Calcium Microscope

KING'S College LONDON

Wohl Cellular Imaging Centre

Nikon Ti-E 3-Camera Calcium Widefield Microscope

Calcium Mic - STEP BY STEP INSTRUCTIONS CONTENTS PAGE

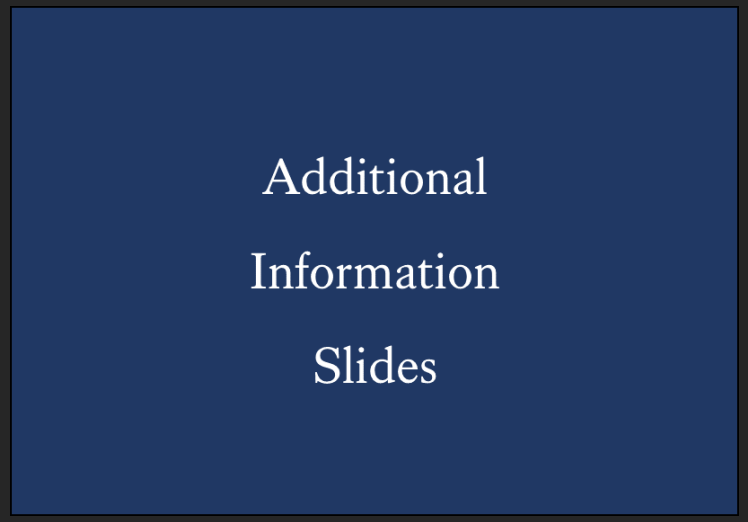
Before Using The Facility…

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 (at a later date)
- Access to booking system: http://ppms.eu/kcl-wohl

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

The Equipment

DAPI/GFP/RFP/Far-red LAMP

Brightfield

DG4 FURA-2 LAMP

Camera 7C

Camera 7B

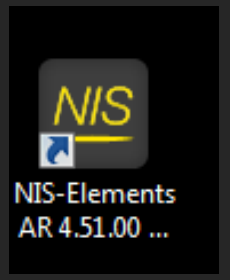
Camera 7A

System On

1. Switch on the Calcium Microscope depending on the function you need **(consult the flowchart on the wall)** and follow 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.**

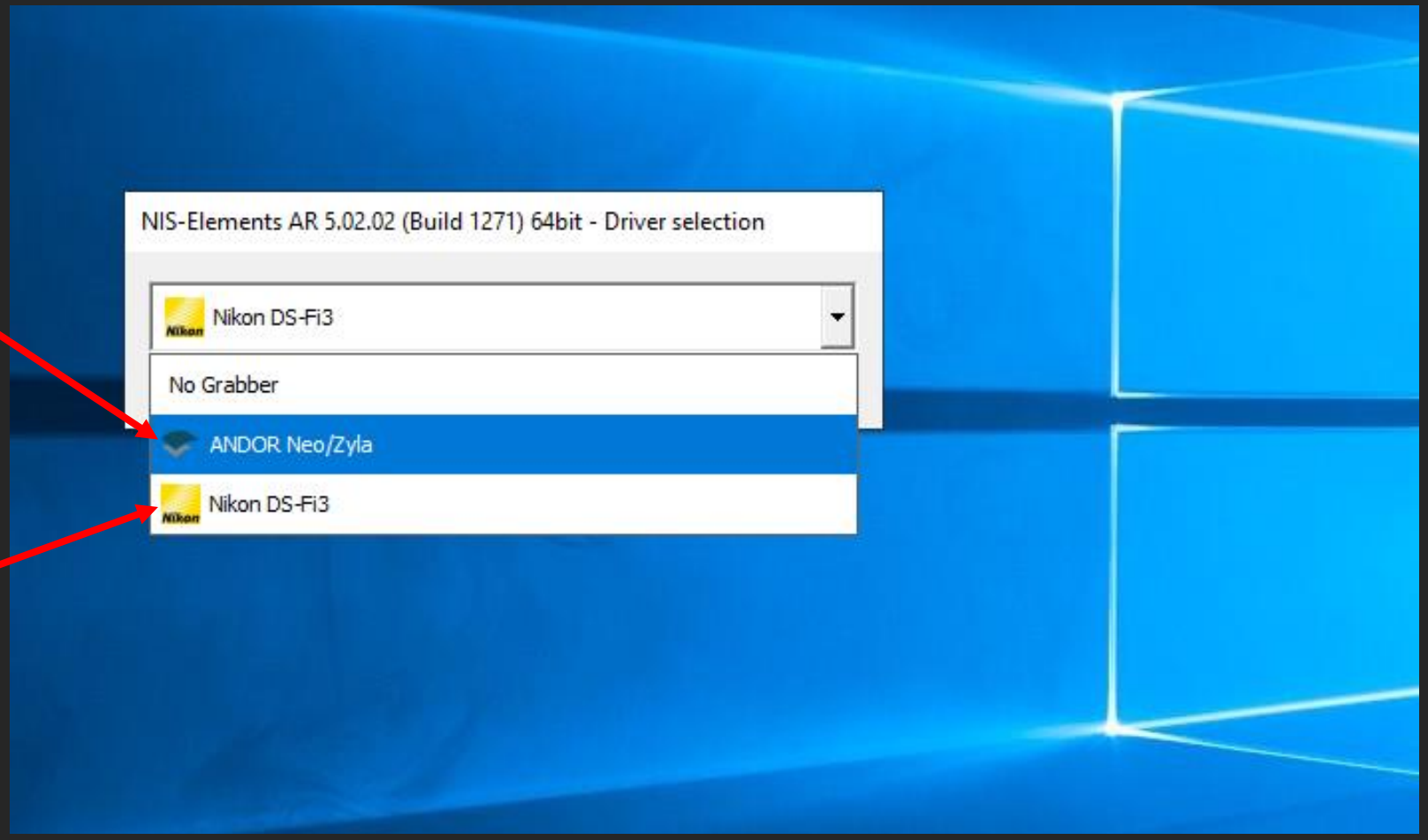


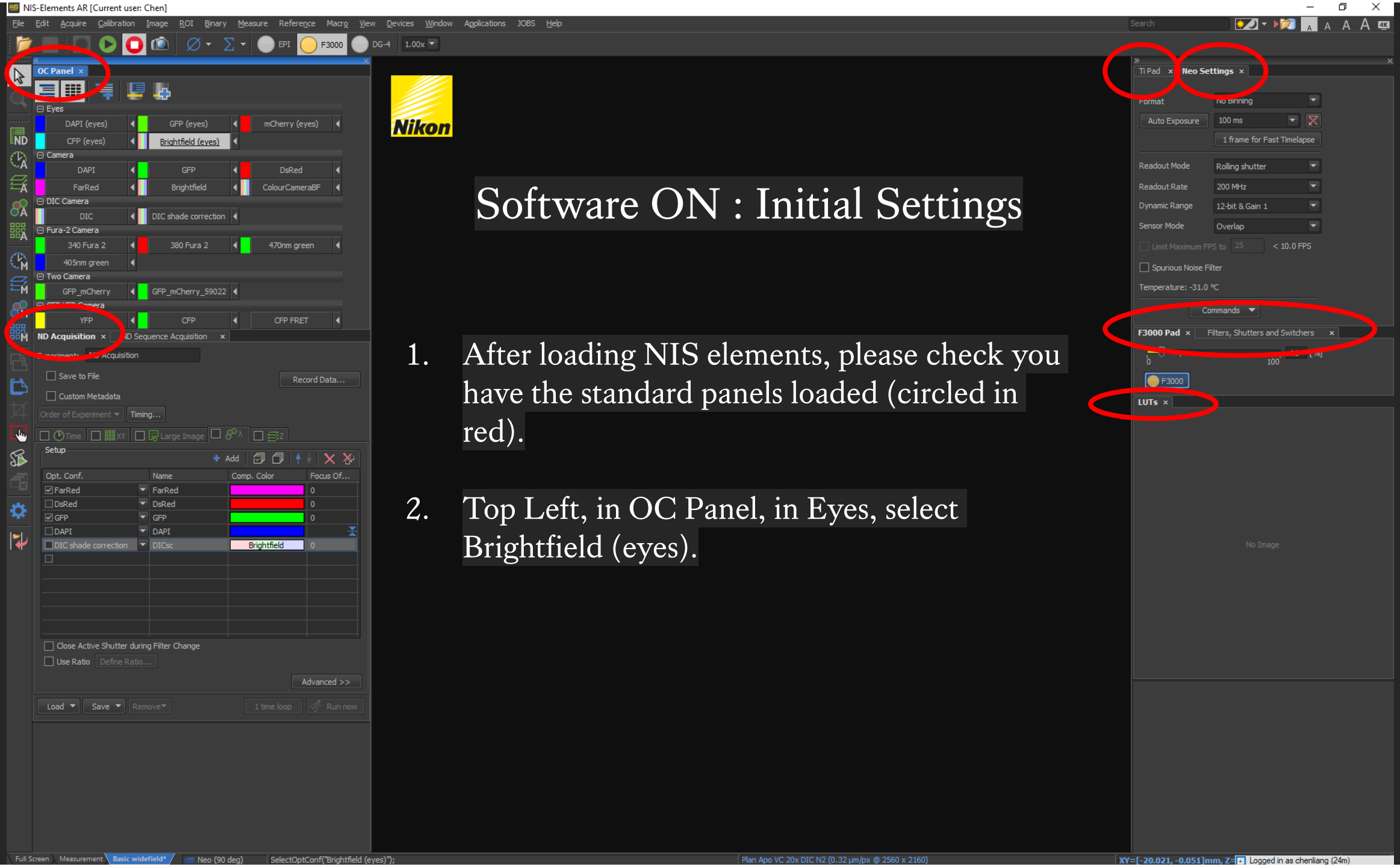
Software ON : Choose A Camera

NIS elements will ask you to choose a camera to use:

ANDOR Neo/Zyla
Switch number 7A
(7A and 7B if you are doing two-camera imaging) Is for Brightfield / DIC / DAPI / GFP / RFP / Far-Red / CFP / YFP / FURA-2

Nikon DS-Fi3
Switch number 7C
Is for coloured imaging.





Software ON : Initial Settings

1. After loading NIS elements, please check you have the standard panels loaded (circled in red).

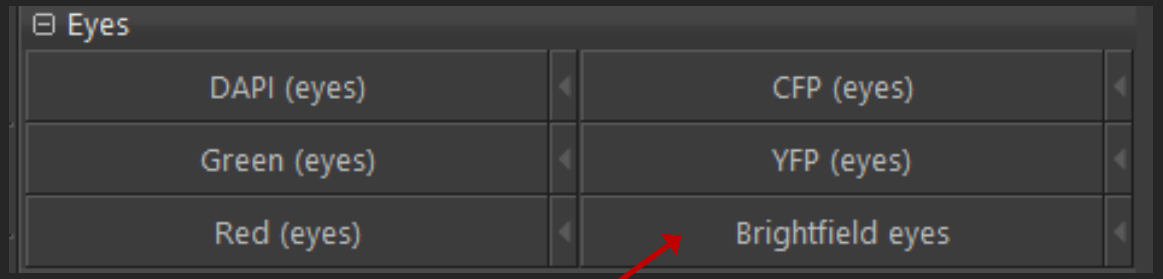
2. Top Left, in OC Panel, in Eyes, select Brightfield (eyes).

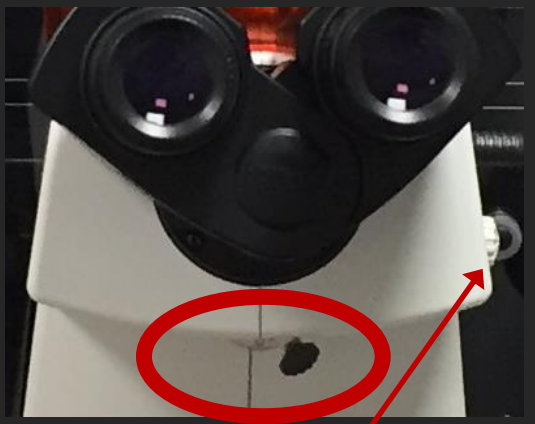
STEP BY STEP INSTRUCTIONS

STEP 2

Lens And Focus

Checking Lens for Damage and Cleanliness





Make sure you are on brightfield eyes and white light is coming through. 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

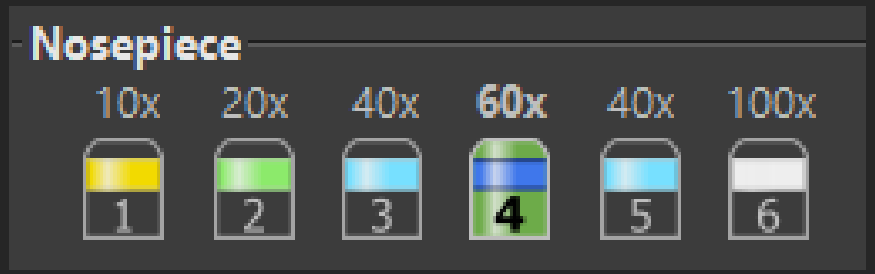
after you're done and before you start to focus.

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 ethanol 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>.

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 to when the light just flash on 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.
- 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.

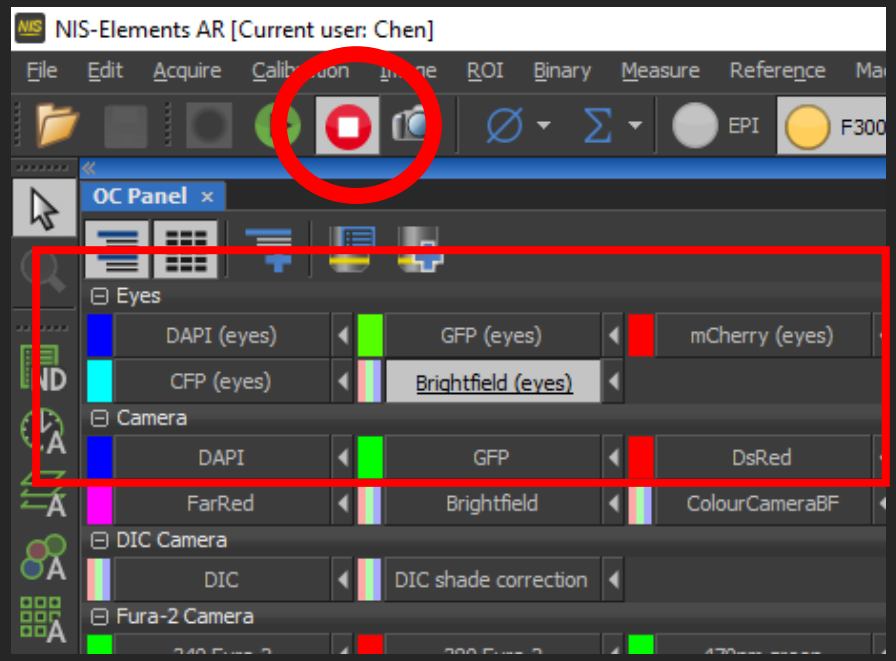
STEP BY STEP INSTRUCTIONS

STEP 3

Change from Eyes to Camera View

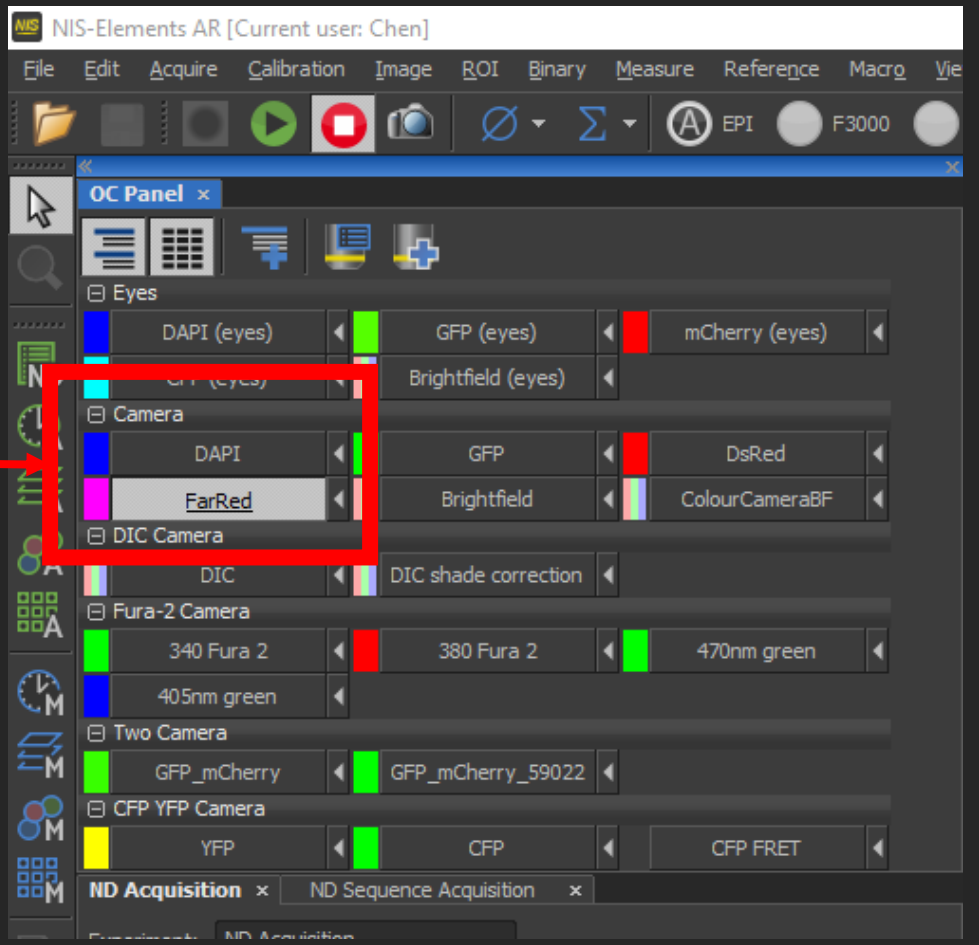
From this point forward we will be adjusting things on the computer screen so we need to work in camera mode instead of looking down the eye piece.

Remain on STOP setting.



Click on a ‘camera’ option

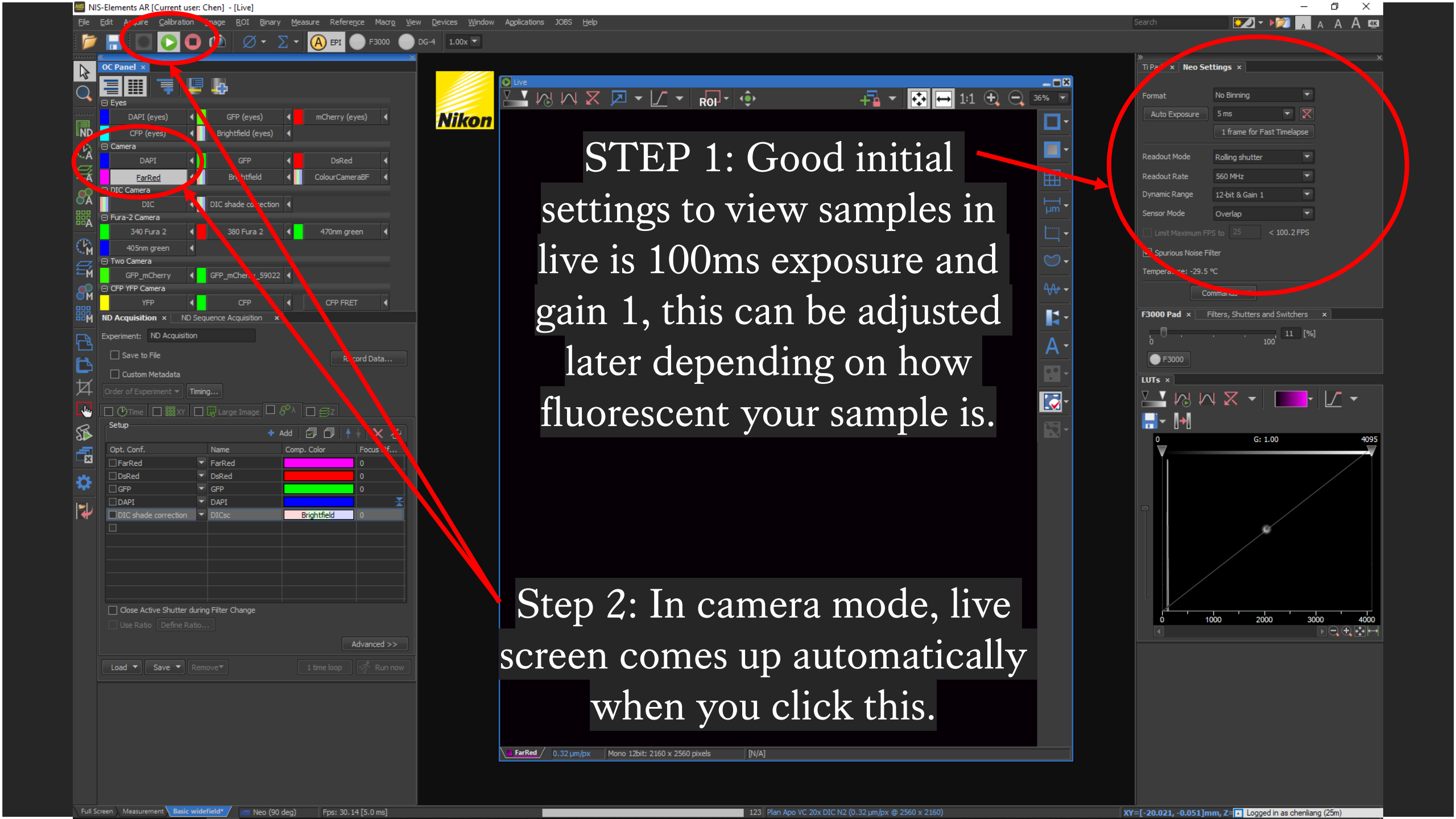
This moves from eyes to camera.

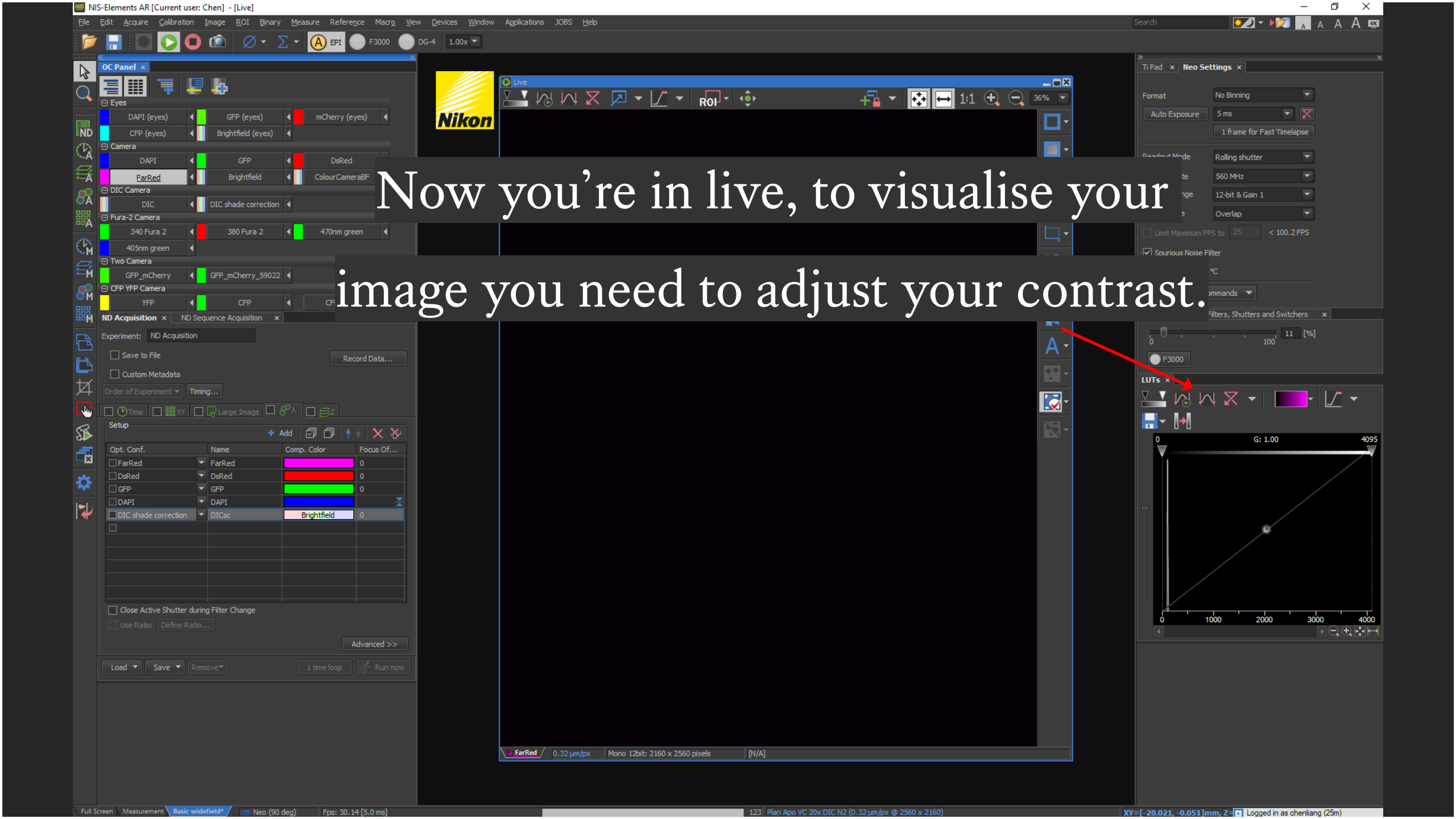


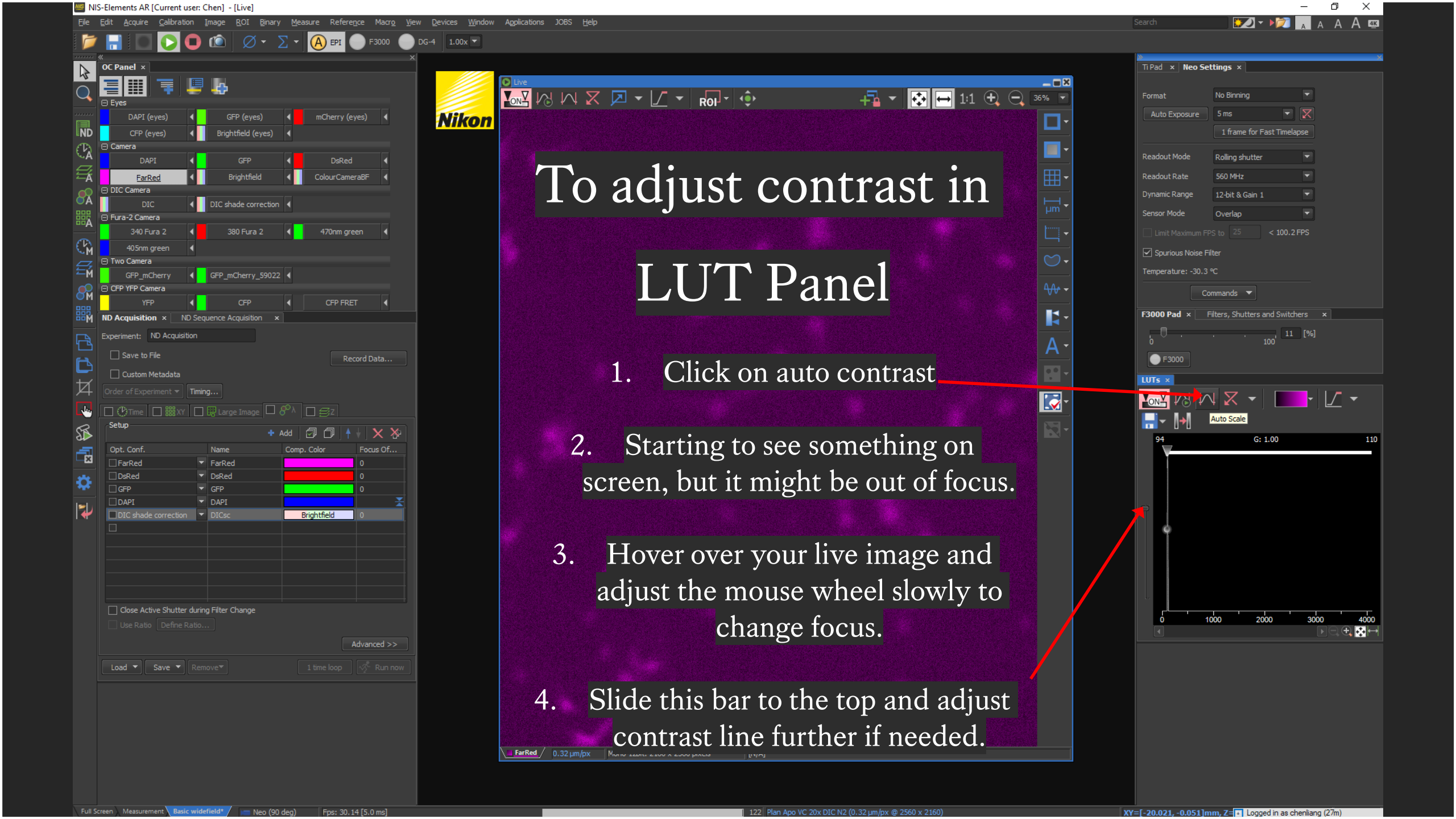
STEP BY STEP INSTRUCTIONS

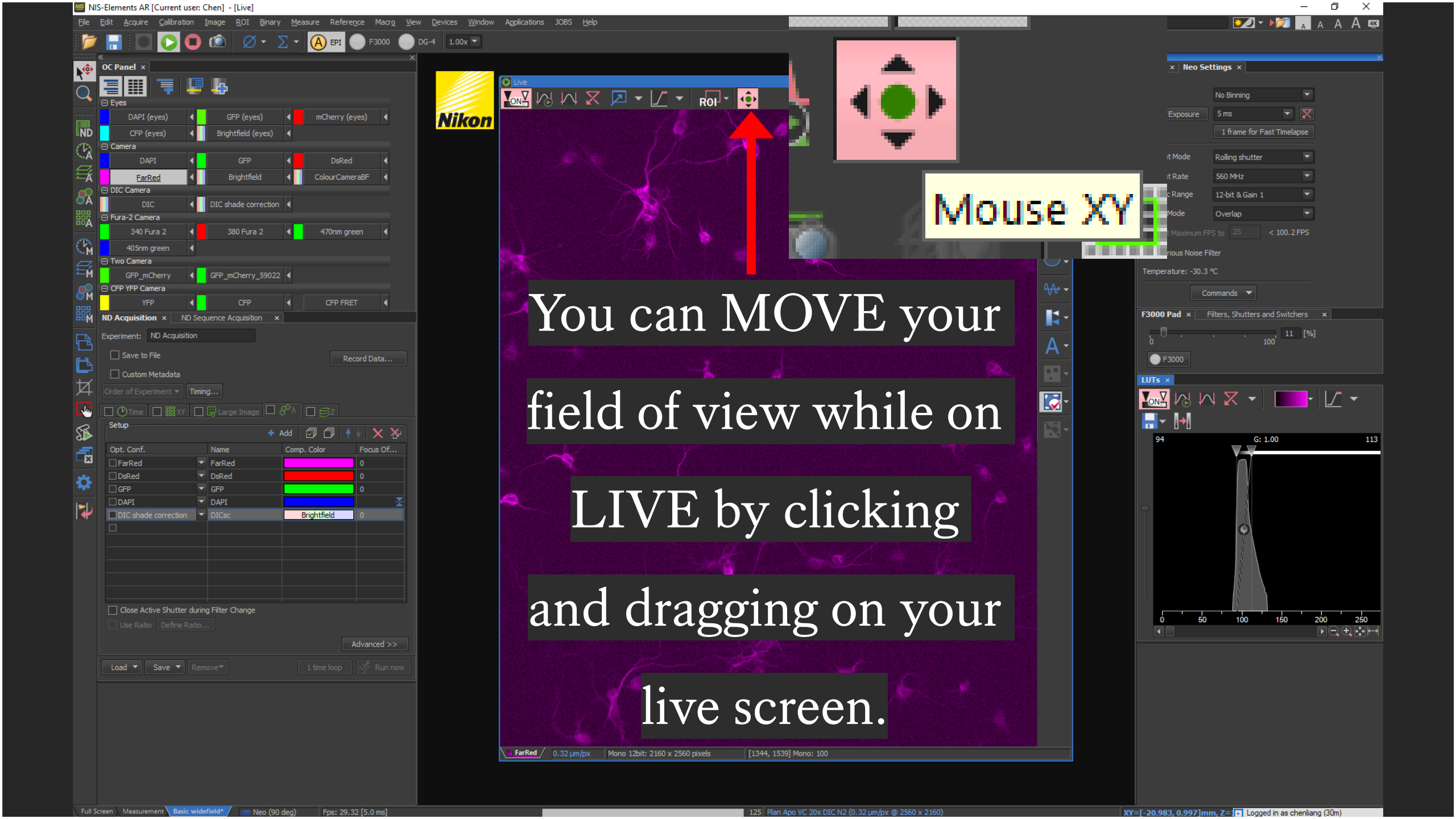
STEP 4

Setting Up Initial Live View


STEP 1: Good initial settings to view samples in live is 100ms exposure and gain 1, this can be adjusted later depending on how fluorescent your sample is.
Step 2: In camera mode, live screen comes up automatically when you click this.


Now you're in live, to visualise your
image you need to adjust your contrast.


To adjust contrast in
LUT Panel
1. Click on auto contrast
2. Starting to see something on screen, but it might be out of focus.
3. Hover over your live image and adjust the mouse wheel slowly to change focus.
4. Slide this bar to the top and adjust contrast line further if needed.
Auto Scale


NIS-Elements AR [Current user: Chen] - [Live]
File Edit Acquire Calibration Image ROI Binary Measure Reference Macro View Devices Window Applications JOBS Help
OC Panel
Eyes
DAPI (eyes)
GFP (eyes)
mCherry (eyes)
CFP (eyes)
Brightfield (eyes)
Camera
DAPI
GFP
DsRed
FarRed
Brightfield
ColourCameraBF
DIC Camera
DIC
DIC shade correction
Fura-2 Camera
340 Fura 2
380 Fura 2
470nm green
405nm green
Two Camera
GFP_mCherry
GFP_mCherry_59022
CFP YFP Camera
YFP
CFP
CFP FRET
ND Acquisition
ND Sequence Acquisition
Experiment: ND Acquisition
Save to File
Record Data...
Custom Metadata
Order of Experiment
Timing...
Setup
Add
Opt. Conf.
Name
Comp. Color
Focus Of...
FarRed
DsRed
GFP
DAPI
DIC shade correction
DICsc
Brightfield
Close Active Shutter during Filter Change
Use Ratio
Define Ratio...
Advanced >>
Load
Save
Remove
1 time loop
Run now
Nikon
Live
ROI
Mouse XY
You can MOVE your
field of view while on
LIVE by clicking
and dragging on your
live screen.
FarRed
0.32 µm/px
Mono 12bit: 2160 x 2560 pixels
[1344, 1539] Mono: 100
Neo Settings
No Binning
Exposure
5 ms
1 frame for Fast Timelapse
Rolling shutter
560 MHz
12-bit & Gain 1
Overlap
Temperature: -30.3 °C
Commands
F3000 Pad
Filters, Shutters and Switchers
F3000
LUTs
G: 1.00
Full Screen
Measurement
Basic widefield*
Neo (90 deg)
Fps: 29.32 [5.0 ms]
Plan Apo VC 20x DIC N2 (0.32 µm/px @ 2560 x 2160)
Logged in as chenliang (30m)

LUTs

Additional Information Slides...

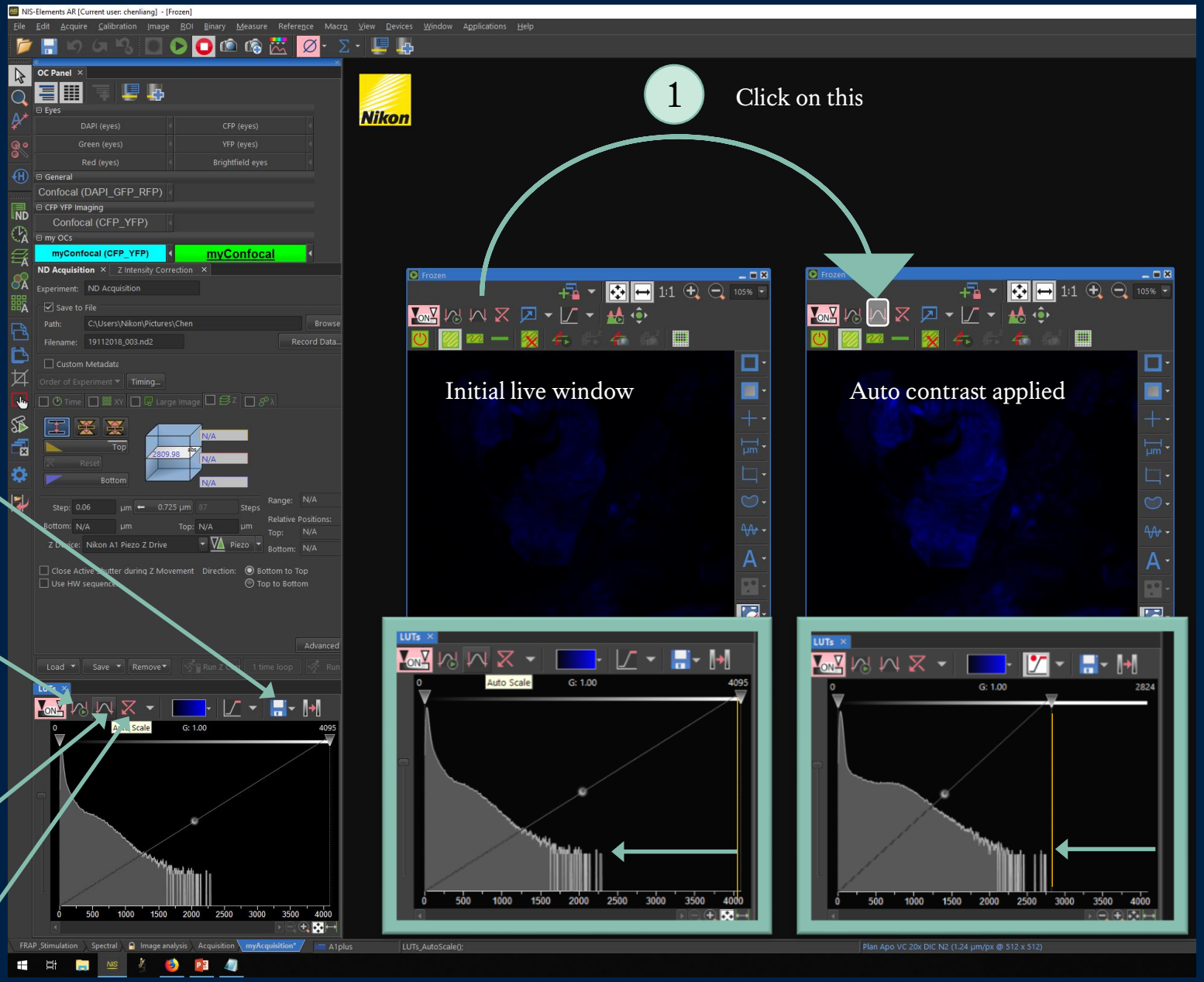
LUTs And Contrast

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.

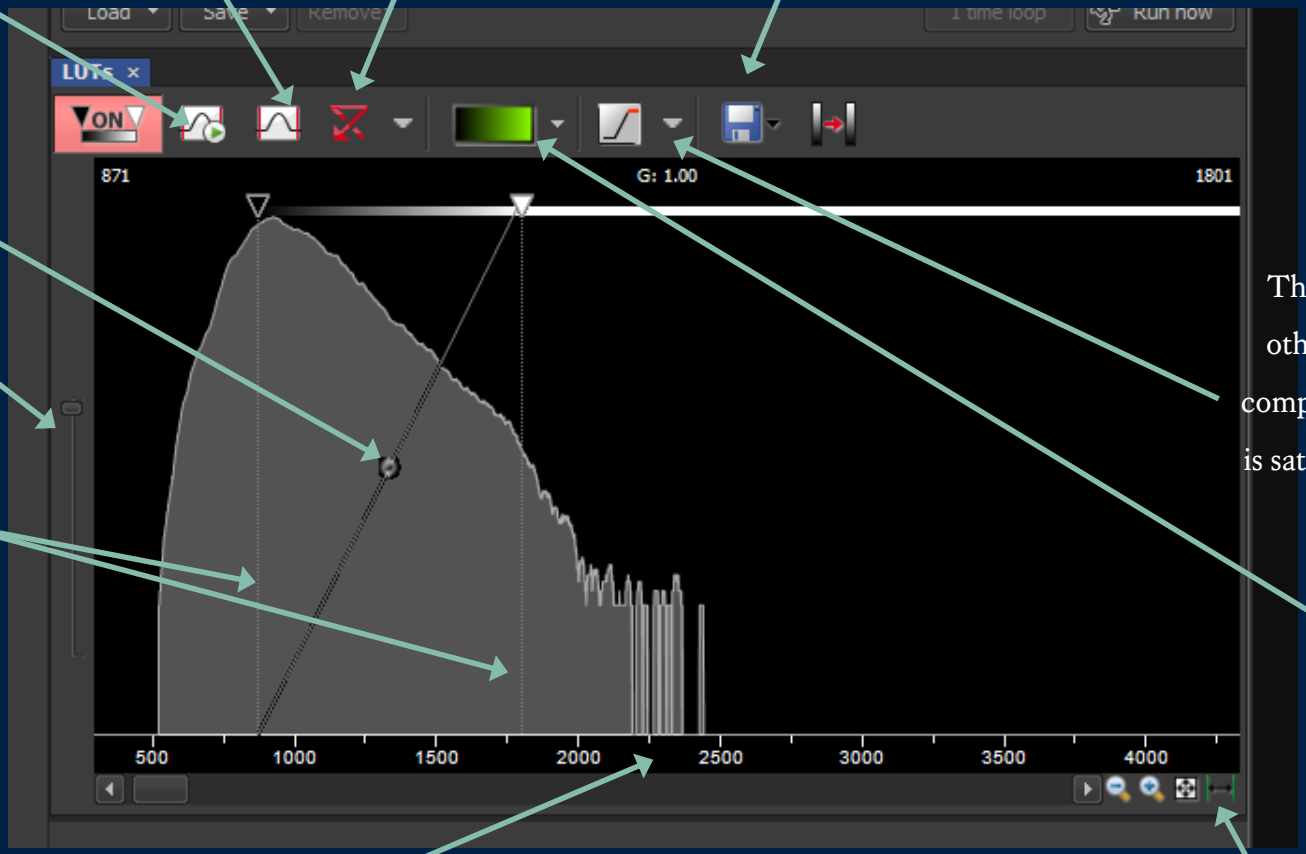
LUTs in more detail…

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 5

Optimising your camera settings

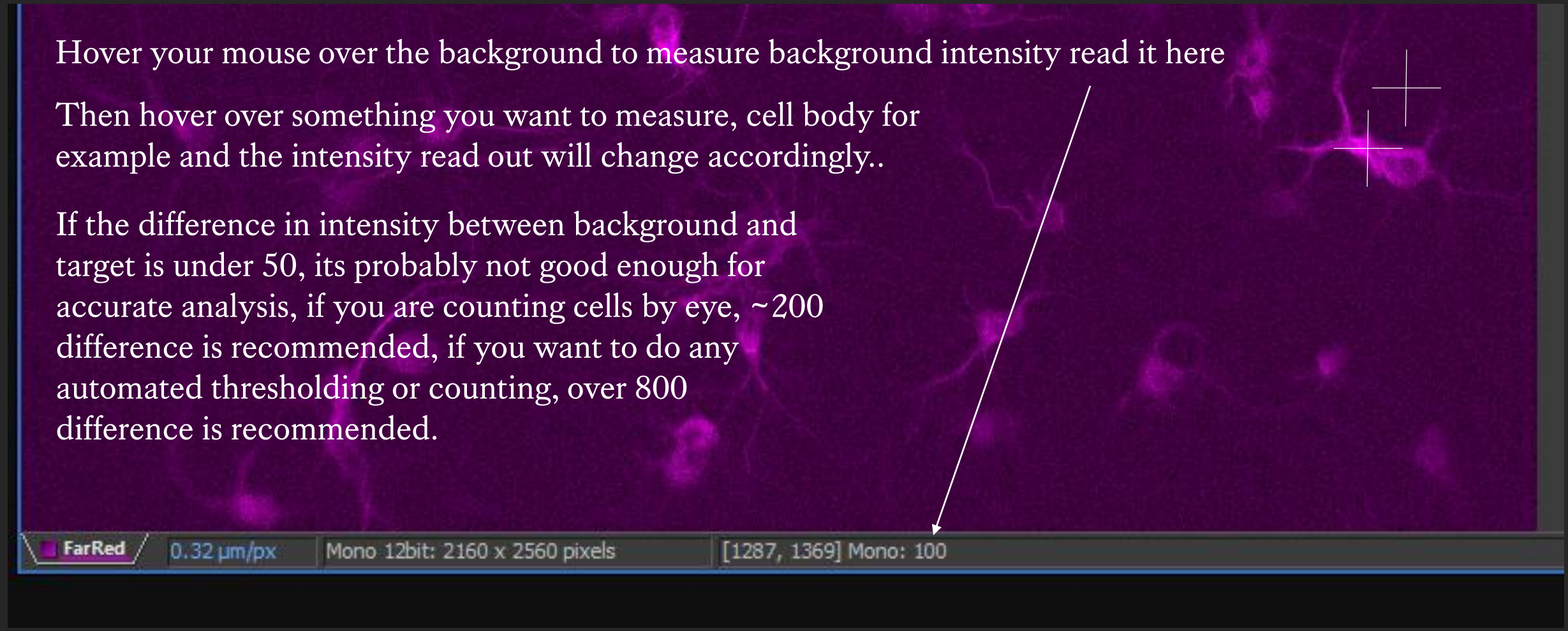
...when you don't have enough signal

Changing LUTs such as contrast does not change your raw data (i.e. it does not affect how much signal your sample is giving off)

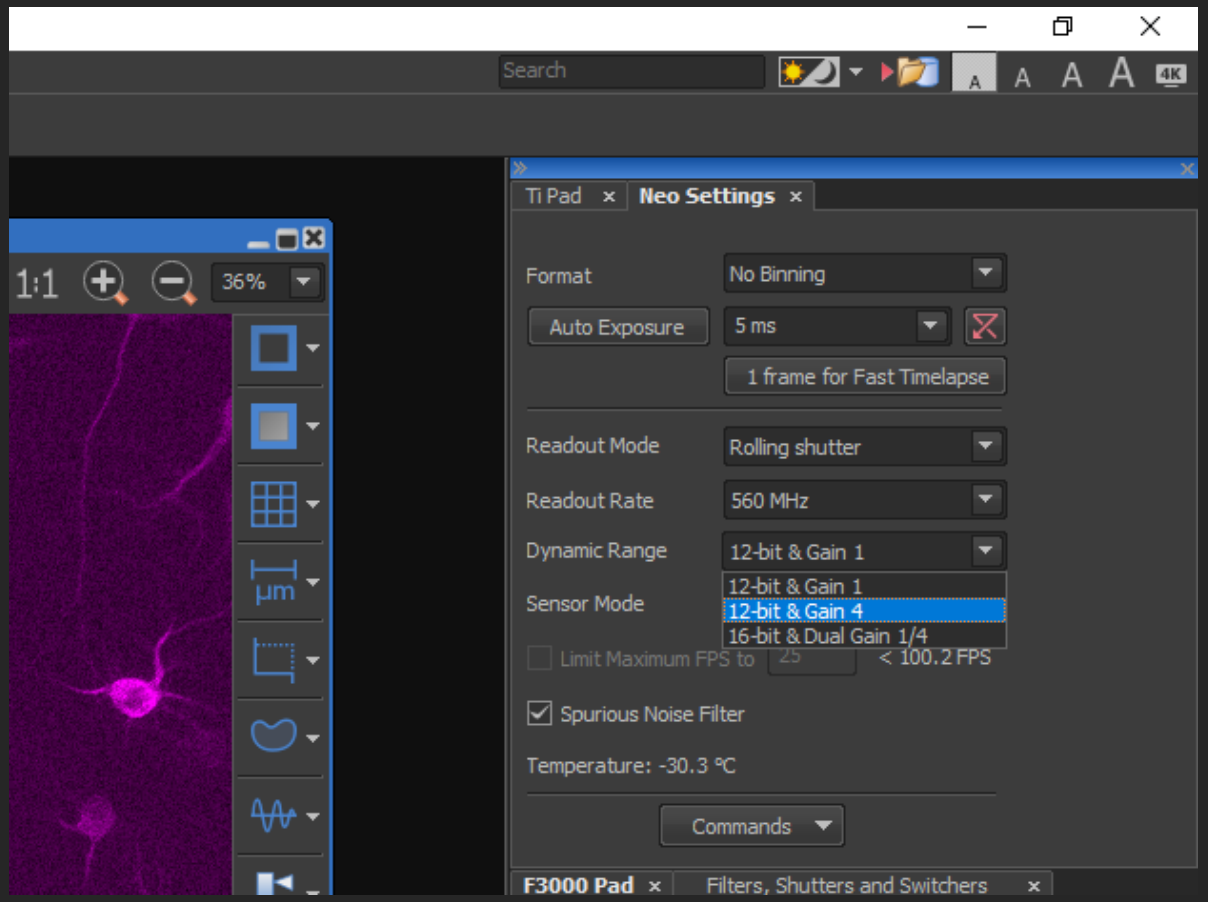
To optimise your signal, we need to optimise the camera settings.

How much signal is enough signal?

That really depends on what you want to measure…



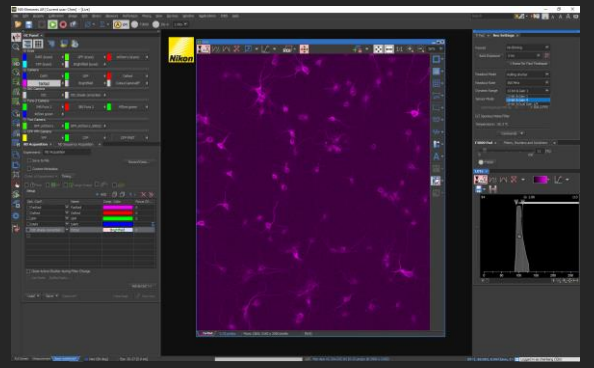
To increase signal intensity…



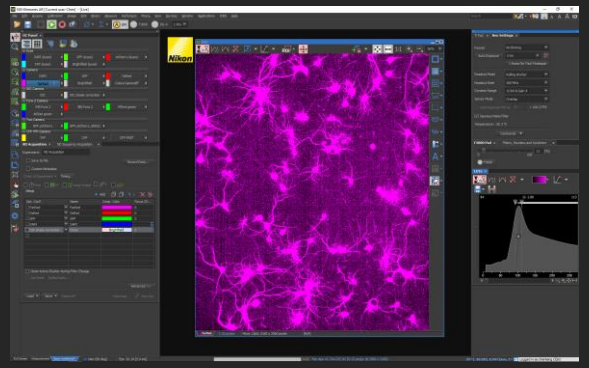
Step 1: Reduce exposure to ~10ms

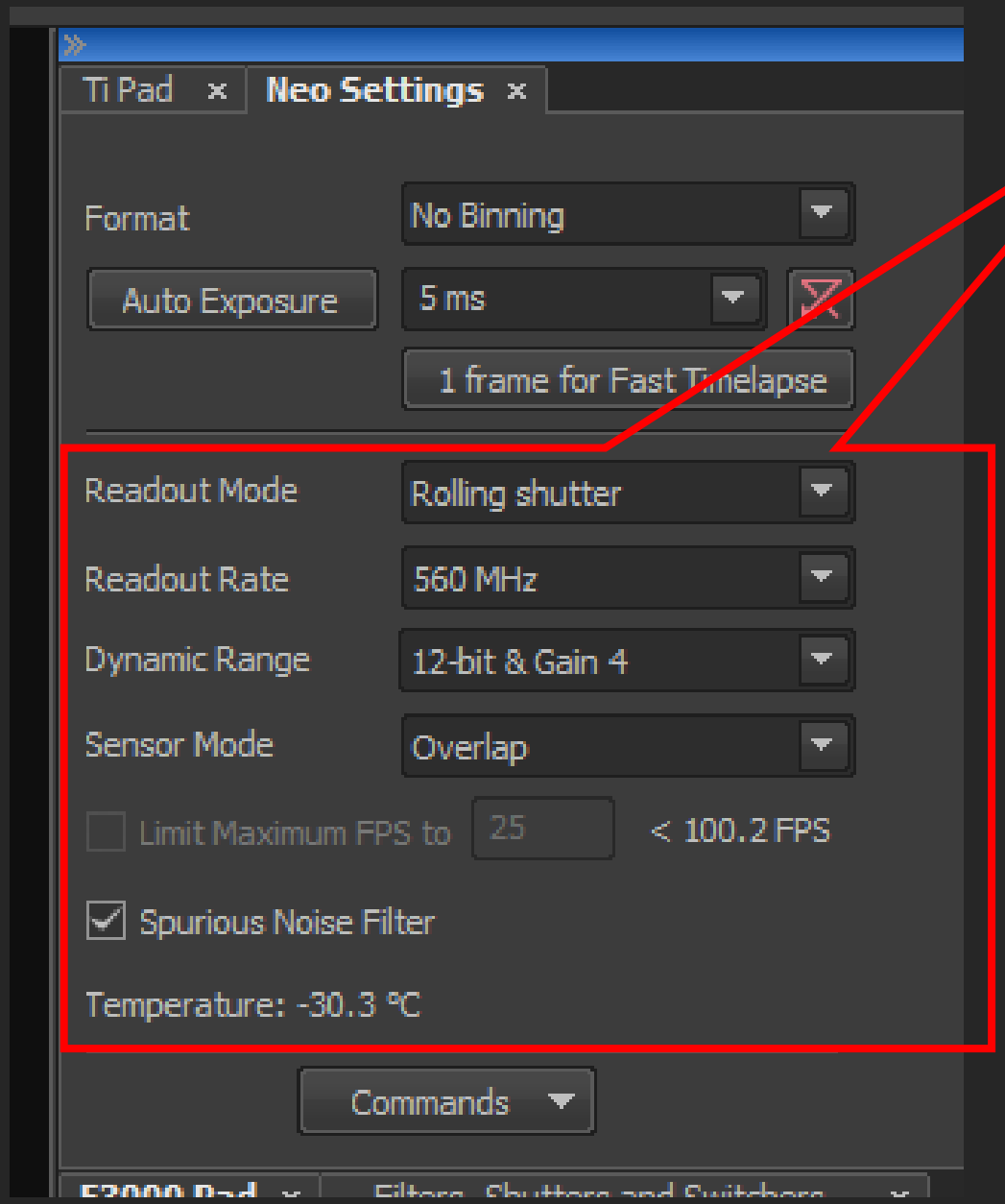
Step 2: Change Gain1 to Gain4 (this amplifies your signal drastically)

Gain 1



Gain 4



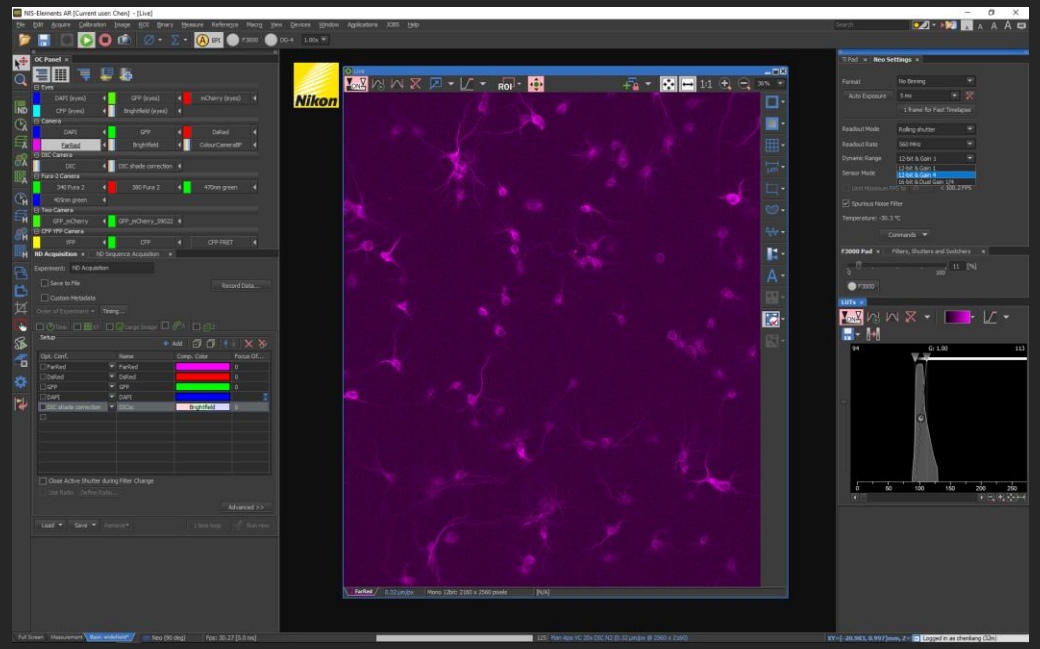


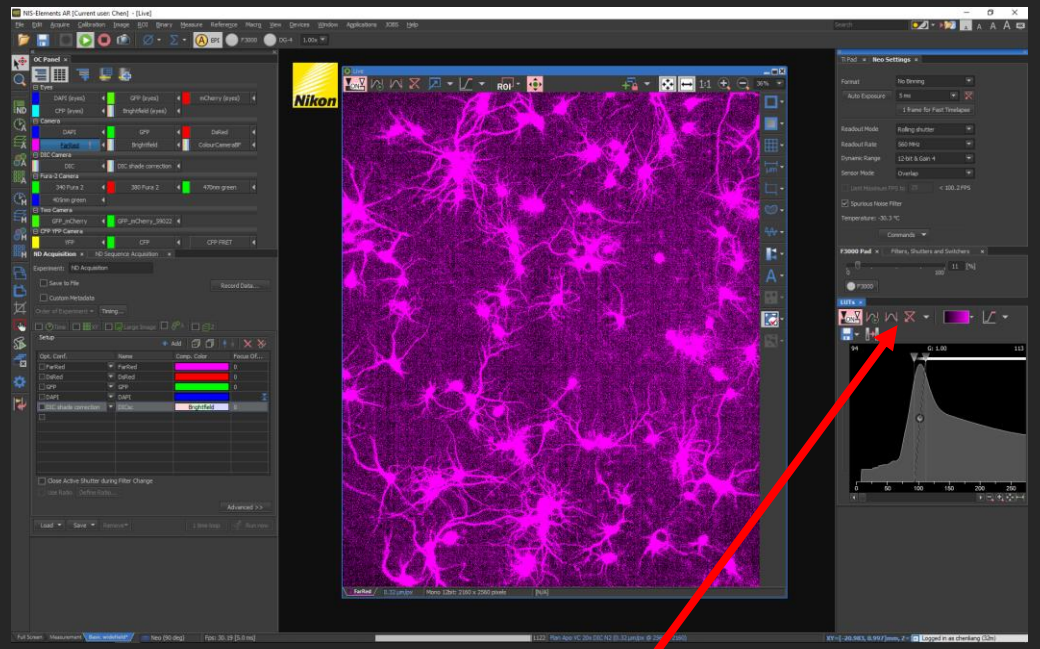
Gain (everything in this box) need to be the same across ALL the channels you are going to use.

I recommend using your weakest channel to set your gain, then for other channels if you have too much signal, just reduce your exposure time.

Gain 1

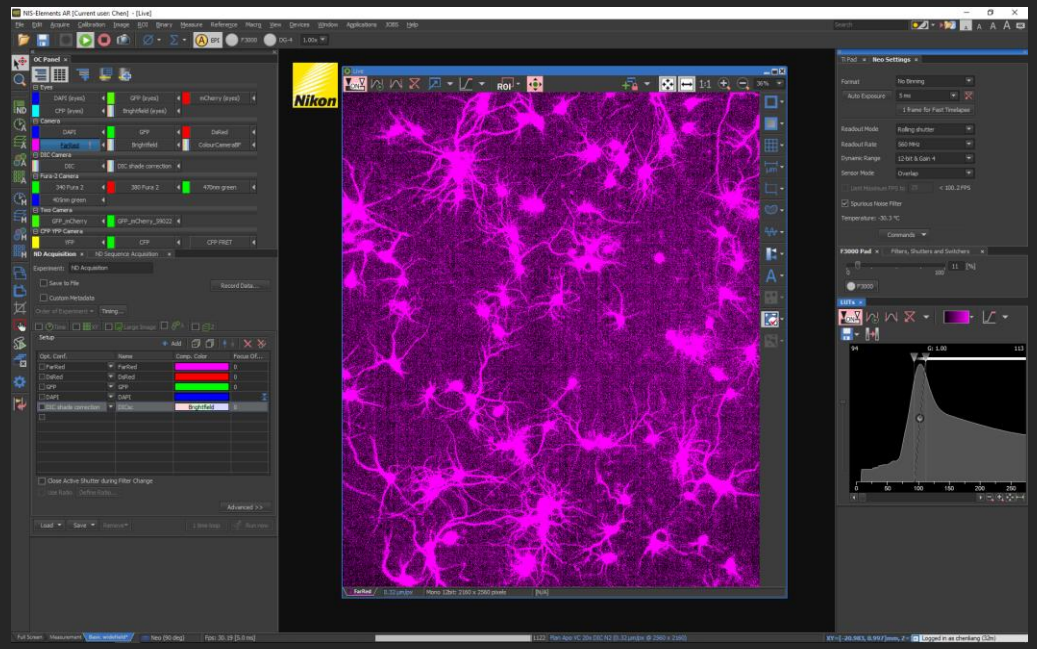
Gain 4



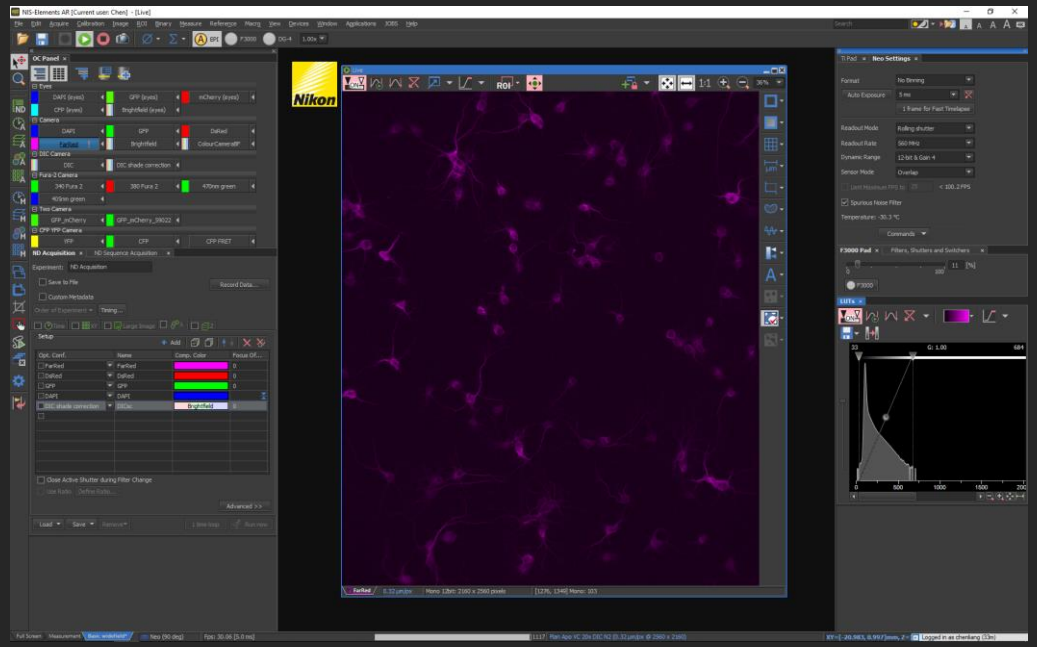


Remember to click on auto-contrast

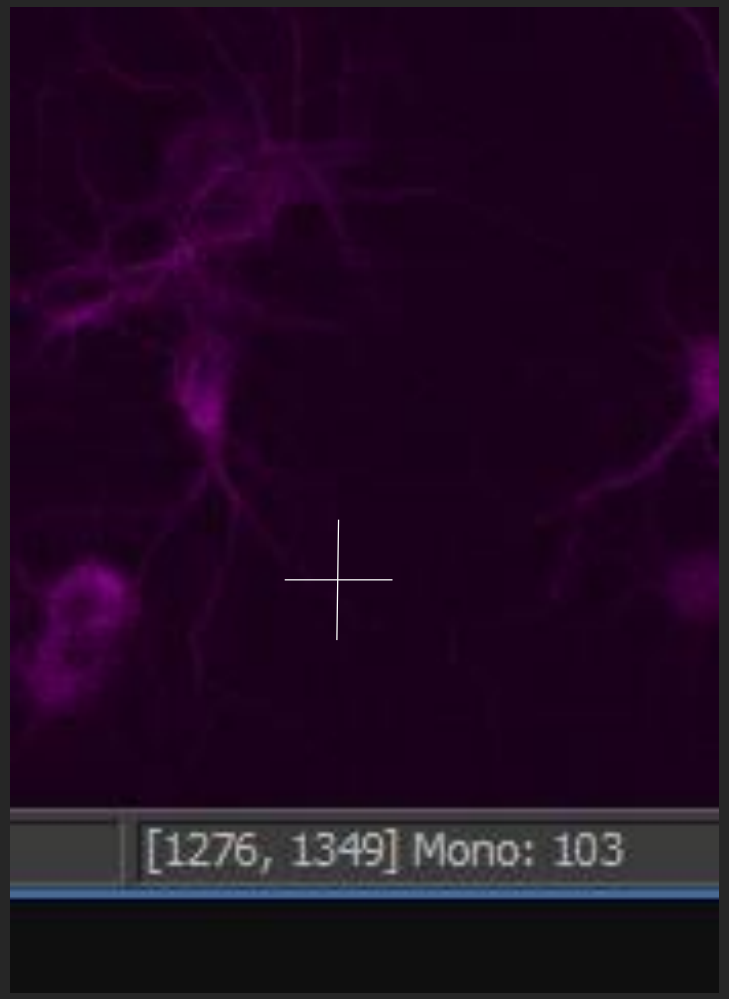
Gain 4

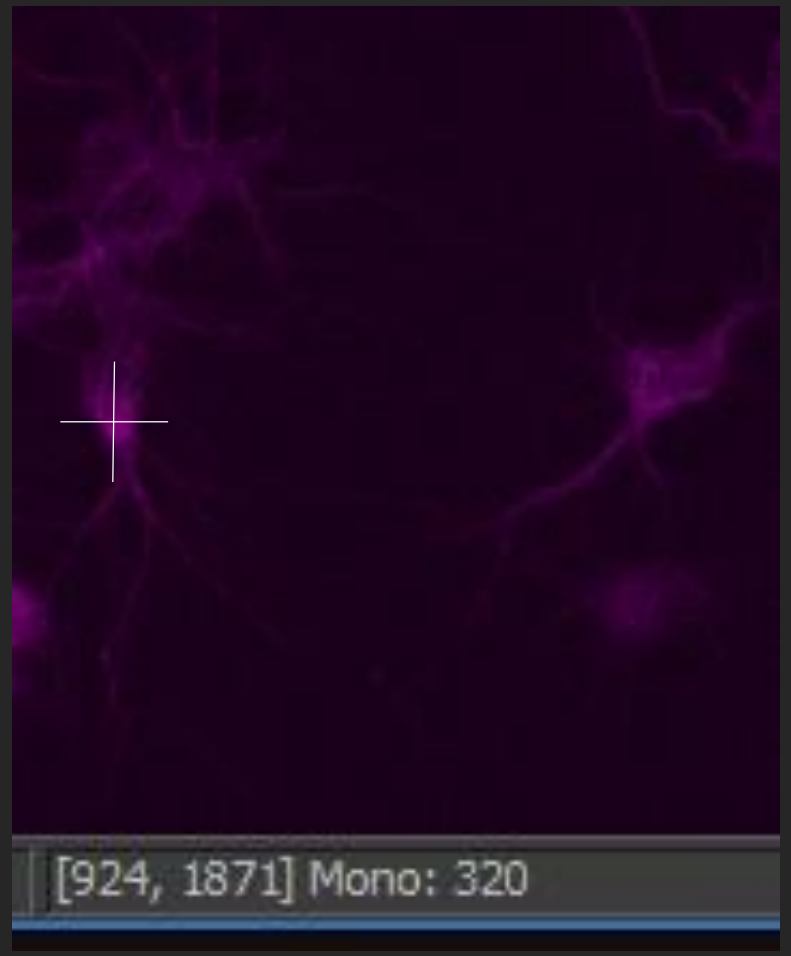


Gain 4



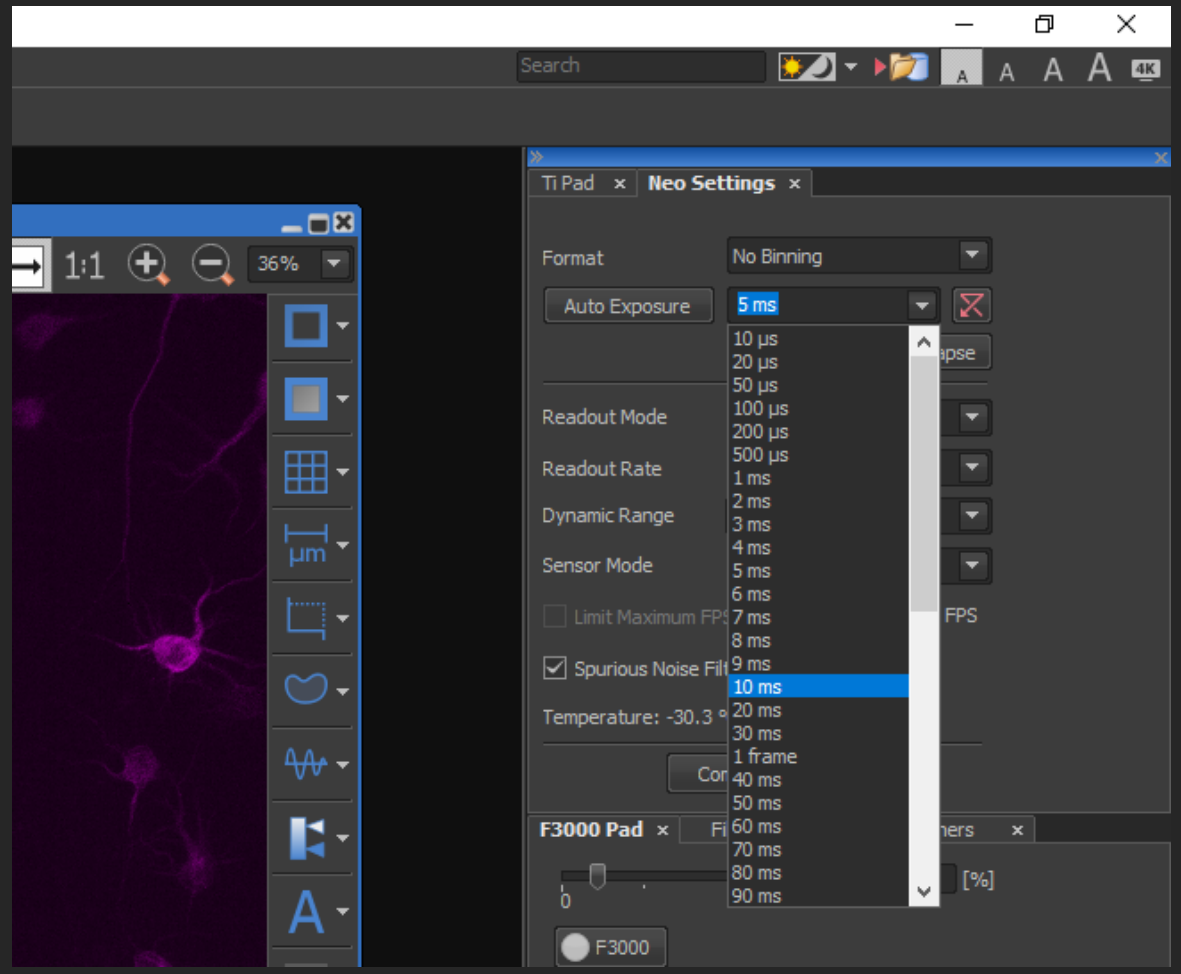
After auto-contrast

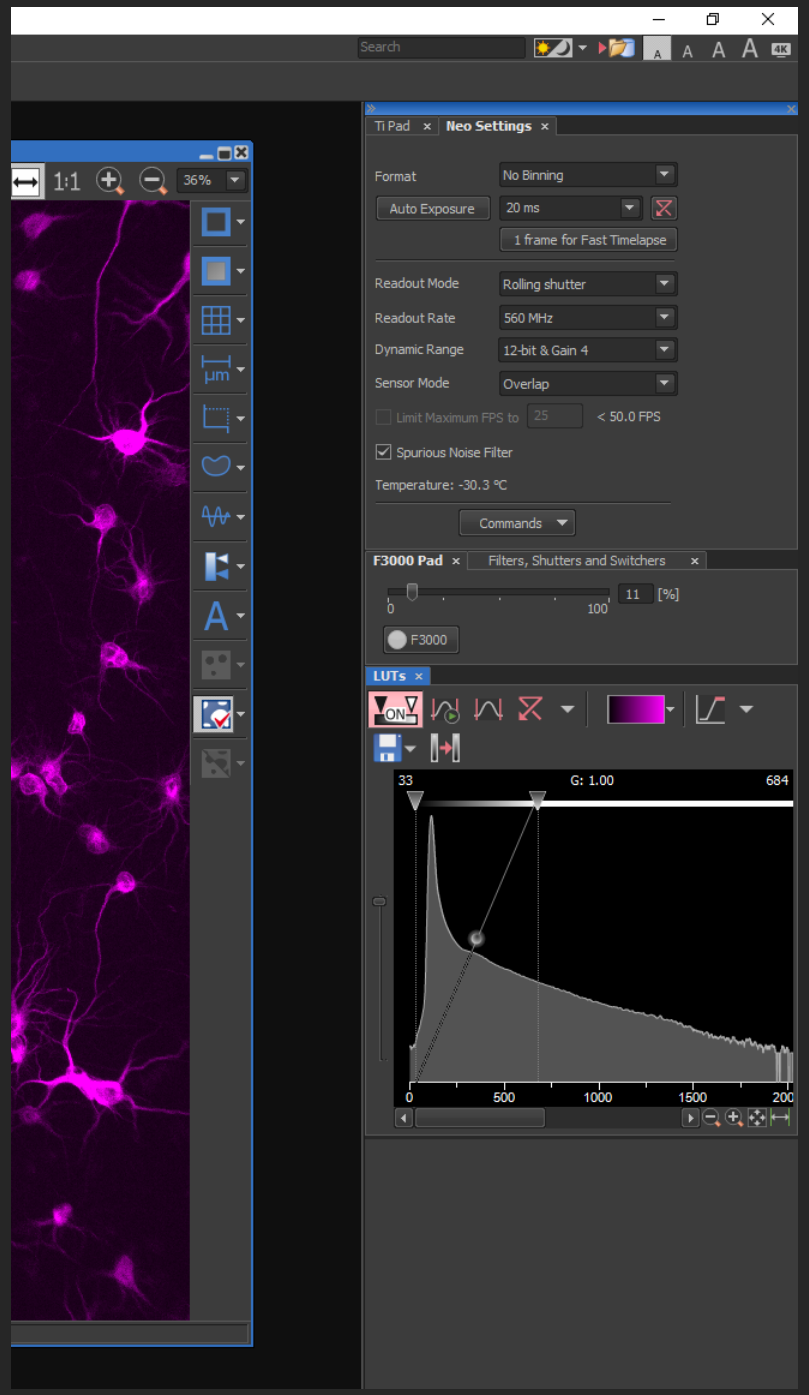




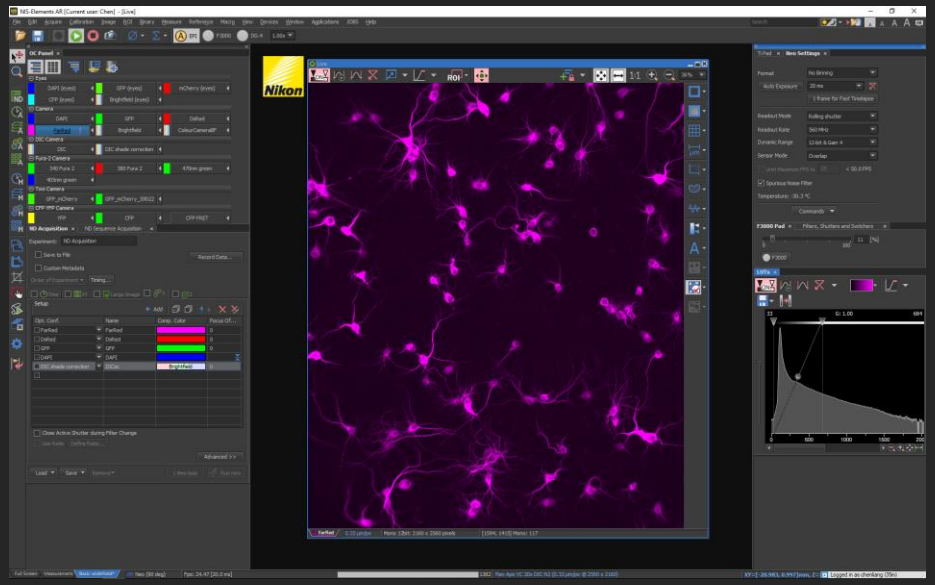
Now check background intensity and target intensity again, difference of ~200 is good for counting by eye but not for automated counting. Adjust the exposure to optimise further.

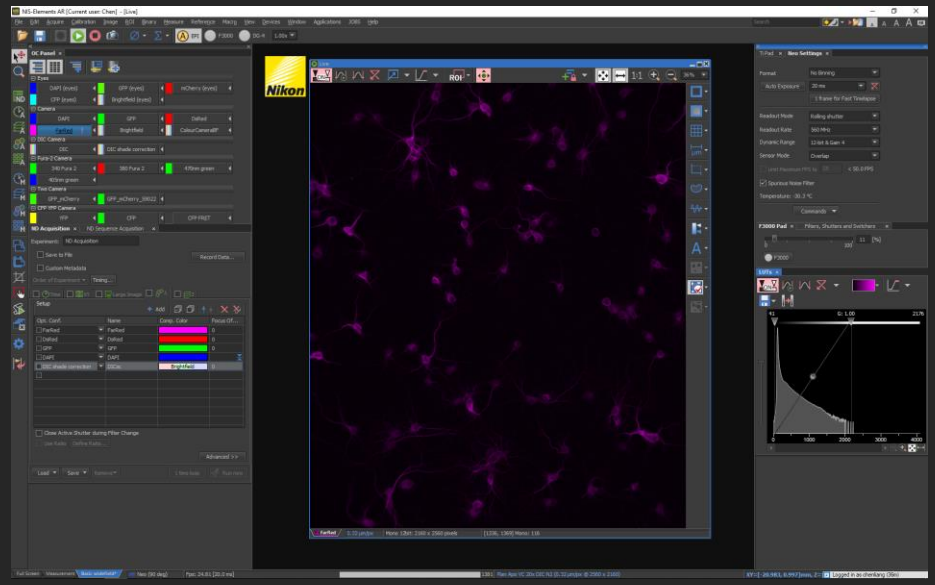
Increase exposure time to 20ms…



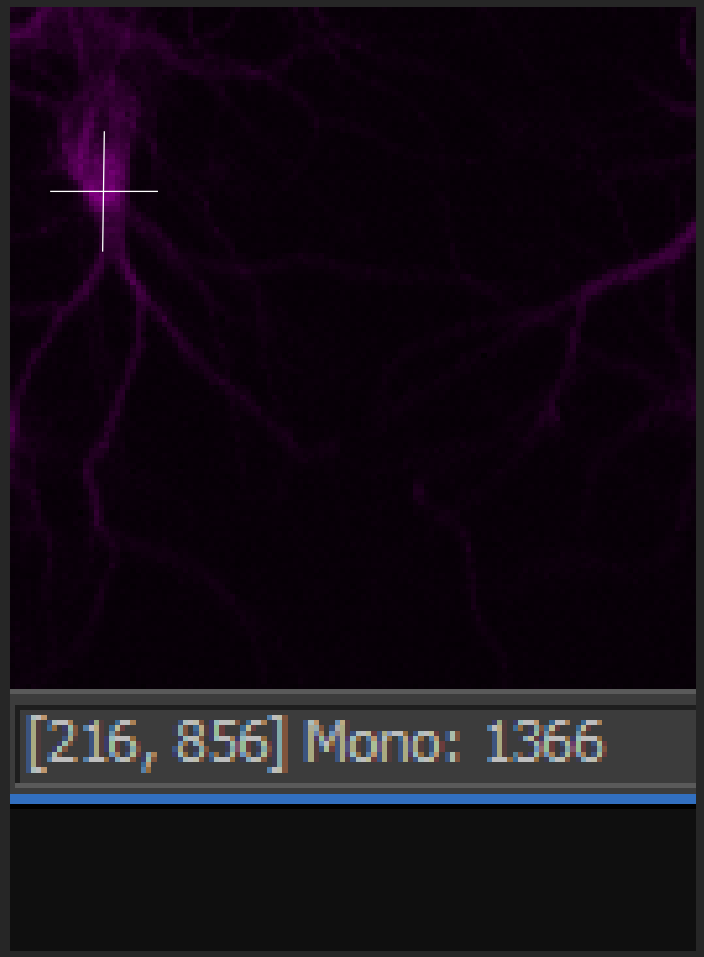


Auto-contrast and check intensity difference again.





>1000 difference between background and target intensities, usable for auto thresholding.



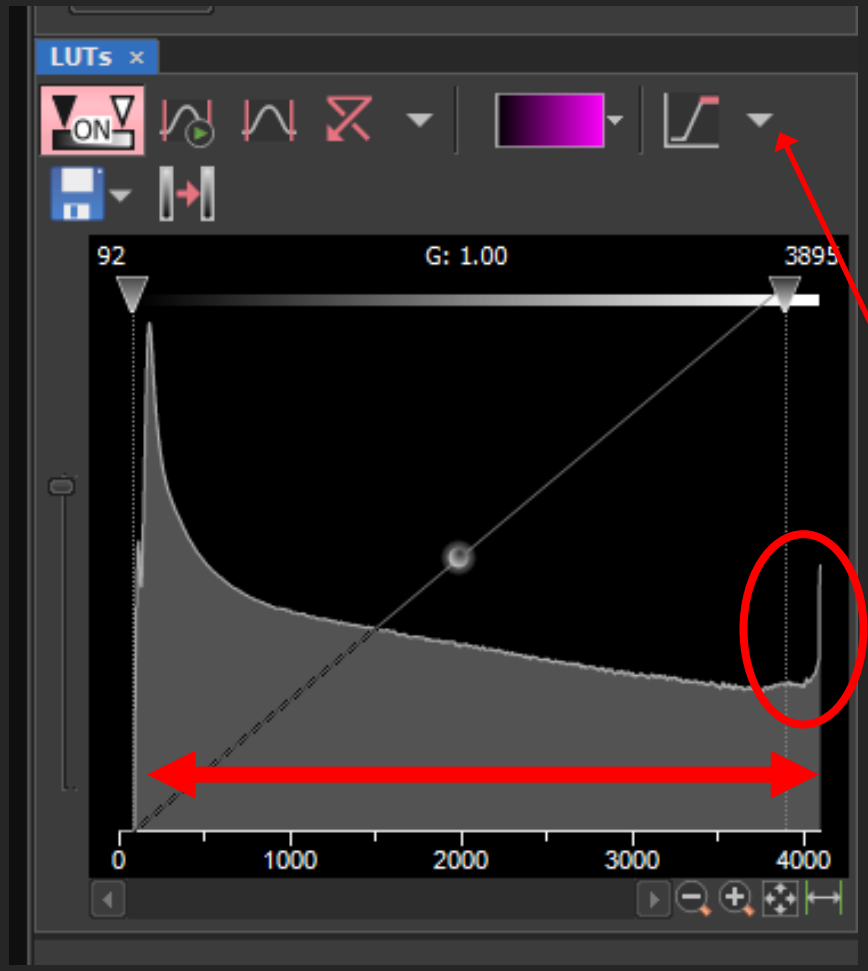
STEP BY STEP INSTRUCTIONS

STEP 5

Optimising your camera settings

...when you have too much signal

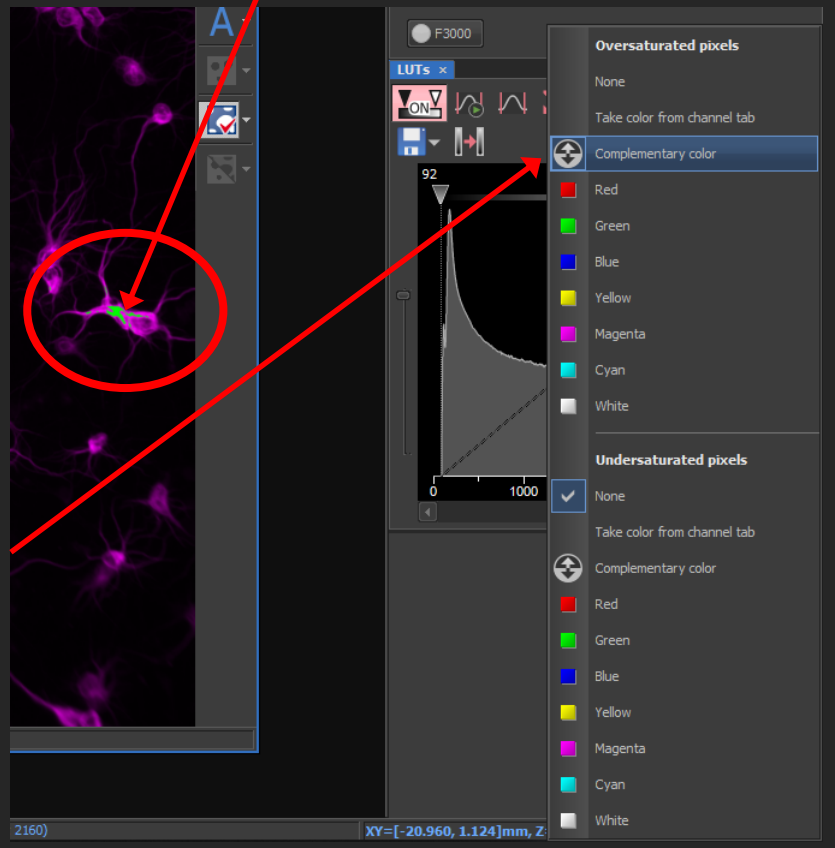
How to tell if you have too much signal?

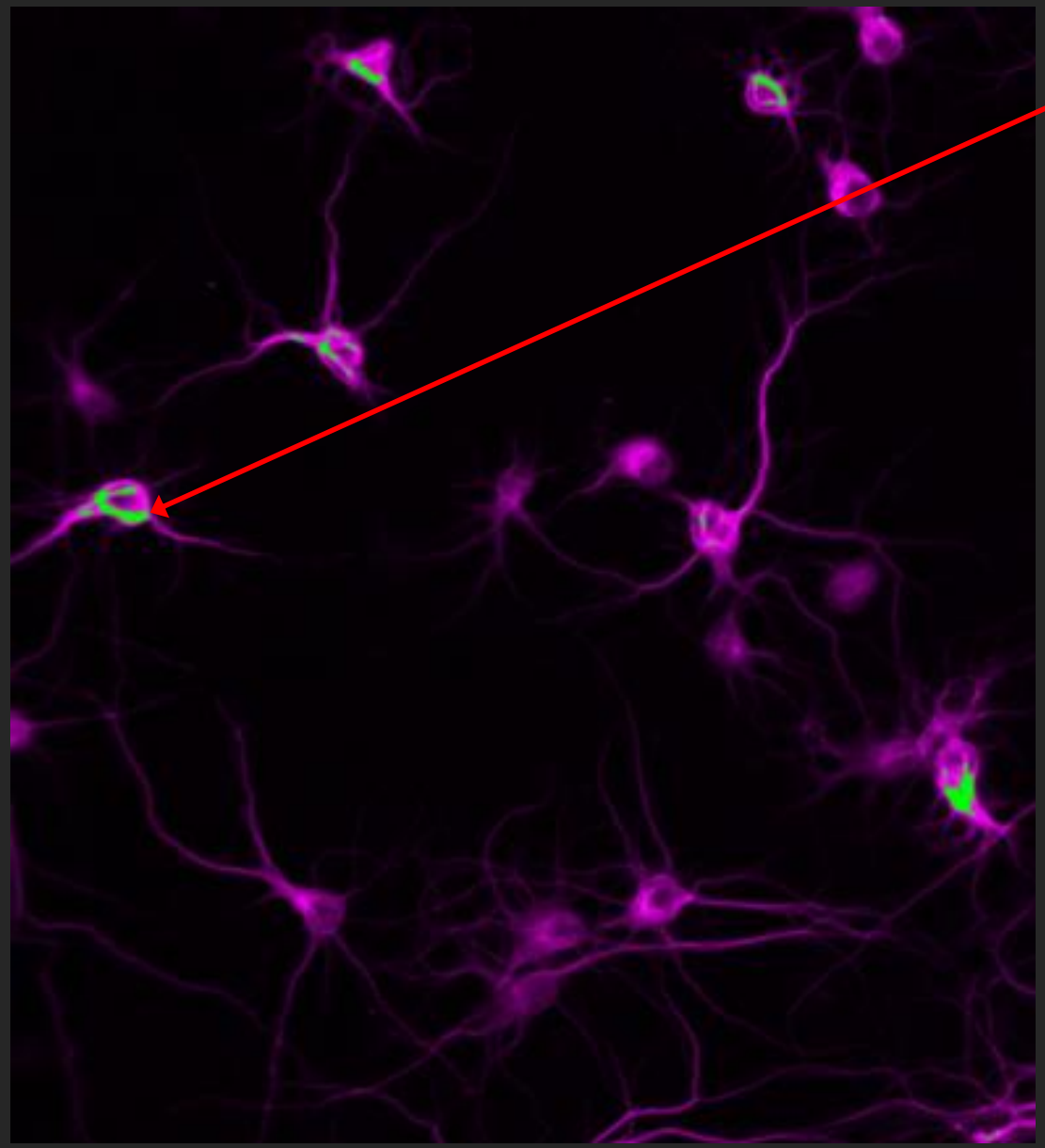


When your LUTs graph is filled up like this with a peak at the end, you may be oversaturating your sample.

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

Oversaturated pixel





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…

1. Reduce exposure time.

2. You can also reduce Gain - but if you can't change this because you are limited by another channel (see rule on page 32) then just reduce exposure time further.

STEP BY STEP INSTRUCTIONS

STEP 5

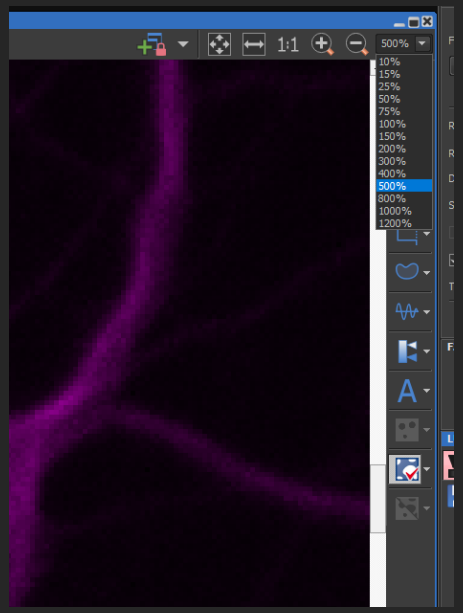
Optimising your camera settings

...when there is too much background NOISE

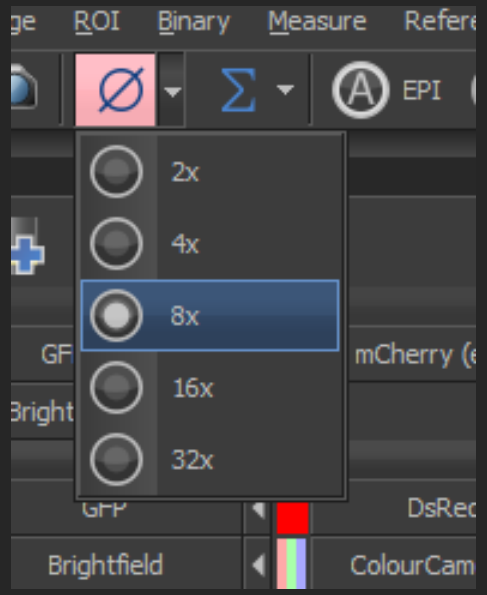
use AVERAGING.

Averaging

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



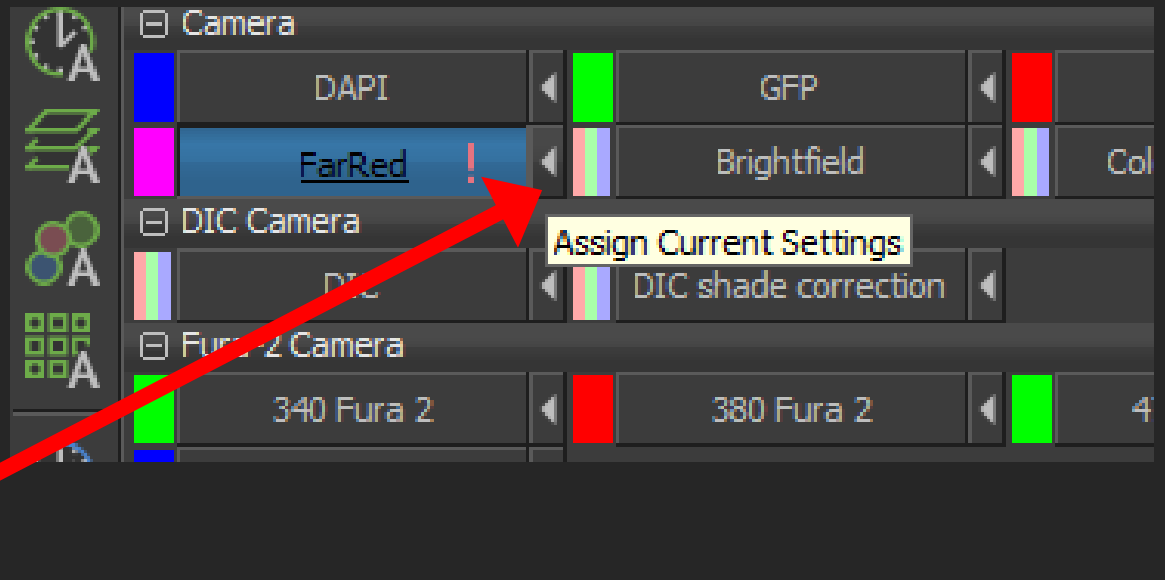
Step 1: Zoom In and then go on LIVE to better see if your image is noisy and need averaging.



Step 2: Determine if you need averaging and how many time you need to average your image.

Happy with camera settings… remember to SAVE!!!

! means there's been a change in your camera settings, such as gain and averaging, exposure time is automatically updated and saved.



SAVE current camera settings for Far-Red channel into the Far-Red optical configuration by clicking on the  make sure you click on the right button.

STEP BY STEP INSTRUCTIONS

STEP 5

Optimising your camera settings

...now do STEP 5 for every channel you want to use.

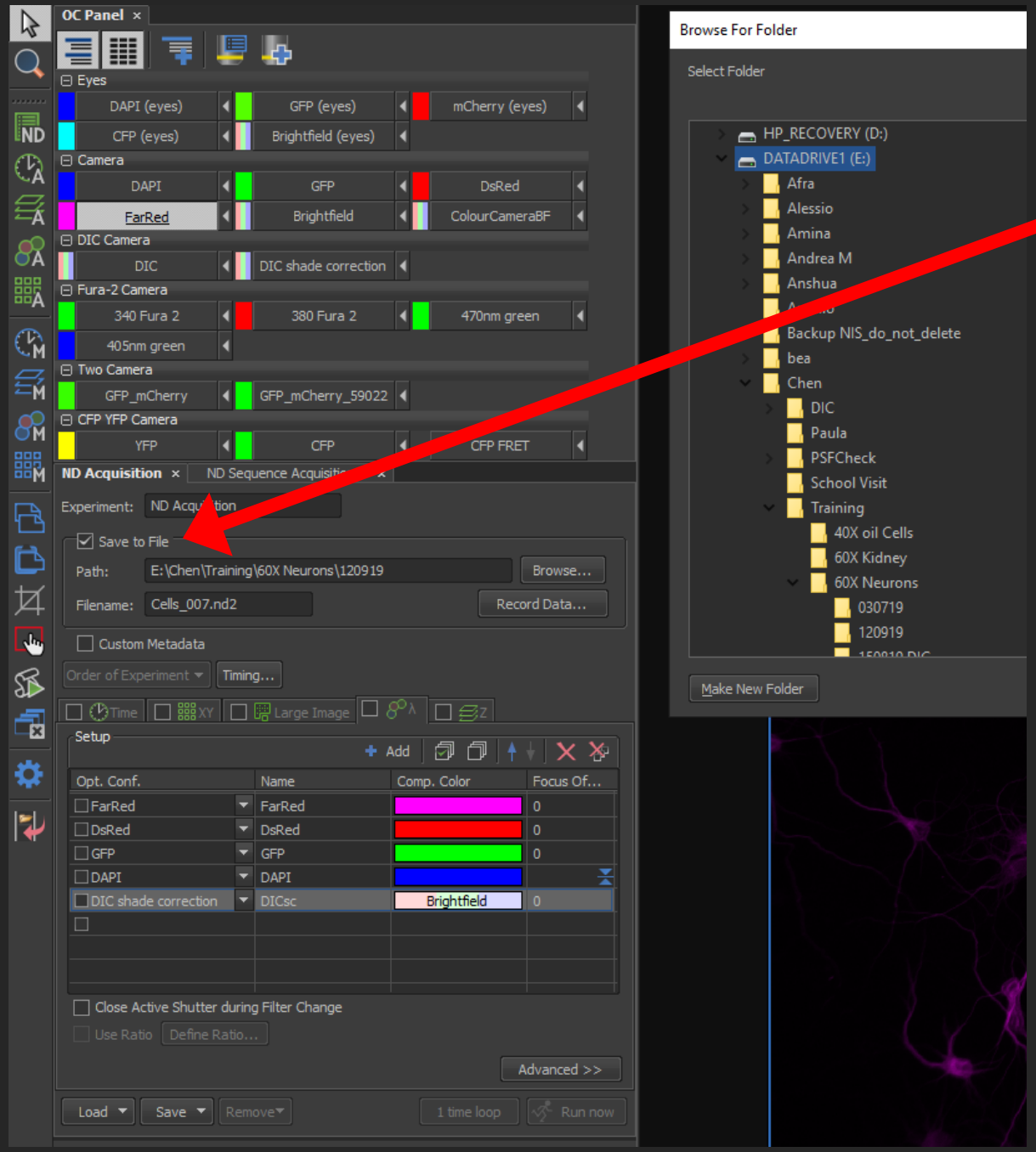
STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... Save to File

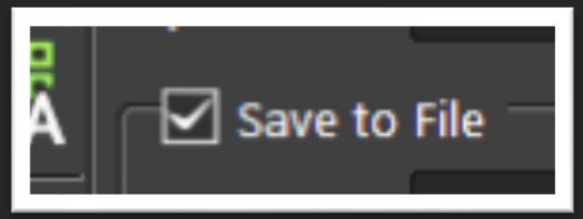
Set Up File Path (SAVE)

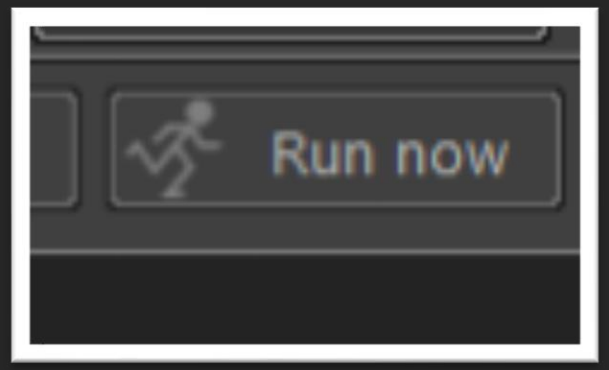


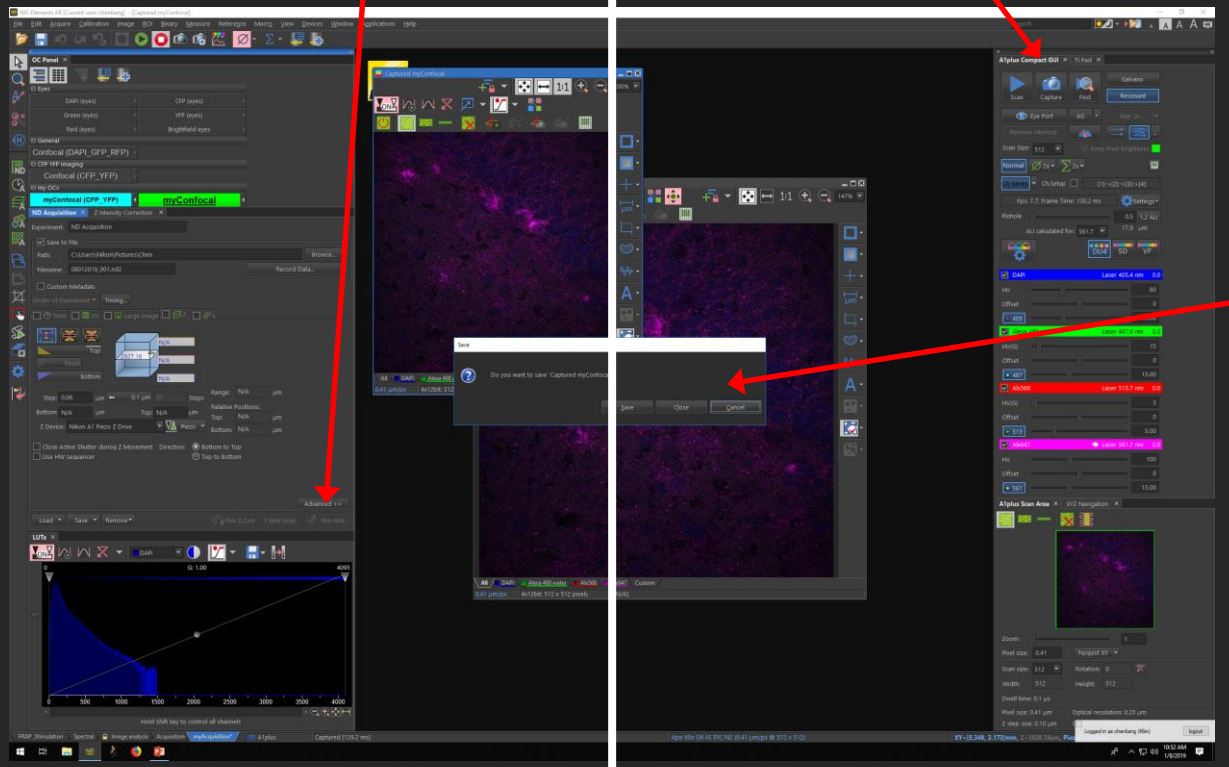
1) Check Save to file option
2) Go to Browse and select DATADRIVE1:
3) Create/find your folder, set up new folder for this session if needed.
4) Recommended file name:
   Experiment_Name_Date_001
5) Finished, anytime you press ”Run now” a new file will be automatically saved in the nd2 format.

Run VS Capture

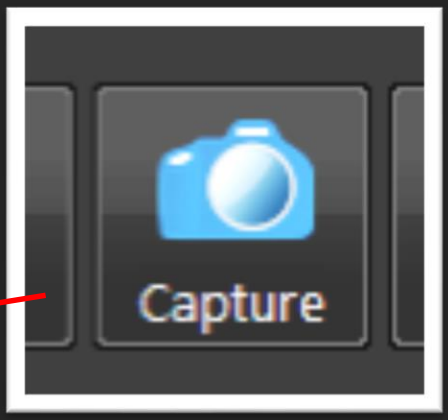
Automatically saved if ‘Save to File’ is ticked







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.

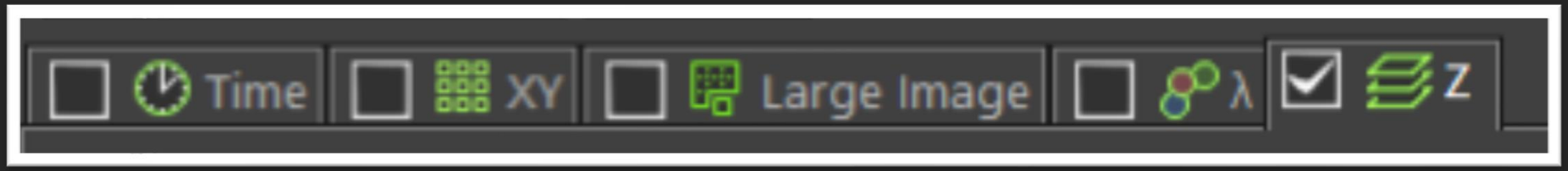
STEP BY STEP INSTRUCTIONS

STEP 6

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.

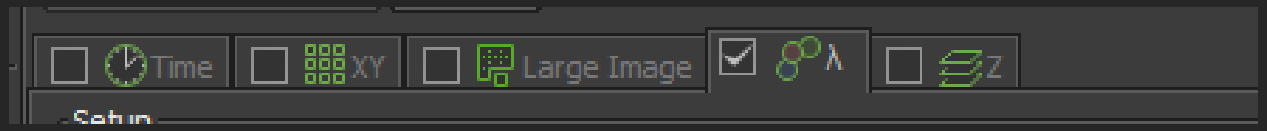
Tick the box for all acquisition functions you want to use.

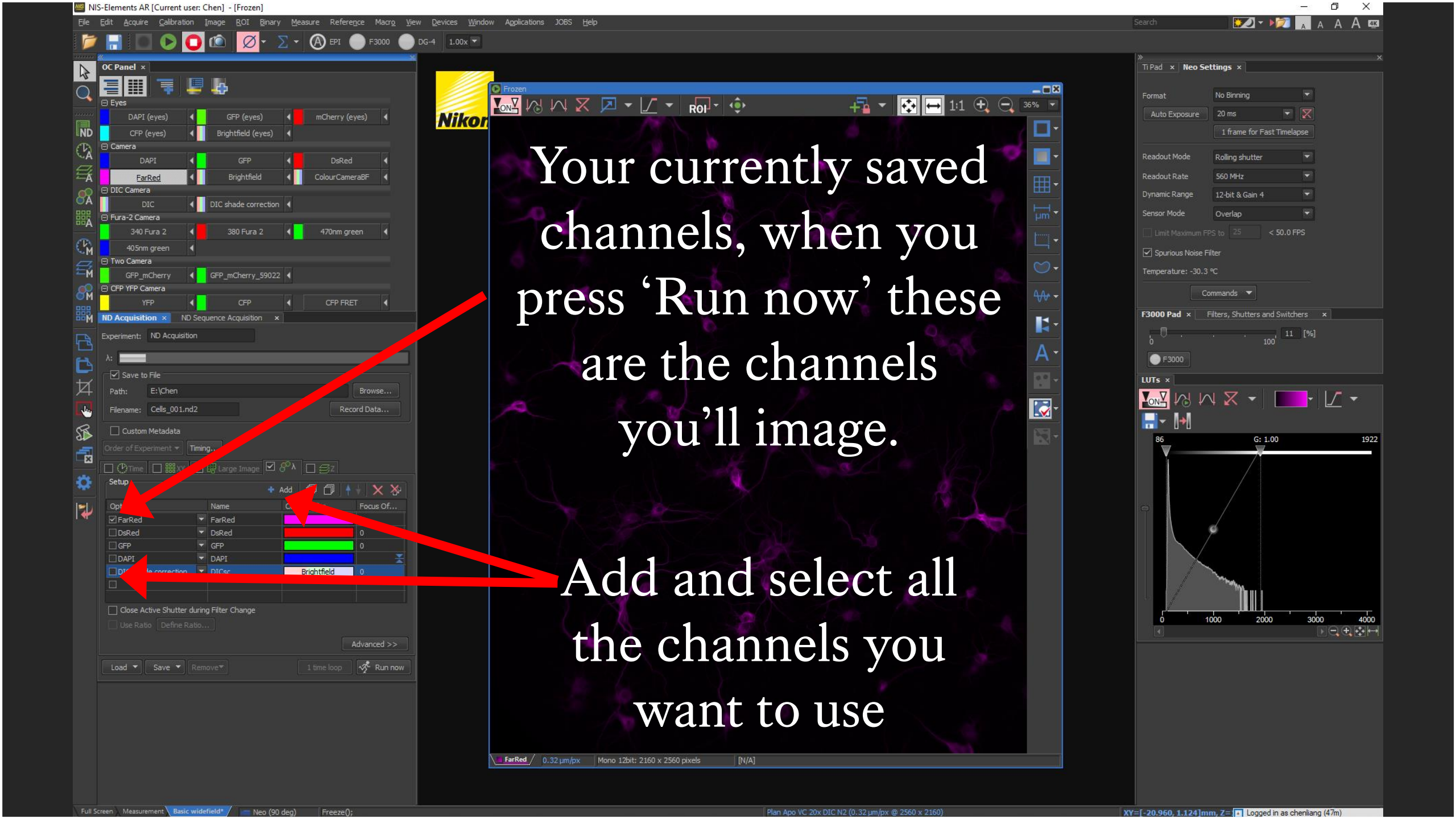
STEP BY STEP INSTRUCTIONS

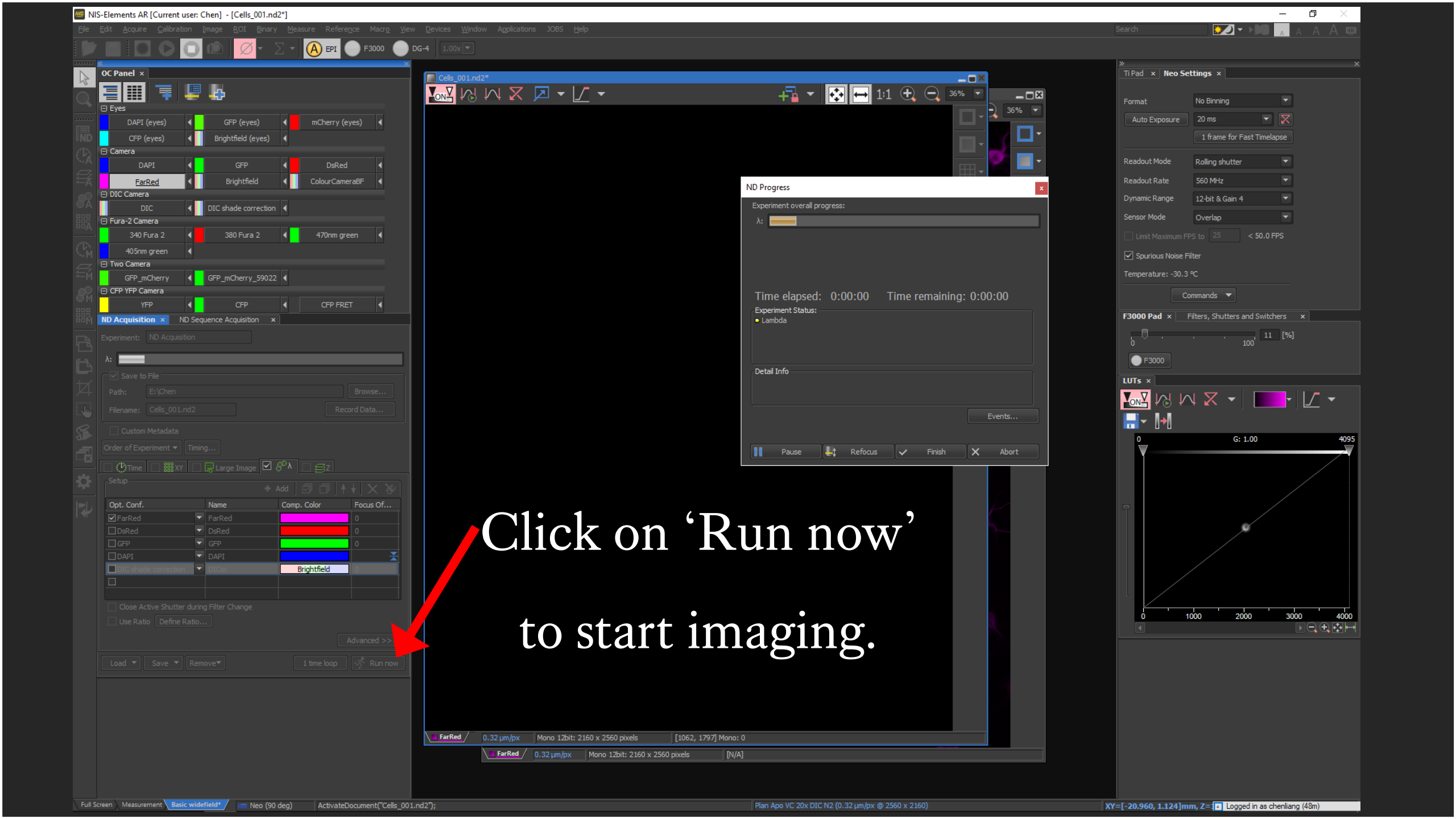
STEP 6

Acquisition Settings

... Lambda (channel selection)




Your currently saved channels, when you press ‘Run now’ these are the channels you’ll image.
Add and select all the channels you want to use


NIS-Elements AR [Current user: Chen] - [Cells_001.nd2*]
OC Panel
Eyes
DAPI (eyes)
GFP (eyes)
mCherry (eyes)
CFP (eyes)
Brightfield (eyes)
Camera
DAPI
GFP
DsRed
FarRed
Brightfield
ColourCameraBF
DIC Camera
DIC
DIC shade correction
Fura-2 Camera
340 Fura 2
380 Fura 2
470nm green
405nm green
Two Camera
GFP_mCherry
GFP_mCherry_59022
CFP YFP Camera
YFP
CFP
CFP FRET
ND Acquisition
ND Sequence Acquisition
Experiment:
ND Acquisition
Save to File
Path:
E:\Chen
Browse...
Filename:
Cells_001.nd2
Record Data...
Custom Metadata
Order of Experiment
Timing...
Setup
Add
Opt. Conf.
Name
Comp. Color
Focus Of...
Close Active Shutter during Filter Change
Use Ratio
Define Ratio...
Advanced >>
Load
Save
Remove
1 time loop
Run now
Cells_001.nd2*
ND Progress
Experiment overall progress:
λ:
Time elapsed: 0:00:00
Time remaining: 0:00:00
Experiment Status:
Lambda
Detail Info
Events...
Pause
Refocus
Finish
Abort
Click on 'Run now'
to start imaging.
FarRed
0.32 µm/px
Mono 12bit: 2160 x 2560 pixels
[1062, 1797] Mono: 0
[N/A]
Ti Pad
Neo Settings
Format
No Binning
Auto Exposure
20 ms
1 frame for Fast Timelapse
Readout Mode
Rolling shutter
Readout Rate
560 MHz
Dynamic Range
12-bit & Gain 4
Sensor Mode
Overlap
Limit Maximum FPS to
< 50.0 FPS
Spurious Noise Filter
Temperature: -30.3 °C
Commands
F3000 Pad
Filters, Shutters and Switchers
F3000
LUTs
G: 1.00
Full Screen
Measurement
Basic widefield*
Neo (90 deg)
ActivateDocument("Cells_001.nd2");
Plan Apo VC 20x DIC N2 (0.32 µm/px @ 2560 x 2160)
XY=[-20.960, 1.124]mm, Z=
Logged in as chenliang (48m)

STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... Z stack

Z stack Basic Options

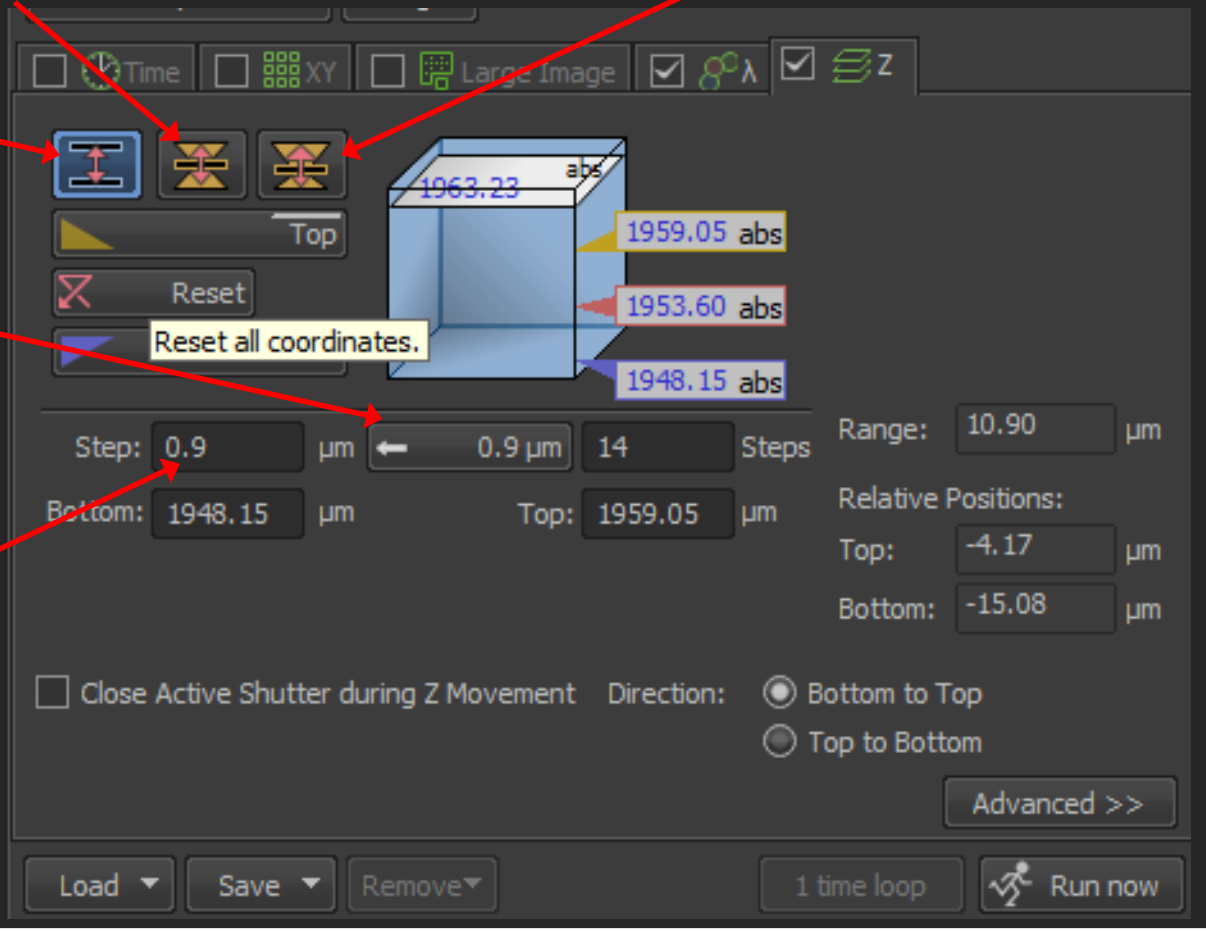
Set top and bottom: use mouse wheel to focus and define the exact range of your Z stack.

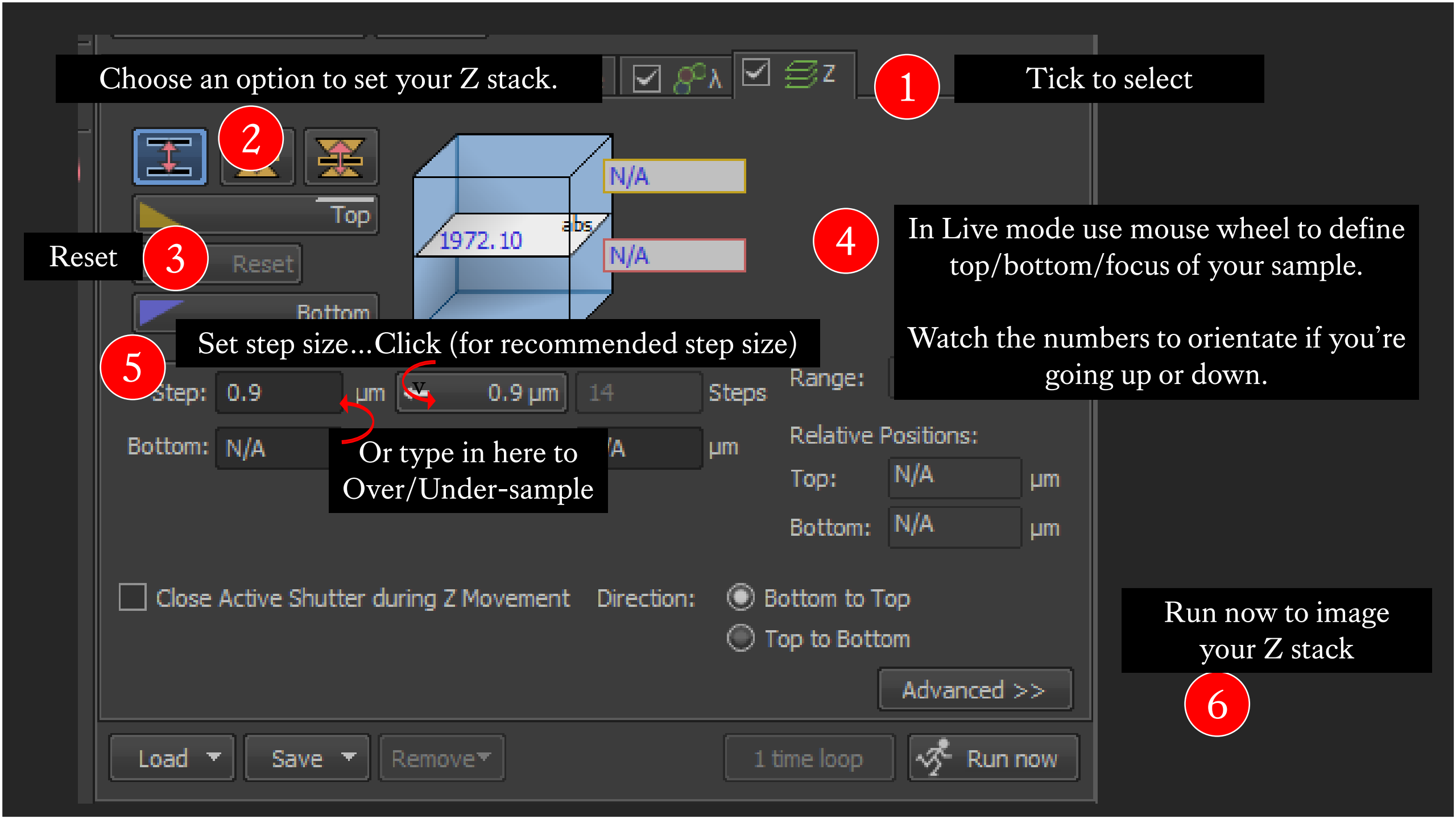
Set Middle: use mouse wheel to find the mid point of your Z focus and set equal distance above and below the focal plane. (Useful if your sample is symmetrical along the Z axis)

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

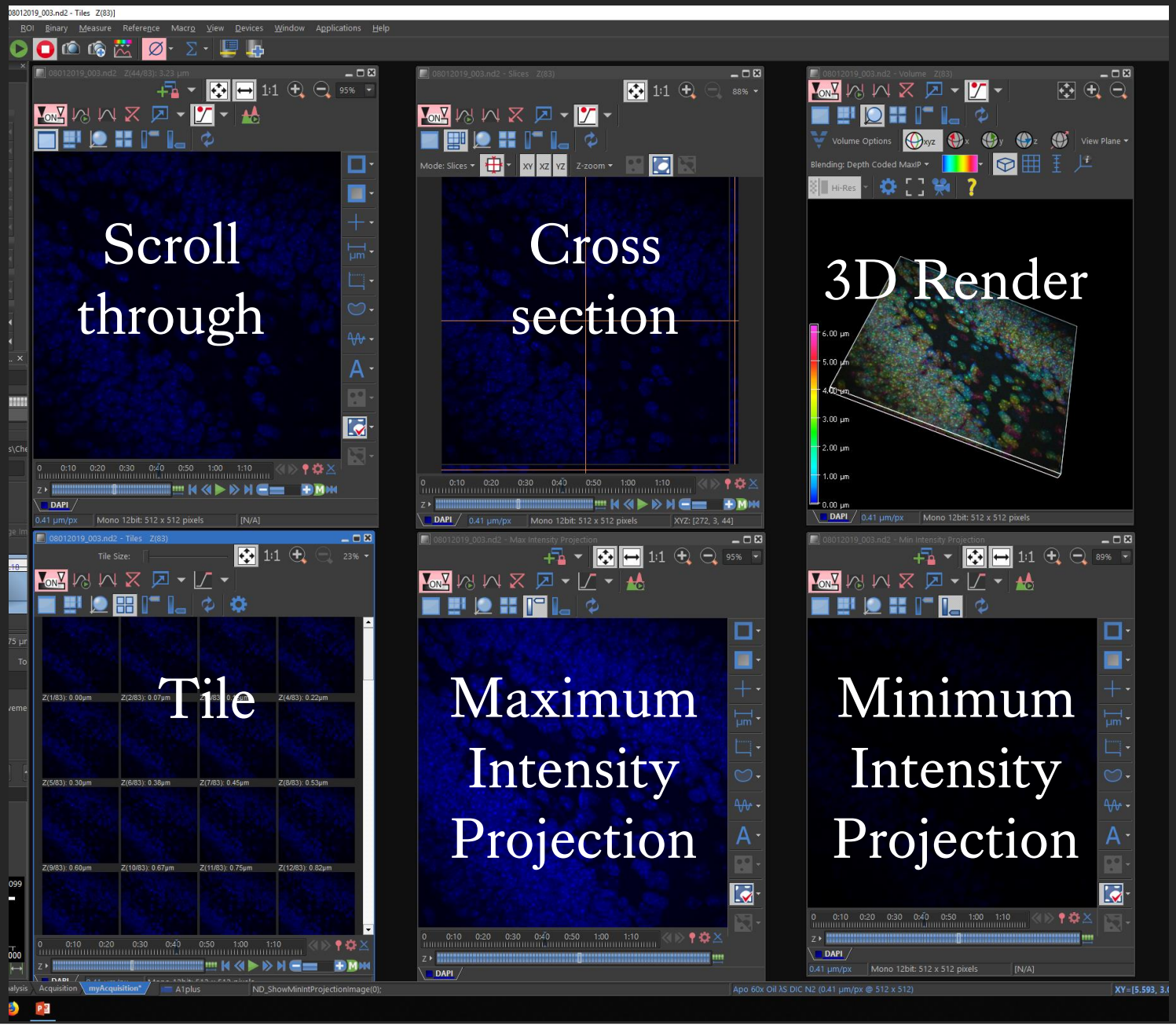
Step Size

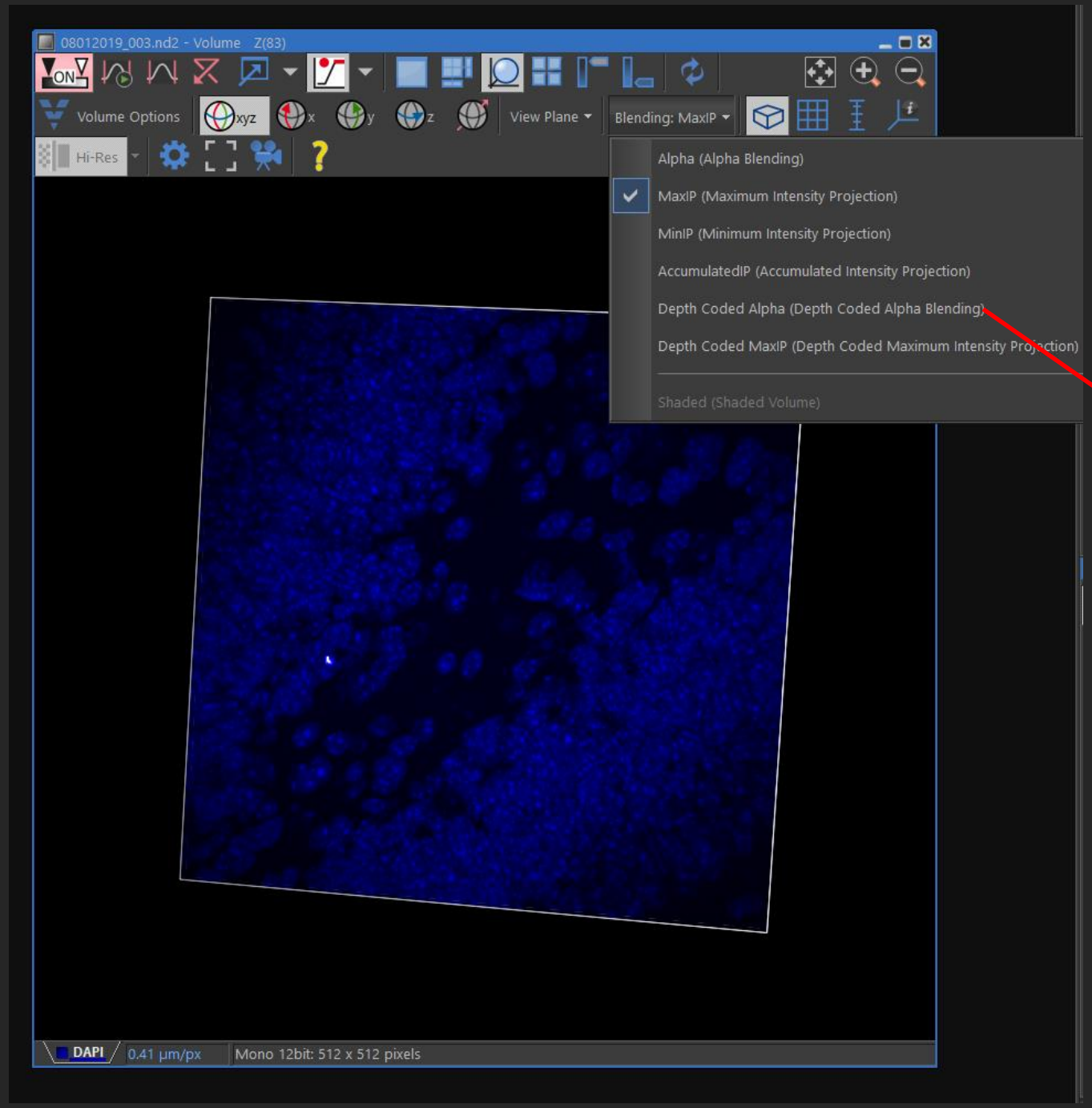
- You can set step size or number of steps.
- Use recommended step size to capture all the information.
- Fewer steps than the recommended is called under-sampling and you may loose information.
- More steps than the recommended is called over-sampling (typed in here) which is required for 3D deconvolution.



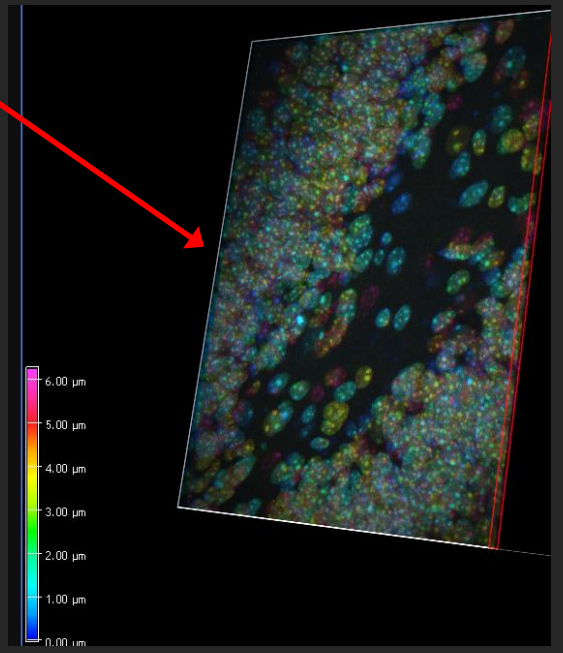

Choose an option to set your Z stack.
1
Tick to select
2
Top
Reset
Bottom
3
Reset
1972.10
N/A
N/A
4
In Live mode use mouse wheel to define top/bottom/focus of your sample.
Watch the numbers to orientate if you're going up or down.
5
Set step size…Click (for recommended step size)
Step: 0.9 µm
0.9 µm
14 Steps
Range:
Bottom: N/A
Or type in here to Over/Under-sample
Relative Positions:
Top: N/A µm
Bottom: N/A µm
Close Active Shutter during Z Movement
Direction:
Bottom to Top
Top to Bottom
Advanced >>
Load
Save
Remove
1 time loop
Run now
Run now to image your Z stack
6

Z stack View Modes





In 3D rendering you have different rendering modes.



STEP BY STEP INSTRUCTIONS

STEP 6

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

X axis x Y axis fields

5.0 x 9.0 mm

Pattern

2

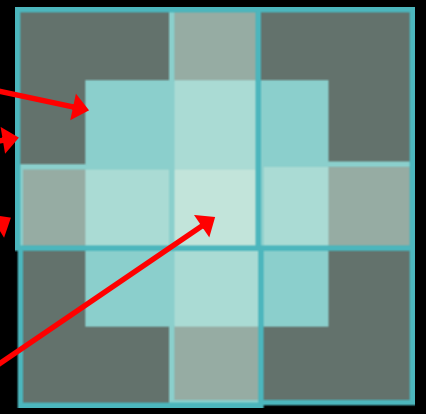
In Live mode, move to the centre of your field of view.

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!

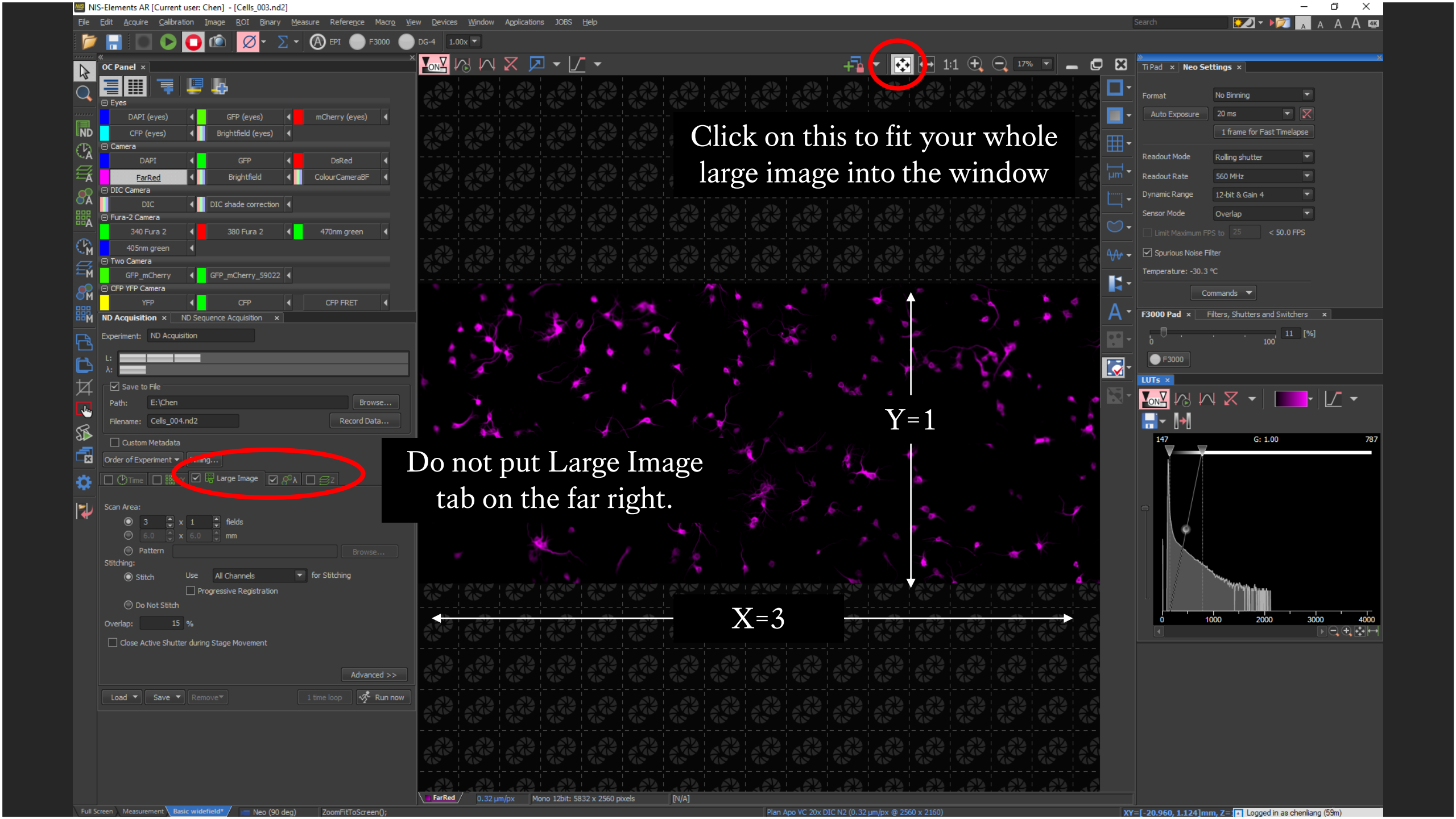
Close Active Shutter during Stage Movement

Run now to image your Large Image

Advance

6

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

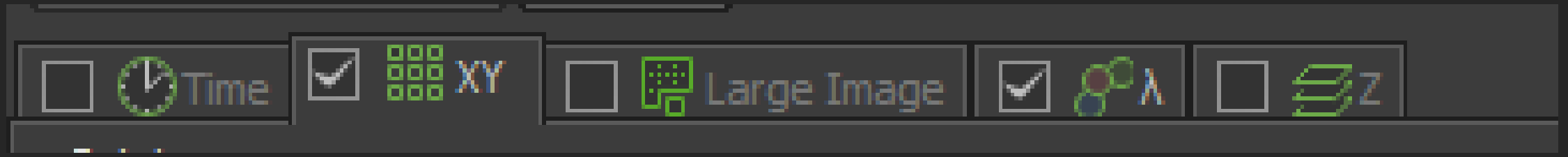

Click on this to fit your whole large image into the window
Do not put Large Image tab on the far right.
Y=1
X=3

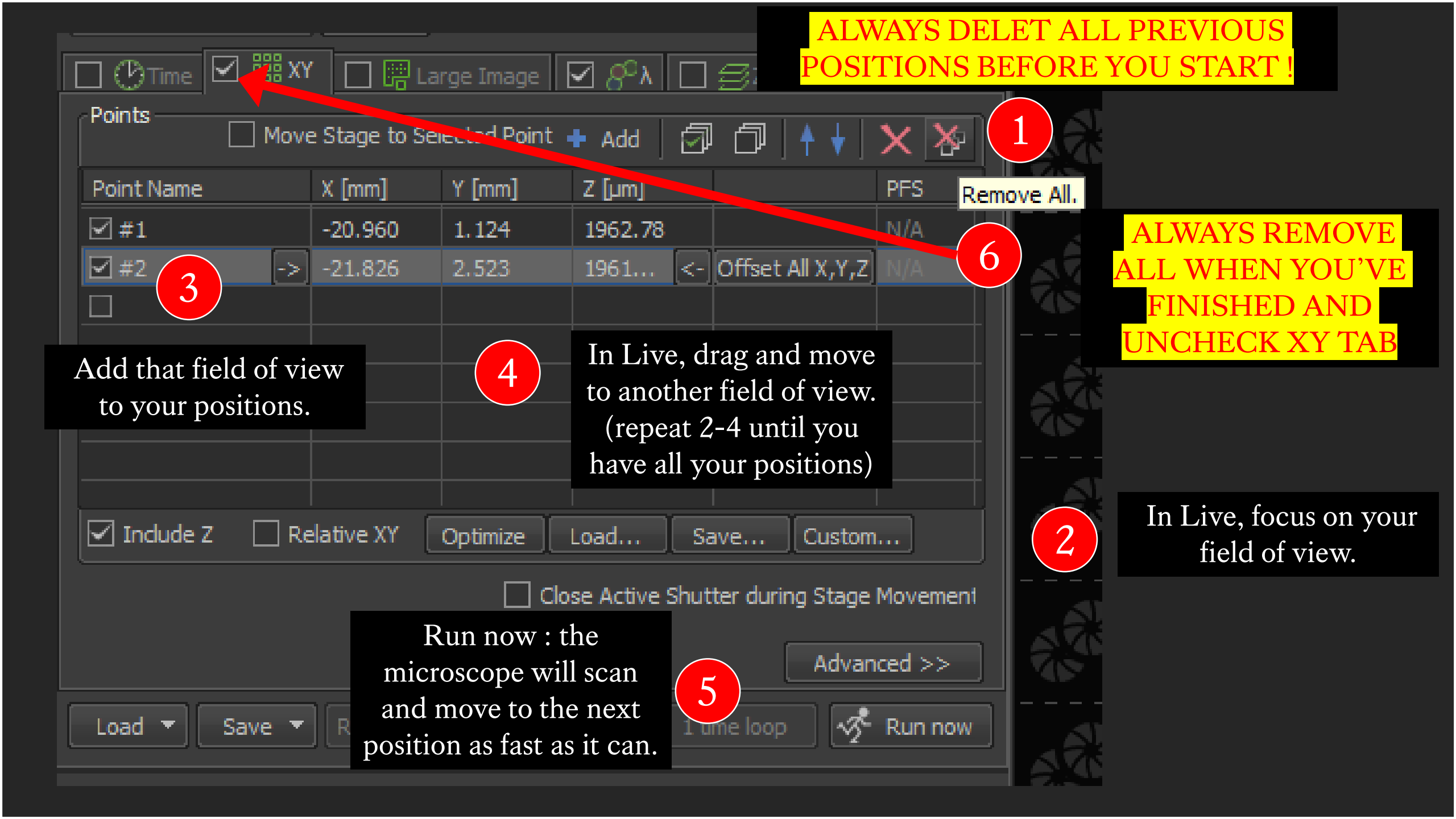
STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... XY Positions



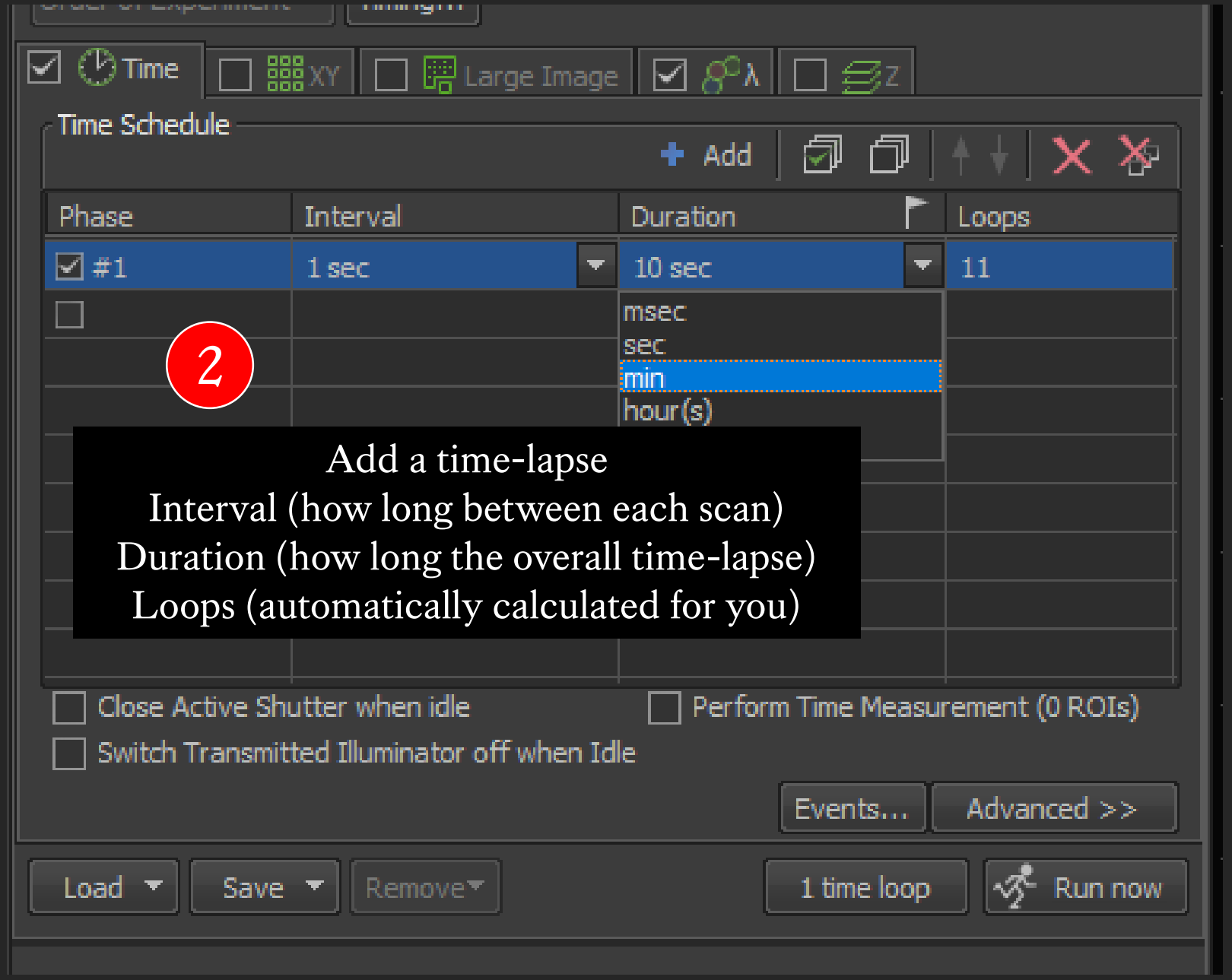

ALWAYS DELET ALL PREVIOUS POSITIONS BEFORE YOU START !
Time
XY
Large Image
Points
Move Stage to Selected Point
Add
Remove All.
Point Name
X [mm]
Y [mm]
Z [µm]
PFS
#1
-20.960
1.124
1962.78
N/A
#2
-21.826
2.523
1961...
Offset All X,Y,Z
N/A
1
2
3
4
5
6
ALWAYS REMOVE ALL WHEN YOU'VE FINISHED AND UNCHECK XY TAB
Add that field of view to your positions.
In Live, drag and move to another field of view. (repeat 2-4 until you have all your positions)
In Live, focus on your field of view.
Include Z
Relative XY
Optimize
Load...
Save...
Custom...
Close Active Shutter during Stage Movement
Advanced >>
Run now : the microscope will scan and move to the next position as fast as it can.
Load
Save
1 time loop
Run now

STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... Time

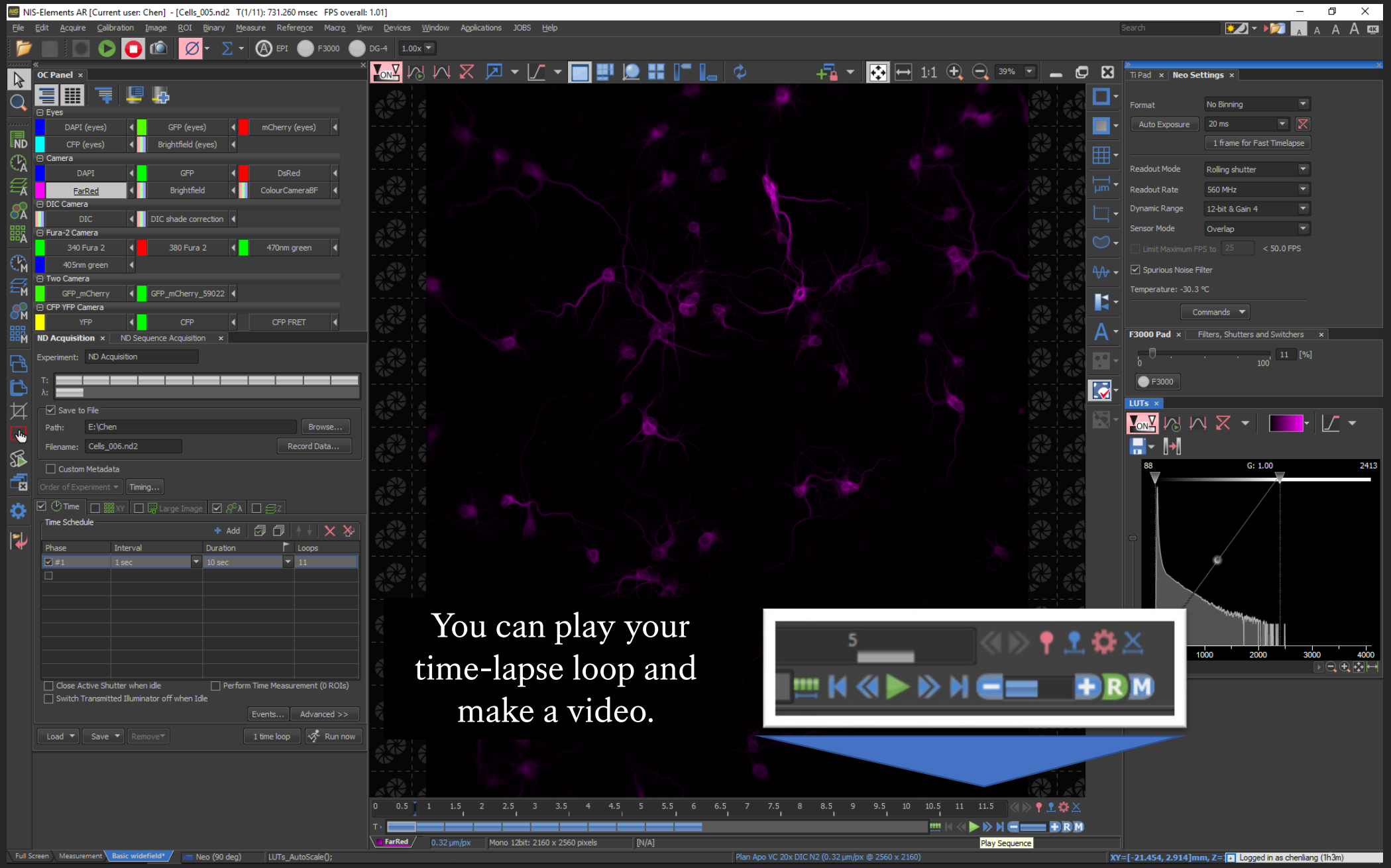


1

In Live, focus on your field of view.

3

Run now


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

Continue with your imaging...

STEP BY STEP INSTRUCTIONS

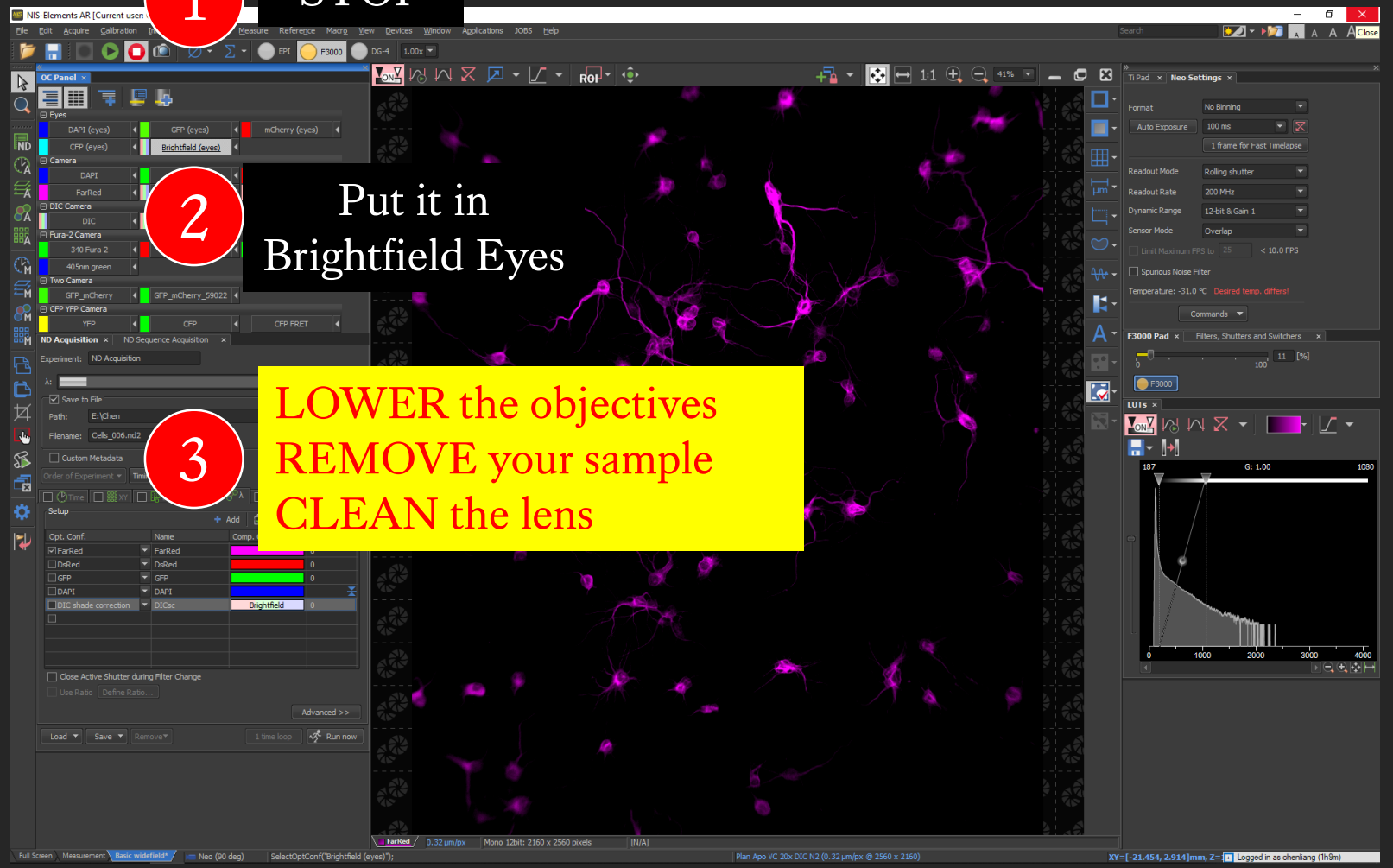
STEP 7

At the end of your session

... Shut down procedure

STEP 1 : Clean Up and Close Software

1 STOP



2 Put it in Brightfield Eyes

3 LOWER the objectives
REMOVE your sample
CLEAN the lens

4 Close Software

Your data should already be automatically saved, check your DATADRIVE1 folder if you want to be sure before shutting off the software.

STEP 2 : Transfer Data to WCIC Shared Network Drive

1

Open file
Find and cut/copy your saved data in DATADRIVE1

2

Right click to open WCIC Shared Drive in new window

3

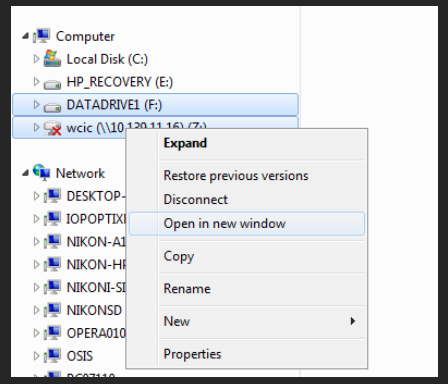
You need to login to this pop-up window, user name is normally: kclad\k number
DO NOT click on remember my credentials

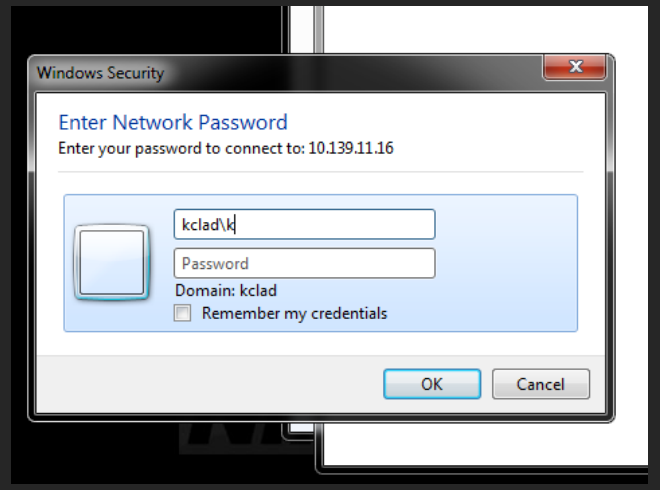
4

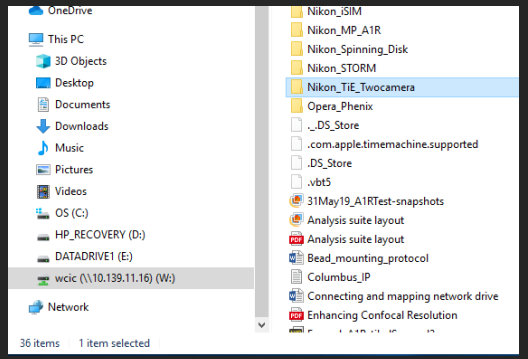
In the Network drive, open the Nikon_TiE_Twocamera folder

5

Paste data into your personal folder





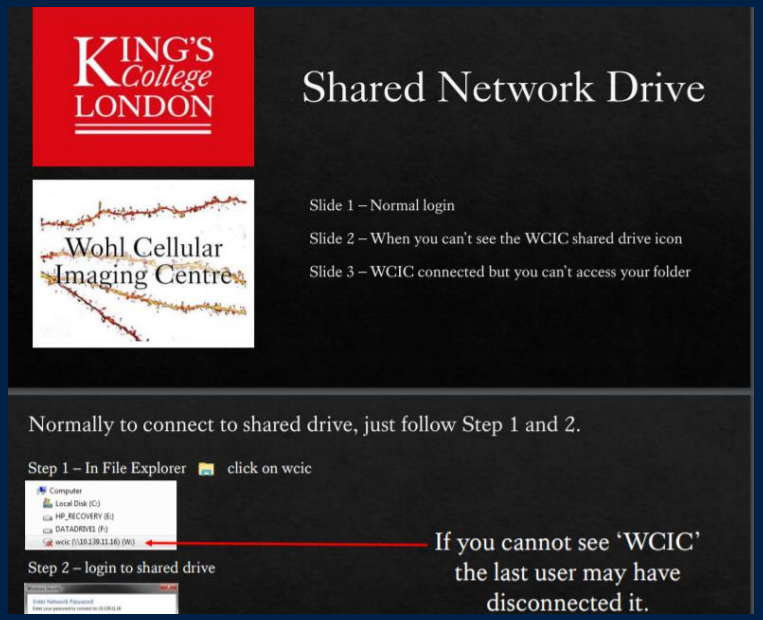


- **DO NOT USE USBs ON ANY COMPUTERS IN THE MICROSCOPE ROOMS!**

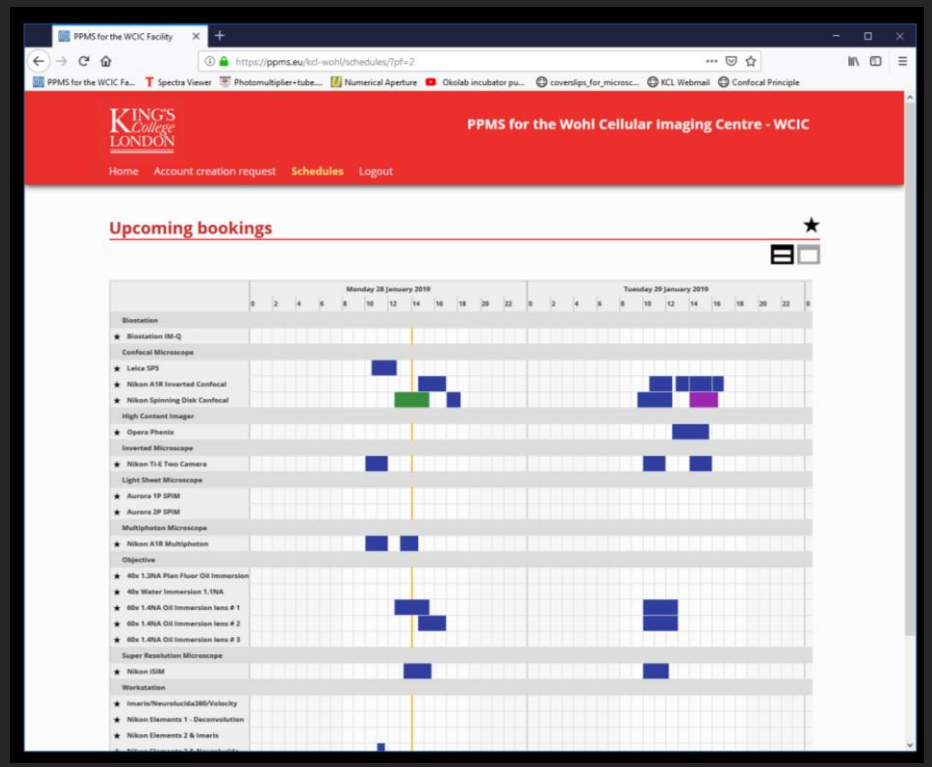
- The Shared Drive can be accessed from the workstations (where you can use USBs), or your personal computers, from there please BACK-UP your data.

If you have trouble connecting to the shared drive, or need to re-map the network drive, please follow the instructions in this link or contact us.

https://f59fa4a0-6222-454a-9101-2ba099e49b58.filesusr.com/ugd/0c5e54_9b32bb45a8fc450bb3e65e9c79de3b44.pdf



STEP 3 : Check the booking schedule!



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

If no one is using the system within 2 hours, shut down the system in REVERSE numerical order.

If the incubator is on... shut down as usual but do not turn the incubator off, another user might be pre-warming the system.

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

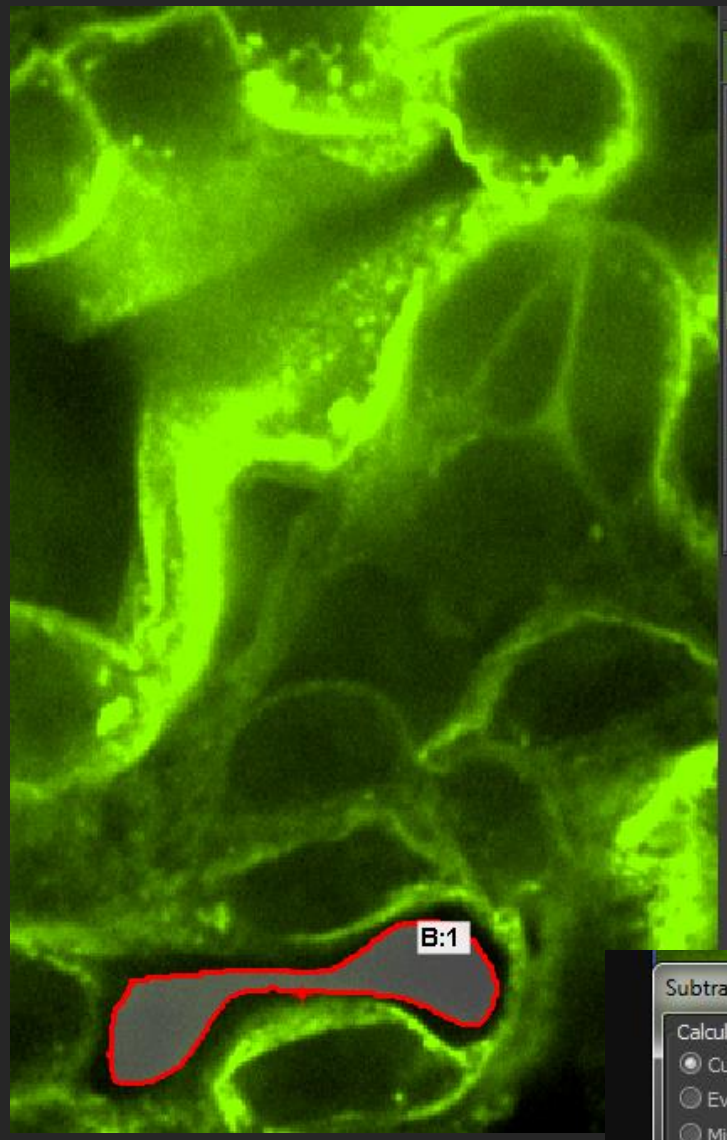
3) Select reuse camera settings

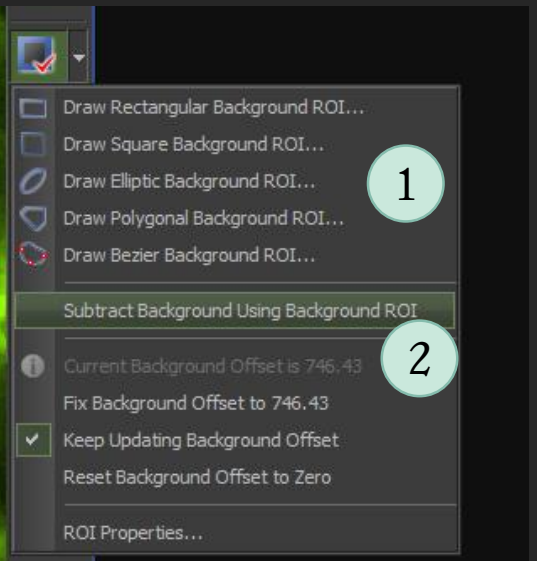
4) Be aware, this uploads camera settings only, acquisition setting (Z-stack, large image etc.) will not be reloaded

Time measurement

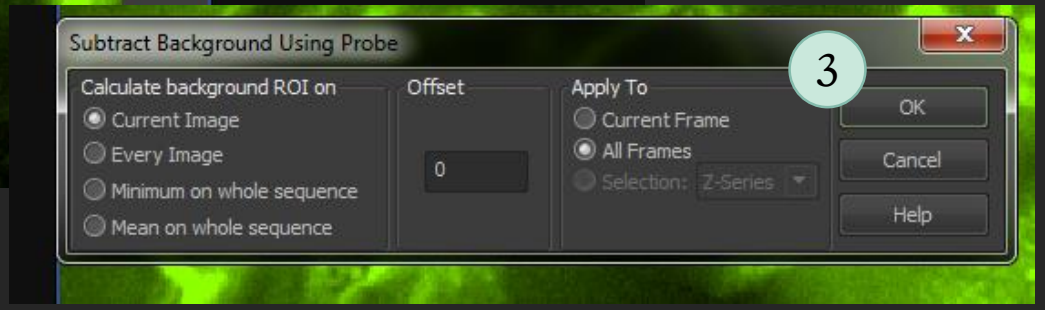
Time measurement (found under the measurement tab) can be used after time-lapse imaging to determine the rate of sample bleaching or recovery.

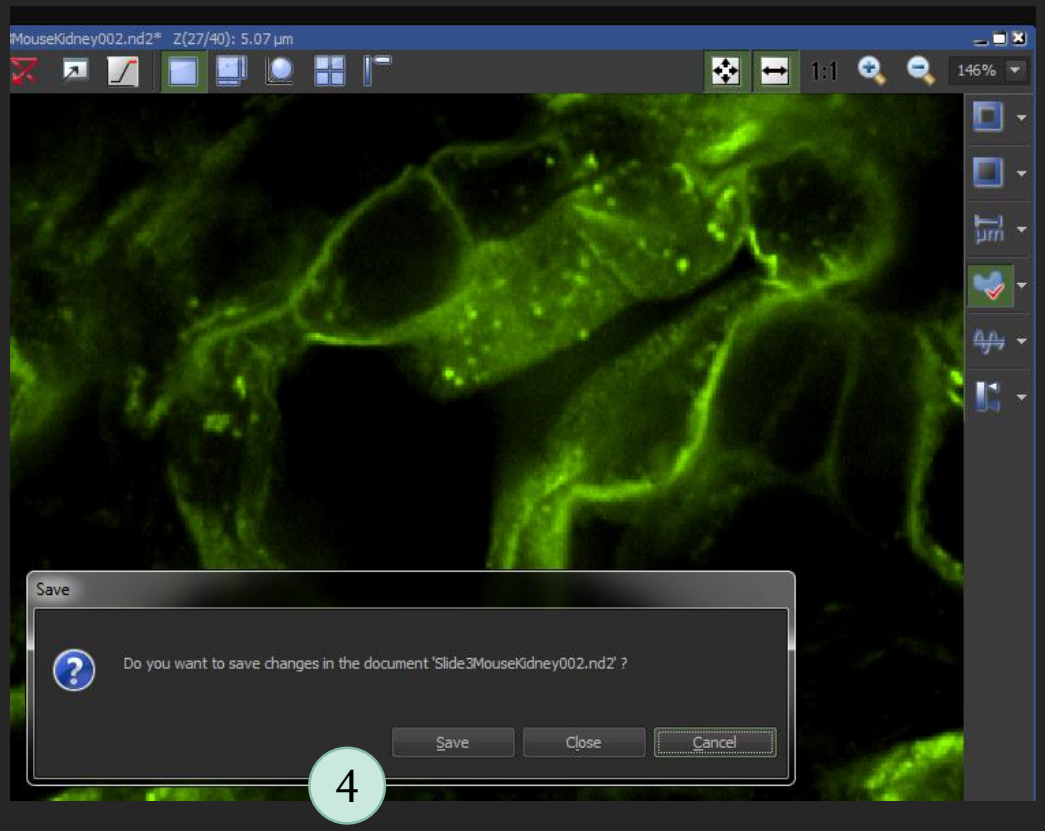
Removing Background (Changes to ROIs can be implemented at workstations/FIJI after your session)





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.

GAIN need to remain the SAME for all Channels!!!

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!