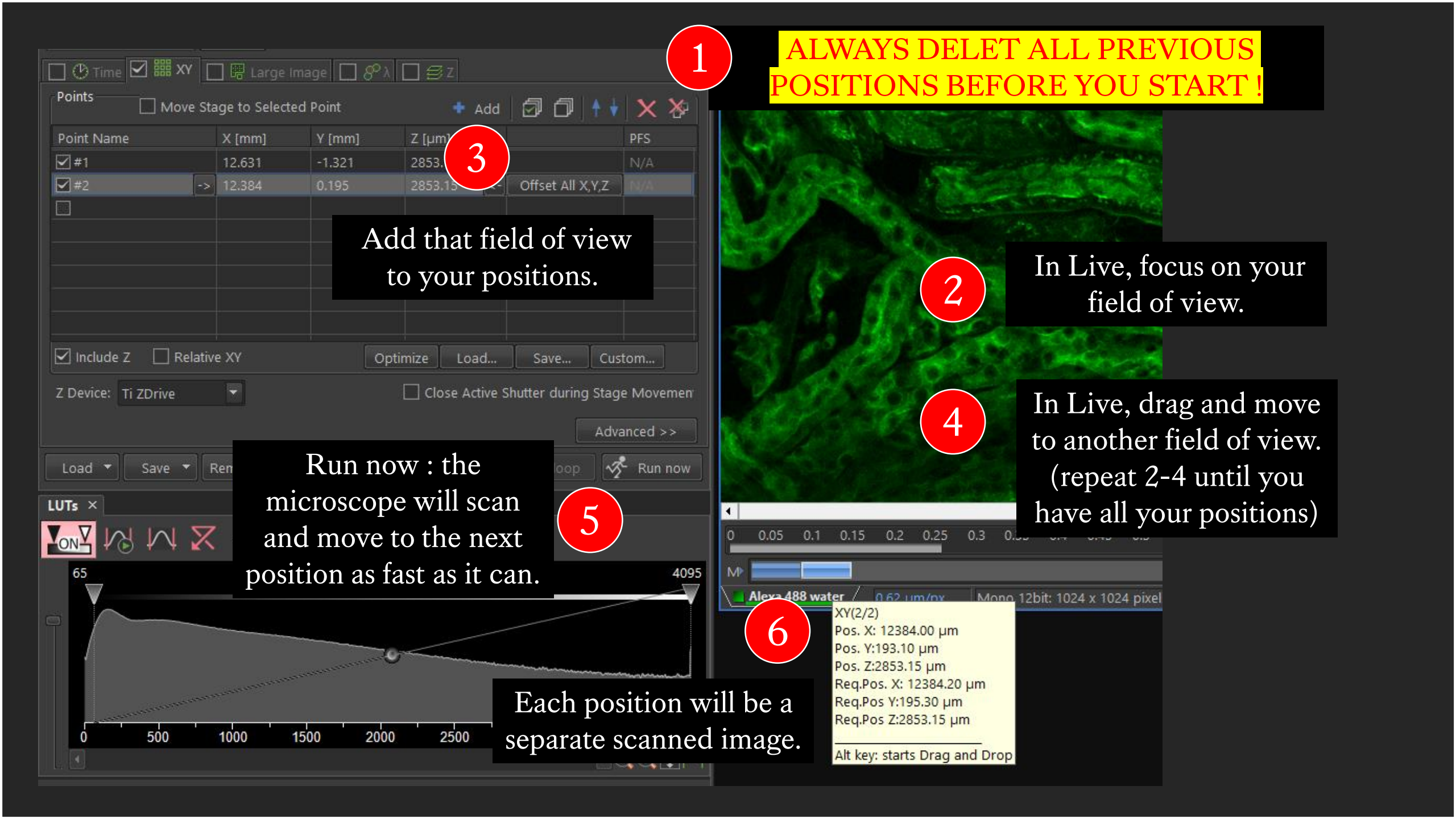
STEP BY STEP INSTRUCTIONS

STEP 9

Acquisition Settings



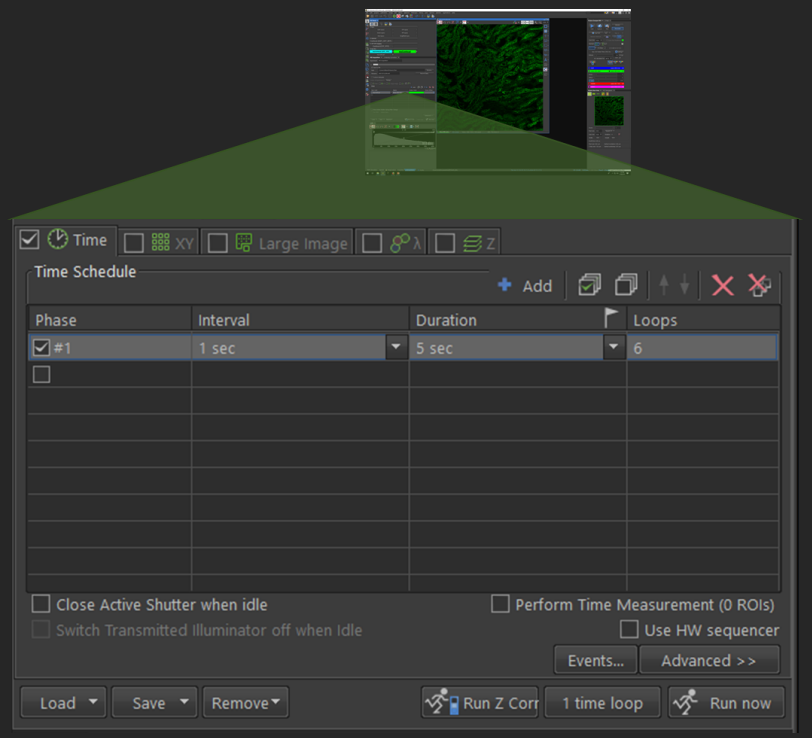
... XY Positions


1
ALWAYS DELET ALL PREVIOUS POSITIONS BEFORE YOU START !
Time
XY
Large Image
Z
Points
Move Stage to Selected Point
Add
Point Name
X [mm]
Y [mm]
Z [µm]
PFS
#1
12.631
-1.321
2853.
N/A
#2
12.384
0.195
2853.15
Offset All X,Y,Z
3
Add that field of view to your positions.
Include Z
Relative XY
Optimize
Load...
Save...
Custom...
Z Device:
Ti ZDrive
Close Active Shutter during Stage Movemen
Advanced >>
Load
Save
Run now
2
In Live, focus on your field of view.
4
In Live, drag and move to another field of view. (repeat 2-4 until you have all your positions)
5
Run now : the microscope will scan and move to the next position as fast as it can.
LUTs
ON
65
4095
0
500
1000
1500
2000
2500
Each position will be a separate scanned image.
0
0.05
0.1
0.15
0.2
0.25
0.3
Alexa 488 water
Mono 12bit: 1024 x 1024 pixel
6
XY(2/2)
Pos. X: 12384.00 µm
Pos. Y:193.10 µm
Pos. Z:2853.15 µm
Req.Pos. X: 12384.20 µm
Req.Pos Y:195.30 µm
Req.Pos Z:2853.15 µm
Alt key: starts Drag and Drop

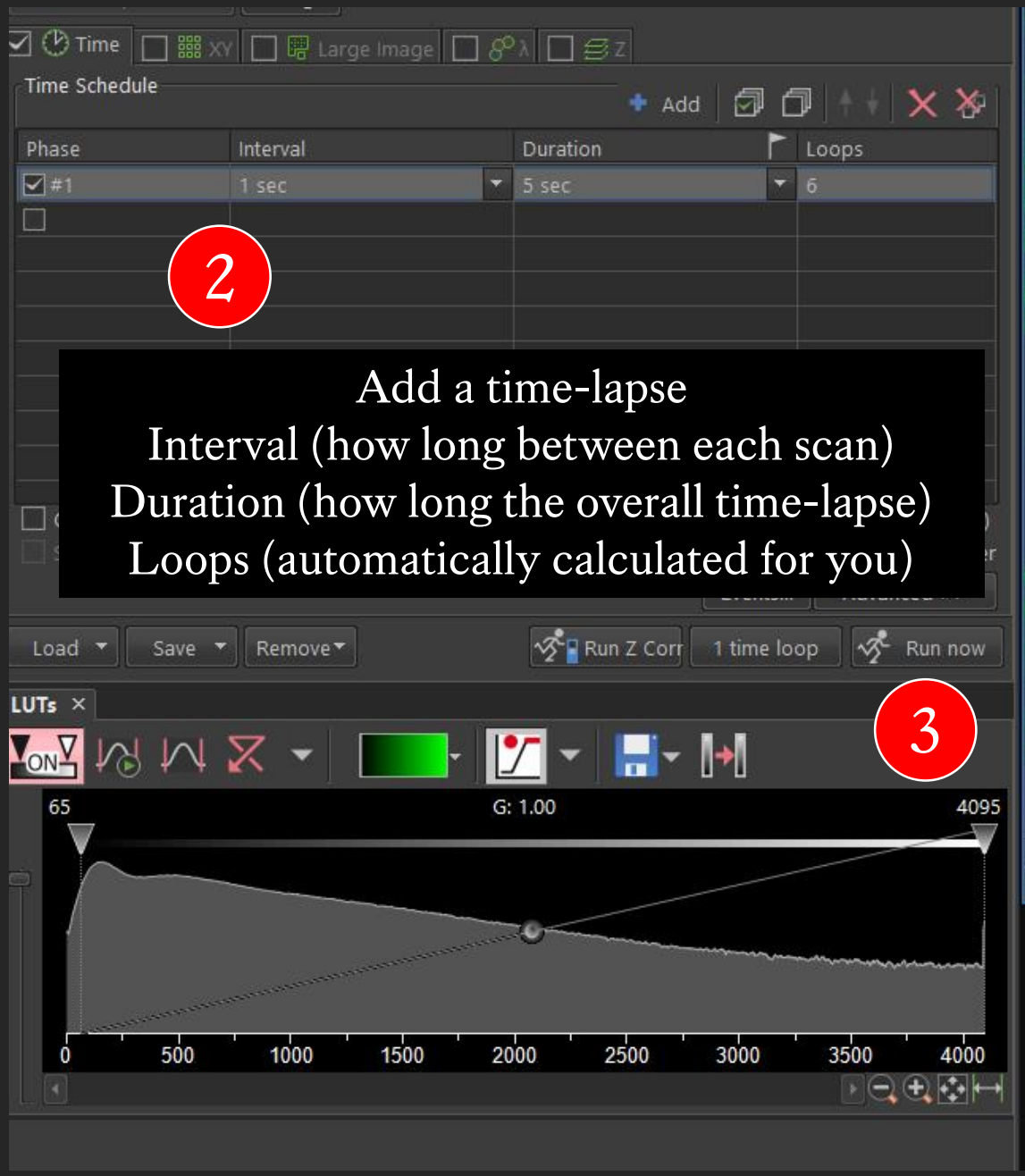
STEP BY STEP INSTRUCTIONS

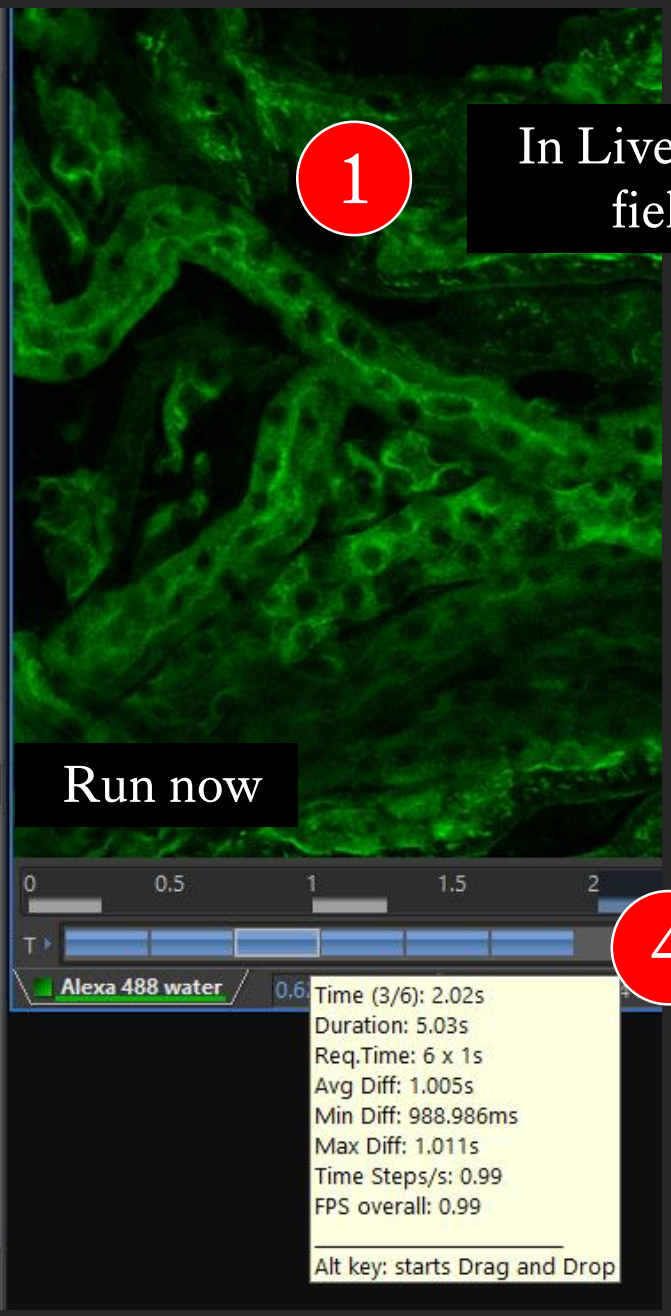
STEP 9

Acquisition Settings

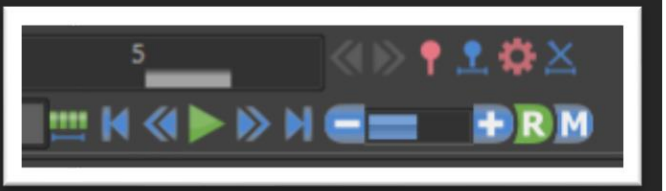


... Time





You can play your time-lapse loop and make a video.



Continue with your imaging...

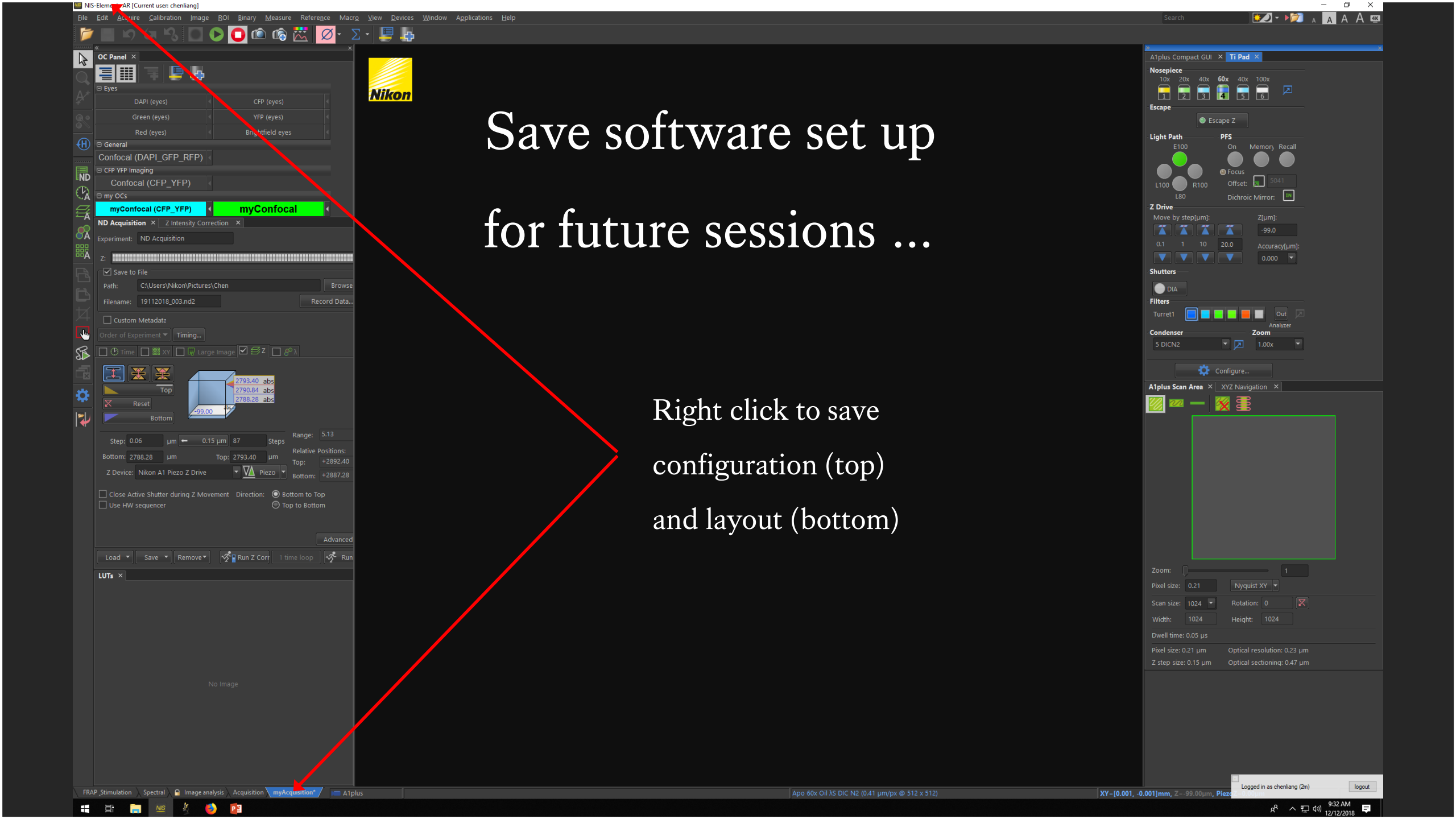
STEP BY STEP INSTRUCTIONS

STEP 10

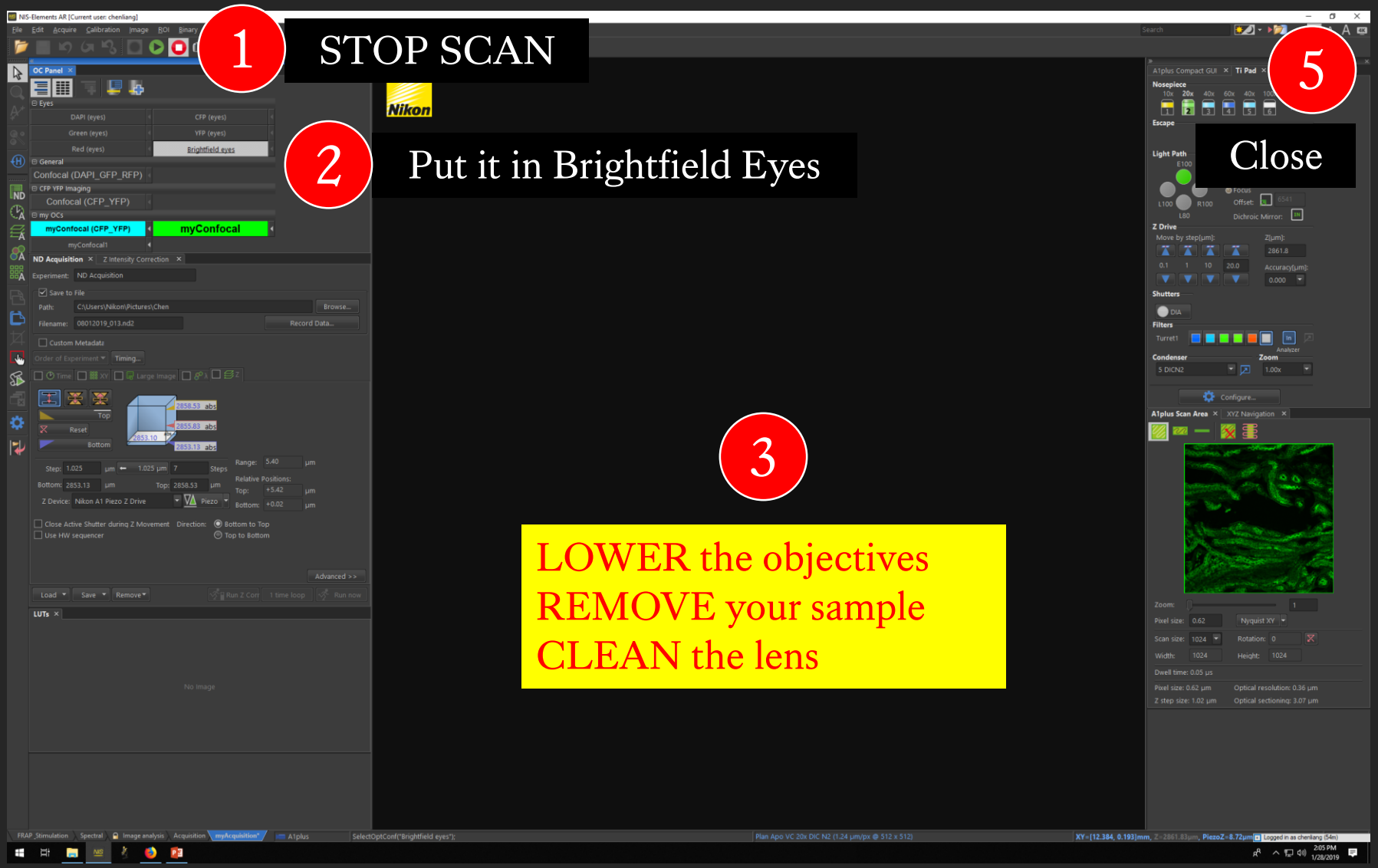
At the end of your session

... Save your software settings

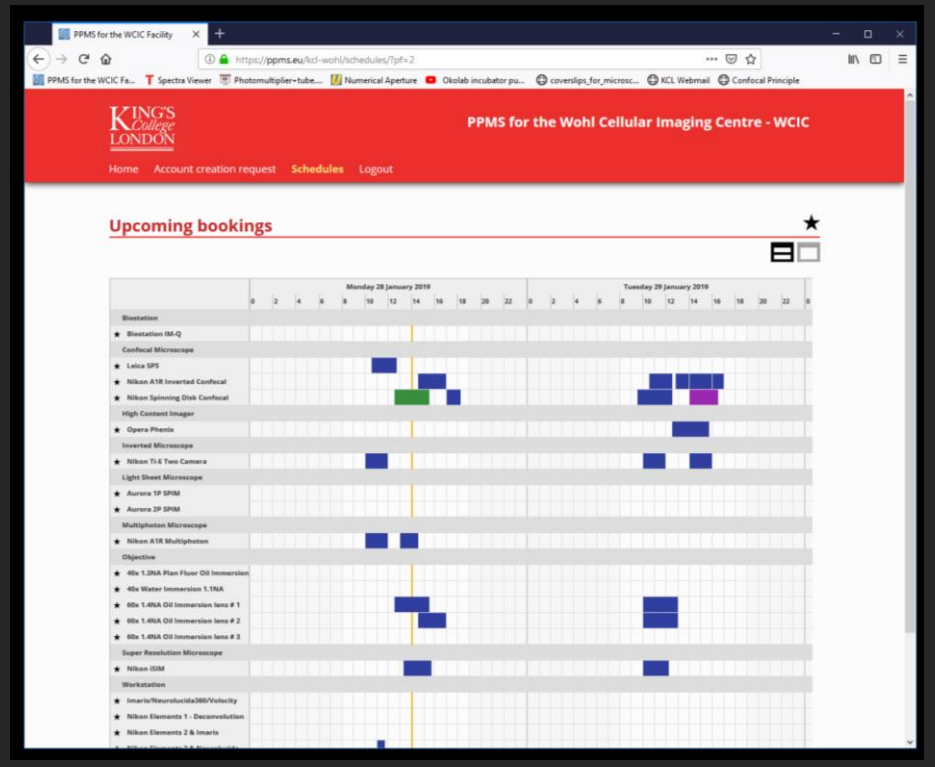
... Shut down procedure


Save software set up
for future sessions ...
Right click to save
configuration (top)
and layout (bottom)

Shut down procedure



Check the booking schedule!



If no one is using the system within 2 hours, shut down the system.

If someone is booked on within 2 hours, leave the system on.

Transfer Data To Shared Drive (1 of 3)

1

Open file
Find your saved data in Pictures

2

Right click on Shared Drive (WCIC) and select Open in new window.

3

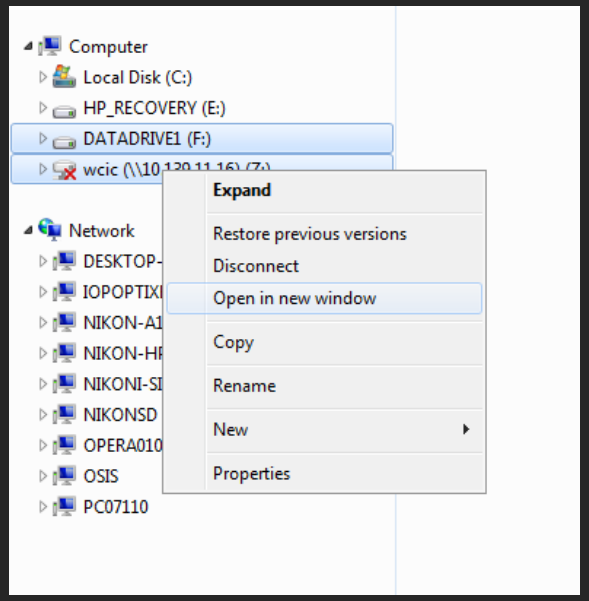
You need to login to this pop-up window, user name is normally: kclad\k number
DO NOT click on remember my credentials

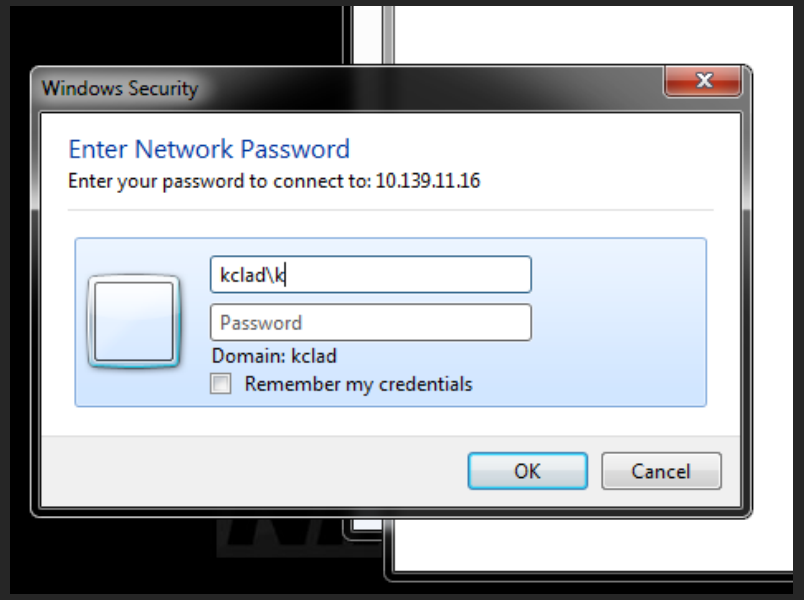
4

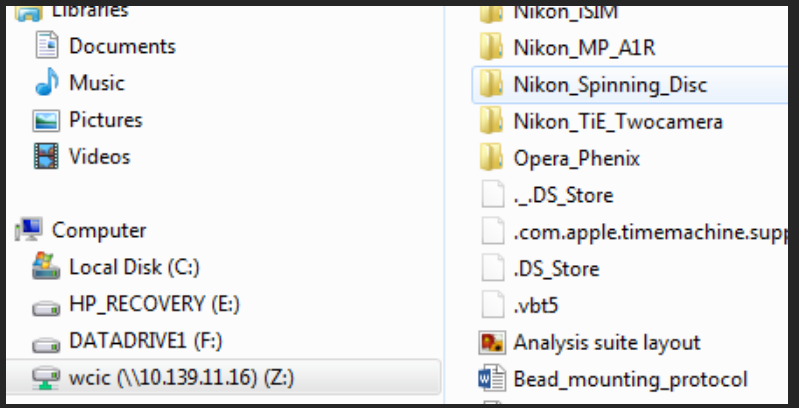
In the Network drive, open the Nikon_A1R folder

5

In the Nikon_A1R folder open your personal folder





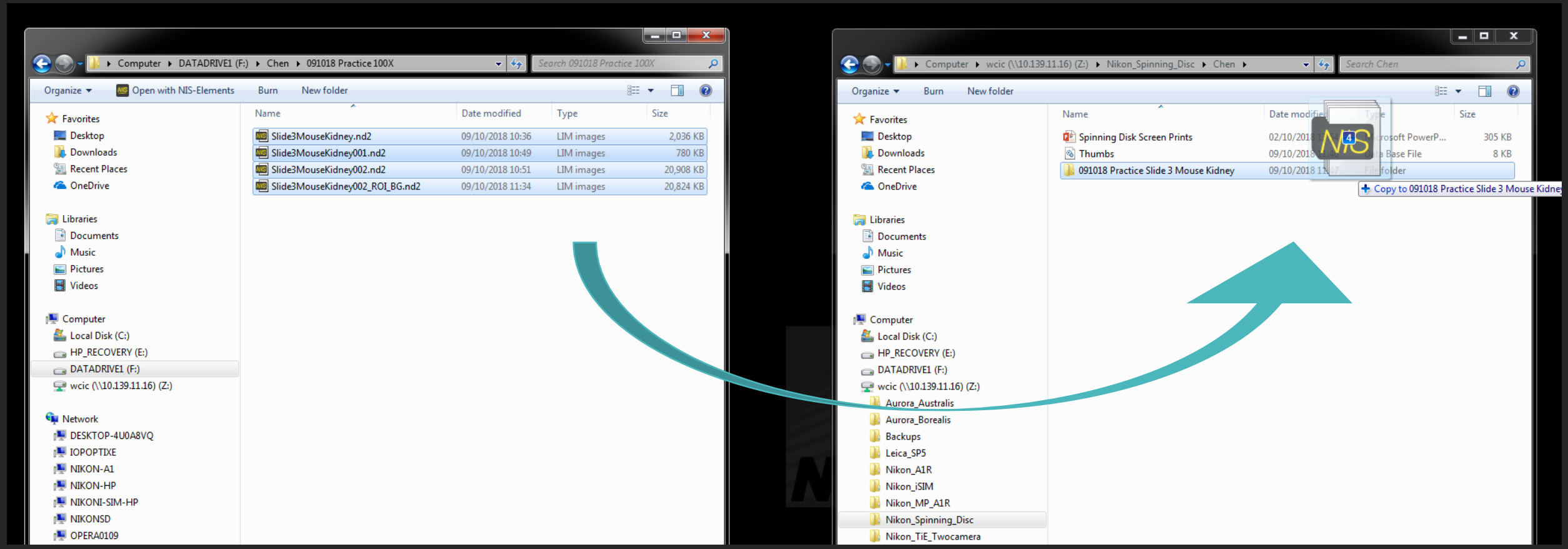


Transfer Data To Shared Drive (2 of 3)

- **DO NOT USE USBs ON ANY COMPUTERS IN THE MICROSCOPE ROOMS!**

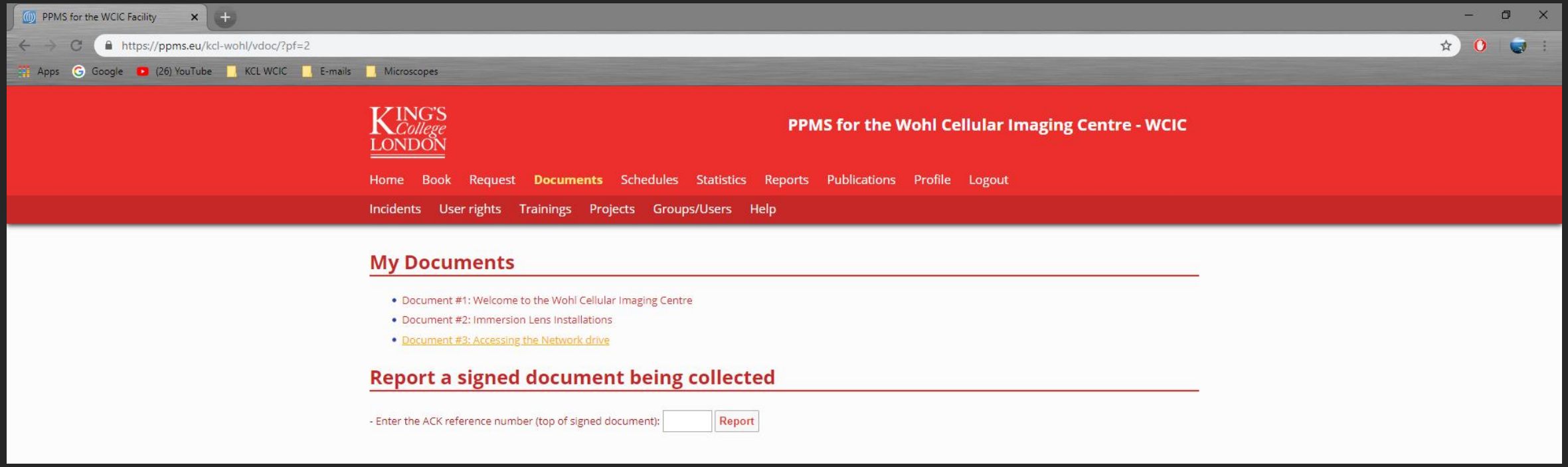
- Drag and drop the files you saved in this session.

- The Shared Drive can be accessed from the workstations (where you can use USBs), or your personal computers, from there please BACK-UP your data.



Transfer Data To Shared Drive (3 of 3)

If you have trouble connecting to the shared drive, or need to re-map the network drive, please login to the booking system, go to documents, the 3rd document contains instructions.



At The End Of The Session…

Remove sample, clean lens, select “Brightfield Eyes”

Save all data on local drive.

Move data from local drive to shared network drive

Close down software

Check the booking system

Is anyone booked on the system after you within 2 hours?

YES → Leave system on.

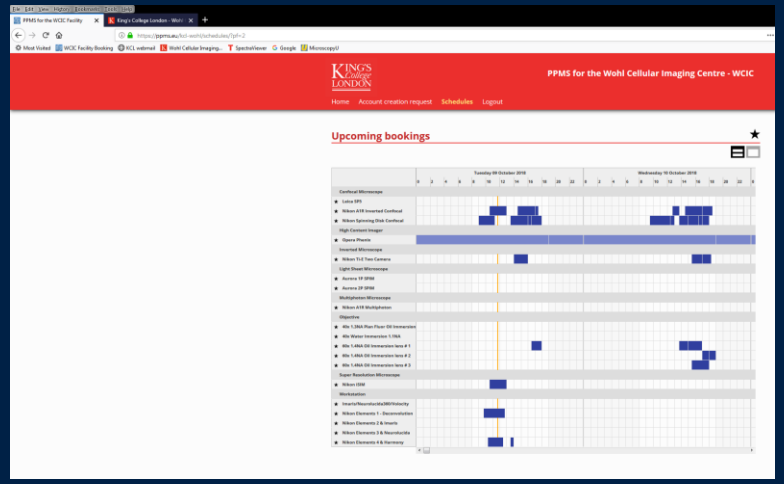
NO → Turn off system in reverse order.

If the incubator is on do not turn the incubator off.

This can then be accessed from the workstations and personal computers.

If you have trouble connecting to the shared drive, please login to the booking system, go to documents and the 3rd document contains instructions.

Connect to internet and click on booking system in favourites, no need to login, go to schedules.



STEP BY STEP INSTRUCTIONS

More advanced instructions

...reuse previous camera settings

What if you are imaging similar samples and want to re-use camera settings you've optimised before...

1) In NIS Elements software, open a previous image with camera settings you want to mimic.

2) Right click on the image once it's open

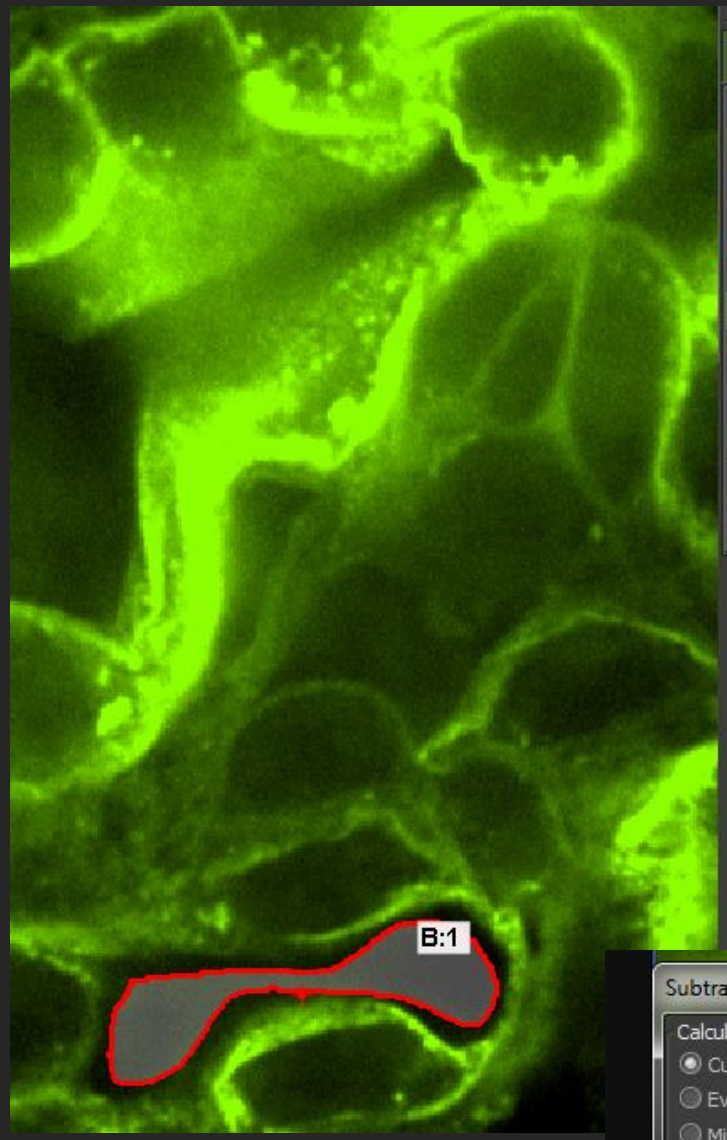
3) Select reuse camera settings

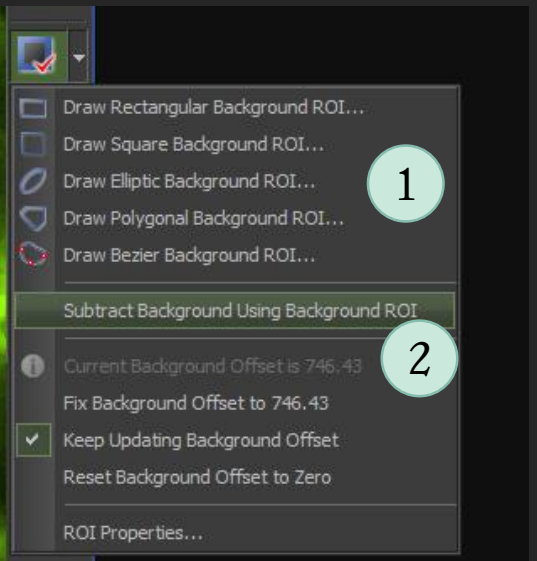
4) Be aware, this uploads camera settings only, acquisition setting (Z-stack, large image etc.) will not be reloaded

Time measurement

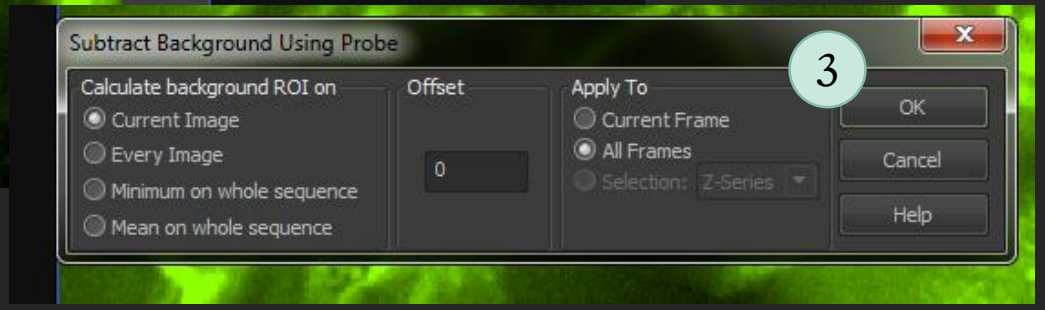
Time measurement (found under the measurement tab) can be used after time-lapse imaging to determine the rate of sample bleaching or recovery.

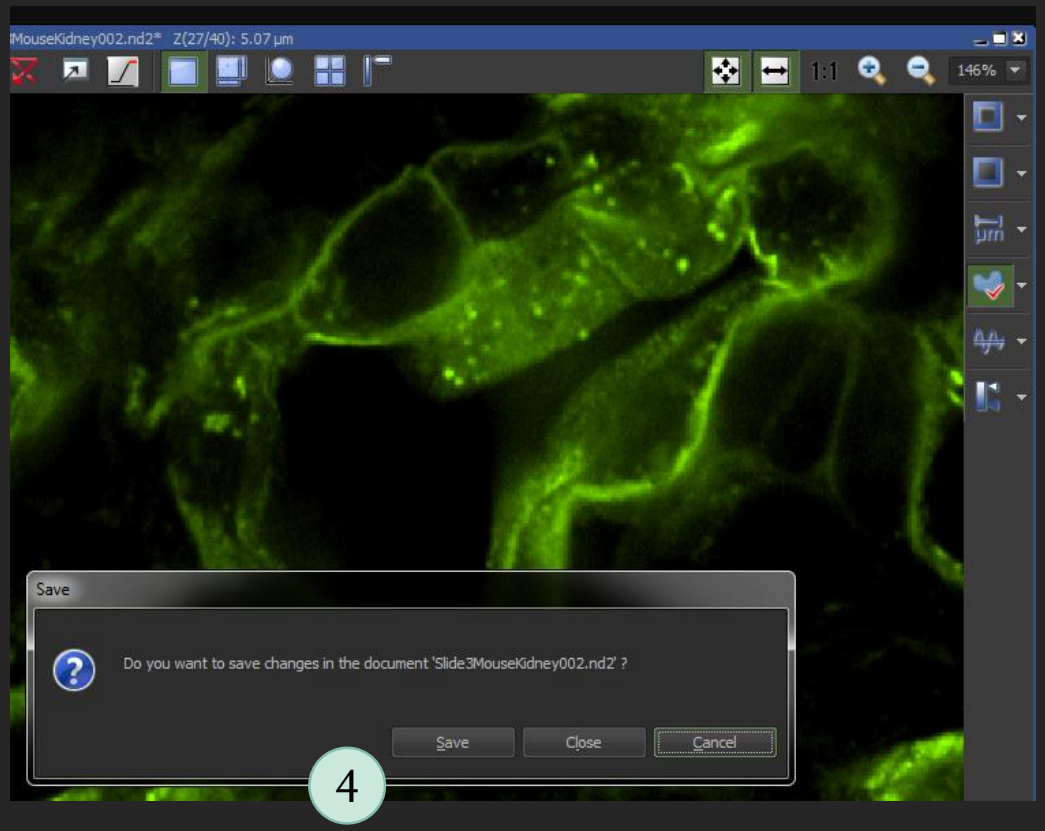
Removing Background (Changes to ROIs can be implemented at workstations/FIJI after your session)





Use the ROI options on the right of your captured image. This option allow you to subtract any background auto-fluorescence.





After any ROI changes, the new image will not be automatically saved. Form a good habit of “Save As” the new image with a new file name reflecting what changes you made to the raw data.

Just remember…

Make sure the stage is EMPTY before turning on the microscope switch at the right, far back.

Always put the toggle back to the RIGHT after you're done and before you start to focus.

Always LOWER the lenses as far as they can go before inserting the stage and/or clicking on another lens.

Always switch from COARSE to FINE when oil / water is in contact with lens.

Increase gain (HV) and laser power. (In SMALL INCREMENTS for green and red channels)

DO NOT increase gain (HV) for green and red channels to above 50 !!!

Acquisition panel XY positions: always DELETE ALL previous positions before you start !

DO NOT USE USBs on any computers in the microscope rooms!

If you need any help, please contact:

George Chennell (07771926760)

or

Chen Liang (07883166321) via WhatsApp

Happy Imaging!