

Nikon NSTORM

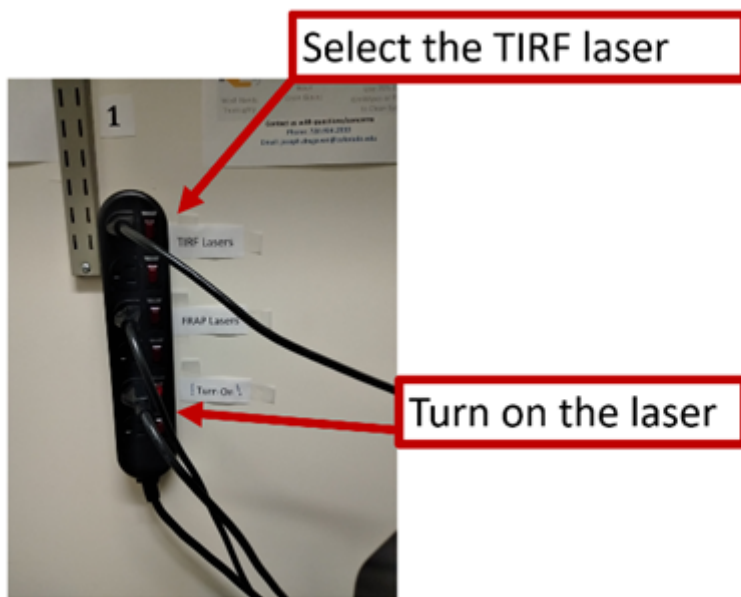
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Nikon NSTORM - Basic Set-up

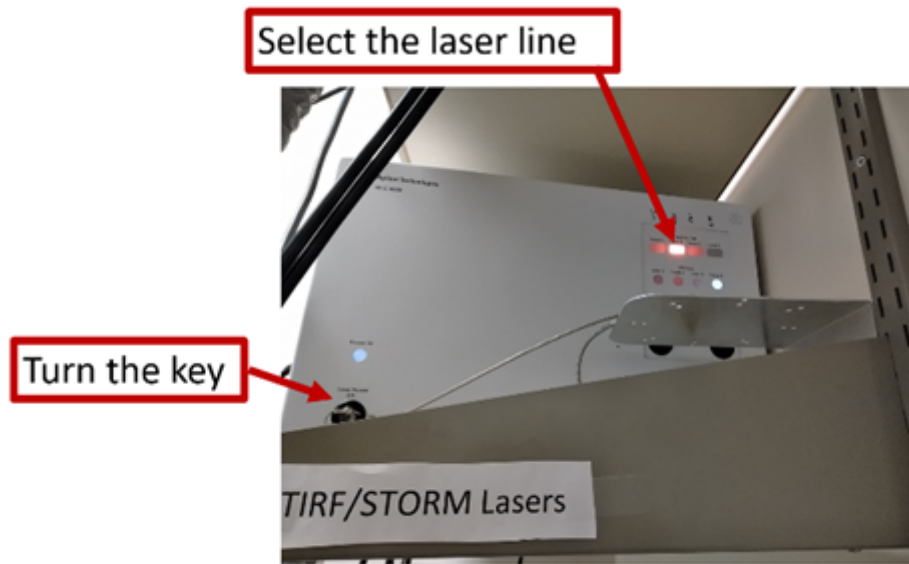
Getting the system ready

- If the microscope is off, turn on the microscope following the protocol taped to the wall:

A. Turn on the Laser Power Strip #1.

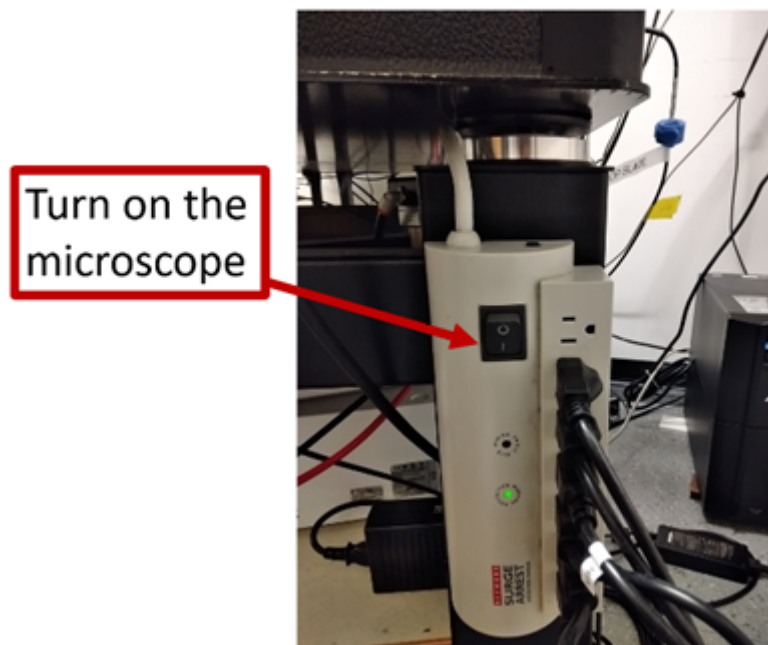


- a. Turn on the appropriate laser boxes by turning the key to the ON position. Then press the grey buttons on each laser box for the laser lines you intend to use ONLY. Please leave the others in their standby (grey) state.



b. Turn on only the laser boxes you intend to use. For this training use the TIRF laser.

B. Turn on the Microscope Power Strip #2. This also turns on the Nikon NSTORM arm and the cameras.

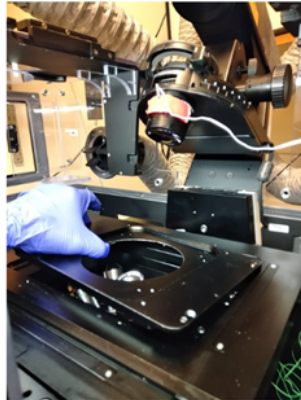


C. Turn on the Computer.

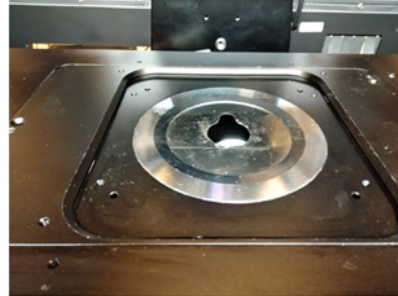
- Insert the sample plate holder

Depending on your experiment you may choose a different plate. You can turn on the light in the environmental chamber using the pedal switch on the floor under the microscope. The intensity can be adjusted with the white knob on top of the environmental chamber. If there are any oil stains on the equipment please clean up with ethanol before starting your experiment.

Insert sample
plate holder



Insert sample plate



Turn on light in
environmental
chamber



Intensity
control

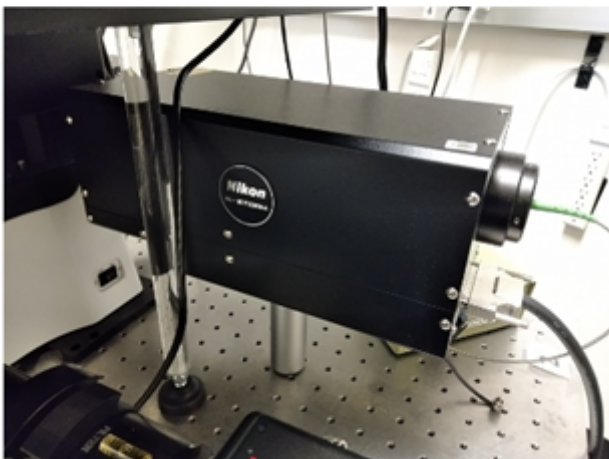


- Nikon N-STORM microscope features

This microscope offers several capabilities, it has 3 light sources and 3 cameras. This manual will describe how to use the TIRF lasers but you could also use the FRAP lasers or the brightfield mode with the Lumencor SOLA Light Engine.

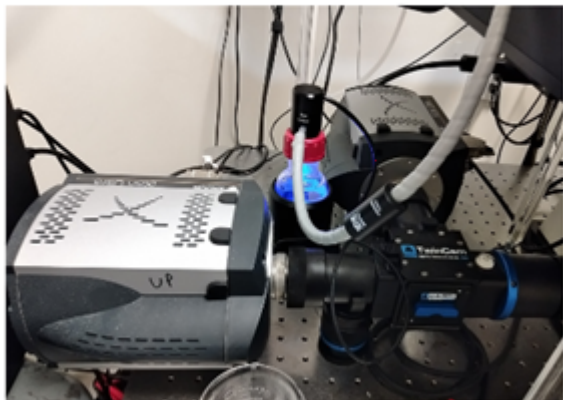
The Nikon motorized TIRF arm controls the TIRF angle.

Nikon STORM arm

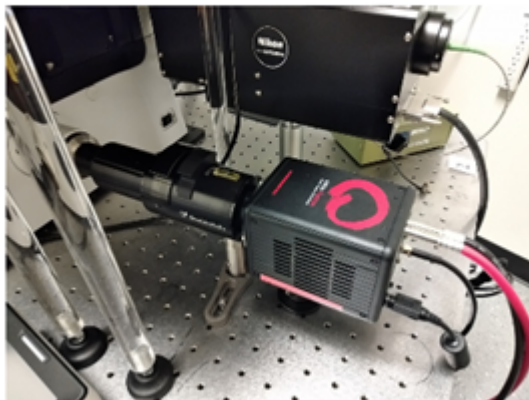


There is a sCMOS camera, Hamamatsu ORCA-Fusion with 2304x2304 pixels and two EMCCD cameras, Andor iXon 897 with 512x512 pixels. You can use a single EMCCD camera or both of them for simultaneous two channel imaging.

Andor EMCCD cameras



Hamamatsu sCMOS camera



To trigger the acquisition with the correct camera(s), select it on the BNC switch box.

Select your
camera for
triggering



The system is now ready for laser alignment and imaging.

Nikon NSTORM - Mode of operation

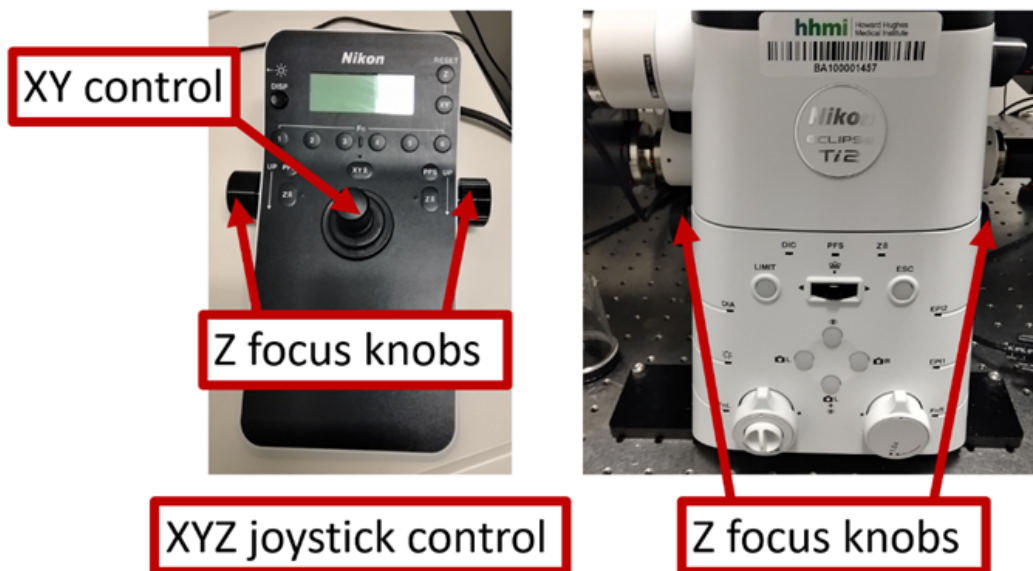
Microscope Controls

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.

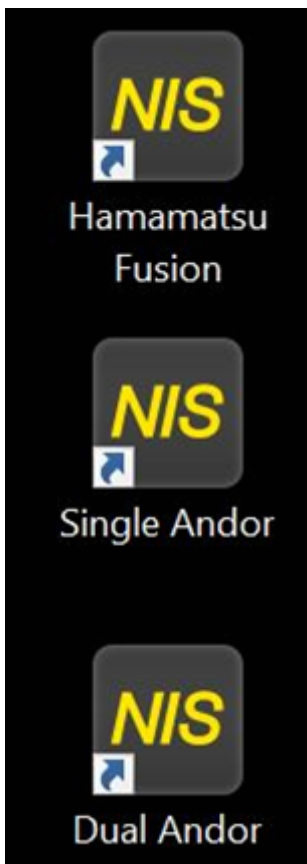
The two knobs on the sides of the joystick can be used to lower or raise the objectives and bring the sample into focus. You can also do this by using the two knobs on each side of the microscope itself.

Note: On Nikon microscopes, when you turn your fingers UP the objective goes UP and vice versa.

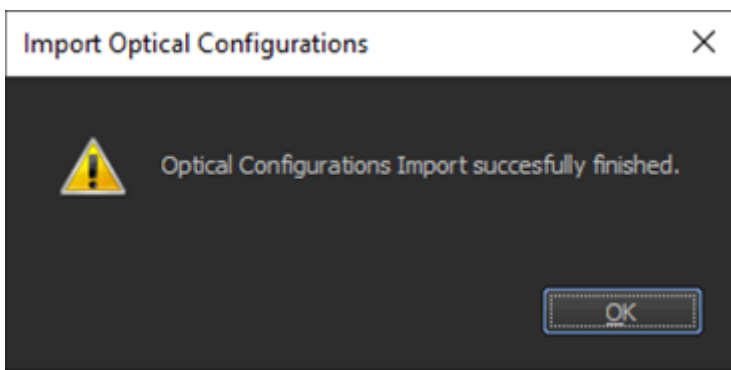
Microscope controls



- Select one of the Nikon Elements versions on the desktop. During the training you used the Hamamatsu Fusion and we will use this one for this manual as well.



- Click OK when the dialog box appears saying “Optical Configurations Import successfully finished.”

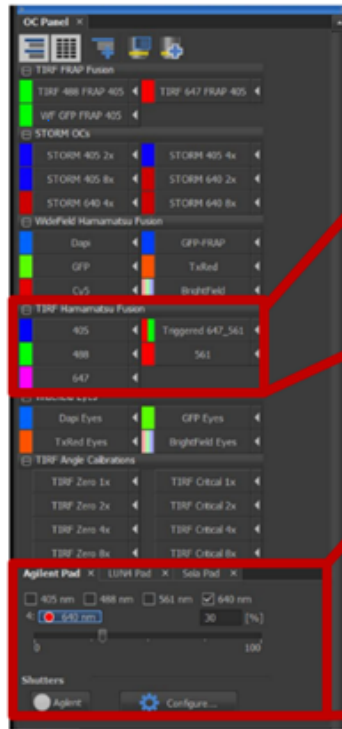


Overview of the Optical Configuration (OC) panel

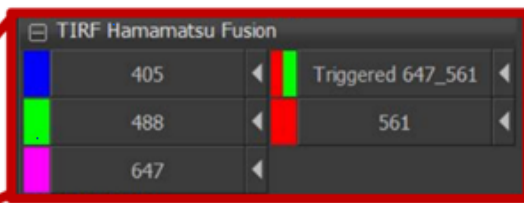
The OC Panel contains the different light source configurations. For each light source, you can select a different fluorescence channel which is a combination of a laser line and a filter set. The TIRF Hamamatsu Fusion lasers is what you will need to work with in this example.

The light source is controlled by the Agilent Pad for the TIRF lasers. In this Pad you can adjust the laser power. Make sure you selected the wavelength that is actually turned ON on the laser box.

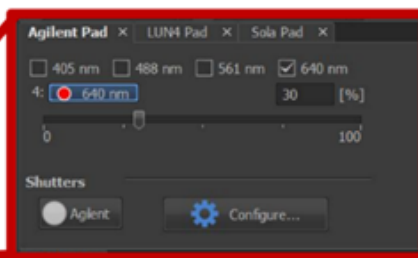
OC panel



TIRF Laser settings



Agilent Pad



• Ti2 Pad

The Ti2 Pad allows you to change objective. Every time you click on an objective, the objective plate lowers itself before turning to the next one for safety.

It lets you choose the light path you need and choose to use the PFS (perfect focus system) or not. You can then drive the objective up and down with the Z Drive, adjust the intensity of the transmitted light with the DIA control slider. There is also an option to change the filters.

Ti2 Pad



Objectives

Light Path

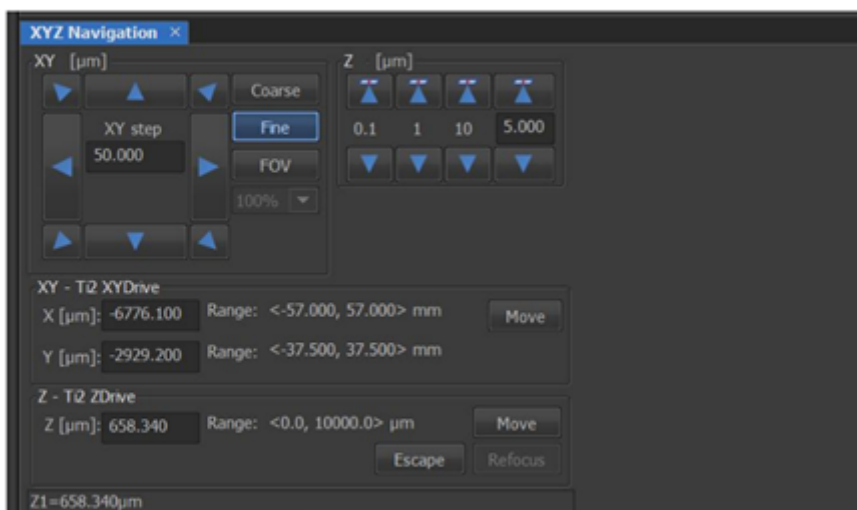
Focusing

Filters

- XYZ Navigation panel

To control the microscope stage and move your sample above the objective you can also use the XYZ navigation panel. This allows you to finely control the steps or set the same position from one experiment to the next.

XYZ Navigation



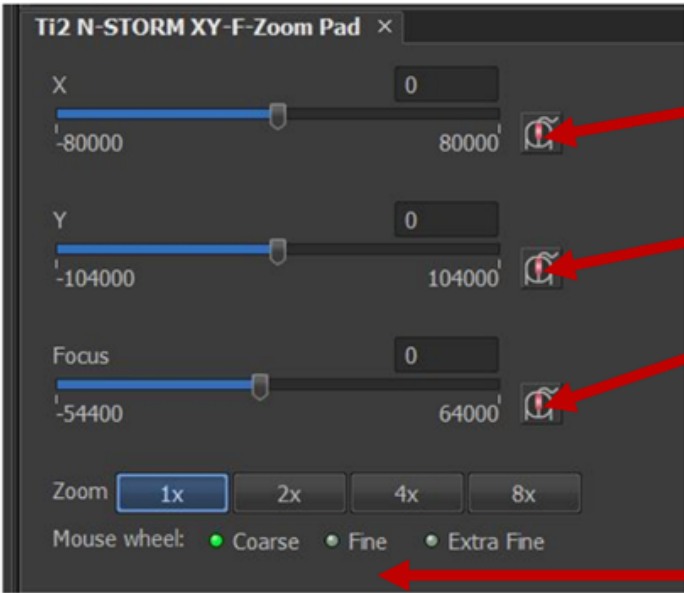
Align the TIRF laser

- TIRF Laser alignment procedure

During your training you practiced aligned the TIRF laser without a sample at first. This is a reminder of the procedure to get the laser going straight up when you position your sample on the microscope. Once you place the sample on the microscope it will only require minor adjustments.

You can control the TIRF laser angle with the Ti2 N-STORM XY-F-Zoom Pad. Click on the mouse cursor button to control the X and Y directions with the mouse wheel. When you click on the mouse cursor button for the focus adjustment you will bring your beam in focus. You can change the mouse wheel speed from coarse (fast) to fine and extra fine (slower).

Ti2 N-STORM XY-F Zoom pad



The screenshot shows the 'Ti2 N-STORM XY-F-Zoom Pad' window. It contains three horizontal sliders: 'X' (range -80000 to 80000), 'Y' (range -104000 to 104000), and 'Focus' (range -54400 to 64000). Each slider has a blue bar and a white knob. To the right of each slider is a small square button with a mouse cursor icon. Below the sliders are four zoom buttons: '1x' (selected), '2x', '4x', and '8x'. At the bottom, there are three radio buttons for 'Mouse wheel' speed: 'Coarse' (selected), 'Fine', and 'Extra Fine'. Four red callout boxes with arrows point to these controls: 'Click to adjust X angle' points to the X slider's mouse button, 'Click to adjust Y angle' points to the Y slider's mouse button, 'Click to adjust focus' points to the Focus slider's mouse button, and 'Click to change the speed of the mouse wheel' points to the 'Coarse' radio button.

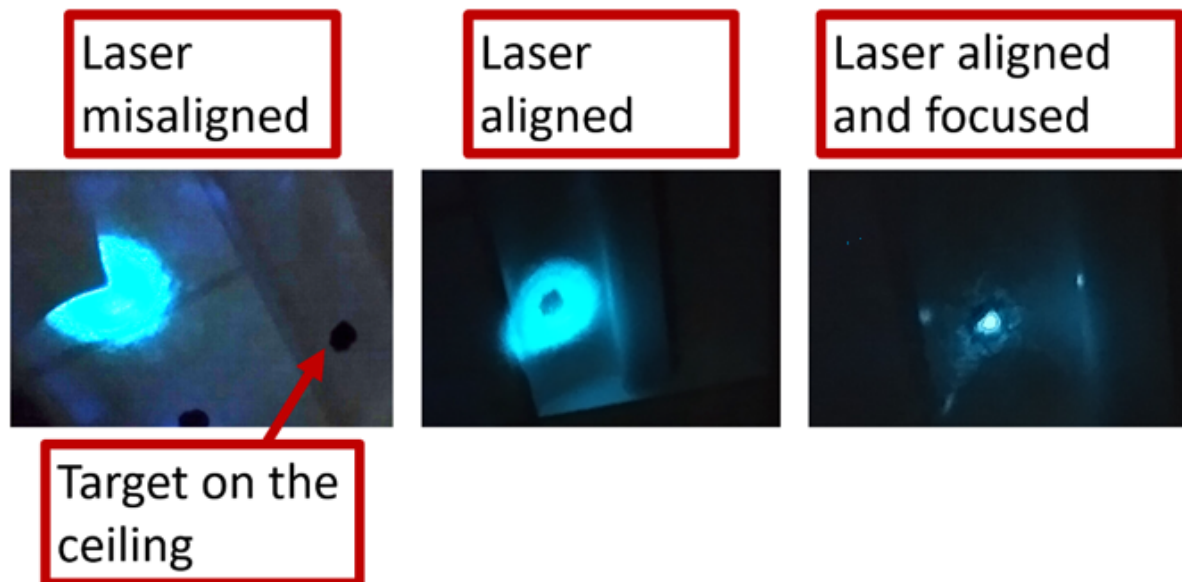
Click to adjust X angle

Click to adjust Y angle

Click to adjust focus

Click to change the speed of the mouse wheel

When you turn on the TIRF laser (using the controls in the OC Panel) it will be misaligned and out of focus, far from the blue tape target on the ceiling straight above the microscope objective. Use the X and Y alignment directions to bring the beam centered on the target on the ceiling and then use the focus button to focus the beam in the center of the target.

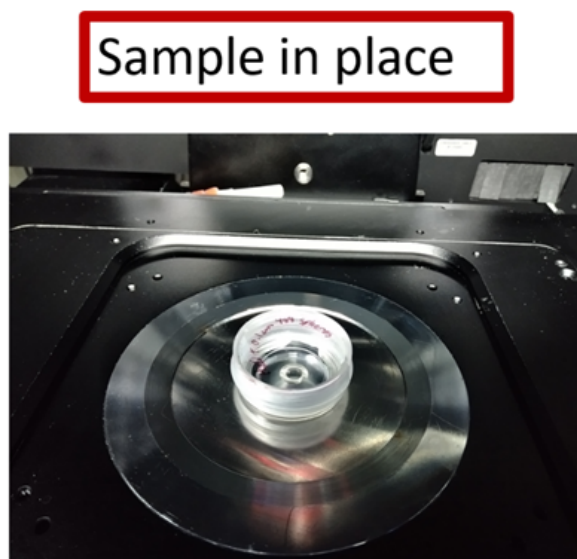


IMPORTANT NOTE: to be able to scroll the mouse wheel to control the TIRF laser you MUST click somewhere on the Ti2 N-STORM Pad. If you happen to click somewhere else on the Nikon Elements software, the mouse wheel won't be connected to the TIRF laser control anymore.

STORM Imaging

At this point in your training it was time to look at the sample. We used the demo sample made of fluorescent beads in water. 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](#).

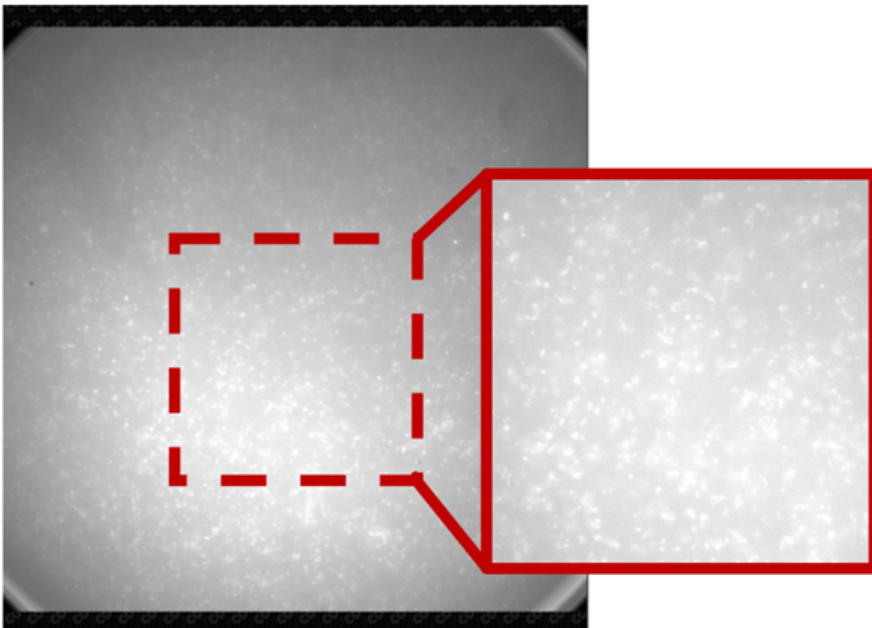
You are now ready to delicately place the sample on the sample plate holder.



- Bring the sample into focus

1. 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.
2. 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.
3. Click “Live” in Nikon Elements to see what the sample looks like and adjust the focus using either the external knobs or the controls in the software. The demo sample has many beads floating in water, you will be able to focus on one plane but won’t see all the beads in focus.

Bring sample into focus



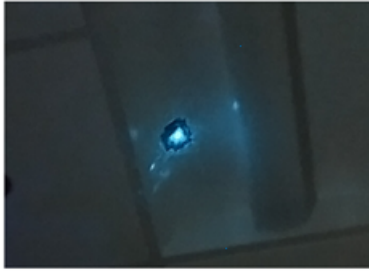
- Re-align the TIRF laser

Following the same procedure as above, bring the laser straight and focused on the blue target on the ceiling.

Laser
misaligned



Laser aligned
and focused



- Finding the TIRF angle

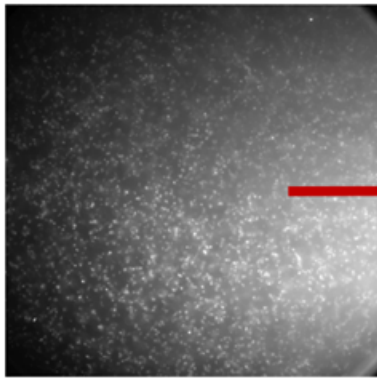
Using the Ti2 NSTORM controls, lower the laser in the direction of the wall until it is almost parallel to the surface of your sample.

Lowering TIRF angle on the wall



While you are lowering the laser beam, keep looking at your sample on the Nikon Elements viewer. As you get close to the critical TIRF angle, the beads will become brighter. When you are just past the critical angle the signal will disappear. You can then adjust the angle using the fine control until you have optimized your signal and it is blinking.

Finding the critical TIRF angle



At the critical TIRF angle the signal increases



Just past the critical TIRF angle the signal disappears

Optimizing the image

- Understanding the Look Up Table (LUT)

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.

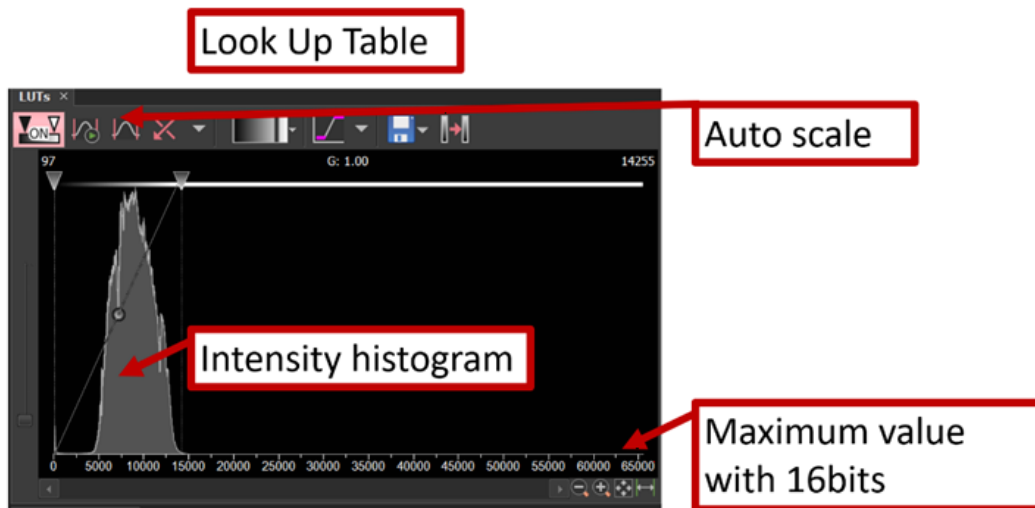


This button will adjust the lookup table for each frame (useful in live view mode)



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 (65535 for the Hamamatsu Fusion which has a 16bits dynamic 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

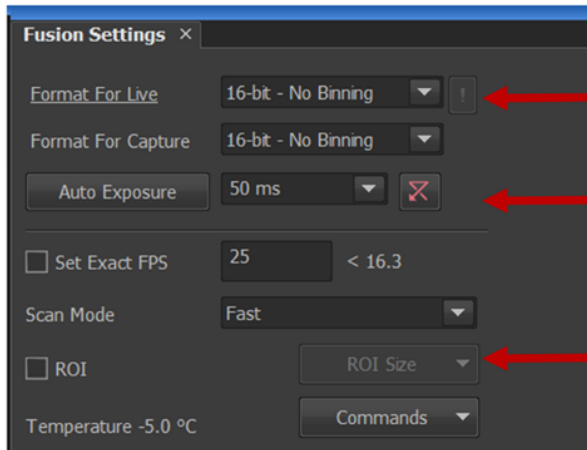
In the Fusion Settings Pad you can adjust the exposure of your image.

For successful STORM imaging you should pick an exposure time under 100ms, 20ms is typical of many STORM experiments.

You can also choose to bin your image (i.e clumping the pixels together to make bigger pixels). The scan mode can be adjusted as well to optimized your data. If you don't see many blinking events you might be scanning your sample too fast.

Adjusting the ROI (i.e Region Of Interest) is a good idea if you do not need to acquire the entire field of view.

Hamamatsu Fusion settings



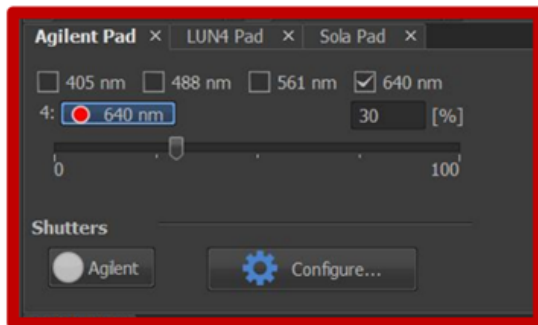
Bit depth and binning

Exposure

Adjust ROI

In the Agilent Pad you can adjust the laser power, expressed as a percentage of the maximum power for each laser.

Adjust laser power



When your image is optimized and you have a good signal with blinking fluorophores it is time to look at the acquisition settings.

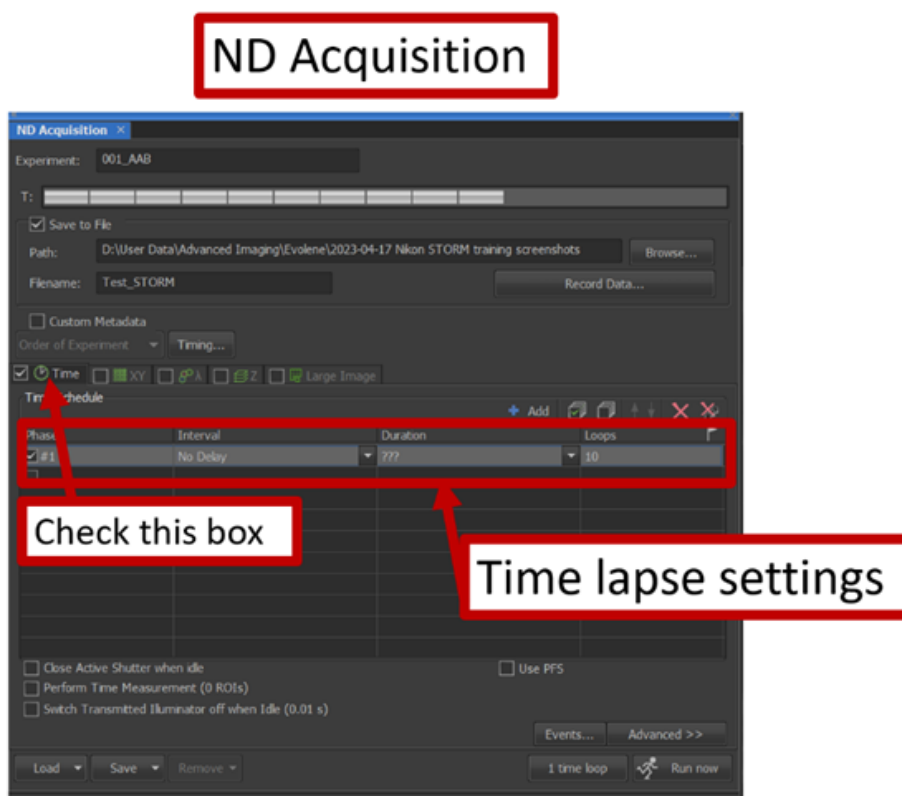
Image acquisition and saving

The Nikon Elements software offers many options for acquiring and saving your data. For STORM experiments you will typically want to acquire a time lapse image stack.

- Acquiring a time lapse image

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 Time.
- b. Then you can define your acquisition settings. The number of loops is the number of images in your time lapse stack.
- c. When you are ready to image click “Run now”



Nikon NSTORM - 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 as it has microscope oil on the bottom.

Place sample on a kimwipe!!



To clean the objective please refer to the [instructions](#) of Dr. Dragavon.

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

- Save your beautiful images and transfer them to your shared drive or flash drive.
- Remove your sample from the stage and clean up the microscope (remove oil from the objective, put any trash in the garbage, wipe with ethanol any areas that may have been contaminated...). Manually lower the objective to a position below 1000 as indicated in the software.
- Exit Nikon Elements and disconnect from your shared drive.
- Open the NSTORM reservation calendar. If there is a user within 2 hours of your session, leave the microscope on. If the next user is more than 2 hours after your session, turn off everything.

Turn off the Microscope power Strip #2

Turn off the laser

- Deselect the laser lines on each box
- Turn the key(s) to the OFF position
- Turn off the Laser Power Strip #1

Turn off the computer

Nikon NSTORM

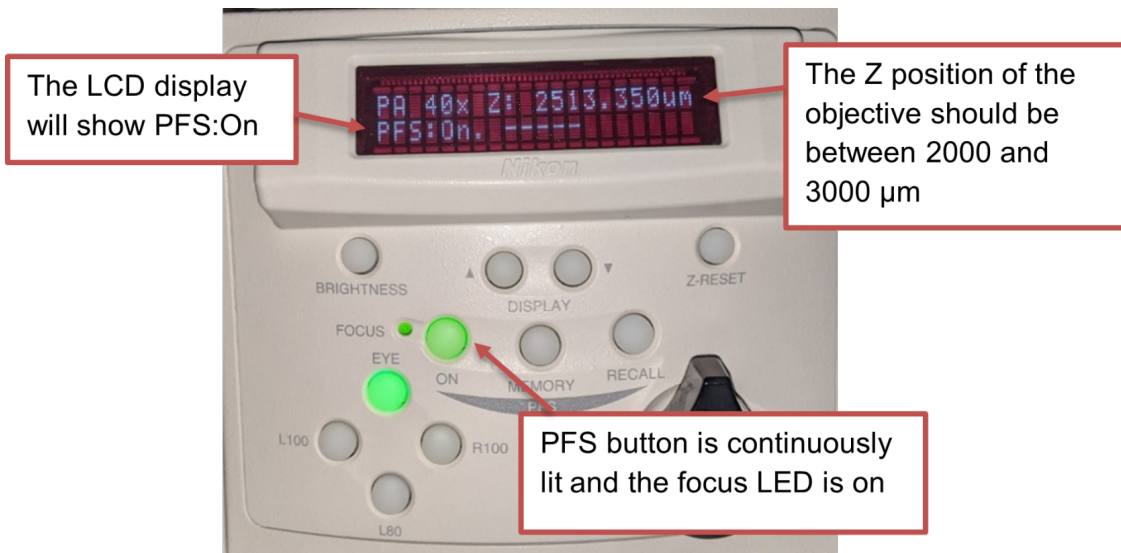
Specifications

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.

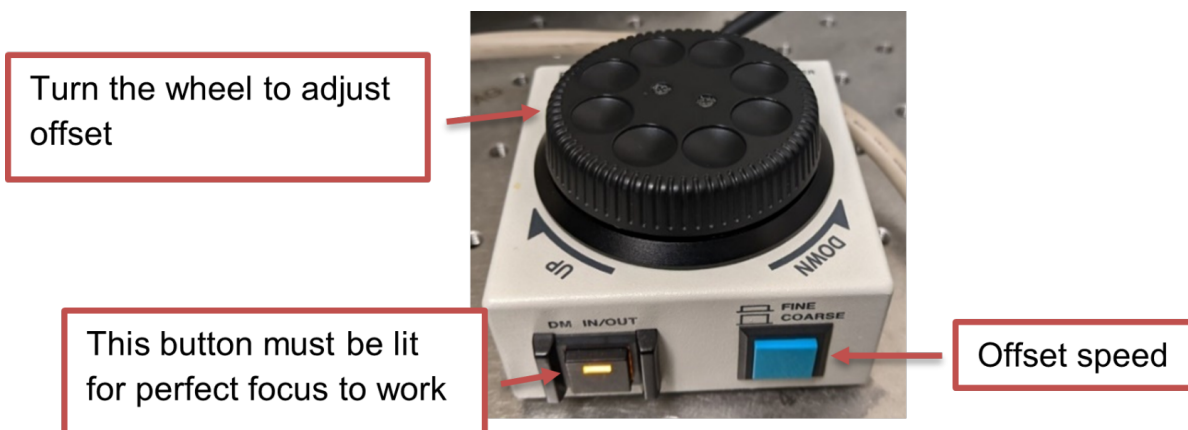
Engaging the PFS


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.



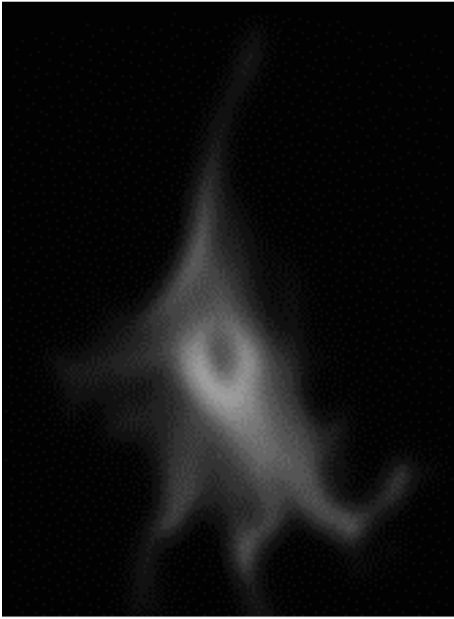
Refining the perfect focus offset

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.

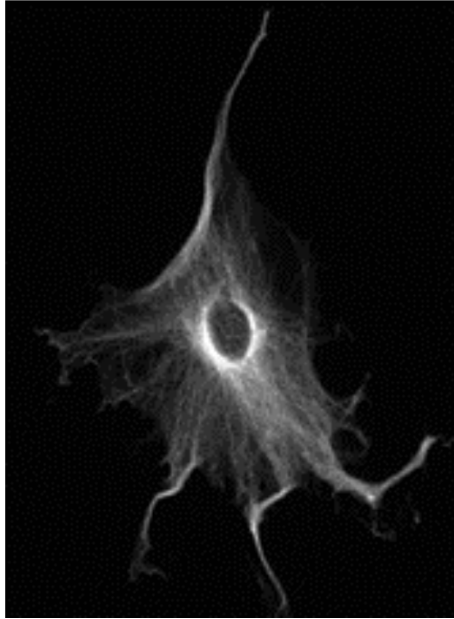


1. Start Live View.
2. Adjust the perfect focus offset by rotating the perfect focus wheel.
3. The blue button can be used to change the offset change speed. When the button is pressed in, turning the wheel changes the offset a little bit. When the button is out, the turning the wheel changes the offset 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."