

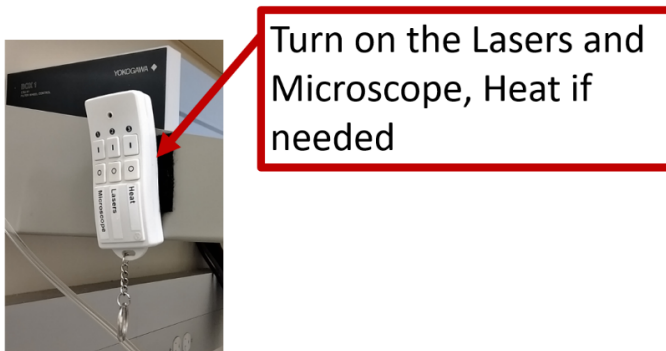
Nikon Spinning Disc Confocal

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Nikon SDC - Basic set-up

This protocol covers the basics of turning on the microscope, mounting your sample, and acquiring a simple image.

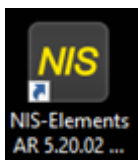
1. If the microscope is off, turn on the microscope (the protocol is also taped on the wall in the facility):
 1. Locate the remote control - it should be attached using velcro to the shelf on the left.
 2. Point the remote in the rough direction of the laser boxes and press the | button to turn the lasers and microscope on.
 3. Press the | button for "heat" if you are planning to use the environmental chamber.
 4. Wait about a minute for everything to start up.



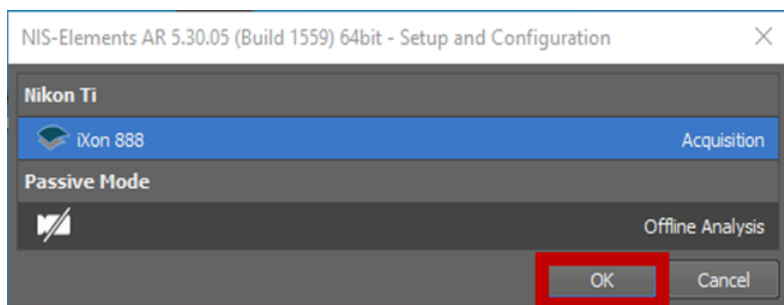
2. Locate the laser box on the left and ensure that the key is set to on.
3. Turn on the laser lines that you need by pressing the corresponding button. The button will blink while the laser line is warming up and will eventually stay solidly lit when the laser is ready. Note: If the button is blinking, that laser line is not yet available for the microscope to use.
 - You can turn off the laser lines that you are not planning to use by pressing the corresponding button.



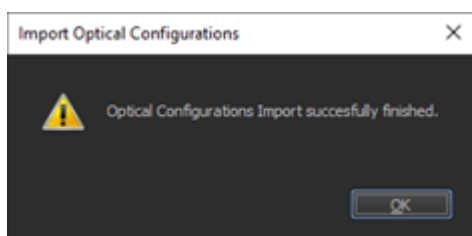
4. Start Nikon Elements by clicking on the icon on the desktop



5. A dialog box will pop up. Make sure the "iXon 888" camera is selected, then click ok.

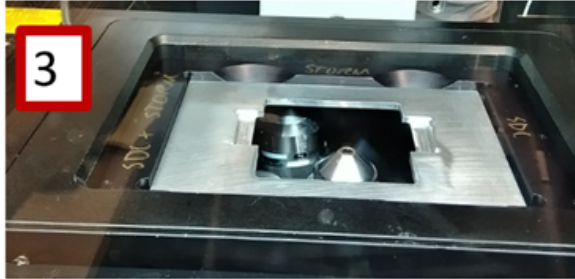
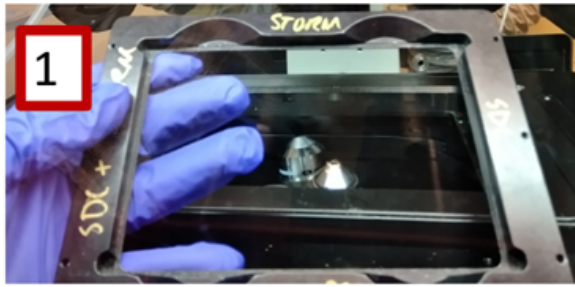


6. Wait for Elements to start up and the dialog box below to show up.



Note: If an error occurs, please [contact us](#) as the microscope might not work correctly.

7. Insert the appropriate sample plate holder in the sample chamber. The available sample chambers are:
1. 96-well plate holder
 2. Slide holder
8. Note: Other holders, including round dish holders, are available in the facility. Please contact us for assistance.



9. You can turn on the lamp using the foot switch if necessary. The intensity can be adjusted with the white knob on top of the environmental chamber.

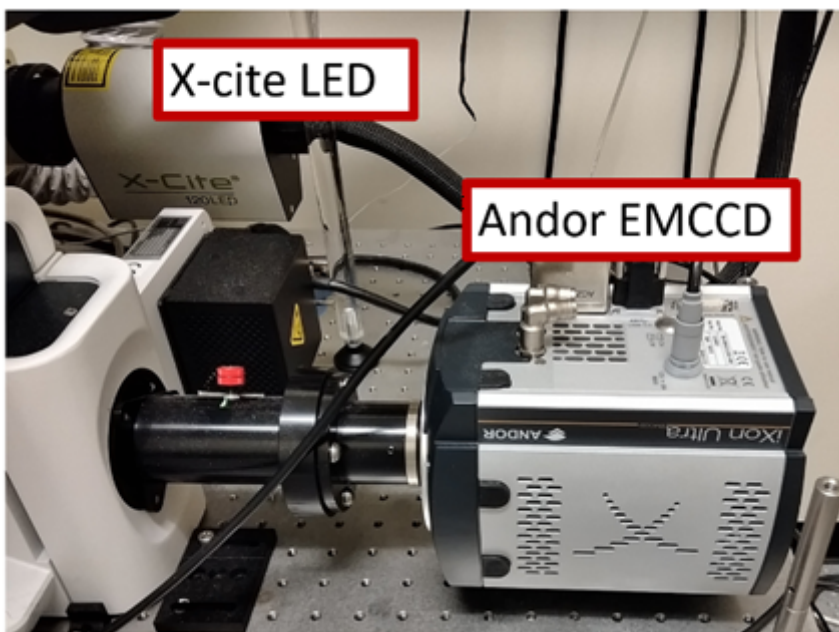


10. If you are using heat, allow the entire chamber to warm up for about an hour to allow the temperature to equilibrate. If you don't allow this time, the sample may lose focus due to thermal drift.
11. Place your sample in the holder. **Ensure that the coverslip is facing downwards.**
12. You are ready to image.

Nikon SDC - Mode of operation

Nikon SDC microscope features

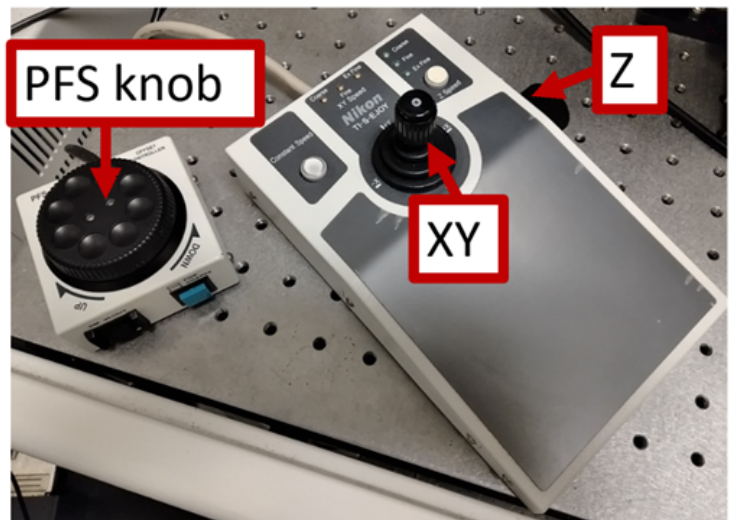
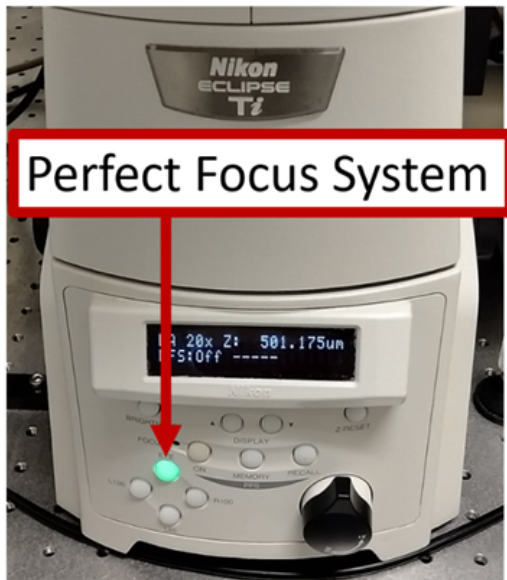
This microscope offers several capabilities, it has two light sources and one camera. In addition to the laser system, you can use the X-cite LED in widefield mode. The camera installed is an EMCCD iXon 888 with 13 μm pixels in a 1024 x 1024 format.



Microscope controls

To control the microscope stage, you will use the joystick. The central knob controls the X and Y directions, you can change the speed by twisting the top of it.

The knob on the side of the joystick can be used to lower or raise the objective and bring the sample into focus. You can also do this by using the two knobs on each side of the microscope itself. **Note:** On Nikon microscopes, when you turn your fingers UP the objective goes UP and vice versa.



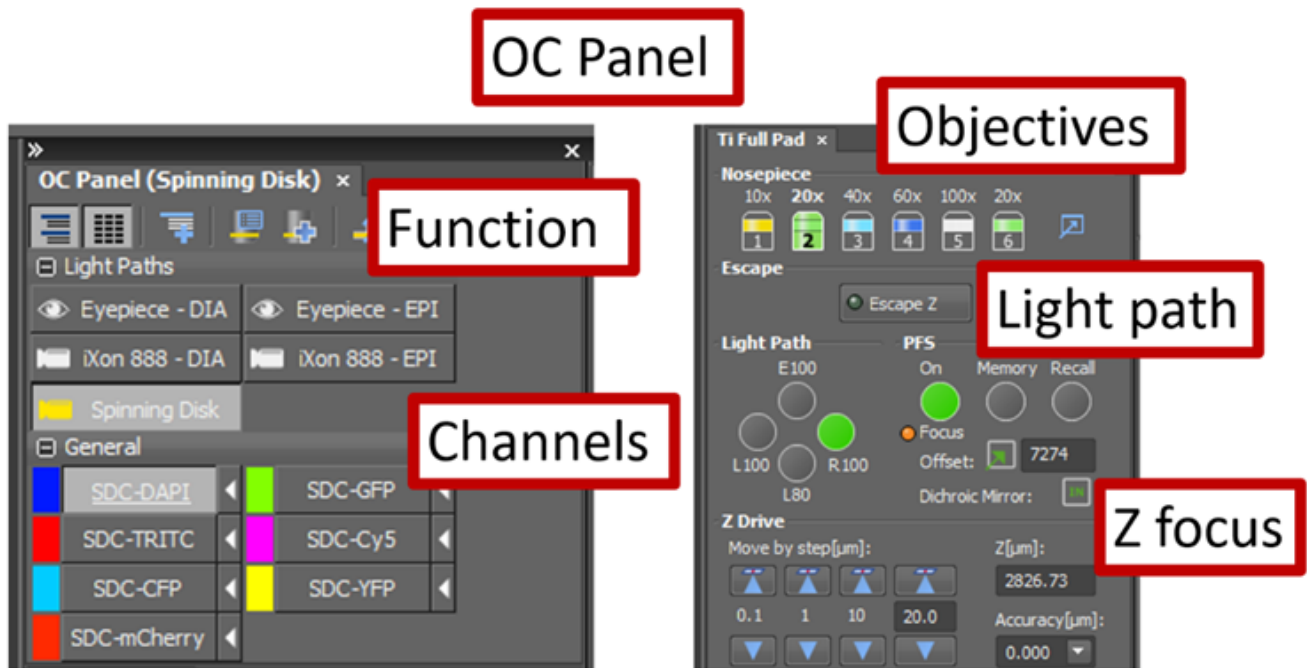
This microscope has a Perfect Focus System that facilitates focus finding. Please refer to [Using the Perfect Focus System \(PFS\)](#) page to use it.

Overview of the Optical Configuration (OC) control panel

The OC Panel contains the different light source configurations (referred to as “Light Paths” and their corresponding preset parameters (listed as “General” but referred to as “Optical Configurations”). You most likely will use the Spinning Disk configuration to observe your sample using the lasers and camera, and possibly the Eyepiece – EPI configuration to find the sample in widefield mode with your eyes if needed. Just below Light Paths you can find the selection of channels (OCs). Select the OC that corresponds to your fluorophores. To help, you can find the list of OCs and their corresponding fluorophores on the wall behind the computer monitor.

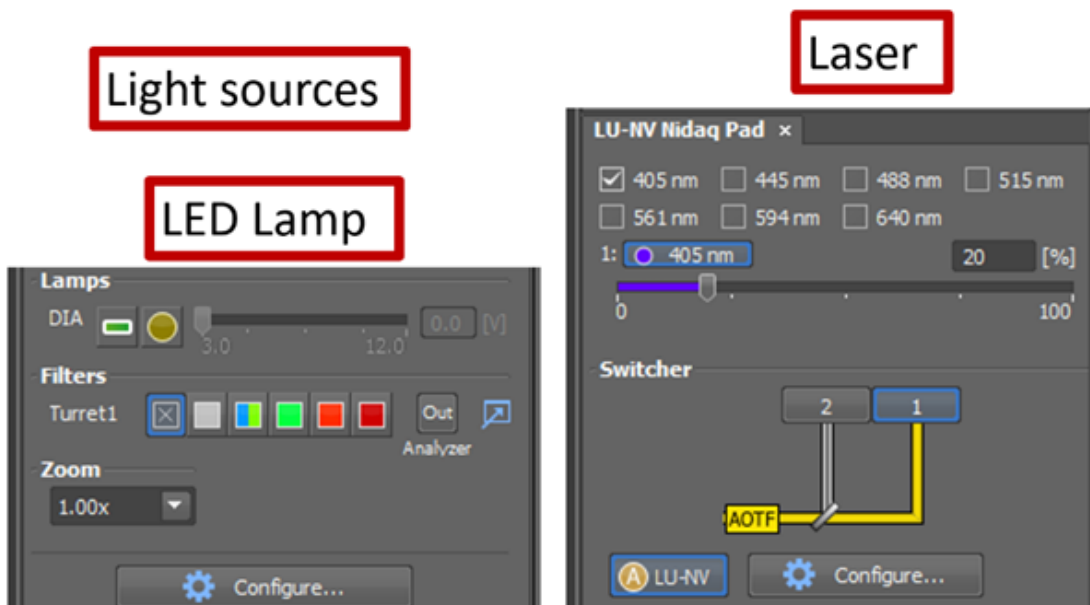
- The Ti Full Pad

The Ti Full Pad allows you to choose the objective you need, orient the light path to the detector (here it is on the right port) and adjust the focus with the software. Note: If you adjust the Z height in the software, the PFS will turn off.



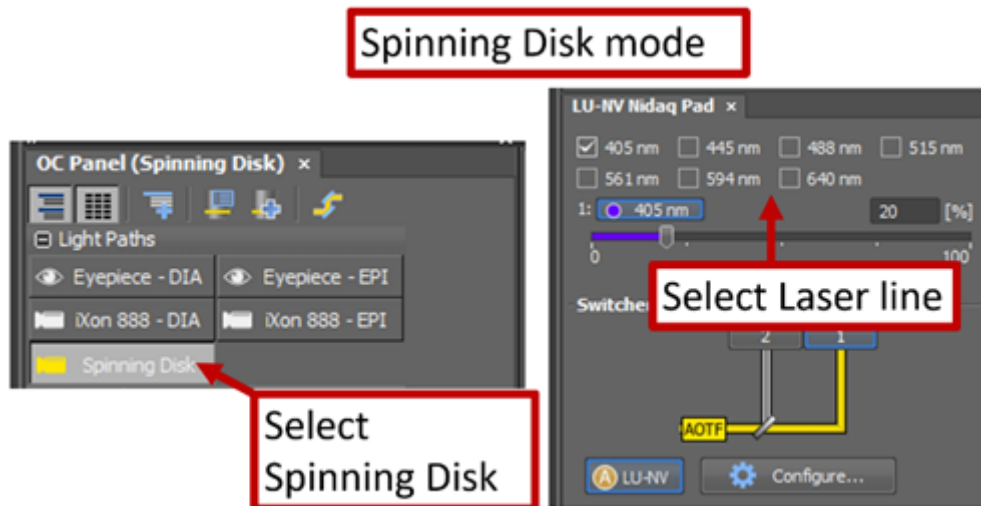
- Light Sources

The OC panel has the different light sources you may use. You can adjust the intensity of the diasopic transmitted light (DIA) for general widefield transmitted light or select the laser line you are using and change its intensity. **Note:** The laser must be turned on (illuminated) on the laser combiner for the control to occur.



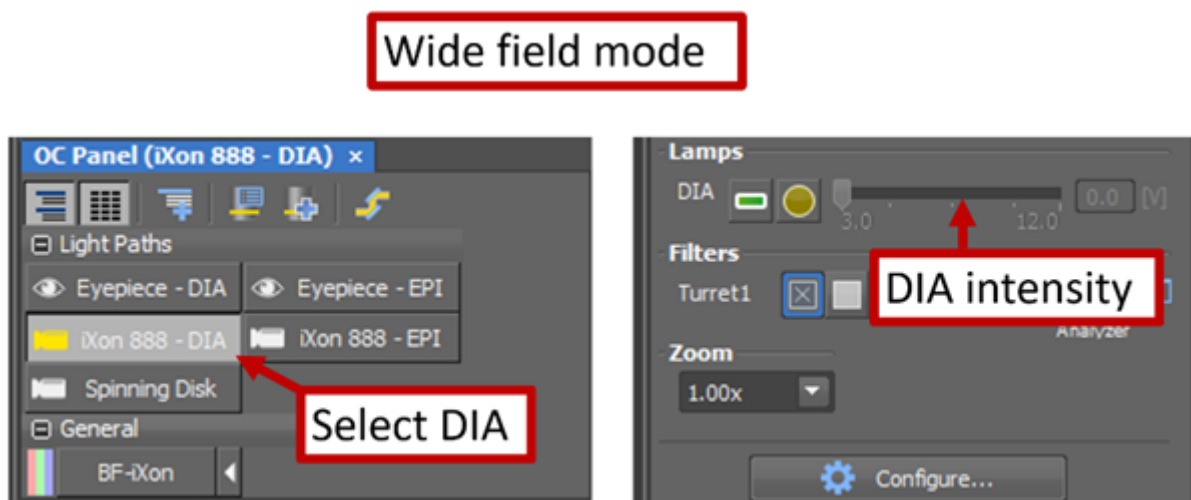
- Spinning Disk Mode

The Spinning Disk mode is the mode you will principally use on the Nikon Spinning Disk Confocal. Make sure you selected this configuration to be able to control the laser lines.



• Widefield Mode

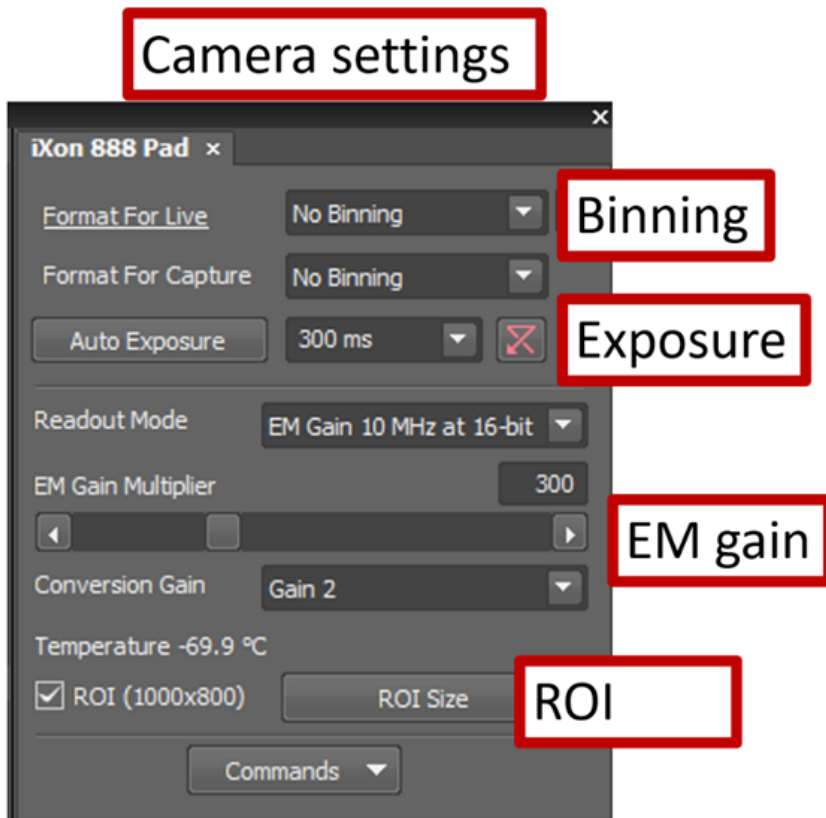
It is sometimes useful to use the widefield mode to find the sample and bring it into focus. Select iXon 888 – DIA as a configuration and then you can adjust the lamp intensity.



• EMCCD Controls

The iXon 888 Pad contains the camera settings. You have the option to apply binning to the images. However, remember this will lower the image quality. The exposure can be adjusted here to have more signal from your sample or avoid saturation. The EMCCD can be used as a regular CCD if you leave the EM gain off. To benefit from the electron multiplying gain you need to set it to a specific value. **Note:** Do not exceed a gain value of 300 as there is a diminished benefit to the image quality as well as the linearity of the signal amplification is lost. The Camera Region of

Interest (ROI) is defined to match the usable field of view of the camera.



Scan Acquisition

At this point in your training it was time to look at the sample and start an acquisition. We used a demo sample with the 20X objective, air immersion. If you are using the 100x oil immersion objective, start by dropping a drop of oil on the top of the objective, as described in this [tutorial video](#).

Note: You cannot use an air objective after you have imaged your sample with an oil objective. This is because the oil can transfer from the sample to the objective, and can result in both diminished image quality as well as damage the objective itself. Please thoroughly clean your sample and the 100x objective prior to using the air objective.

Now that you have delicately placed your sample on the sample plate holder you need to:

- Bring the sample into focus using the PFS:
 - A. Press the PFS button on the microscope base. You will hear several beeps and the light will start blinking, indicating that the microscope is searching for the focal plane.

- B. Raise the objective *slowly* using the black knob on the side of the joystick until you hear a beep. The PFS button will be continuously lit when perfect focus is engaged correctly and the LED next to the FOCUS display will be lit. Be careful not to push the objective into your sample - this will damage the objective and lead to costly repairs.
- C. Click “Live” in Nikon Elements to observe the sample
- D. Autoscale your Look-Up Table (LUT)
- E. Adjust the focus using either the external knobs or the controls in the software.
- F. Adjust the Laser Power, Exposure Time, and/or EM Gain to refine your image quality.

• Optimizing the image

Depending on the camera you are using and how much signal your sample emits with the laser settings that you chose, your image may be very dim to start with, to the point that you don't see anything. The first thing you should do is adjusting the Look Up Table.

To auto-adjust the lookup tables, clicking on the Auto Scale buttons above the image of interest.

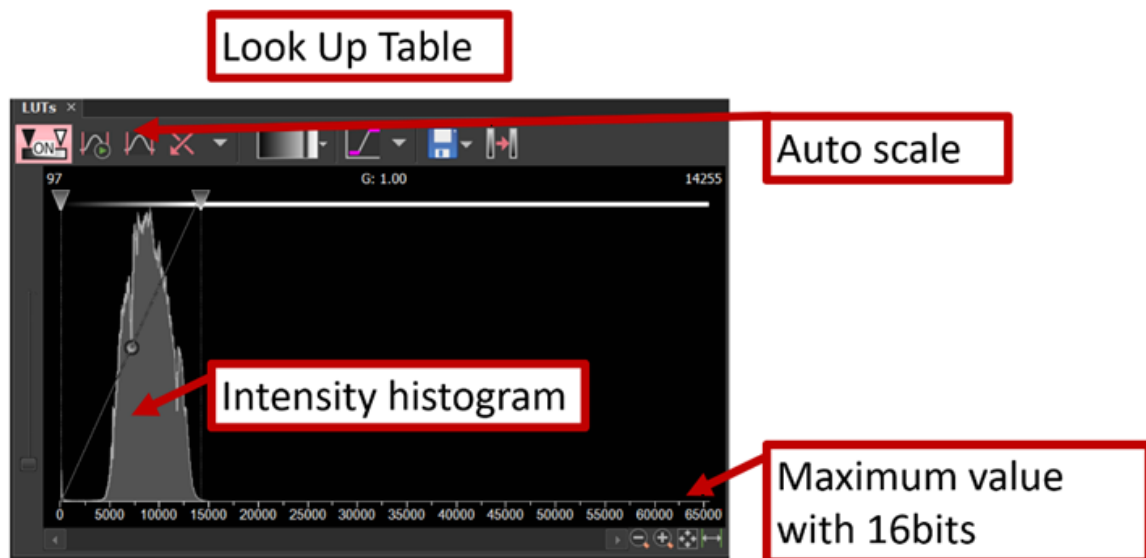


This button will adjust the lookup table for each frame (useful in live view mode)



This button will adjust the lookup table for the current image

You can also set the range of the Look Up Table by dragging the lines on either side of the intensity histogram with the little triangular cursor on the top. If the intensity histogram reaches the end of the range that means your image is saturated and you need to reduce the laser power or the exposure time or both.



- Adjusting the exposure and laser power

In the Camera Settings Pad you can adjust the exposure of your image. You can also choose to bin your image (i.e clumping the pixels together to make bigger pixels). With the EMCCD camera, binning will improve the sensitivity of the pixels but they are now twice as big so you will lose in image sharpness (resolution).

Now that you found the sample, brought it in focus and optimized its signal, you may want to select a specific cell and center it in the image. You can always do this using the joystick of course. But there is another useful way to **perfectly center** the cell you choose. If you right click on the image viewer where the cell is located you can find the option to center it and the stage will move accordingly.

Another useful feature of Nikon Elements is the option to save your field of view in the image format of your choice. This will be a **view only image**, it won't allow you to process the different channels separately and such. But you may find this useful to show your colleagues or for later comparison with other data sets. To do so, **click on the "x" letter** on the keyboard and you will find the option to save in the format of your choice.

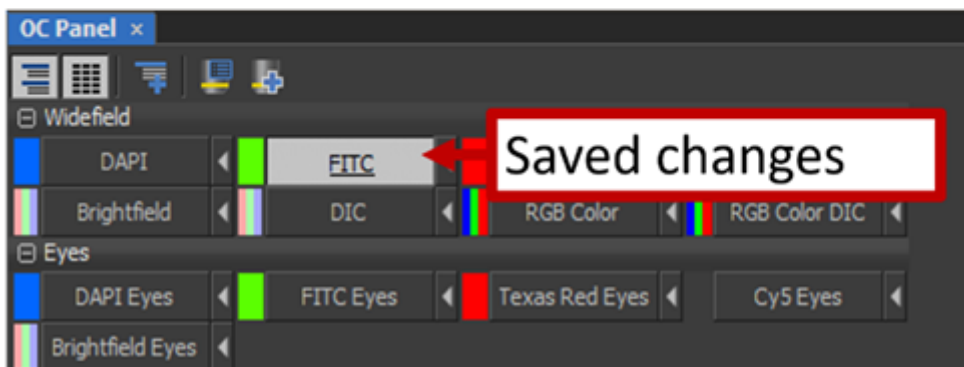
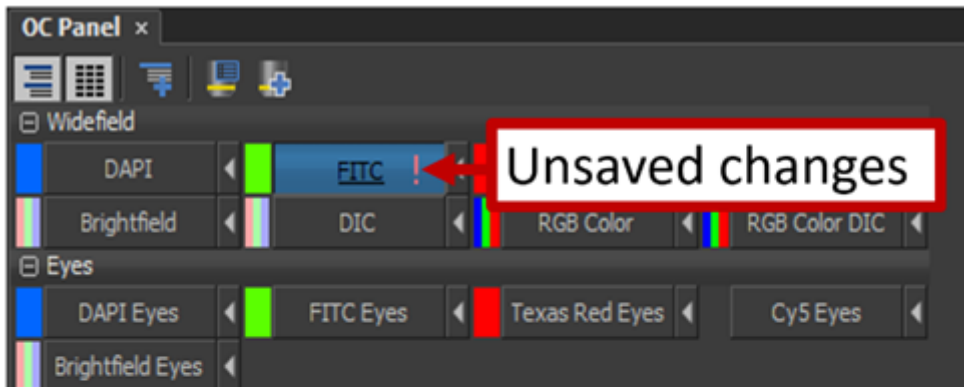
Image acquisition and saving

The Nikon Elements software offers many options for acquiring and saving your data. For SDC experiments you will typically want to acquire a z-stack and possibly for different channels at different wavelengths.

- Acquiring a z-stack image for different channels

Before you start acquiring and saving data, you need to assign the settings you chose. When you open the software, each channel is set at its default settings. After finding your sample, you probably changed some settings so that the image looks as good as it can.

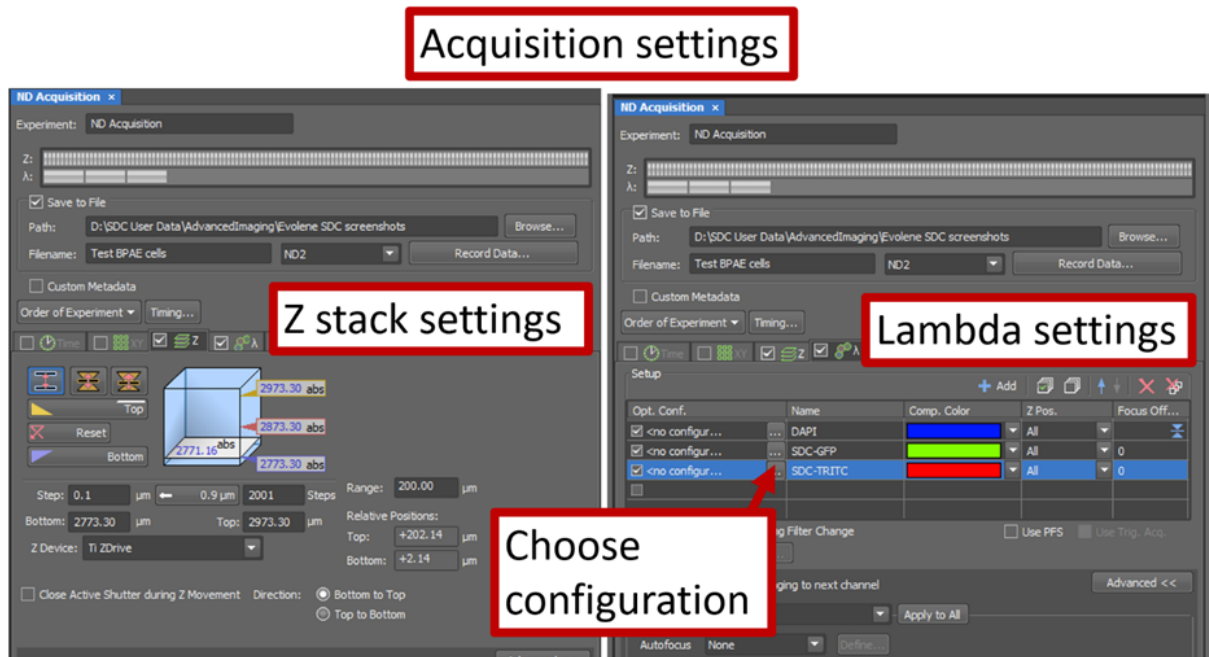
You should see a little “!” in the corresponding channel buttons. Click on the arrow next to it to assign the settings. When the “!” mark disappears your changes should be saved and you are ready to move on to the acquisitions.



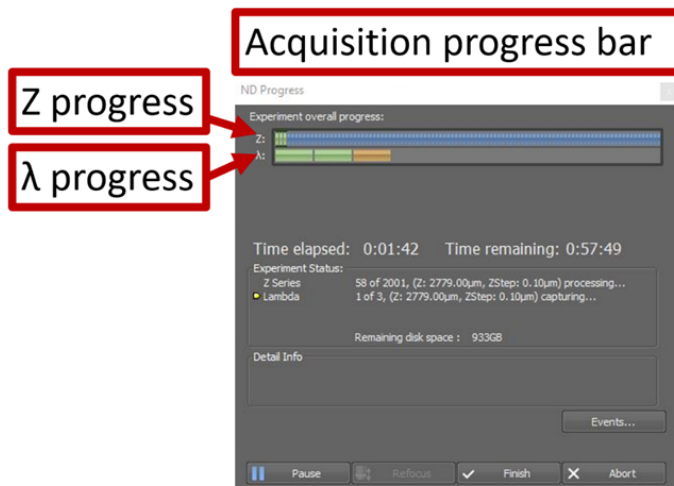
In the ND Acquisition Panel, check the box to “Save to File” and choose your destination and filename.

- A. To acquire a time lapse image stack, you need to check the box that says Z and the box that says λ .
- B. Then you can define your acquisition settings for Z: number of steps, start and end point, range, step size and order of acquisition.
- C. Define the acquisition settings for the different wavelengths. You need to choose a configuration for each channel (those are pre-set in the software).
- D. After you defined all the settings, click “Order of Experiment” to define in which order you want the scan to proceed.

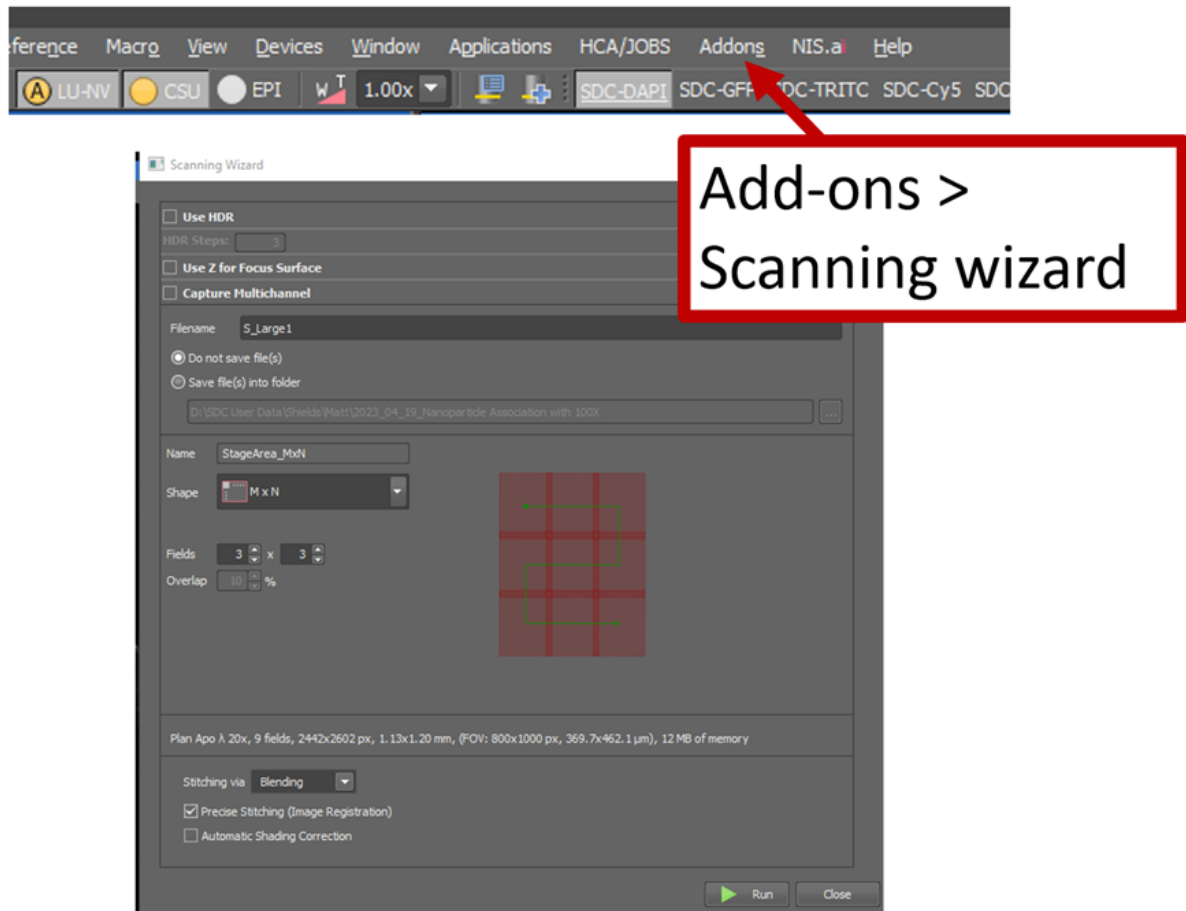
E. When you are ready to image click “Run now”



While the scan is in progress you will see this window:



Finally, you can use the scanning wizard to tile several scan areas together. This option is found in the Addons menu. Select “Scanning wizard”. You can find options for the scan there, define the number of tiles and in which order the scan goes.

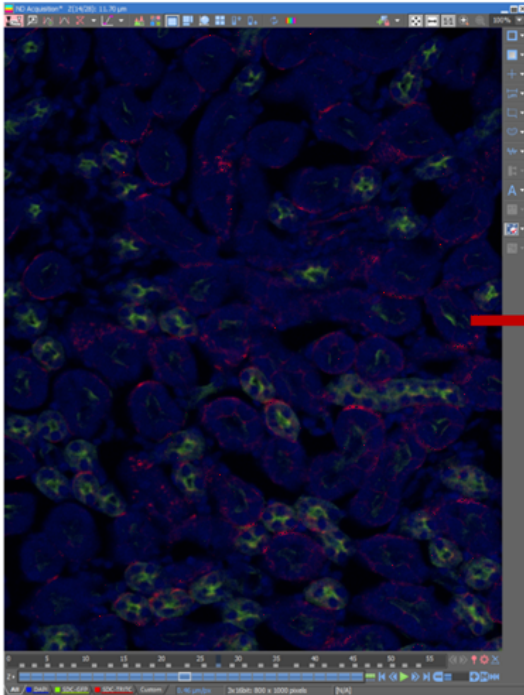


Look at your z-stack in Nikon Elements

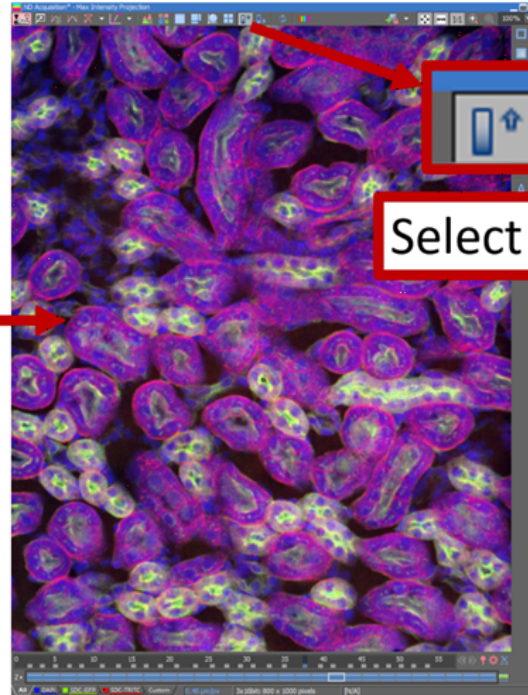
- Maximum Intensity Projection

Once the acquisition is complete you can look at its maximum intensity projection.

Acquisition completed



Max Intensity Projection

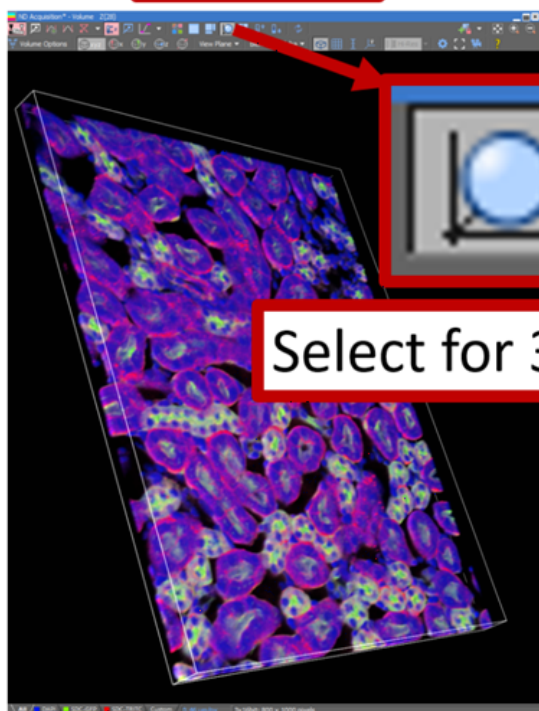


Select for MIP

- 3D View of the sample

The 3D viewer allows you to look at the full z stack in all directions. It can be useful for nice renderings too!

3D view



Select for 3D view

Nikon SDC - Shut-down and clean up

When you are done imaging, remove your sample from the microscope and place it on a kimwipe or other paper towel if it has oil or water.

If you used an oil objective, please refer to the [instructions](#) of Dr. Dragavon to clean it:

Shut down the system according to the shut-down procedure taped on the wall:

1. Save your beautiful images to your Network drive.
2. Exit Nikon Elements and disconnect from your Network drive
3. Remove your sample from the stage and clean up the microscope

Clean oil from the objective and remove your waste, wipe everything with ethanol

4. Open the reservation calendar

If there is a user within 1 hour of your session, turn off the environmental chamber (if used) and leave the system on.

If the next user is more than 1 hour after your session, turn everything off (step 5).

5. Using the remote control ONLY:

Turn off the Microscope

Turn off the lasers

Turn off the environmental chamber (follow the instructions on the wall)

Nikon SDC - Specifications

Type: Spinning disc confocal

Purpose: Rapid 2D and 3D imaging of samples

Fixed cells: Yes

Live cells: Yes

FPBase link: <https://www.fpbases.org/microscope/X6ne45wr4DYFPsgc5nPrKo>

Tag: None

Room: C350A

Software:

- NIS Elements - v5.42.03

HASP: 293CDCAA

Base: Nikon TiE

XY Stage: Motorized

Z:

- Ti Z drive
- MCL Piezo Z - 100 μ m

Autofocus: PFS 3

Environment control:

- Oko Labs enclosure: CO₂, Humidity, Temperature

Scan head: Yokogawa CSU-X1

Light source:

- LUNV22 70186
- Lasers: 405, 445, 488, 515, 561, 594, 640

Emission Filters

Vendor	Model	Center	Width	Min	Max	Common Fluorophores
Chroma	ETDAPI-Cy5 m Lot 314725	437	18	428	446	DAPI. BFP

Chroma	ET482/27m	482	34	465	499	CFP
Chroma	ET525/50m	525	50	500	550	GFP, FITC, Alexa Fluor 488
Chroma	ET540/30m	540	30	525	555	YFP
Chroma	ET620/60m	620	60	590	650	TRITC, Texas Red, dsRed, Alexa Fluor 568
Chroma	ET630/30m	630	30	615	645	mCherry
Chroma	ETDAPI-Cy5 m Lot 314725	705	68	671	739	Cy5, Alexa Fluor 647, Draq5

Dichroics

- Chroma ZT405/488/561/640rpcv2
- Chroma ZT44/514/594rpc

Objectives

Magnification	NA	Medium
4	0.2	Air
10	0.45	Air
20	0.75	Air
40	0.95	Air
100	1.4	Oil

Detectors

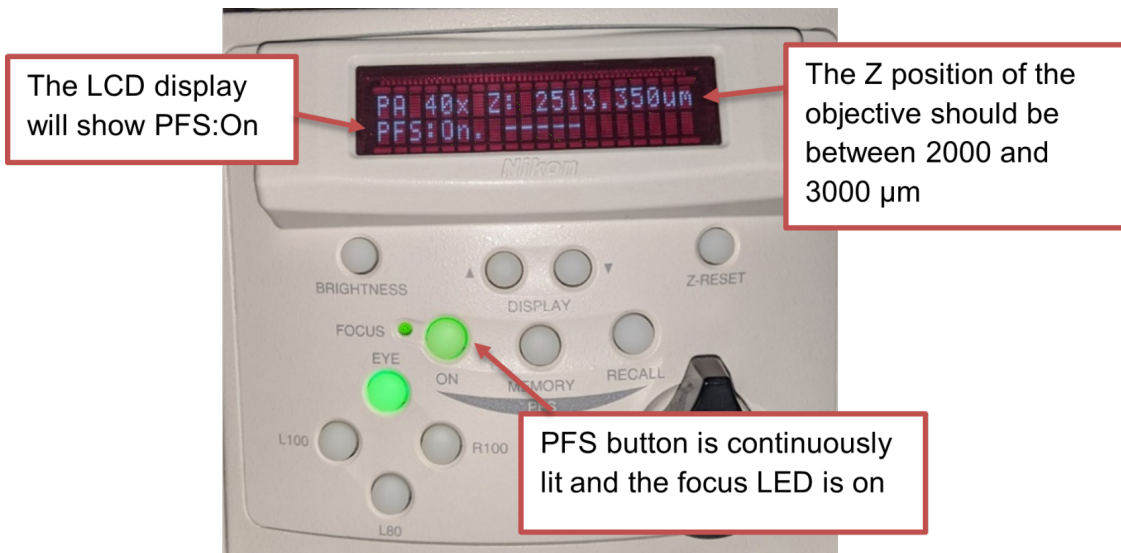
- EMCCD
- Andor IXon 888 Ultra
- S/N: X-9963

Using the Perfect Focus System (PFS)

The perfect focus system locates the coverslip and continually adjusts the objective height to follow any drift in the system.

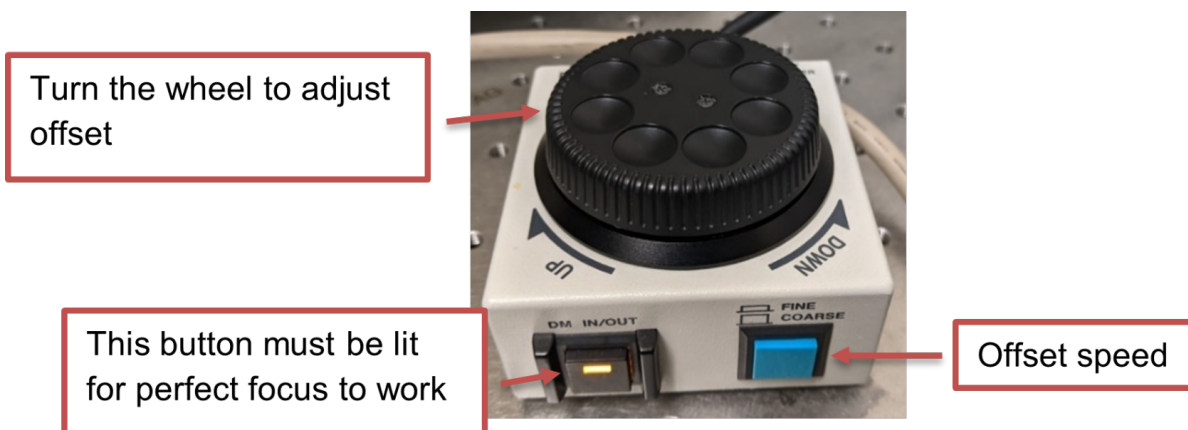
Engaging the PFS


1. **Only if using the 100x oil objective**, first raise the objective by turning the z-axis control until the oil contacts the sample. This step is not necessary for the air objectives.
2. Press the PFS button on the microscope base. You will hear several beeps and the light will start blinking, indicating that the microscope is searching for the focal plane.
3. Raise the objective *slowly* until you hear a beep using the black knob on the right side of the joystick. The PFS button will be solidly lit when perfect focus is engaged correctly and the LED next to the FOCUS display will be lit. **Be careful not to push the objective into your sample - this will damage the objective and lead to costly repairs.**
 1. The display on the LCD screen on the microscope base indicates the Z position of the objective. The typical Z-position is between 2000-3000 μm .
 2. If you turn the knob the wrong way (i.e., lowering the objective), you might hear a continuous tone. This is fine - you just have to reengage the PFS system by pressing the button.
 3. If you are having trouble getting perfect focus to engage, see Troubleshooting Perfect Focus below.



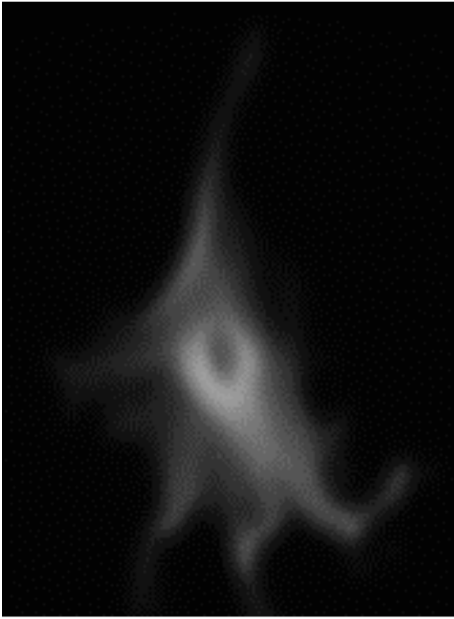
Refining the perfect focus offset

Once perfect focus is engaged, you cannot change the z-position of the microscope. However, to get cells in focus, you can change the perfect focus offset using the perfect focus wheel.

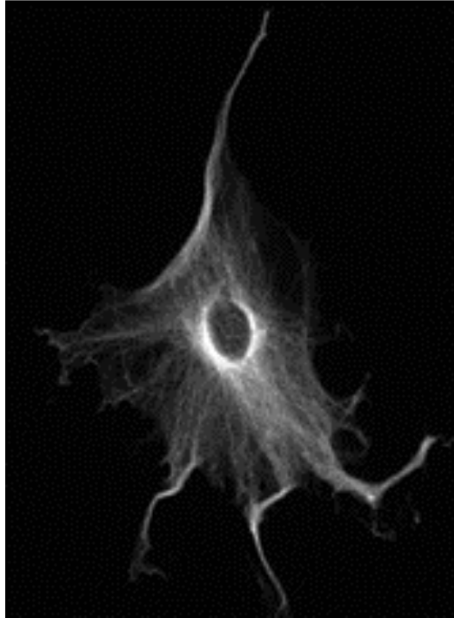


1. Start Live View.
2. Adjust the perfect focus offset by rotating the perfect focus wheel.
3. The blue button can be used to change the offset change speed. When the button is pressed in, turning the wheel changes the offset a little bit. When the button is out, the turning the wheel changes the offset a lot. You should start with coarse adjustment until you start to see some cells, then switch to fine.
4. When the cells are in focus, click on the  button next to the selected optical configuration to save the settings.

Out of focus



In focus



Troubleshooting tips

- The perfect focus system looks for a reflection off the coverslip. This can be formed by a glass-air or a glass-water (e.g. sample mounted under an agar pad) boundary. If your sample is fixed with mounting media, the perfect focus will not work.
- Check that the coverslip is facing downwards towards the objective. The coverslip thickness should be no thicker than 1 mm.
- Are you using the correct objective? Did you add oil to an air objective or forget the oil for the 100x objective? If the latter is true, clean the oil off and let Joe know asap.

Online learning resources by Nikon

Nikon is now offering learning resources available [online](#). To request a login password, please email Joe Dragavon at biof-imaging@colorado.edu

[Nikon e-learning center](#)

"The Nikon Instruments Learning Center provides interactive tutorials on a variety of topics ranging from basic to advanced. Novice users can follow step-by-step videos on how to perform DIC alignment on their Nikon microscope, carry out basic measurements in [NIS-Elements](#), and learn how to choose the best objective for their experiment. Experienced users will learn how to leverage advanced features of their Nikon system and learn tips and tricks for brushing up on their NIS-Elements skills. Current Nikon Instruments customers can enjoy exclusive access to this resource at no additional cost."