KING'S College LONDON

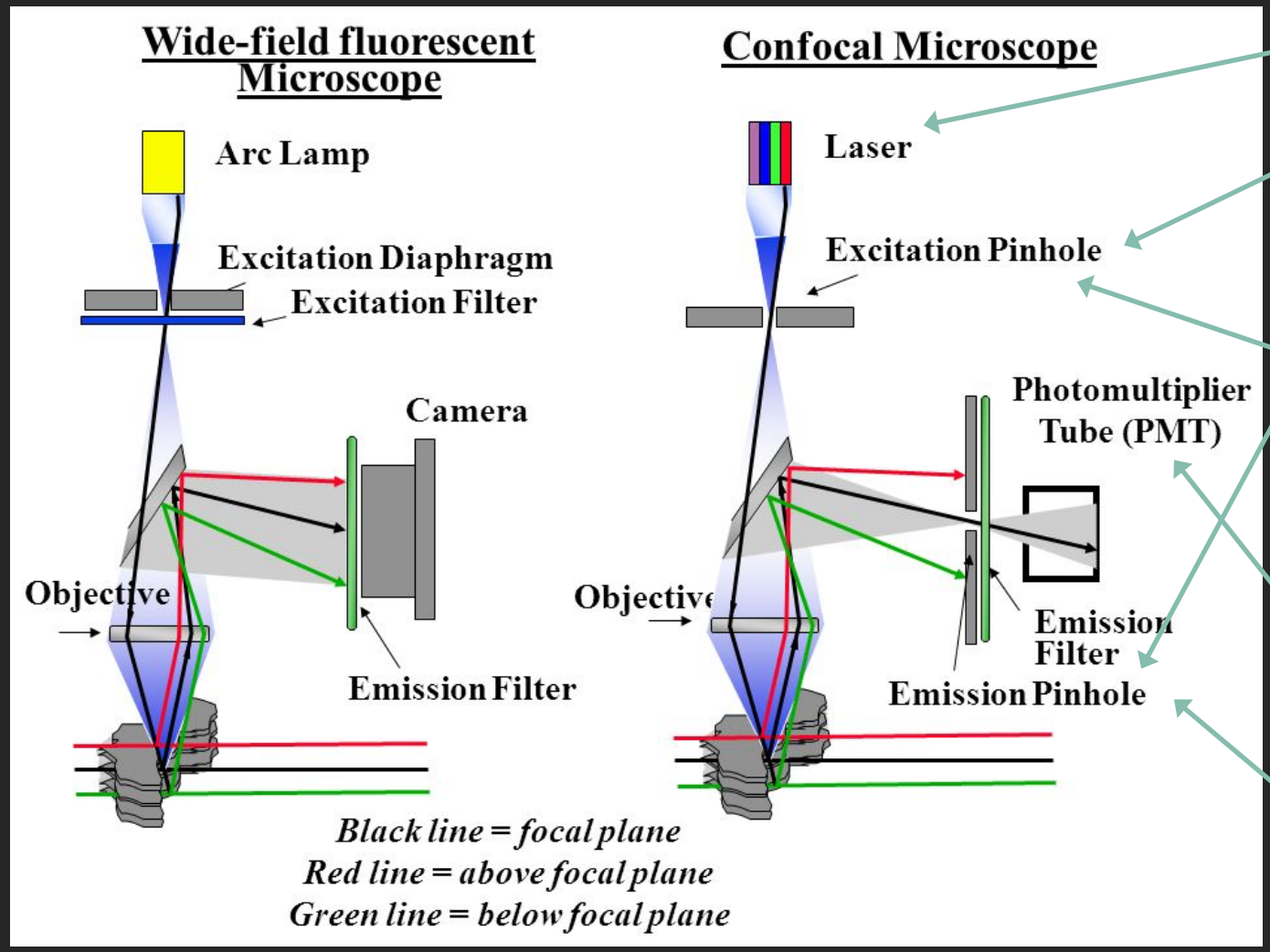
Wohl Cellular Imaging Centre

Nikon Spinning Disk Confocal

SD - STEP BY STEP INSTRUCTIONS CONTENTS PAGE

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.

Spinning Disk Confocal

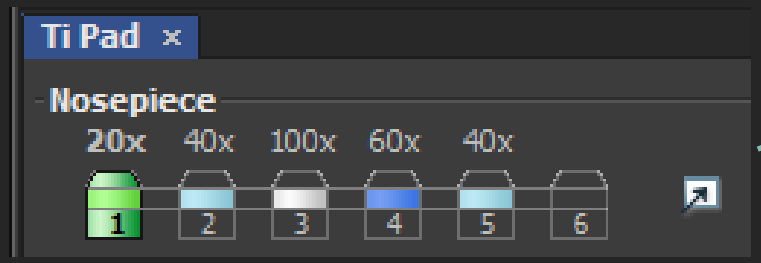
This multiple pinholes system uses very sensitive camera detectors for high resolution image capture.

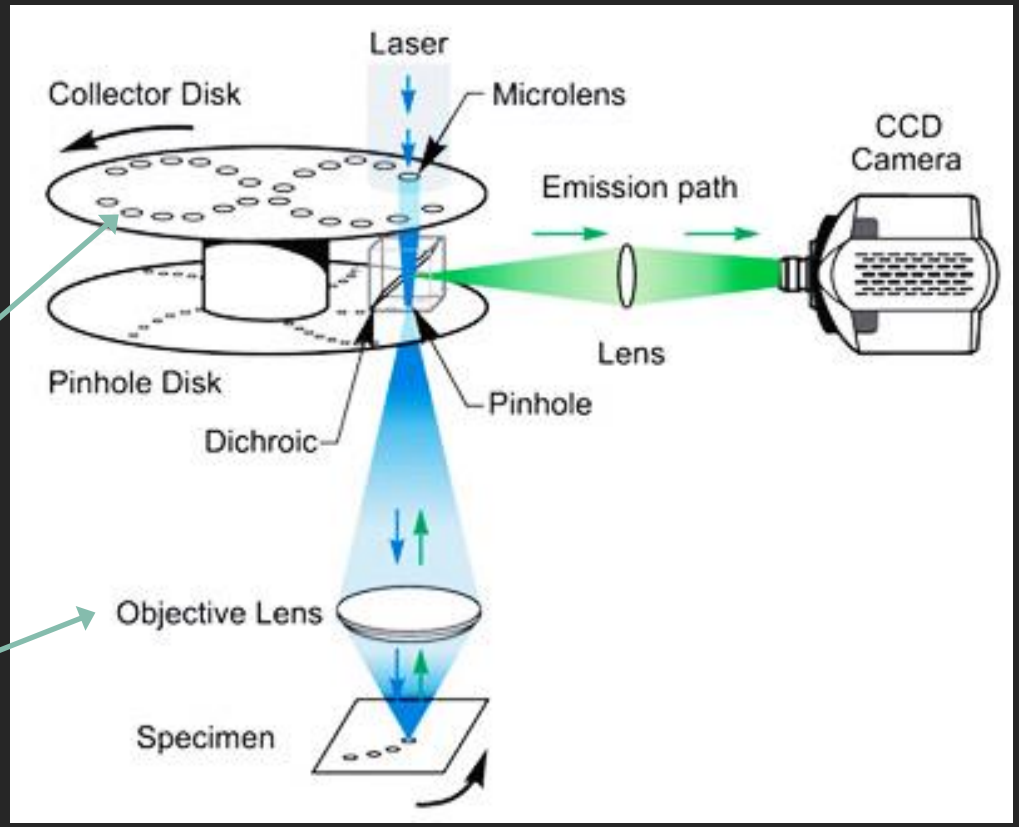
This system is fast and very gentle on samples, highly recommended for live imaging.

Be careful:

With 10X and 20X lenses, the Spinning Disk is closer to a widefield than a confocal microscope.

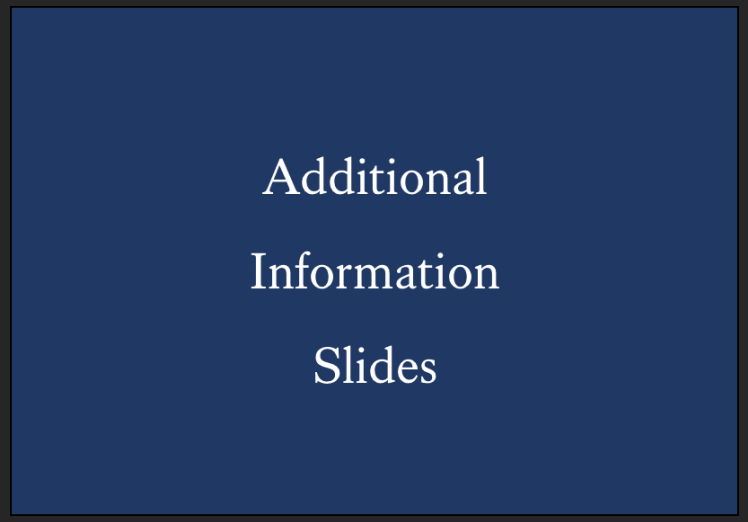
Pinhole size is critical for confocal efficacy, the smaller the pinhole the higher the resolution, but because the pinholes in the spinning disk are at a fixed size, the objective lenses determine confocal efficacy.





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!

This usually means the next user after you need the incubation and is pre-warming the incubation box.

Keep the incubator doors closed as much as you can during your session and always check the incubation box doors are closed before you leave at the end of your session.

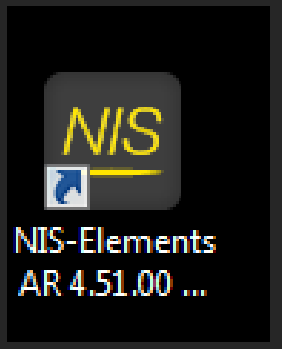
System On

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

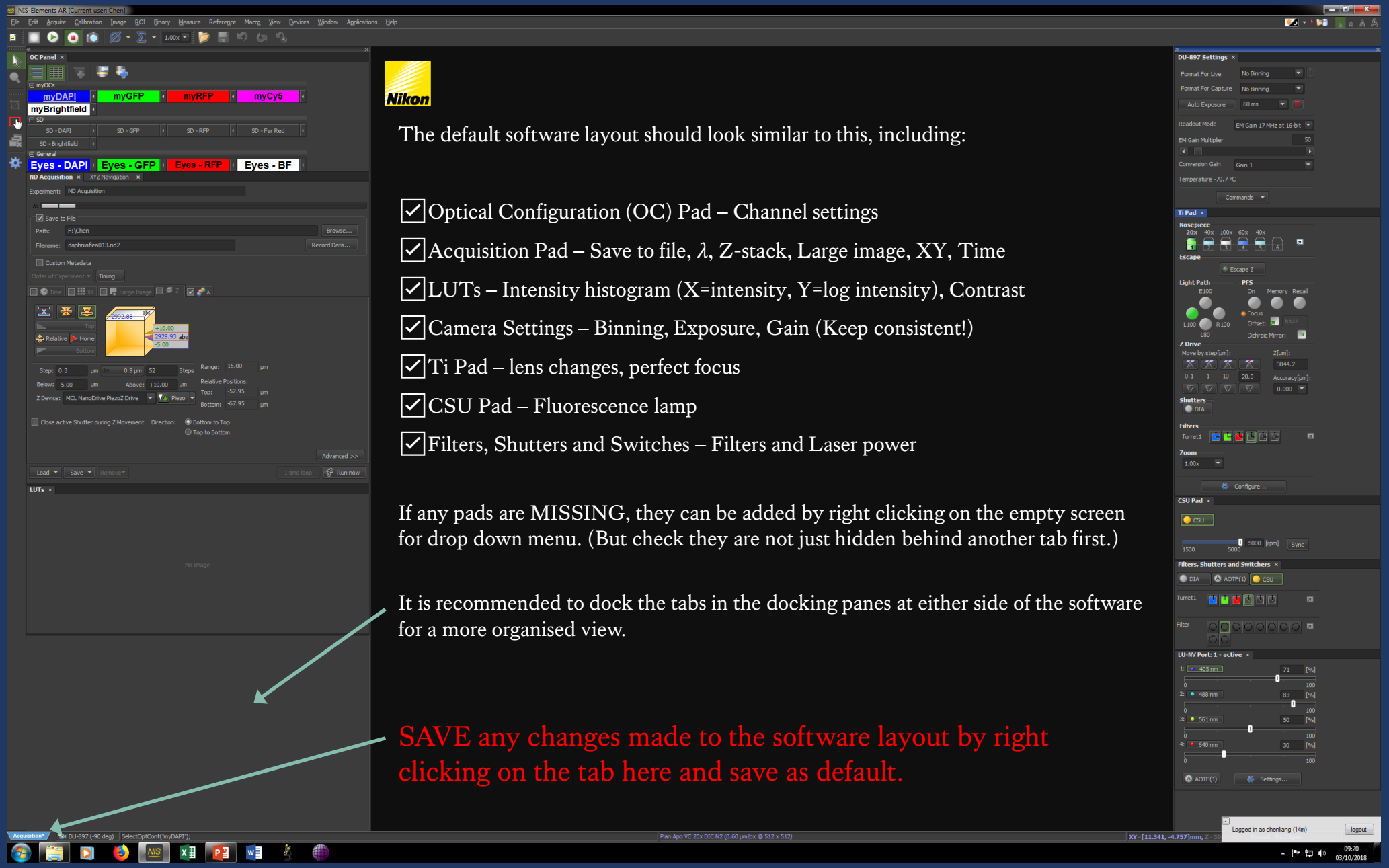
1. Switch on the Spinning Disk confocal system by following the numbered switches.

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.**



Software Set Up



The default software layout should look similar to this, including:

- ☑ 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 – Binning, Exposure, Gain (Keep consistent!)
- ☑ Ti Pad – lens changes, perfect focus
- ☑ CSU Pad – Fluorescence lamp
- ☑ Filters, Shutters and Switches – Filters and Laser power

If any pads are MISSING, they can be added by right clicking on the empty screen for drop down menu. (But check they are not just hidden behind another tab first.)

It is recommended to dock the tabs in the docking panes at either side of the software for a more organised view.

SAVE any changes made to the software layout by right clicking on the tab here and save as default.

Software Layout

Live

Stop

Capture: This is not automatically saved

Averaging

Camera Settings

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

Shared Default DO NOT CHANGE

Camera view.

Exposure/Gain/Laser/averaging settings can be saved for each channel in "my" OCs

LOWER the OBJECTIVES

Lens Options

Perfect Focus

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

Eye View – For location/focus finding

Zoom

DIA Brightfield Lamp shutter

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.

Confocal Scanning Unit (CSU) Shutter

LUTs

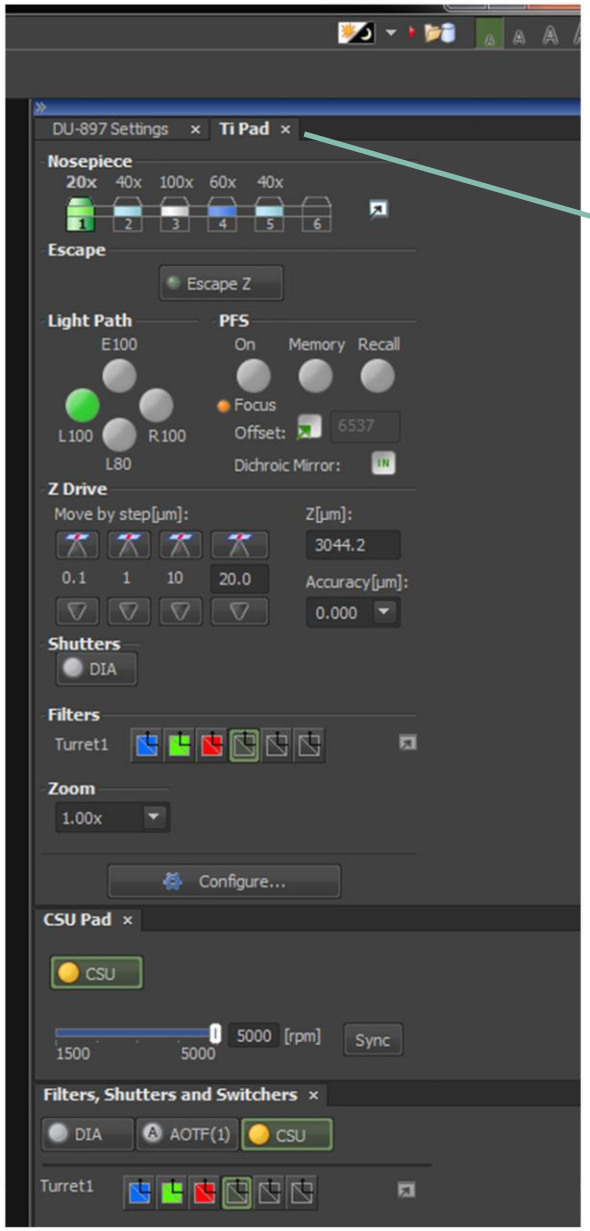
Show intensity in a histogram format

Contrast manipulation

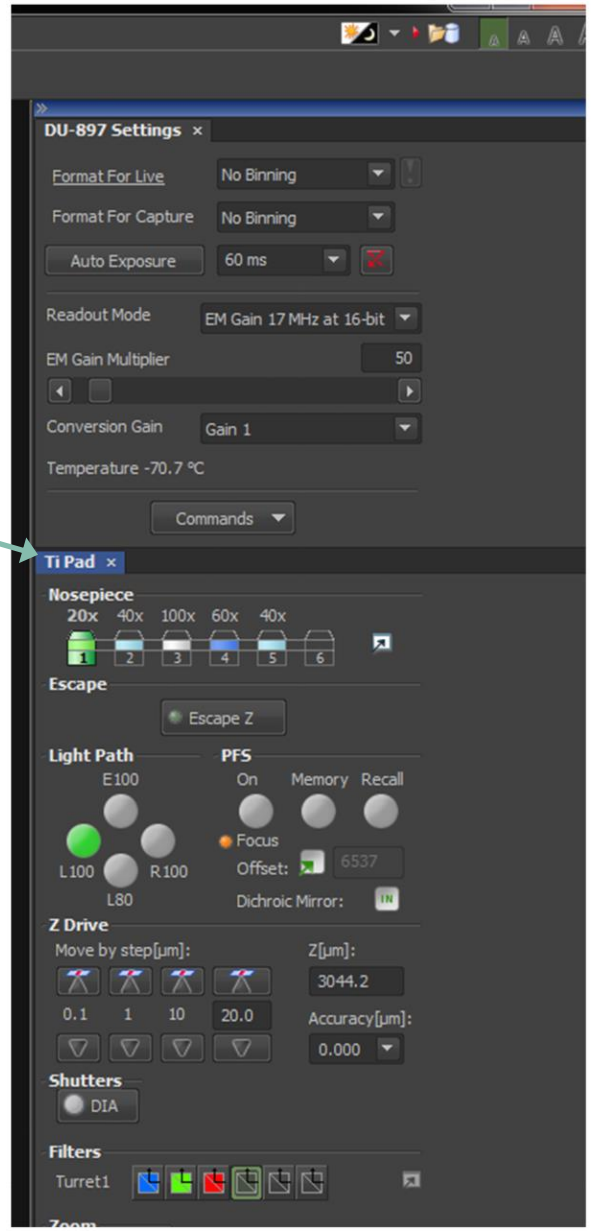
Saturation indicator options

Laser Power

Software Hidden Panels



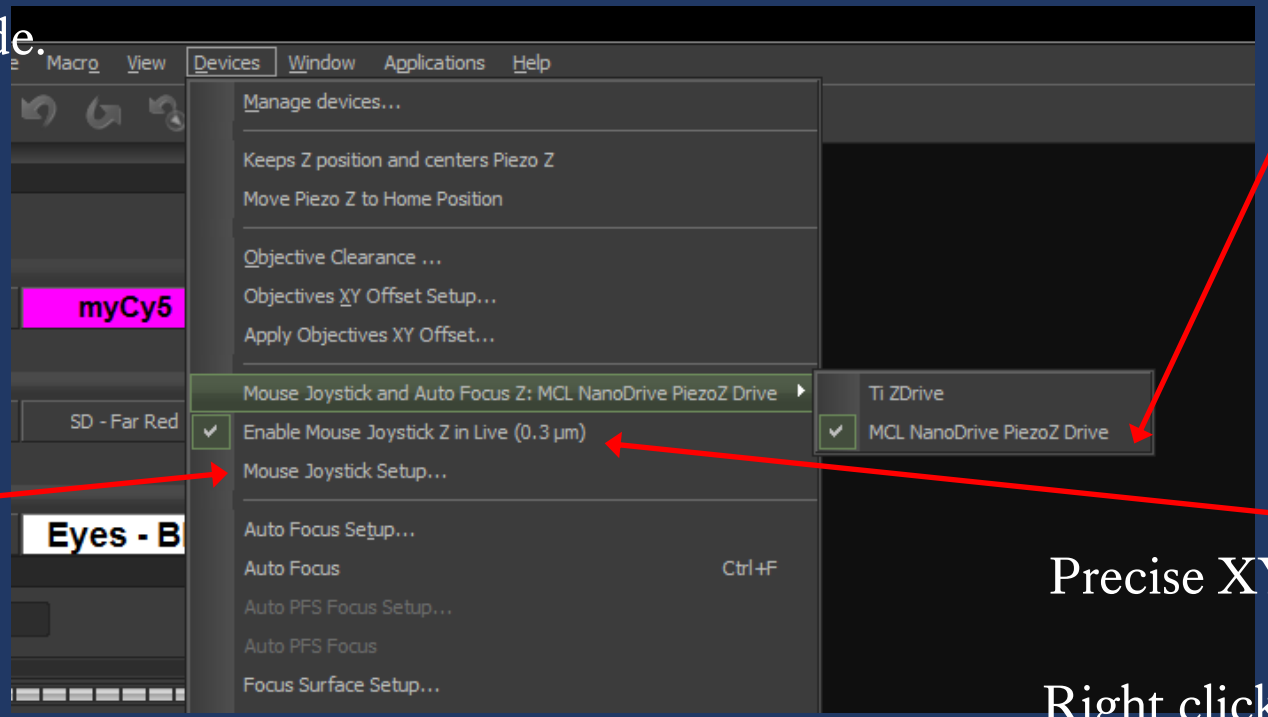
Mouse left click hold, drag and drop to rearrange docking pane.



Use Mouse Wheel To Focus And Move In Live Mode

This will be set up for you in your 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.

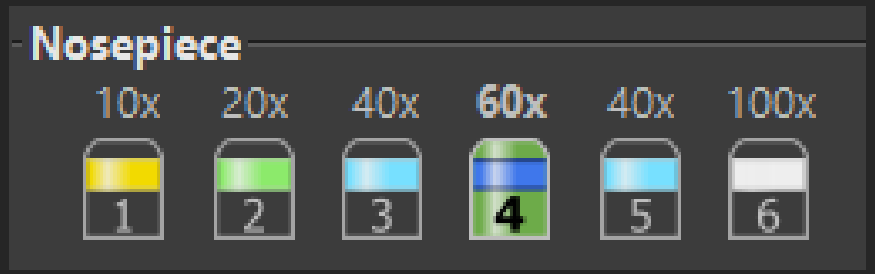
Right click for move object to centre option.

STEP BY STEP INSTRUCTIONS

STEP 2

Lens And Focus

Changing Lens



ALWAYS

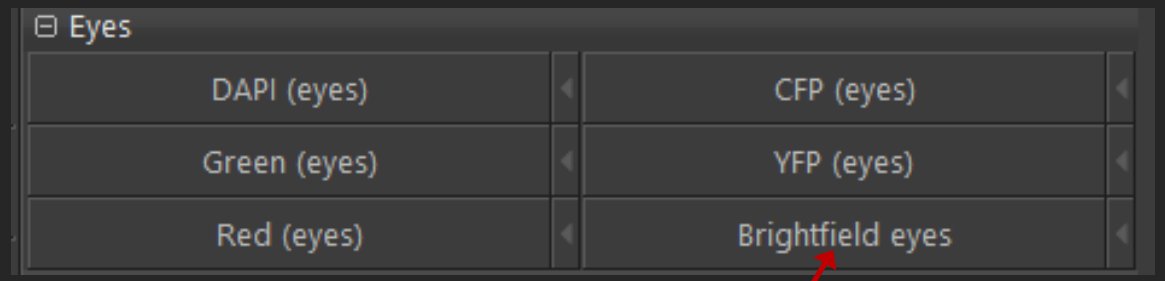
Lower the lenses as far as they can go before inserting the stage and/or clicking on another lens.

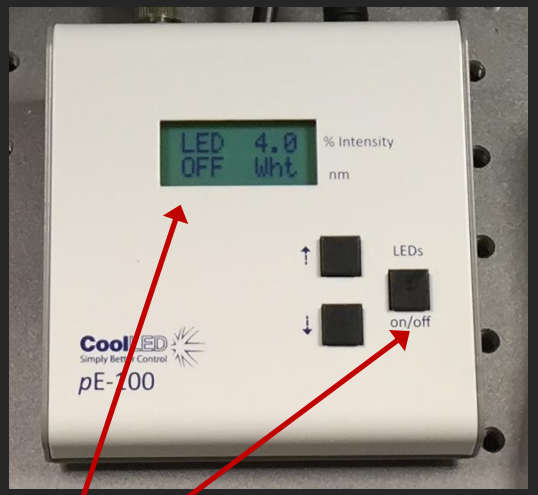
To lower lens…

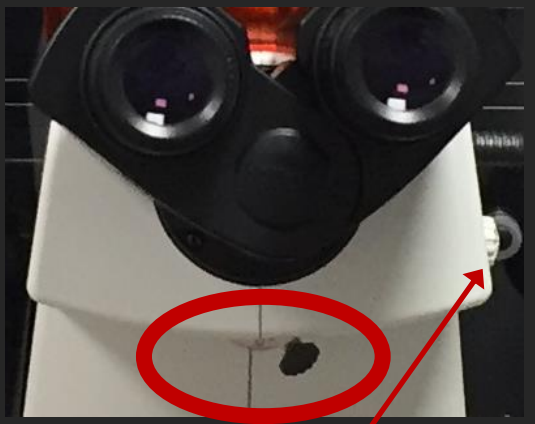
- Rotate wheel clock-wise
- Bottom = this number no longer reduces
- Be careful - zero doesn't mean it's at the bottom

Lower the lenses between changing slides.

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

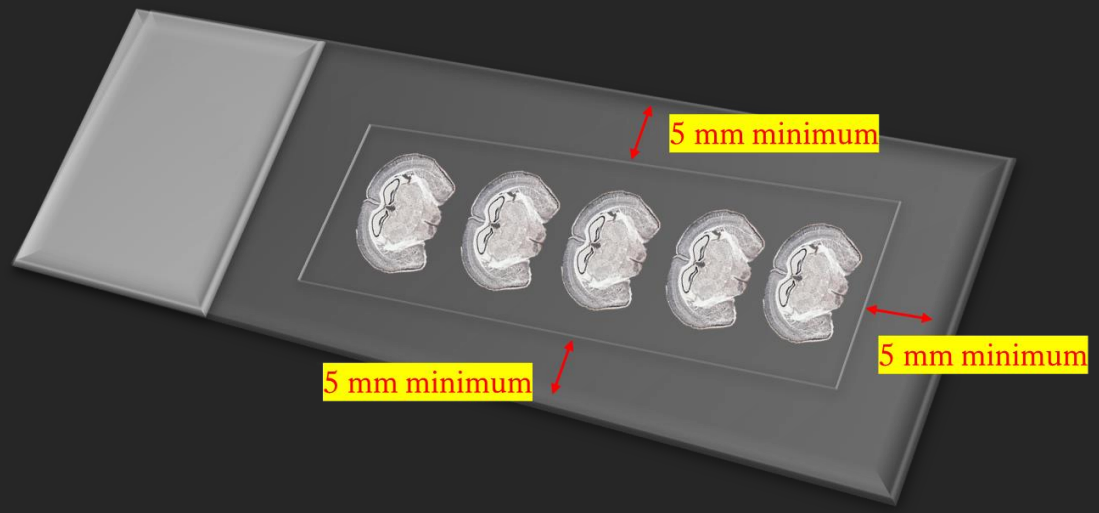
after you're done and before you start to focus.

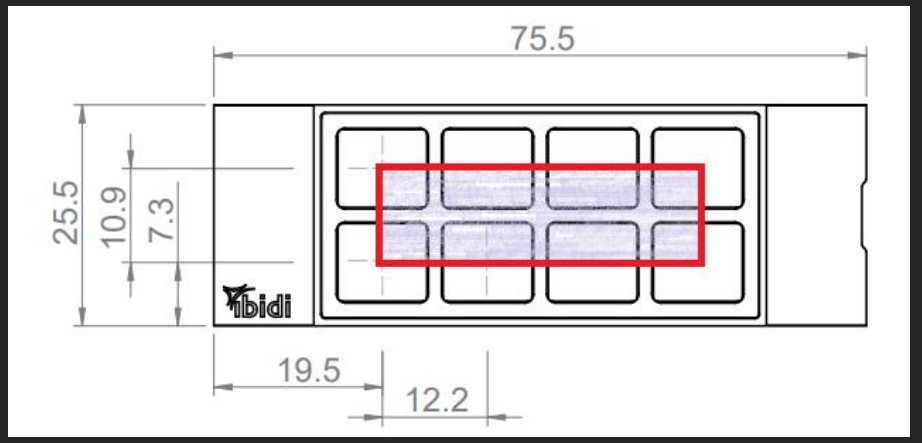
Cleaning OIL Lens

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 ethanol and clean lens from centre outwards (REPEAT 3 TIMES)
3. Clean once more with dry lens tissue

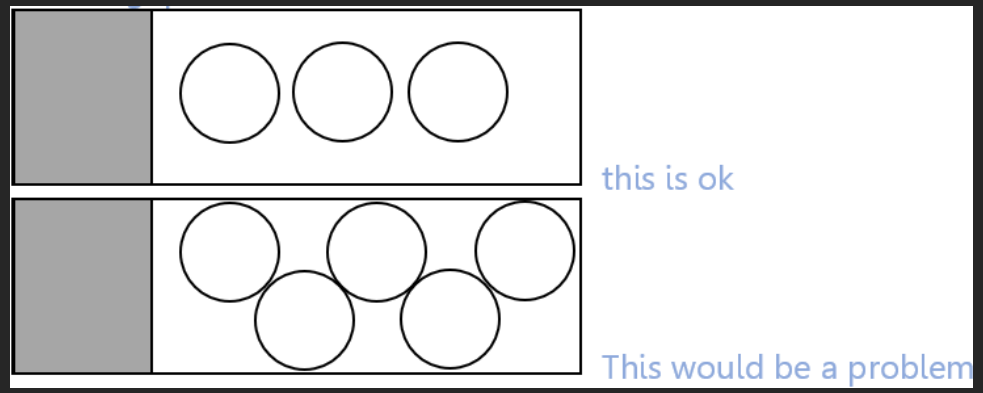
- During your session if you are switching between oil/dry lenses, just wipe away excess oil on the lens and your slide, lower your objectives down, before switching.

Load your sample into the stage





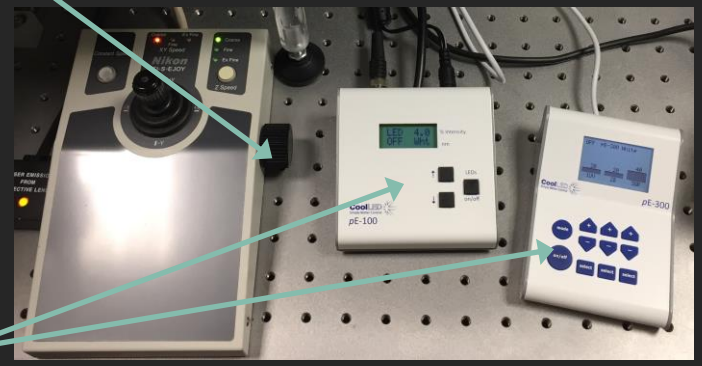
Make sure to mount your sample 5mm away from the edge of the slide! You cannot image too close to the edge of the slide, it can damage the lens!



Focus

DRY LENS ONLY (20X)

- Use Wheel and joystick on the control pad to adjust and focus.
- Make sure you are on the specimen.
- Focus indicator comes on approximately 2800µm when moving up from the lowest setting. (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 any of the “EYE” options in the software (DAPI/GFP recommended).
- Turn on and adjust the brightfield or Epi-fluorescence illumination intensity.
- Find focus using eye piece.



OIL LENS

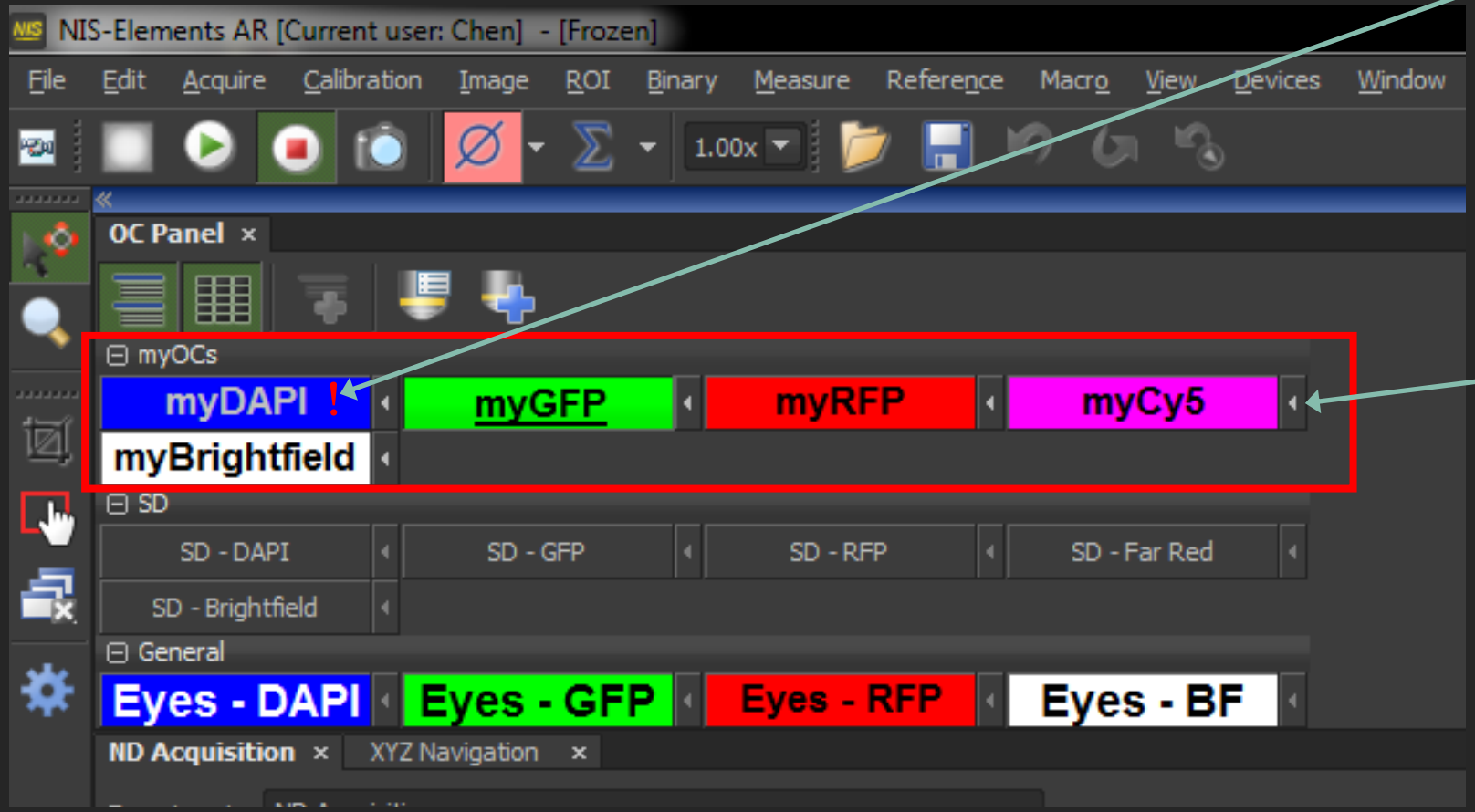
- Focus indicator does NOT work for oil lens.
- Wheel upwards on coarse ONLY until lens comes in contact with oil.
- Switch coarse XZ to fine, coarse XY to fine (twist the joystick)
- Find focus using eye piece.
- Find focus on camera.

STEP BY STEP INSTRUCTIONS

STEP 3

Change from Eyes to Camera View

Switch from EYE buttons to myOCs buttons



If after you click on one of your myOCs buttons, e.g. myDAPI and you immediately see a !

This is a glitch in the software, all you need to do is click on another button in myOCs e.g. click on myGFP and then click back on myDAPI, the ! Should disappear.

This will switch to the camera pathway. You will no longer be able to see anything down the eye piece.

STEP BY STEP INSTRUCTIONS

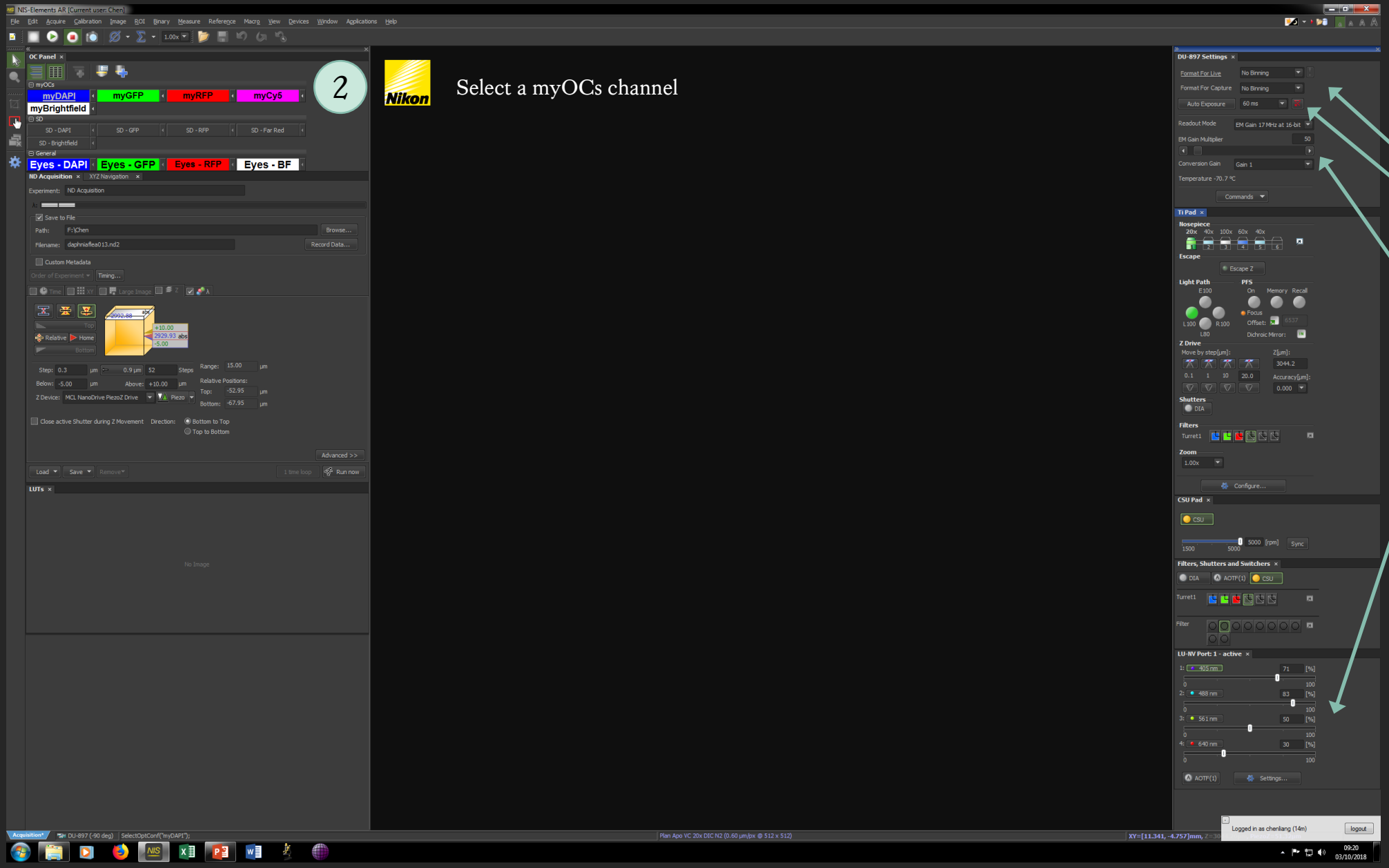
STEP 4

Setting Up Initial Live View

Setting Up For Live View

3 4

Click on live then stop, a frozen image should appear on your screen.



Nikon

Select a myOCs channel

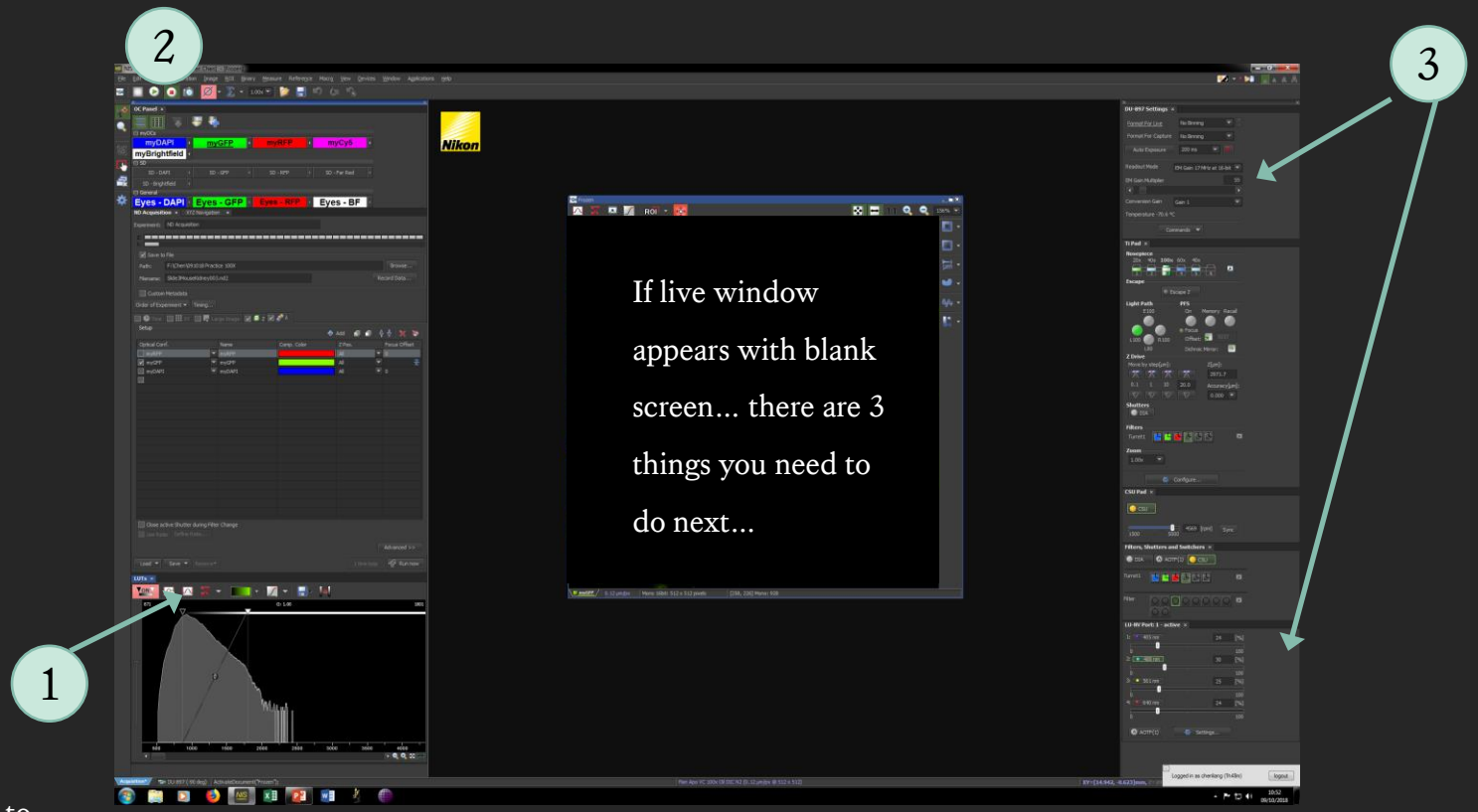
1

Camera Settings for initial live view:

- No Binning
- Exposure: ~100ms
- Readout: EM Gain 17MHz at 16 bit
- EM Gain Multiplier: 50
- Conversion Gain: 1
- Laser Power: ~20%

To see your image

Still can't see anything? Then adjust your focus a little by going back on live (click on play button) then click on your live image, then slowly turn your mouse wheel in one direction then the other to see if anything comes into focus. If nothing appears, then go back on your Eye button and bring your sample back in focus and make sure your sample is right in the centre of your field of view and come back to myOCs button.

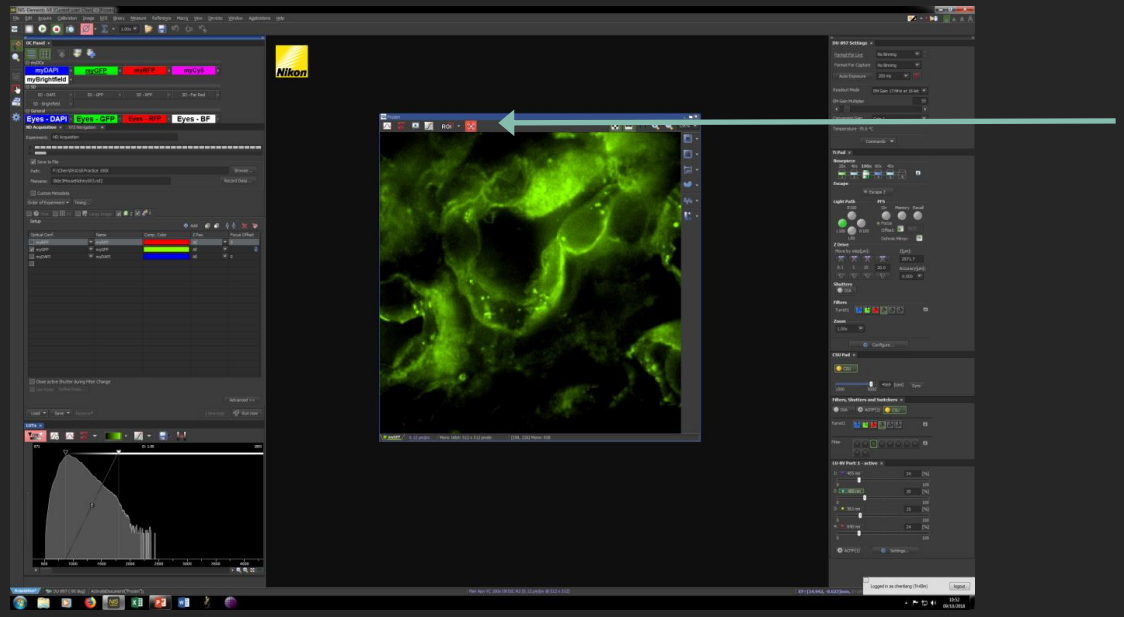


If you still cannot see anything then you might not have enough signal, you want to increase your exposure time or gain or laser power until something appears on screen.

After this stage, if you still cannot see anything on screen, please contact us and we will troubleshoot the issue.

Click on auto contrast to adjust contrast to your signal intensity range.

To move your field of view



Once you have your sample on screen, stay in live (green play button), adjust your focus using your mouse wheel, once you are happy with your focus, click on this button  and click and drag on your live screen to move your field of view.

Stop scanning once you're happy with your live view.

STEP BY STEP INSTRUCTIONS

STEP 5

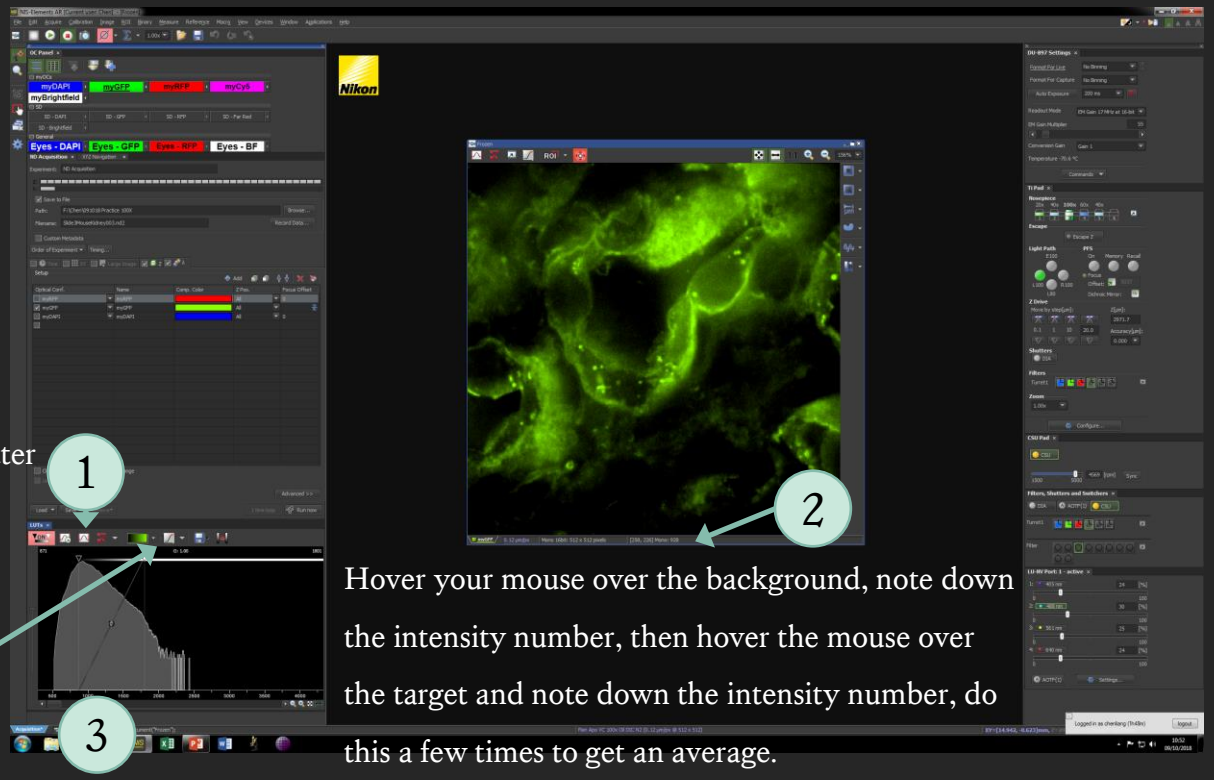
Optimising your camera settings

...adjust exposure time, gain and laser

Do You Need To Optimise Your Camera Settings?

Intensity is used as a guide to determine if there is enough exposure, gain and laser power to form a quantifiable image for different analysis needs.

This slide demonstrates how to check the intensity of your target against background intensity values.



Select automatic contrast to better visualise your target.

The saturation indicator does not work, please just make sure your intensity is NOT over 50,000, this is the oversaturation threshold.

Hover your mouse over the background, note down the intensity number, then hover the mouse over the target and note down the intensity number, do this a few times to get an average.

See next slide for more detailed manipulations of LUTs

Intensity value difference between background and target in the:

100s (Not good, try increase your exposure time or laser power or gain)

1000s (Good)

10,000s (Good and highly quantitative)

This gives you an idea if you need to change the camera settings.

Should NOT go over 50,000 !!!

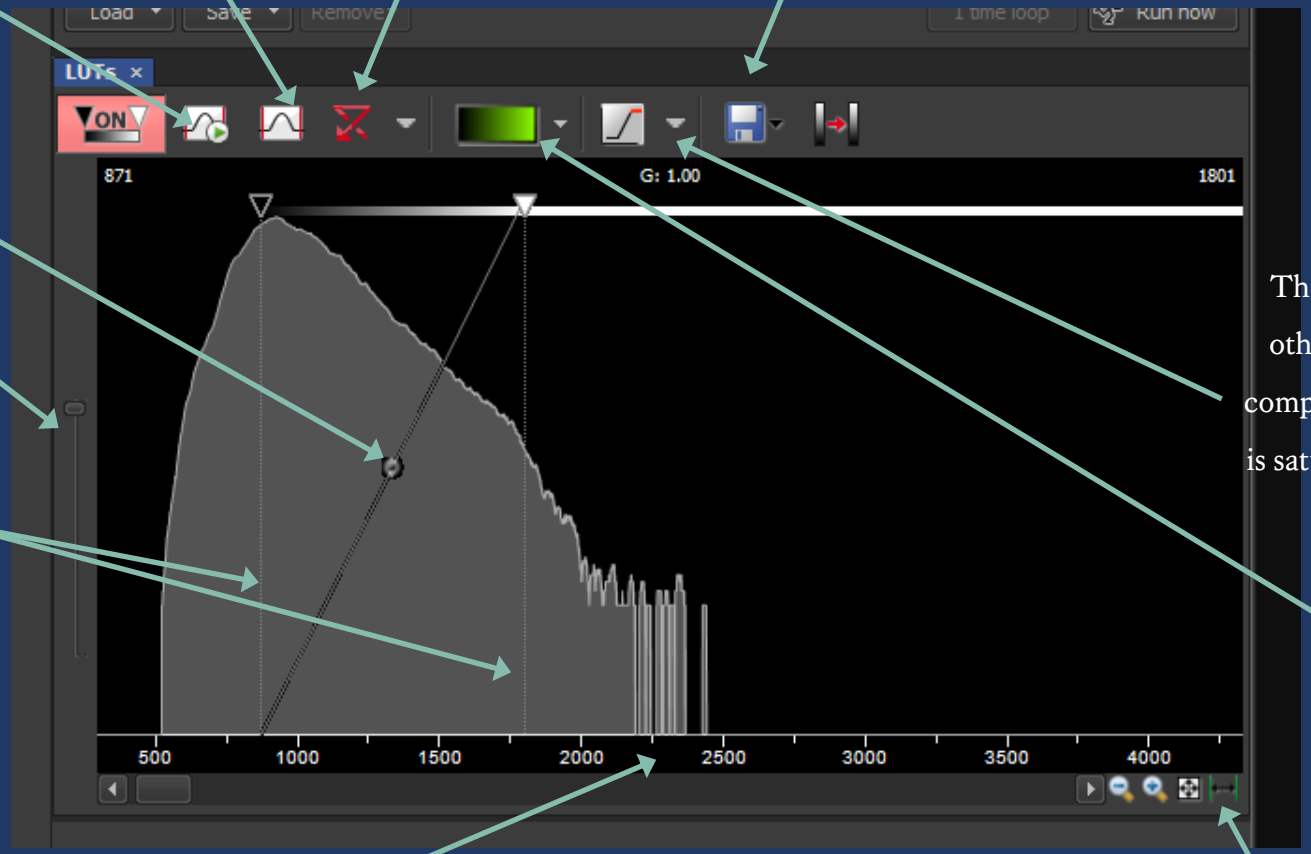
LUTs in more detail... LUTs help you visualise your image, changing it does NOT affect your signal levels.

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.

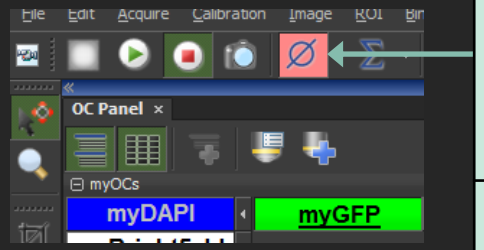
The camera is a black and white camera. You can assign any colour combination to your captured image.

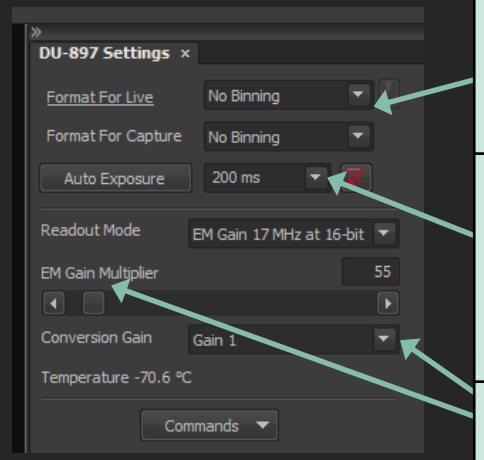
Drag and change the threshold of this histogram for better visualisation.

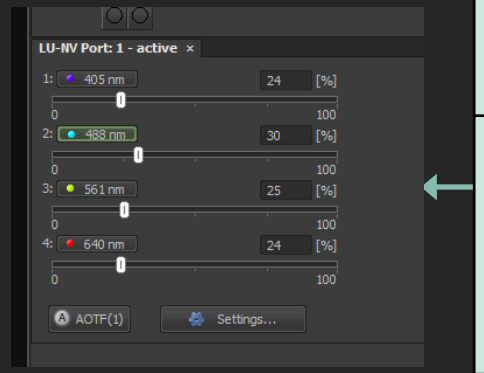
X axis - relative to #-bit camera (e.g. 16-bit CCD camera gives 65,536 different intensity values, of which you should not go over 50,000). Y axis – log intensity scale

Fit the histogram to this space

To increase or decrease signal intensity, you need to change exposure time, gain and laser power.







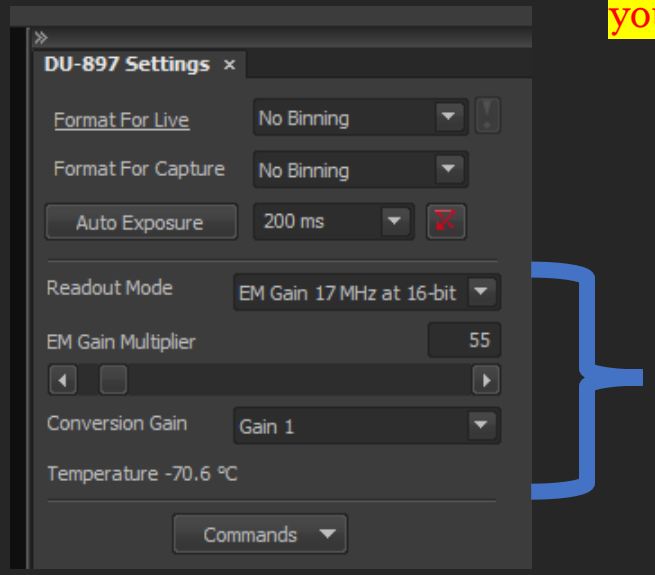
Camera Settings	Concept	Pro	Con	When To Change This...
Averaging	The camera takes multiple images and form an averaged image.	• Reduce noise	• Increase acquisition time drastically	• Increase when your image have lots of noise.
Binning	Combines the charges (signal) from adjacent pixels to form one “super” 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 (Keep consistent across ALL channels)	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 • Need to find a compromise across all channels	• DO NOT GO OVER 300 • 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.

How to change gain (HV)

Keep Gain as low as possible, increasing gain is great for increasing signal intensity, but it also increases background noise, often when users complain about their images not being ‘clear’ or ‘detailed’ it is because they have too much gain.

Once you increased gain please DECREASE your exposure time to 20ms before clicking on scan, gain can cause your signal to jump up a lot, this way you won’t suddenly oversaturate your image.

When you change gain for one myOCs channel, you MUST use this gain setting for ALL the other channels as well. (E.g. once you set your gain on DAPI like in the image, then you switch to your GFP channel, you need to replicate this gain set up , decrease your GFP exposure time to 20ms, decrease your laser to 10%, then click on scan.



These are the gain settings you can change (max EM gain is 300).

Save Your Camera Settings

After you changed gain, laser and averaging, you will see this. !

This means you've changed something in your settings and it is asking if you would like to save these changes.

To save, you just press on the triangle button beside whichever myOCs channel you are on.

Please make sure you press on the correct triangle, you don't want to save your DAPI settings into your GFP button for example.

Optimise your camera settings for every channel you want to use.

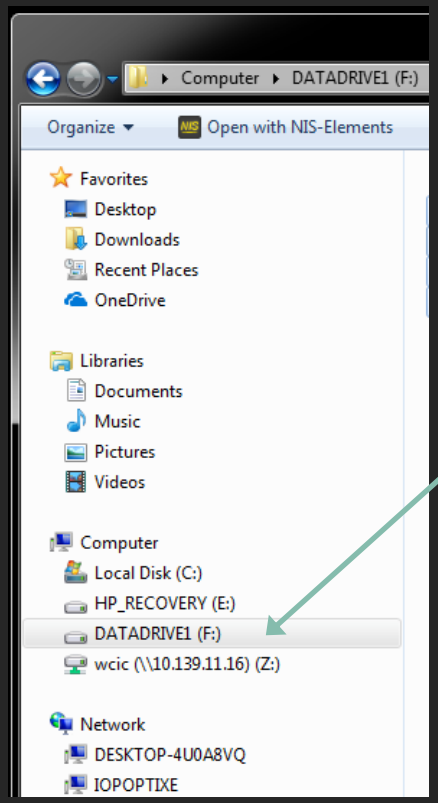
STEP BY STEP INSTRUCTIONS

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 (F:)
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.

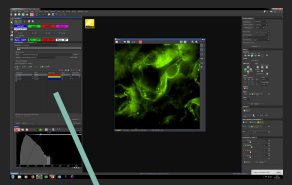
STEP BY STEP INSTRUCTIONS

STEP 6

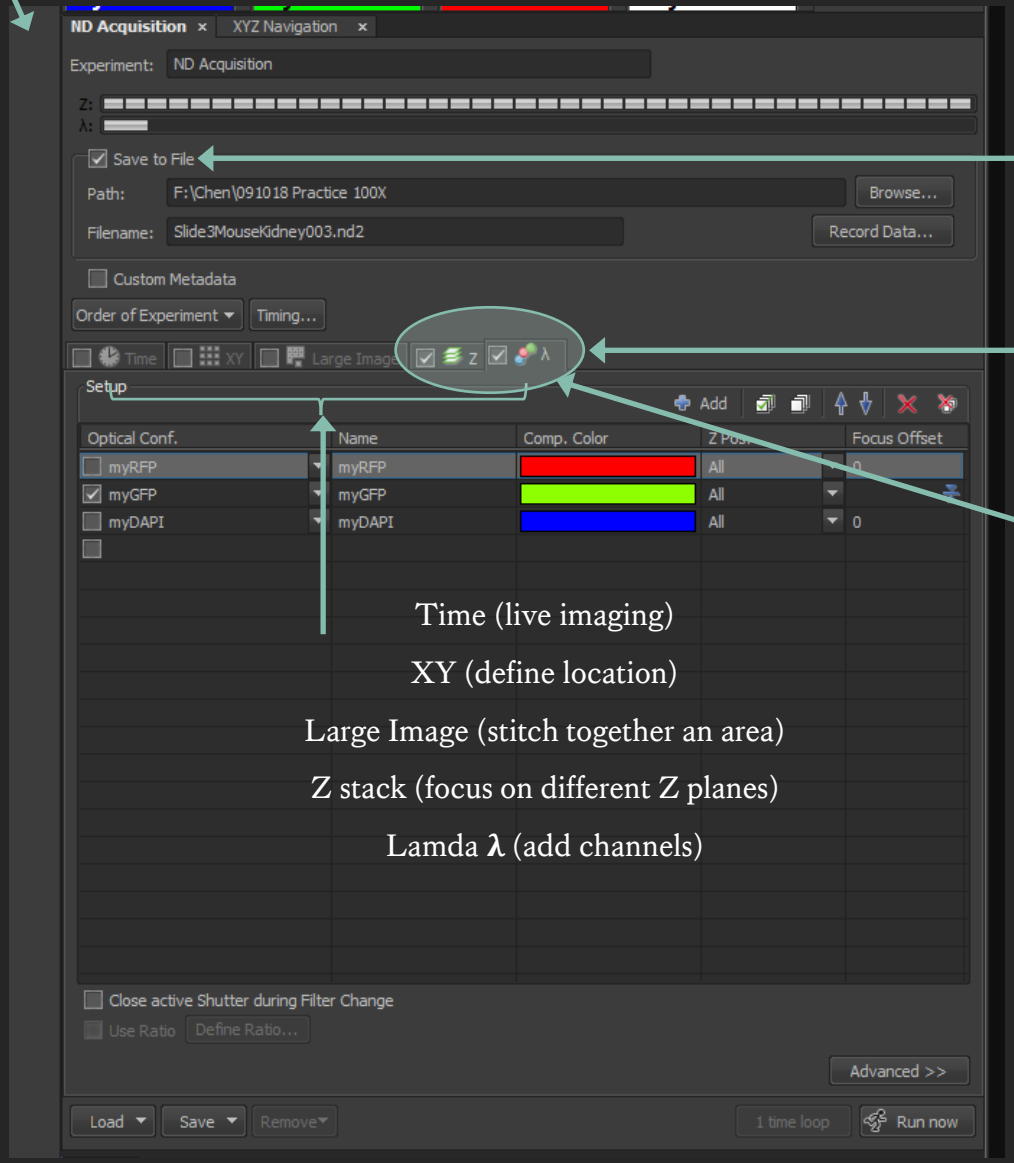
Acquisition Settings

... Order or acquisition tabs

Acquisition panel



You can apply different methods of imaging /acquisition depending on your experimental need.



If Save to file is checked, the acquired image after you press "Run now" will be automatically saved. However any contrast manipulation you apply will not be saved and the image will revert back to default contrast if you open the image again in i.e. FIJI/ImageJ

"Save As" any added ROI as a new file so not to overwrite your raw data.

Make sure only the acquisition methods you want to use are checked!

Time (live imaging)

XY (define location)

Large Image (stitch together an area)

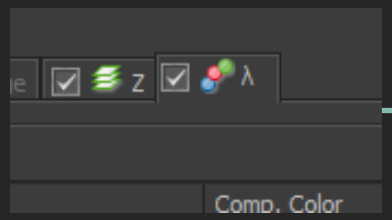
Z stack (focus on different Z planes)

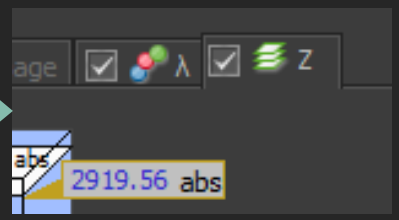
Lamda **λ** (add channels)

What Order To Take Your Image In?

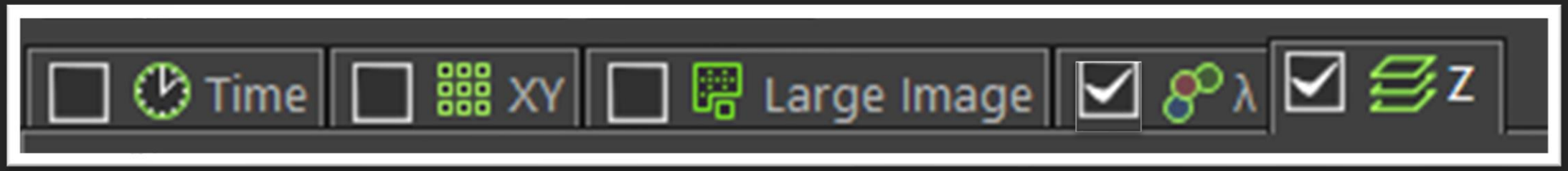
The software prioritise whichever method you put on the **RIGHT HAND SIDE!** (so in this case the system will capture each channel on a single Z plane then move on to the next Z plane and repeat all the channels. This SLOW but can be useful for rapidly changing live imaging. Most users put Z-stack tab on the RIGHT of the **λ** tab, which means a Z-stack of a single channel will be captured before the microscope takes another Z-stack of another channel.

To change the order of acquisition just drag and drop the 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

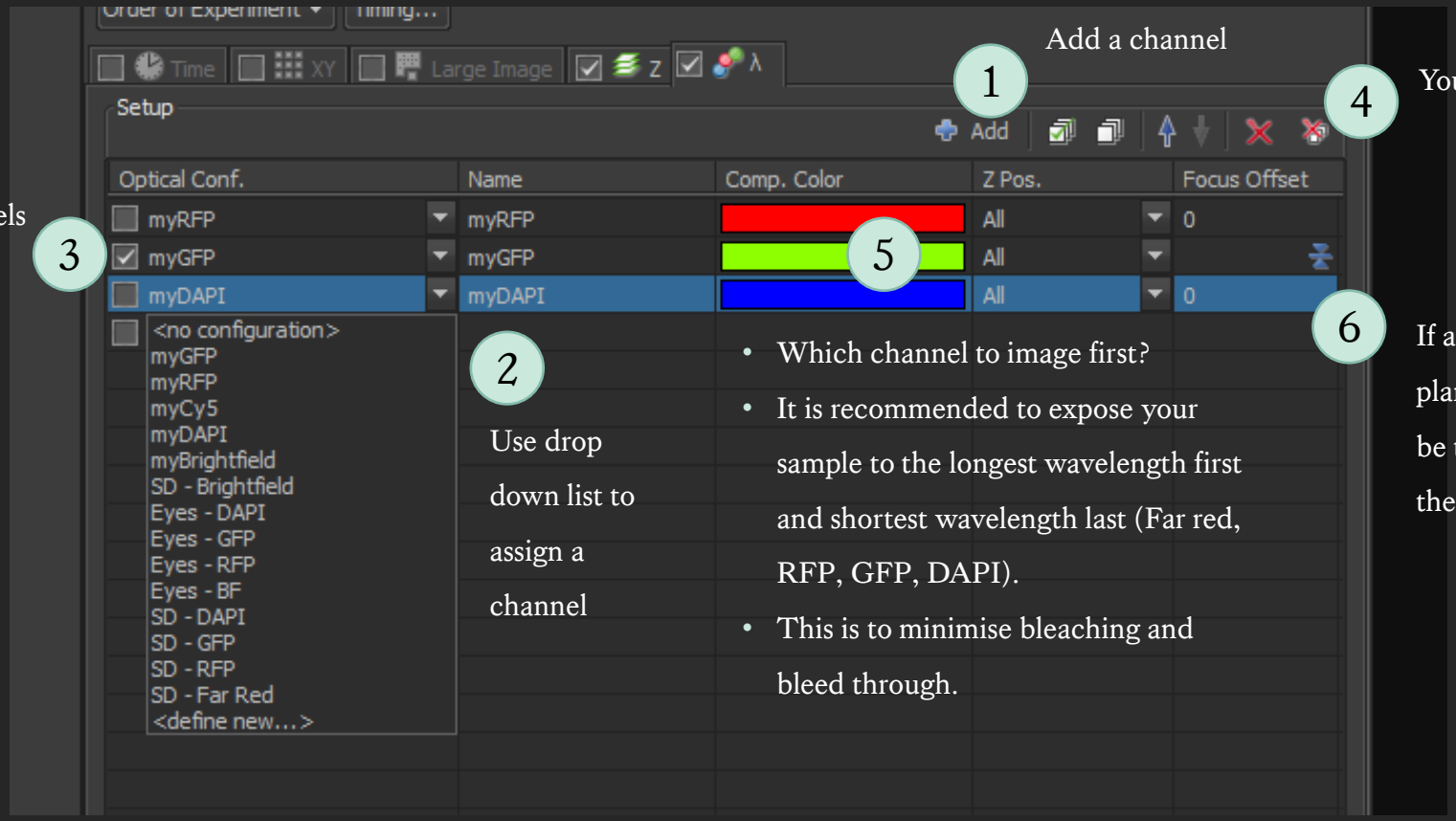
STEP 6

Acquisition Settings

... Lambda (laser channels)

Acquisition panel
Assign Channels In The Lamda λ Tab

Remember to check the channels you want to image.



Add a channel

You can delete channels

Use drop down list to assign a channel

- Which channel to image first?
- It is recommended to expose your sample to the longest wavelength first and shortest wavelength last (Far red, RFP, GFP, DAPI).
- This is to minimise bleaching and bleed through.

If a channel is out of focus on the Z plane compared to other channels (can be tested using beads) then offsetting the Z focus is an option.

STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... Z stack

Z stack Basic Options

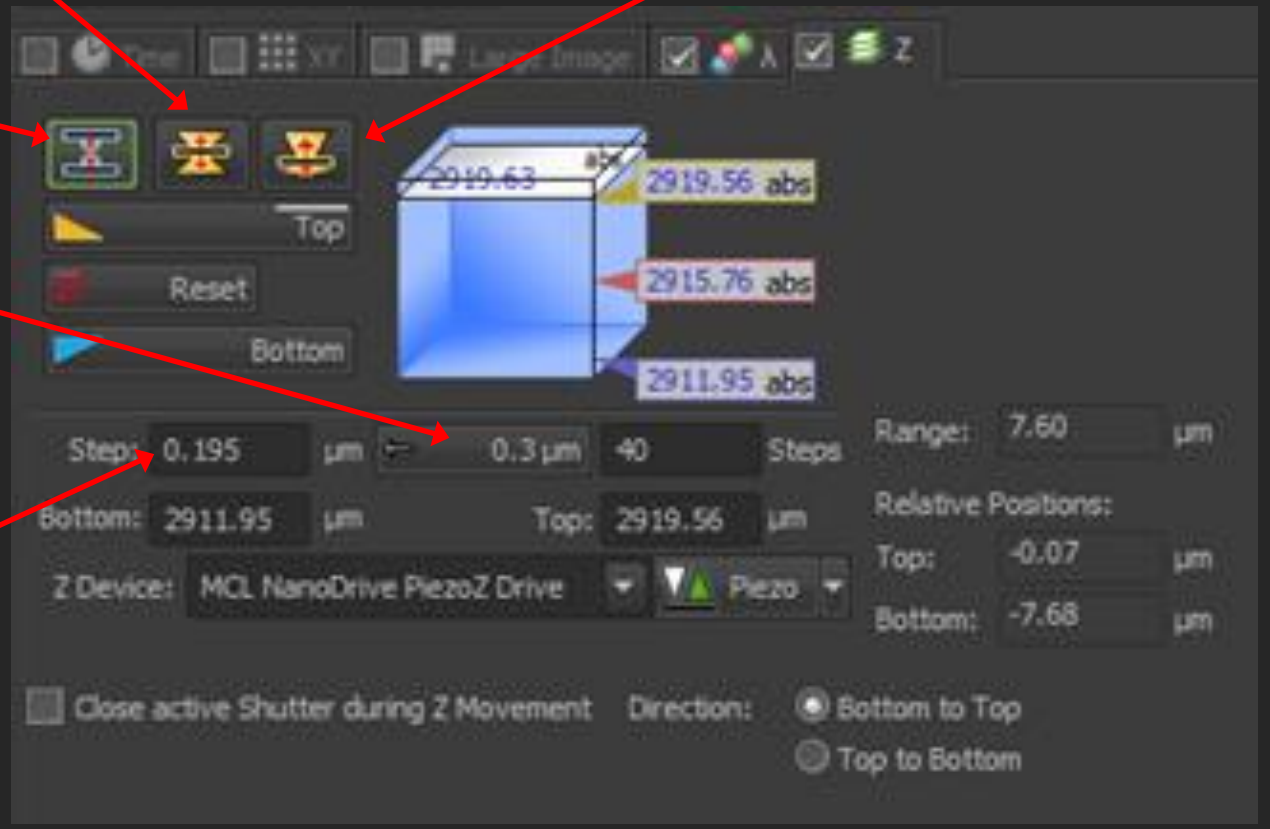
Set top and bottom: use mouse wheel to focus and define the exact range of your Z stack.

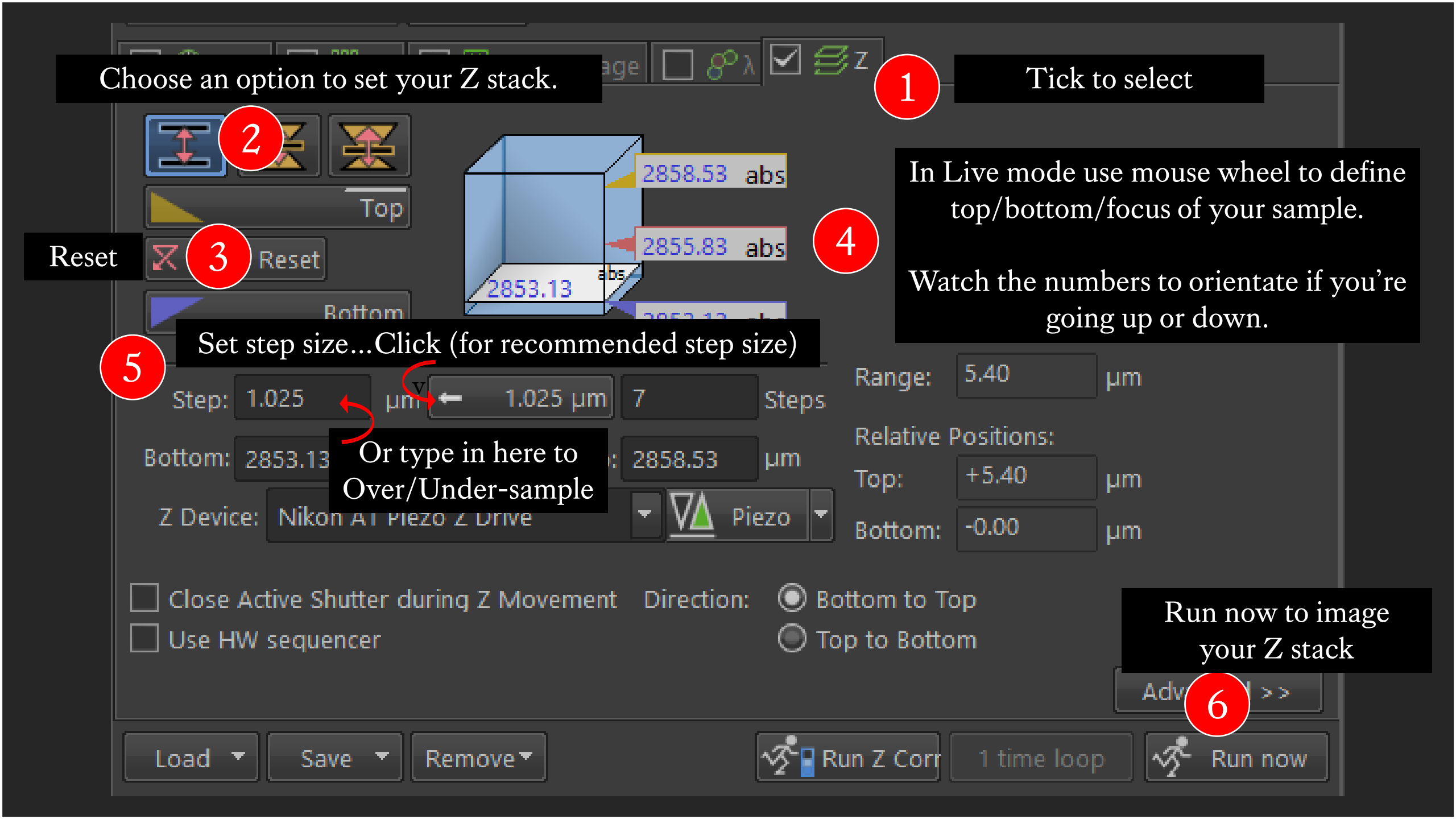
Set Middle: use mouse wheel to find the mid point of your Z focus and set equal distance above and below the focal plane. (Useful if your sample is symmetrical along the Z axis)

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

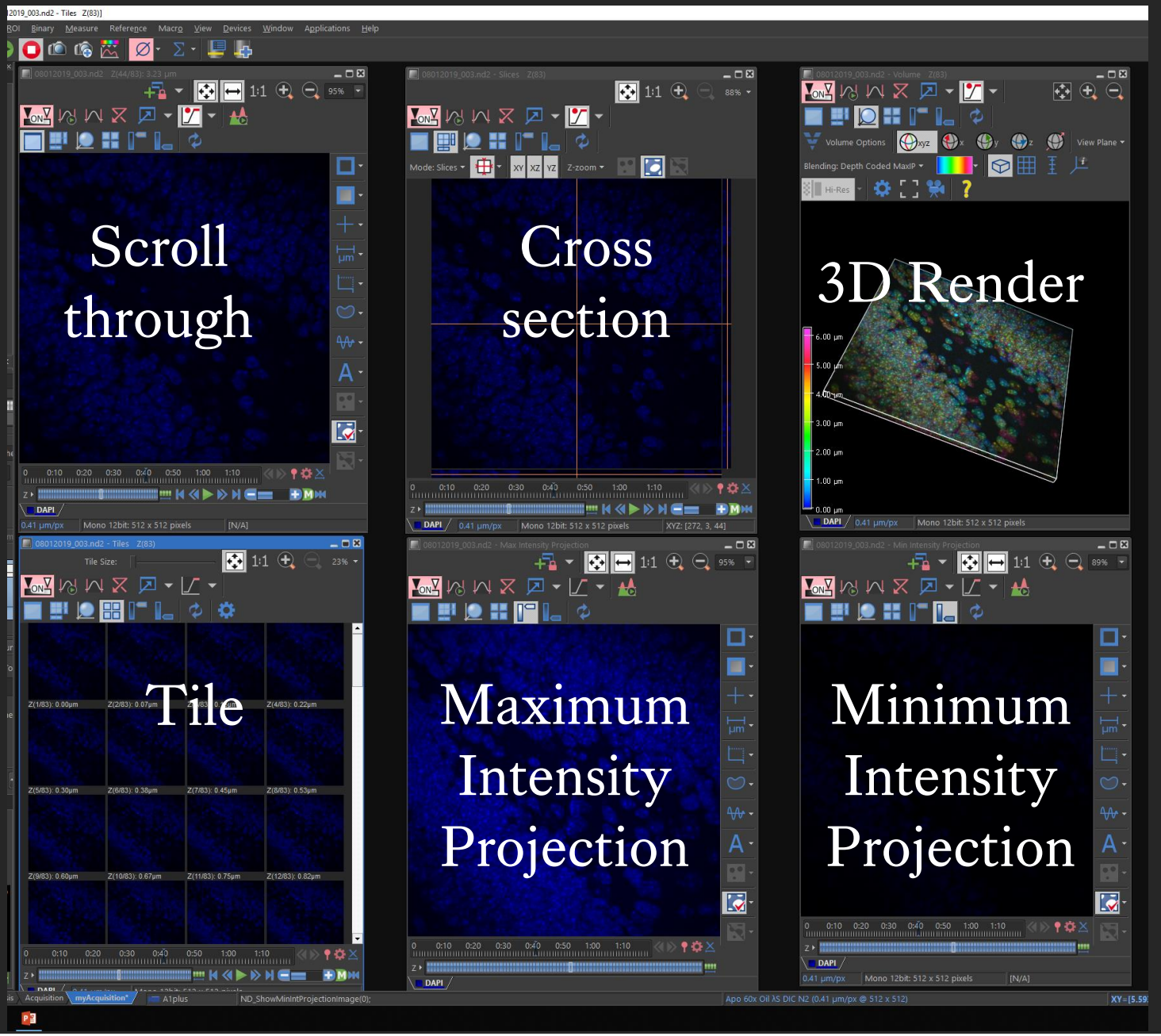
Step Size

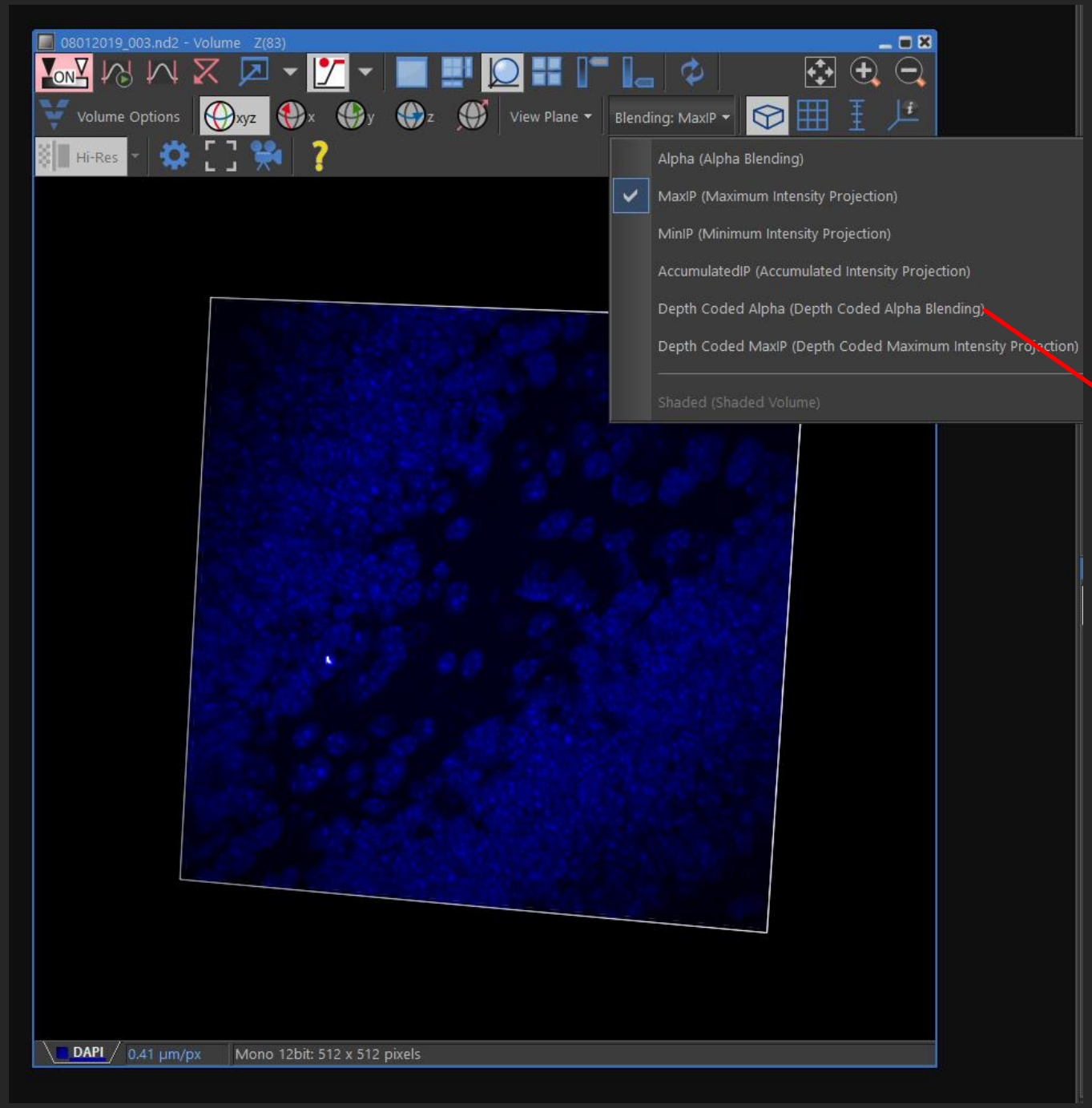
- You can set step size or number of steps.
- Use recommended step size to capture all the information.
- Fewer steps than the recommended is called under-sampling and you may loose information.
- More steps than the recommended is called over-sampling (typed in here) which is required for 3D deconvolution.



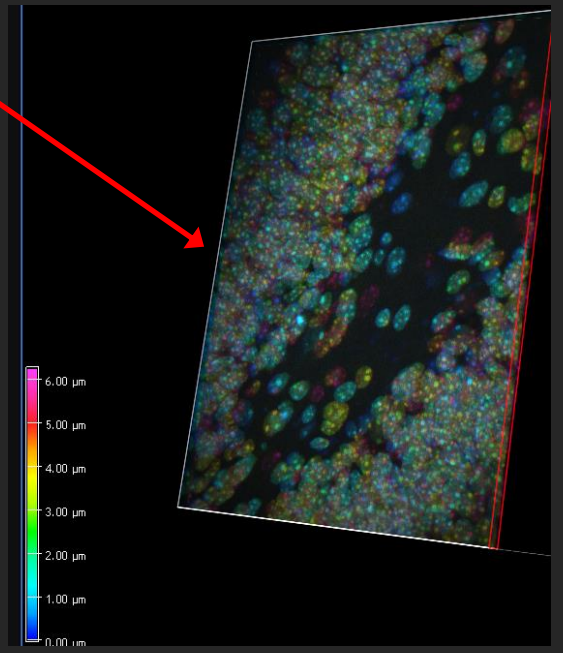

Choose an option to set your Z stack.
1
Tick to select
2
Top
Reset
3
Reset
Bottom
2858.53 abs
2855.83 abs
2853.13
4
In Live mode use mouse wheel to define top/bottom/focus of your sample.
Watch the numbers to orientate if you're going up or down.
5
Set step size…Click (for recommended step size)
Step: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

2 x 3 fields

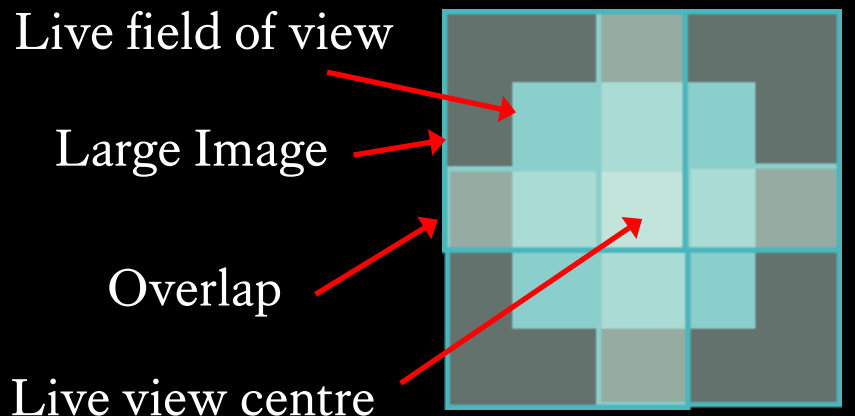
X axis Y axis

5.0 x 9.0 mm

Pattern

2

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



Stitching:

4

Stitch

Use overlapping image edge to stitch

Progressive Registration

Do Not Stitch

Overlap: 15 %

5

15% overlap minimum!

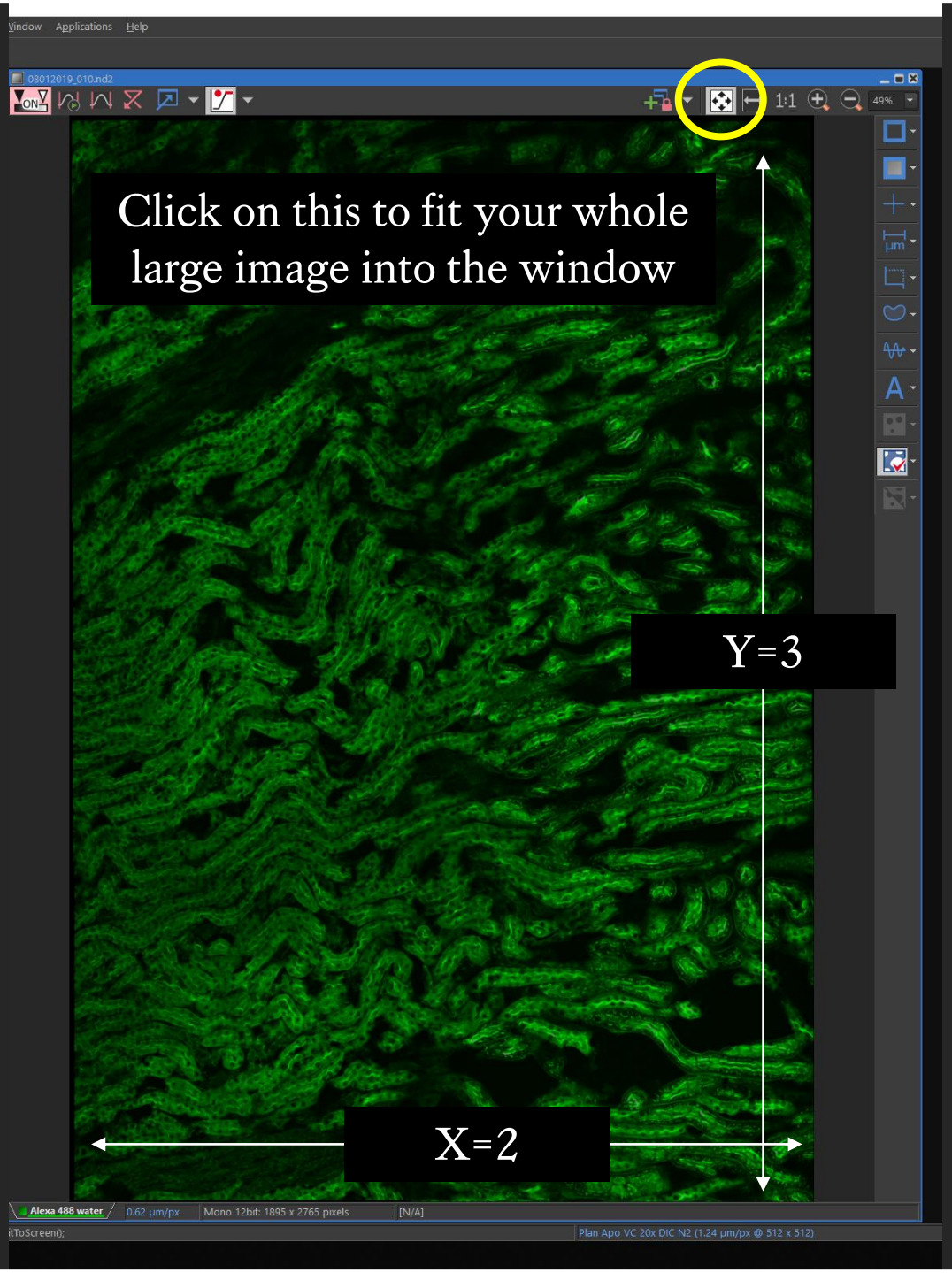
Close Active Shutter during Stage Movement

Run now to image your Large Image

Advance

6

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

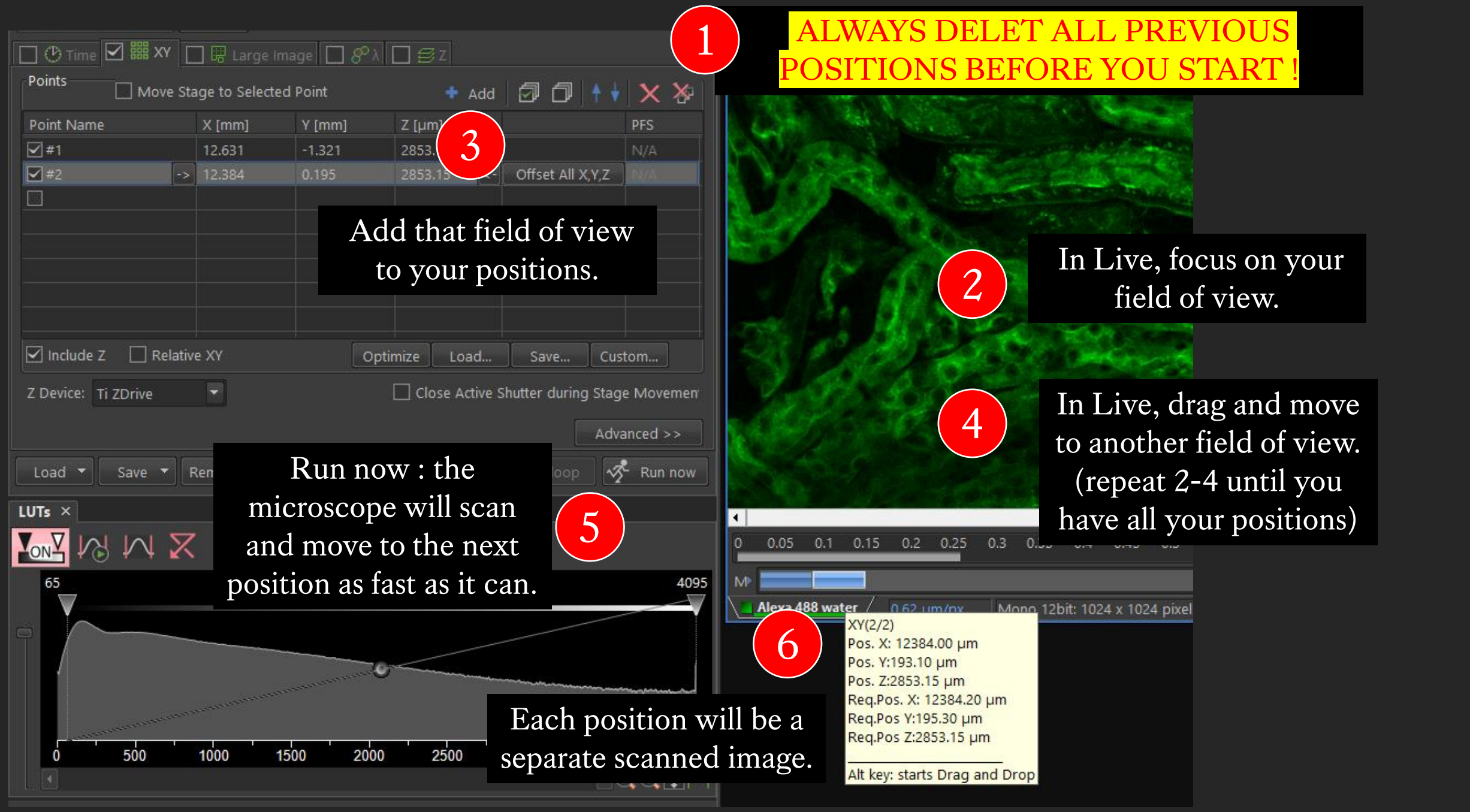

Window Applications Help
08012019_010.nd2
1:1
49%
Click on this to fit your whole large image into the window
Y=3
X=2
Alexa 488 water
0.62 µm/px
Mono 12bit: 1895 x 2765 pixels
[N/A]
itToScreen();
Plan Apo VC 20x DIC N2 (1.24 µm/px @ 512 x 512)

STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... XY Positions

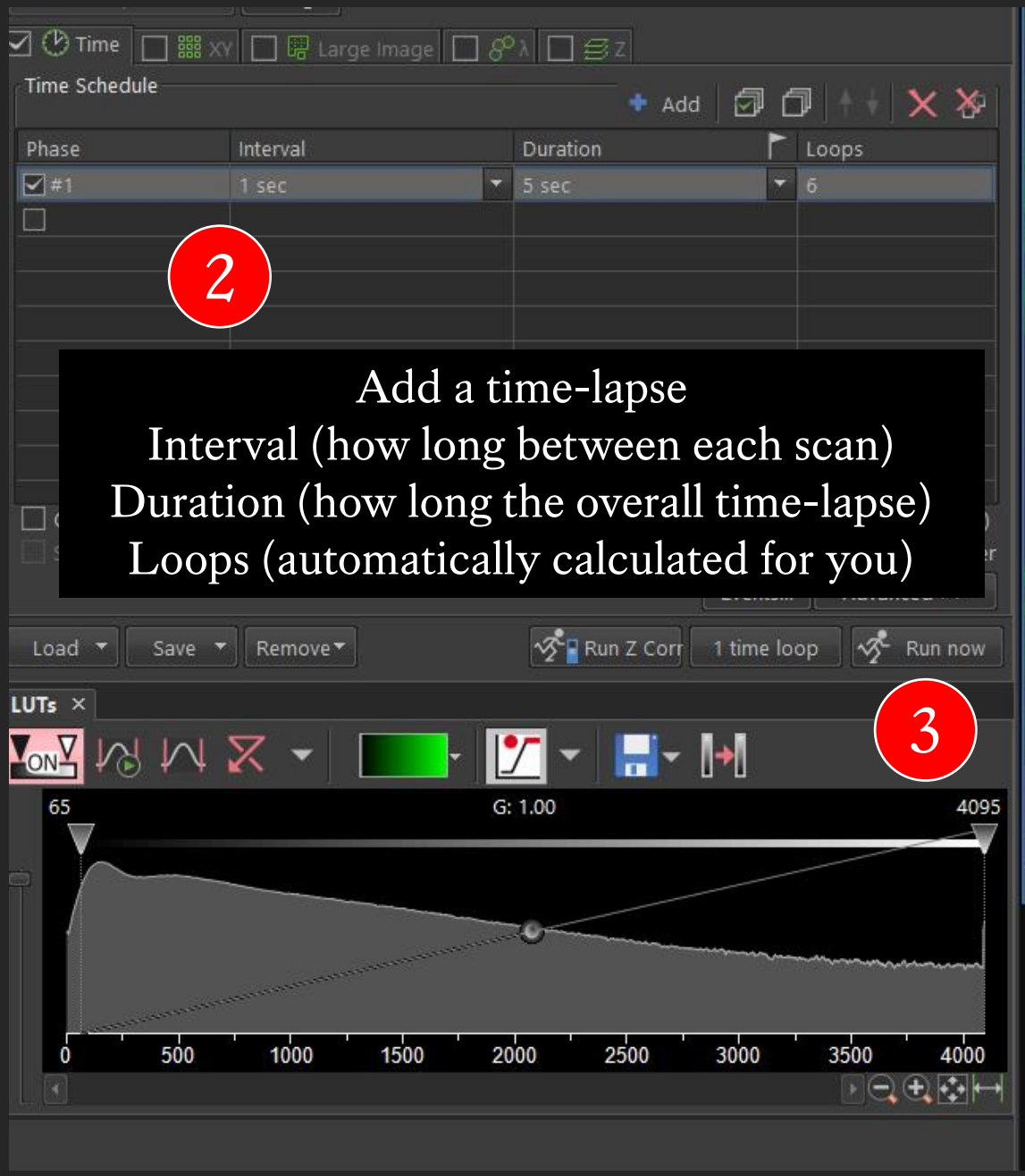

1
ALWAYS DELET ALL PREVIOUS POSITIONS BEFORE YOU START !
Time
XY
Large Image
Z
Points
Move Stage to Selected Point
Add
Point Name
X [mm]
Y [mm]
Z [µm]
PFS
#1
12.631
-1.321
N/A
#2
12.384
0.195
Offset All X,Y,Z
3
Add that field of view to your positions.
Include Z
Relative XY
Optimize
Load...
Save...
Custom...
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)
Run now : the microscope will scan and move to the next position as fast as it can.
5
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

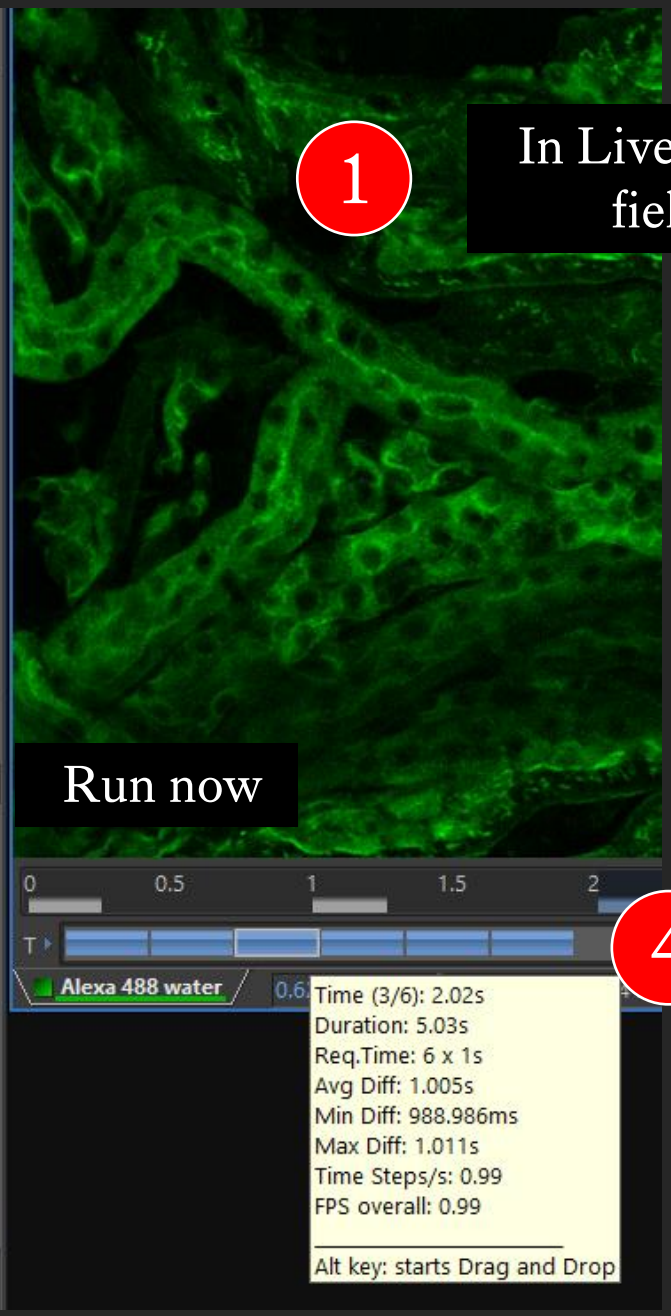
STEP BY STEP INSTRUCTIONS

STEP 6

Acquisition Settings

... Time





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

Continue with your imaging...

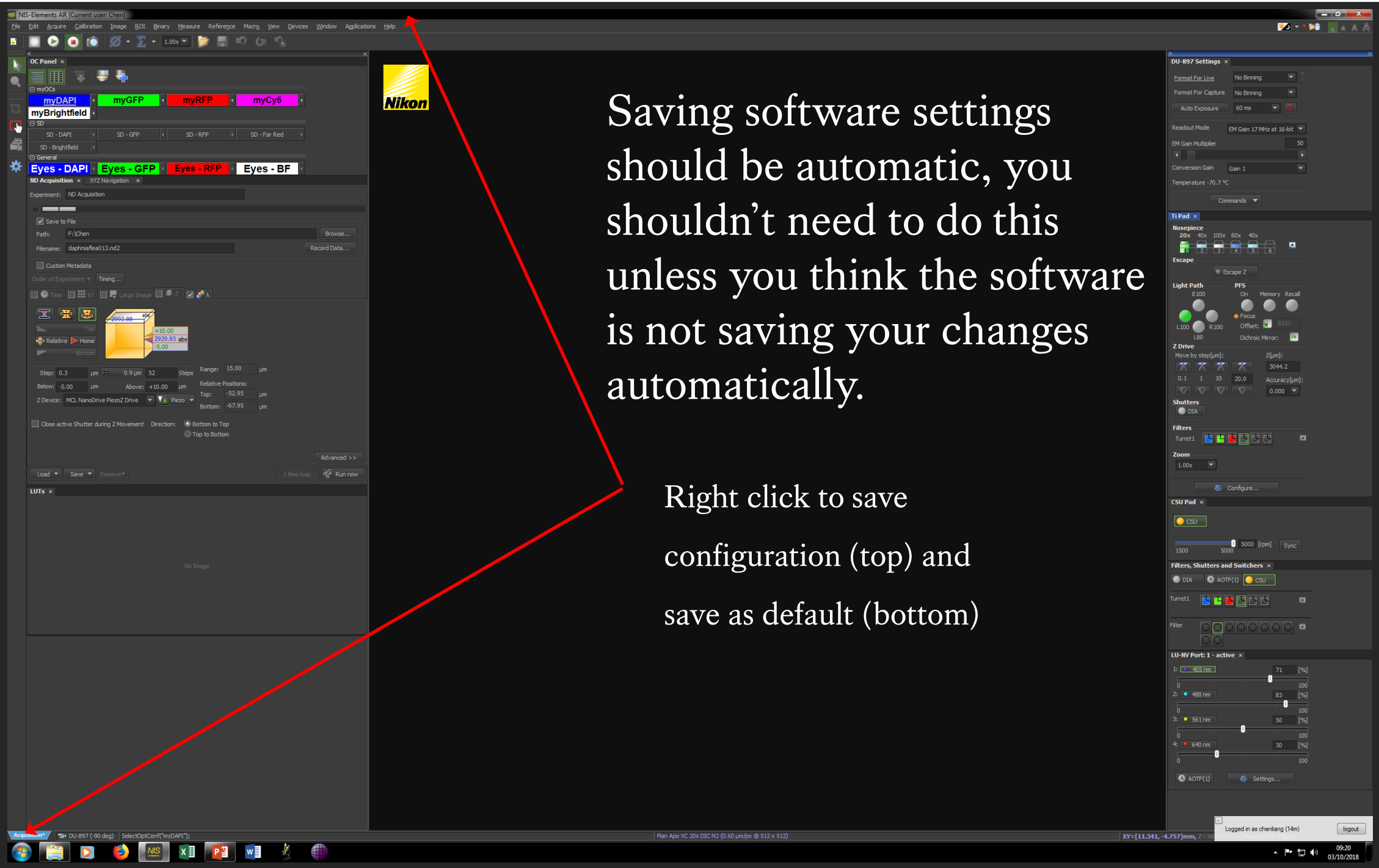
STEP BY STEP INSTRUCTIONS

STEP 7

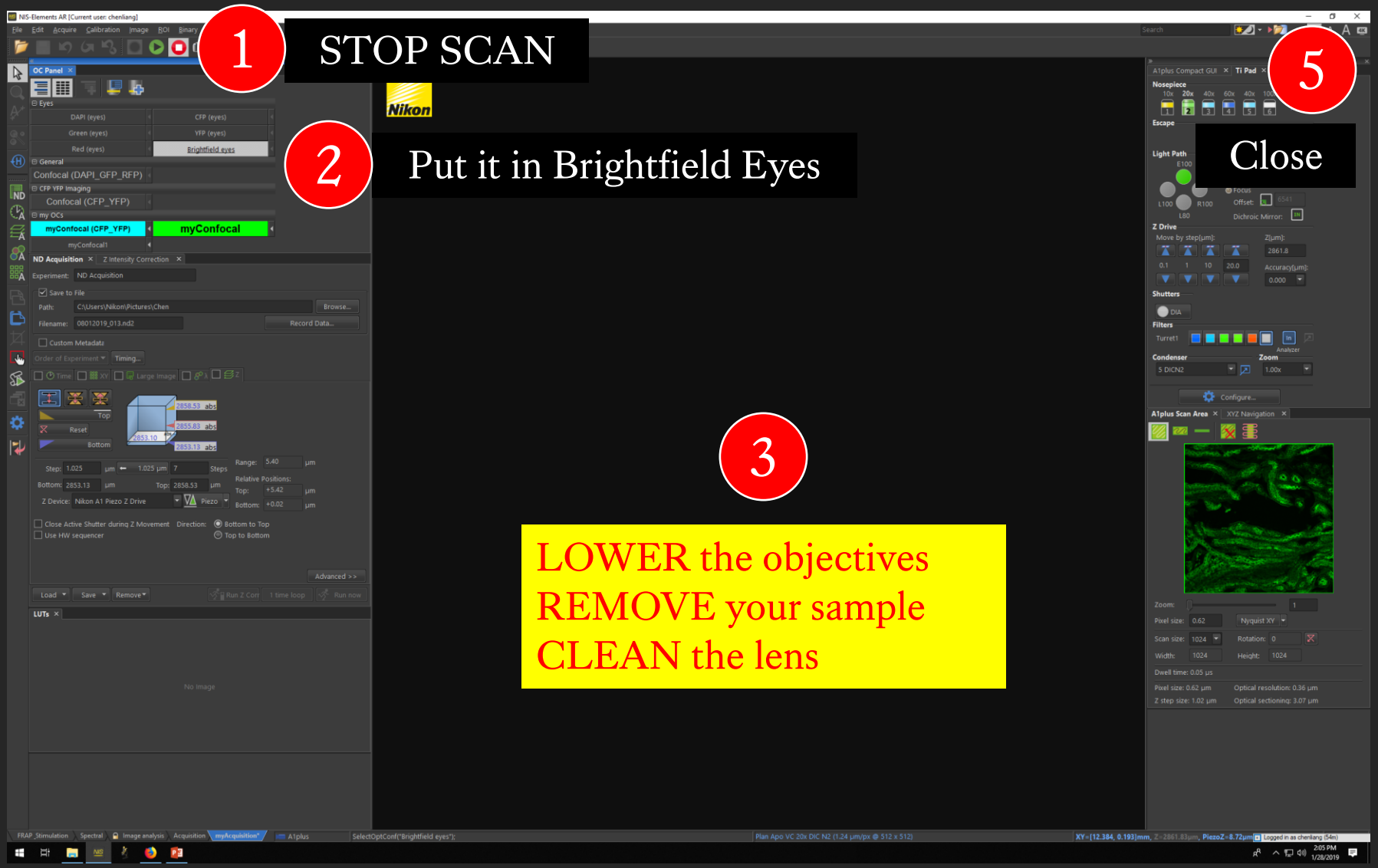
At the end of your session

... Save your software settings

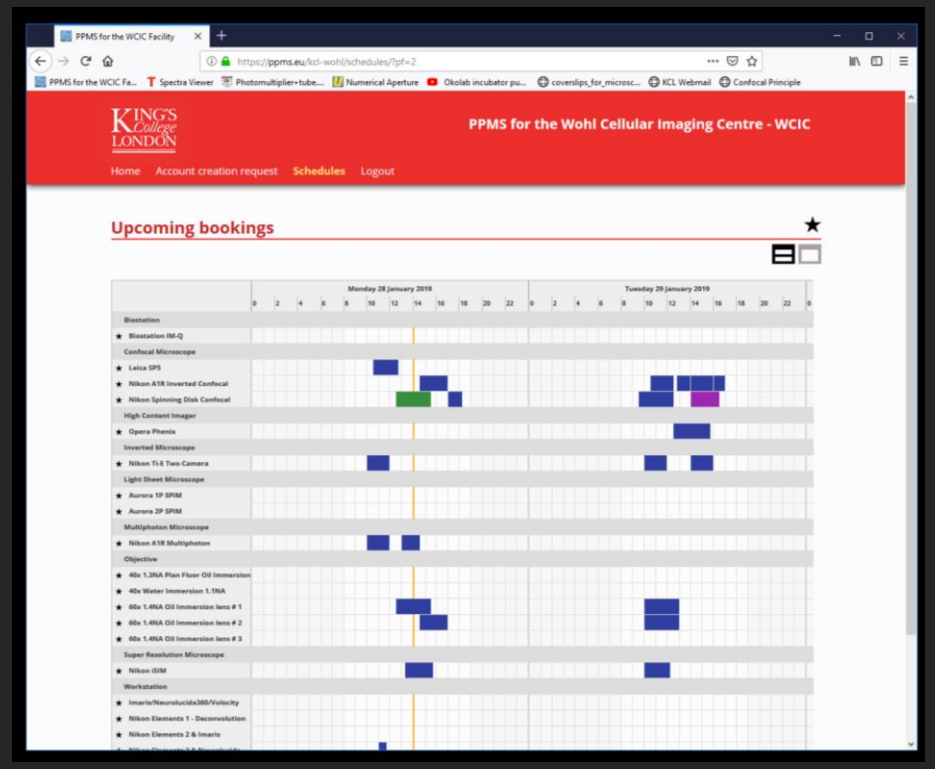
... Shut down procedure


Saving software settings should be automatic, you shouldn't need to do this unless you think the software is not saving your changes automatically.
Right click to save
configuration (top) and
save as default (bottom)

Shut down procedure… shut down the software first.



Check the booking schedule!



If no one is using the system within 2 hours, shut down the system in reverse order number 10 to number 1.

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

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

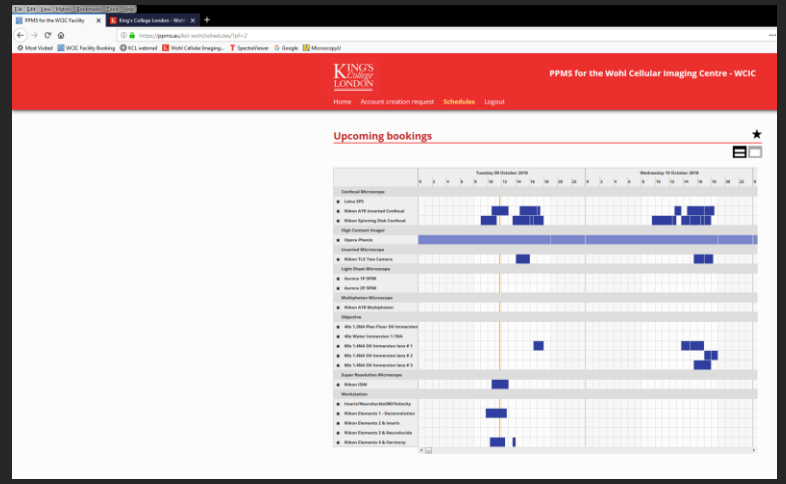
Turn off system in reverse order.
From number 10 to number 1

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.



Transfer Data To Shared Drive (1 of 3)

1

Open file

Find your saved data in DATADRIVE1 (F:)

2

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

3

This should be logged in already, if not, you need to login to this pop-up window, user name is normally: kclad\k number

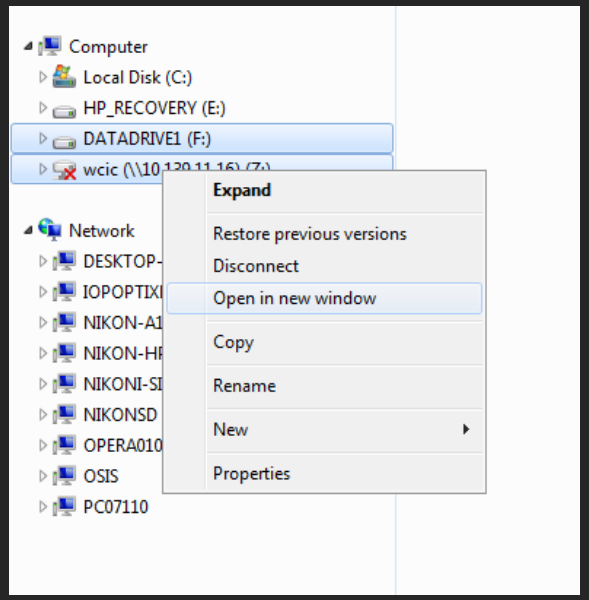
DO NOT click on remember my credentials

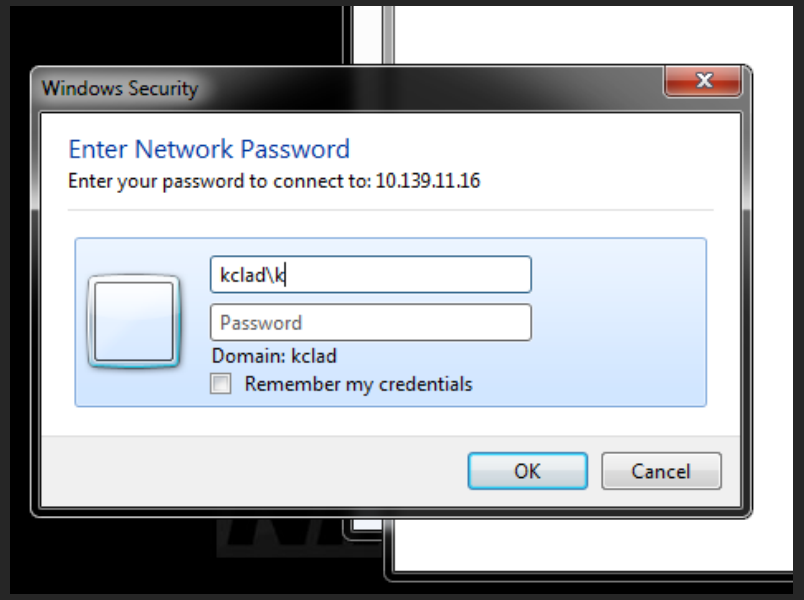
4

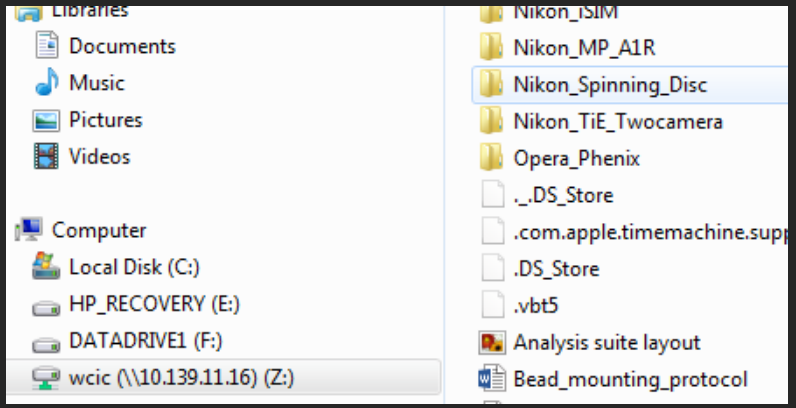
In the Network drive, open the Nikon_Spinning_ Disk folder

5

In the Nikon_Spinning_Disk folder open your personal folder





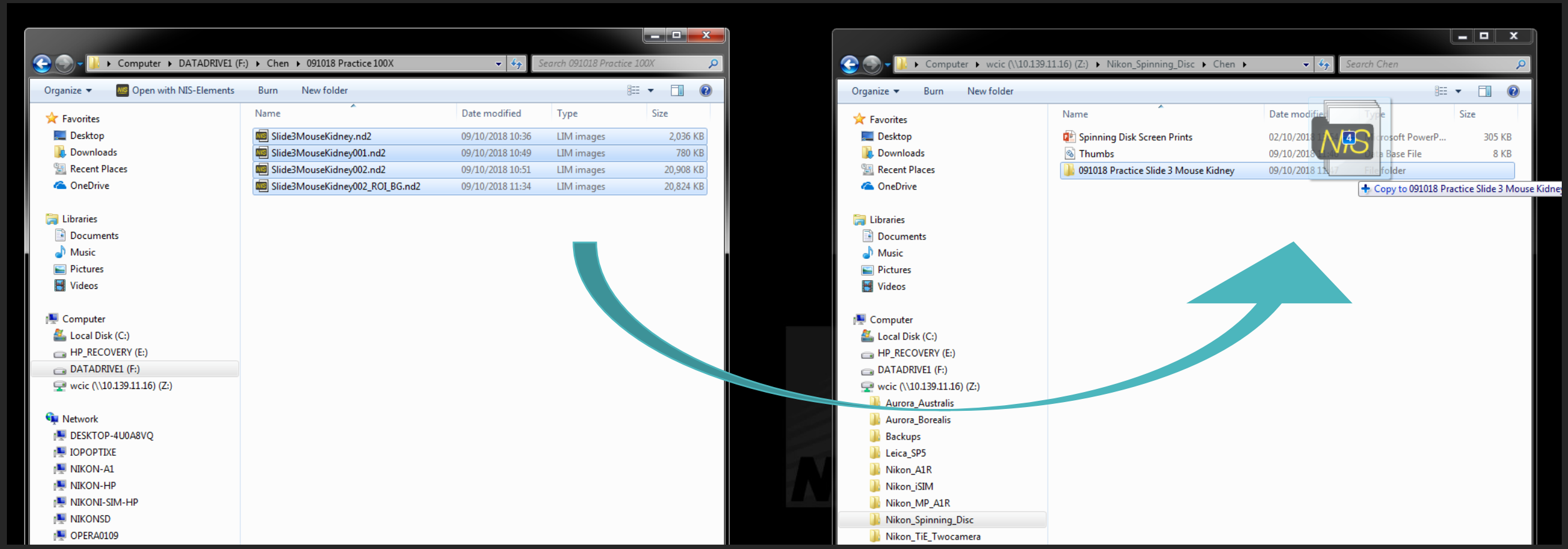


Transfer Data To Shared Drive (2 of 3)

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

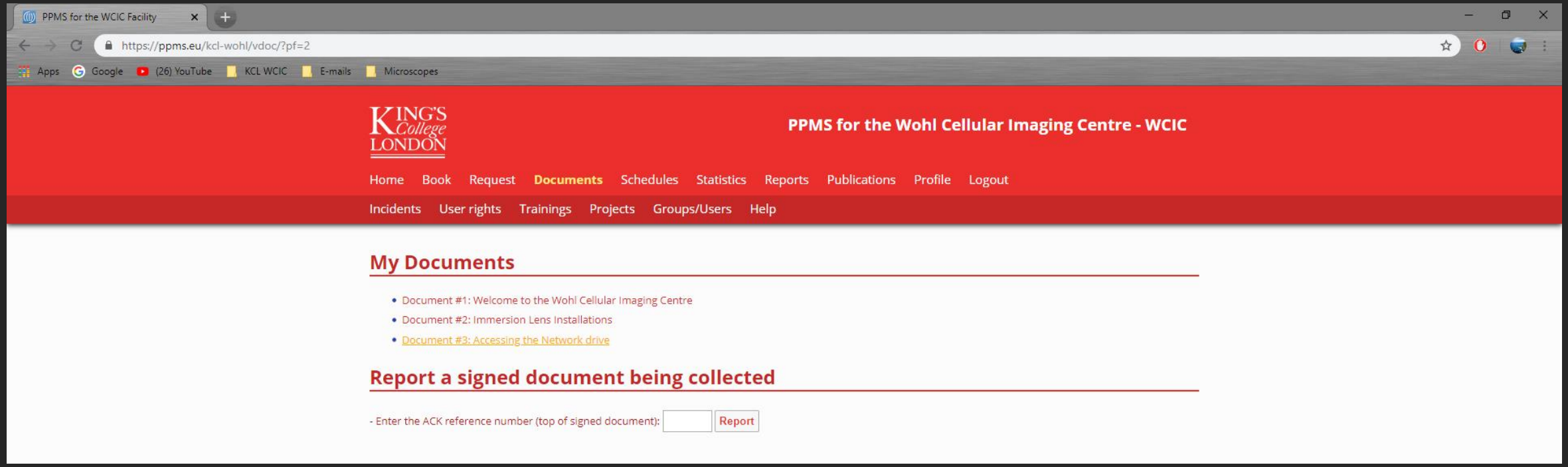
- Drag and drop the files you saved in this session.

- 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.



If you need any help, please contact:

George Chennell (07771926760)

or

Chen Liang (07883166321) via WhatsApp

Happy Imaging!