KING'S College LONDON

Wohl Cellular Imaging Centre

Nikon AX Inverted Confocal Microscope

STEP BY STEP INSTRUCTIONS

STEP 1

System ON

You can follow the AX confocal set up video here:

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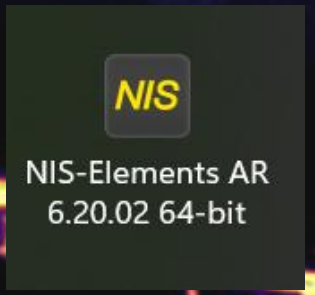
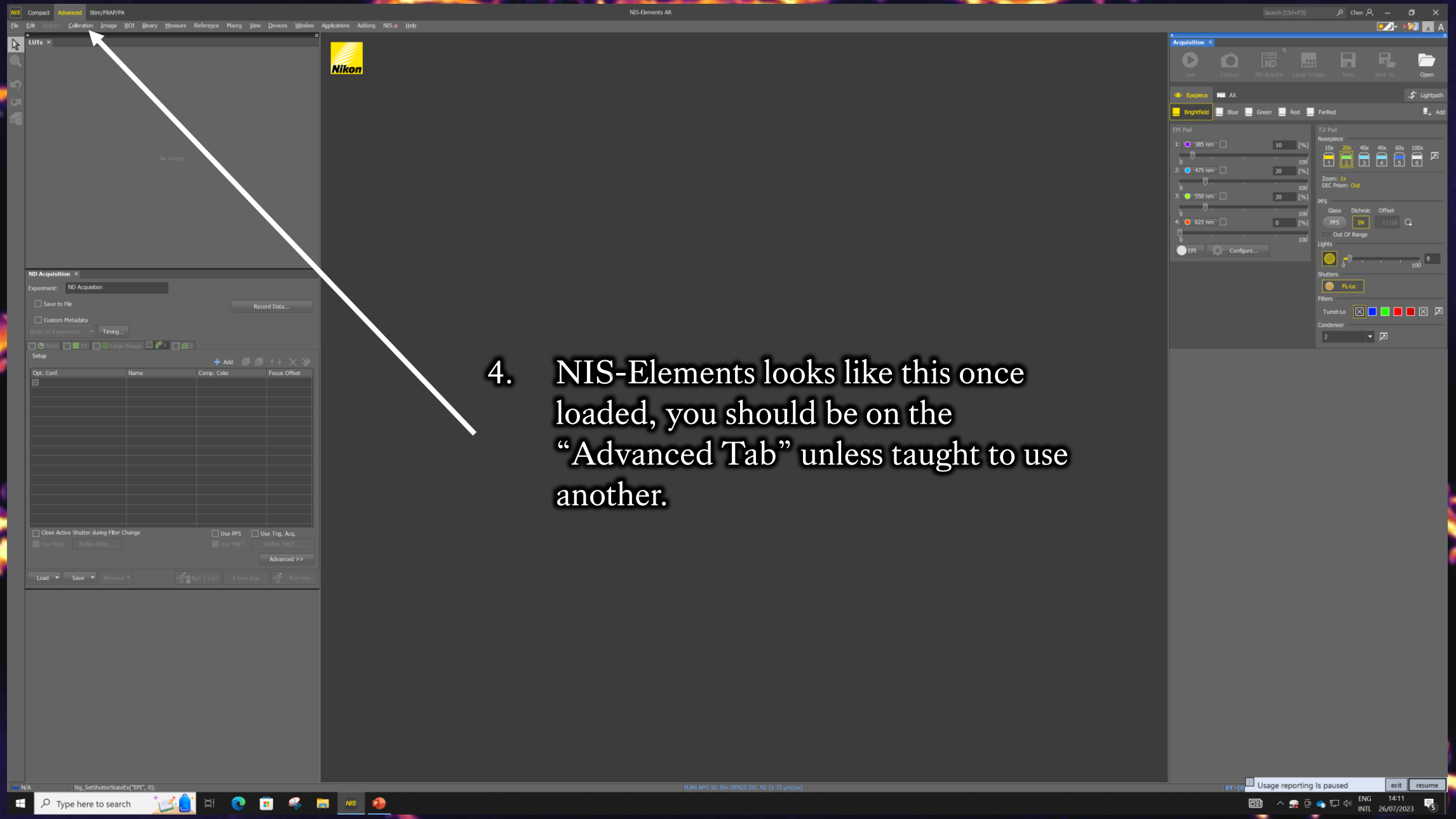
1. Switch on the A1R confocal system by following the numbered switches, wait a 5 seconds between number 1, 2 and 3.

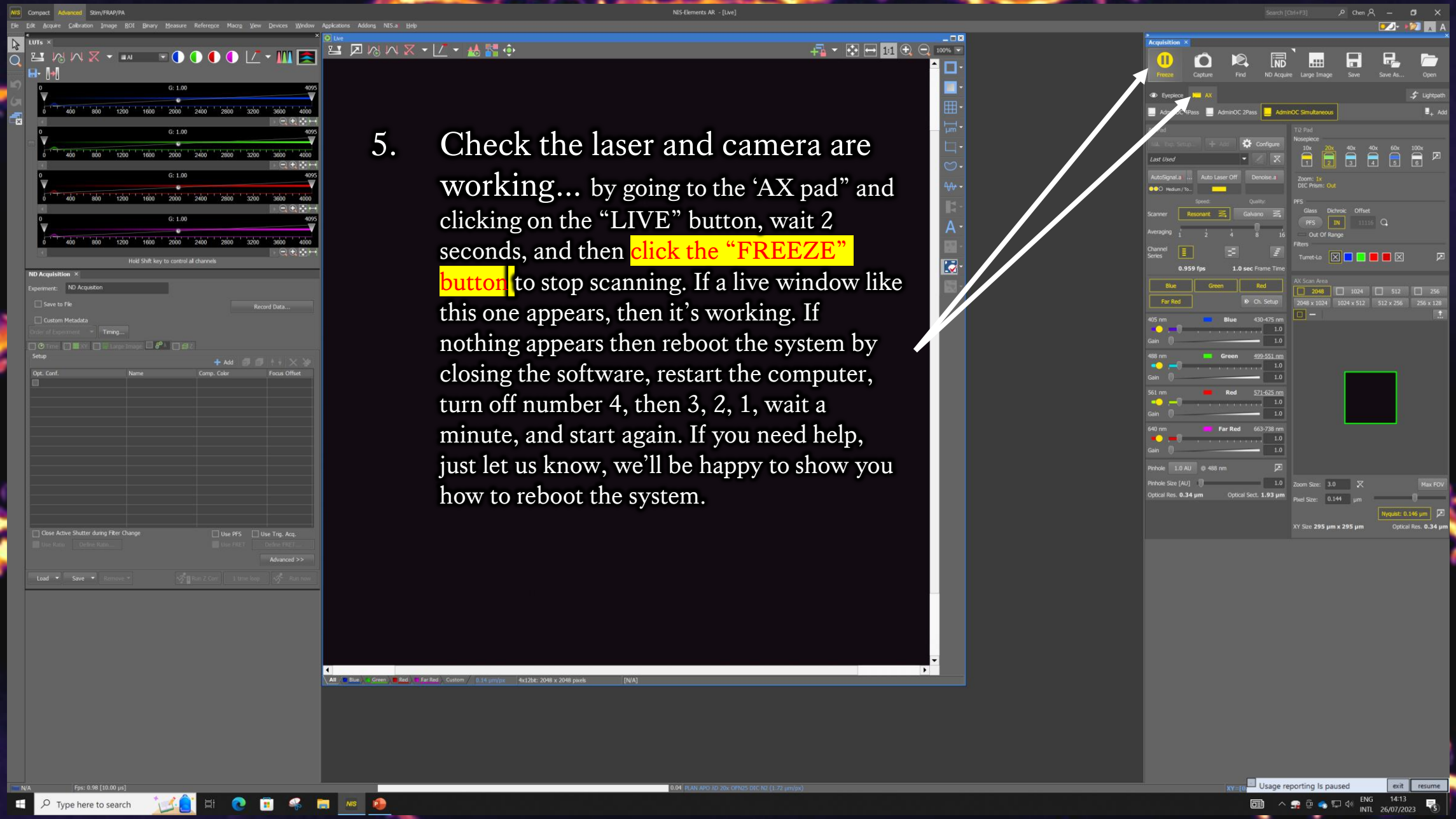
2. **Make sure the sample stage is empty before turning on number 4, the Microscope switch at the right-hand side of the microscope body, towards the back.**



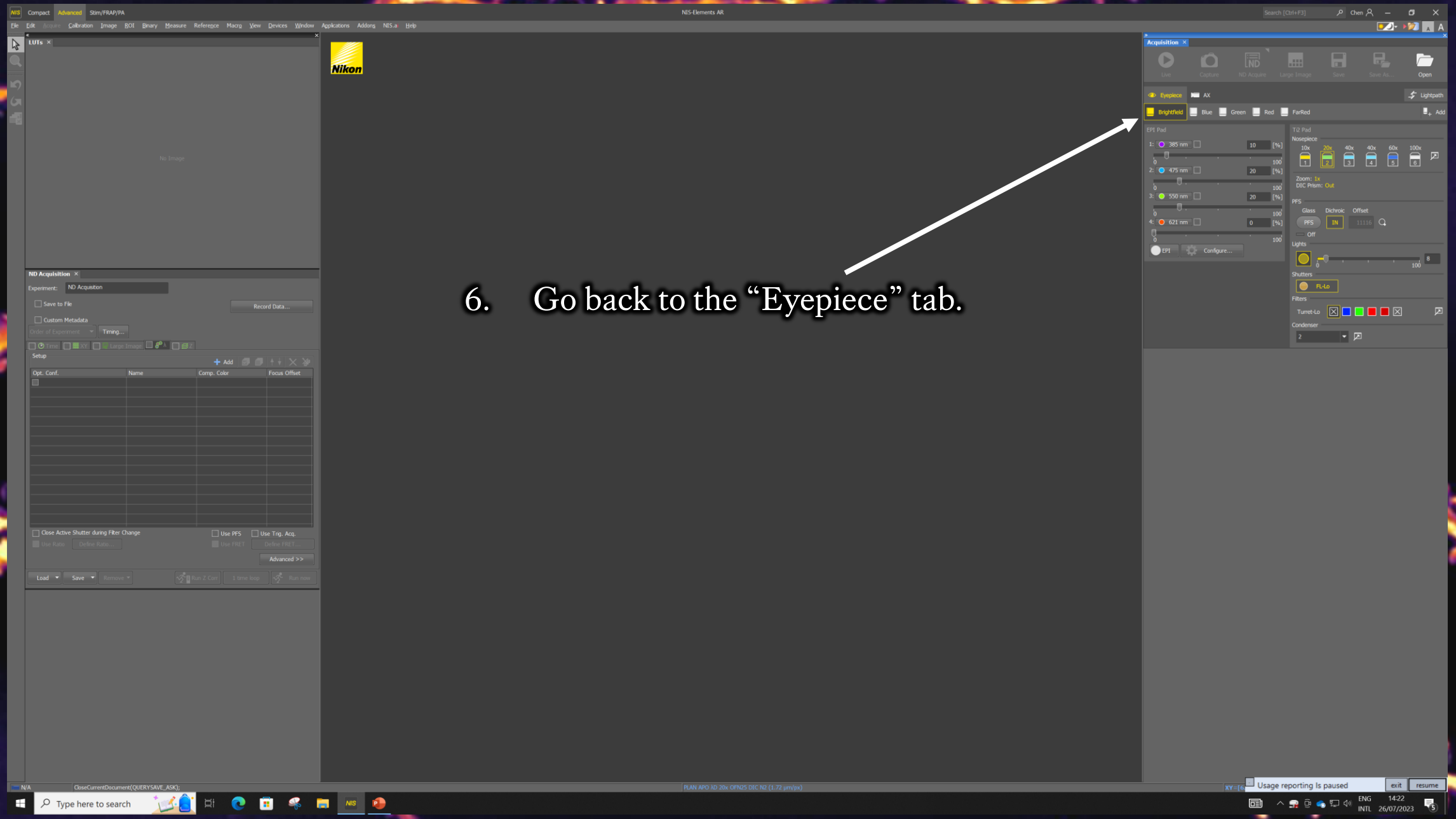
3. ALWAYS login to NIS-Elements Software and wait for it to open, before loading any sample, this checks if all systems are connected.



4. NIS-Elements looks like this once loaded, you should be on the "Advanced Tab" unless taught to use another.



5. Check the laser and camera are working… by going to the ‘AX pad” and clicking on the “LIVE” button, wait 2 seconds, and then click the “FREEZE” button to stop scanning. If a live window like this one appears, then it’s working. If nothing appears then reboot the system by closing the software, restart the computer, turn off number 4, then 3, 2, 1, wait a minute, and start again. If you need help, just let us know, we’ll be happy to show you how to reboot the system.


6. Go back to the “Eyepiece” tab.

STEP BY STEP INSTRUCTIONS

STEP 2

Check Lens And Focus

Please watch this video for instructions:

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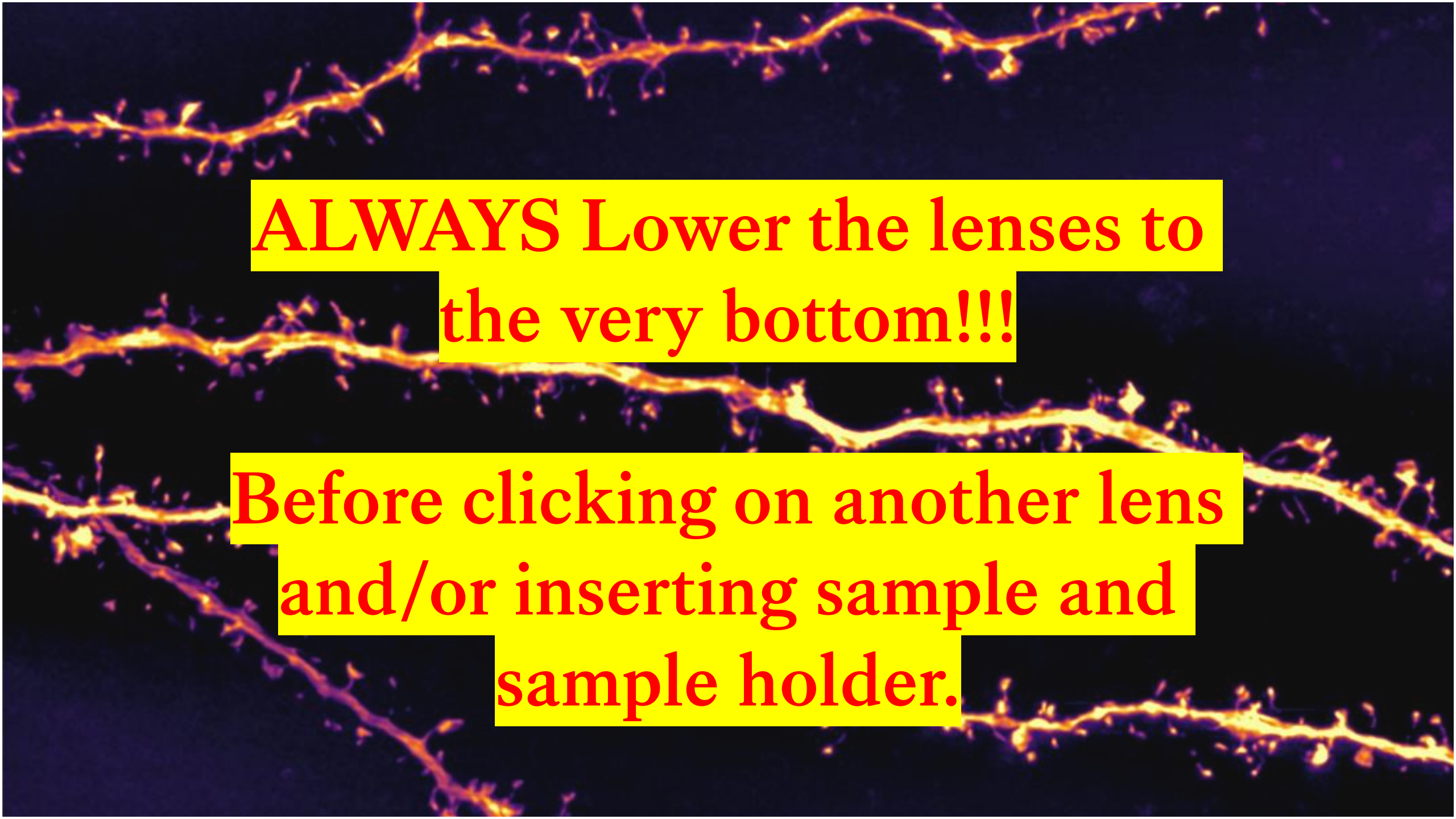

ALWAYS Lower the lenses to the very bottom!!!
Before clicking on another lens and/or inserting sample and sample holder.

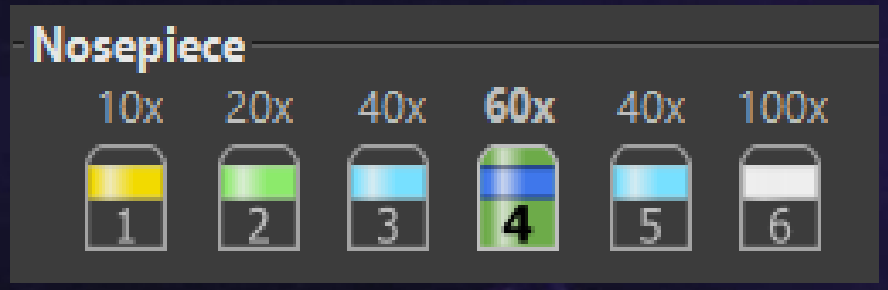
1. With the lenses at the very bottom, check all the lenses you'll be using during your session, if you see any damage, let us know at the start of your session.

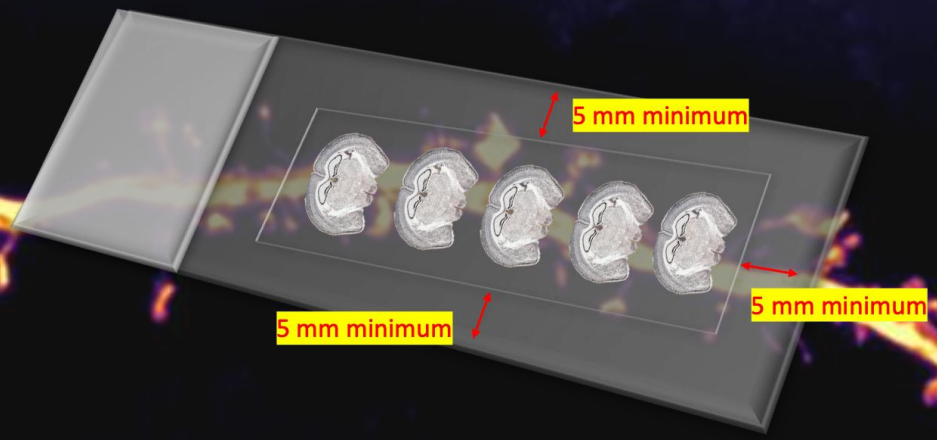
2. Always click and use the 10X or 20X dry lens to start focusing.

3. Insert the sample holder if it's not there already.

4. Clean and load your sample, coverslip side always face towards the lens, the labelling area preferably towards the right hand side, push it into a corner and clip it down. 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!

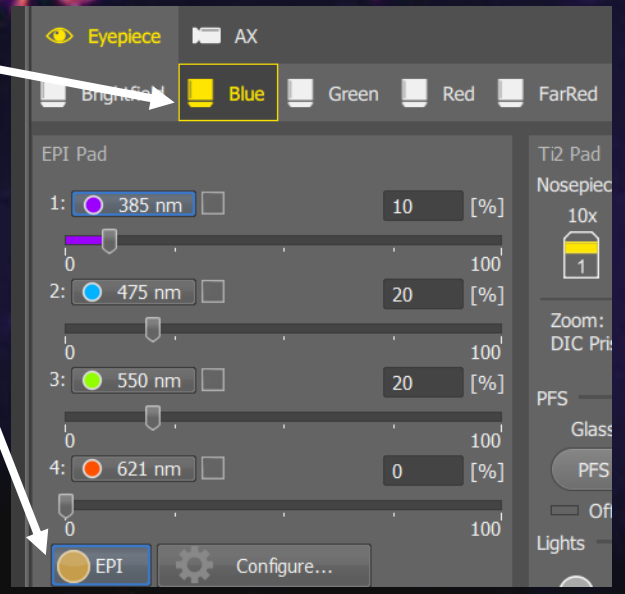
5. Lens still lowered, move the joystick to align lens and sample.





Coverslip should be 0.17mm thick , usually called number 1.5 coverslips, for more information, please read "Which coverslip should I use?" on the WCIC website < Instructions

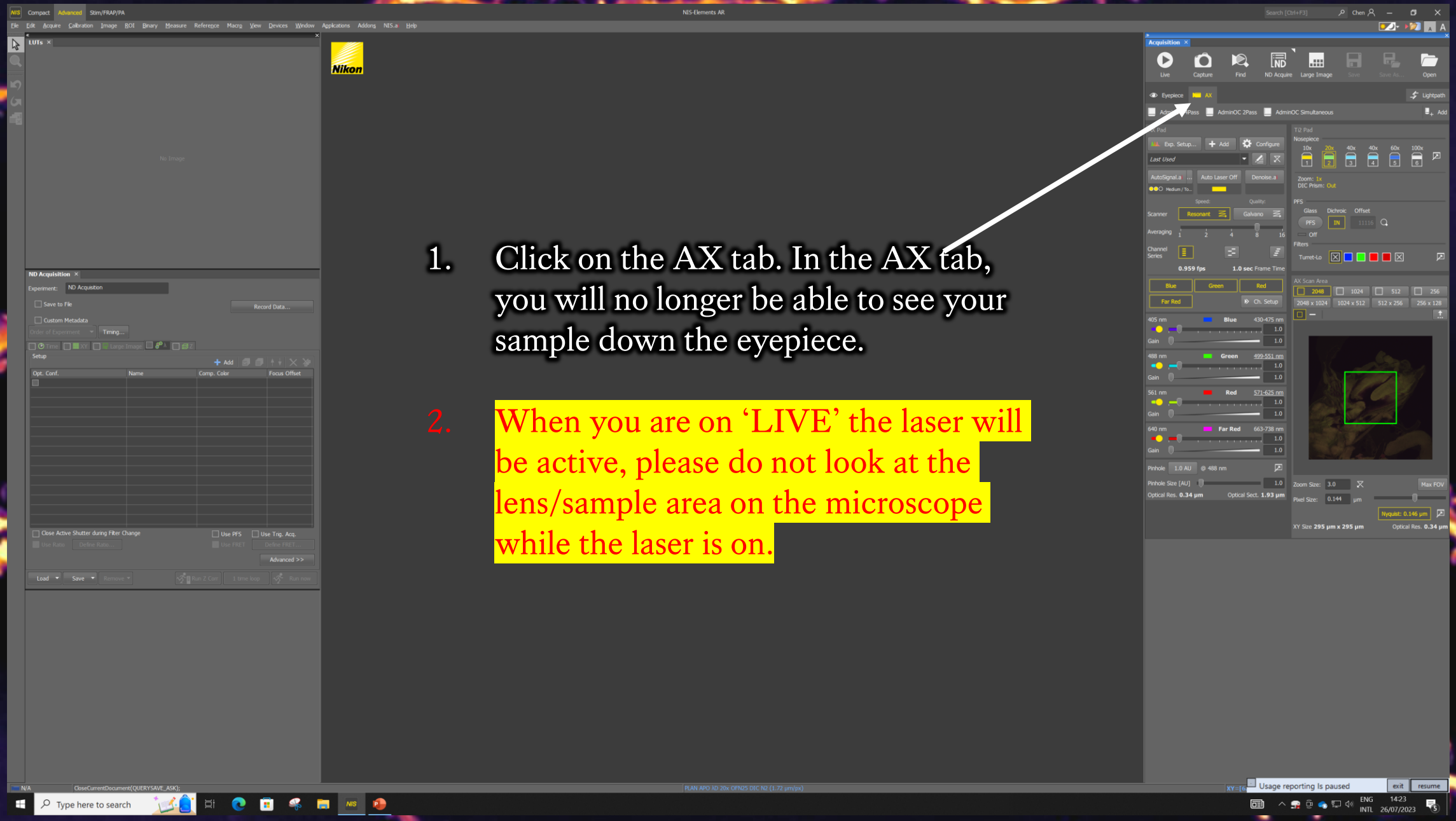
6. Select the Epifluorescence (NOT FarRed) and check it is working.
7. Focus on your sample via eyepiece, then click the shutter to stop bleaching.
8. Z-reset the display number and LOWER THE LENS!!!
9. Remove sample, click and change to higher magnification lens, add oil.
10. Load sample, bring the lens into contact with the oil.
11. Change the XY (joystick) and Z (wheel) speed from FAST (3 arrows) to SLOW (2 arrows).
12. Open the Epifluorescence shutter, focus on your sample down the eyepiece again.
13. DO NOT GO 100 microns over the zero display Z-number (ask us for help instead).
14. Only move the joystick when your sample is in focus down the eyepiece, or if the lens is at the bottom.
15. Check other Epifluorescence channels if needed and place something you want to image right in the middle of your field of view.



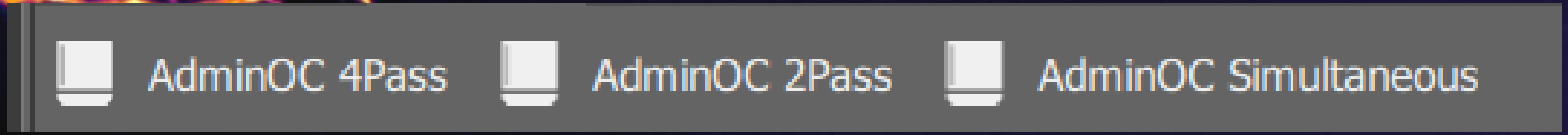
STEP BY STEP INSTRUCTIONS

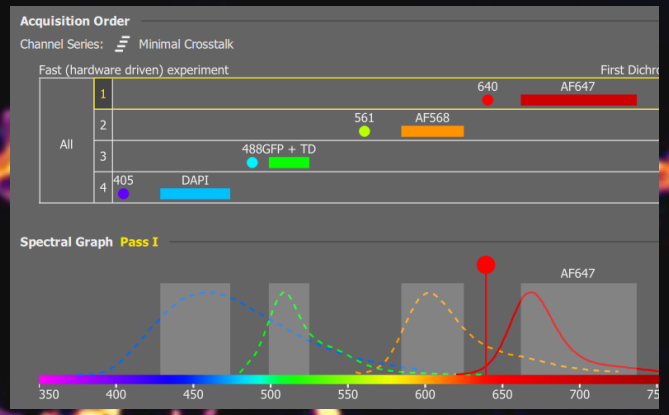
STEP 3

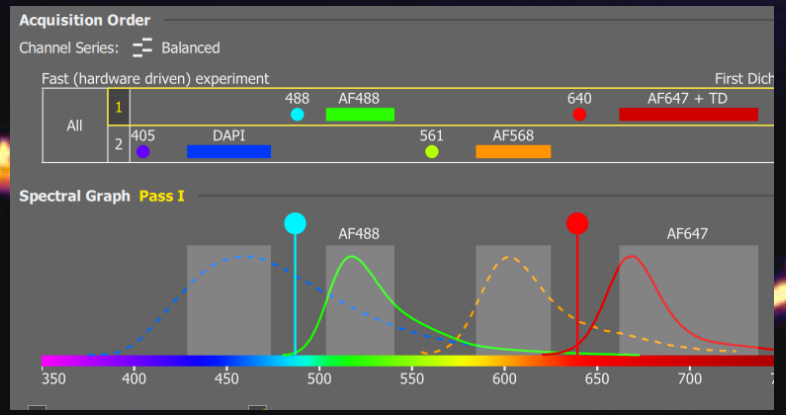
Change from Eyepiece to Camera View

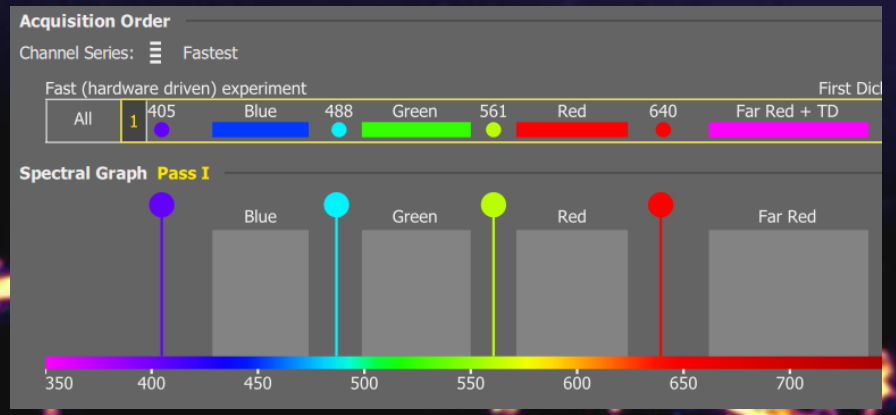

1. Click on the AX tab. In the AX tab, you will no longer be able to see your sample down the eyepiece.
2. When you are on 'LIVE' the laser will be active, please do not look at the lens/sample area on the microscope while the laser is on.

3. Choose an AdminOC button to duplicate. These admin optical configurations are shared defaults, you cannot make any changes to these, but you can optimise the duplicated one.









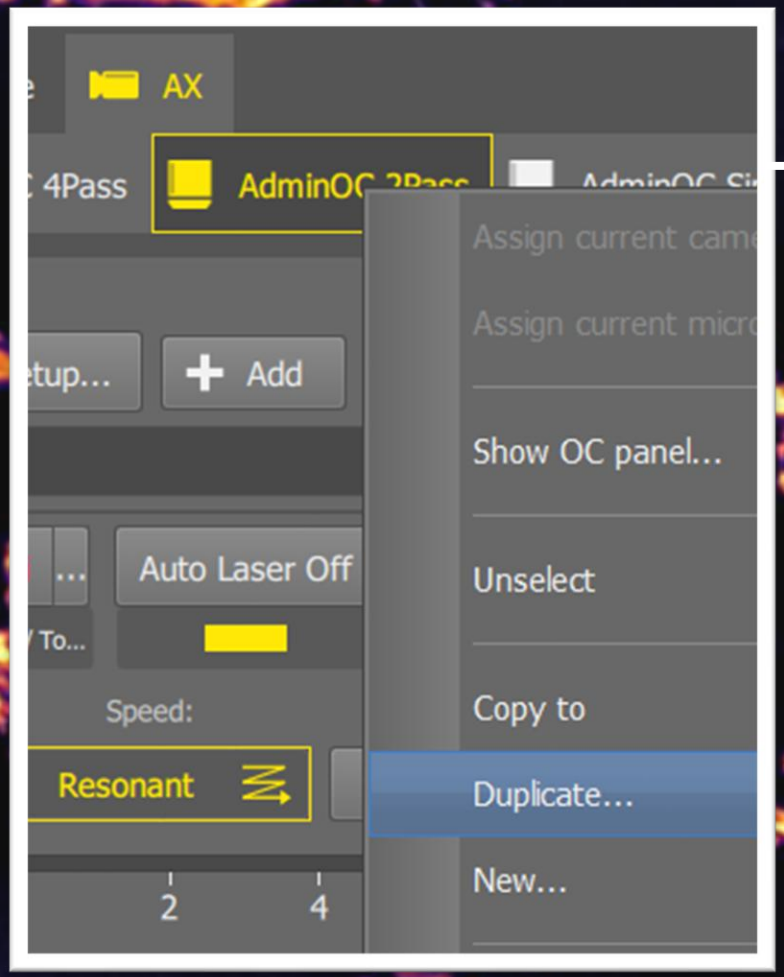
AdminOC 4Pass uses each laser line one after another, this is the slowest option but it minimises crosstalk.

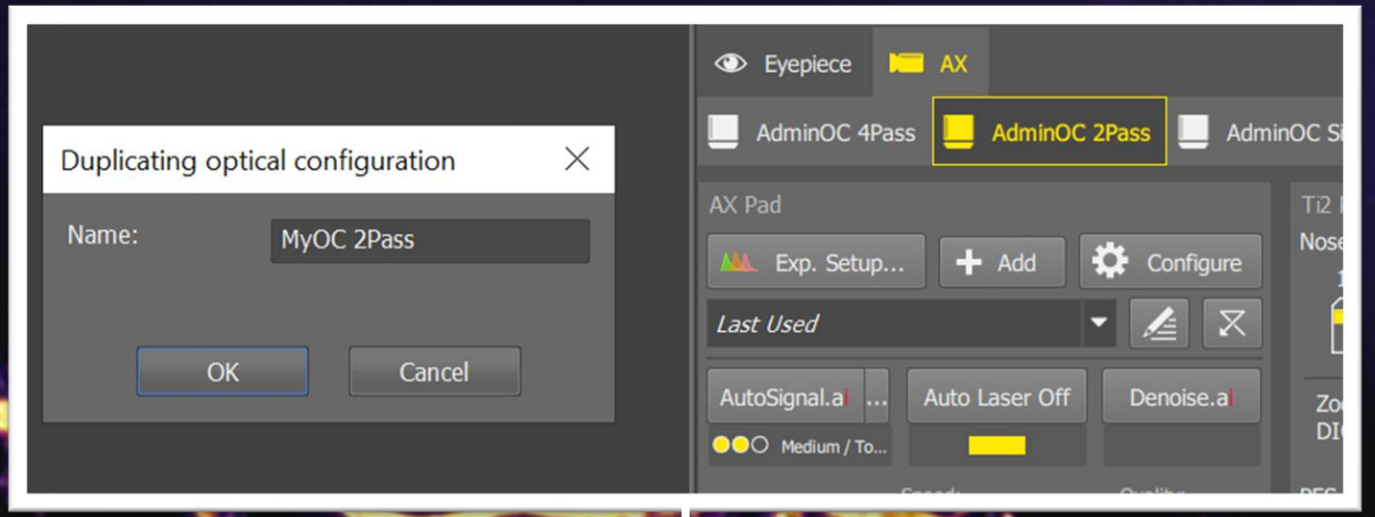
AdminOC 2Pass uses the 488 and 640 lasers at the same time, then switches to the 405 and 568 lasers, this avoids using laser lines that are close together, it is a good balance between speed and minimalizing crosstalk.

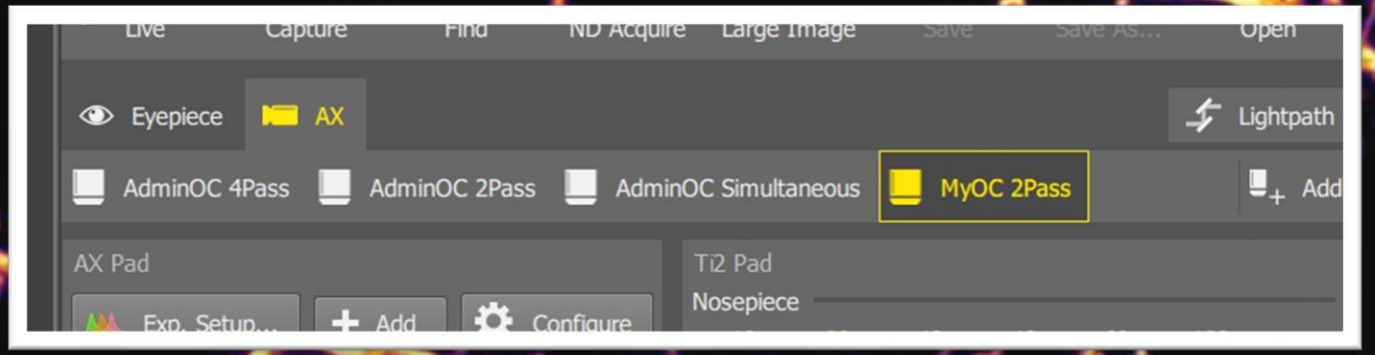
AdminOC Simultaneous uses all the lasers at the same time, this is the fastest option, however crosstalk will likely be an issue.

If you cannot decide on an option, please let us know and we can help.

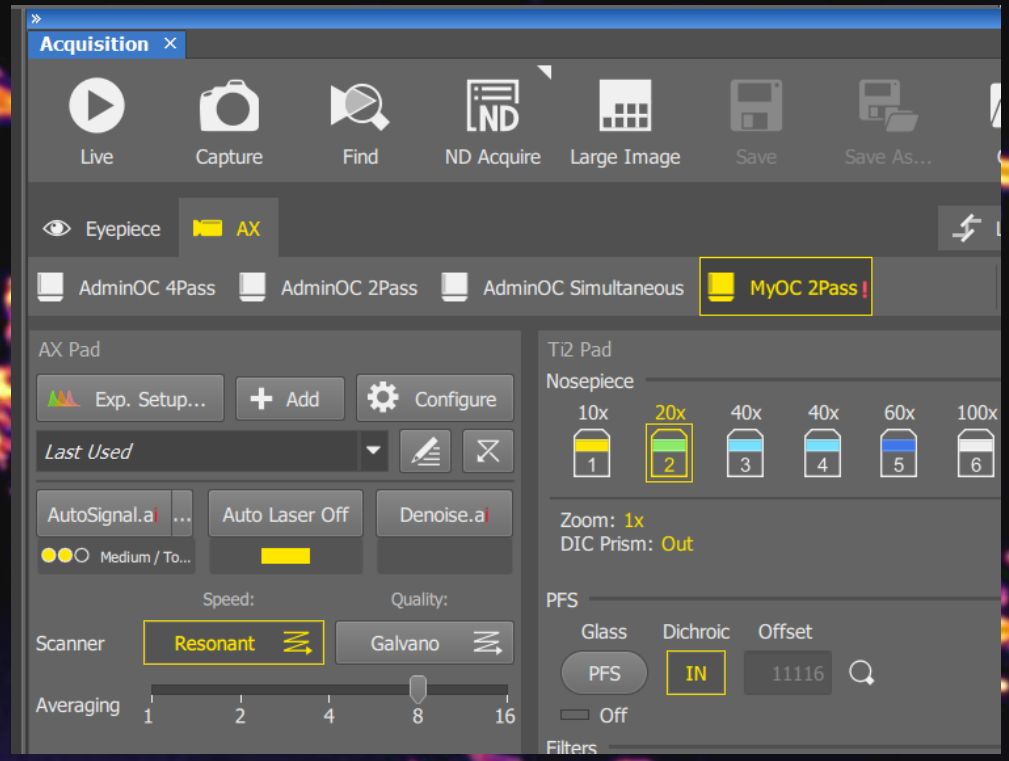
4. Right click on a ‘AdminOC’ button to duplicate and name, I’ll refer this as ‘MyOC’ for the rest of this guide.

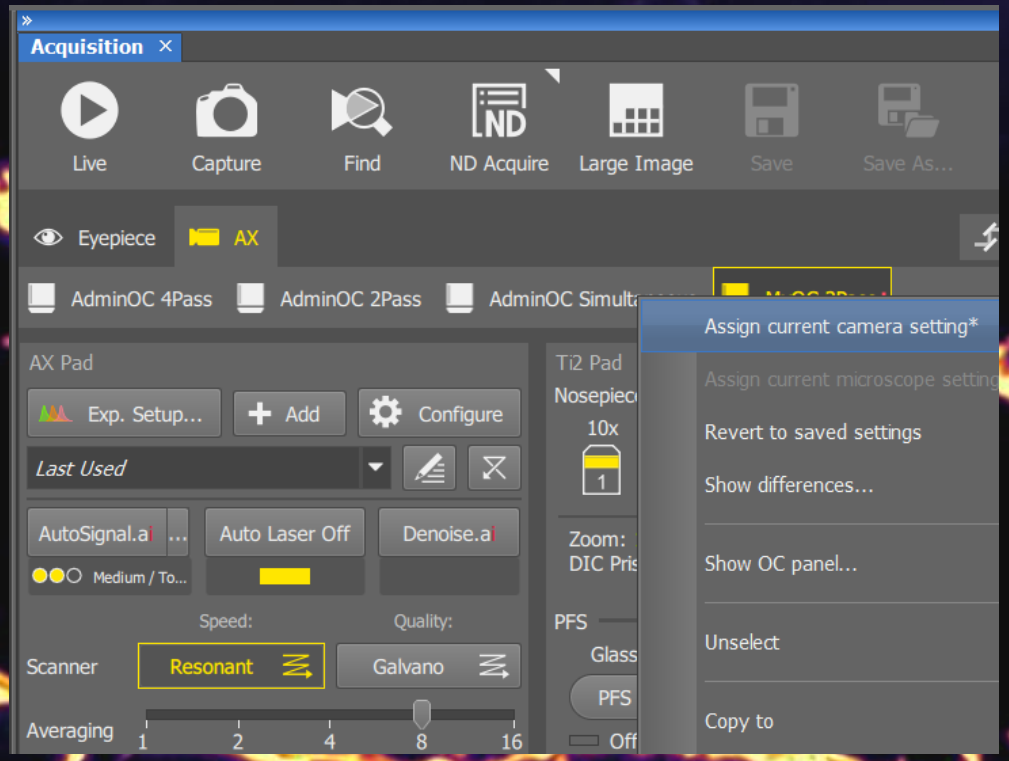






5. When you make any changes in the AX pad and AX scan area, ! will appear next to the 'MyOC' button. Anytime you want to save any changes you've made, right click and 'assign current camera settings'. If you do not save the changes before imaging, the settings will revert back to the last saved configuration.





STEP BY STEP INSTRUCTIONS

STEP 4

Setting Up Initial Live View

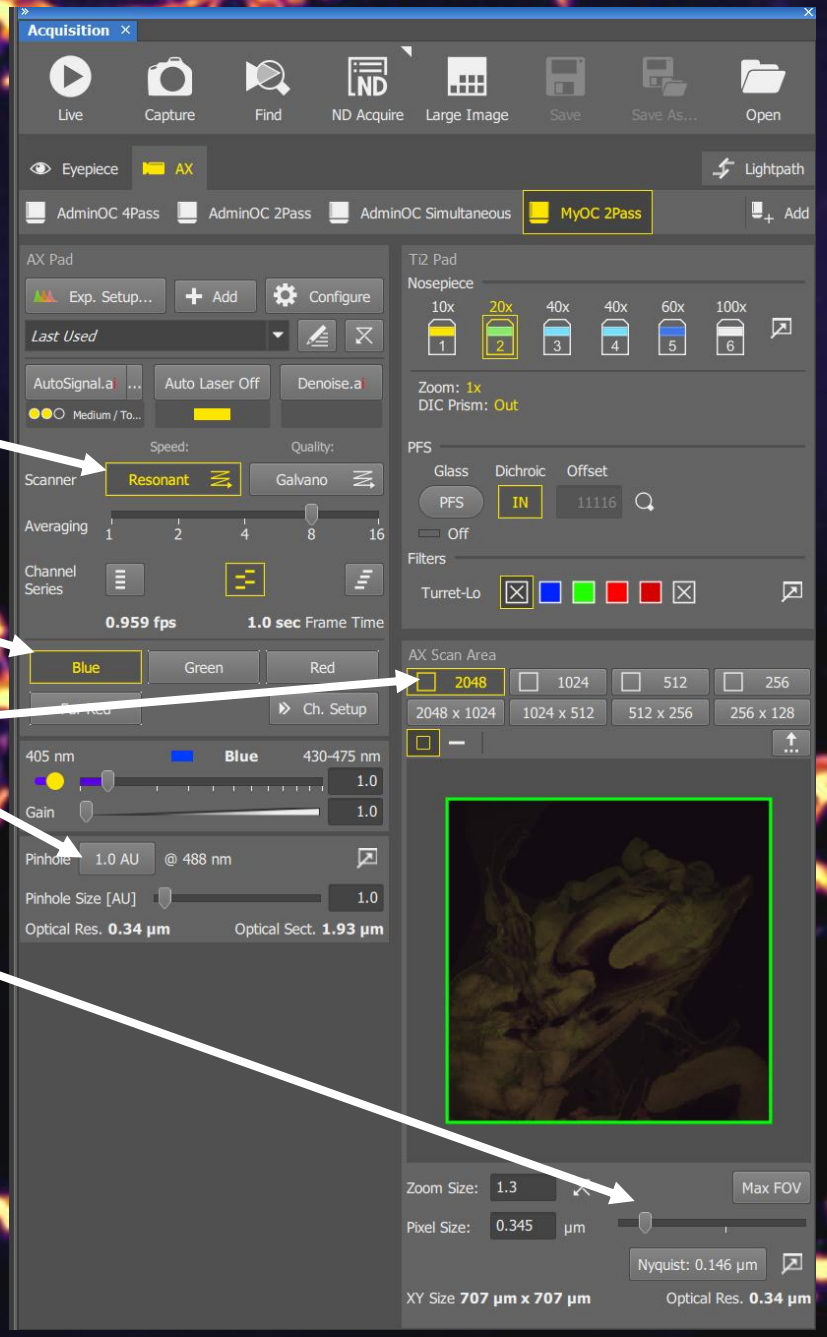
1. Start with Resonant scanning mode.

2. Start with the channel you used to focus on the eyepiece, de-select all other channels.

3. Start with 1.0 AU Pinhole size by clicking on this.

4. Start with 2048 AX Scan Area

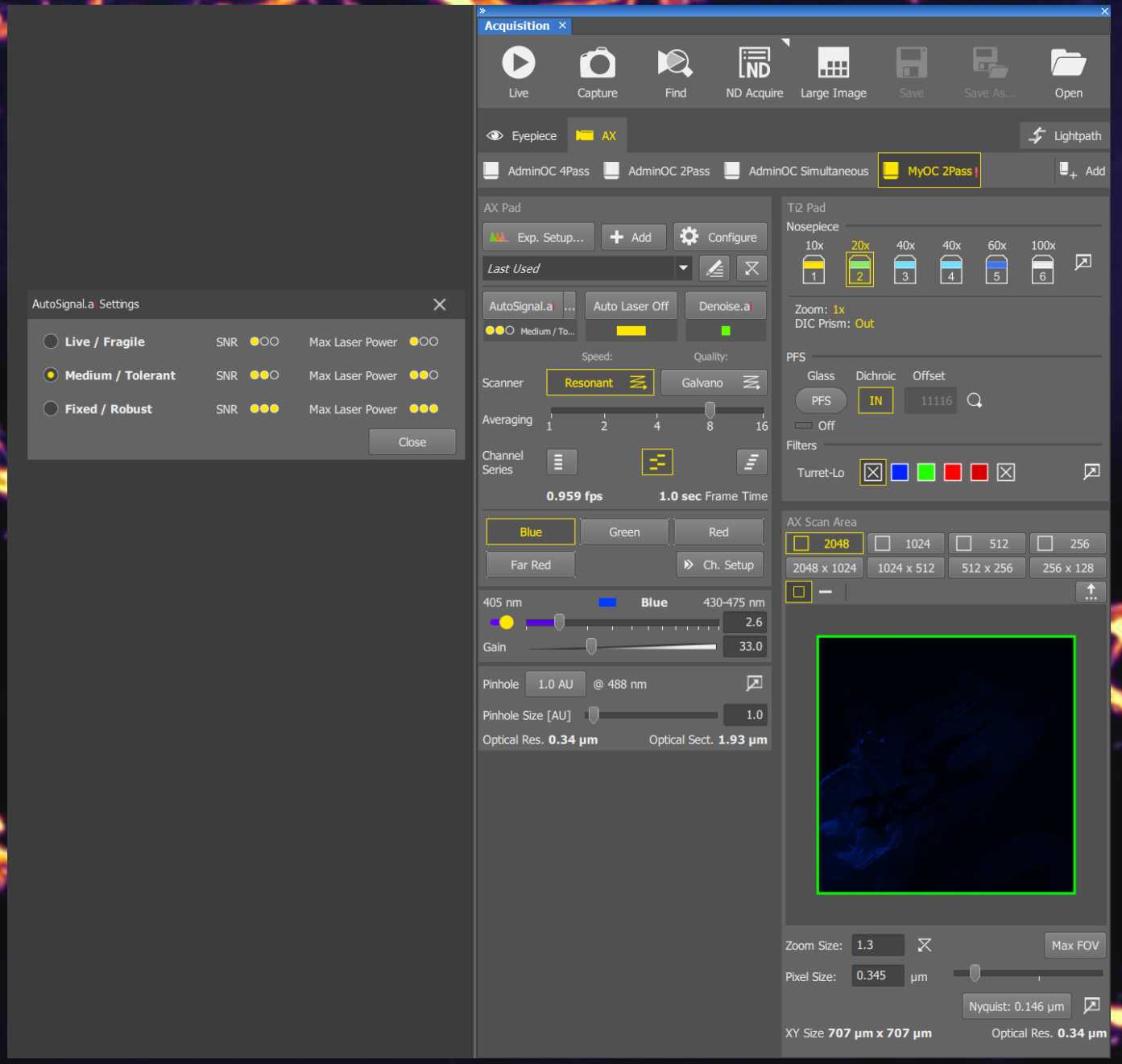
5. Start with Zoom Size bar slider here, so you're not too zoomed in and therefore have a bigger field of view. If you change the bar slider, right click on the Scan Area view box to apply the change, the square should have a green boarder around it once applied.

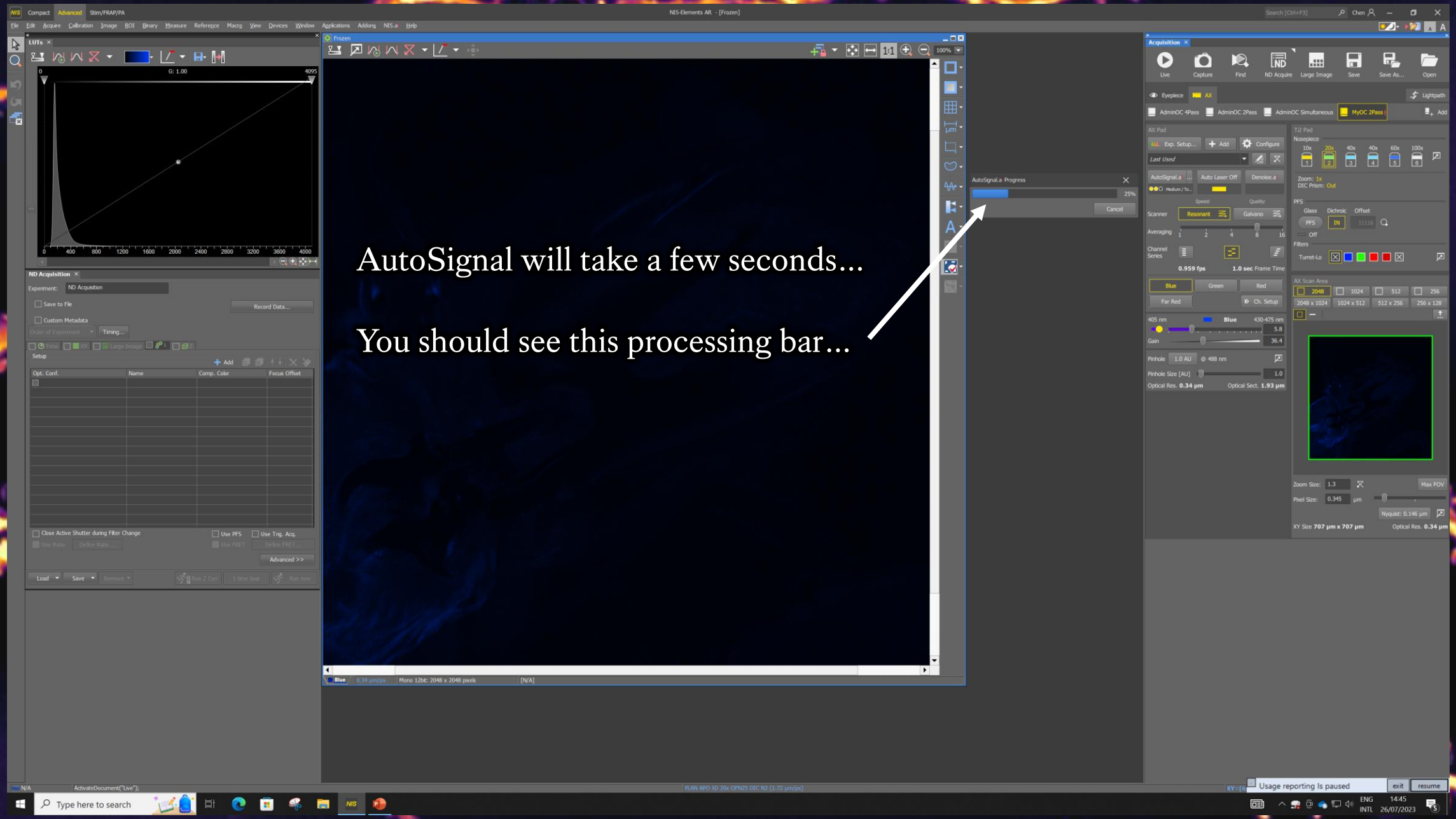


6. ‘AutoSignal’ allows the software to try different laser powers and gain settings, this will provide a decent set up for you to view your sample.

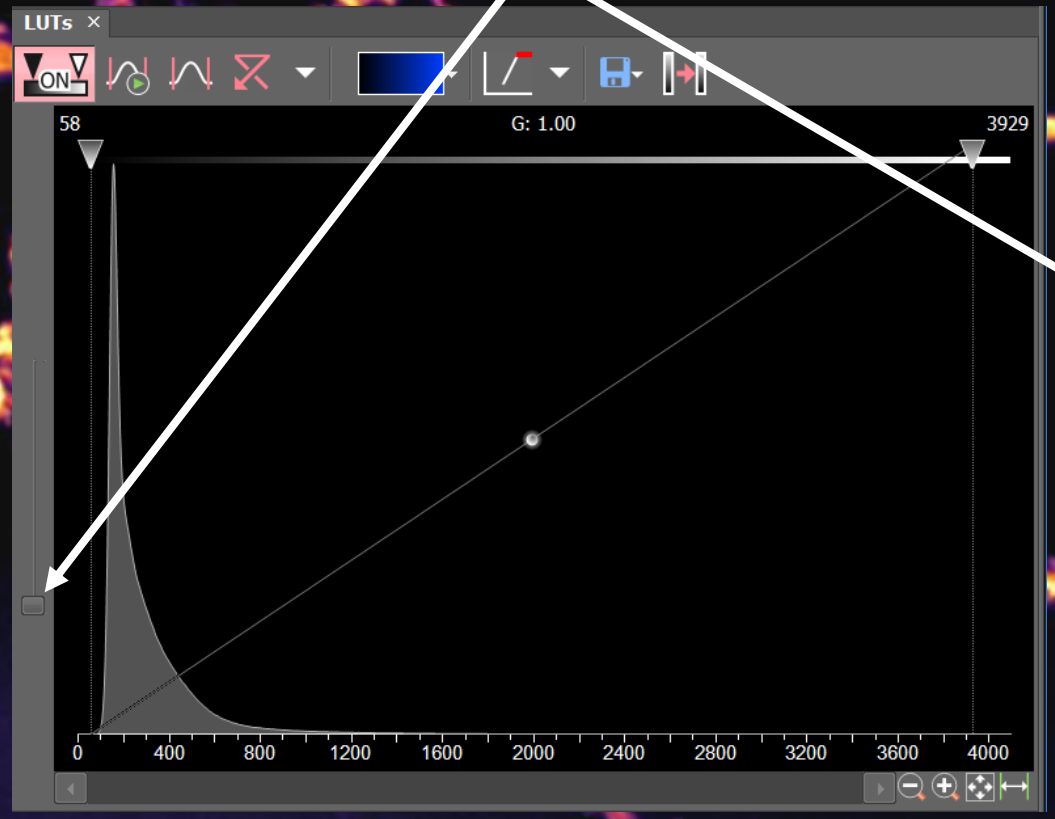
7. Click on the ‘...’ next to ’AutoSignal’ and choose between Live, Medium and Fixed, depending how easily bleached your sample is.

8. Close the box and click on the ‘AutoSignal’ button.

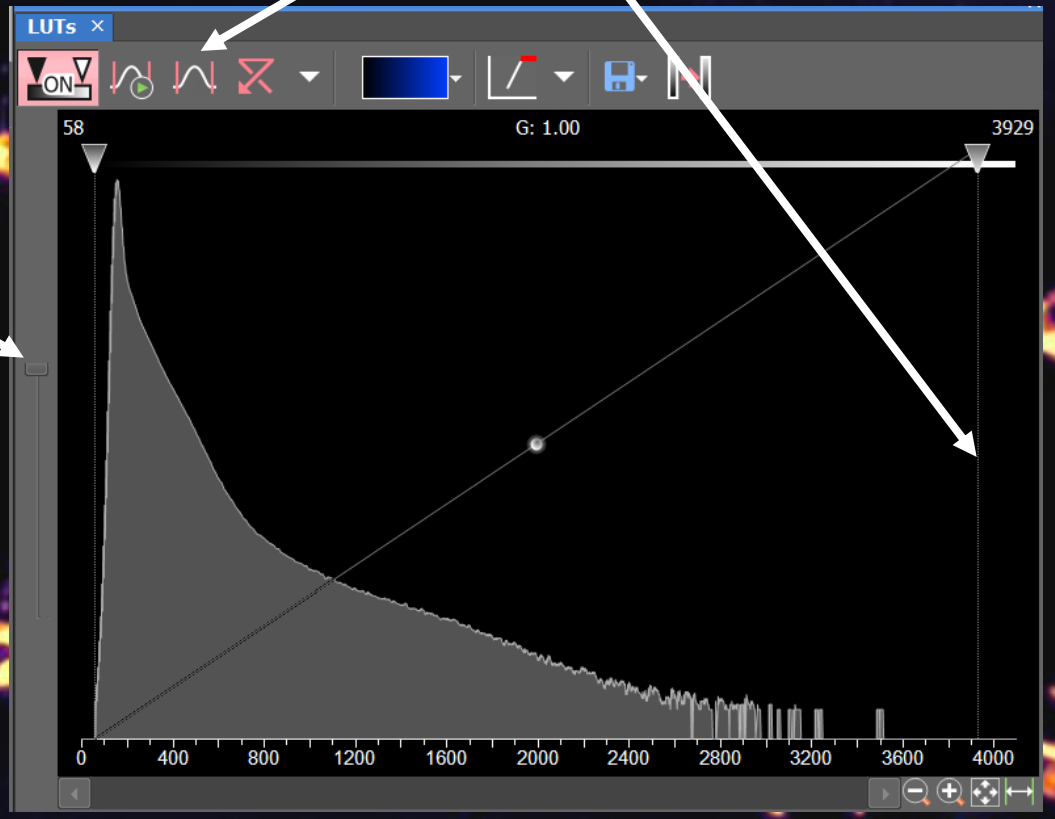



AutoSignal will take a few seconds…
You should see this processing bar…

9. Once the AutoSignal is done, you now need to adjust your Look Up Table (LUTs). <u>LUTs DOES NOT change your raw signal intensity at all, just how your sample looks on screen.</u> First you want to see your entire signal intensity graph by dragging this bar to the top.



10. Adjust your contrast to better view your sample. Click on Auto Contrast or you can manually increase contrast by dragging this line towards the left.



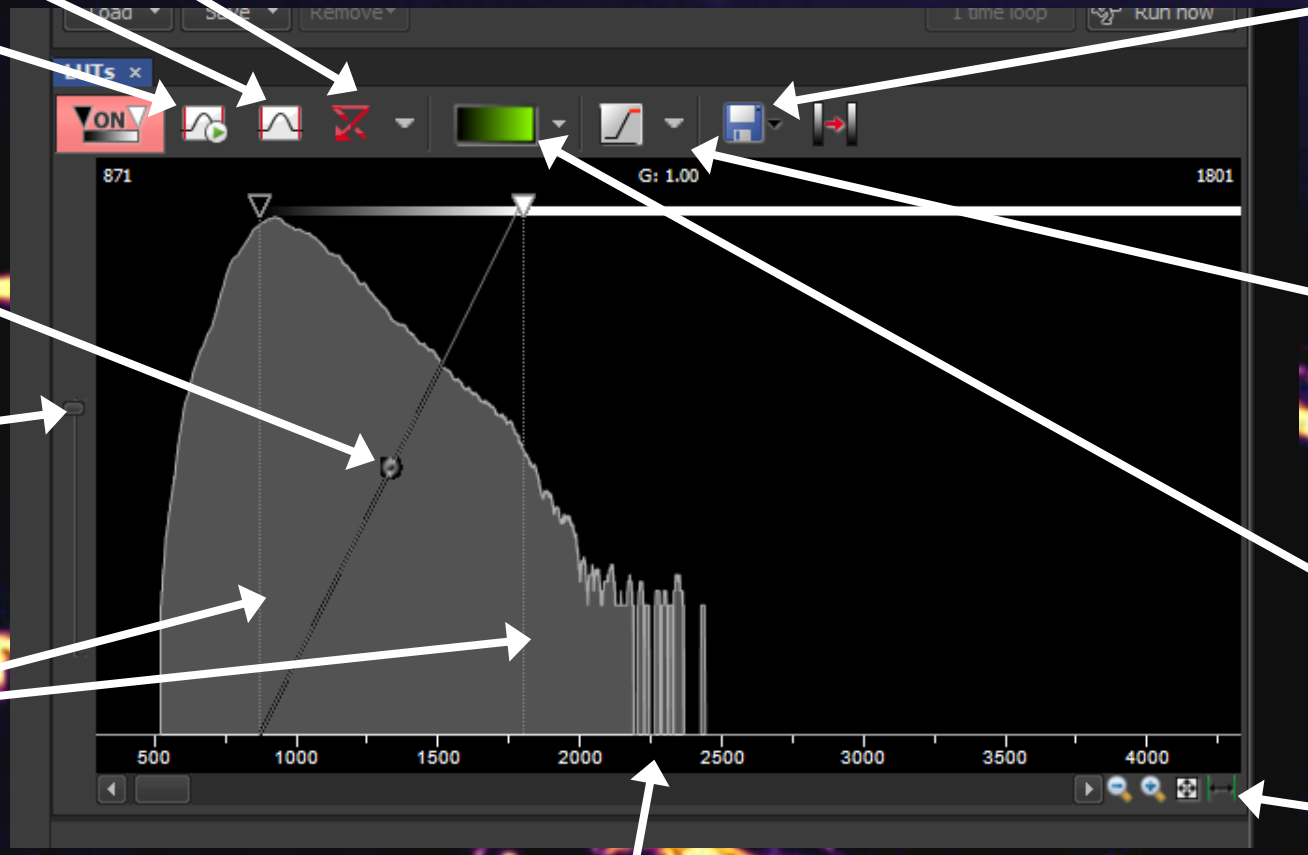
LUTs in more detail…

Auto-contrast

Delete all contrast adjustments

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

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



Makes dim targets brighter while keeping bright target the same.

For viewing saturation, complementary colour recommended. Once an area is saturated, it loses any intensity value information.

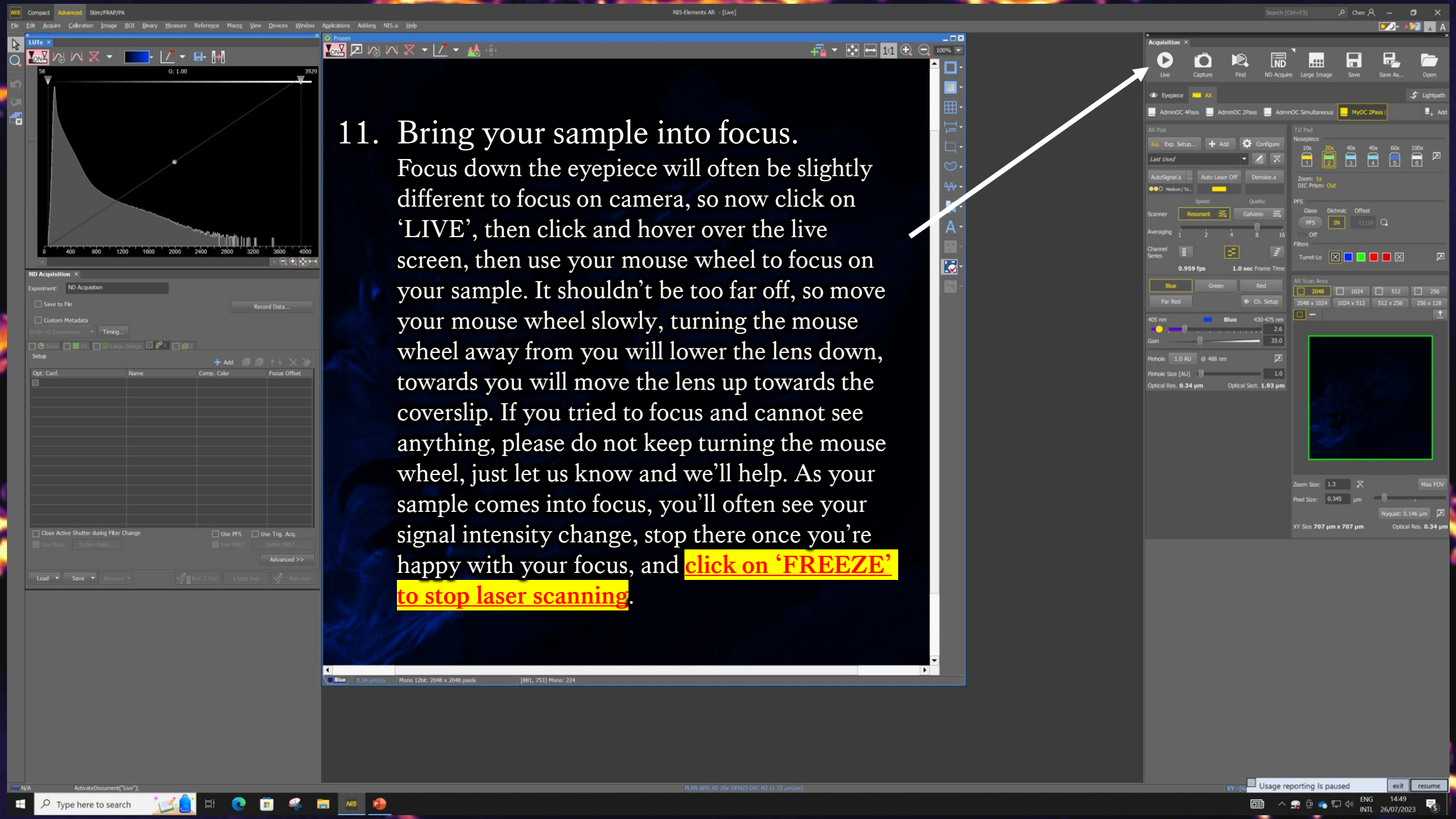
Controls the Y axis log graph

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

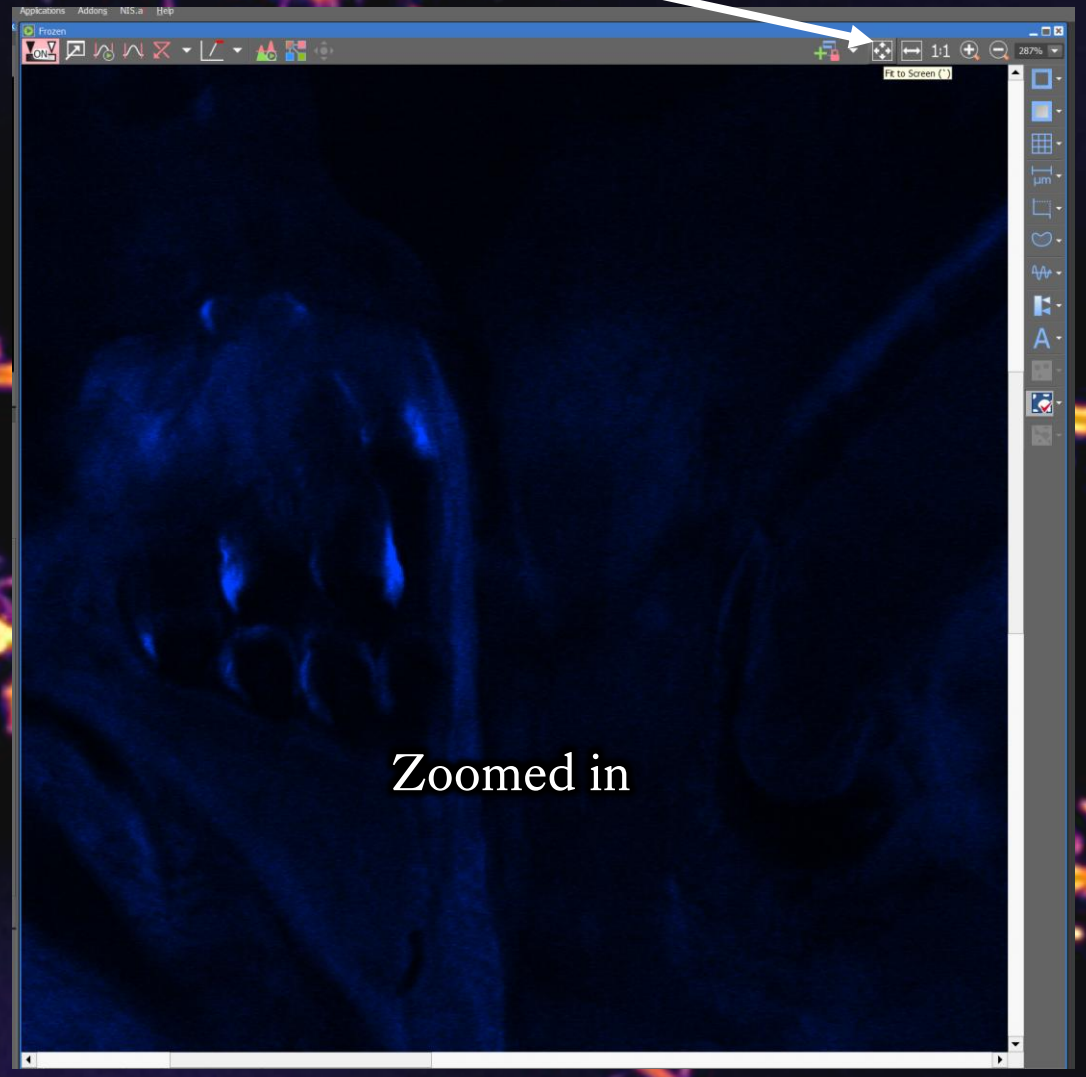
Controls the X axis contrast, this doesn't change the captured raw data.

Fit the histogram to this space

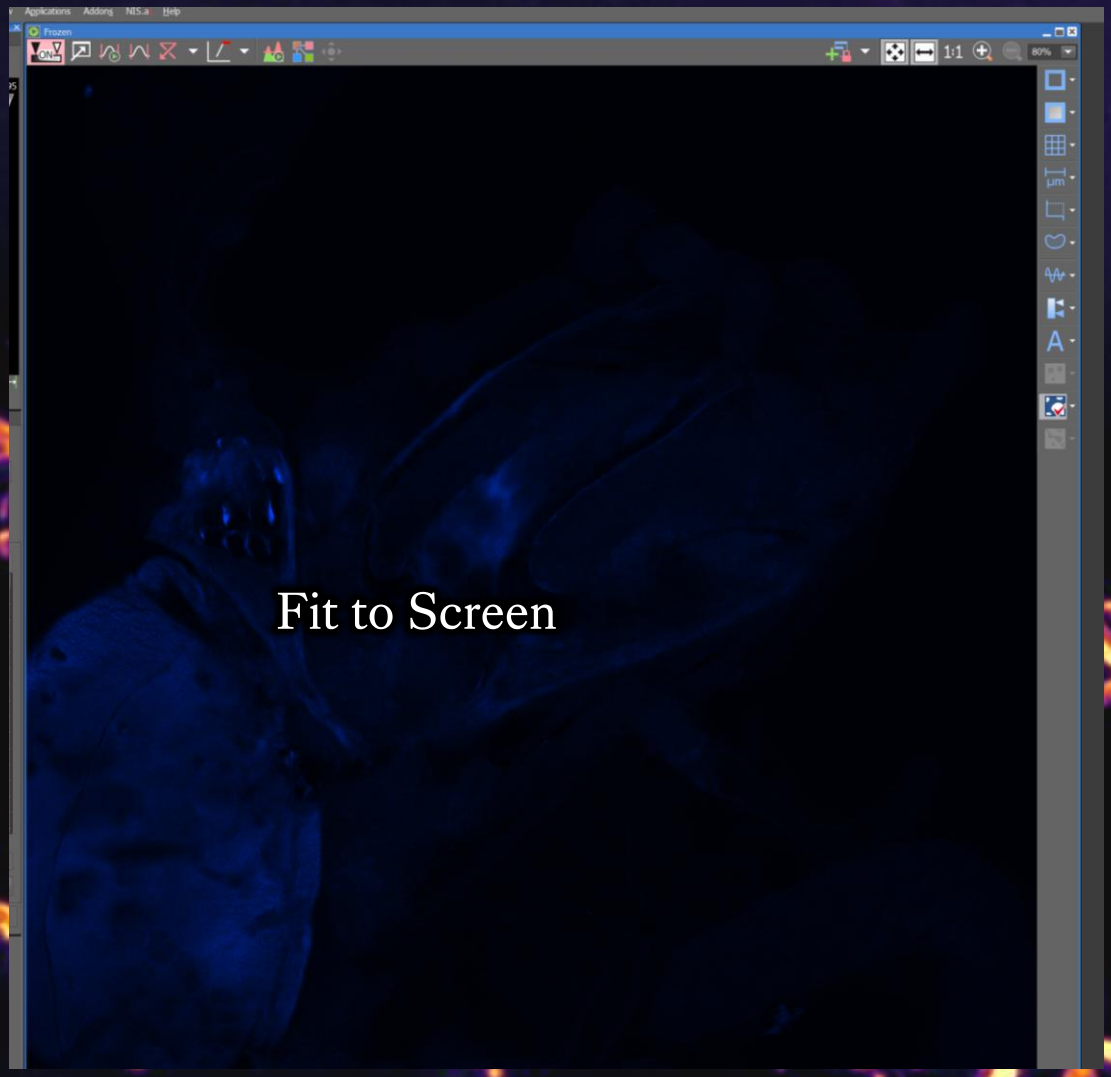
Drag and change the threshold of this histogram for better visualisation. X axis - relative to #-bit camera (e.g. 16-bit CCD camera gives 65,536 different intensity values, of which you should not go over 50,000). Y axis – log intensity scale


11. Bring your sample into focus.
Focus down the eyepiece will often be slightly different to focus on camera, so now click on 'LIVE', then click and hover over the live screen, then use your mouse wheel to focus on your sample. It shouldn't be too far off, so move your mouse wheel slowly, turning the mouse wheel away from you will lower the lens down, towards you will move the lens up towards the coverslip. If you tried to focus and cannot see anything, please do not keep turning the mouse wheel, just let us know and we'll help. As your sample comes into focus, you'll often see your signal intensity change, stop there once you're happy with your focus, and click on 'FREEZE' to stop laser scanning.

12. Click here, to fit your image to screen if you want to see everything in your scan area.

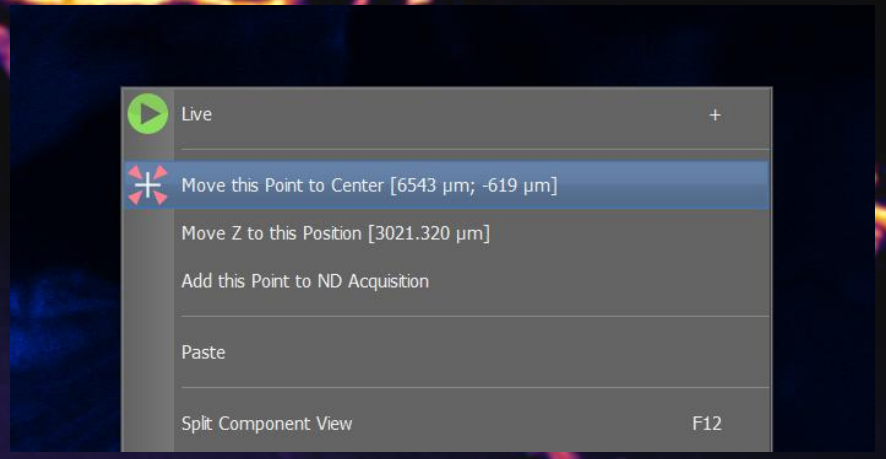


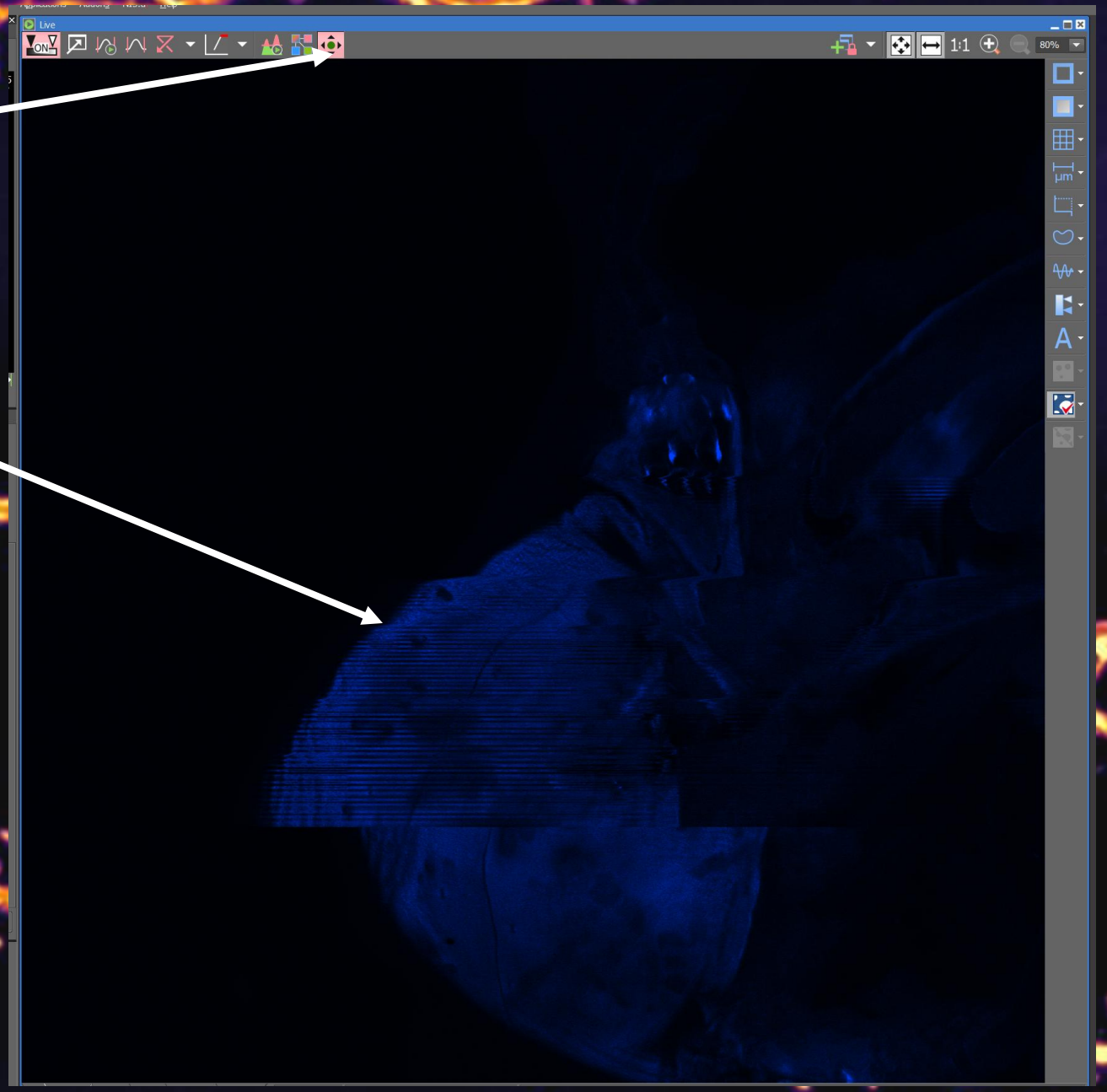
Zoomed in



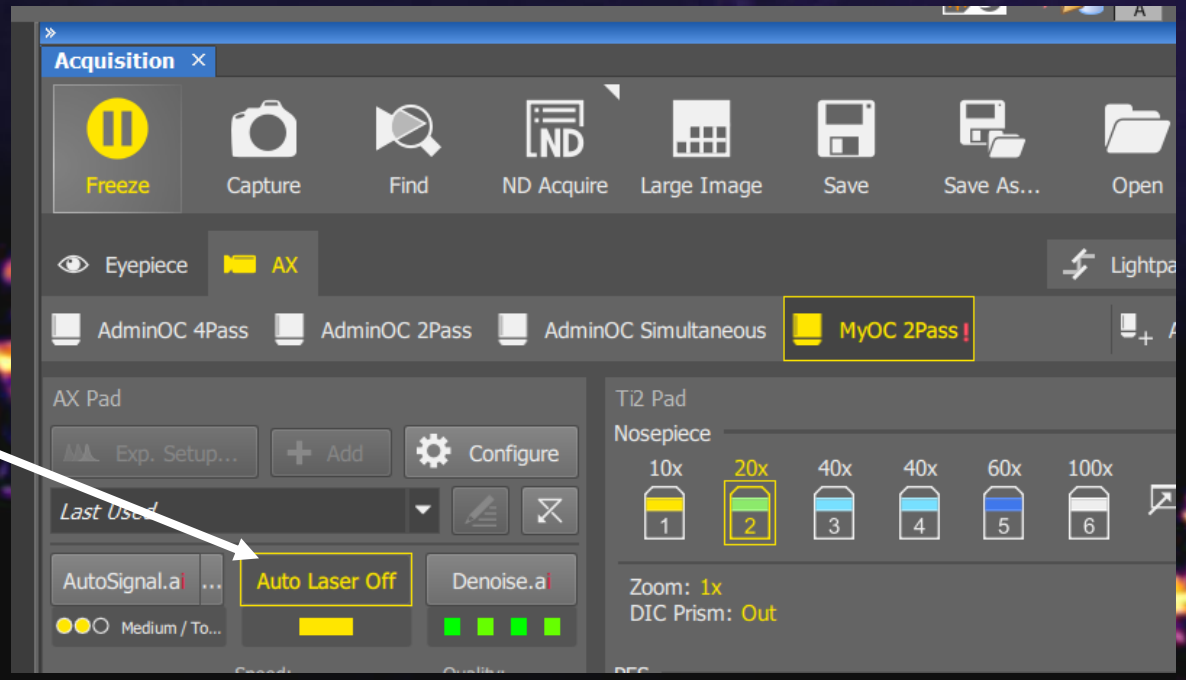
Fit to Screen

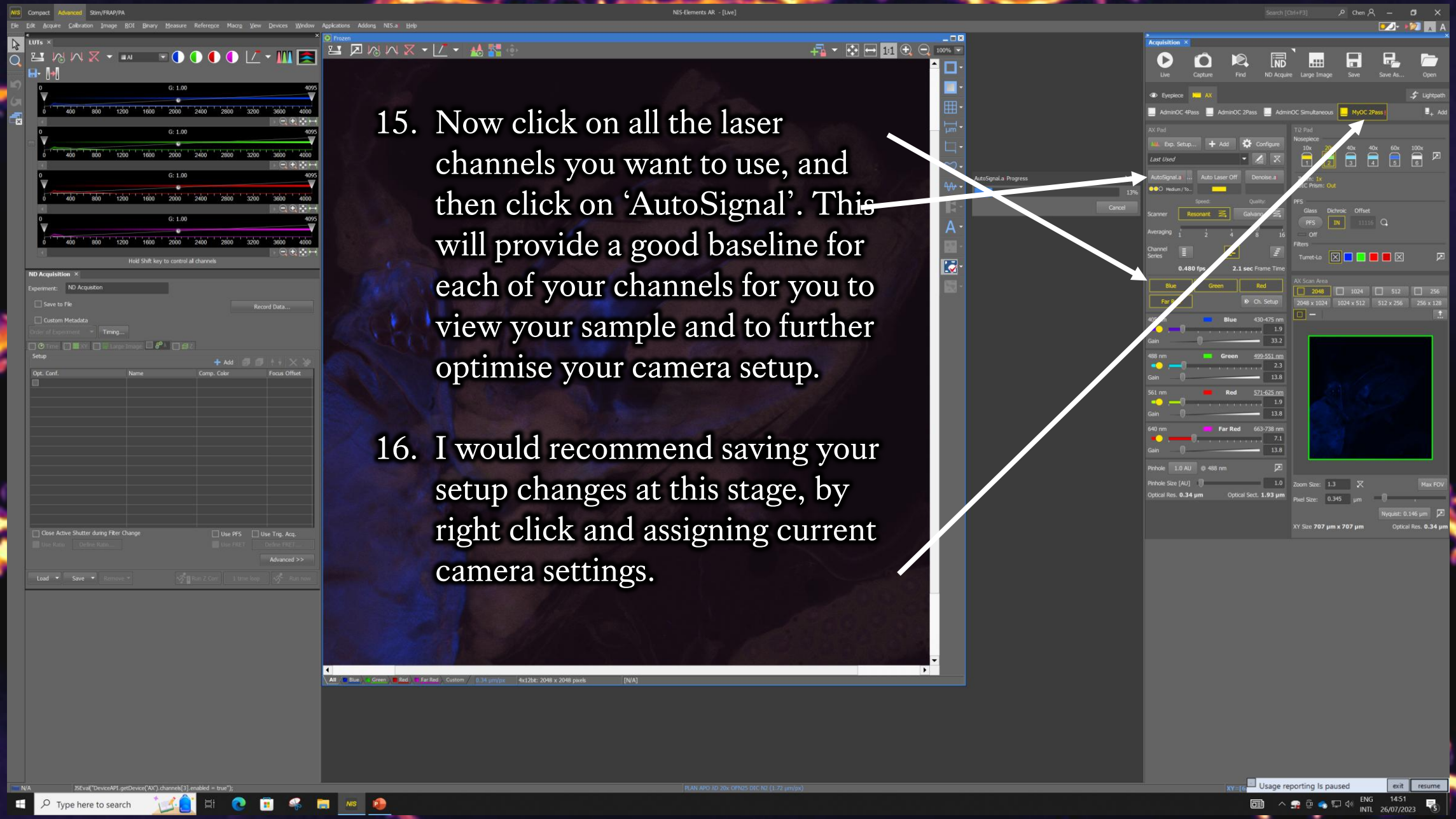
13. Click on LIVE and then click here, this enables ‘click and drag’ on your live screen to move positions. You might see some lag as you drag, this is normal. If you would like to place a particular target in the centre of your frame, then right click on the target and select ‘Move this Point to Centre’.





14. The LIVE and FREEZE button is used constantly, because the longer you stay on LIVE, the longer you expose your sample to laser, this leads to phototoxicity and/or bleaching. If you find yourself forgetting to click on FREEZE to stop scanning, there is an automatic method. Click here to apply ‘Auto Laser Off’, this will turn the laser on when you make any changes to your frame, such as moving or focusing, then it will detect when you stop moving and automatically pause the laser to minimise bleaching.

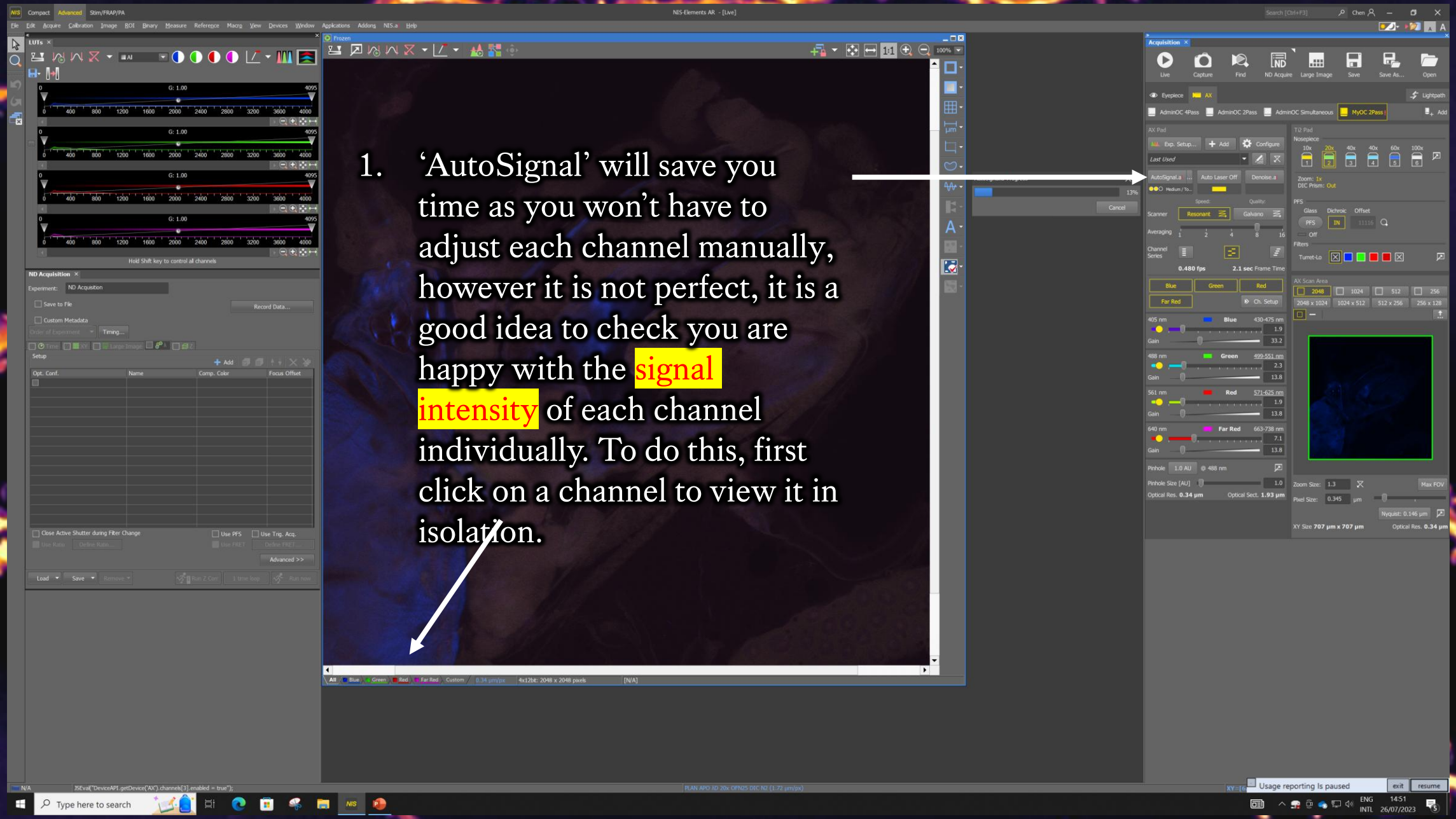


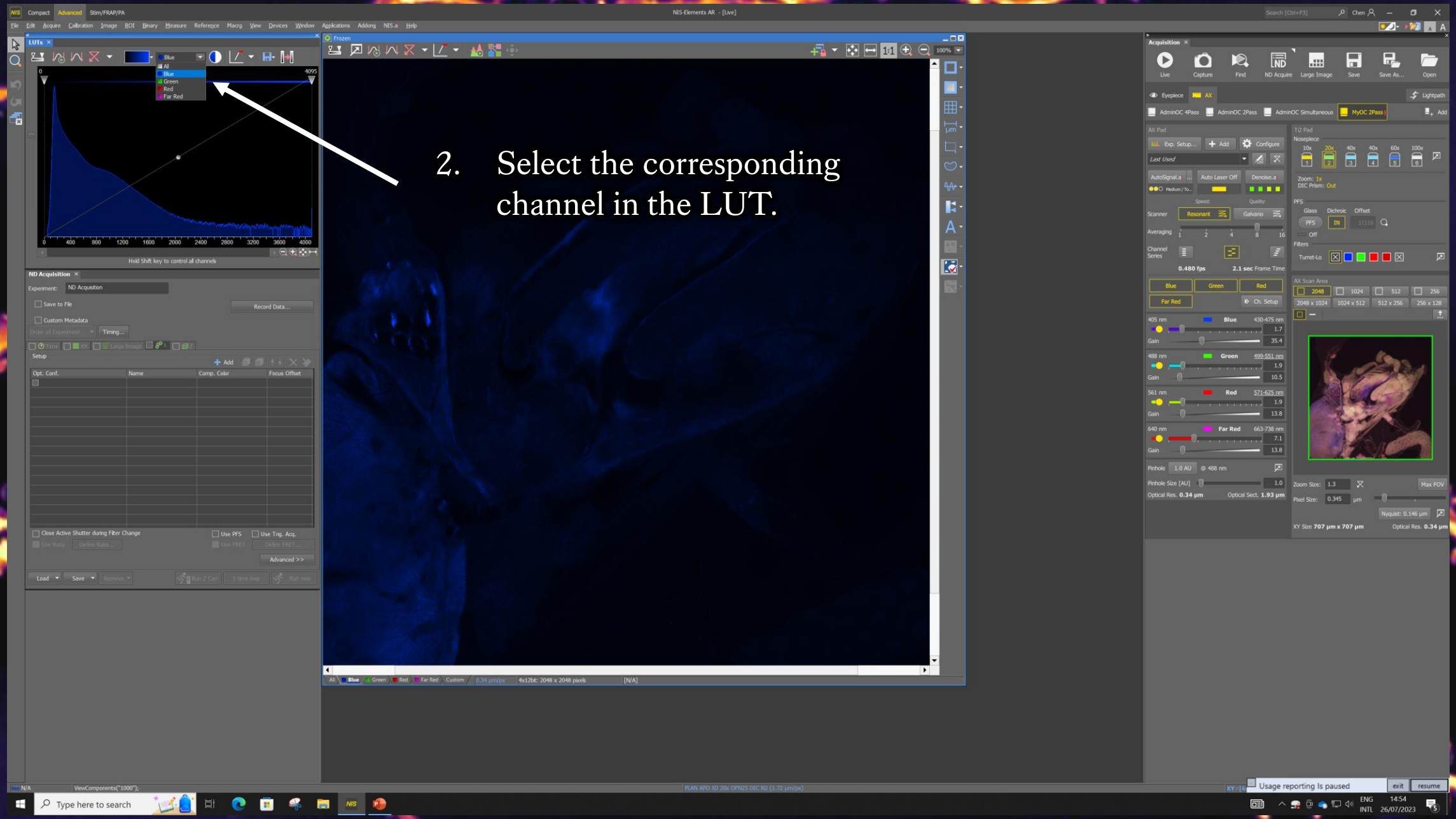

15. Now click on all the laser channels you want to use, and then click on ‘AutoSignal’. This will provide a good baseline for each of your channels for you to view your sample and to further optimise your camera setup.
16. I would recommend saving your setup changes at this stage, by right click and assigning current camera settings.

STEP BY STEP INSTRUCTIONS

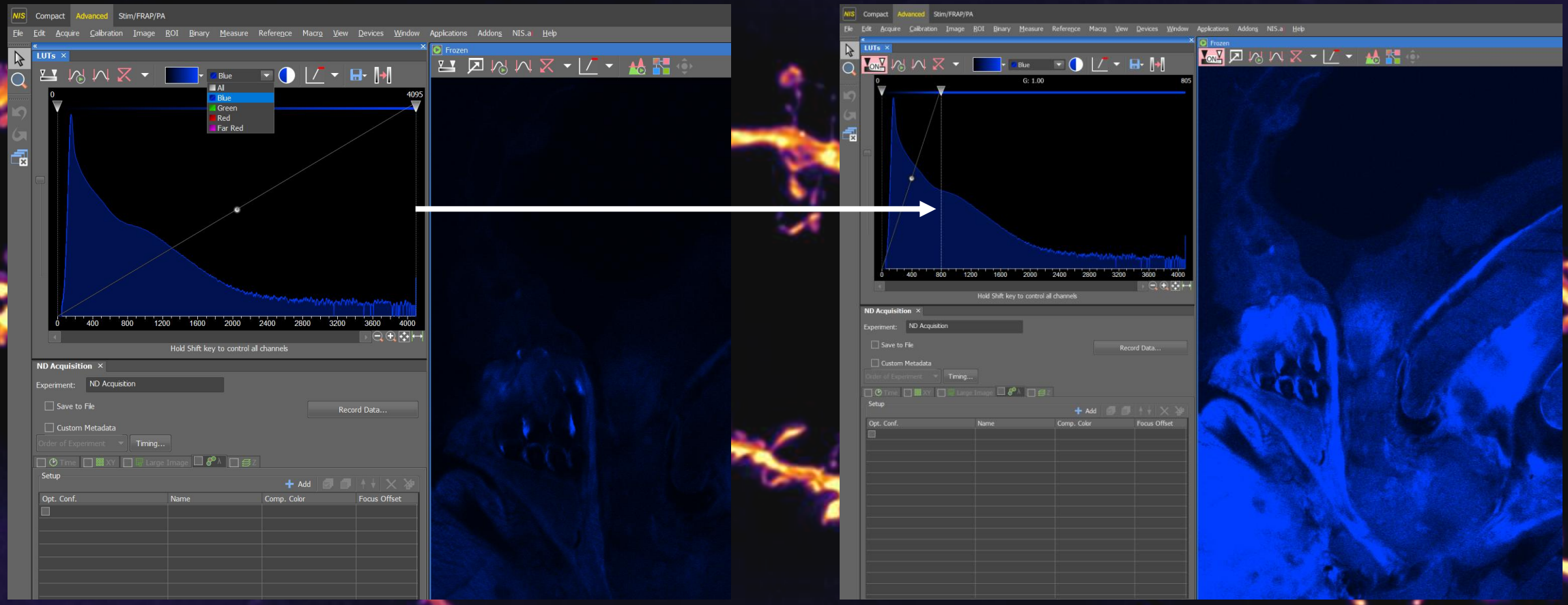
STEP 5

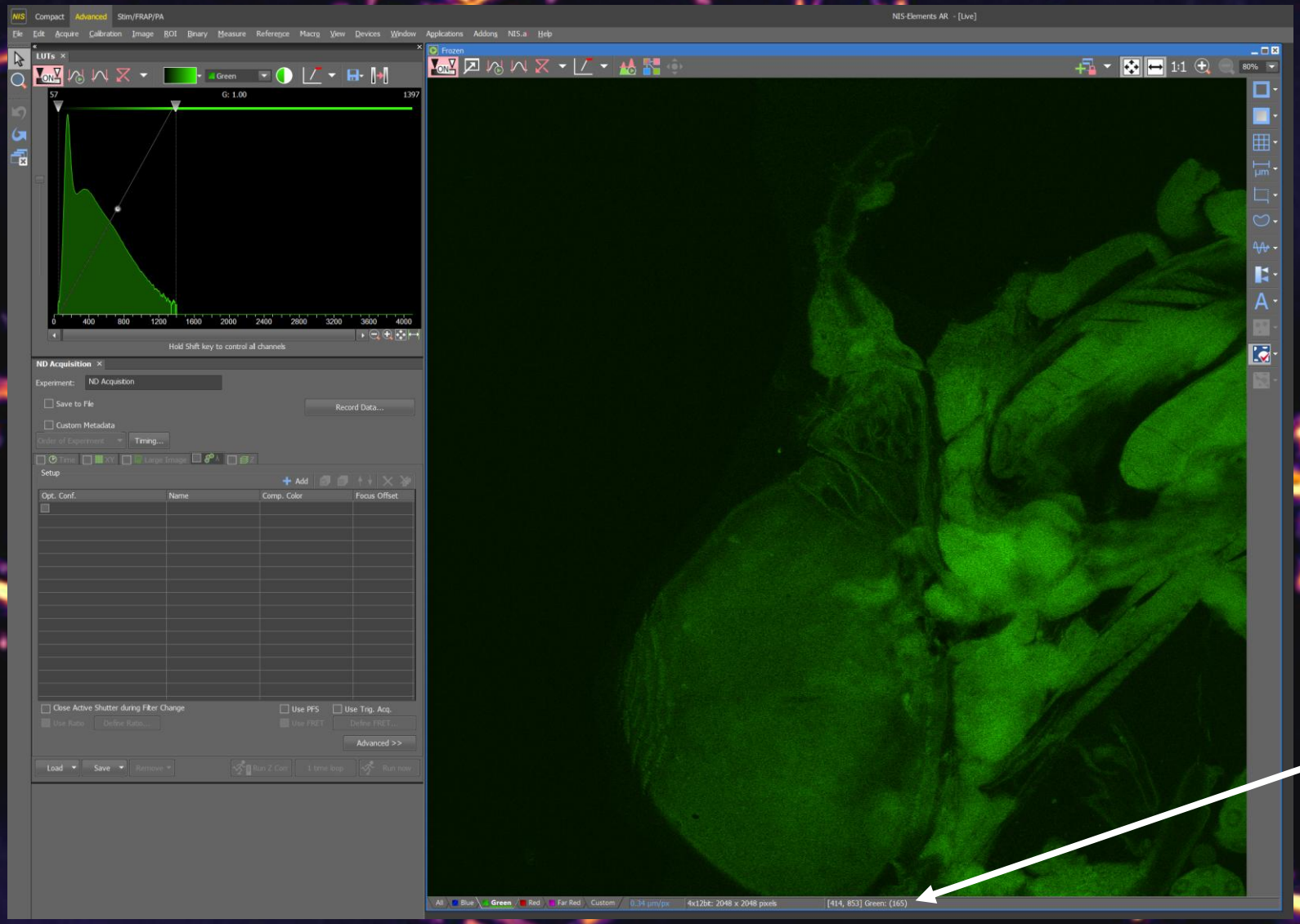
Optimising Your Camera Settings When You Do Not Have Enough Signal


1. ‘AutoSignal’ will save you time as you won’t have to adjust each channel manually, however it is not perfect, it is a good idea to check you are happy with the signal intensity of each channel individually. To do this, first click on a channel to view it in isolation.


2. Select the corresponding channel in the LUT.

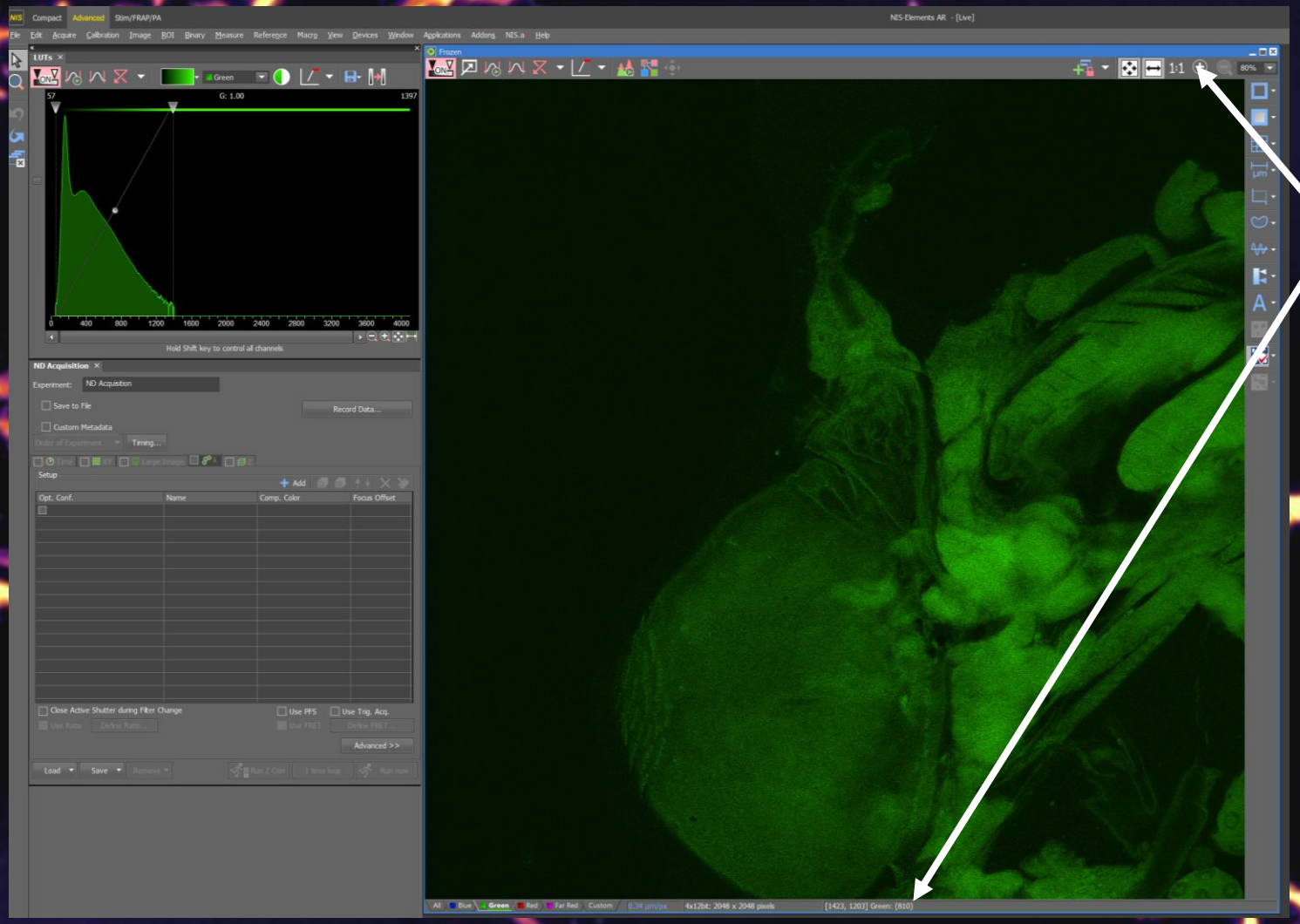
3. Drag the contrast line towards the left if you need to visualise your sample better. Changing the LUT contrast DOES NOT change the raw signal intensity in any way, the sample will look a lot brighter on screen, but your signal intensity, which is what your automated analysis relies upon, remains exactly the same.





I'm going to use the green channel to demonstrate how to check if you have enough signal intensity for analysis and how to optimise the setup if you don't have enough or have too much signal.

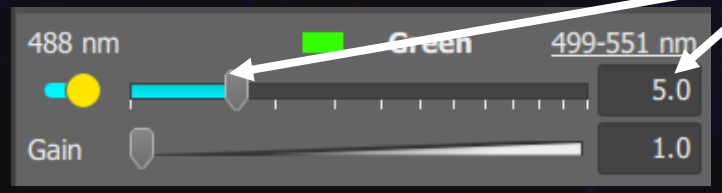
4. Hover your mouse over an area you consider background and read the signal intensity number here (165). Repeat in a few places to work out a rough average background.



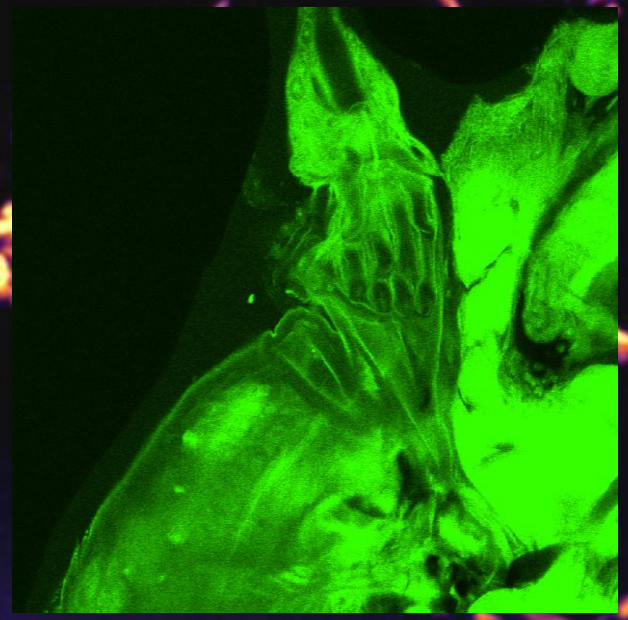
5. Now hover your mouse over a few focused targets you'd like to analyse, and read it's signal intensity (810) and work out the rough average for your targets. It is important to measure the correct intensity, so if you need to zoom in to be more accurate, you can use this or if you are not on LIVE, then you can use your mouse wheel as a zoom instead of focus.

6. Now work out the rough difference between your target and background (800 – 150 = 650). Target signal intensity is 650 above background, if you want to do any automated analysis, this difference needs to be 1000 minimum.

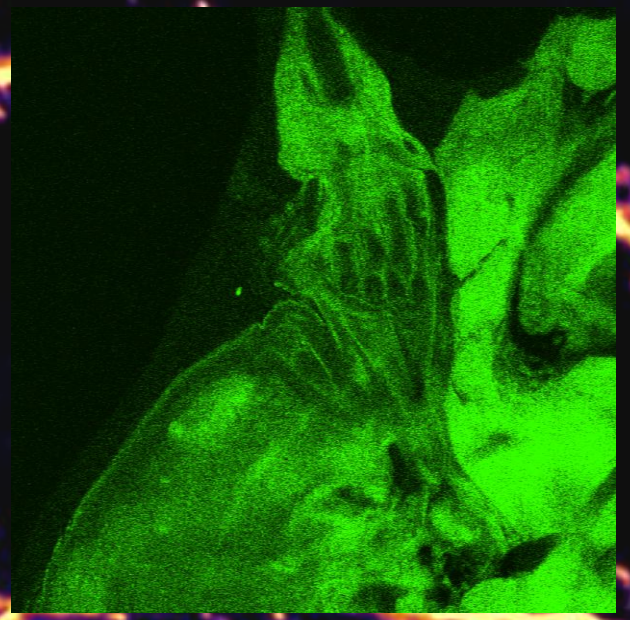
7. Increase the laser power and/or gain for more signal intensity. Please change a single channel at a time, increase by SMALL INCREMENTS, no more than 5 at a time, and always go on LIVE to check your signal before making another change. Increasing laser power can increase bleaching and phototoxicity but will maintain your image resolution. Increasing gain will amplify your signal intensity, without using more laser on your sample, however this will also amplify noise and background signal and therefore can affect the clarity of your image.



Increased laser power



Increased gain

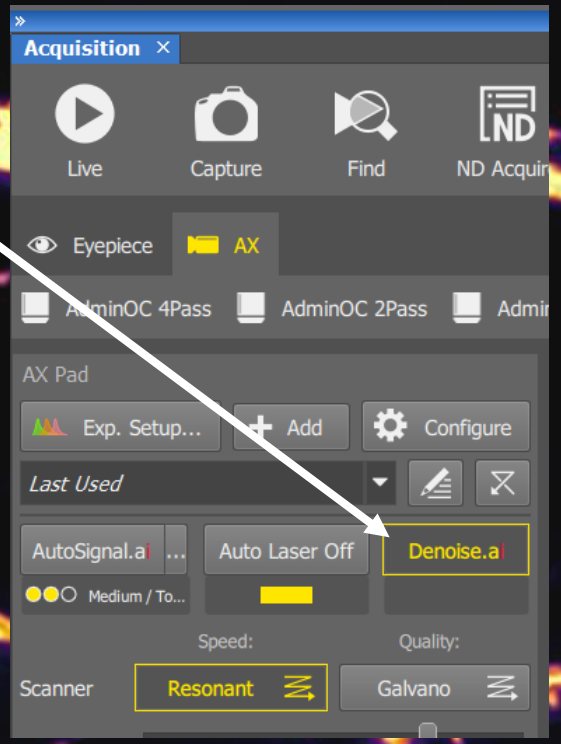


8. If you need to protect your sample from bleaching but also need to reduce noise after applying more gain, you can increase averaging.

No Averaging

8X Averaging

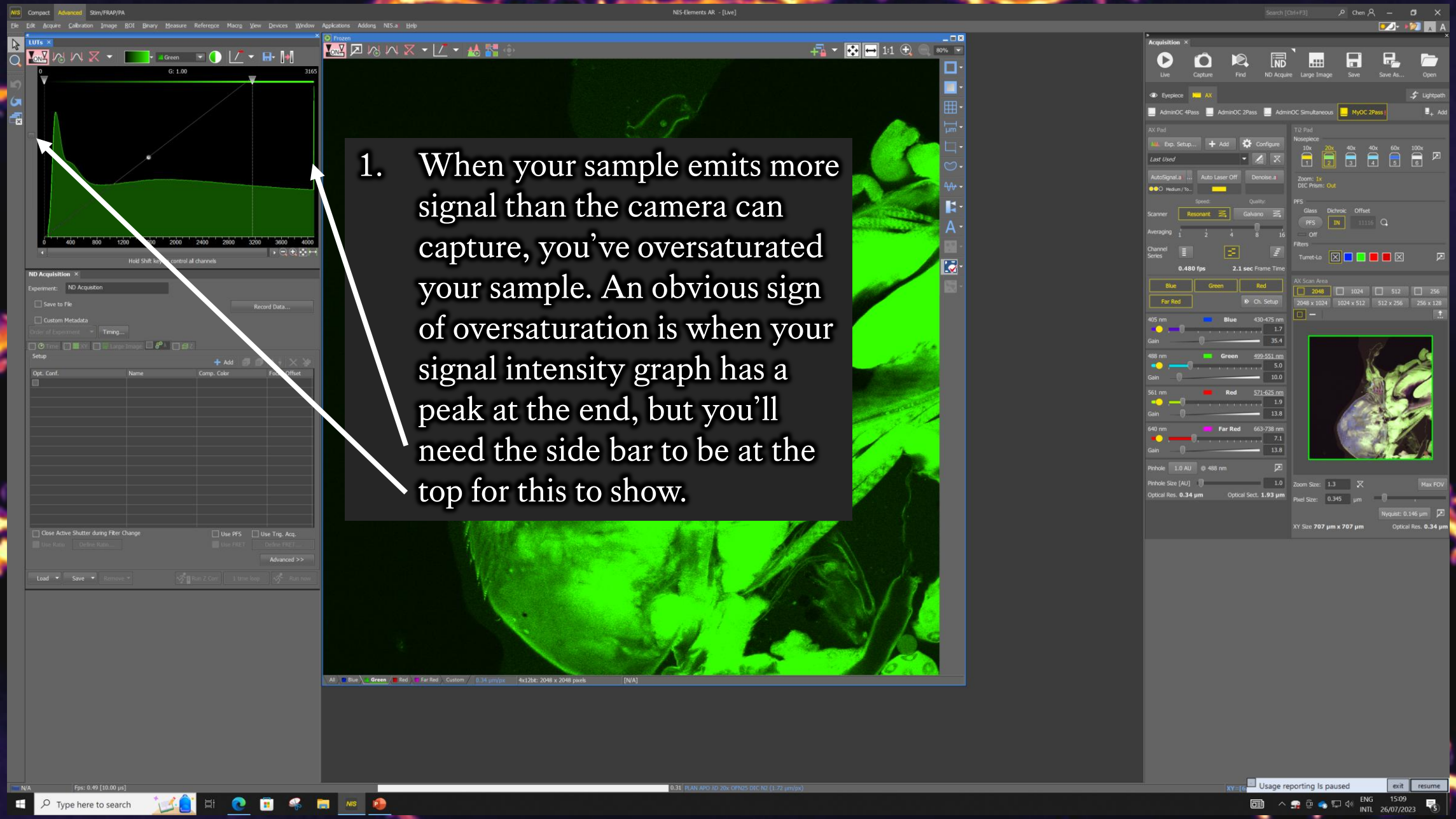
9. You can also use ‘Denoise.ai’. Click this button, then click live to view what your image will look like after applying ‘Denoise.ai’, now click the button again to turn off ‘Denoise.ai’. This is a post-imaging process, meaning you do not need to apply this during imaging, after you have your images, go to our workstations with NIS-Elements and apply ‘Denoise.ai.’ to all comparable images.

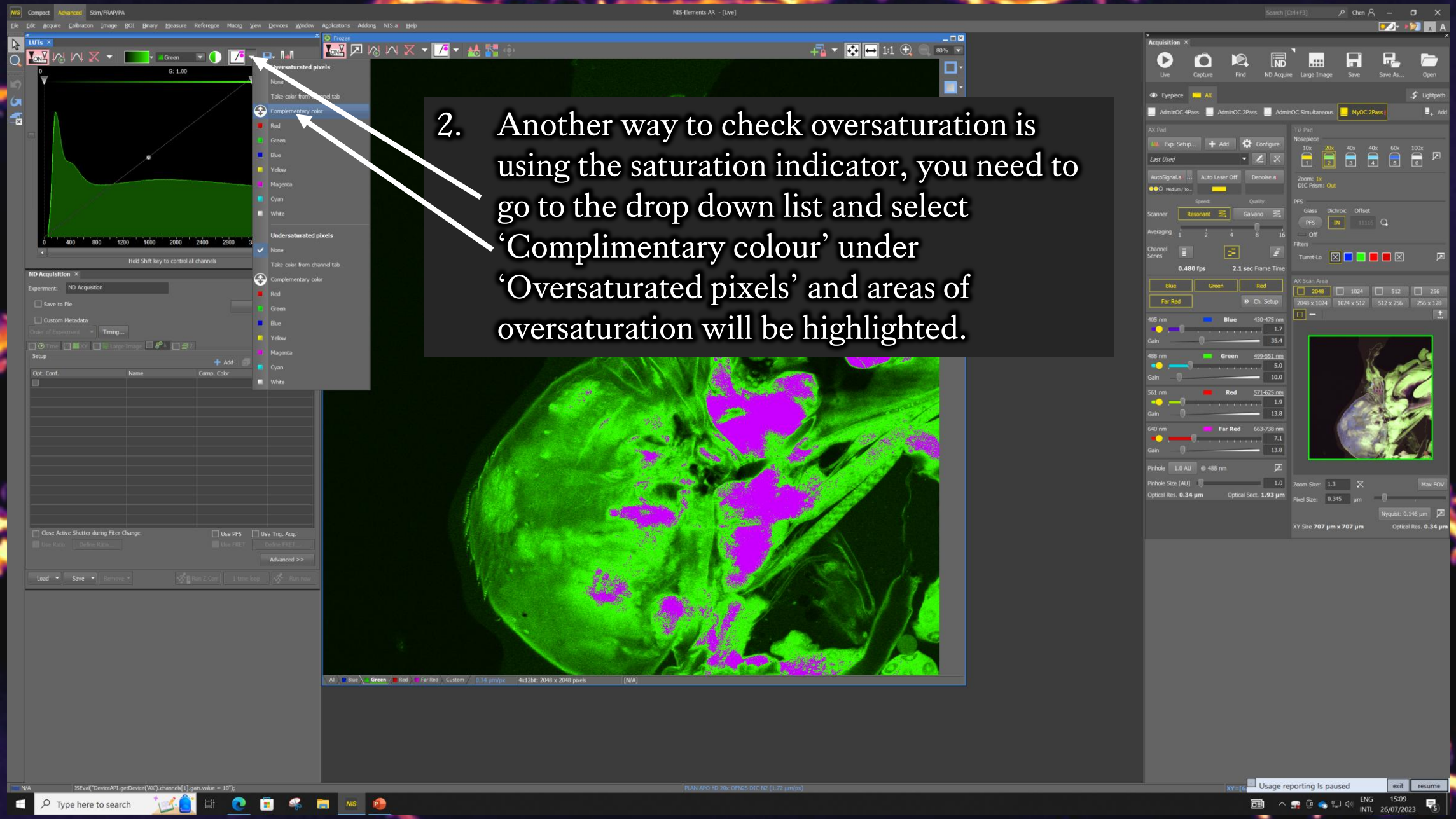


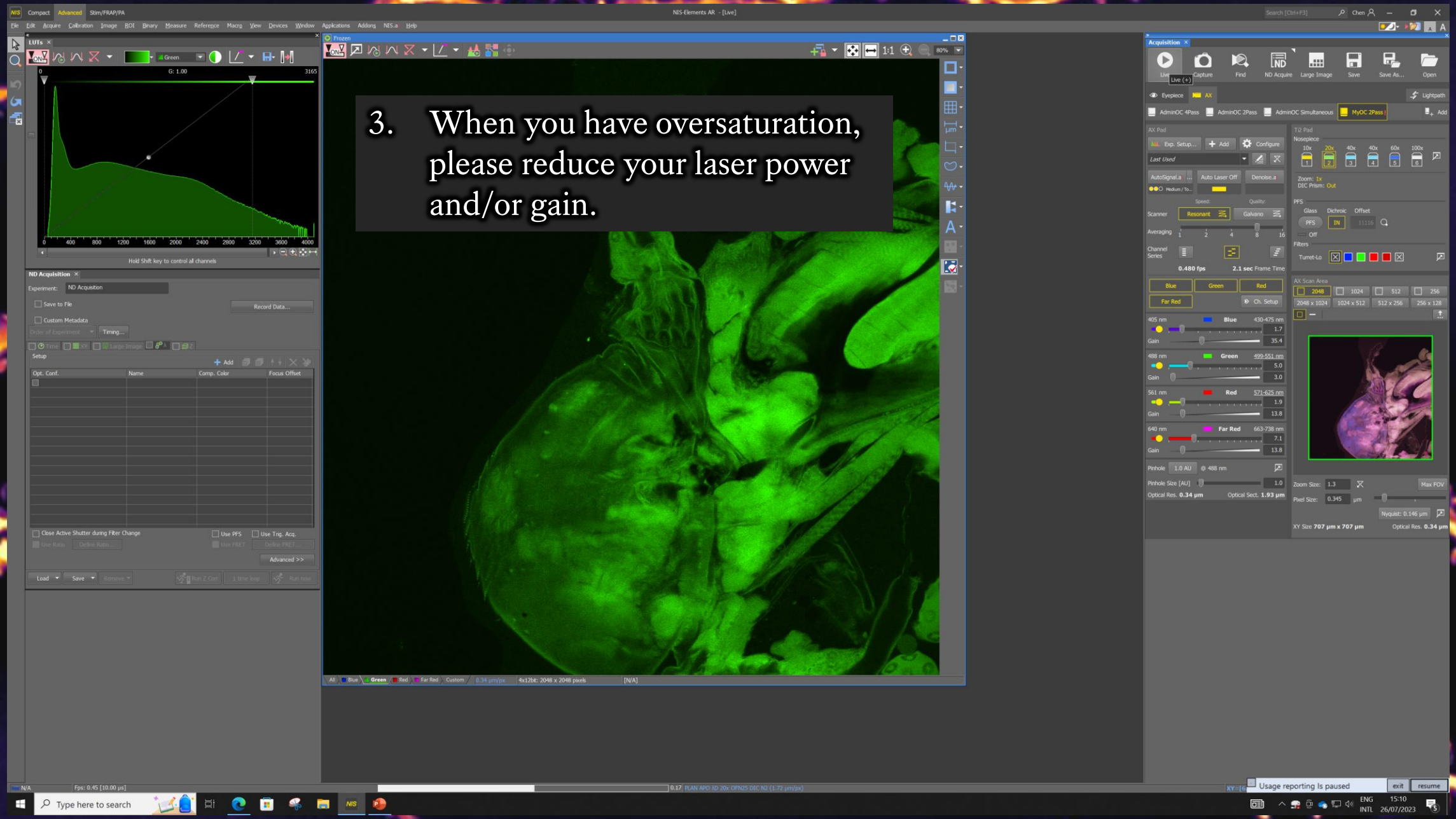
STEP BY STEP INSTRUCTIONS

STEP 6

Optimising Your Camera Settings When You Have Too Much Signal


1. When your sample emits more signal than the camera can capture, you've oversaturated your sample. An obvious sign of oversaturation is when your signal intensity graph has a peak at the end, but you'll need the side bar to be at the top for this to show.


2. Another way to check oversaturation is using the saturation indicator, you need to go to the drop down list and select ‘Complimentary colour’ under ‘Oversaturated pixels’ and areas of oversaturation will be highlighted.


3. When you have oversaturation, please reduce your laser power and/or gain.

STEP BY STEP INSTRUCTIONS

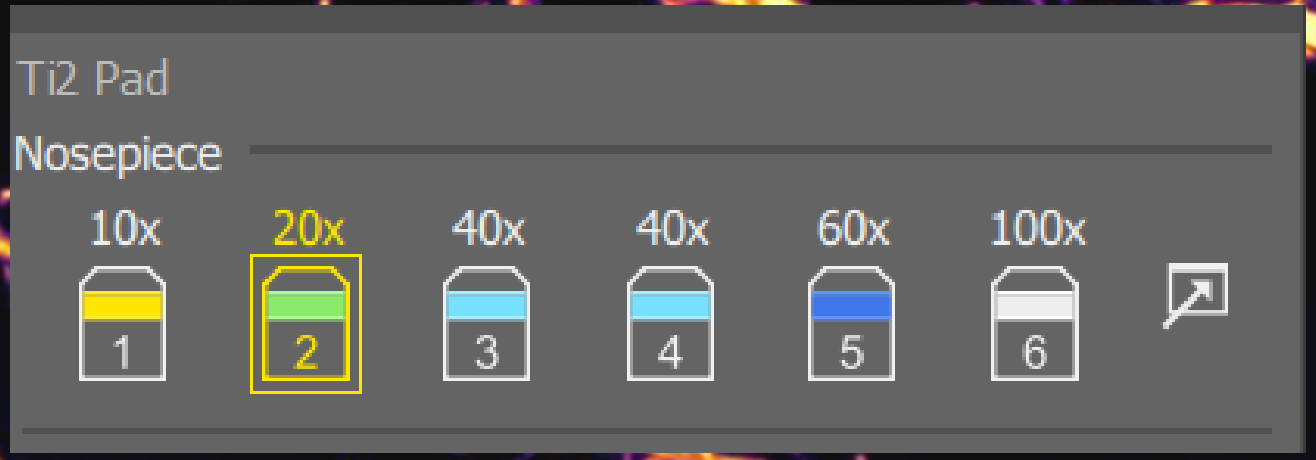
STEP 7

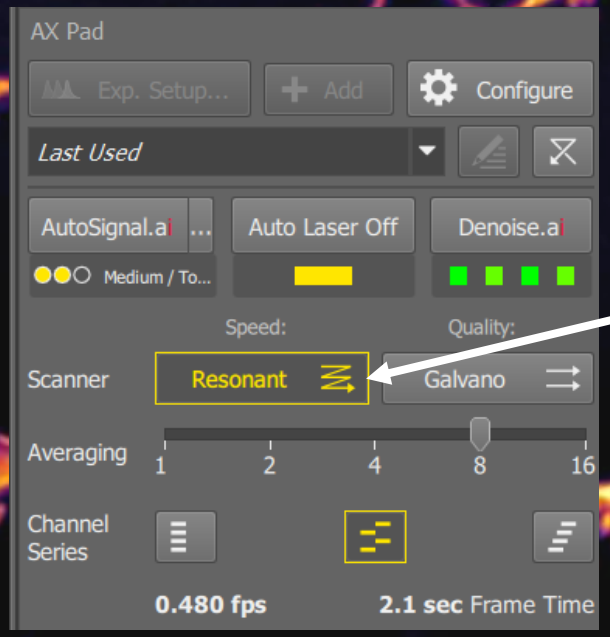
Optimising Your Image Resolution

If you would like to read more on image resolution

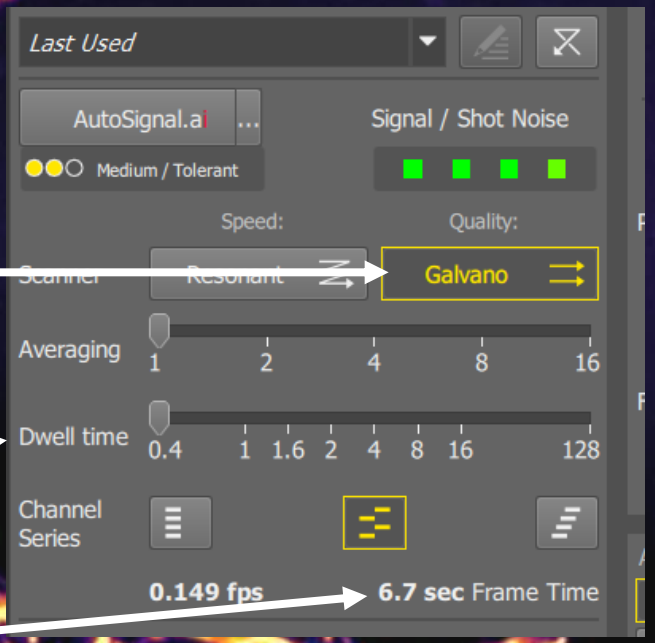
please read our guide here: _____

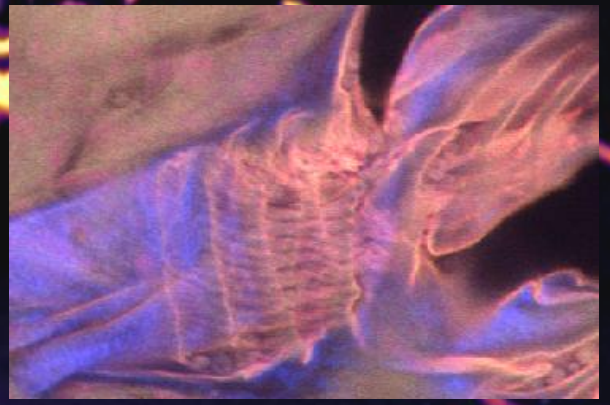
1. Are you using the correct lens for your needs, if you are unsure, you can ask us. The oil and water lenses (40X, 60X and 100X) will provide a higher image resolution than the air lenses (10X and 20X).



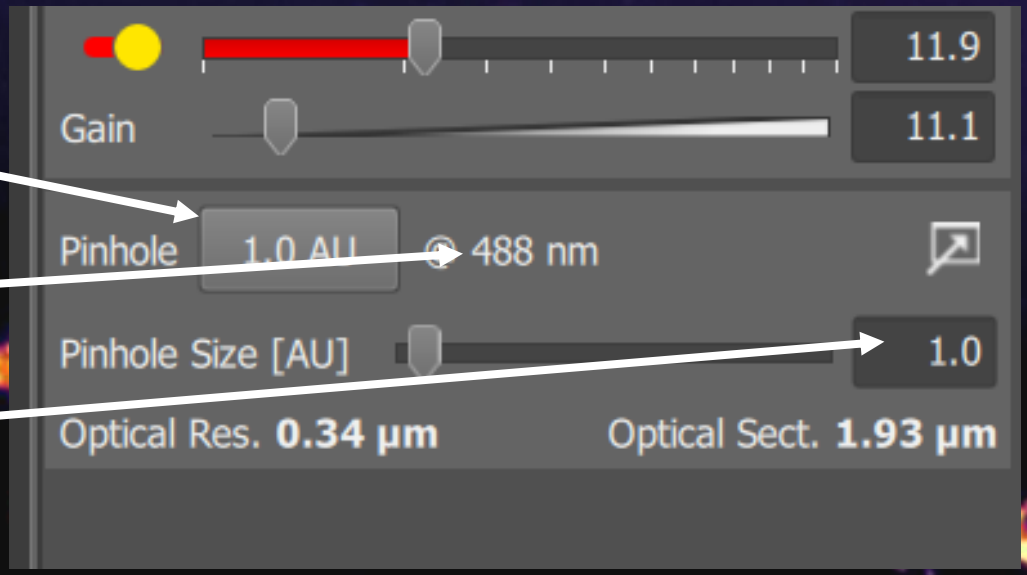


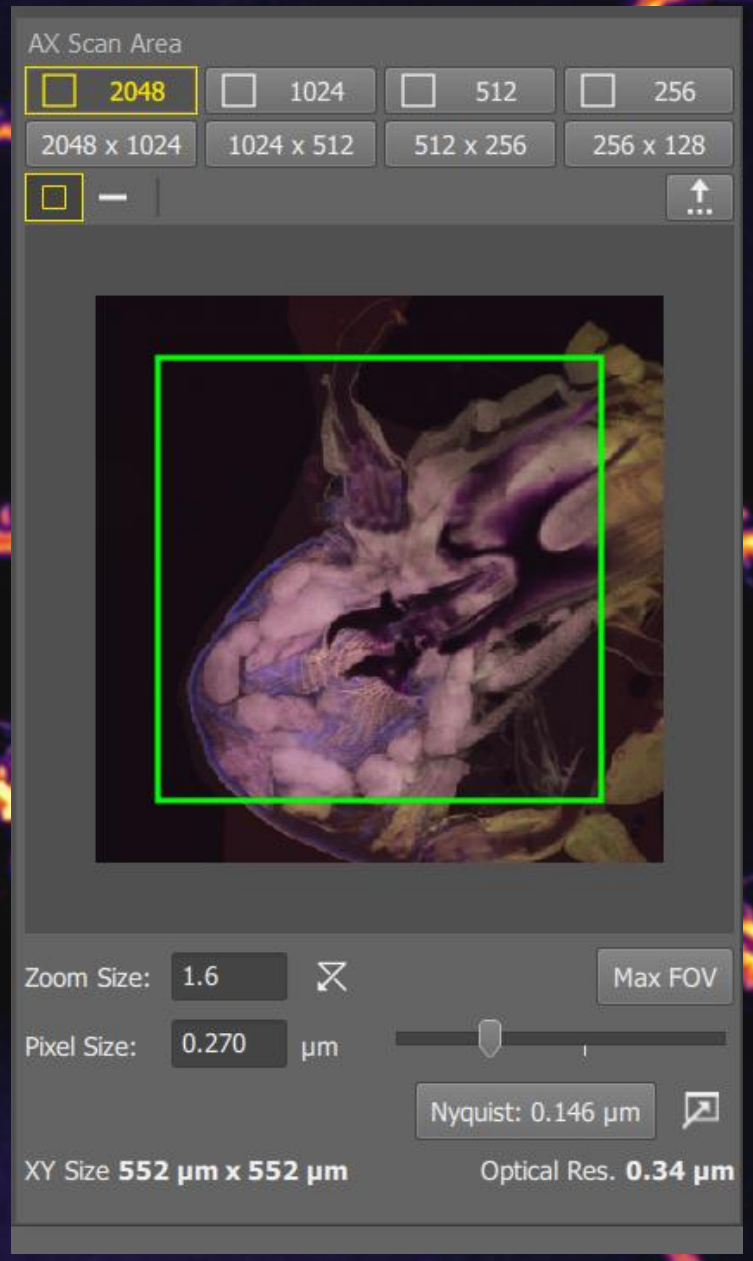
2. Decide between Resonant or Galvano scanning modes. In Galvano, you can change how long you want to expose your sample to the laser in 'Dwell time'. Galvano can result in higher resolution but it can take significantly longer to image. With averaging and denoise options, resonant scanning can provide a good enough resolution for most users' analysis needs.





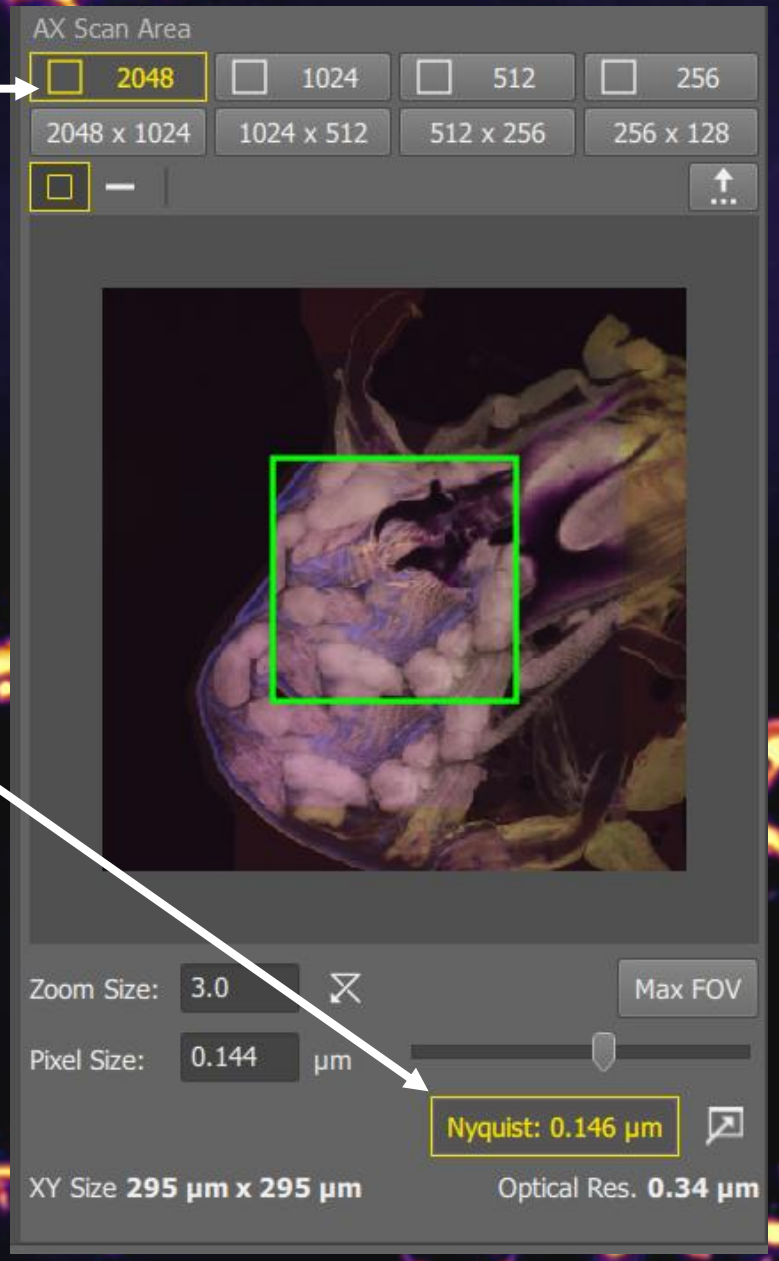
3. Set the pinhole size. Click on this button for the recommended pinhole size. Pinhole can be set using any wavelength but it should be set to 488 nm by default. You can manually change the pinhole size by typing here. The smaller the pinhole the higher the resolution. Do not go smaller than 0.6, you will restrict too much signal. Do not go bigger than 1.5, you will eliminate the benefits of a confocal microscope.





4. Set the scan area to 2048 (meaning 2048x2048 pixels) this will give you the largest field of view compared to the other options.

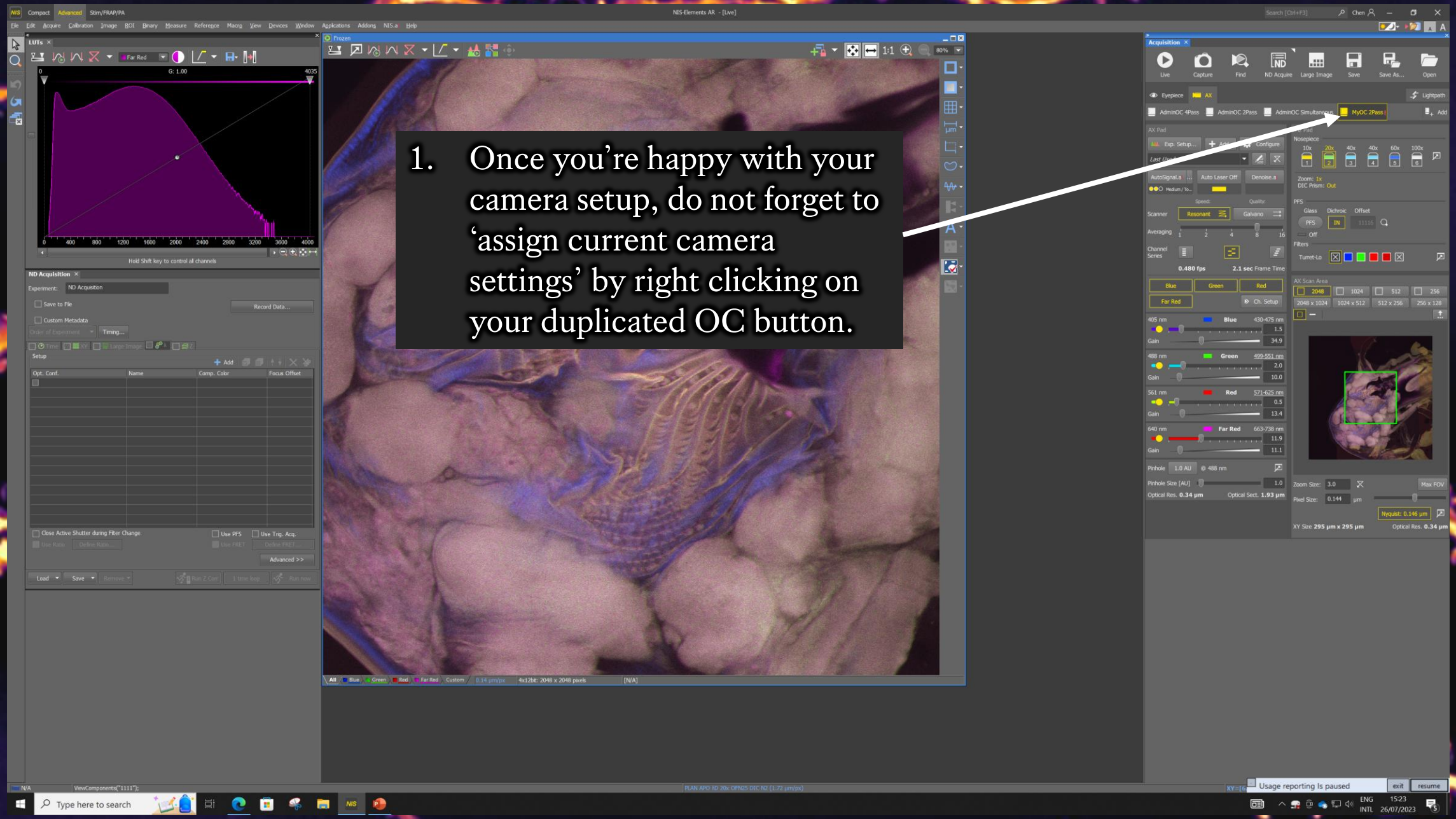
5. If you want the highest resolution, set zoom size by clicking on 'Nyquist'. This will allow you to see details on the resolution limit this microscope can provide.



STEP BY STEP INSTRUCTIONS

STEP 8

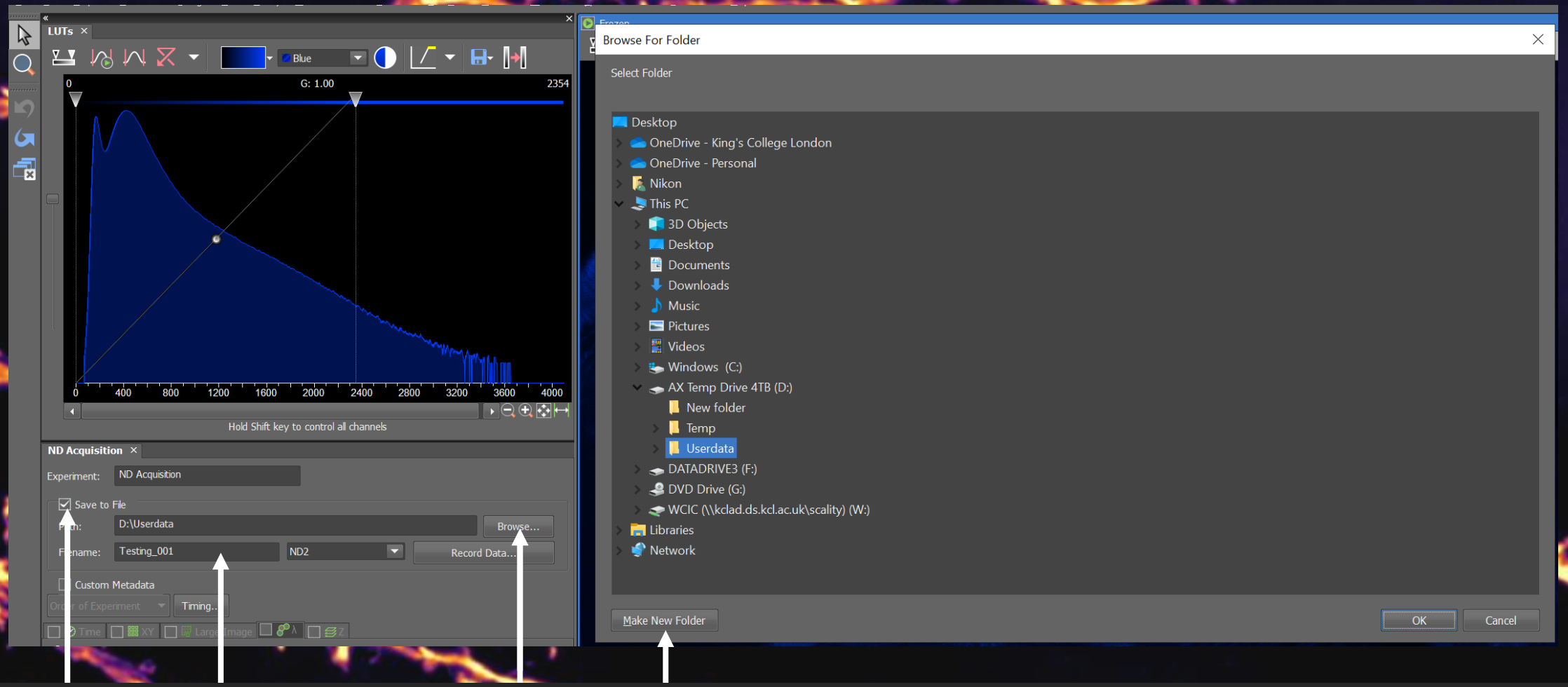
Save Your Optical Configuration Changes


1. Once you're happy with your camera setup, do not forget to 'assign current camera settings' by right clicking on your duplicated OC button.

STEP BY STEP INSTRUCTIONS

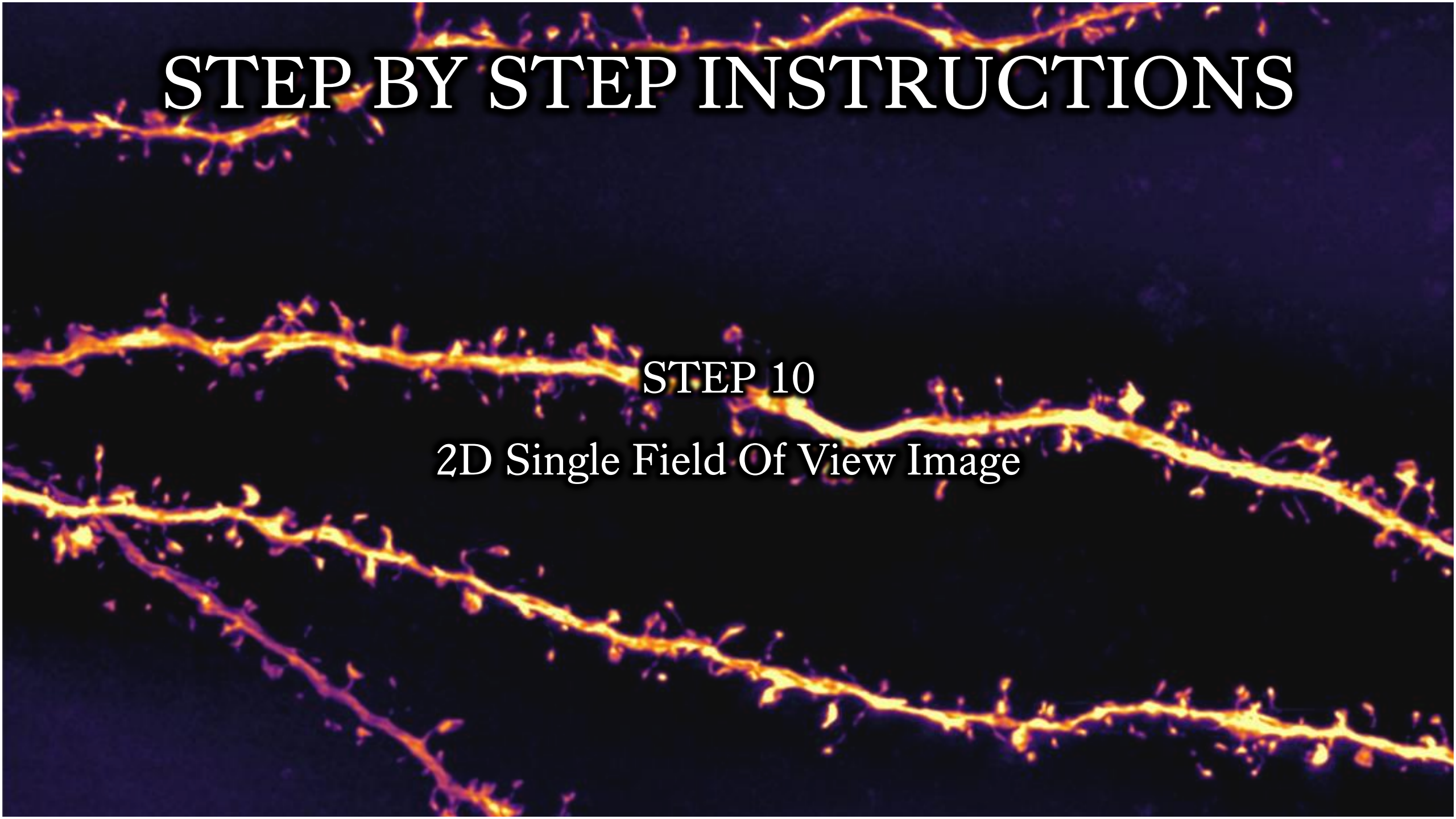
STEP 9

Set Up ‘Save To File’



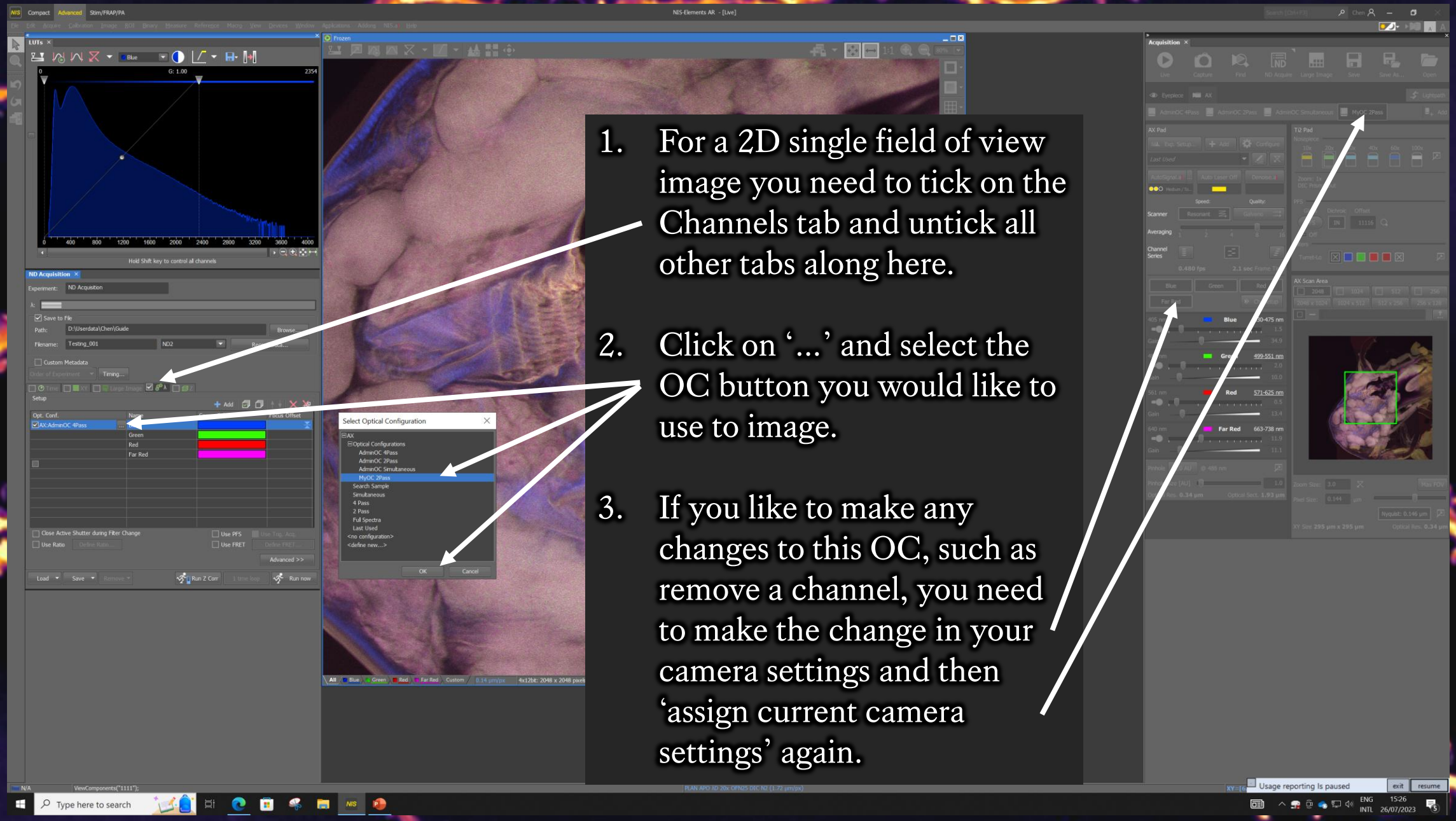
1. ‘Save to File’ will save your images automatically, always make sure the box is ticked and then go to ‘Browse’ to select This PC < AX Temp Drive 4TB (D:) < User Data < Your Folder (or ‘Make New Folder) < Then make a new folder for your current session. Then give your next image a name in ‘Filename’, please do not use any symbols, if you need a space use underscore, put _001 at the end so your second image will be _002. You do not need to click on anything else, ‘Save to File’ is now applied.

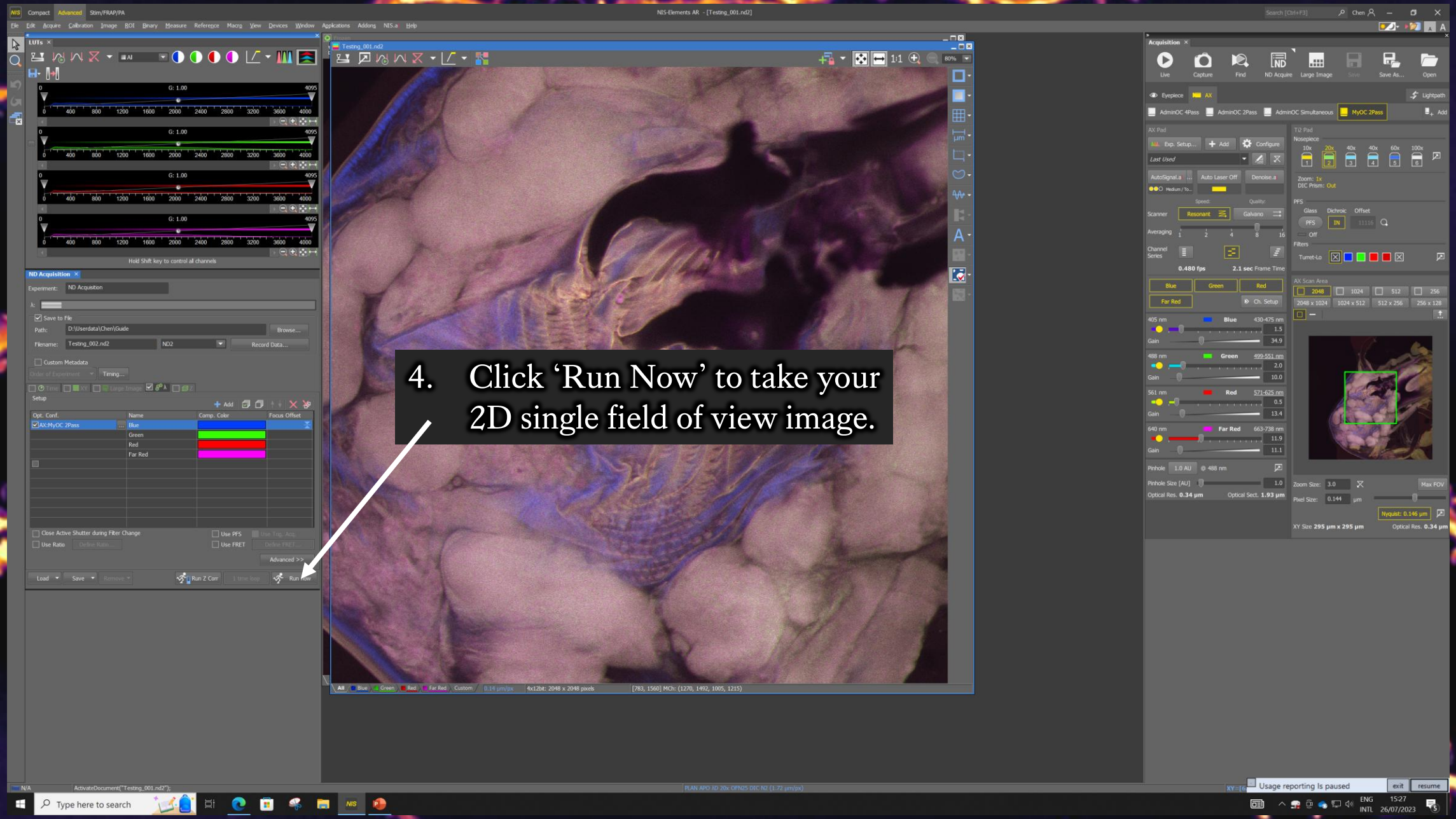
STEP BY STEP INSTRUCTIONS



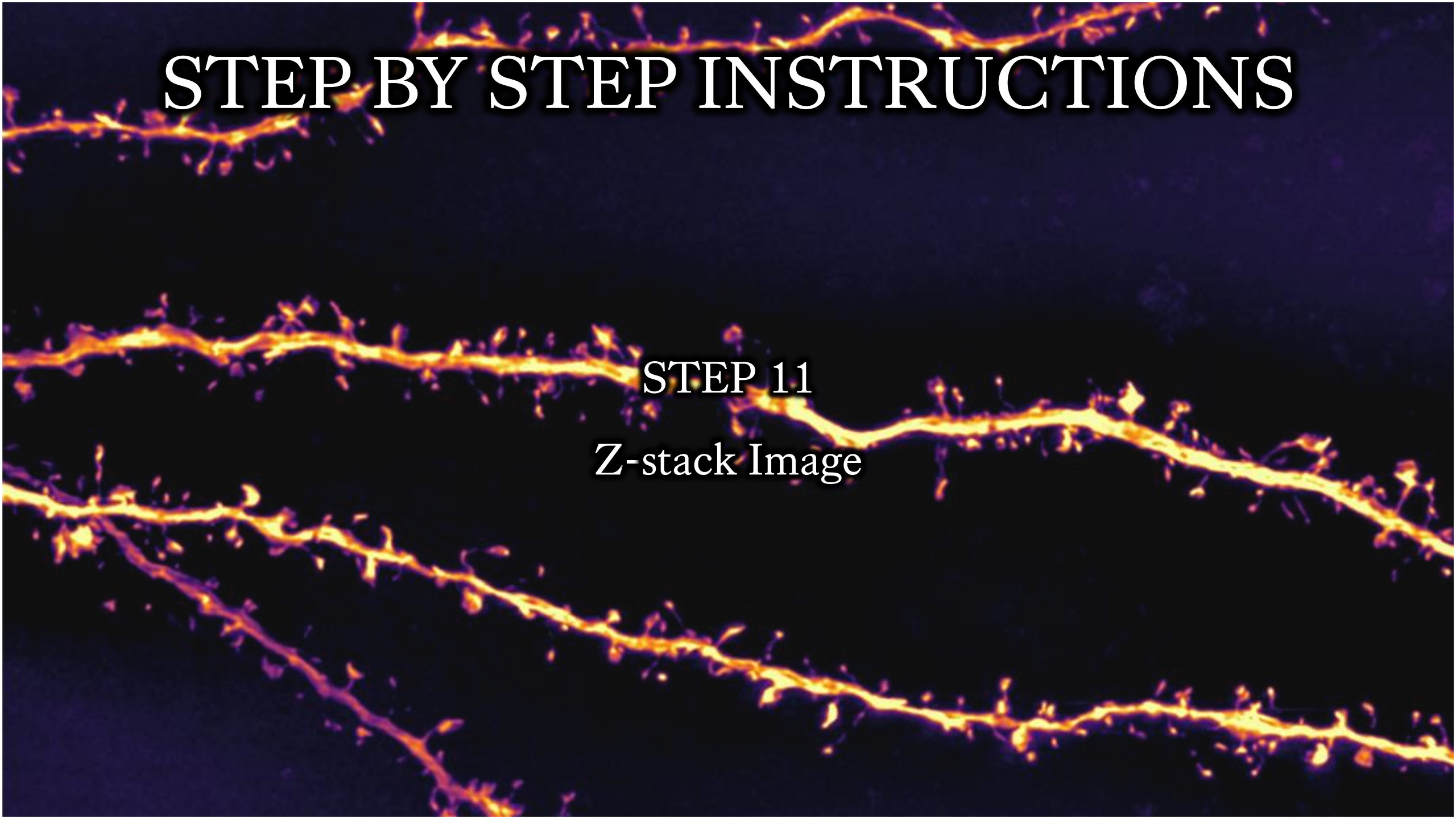
STEP 10

2D Single Field Of View Image


1. For a 2D single field of view image you need to tick on the Channels tab and untick all other tabs along here.
2. Click on '...' and select the OC button you would like to use to image.
3. If you like to make any changes to this OC, such as remove a channel, you need to make the change in your camera settings and then 'assign current camera settings' again.
Select Optical Configuration
Optical Configurations
AdminOC 4Pass
AdminOC 2Pass
AdminOC Simultaneous
MyOC 2Pass
Search Sample
Simultaneous
4 Pass
2 Pass
Ful Spectra
Last Used
<no configuration>
<define new...>
OK
Cancel


4. Click ‘Run Now’ to take your 2D single field of view image.

STEP BY STEP INSTRUCTIONS



STEP 11

Z-stack Image

Z stack Basic Options

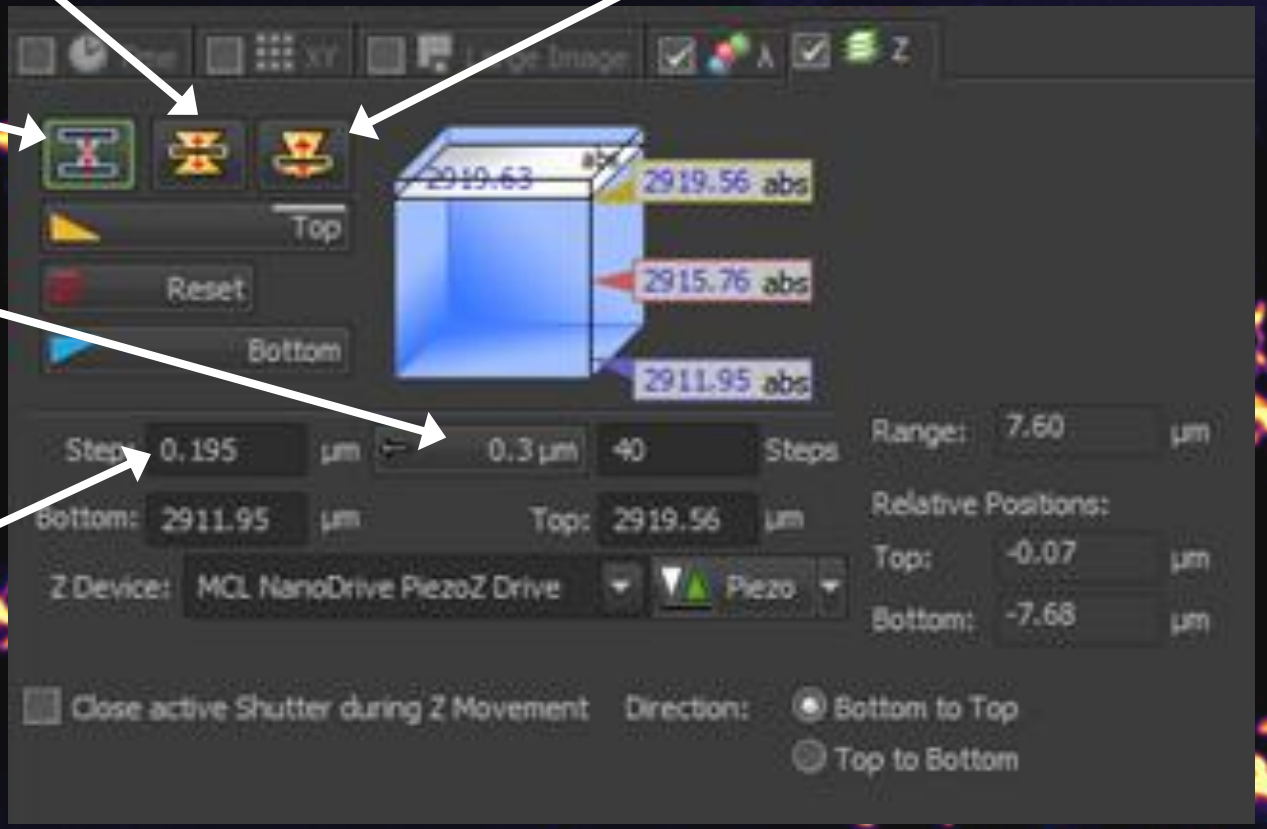
Set top and bottom: use mouse wheel to focus and define the exact range of your Z stack.

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)

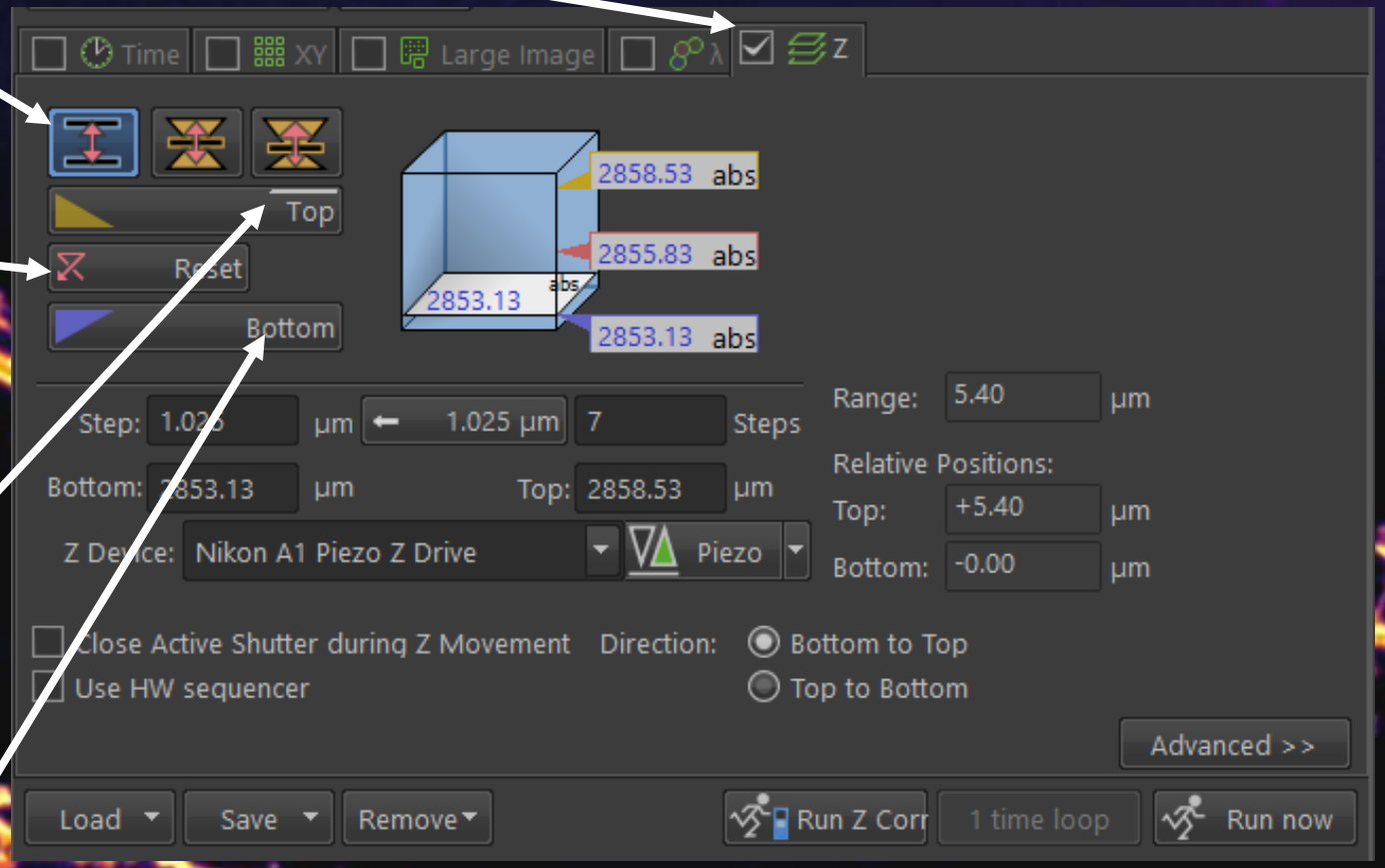
Asymmetrical: find focal plane and then set different distances above and below. (Useful for  like cells)

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.



1. Tick the Z tab and untick others.
2. Choose your method, I'll use the first one for this guide.
3. Press reset.
4. Go on LIVE and click and hover your mouse on your live window and scroll your mouse wheel towards you (lens going up) until the top of where you want to image, and click on 'Top'.
5. Hover your mouse over your LIVE window again and scroll your mouse wheel away from you (lens going down) and scroll through your sample and click 'Bottom'.



6. Click on FREEZE to stop scanning.

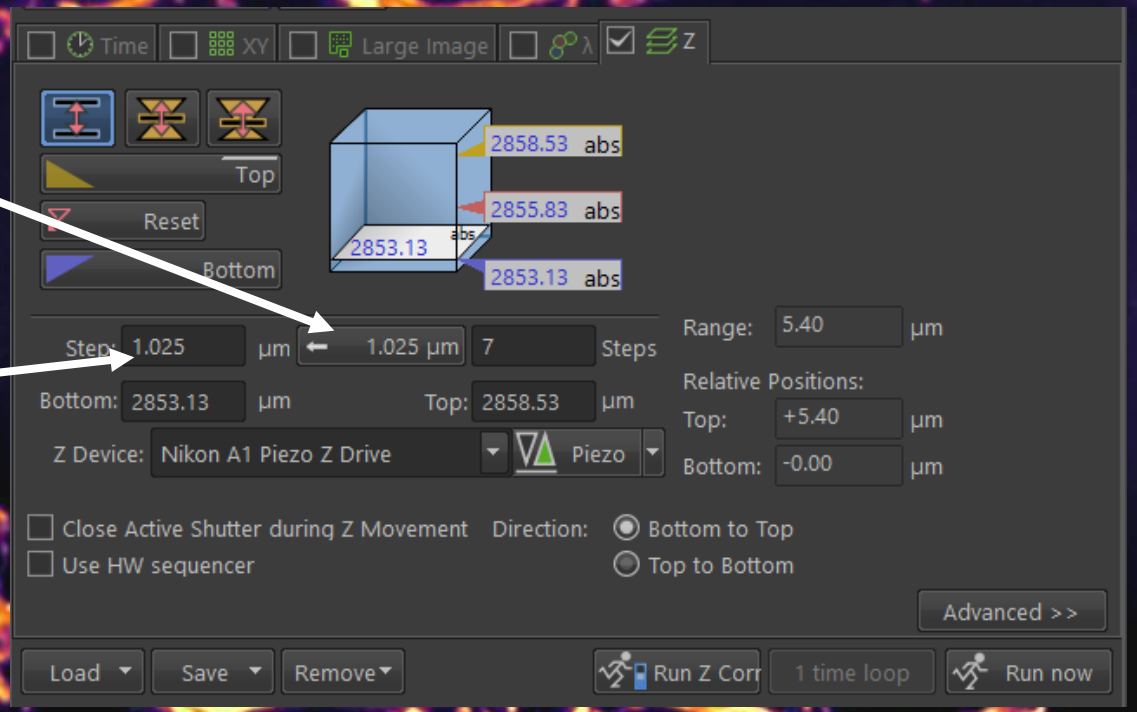
7. Click on the recommended step size, this will give you the optimum image resolution in the Z range.

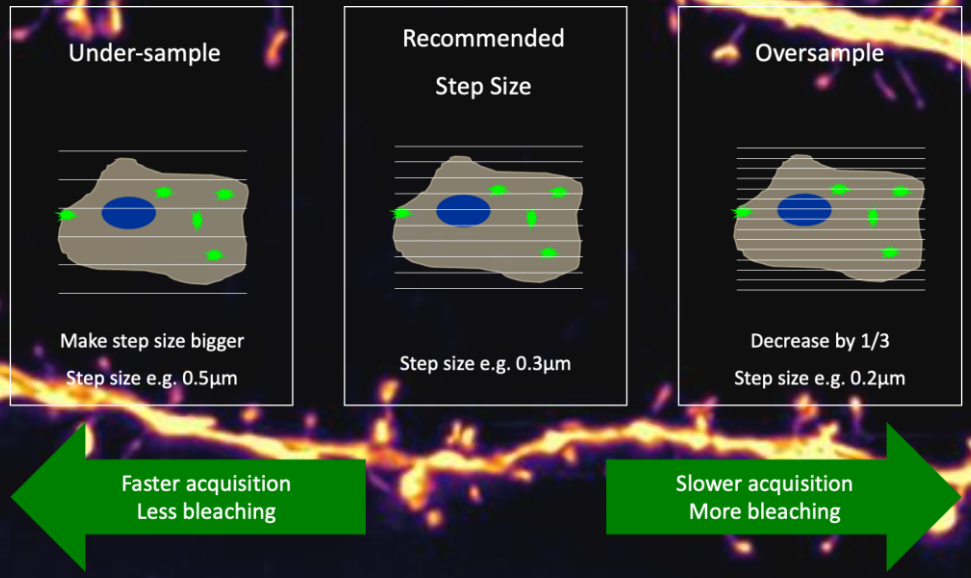
8. You have the option to manually change the step size by typing here. Longer distance (Under-sampling) between each step will result in fewer steps in total, fewer steps takes less time to image but you may miss out on information. Shorter distance (Oversampling) between each step will result in more steps in total, this will take longer to image, but you can use these extra information in 3D deconvolution on the workstations after imaging to increase your image resolution even further. If you are interested further information on this, please see out Image resolution guide here:

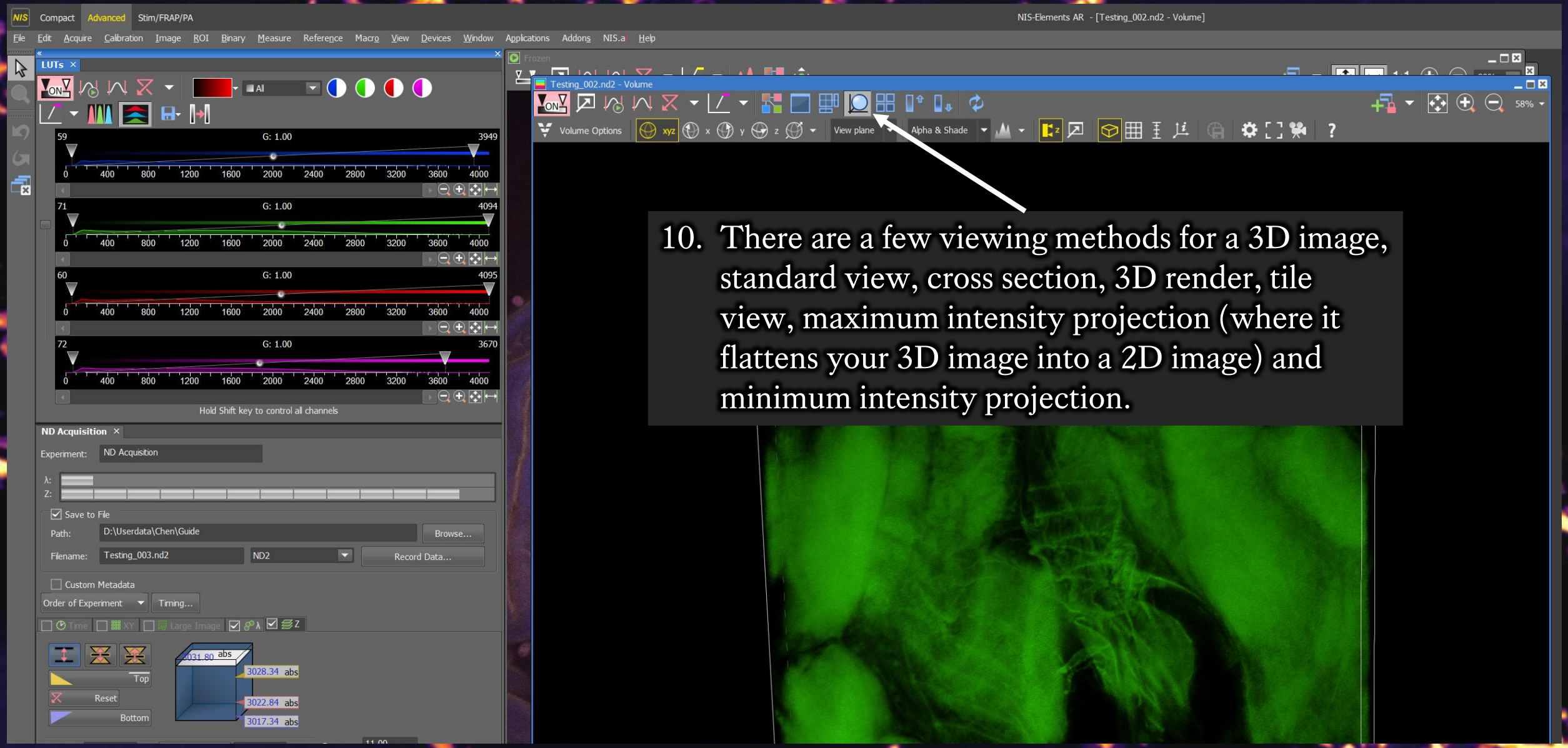
_____ and our Deconvolution guide here:

_____ .

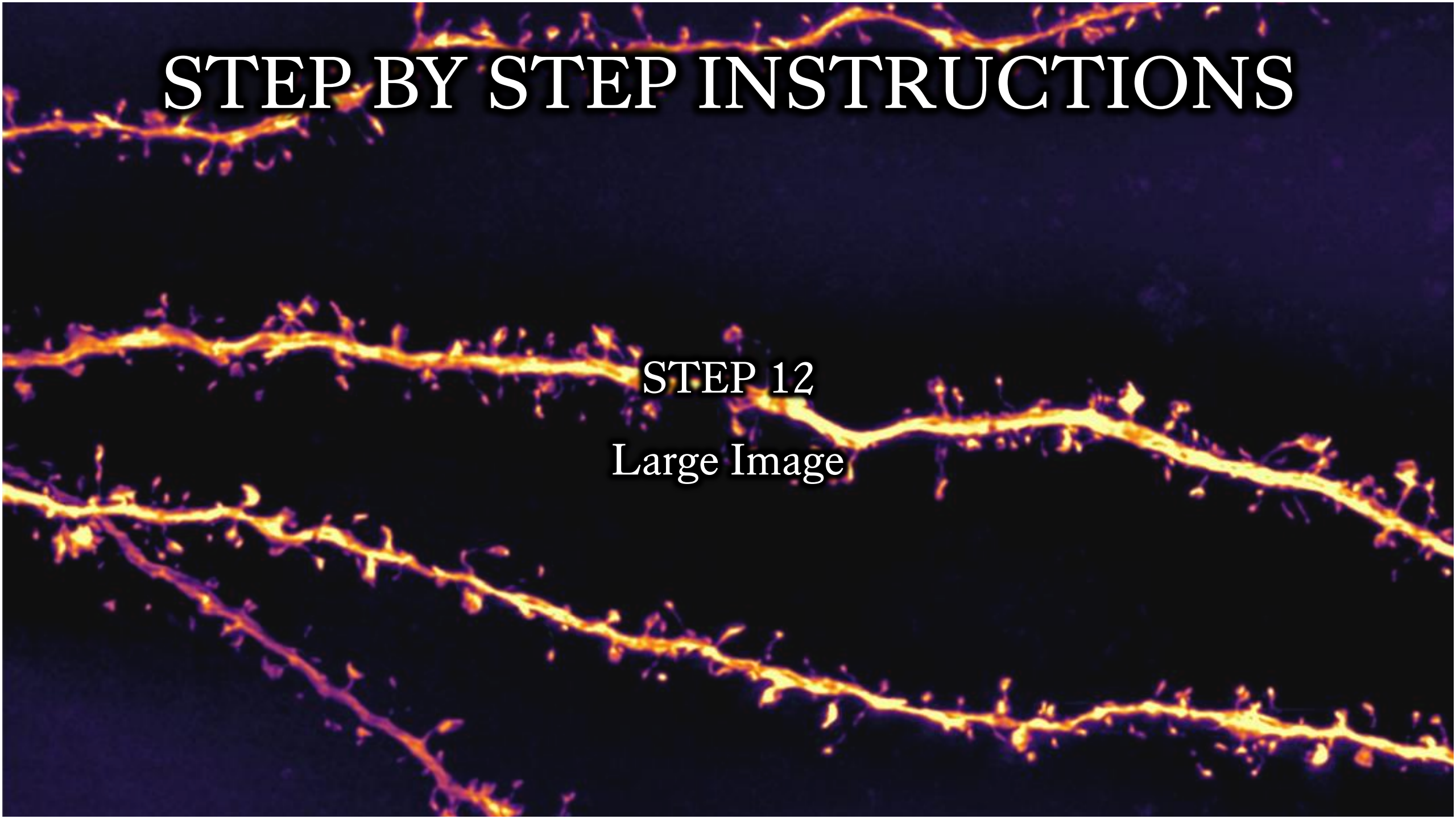
9. Click on 'Run Now' to take your Z-stack image.






Compact
Advanced
Stim/FRAP/PA
NIS-Elements AR - [Testing_002.nd2 - Volume]
File Edit Acquire Calibration Image ROI Binary Measure Reference Macro View Devices Window Applications Addons NIS.a Help
LUTs
Hold Shift key to control all channels
ND Acquisition
Experiment: ND Acquisition
Save to File
Path: D:\Userdata\Chen\Guide
Browse...
Filename: Testing_003.nd2
ND2
Record Data...
Custom Metadata
Order of Experiment
Timing...
Top
Reset
Bottom
Testing_002.nd2 - Volume
Volume Options
View plane
Alpha & Shade
10. There are a few viewing methods for a 3D image, standard view, cross section, 3D render, tile view, maximum intensity projection (where it flattens your 3D image into a 2D image) and minimum intensity projection.

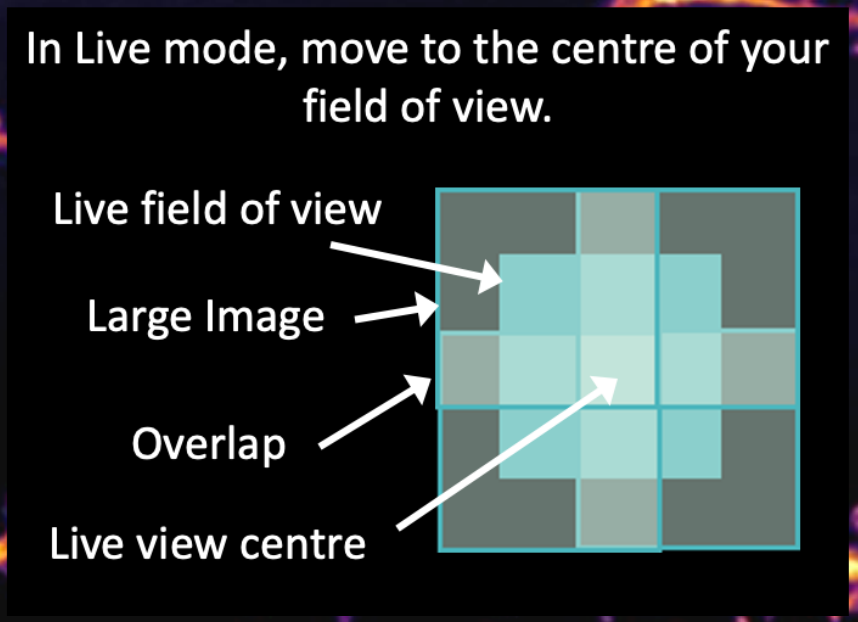
STEP BY STEP INSTRUCTIONS



STEP 12

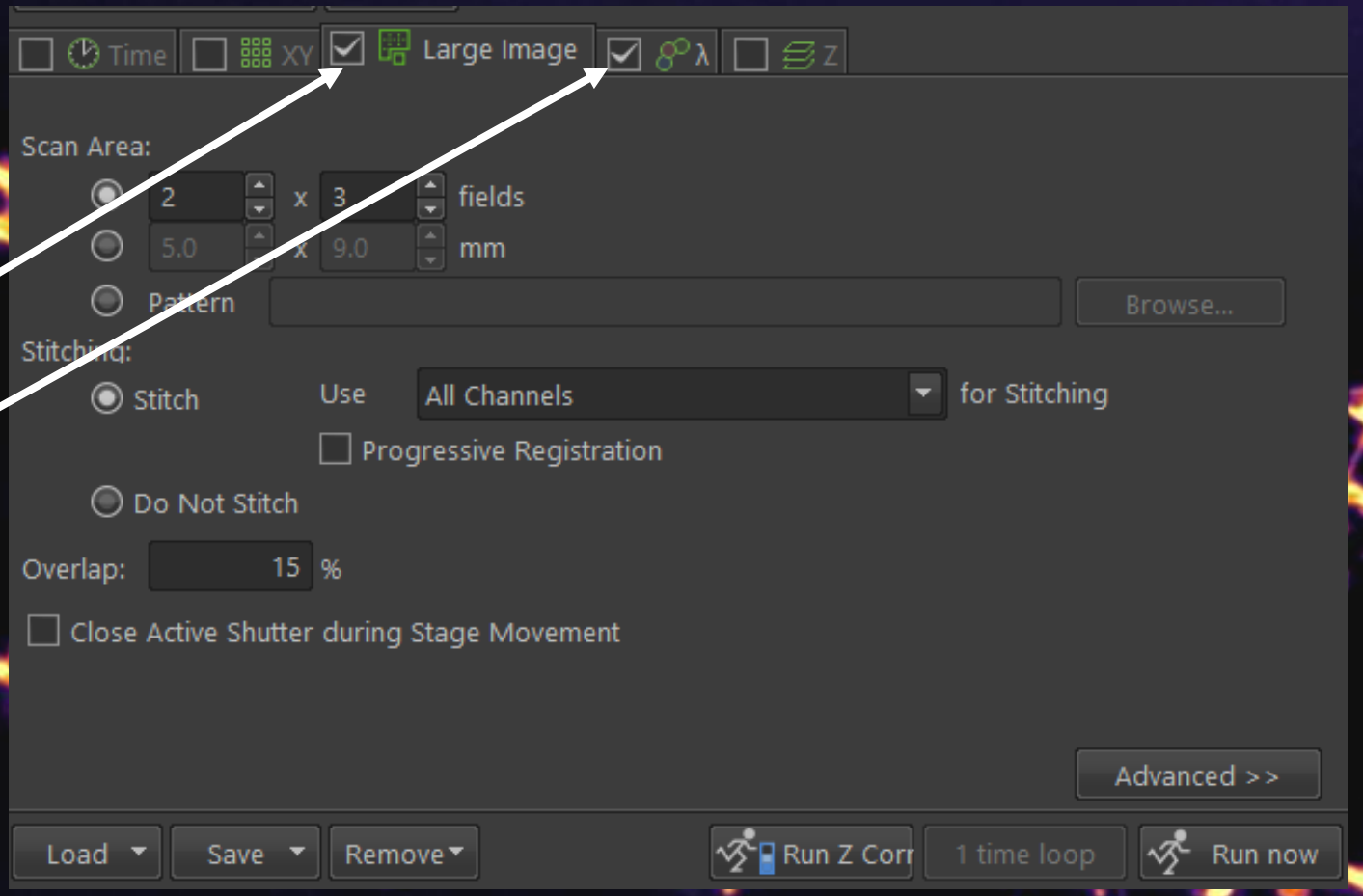
Large Image

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



1. Click on LIVE and focus on your sample (especially after doing a Z-stack), your live view will be the centre of your large image, this is a diagram of a 2 by 2 large image.

2. Tick on 'Large Image' and 'Channels' tabs, make sure the Large Image tab is on the left hand side of the Channels tab, you and click and drag these tabs to re-order them.

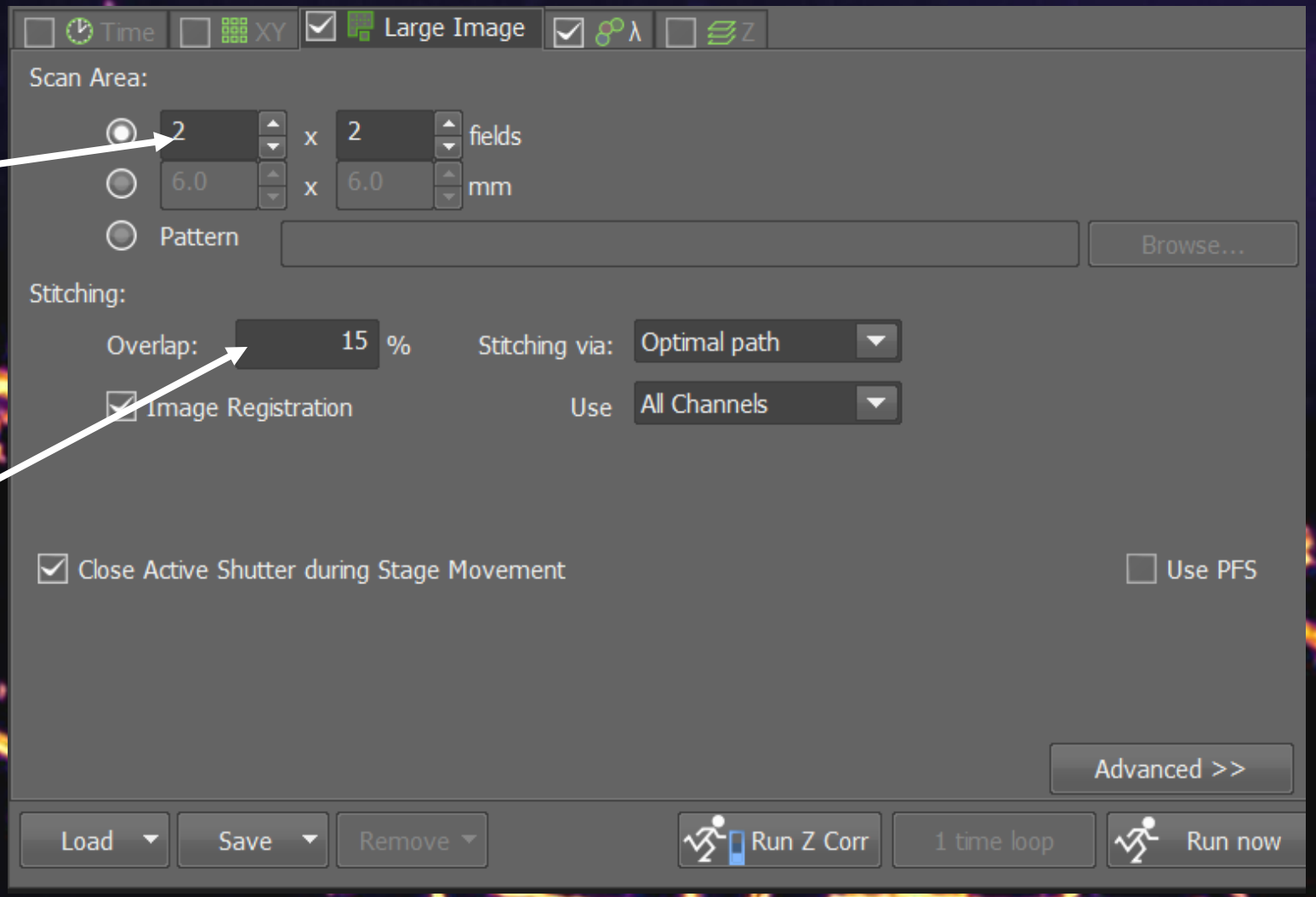


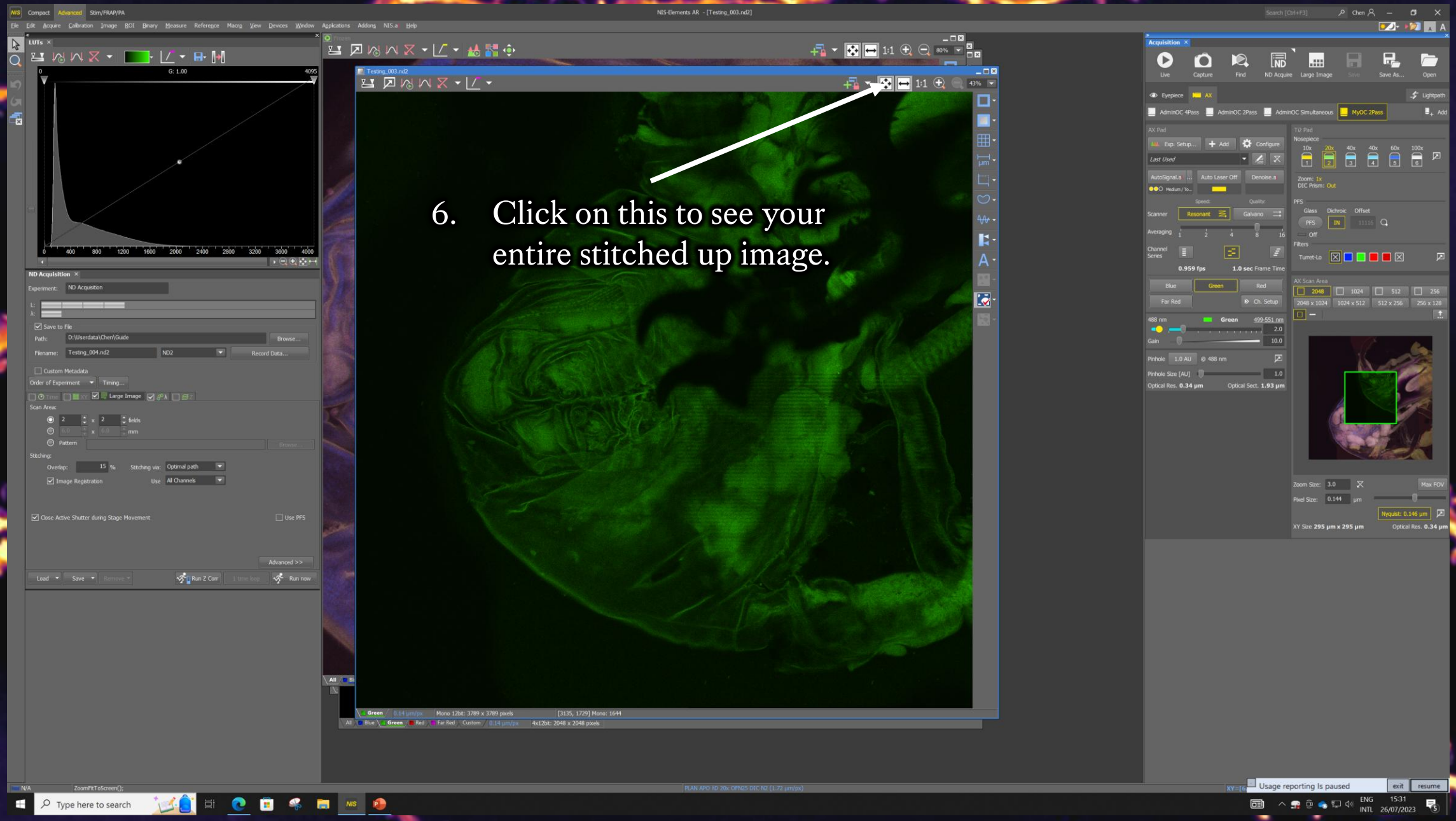
3. Enter in the Scan Area, how many fields of view you need in the X and Y axes respectively.

4. Be mindful where you are on your coverslip, make sure you're not at the edge.

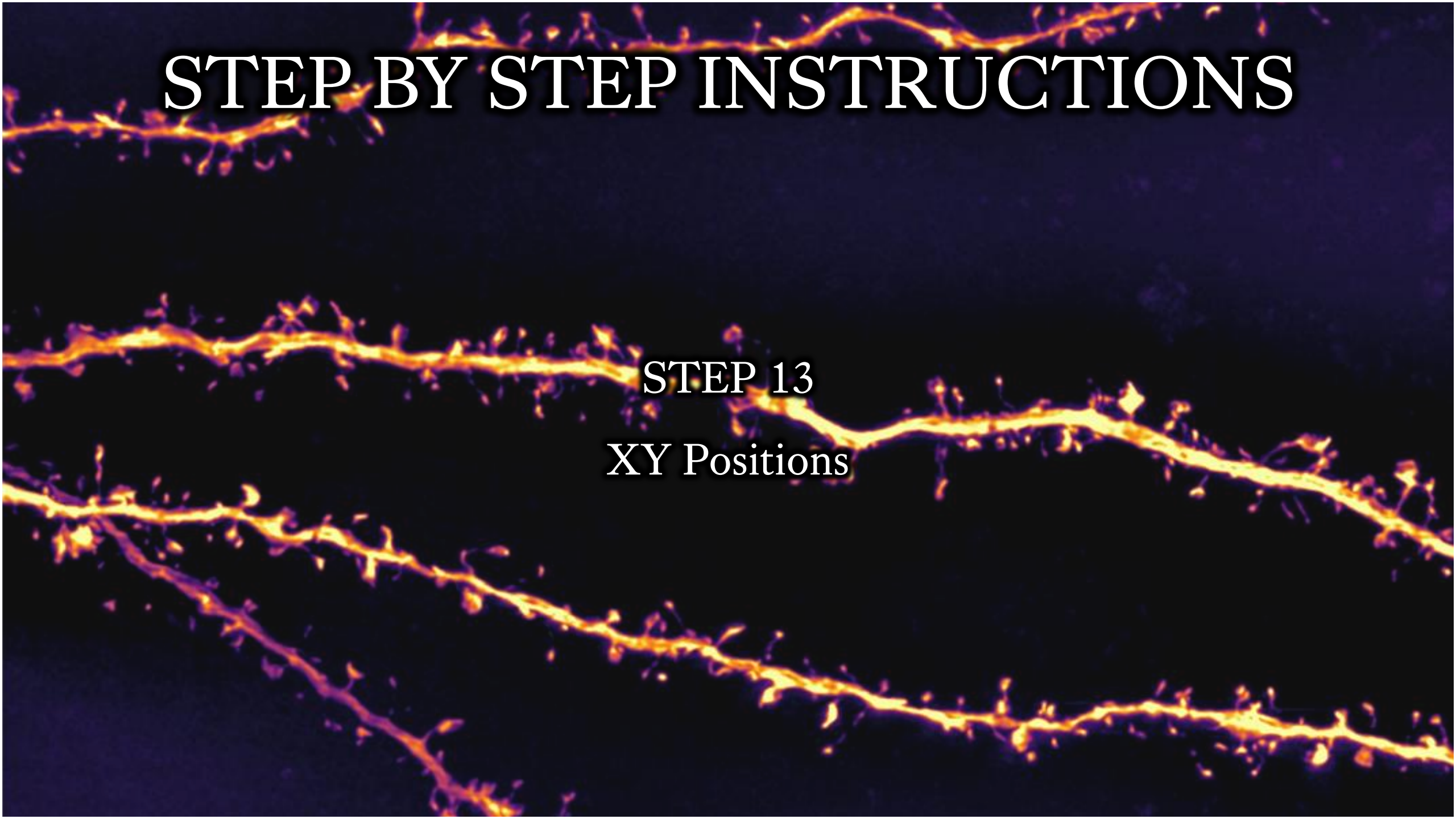
5. If you want to stitch up your large image, you need 15% overlap minimum.

6. Click on 'Run Now' to take your Large Image.



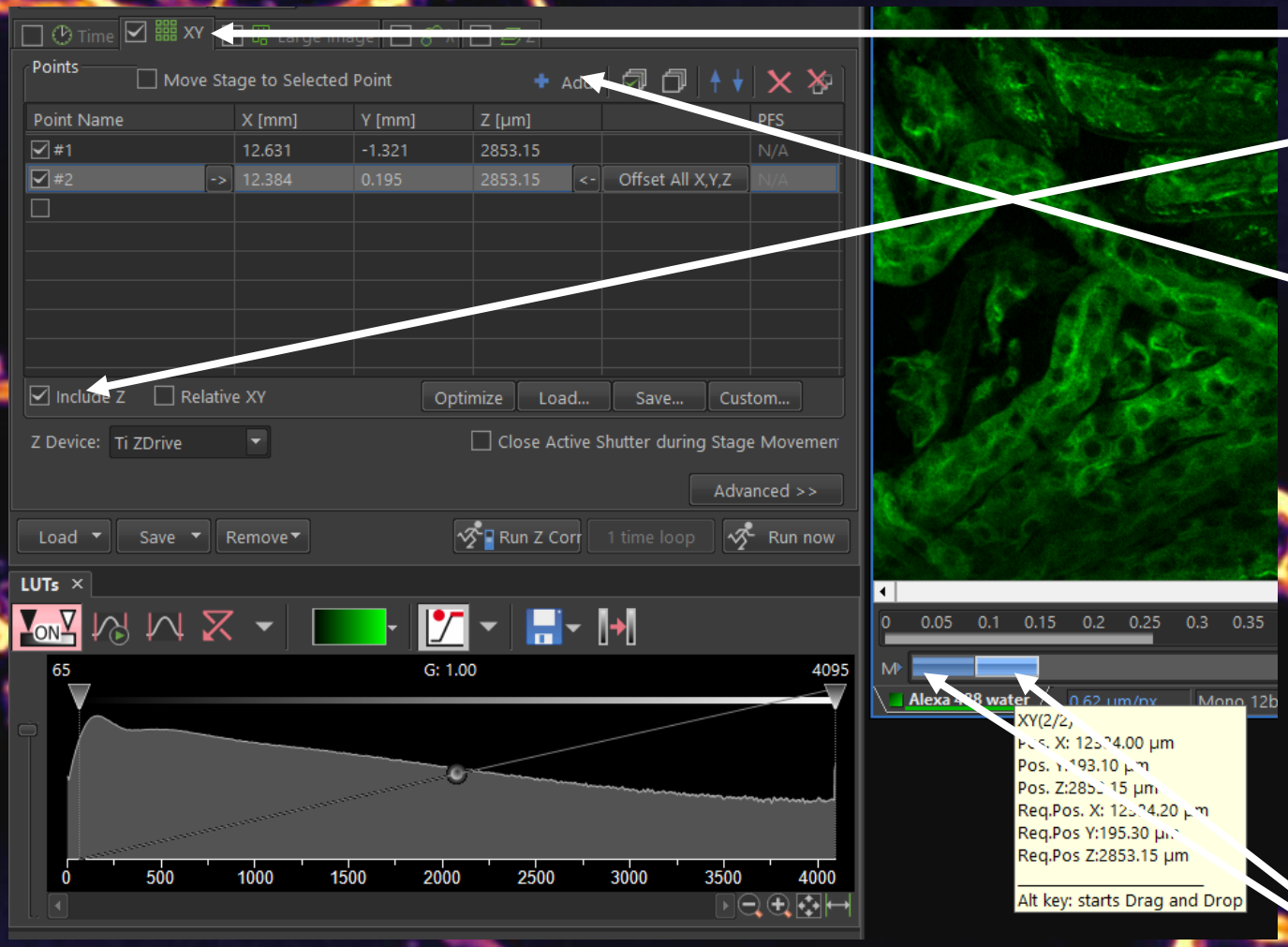

6. Click on this to see your entire stitched up image.

STEP BY STEP INSTRUCTIONS



STEP 13

XY Positions



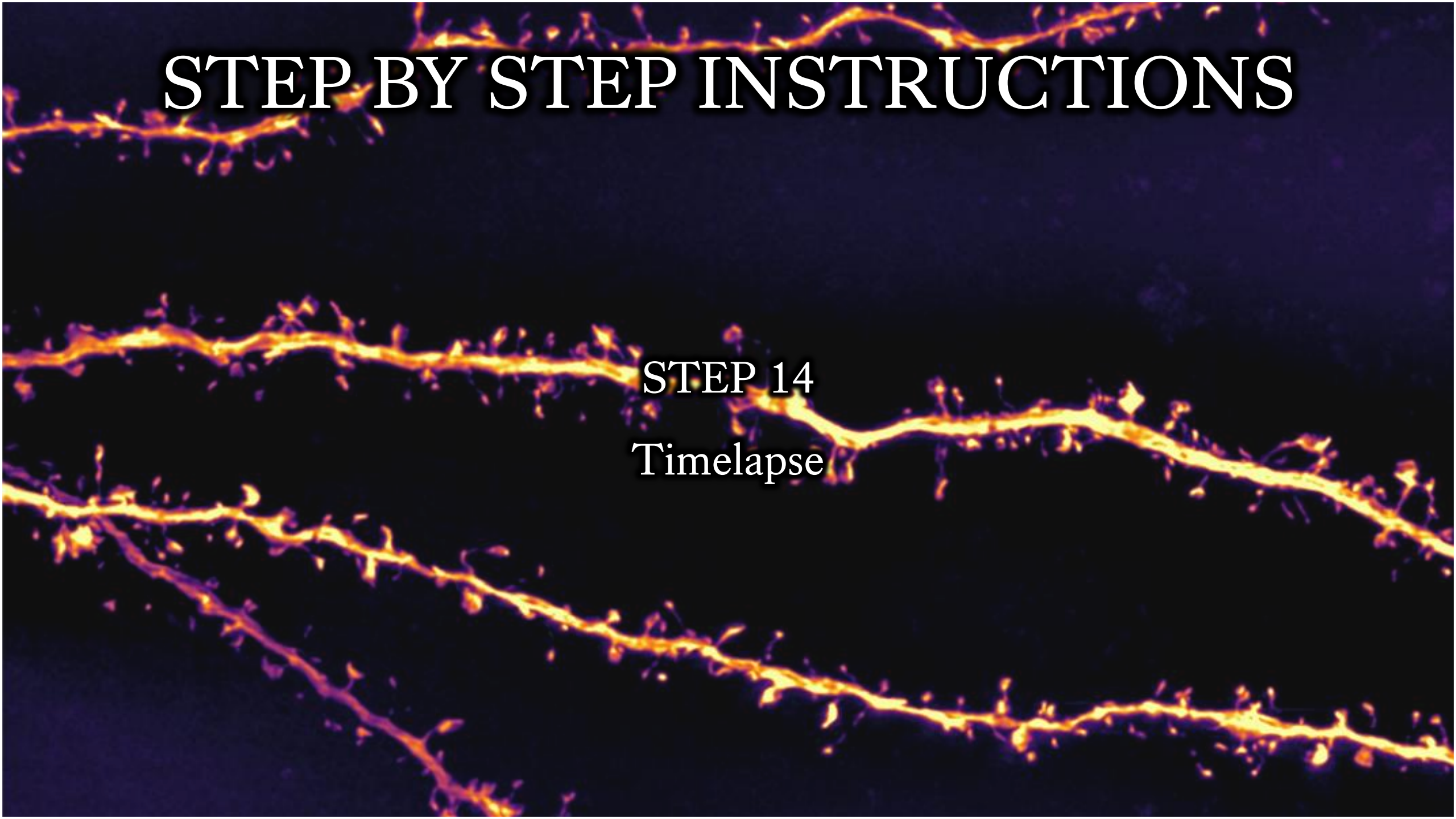
1. Tick on the XY tab and click on ‘Include Z’, also tick on the channels tab.

2. Click on LIVE and focus on your sample, move to a field of view you want to image. Click ‘Add’ to set this XY position.

3. Move to another field of view, be mindful where you are on your coverslip, focus again if you need to and add this position.

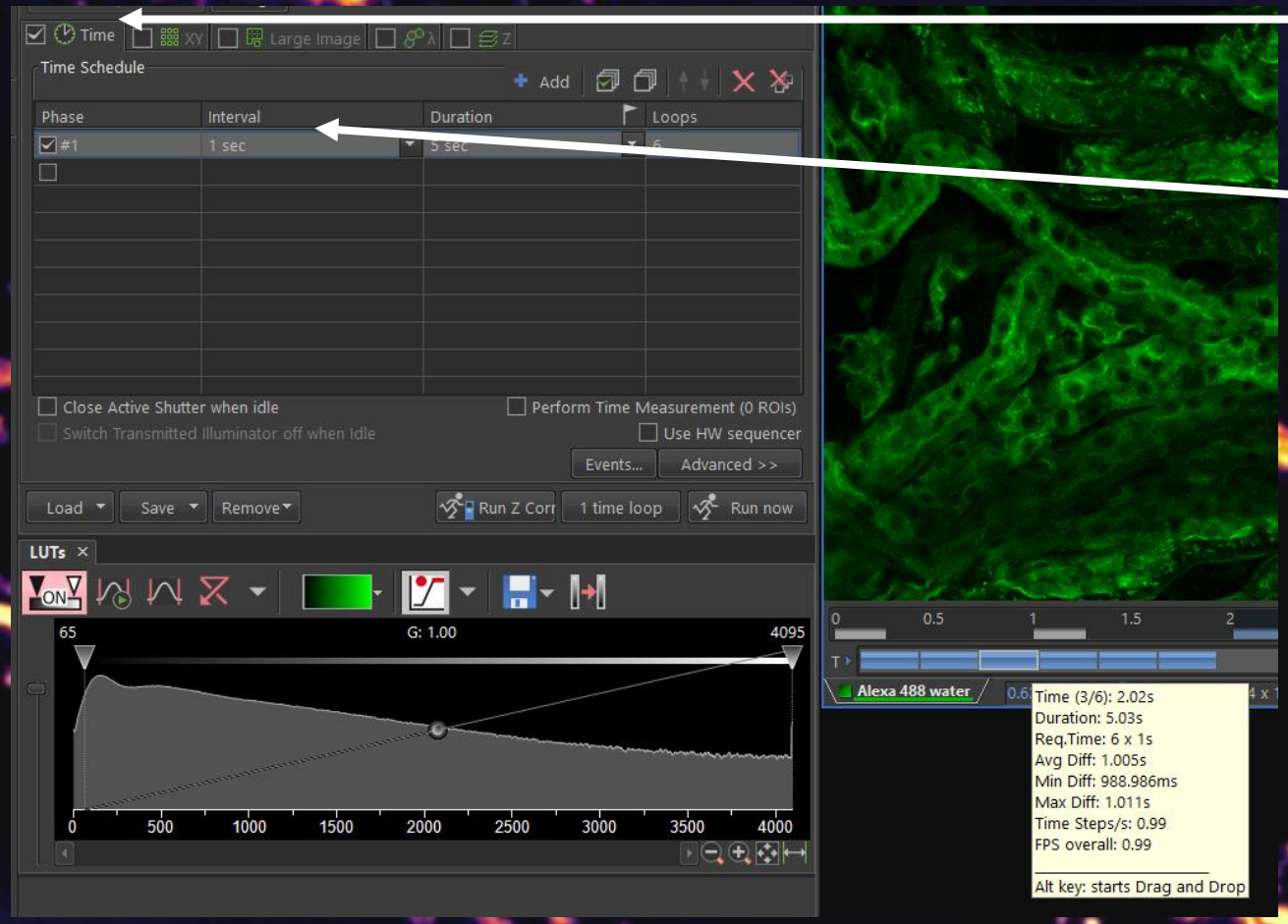
4. Repeat for as many positions as you require on the same coverslip and press ‘Run Now’ to take images at multiple XY positions.

STEP BY STEP INSTRUCTIONS



STEP 14

Timelapse



1. Tick on the Time tab and add a time-lapse, also tick on the channels tab.
2. Interval (how long between each scan)
3. Duration (how long the overall time-lapse)
4. Loops (how many images will be taken)
5. Click on LIVE and focus on your sample, move to a field of view you want to image and click on ‘Run Now’.
6. If you’ve not been trained to use Timelapse or set up live imaging, please let us know and we’ll be happy to help.

STEP BY STEP INSTRUCTIONS

STEP 15

At The End Of Your Session

1. At the end of your session, stop scanning, click on ‘Eyepiece’.

2. LOWER THE LENS to the very bottom!!!

3. Remove your sample and the sample holder.

4. If you’ve only used the dry lens, you do not need to clean anything.

5. If you used the water or oil lenses, then please clean the lens, once with dry lens tissue to wipe as much oil off as you can, then 3 times with fresh lens tissues with ethanol, then once more with dry lens tissue.

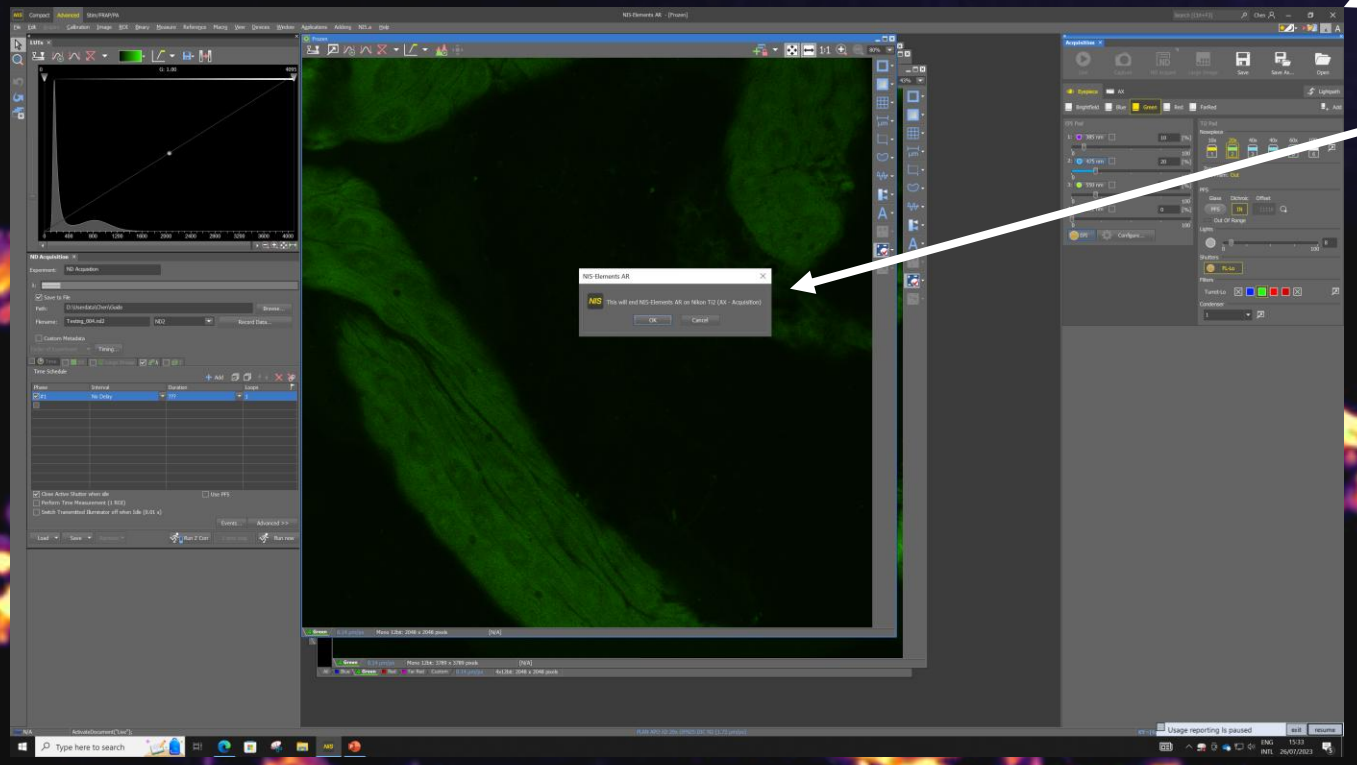
6. Always leave the microscope on 20X lens, lowered to the bottom, for the next user.

7. Please clean up after yourselves. All bins in the WCIC are for PAPER only, if you have any gloves/media/cells/slides/etc. you’d like to throw away, please dispose of it in the wet lab, thank you.

STEP BY STEP INSTRUCTIONS

STEP 16

Close The NIS-Elements Software



1. Close the software from here.

2. Sometimes the software will remind you to save the changes you've made to your images, such as if you added a region of interest or a scale bar, etc. 'SAVE AS' if you do want to save these changes.

3. If you have 'Save To File' set up correctly, your raw images should already have been saved. You can check them in 'file explorer' before closing the software if you'd like.

STEP BY STEP INSTRUCTIONS

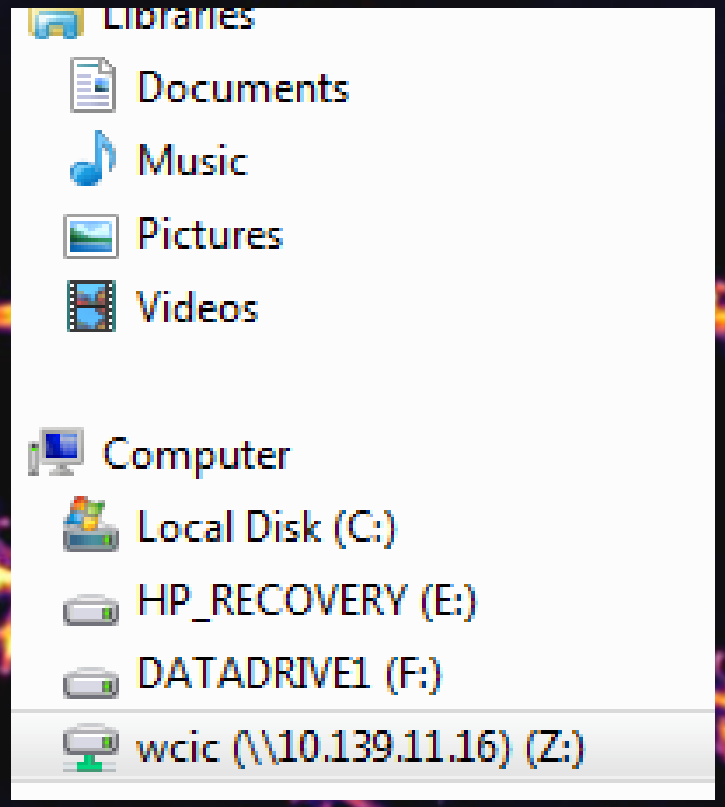
STEP 17

Data Transfer

YOU MAY NOT USE USB DRIVES

ON ANY MICROSCOPE COMPUTER

You can use USBs on the Analysis workstations.



1. Go to File Explorer.
2. Find your data where you saved it in ‘Save To File’ and copy.
3. On the left hand side, click on the WCIC shared drive.
4. Click on ‘Nikon_A1R’ folder.
5. Find your folder within ‘Nikon_A1R’.
6. Paste your data.
7. This shared network is for data transfer only, it is not a backup storage, please connect your own computer to this shared drive (instructions can be found in your PPMS booking system > Documents > Accessing The Network Drive) or book and go to the workstations with a USB, and copy and paste your data into your personal backup storage.

STEP BY STEP INSTRUCTIONS

STEP 18

Check The PPMS Booking System

1. ALWAYS check PPMS booking system at the **END** of your session, to see if anyone if using it after you.

2. If another user is booked on within 2 hours, please leave the microscope on, you just need to make sure your NIS-Elements software is closed, your data transferred and finally, sign out of Windows from 'Start' (bottom left).

3. If no one is booked on within 2 hours, please close the NIS-Elements software if you haven't already, then switch off the computer, then the microscope number 4, then 3, then 2 and finally 1.

4. Before you leave, please help us keep the room clean, use ethanol to clean up any accidental oil and media spills, the bins in the WCIC are for paper waste ONLY, please take any other rubbish with you (i.e. gloves and sample) and dispose of them in the main wet labs.

5. Thank you and enjoy imaging.

Please don’t email us if you need help during your session.
If you need urgent (in session) help, please contact us on WhatsApp or Using Teams:

George Chennell 07435 737 423
Chen Liang 07469 356 576
Josh Kellet: 07553 416 467
Or use Teams Instant Message

Happy Imaging!