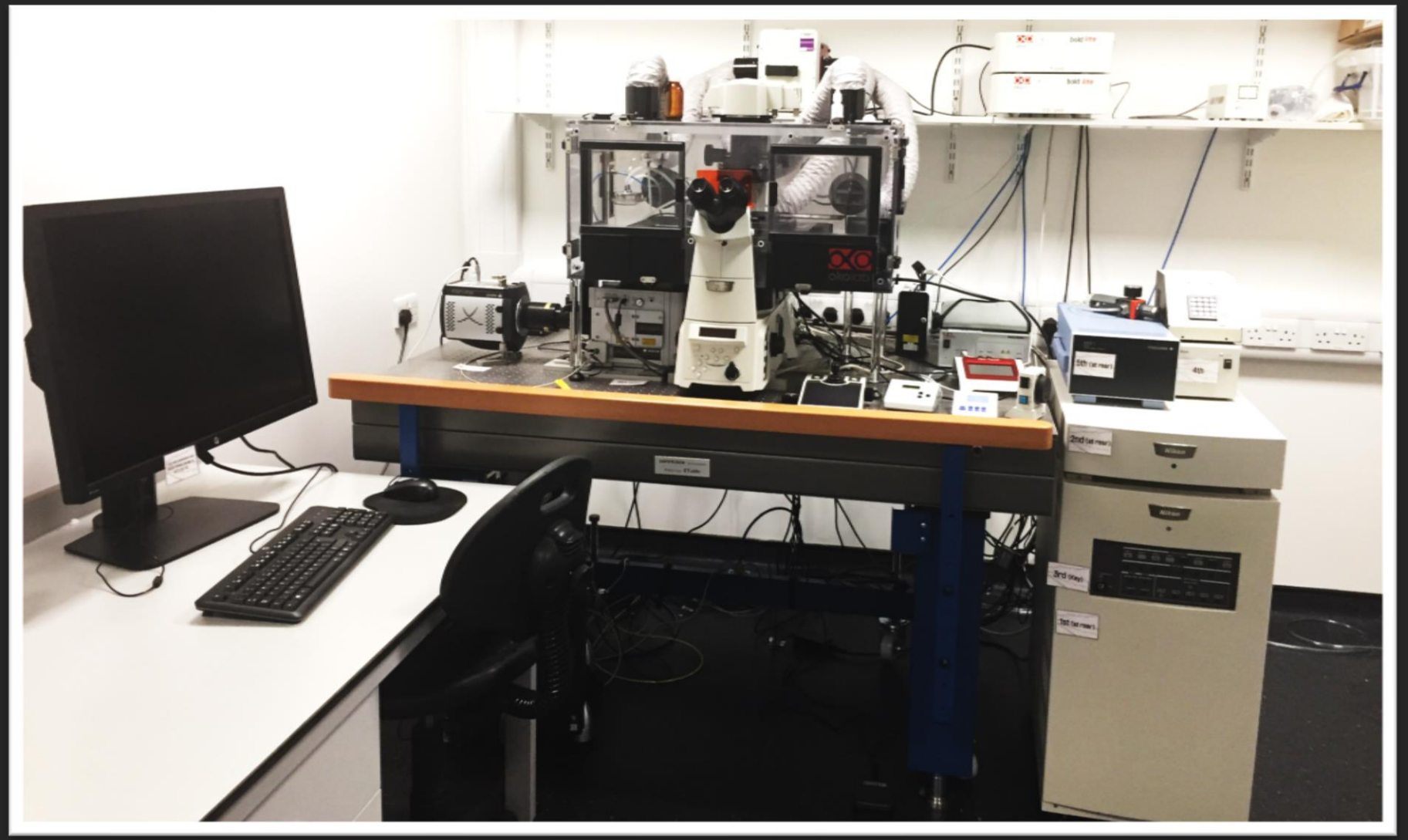
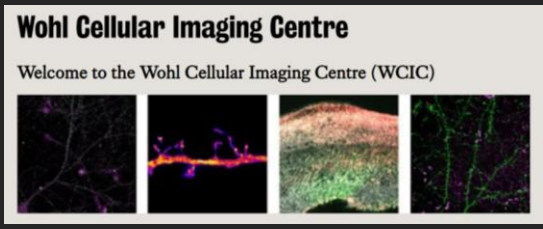
# Nikon Spinning Disk Confocal Microscope






KING'S College LONDON

# Nikon Spinning Disk Confocal Microscope

**Contents:**

# Booking and Fees (current to date November 2018)

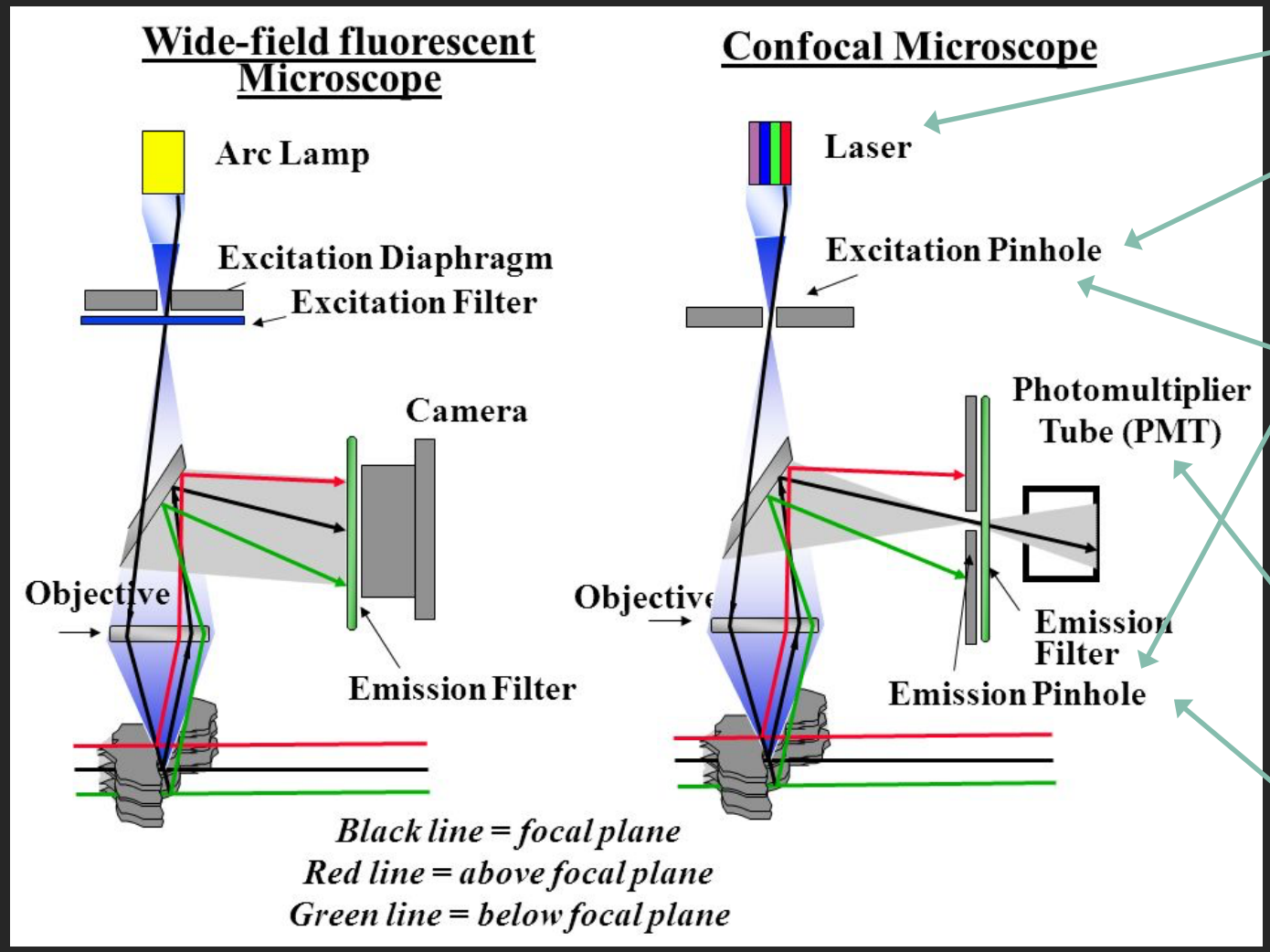
| EQUIPMENT | KCL staff (per hour) | EXTERNAL (per hour) |
|---|---|---|
| A1R Confocal | £25 | £62 |
| i-SIM Super resolution | £18 | £44.50 |
| Inverted Spinning Disk Confocal | £16 | £47 |
| A1R Multiphoton | £29 | £72 |
| Bio-Station IMQ | £6 | £15 |
| Ti-E Live Imaging Systems | £11 | £27 |
| Opera Phenix HSC | £25 | £62 |
| Airy Light sheet microscope single-photon system | £15 | £45 |
| Airy Light sheet microscope multi-photon system | £15 | £45 |

**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 lens installation training (optional)
- Access to booking system: http://ppms.eu/kcl-wohl

# Basic Principles Of A Confocal Microscope

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




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

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

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

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

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

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

# Spinning Disk Confocal

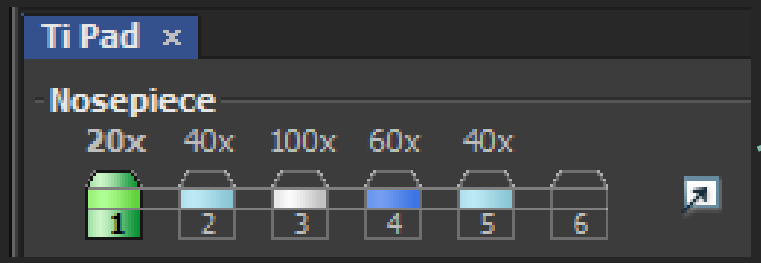
This multiple pinholes system uses very sensitive camera detectors for high resolution image capture.

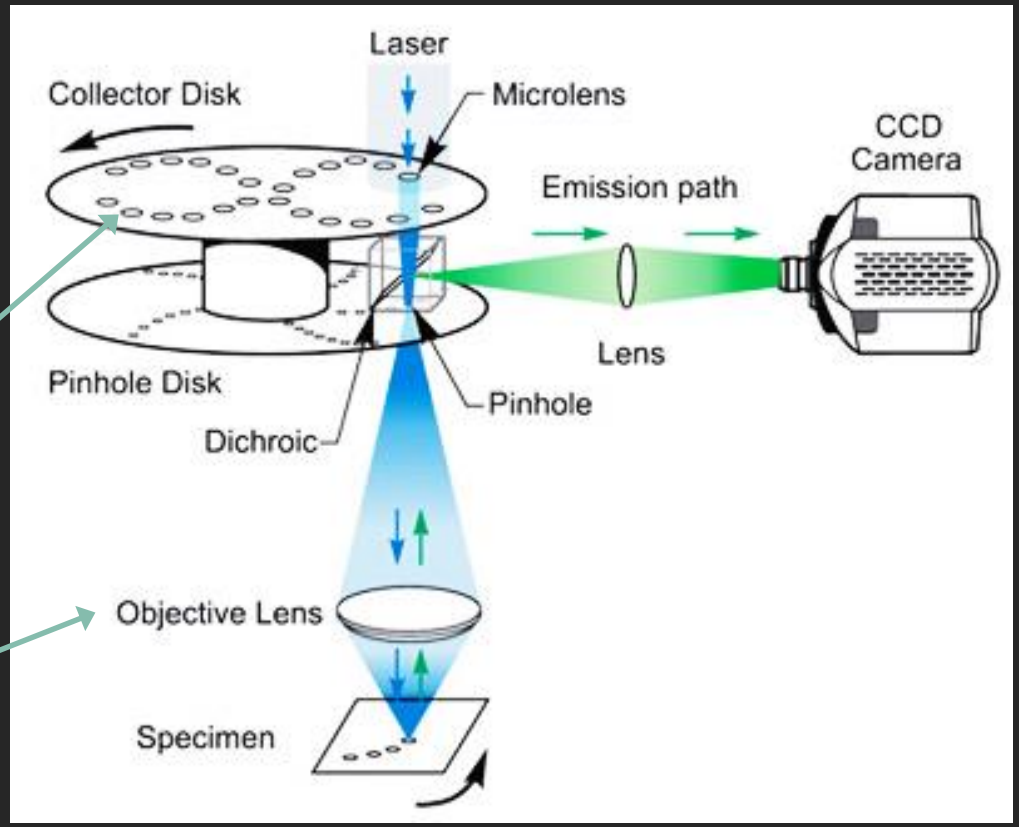
This system is fast and very gentle on samples, highly recommended for live imaging.

## 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.



# System On

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

Switch on the Spinning Disk System by following the numbered switches.

Numbered labels are normally at the same horizontal level as switches at the side or back.

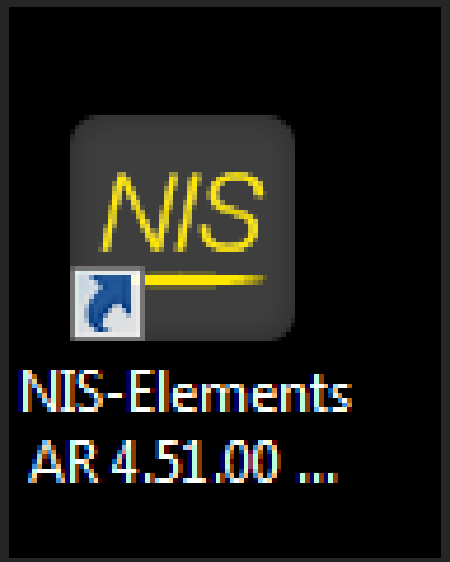
Make sure the stage is empty before turning on the Microscope switch at the right, far back.

Computer / Windows login: Scientist

PPMS Login (username is normally the first part of your KCL e-mail address)

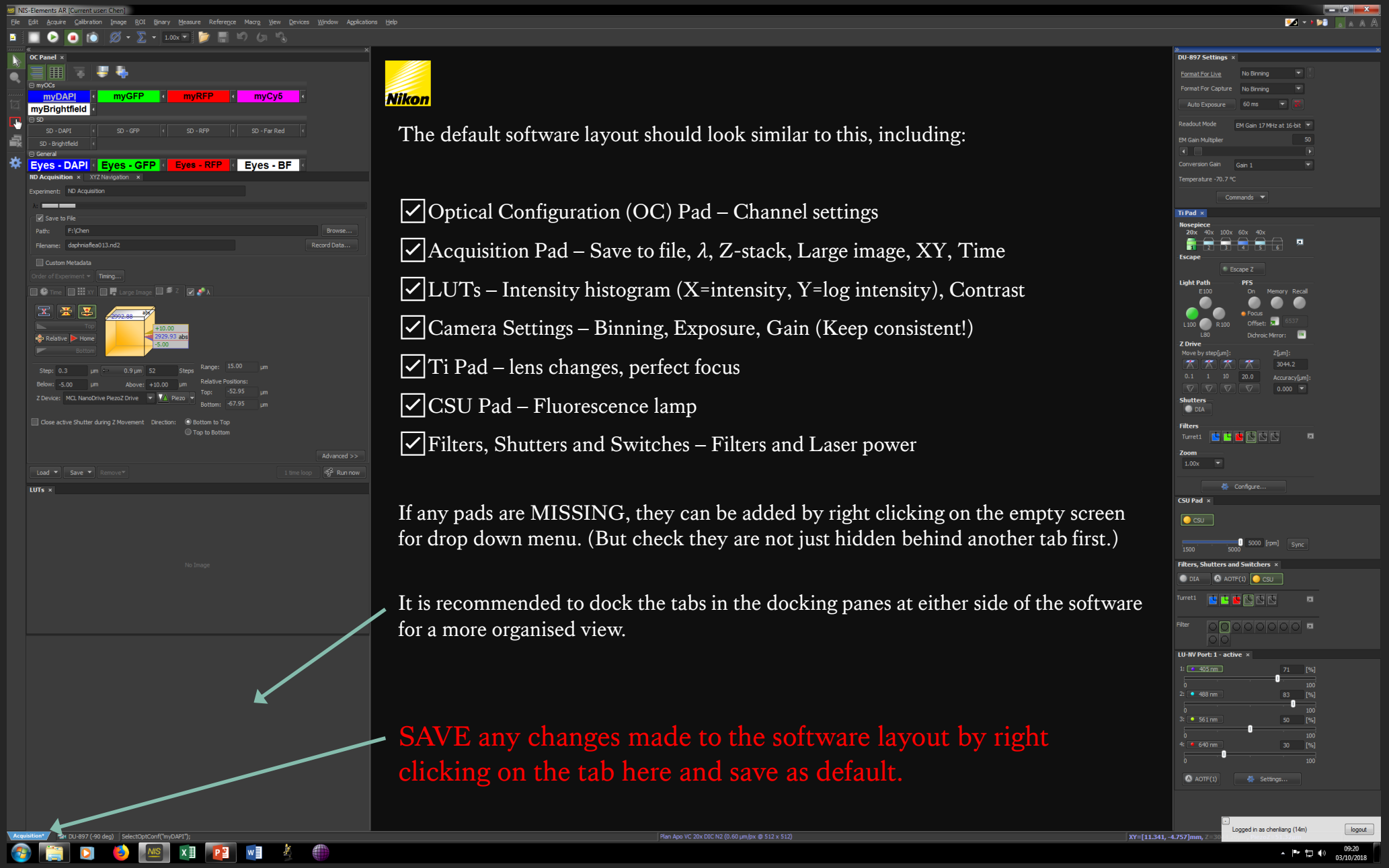
**ALWAYS**
**Login to NIS-Elements Software**
**Before loading any sample**

The software start-up checks each system for connection errors




Desktop Icon

# Software Set Up



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

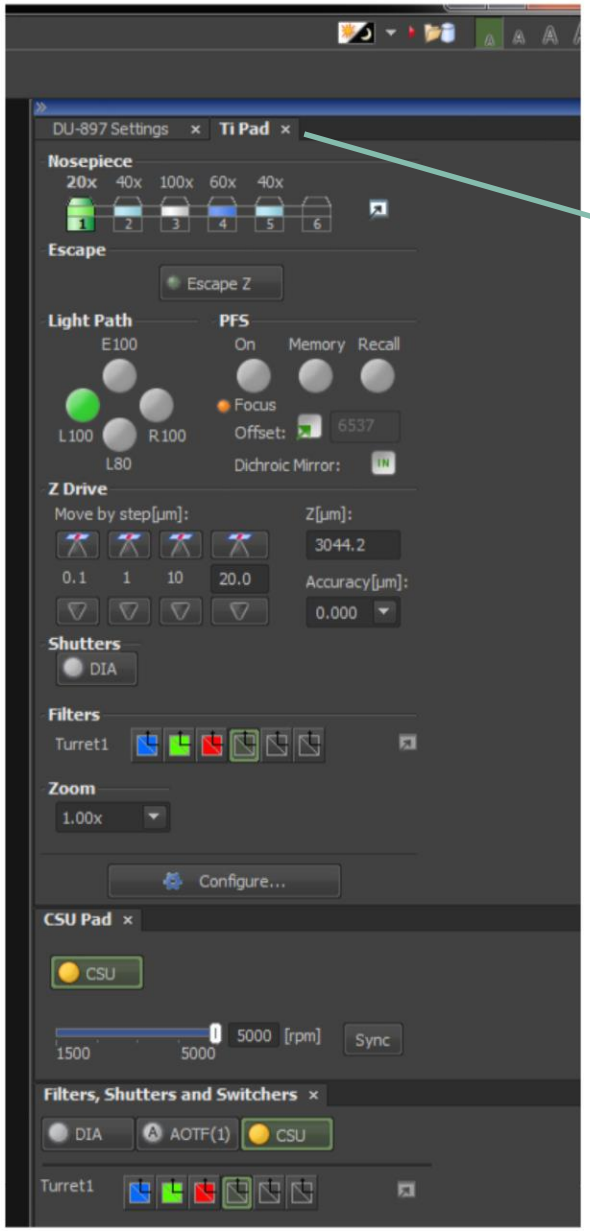
- ☑ Optical Configuration (OC) Pad – Channel settings
- ☑ Acquisition Pad – Save to file, λ, Z-stack, Large image, XY, Time
- ☑ LUTs – Intensity histogram (X=intensity, Y=log intensity), Contrast
- ☑ Camera Settings – Binning, Exposure, Gain (Keep consistent!)
- ☑ Ti Pad – lens changes, perfect focus
- ☑ 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.

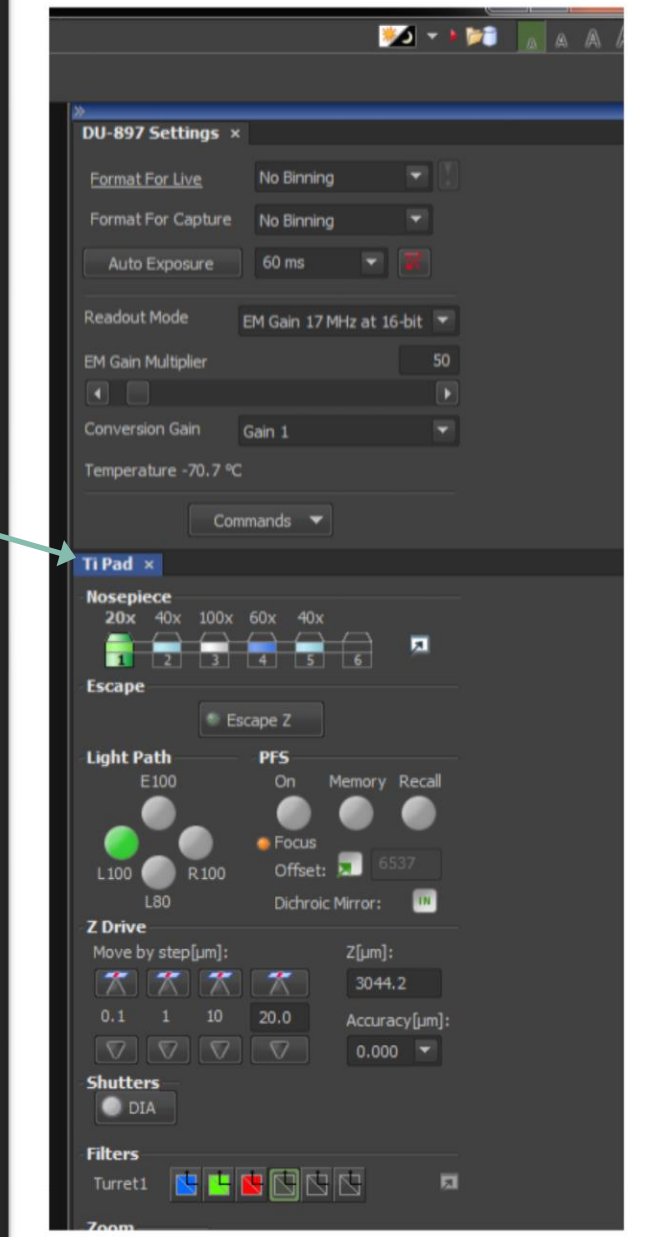
SAVE any changes made to the software layout by right clicking on the tab here and save as default.

# Software Hidden Panels

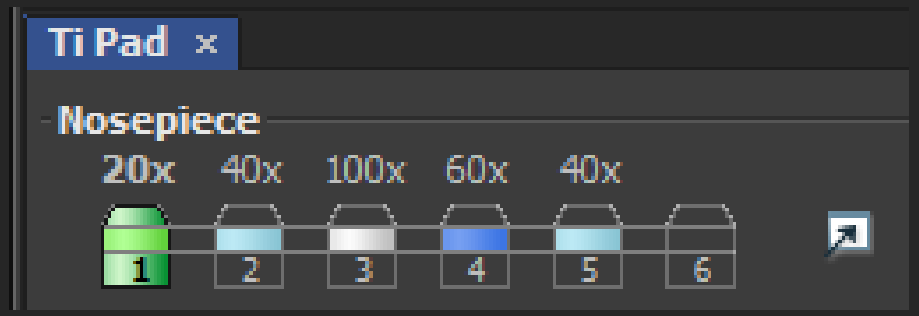



Mouse left click hold, drag and drop

to rearrange docking pane.



# Changing & Cleaning Lens




Cleaning oil lens:

- Check the lens for any damage
- Wipe away any excess oil with dry lens tissue (normally after your session)
- Wrap lens tissue around your finger and soak up some Isopropanol and clean lens from centre outwards (REPEAT 3 TIMES)
- 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 before switching.


**ALWAYS**

**Lower the lenses as far as they can go before clicking on another lens.**

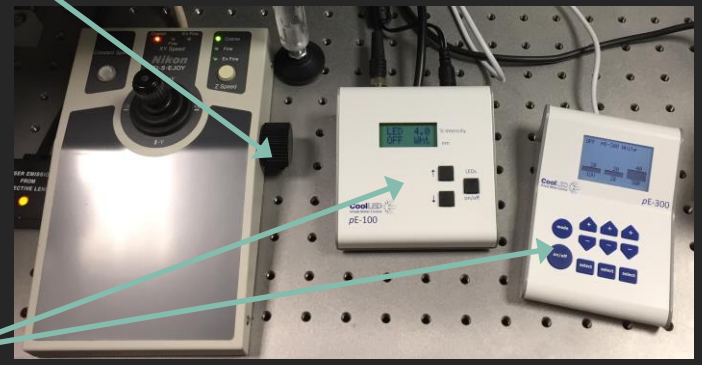
- **Rotate wheel clock-wise**
- **Bottom = this number no longer reduces**
- **Be careful - zero doesn't mean it's at the bottom**


Between changing slides, you need to wheel the lenses slightly down.

# 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.

# 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

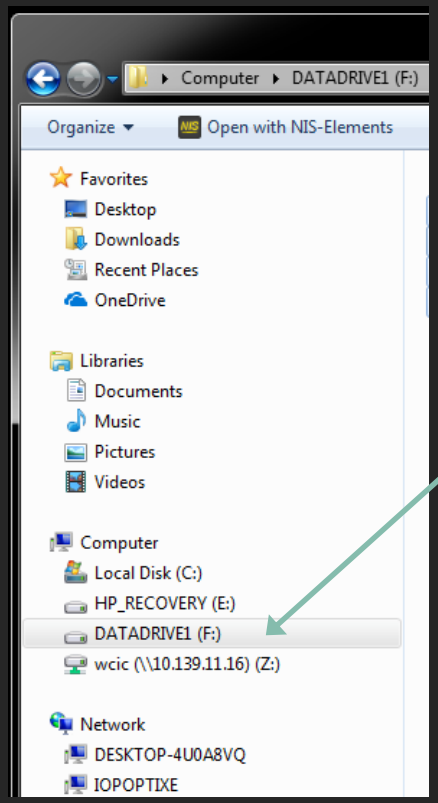
Show intensity in a histogram format

Contrast manipulation

Saturation indicator options

Laser Power

# Set Up File Path (SAVE)

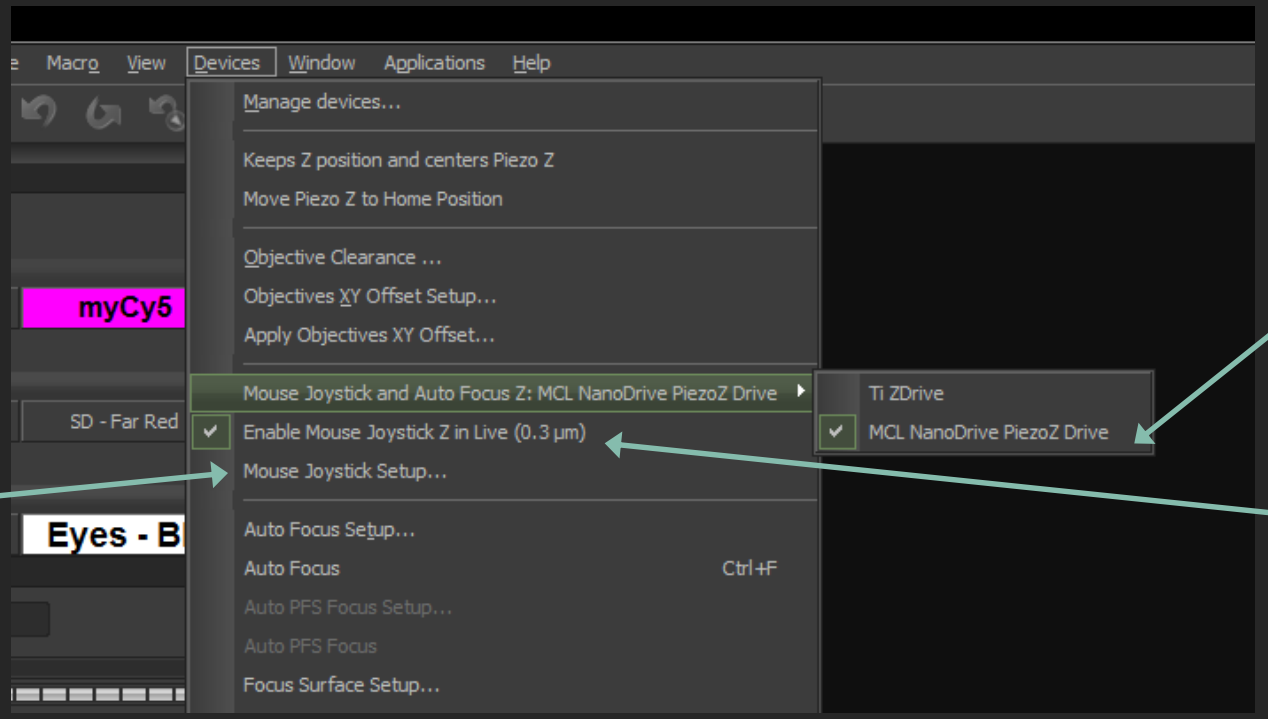

1) Check Save to file option
2) Go to Browse and select DATADRIVE1 (F:)
3) Create/find your folder, set up new folder for this session if needed.
4) Recommended file name: Experiment_Name_Date_001
5) Finished, anytime you press ”Run now” a new file will be automatically saved in the nd2 format.

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.

# Use Mouse Wheel To Focus And Move In Live Mode

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

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




Select Piezo Drive to increase precision.

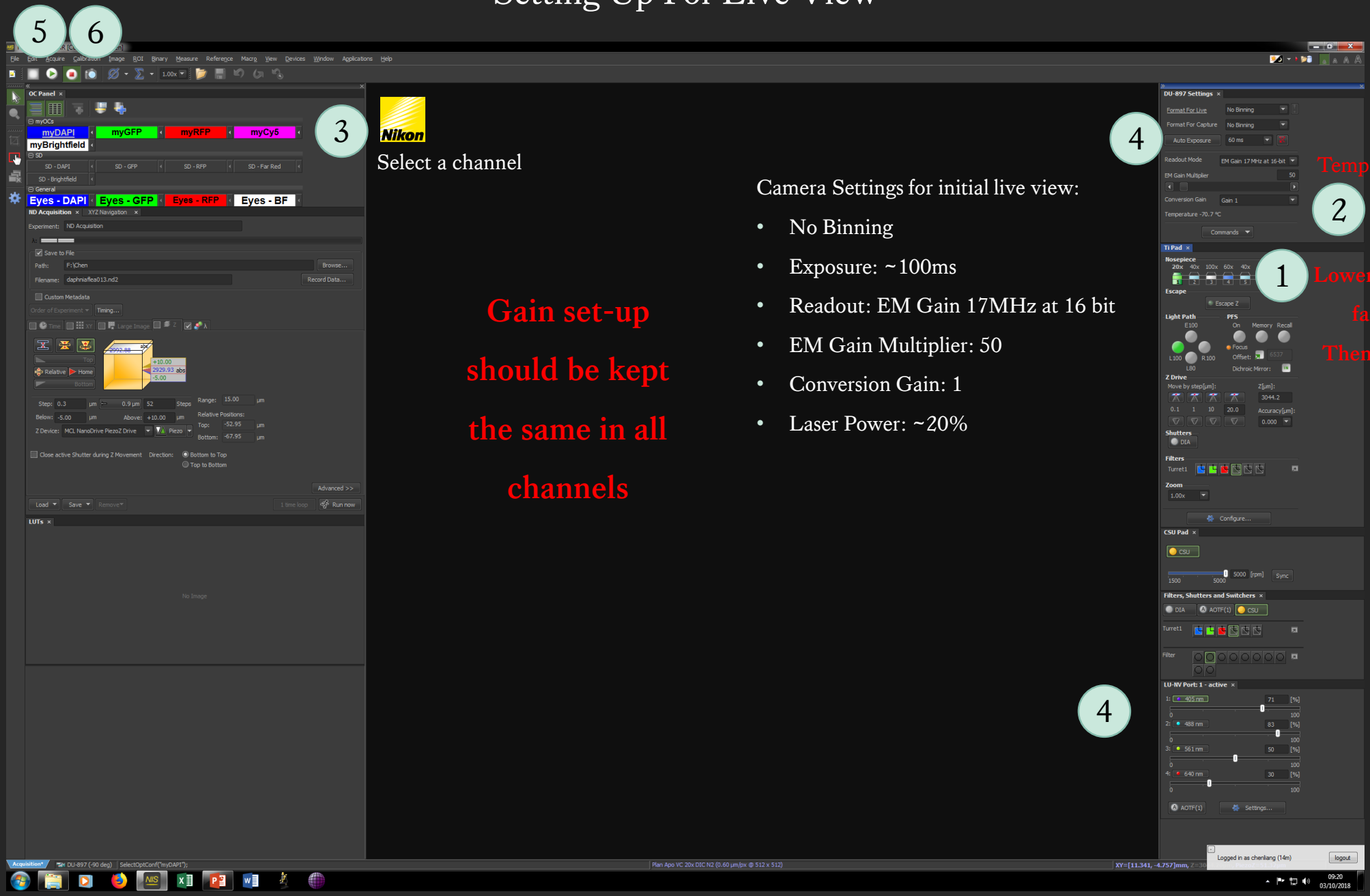
Options for extra fine Z control.

Precise XY movement in live mode.

Right click for move object to centre option.

# Setting Up For Live View

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




Select a channel

Gain set-up should be kept the same in all channels

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%

Temperature at -70°C

Lower the objectives as far as it can go!

Then...select the lens

# Troubleshooting: No Live Image?

**After you've tried these six points there is still no live view, then restart the software.**

**If the restart doesn't solve this issue come and find:**

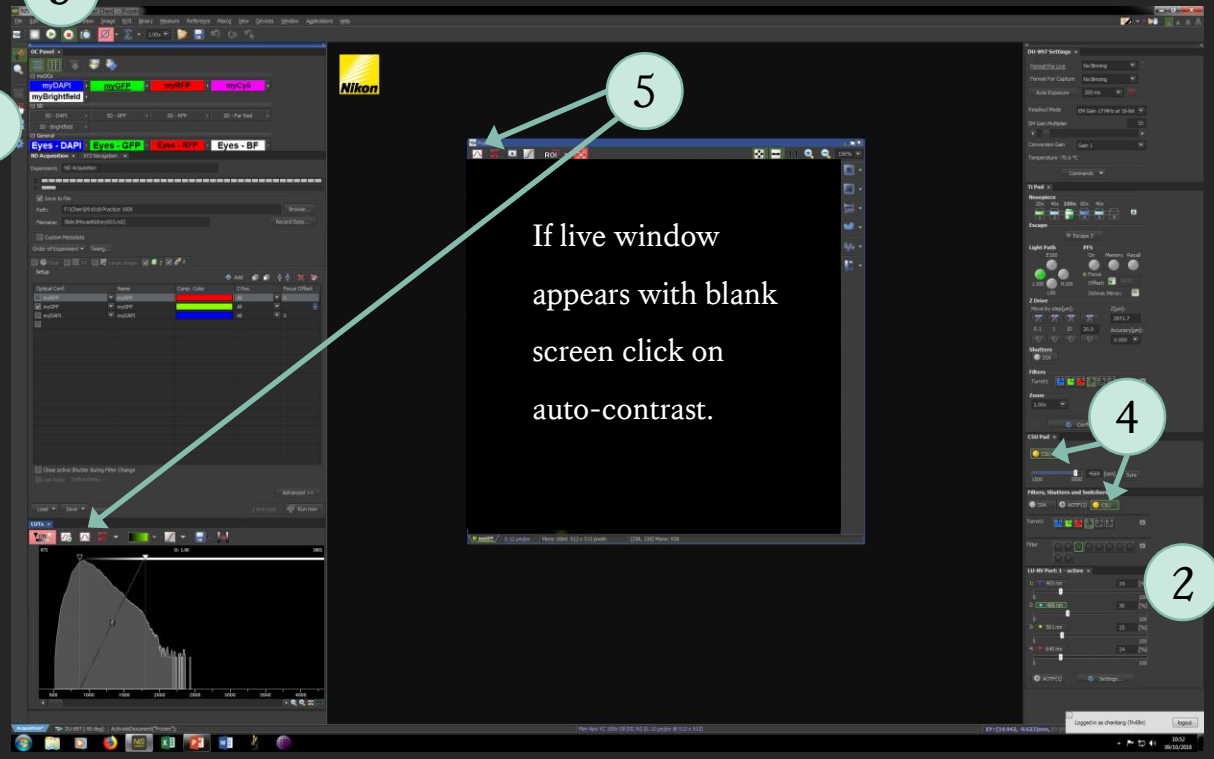
**George Chennell (07771926760) or Chen Liang (07883166321)**

Try clicking live again

3

Are you still on Eyes? Select a camera channel.

1




6

If you do see colour or blurred image on the screen then you might need to optimise your exposure time, gain and laser power. Or try and focus using your mouse wheel in live mode.

Make sure your shutter is open

2

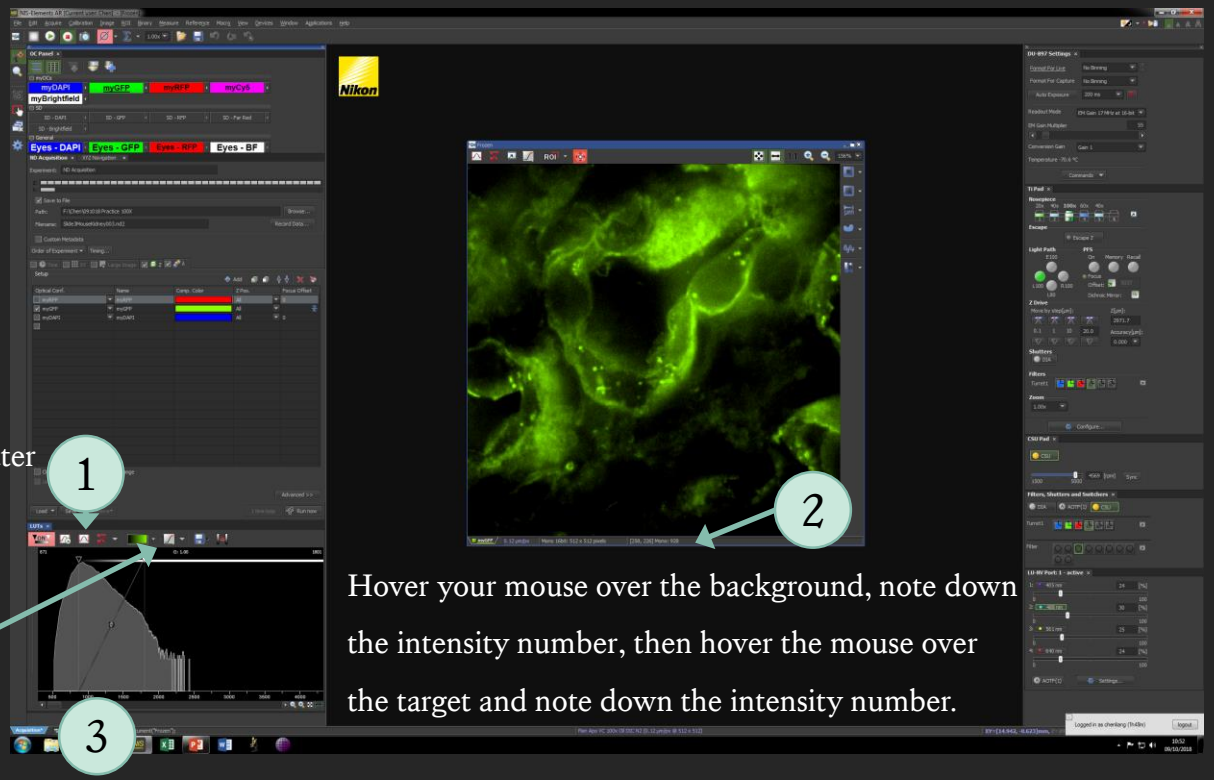
After moving from "Eyes" to "MyOCs"
Check if laser power is turned on or if its at zero!

A system safety measure delays turning lasers on after this change, to solve this issue, click on another "MyOCs" channel tab and then go back to the channel you wanted to view. The laser power slide bar should now be coloured in up to the % bar.

# 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 Spinning disk almost never reaches saturation threshold, there is no need for saturation checks.

Hover your mouse over the background, note down the intensity number, then hover the mouse over the target and note down the intensity number.

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)

Should NOT go over 50,000 !!!

Remember to keep Gain the same across all channels.

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

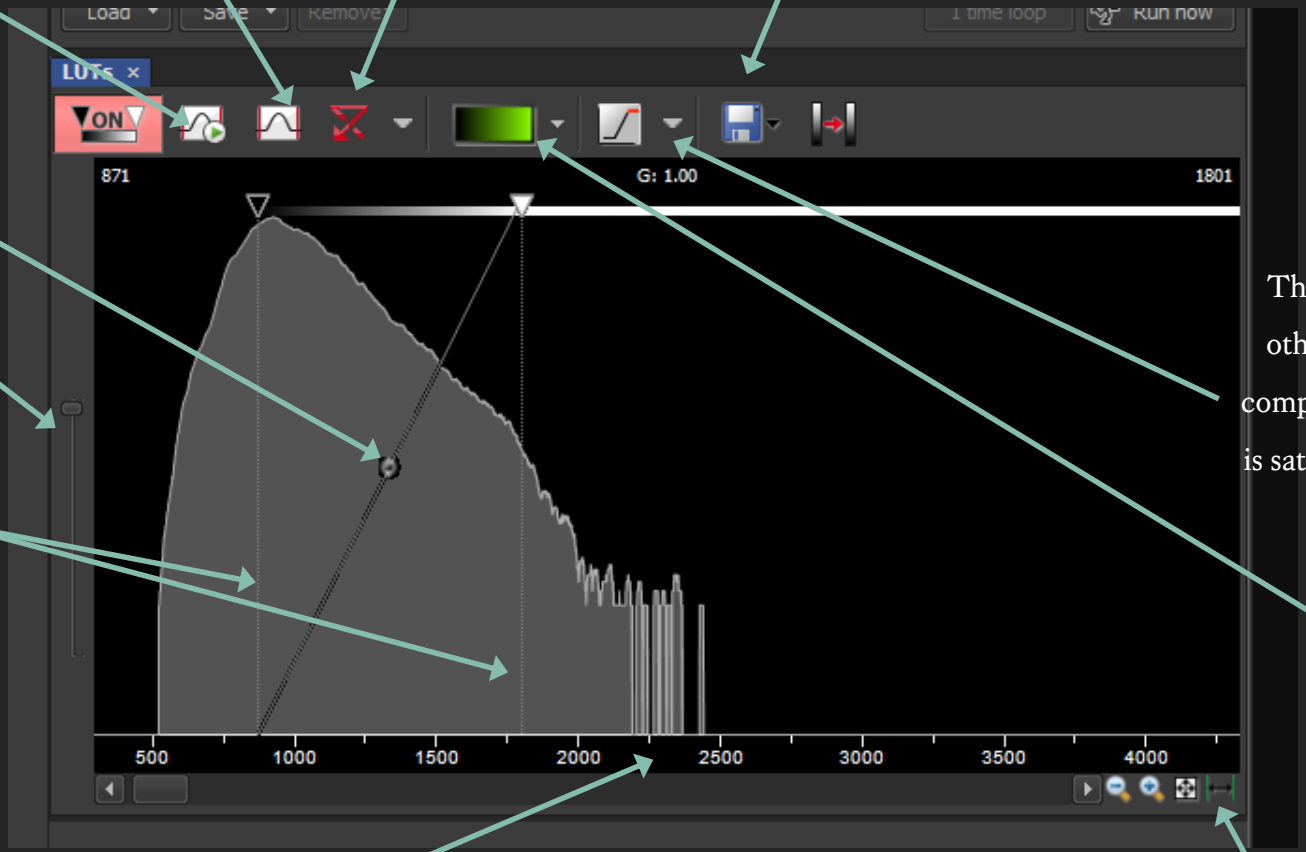
# LUTs in more detail…

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.

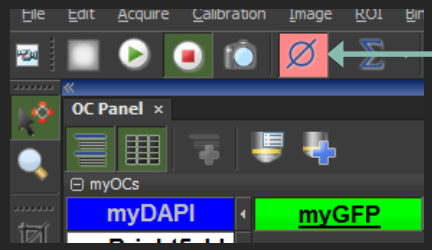
The camera is a black and white camera. You can assign any colour combination to your captured image.

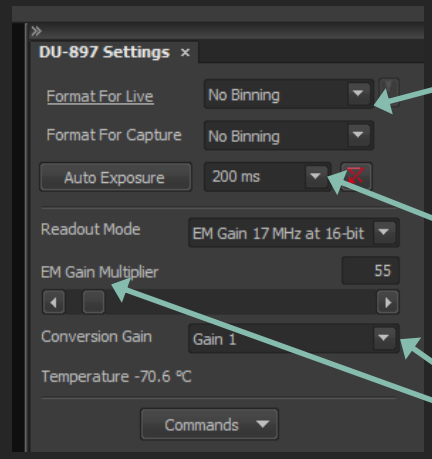
Drag and change the threshold of this histogram for better visualisation.

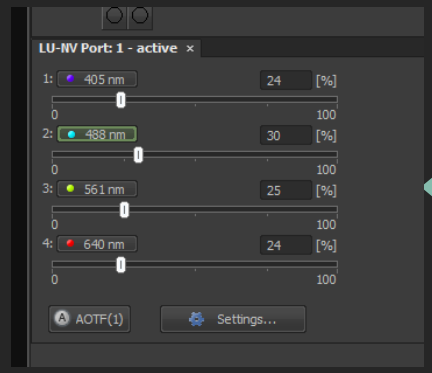
X axis - relative to #-bit camera (e.g. 16-bit CCD camera gives 65,536 different intensity values, of which you should not go over 50,000). Y axis – log intensity scale

Fit the histogram to this space

# Camera Settings – What Does It Do?

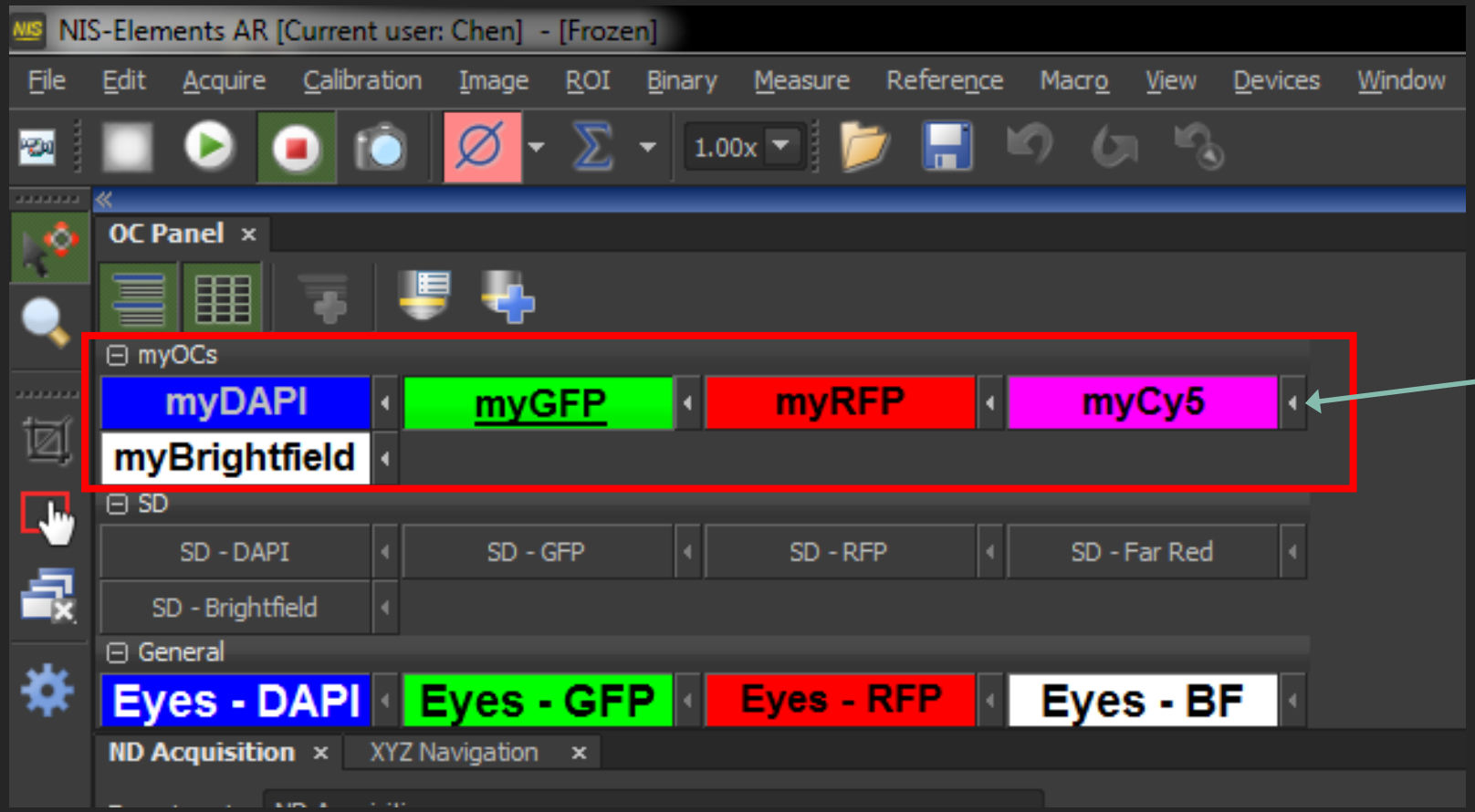



| Camera Settings | Concept | Pro | Con | When To Change This... |
|---|---|---|---|---|
| Averaging | The camera takes multiple images and form an averaged image. | • Reduce noise | • Increase acquisition time drastically | • Increase when your image have lots of noise. |
| Binning | Combines the charges (signal) from adjacent pixels to form one “super” pixel. | • Faster read out<br>• 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<br>• Bleaching<br>• Fade<br>• Acquisition time | • Increase when signal captured is not enough to give you the intensity level you need.<br>• 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<br>• Need to find a compromise across all channels | • DO NOT GO OVER 300<br>• Increase when signal is low and you have sensitive samples.<br>• 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<br>• Increase signal | • Bleaching<br>• Heating<br>• Harmful to sample | • Increase when you have thick samples or need more signal.<br>• Decrease if you have sensitive sample, especially live samples. |

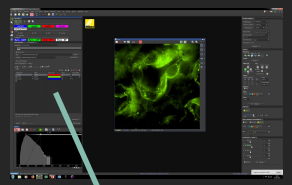
# Save Your Camera Settings



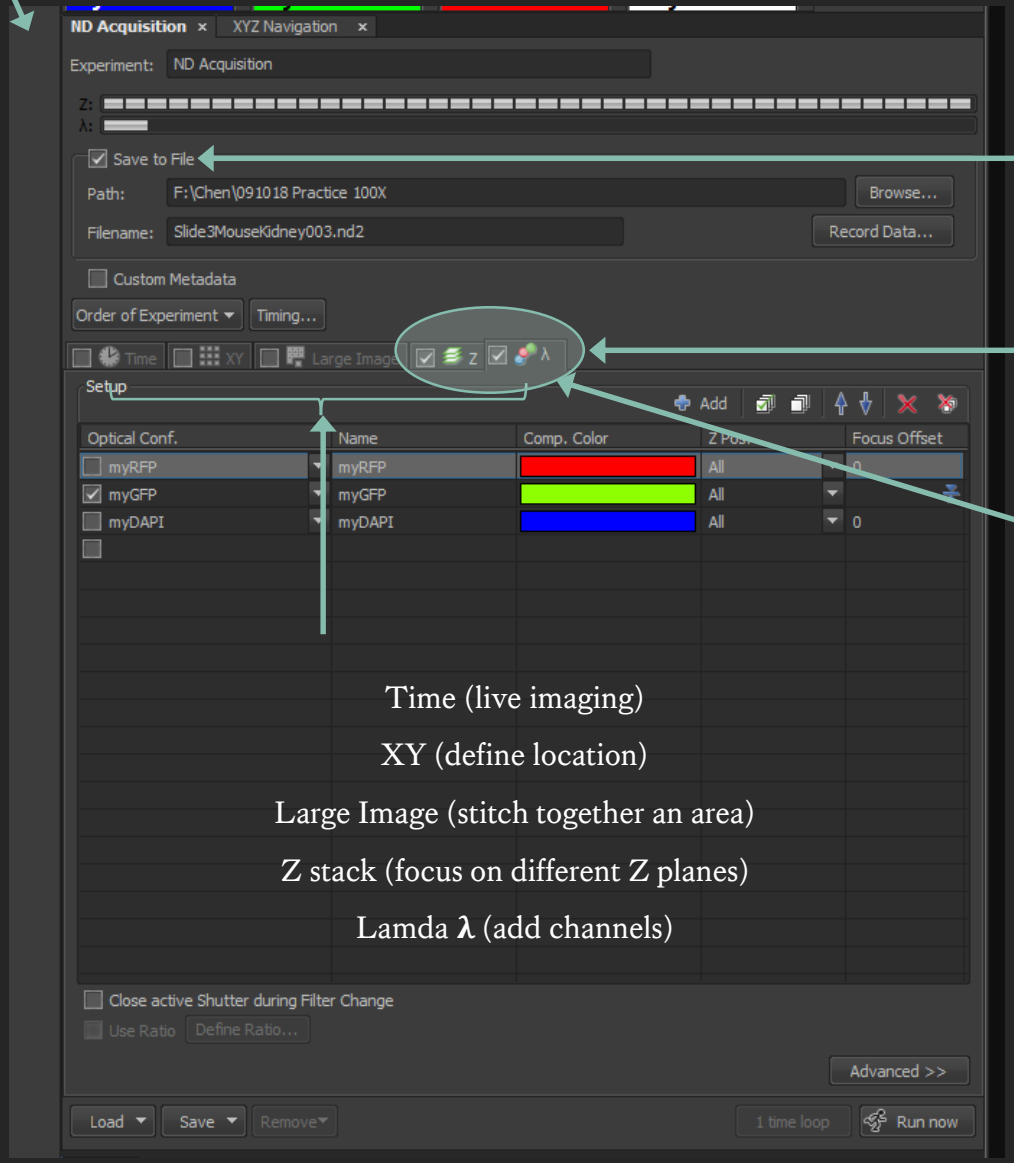

After adjusting the camera settings and average, you can save your settings by clicking on the small arrow button beside the channel tabs.

You can only save in "MyOCs"

# 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)

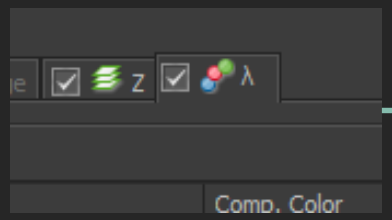
Z stack (focus on different Z planes)

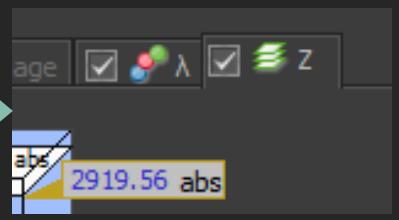
Lamda **λ** (add channels)

**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.

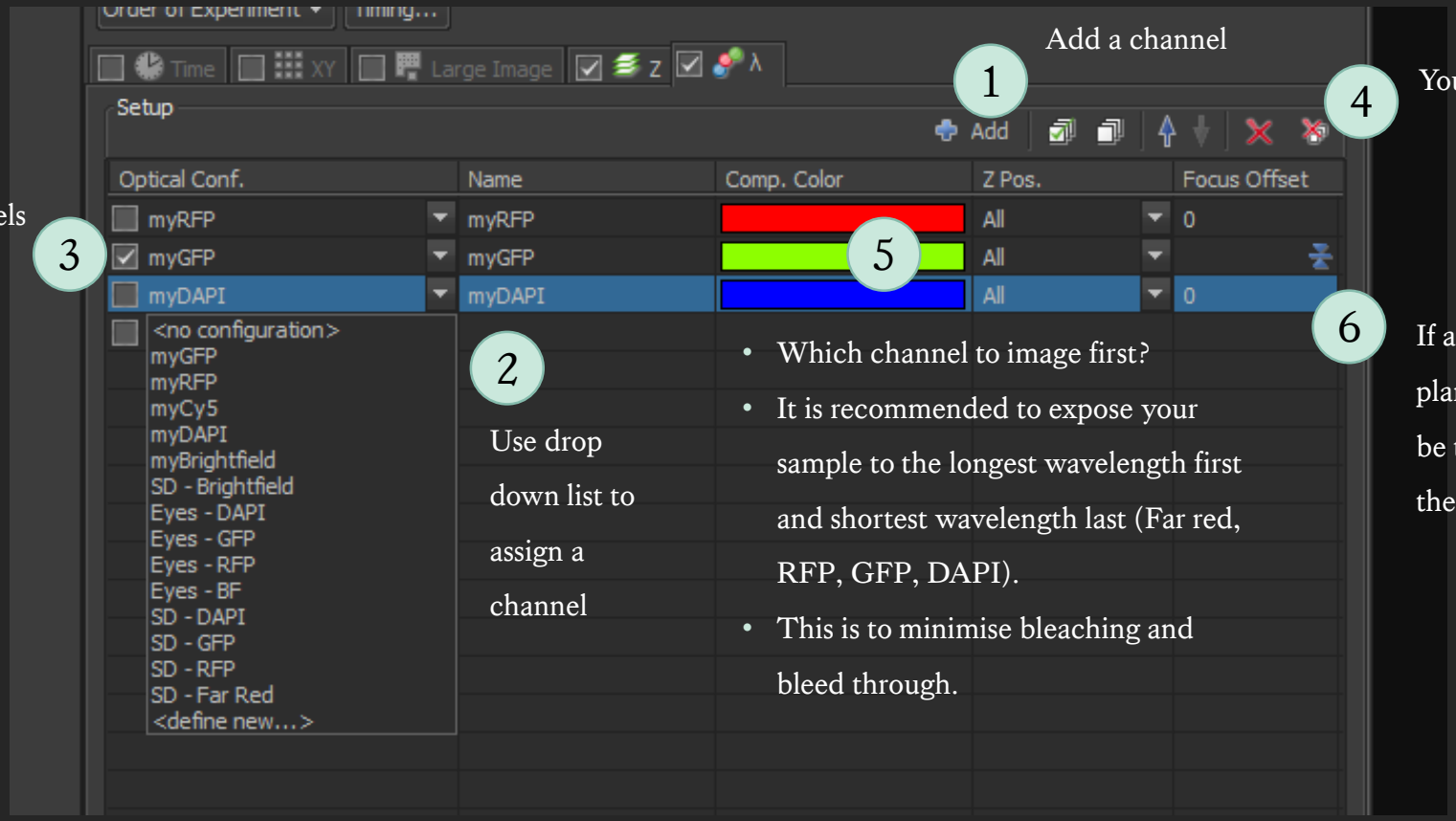
To change the order of acquisition just drag and drop the tabs.



# 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.

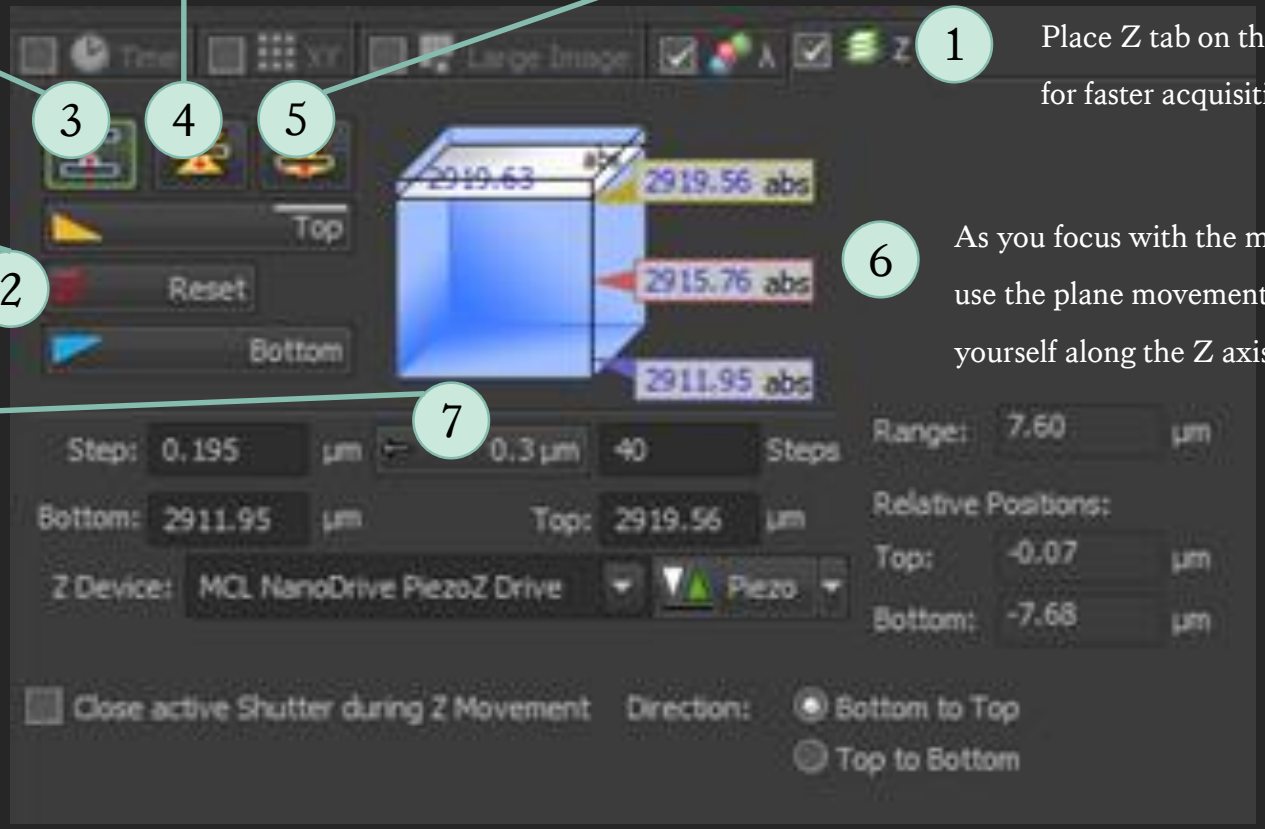
If a channel is out of focus on the Z plane compared to other channels (can be tested using beads) then offsetting the Z focus is an option.

# Acquisition panel
# Set Z-Stack Range And Step Size

**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)




Place Z tab on the right for faster acquisition

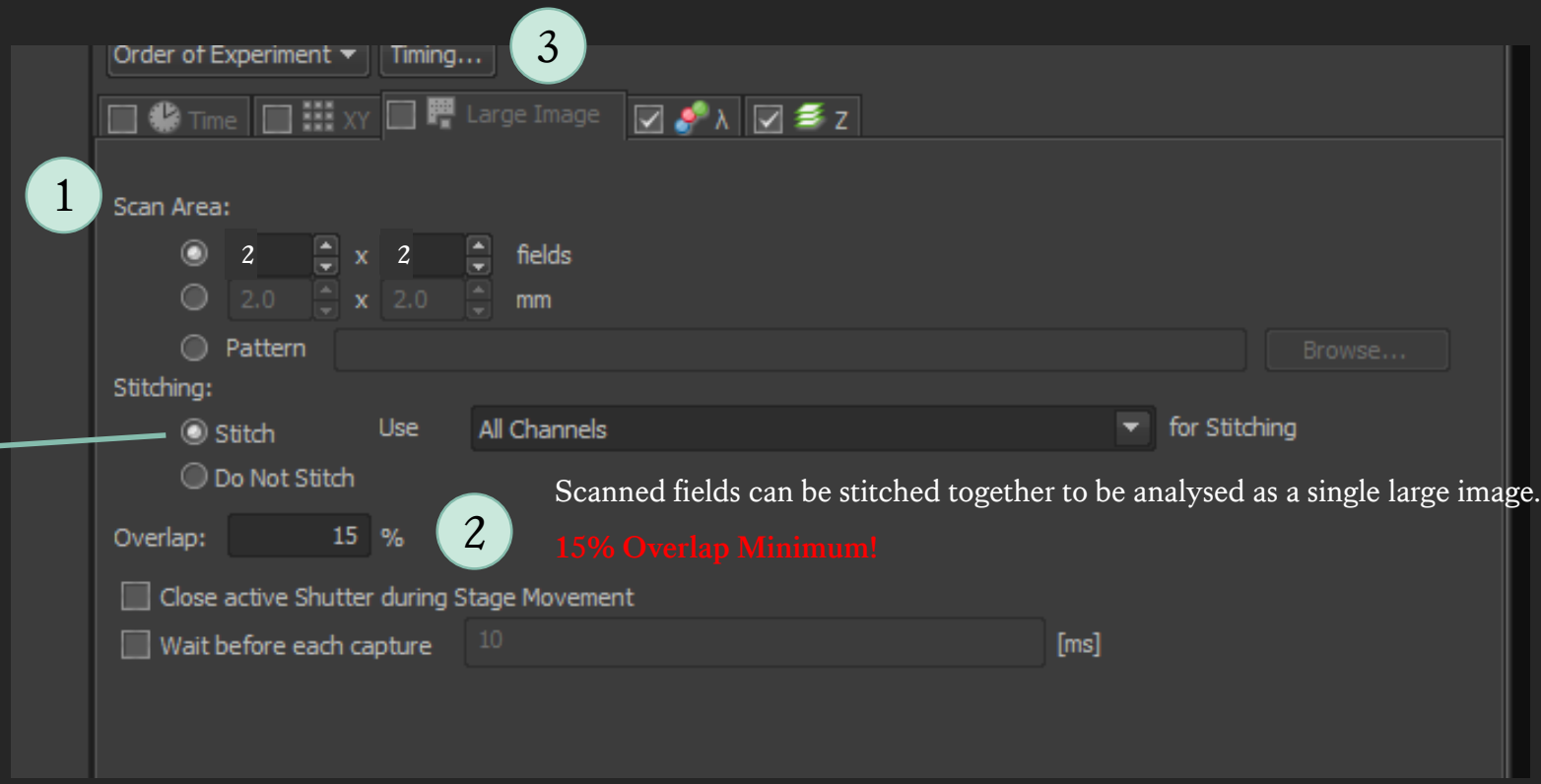
Recommended: RESET the Z positions

As you focus with the mouse wheel, use the plane movement to orientate yourself along the Z axis.

- 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 which is required for 3D deconvolution.

# Acquisition panel
# Set Large Image

**Never put Large Image on the far right of these panels (this well drastically increase your acquisition time)**

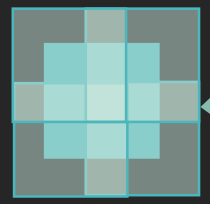


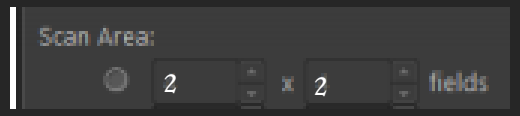

Scanned fields can be stitched together to be analysed as a single large image.

15% Overlap Minimum!

Large image scan area will surround the middle of your field of view.

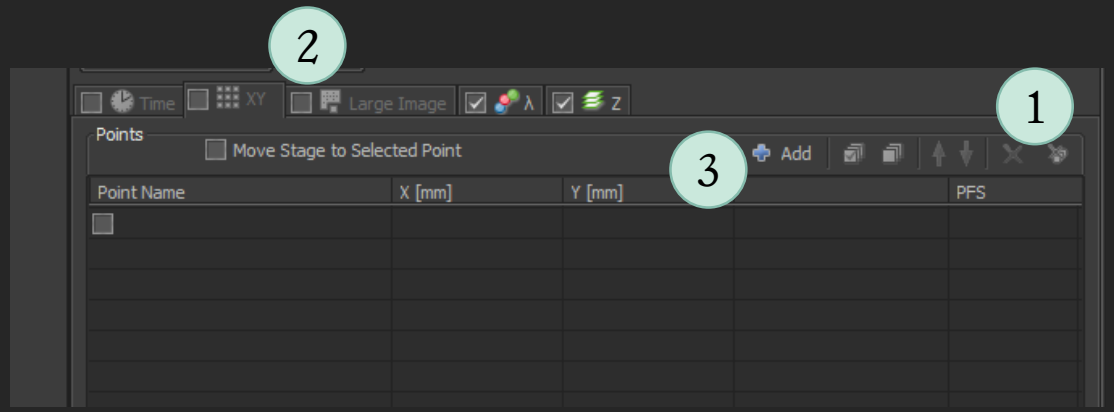
E.g.





# Acquisition panel
# XY Set Position

**ALWAYS UNCHECK the XY position tab unless in use.**



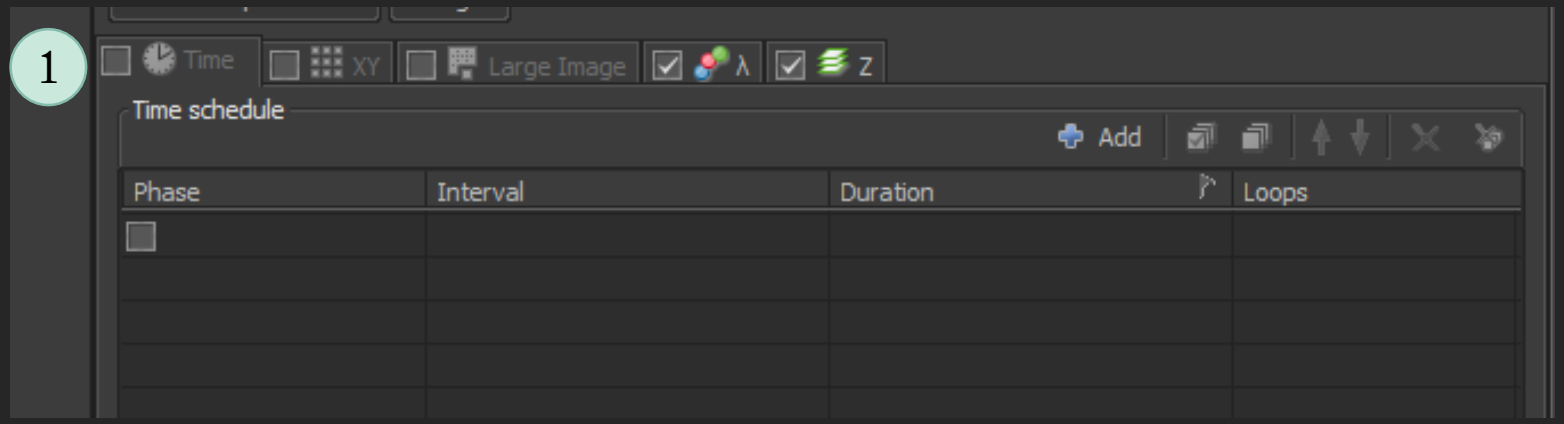

**ALWAYS DELETE XY positions from previous sessions!!! (If you saved positions while using a different stage, moving to the same position by accident is very likely to break the lens!)**

Once you found an area you'd like to come back to, just click on Add.

XY position can be used to save the coordinates of a target of interest.

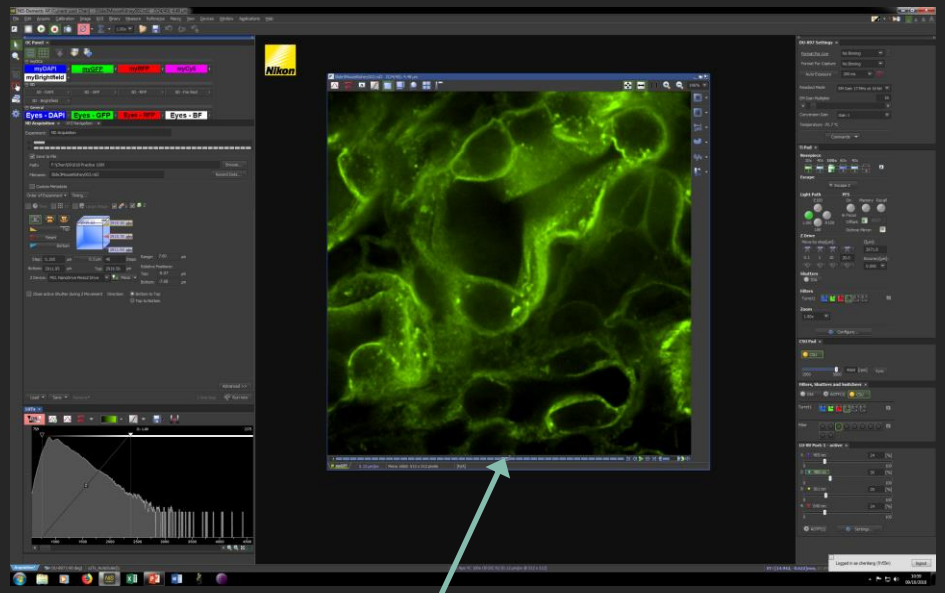
If you need to rapidly take images of two targets not in close proximity.

# Acquisition panel
# Time




Set interval time and duration for your time-lapse and the loops will be automatically calculated.

# View Modes

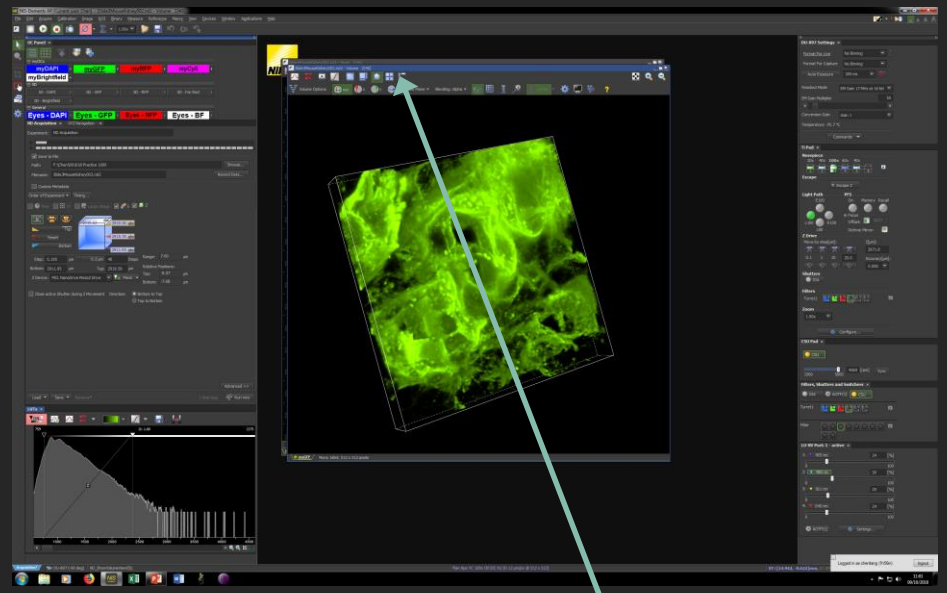


This is the standard view after a z-stack acquisition.

You can move through the Z plane by dragging the bar.


From left to right:

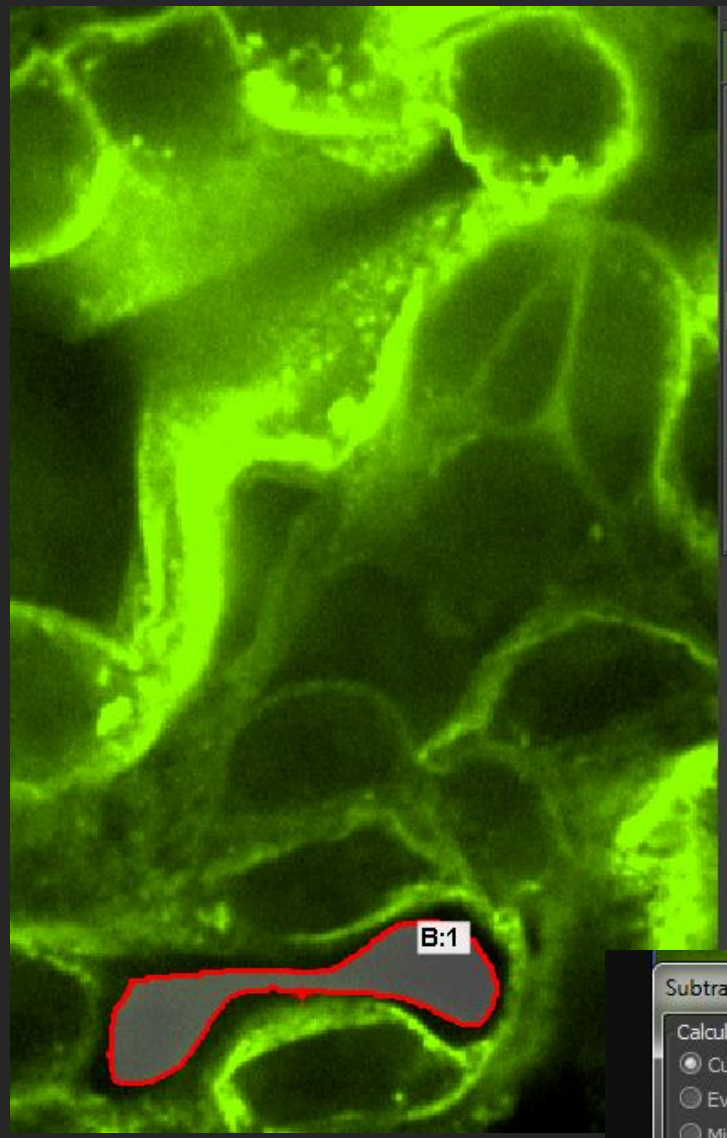
- Standard view
- Side view
- 3D rendering
- Tile view
- Max Intensity Projection flattens the 3D stack into a 2D image, a snapshot of which you can obtain using this view mode.

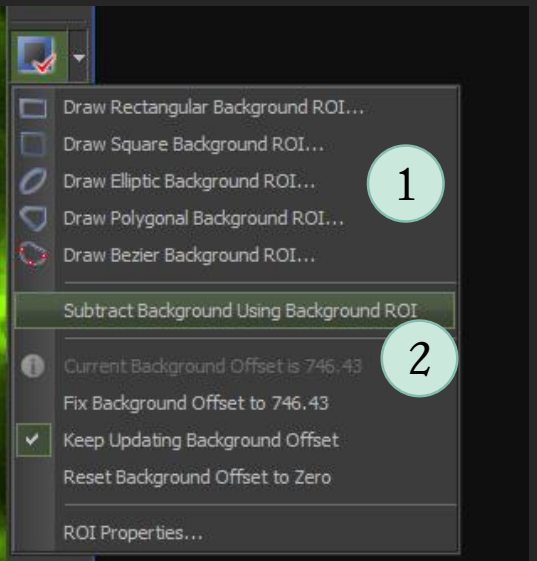


Different view modes can be selected using the options here.

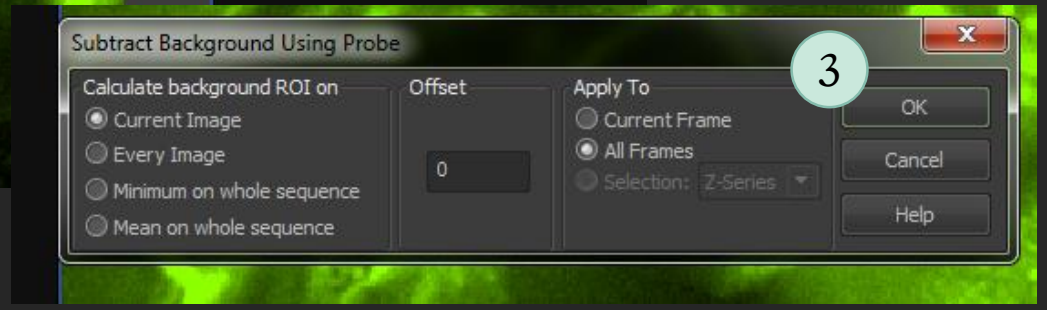
Left click to open view mode in new window, or you can click and drag a view mode into an existing window.

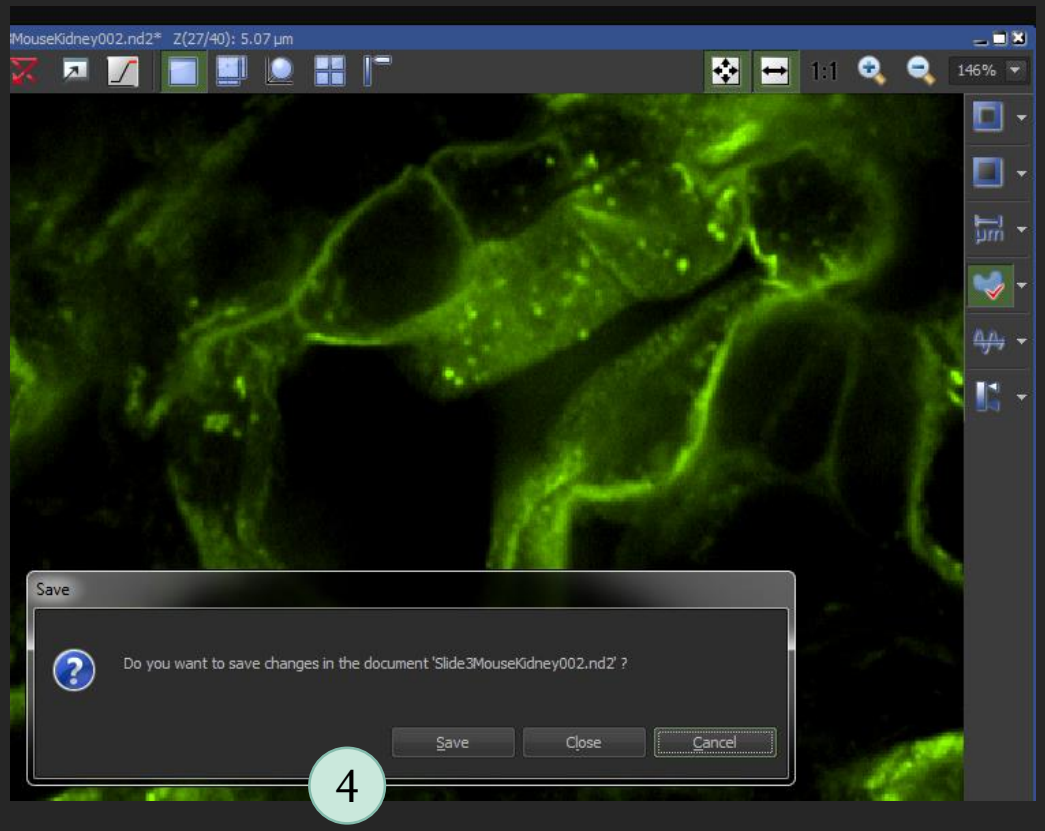
# Removing Background (Changes to ROIs can be implemented at workstations/FIJI after your session)




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.

# At The End Of The Session…

Remove sample, clean lens, select “Brightfield Eyes”

Save all data on local drive.

Move data from local drive to shared network drive

Close down software

Check the booking system

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

YES → Leave system on.

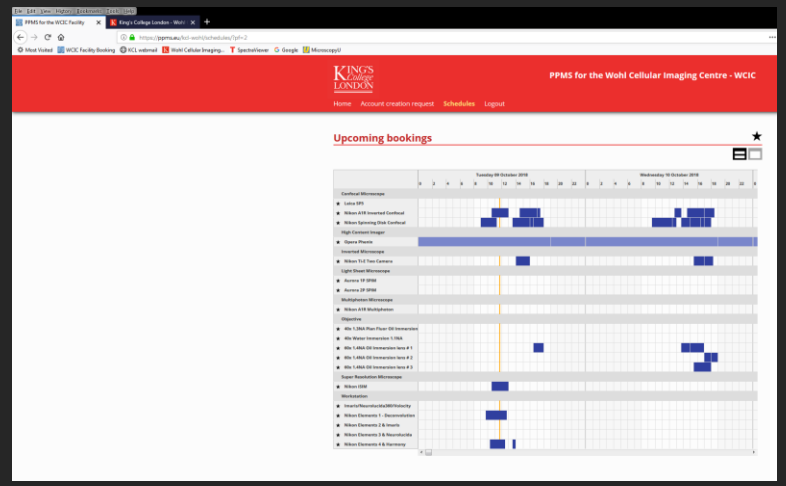
NO → Turn off system in reverse order.

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

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

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

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



# 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

You need to login to this pop-up window, user name is normally: kclad\k number

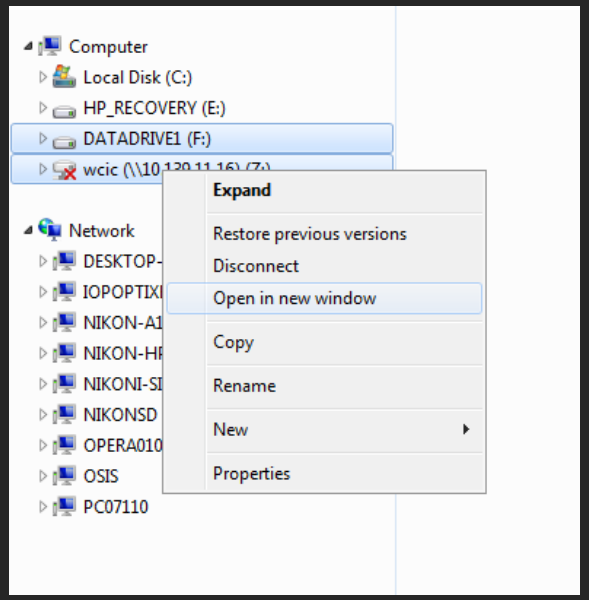
DO NOT click on remember my credentials

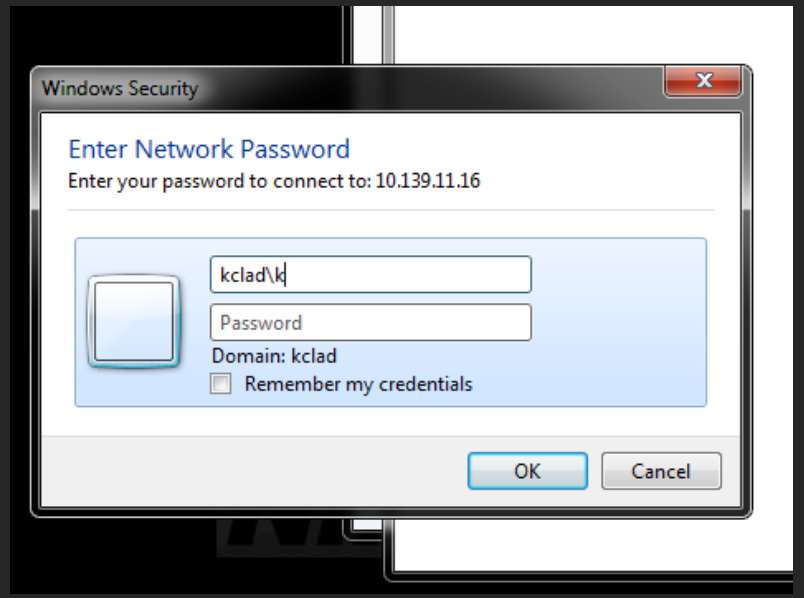
4

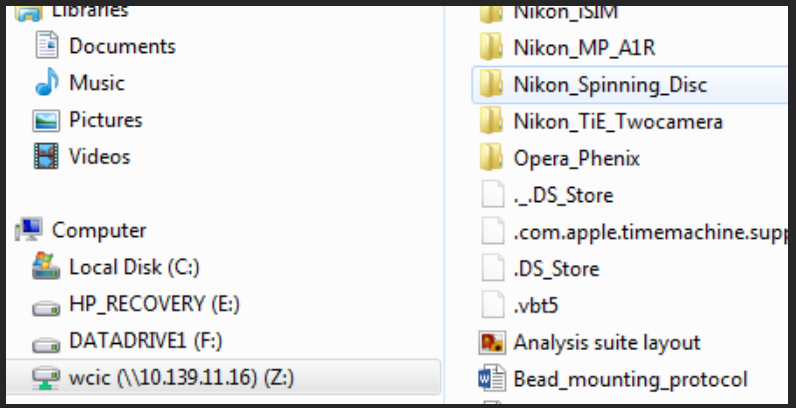
In the Network drive, open the Nikon_Spinning_ Disk folder

5

In the Nikon_Spinning_Disk folder open your personal folder



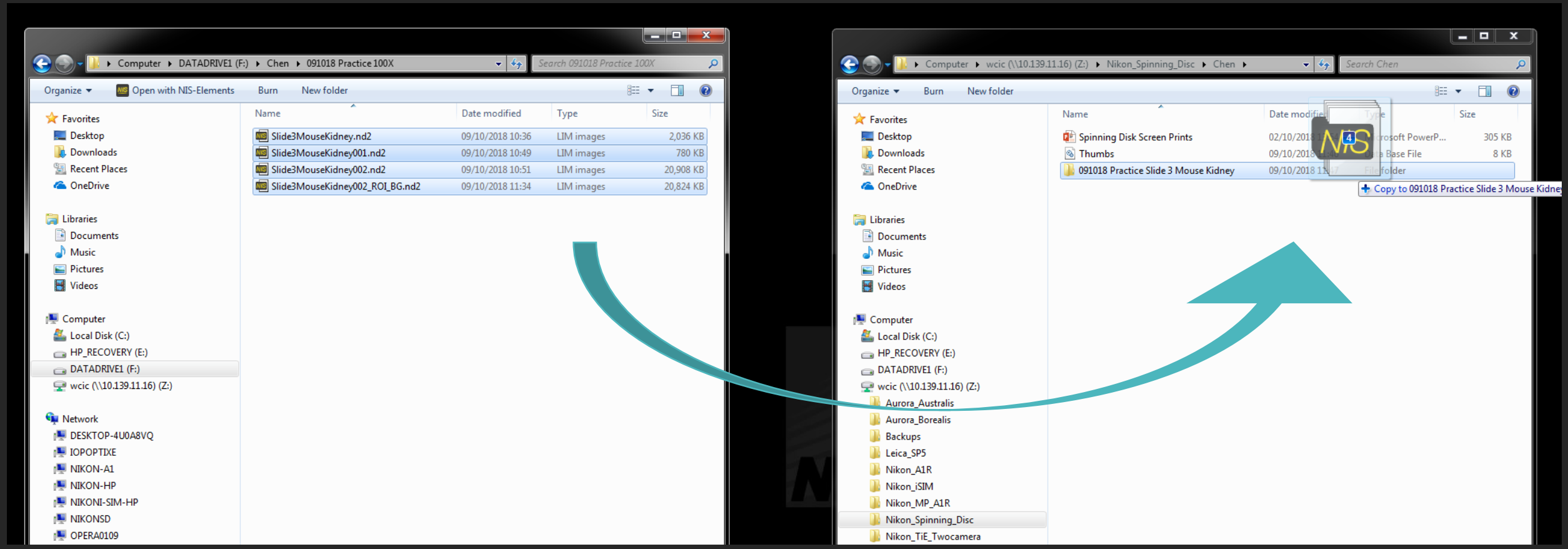



# Transfer Data To Shared Drive (2 of 3)

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

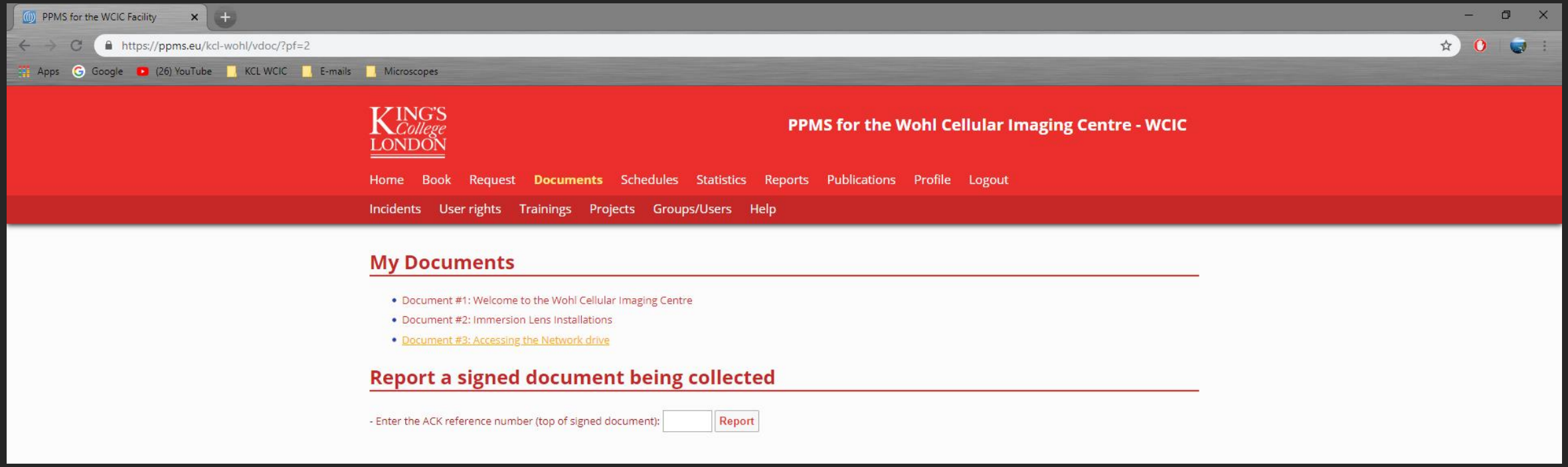
- Drag and drop the files you saved in this session.

- 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!