Black Widow

Nikon Inverted Spinning Disk Confocal Microscope
User Guide

v. 1.0 (06/2015)

Objectives

10x/0.45NA Plan Apo λ
20x/0.75NA Plan Apo λ
40x/0.95NA Plan Apo λ with coverslip thickness correction collar

100x/1.45NA Plan Apo λ **OIL**

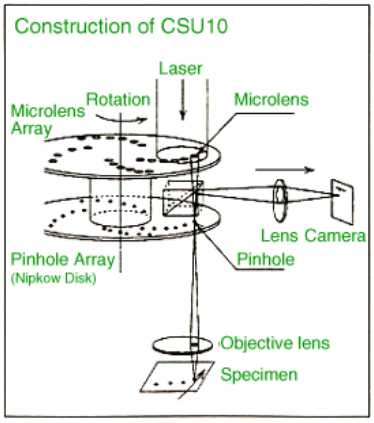
Standard coverslip thickness 0.17 (#1.5 coverslip)

Figure 1

Lumencor Sola light engine (ocular and widefield)

Brightfield, Dapi, Fitc, Texas Red, Cy5

Nikon LUn4 Type A Solid State Lasers
with Yokagawa CSU10B Spinning Disk
405, 488, 561, 640



Cameras

SDC - Andor iXon Ultra (DU-897) EM-CCD camera - 16 μm x 16 μm pixel
WF - Hamamatsu ImagEM (C9100-13) EM-CCD camera - 16 μm x 16 μm pixel

<u>Both have a 1.5x lens</u> (so effective magnification at 10x is 15x and pixels would be 16 μm / 15 = 1.067 μm with no binning)

$$\text{Micron per pixel} = \frac{\text{16 micron pixel camera x binning}}{\text{Objective magnification x 1.5 x zoom lens}}$$

Contents:

Quick Guide:

1) Turn on components in order. Only turn on laser lines you need.
2) Log into Windows with your IdentiKey and start NIS Elements Acquisition software.
3) Put sample on scope coverslip down. If using oil immersion, do not switch back to air objective. No oil on air objectives!
4) Find and focus your sample through the oculars.
5) Acquire images, timelapses, Z-series, etc.
6) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!
7) Leave the microscope on 10x objective for next user.
8) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Server.
9) Close NIS Elements.
10) Log use in Excel sheet, save and close.

CHECK THE MCDB CALENDAR:

➔ **If someone is signed up immediately**

11) Log off Windows.

➔ **If someone is signed up within the next two hours**

11) Log off Windows.

12) Switch OFF all laser lines by pressing the colored buttons.

➔ **If no one is signed up within the next two hours or you are the last of the day:**

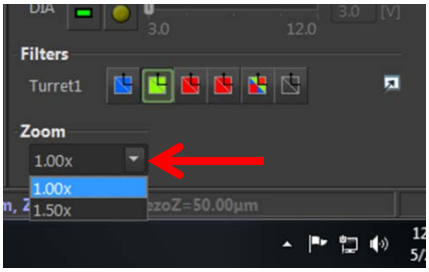
11) Shut down system in reverse numerical order. (Start → Shut down PC, turn keys #4 and #3 to off position, pick up remote and aim to turn off FRAP, #2 , and #1.)

12) Cover the microscope taking care not to put the cover over the cameras or liquid light guide on the back right of the microscope.

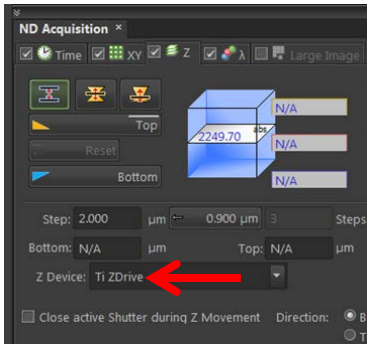
Quick Hints:

- ➔ Focus knob not responding?
  - o Is Perfect Focus on? It must be off to use the focus knob.
  - o Is the speed set to coarse?
  - o Have you removed your sample, hit refocus on the right side of the microscope, and adjusted from there?

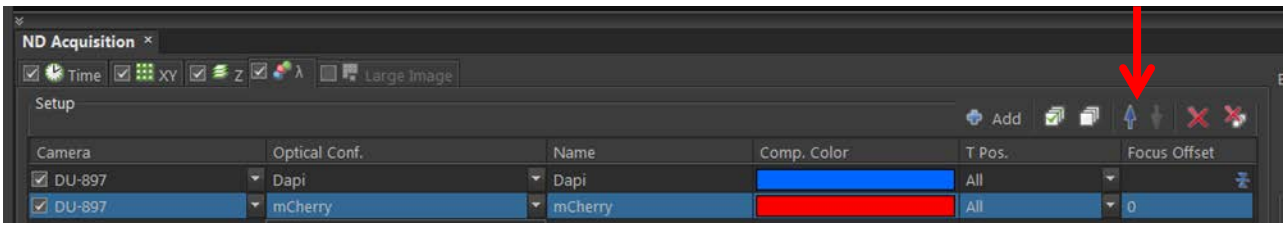
- ➔ Make sure the zoom lens on the front of the microscope base is set to 1x and set to 1x in the software.

- ➔ Make sure the Z drive selected for ND Acquisitions is the Ti ZDrive (and not the Piezo). Normally you will not see this option at all. If you do see it, please let Jolien know.



- ➔ For stitching, make sure that the first color (lambda) you acquire is one with enough detail and structure to permit accurate stitching. Use the arrows to adjust the color acquisition order if needed.



Starting Up the System:

➔ Numerical order

1) Pick up little remote and **aim** to each white box in outlet, and press on (the "I" button). Do this for 1, 2, and FRAP.

2) Turn the key on laser box to on position. Colored lights indicate that the lasers are on and at temperature. Press the colored buttons to turn off lasers you will not be using to preserve laser life span. If you need to turn any lasers back on later, there may be a delay as the laser will not restart if it is still hot.

3) Turn the key on spinning disk unit on left side of microscope all the way to "Laser on" position.

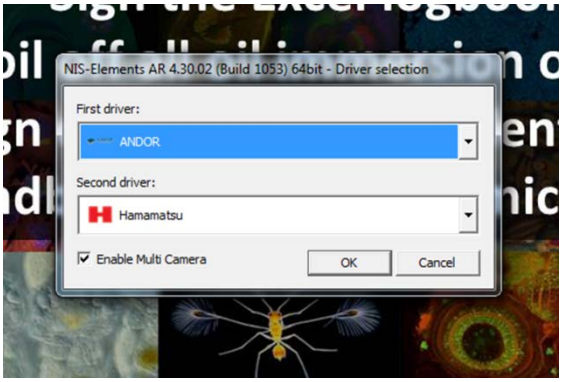
4) Turn on PC.

5) Login to Windows with your IdentiKey.

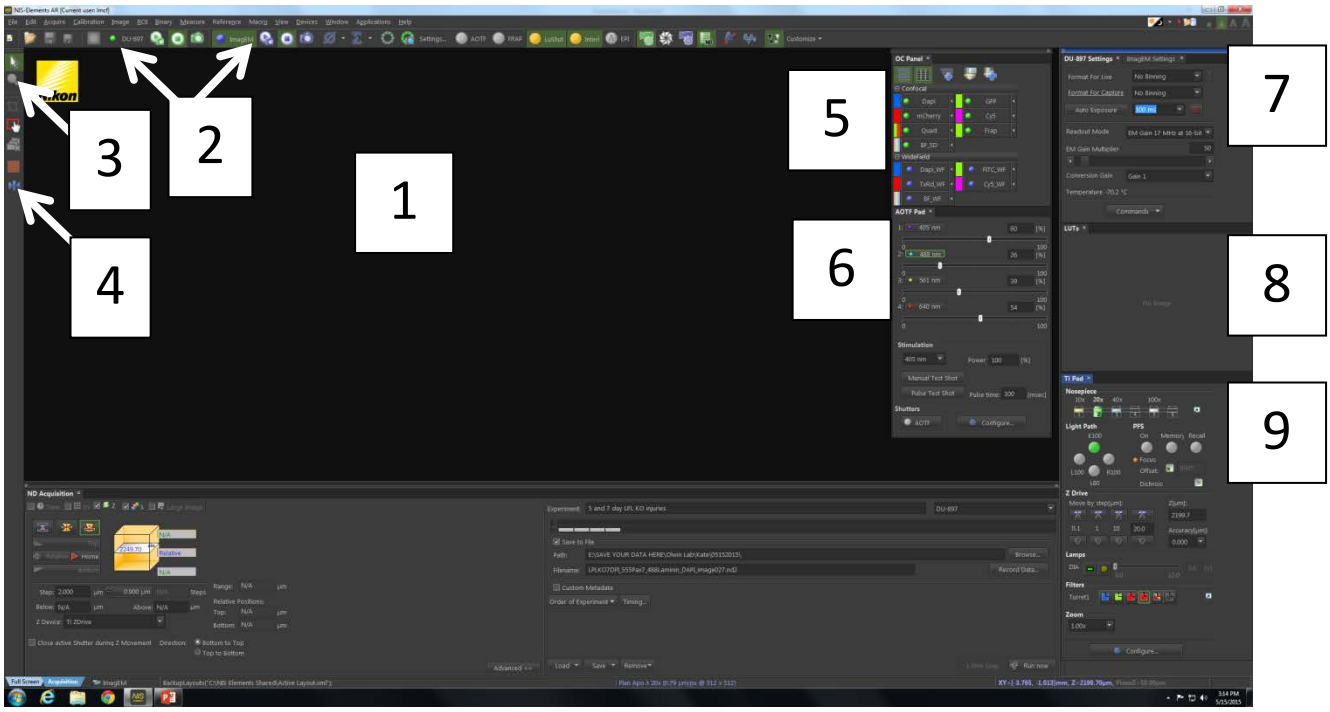
6) Open NIS Elements Acquisition software (not the Off-line shortcut).

(Side note – to look at your images without booting up the microscope, just turn on PC and use the Off-line NIS Elements shortcut. We also have a full off-line license for NIS Elements on separate PC – "the Hulk.")

7) Click OK to launch with both cameras enabled – First Driver: Andor and Second Driver: Hamamatsu.



The NIS Elements User Interface:



1) Open/live images will display here.

2) Camera toolbar – Select live, stop or snap for the appropriate camera here – DU-897 for confocal and ImagEM for widefield/oculars.

3) Magnifying button – Use to mouse over your image and magnify the display.

4) Restore defaults button – Click this is restore default layouts and OC configurations.

5) OC Panel – Preset configurations for confocal imaging to the Andor camera and widefield imaging to the Hamamatsu camera. Use widefield OCs for illumination to the eyepieces.

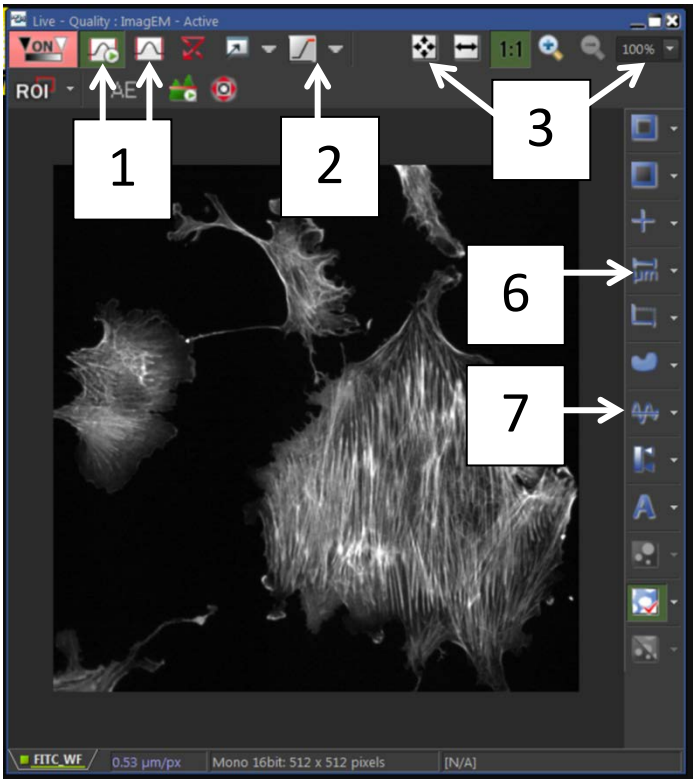
6) AOTF Pad – Laser intensity sliders for confocal imaging. The active laser will be highlighted when a confocal OC is selected.

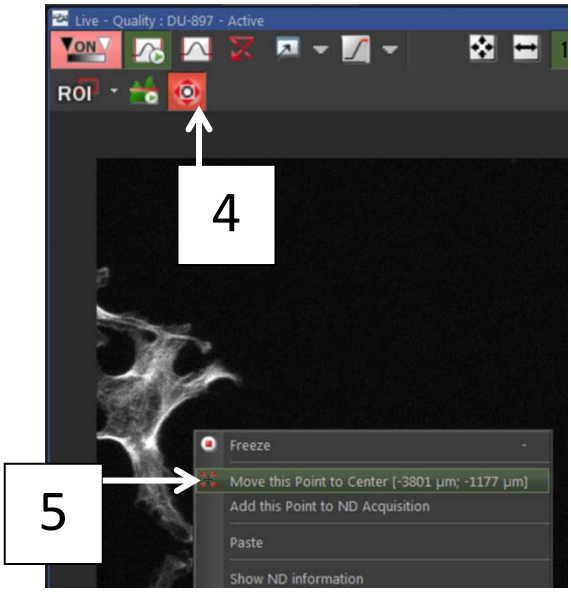
7) Camera Settings Tabs – Choose “DU-897 Settings” for the Andor camera used for confocal imaging and “ImagEM Settings” for the Hamamatsu camera used for widefield imaging.

8) LUTs – The lookup tables for open images will display here.

9) Ti Pad – Use these options to select objective, light path (L100 for confocal, E100 for oculars, R100 for widefield), and brightfield lamp settings. Other options should not be changed (like filter turret position) as these are part of the OC configurations.

The NIS Elements image window:





1) Autoscaling: Press the right autoscale button to scale the image once and press the left autoscale with play button to enable continuous autoscaling of the image.

2) Pixel saturation indicator: Press this button to display over- and under-saturated pixels in color on your image window.

3) Zoom and image window size buttons.

4) Mouse X,Y button: Click here and then click and drag in your image window to move the live image instead of using the joystick. Do not adjust the Z focus at the same time as moving the stage in X, Y.

5) To move a specific spot to the center of the field of view: right-click inside the image window on the spot to move to the center and select "Move this Point to Center." Do not adjust the Z focus at the same time as moving the stage in X, Y.

6) Scale bar button and options.

7) Line profile button and options.

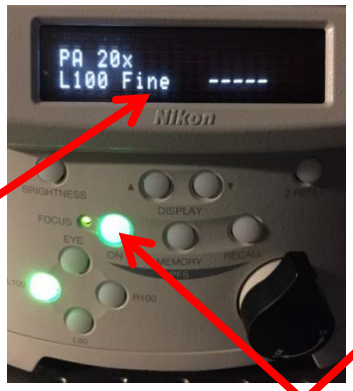
➔ Mouse scroll wheel over image window will change zoom. Do not adjust the Z focus at the same time as moving the stage in X, Y.
➔ Press X to create a non-quantitative snapshot of the image as displayed on the screen

How to look at sample through oculars:

1) Load the correct objective in position.
   - Objectives can be changed by selecting the desired lens in the software.
   - If you are using an air objective, start with 10x at low focus position.
   - If you are using oil immersion, place a small drop of oil on the slide before placing it on the microscope. **Do not switch back to an air objective**. Turn the focus knob until the objective lens is just touching the oil.

➔ <u>If the focus knob is not responding:</u> make sure that it is set at the correct speed (Coarse – Fine – ExFine), that Perfect Focus is not engaged (no green button lit up on the front of the microcope), and that the previous user has not hit the Escape button on the side of the microscope.

Focus speed is displayed on the front microscope panel and on the joystick control.

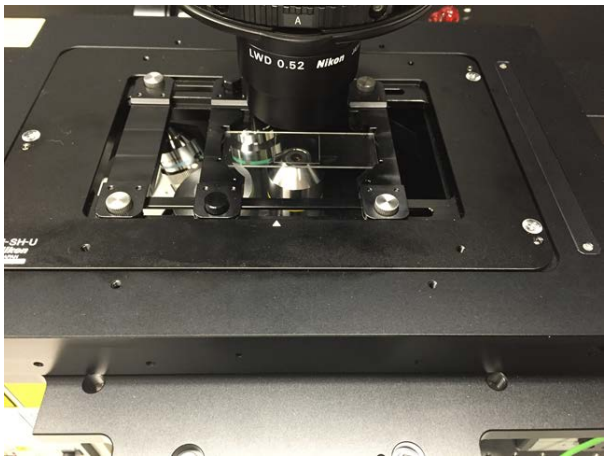


For focus knobs to work, perfect focus (PFS) cannot be engaged. Push "ON" button to turn it off if the button is lit up.

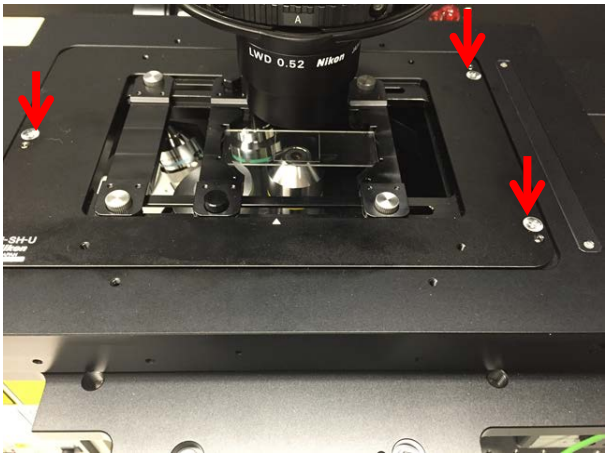
If the previous user used the Escape button, the focus knob will not respond. Remove your sample, hit the refocus button, adjust as needed, and replace your sample on the stage.

2) Adjust stage position with the joystick if needed. To change speed of joystick, gently rotate the top portion clockwise to reduce speed and counterclockwise to increase. The current speed is denoted by the orange light on the joystick (Coarse – Fine – ExFine).

3) Place the slide, coverslip down, onto the slide holders on the stage. If you need more working space, gently lean the top pillar backward. Please be sure that your slide is clean and dry – do not get wet mounting media, sealant, or condensation on the stage. Ensure that your coverslip is clean and dry (free from salts).

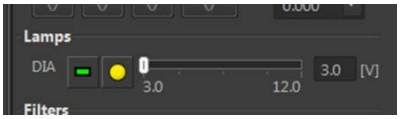


➔ To switch stage insert to the multi-well plate holder, pull down the storage box with inserts and tools. Carefully lean the top pillar back, unscrew the three clear screws using the provided screw driver, remove the universal holder, place the multi-well plate holder on the stage, reattach the three clear screws. <u>NOTE: when you finish, please remember to switch back to the universal holder.</u> Do not over-tighten the clear plastic screws.

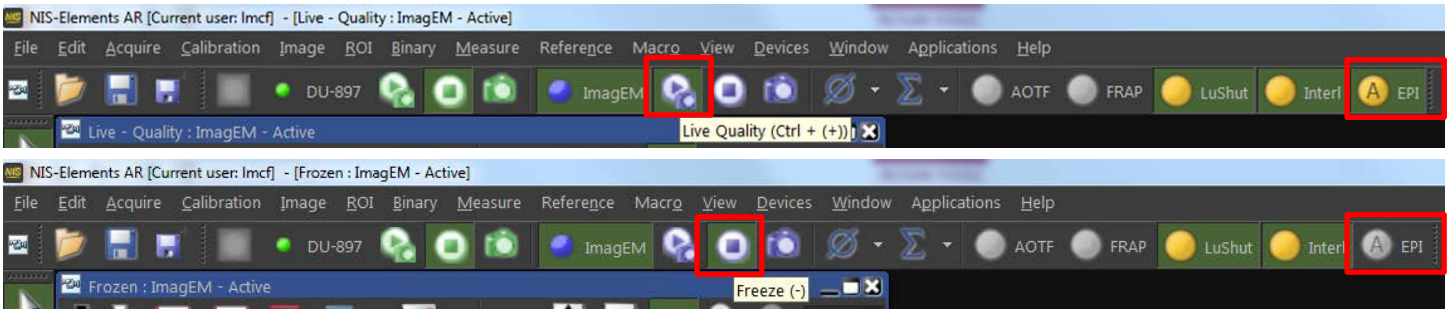


4) Select appropriate widefield illumination optical configuration (OC) – DAPI_WF, FITC_GFP, TxRd_WF, Cy5_WF, or BF_WF. Widefield optical configurations default by sending fluorescence light to the oculars (E100).

- To adjust brightfield lamp intensity, use the DIA slider at the bottom. See Appendix B for instructions on Kohler alignment of the brightfield.



5) To open shutter to send light to sample, click the EPI button or Live on the ImagEM camera controls (short cut: Ctrl+ for live quality or just + for fast live). To turn off, click EPI button again or Stop on the ImagEM camera controls (shortcut: - to stop).

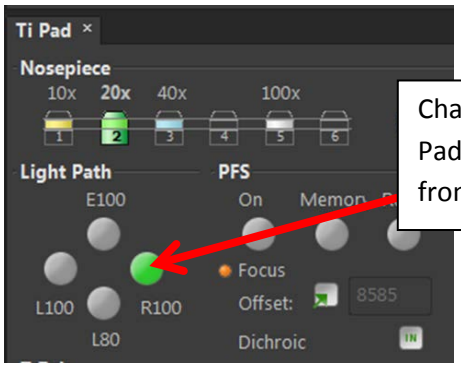


6) Adjust the focus using the focus knob on the joystick or the knobs on the sides of the microscope. To adjust focus speed, press the button on the right side of the joystick to switch from Coarse – Fine – ExFine.

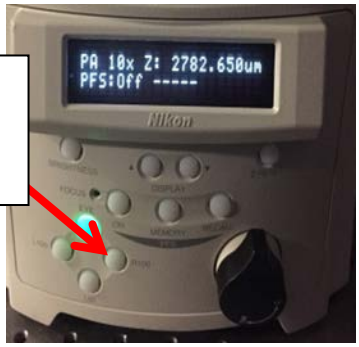
7) Switch objectives, refocus, and reposition to get the field of view you want.

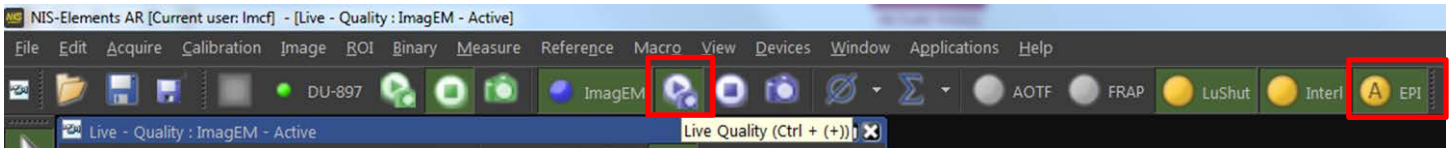
Taking a Widefield Image using the Hamamatsu EM-CCD:

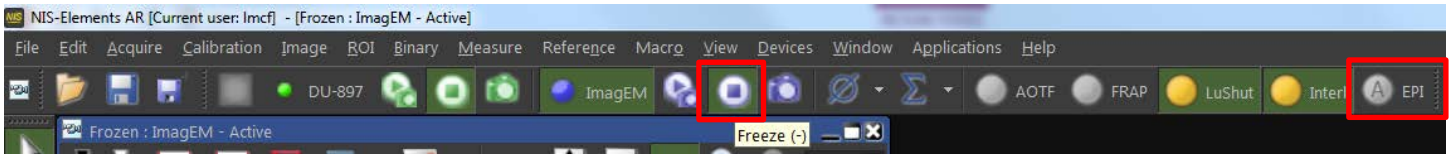
1) Select desired Widefield OC, send the light to the R100 port, and click Open EPI or Live button (short cut: Ctrl+ for live quality or just + for fast live). To turn off, click EPI button again or Stop on the ImagEM camera controls (shortcut: - to stop).



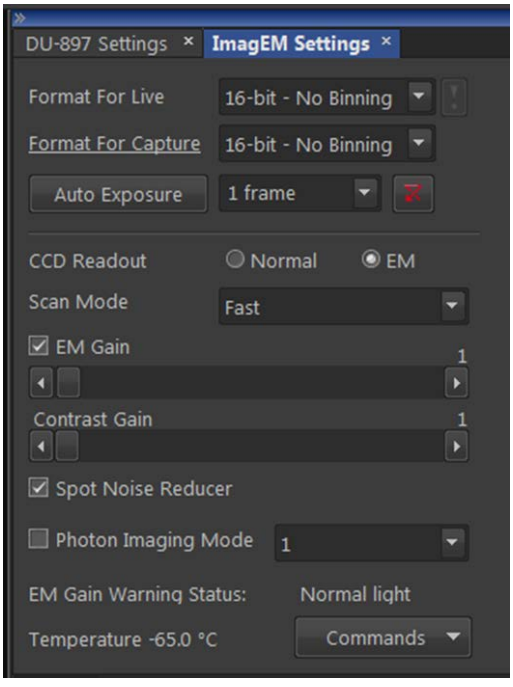
Change light path direction in Ti Pad area of software or on the front panel of the microscope.



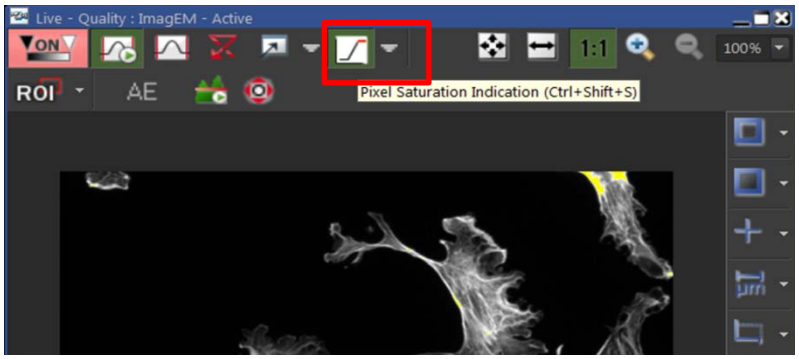




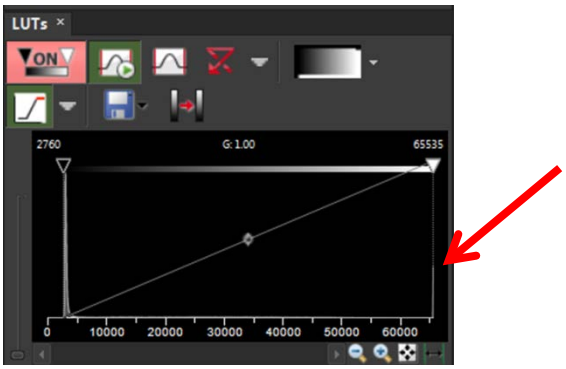
2) Adjust ImagEM camera settings. The Sola light engine is very bright, so for most samples you will use short exposure times and low or no gain. If you hear a beeping noise from the camera, that means you are **way** oversaturating the camera chip. Stop the live exposure, adjust settings down, and restart live. Oversaturating the chip will, in the long run, lead to damage.



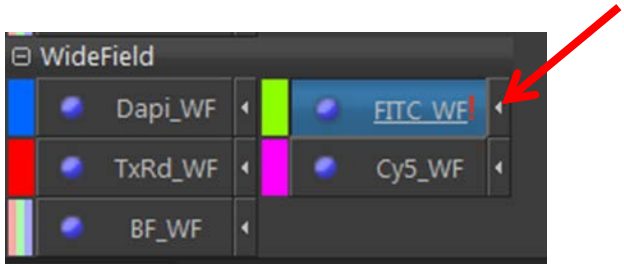
3) To view saturated pixels in the live image window, click on the pixel saturation indication button. Click on the arrow next to this button to change how under- and over-saturated pixels are displayed. In the image below, oversaturated pixels are shown in yellow.



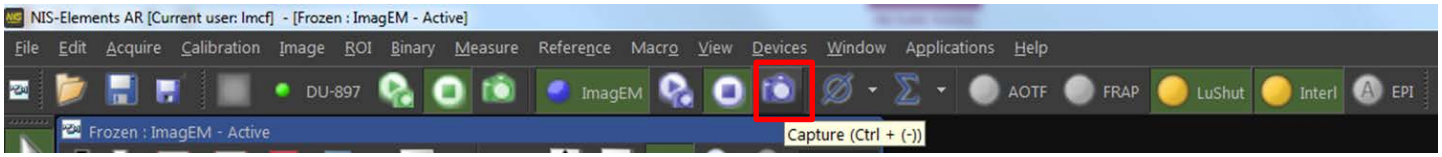
Over-saturation can also be observed in the LUTs panel on the right side of the screen.



4) Once you have created the correct settings for that color, click the arrow next to the OC button to lock in the settings. Repeat these steps for all colors of interest.



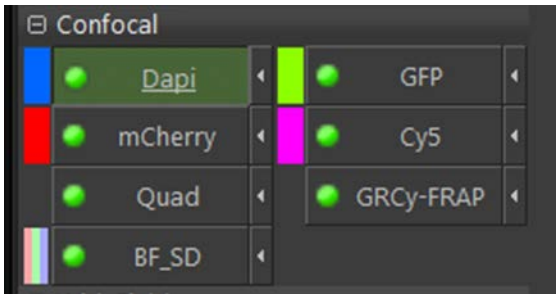
5) To capture a single plane image in one color, click the snap button for the ImagEM camera (<u>shortcut</u>: Ctrl- to snap a single image).



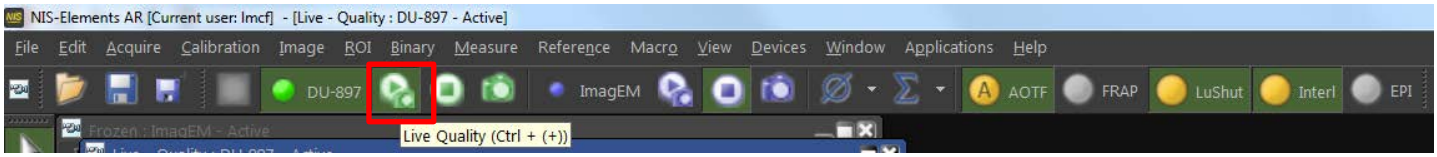
6) To capture multiple timepoints, XY positions, Z positions, wavelengths, colors, or a montage, see section on ND Acquisition set up.

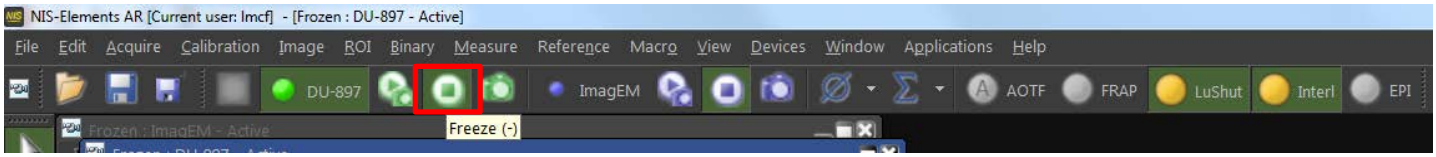
Taking a Confocal Image using the Andor EM-CCD:

1) Select desired Confocal OC, send the light to the R100 port, and click Open EPI or Live button (short cut: Ctrl+ for live quality or just + for fast live). To turn off, click EPI button again or Stop on the DU-897 camera controls (shortcut: - to stop). These OCs will always go to the left port. Laser interlocks will prevent sending light to the eyepieces from the confocal OCs.

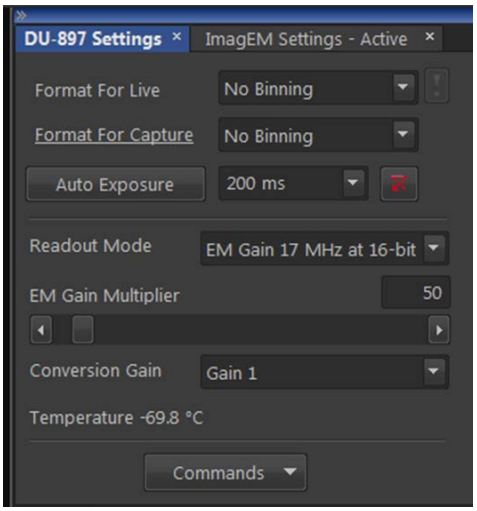


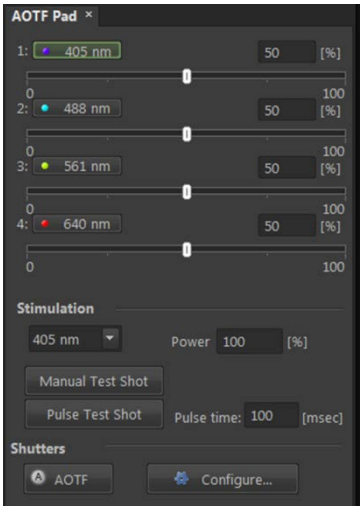
DAPI, GFP, mCherry, and Cy5 go through single pass emission filters. **Quad** is used for Triggered Acquisition – see later section in this guide. **GRCy-FRAP** is used for FRAP – see later section in this guide.





2) Adjust DU-897 camera settings. Adjust laser power on AOTF Pad (you can go up to 100% if necessary). Adjust camera exposure time, readout mode, EM Gain multiplier (do not go over 300 gain), and Conversion Gain. Start at conversion gain = 1 and lower EM Gain and try higher if necessary, but overall SNR is reduced at higher gain multiplication. Try to get a SNR ratio of 20:1 for fixed samples and 2.2:1 for live dynamics, although this may not always be possible. Look at LUT to determine min and max values or mouse over image.





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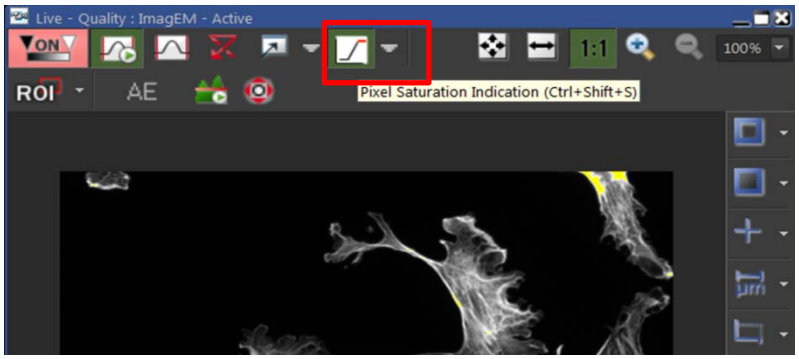

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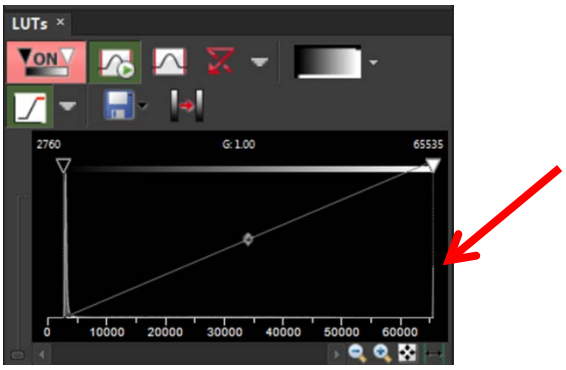

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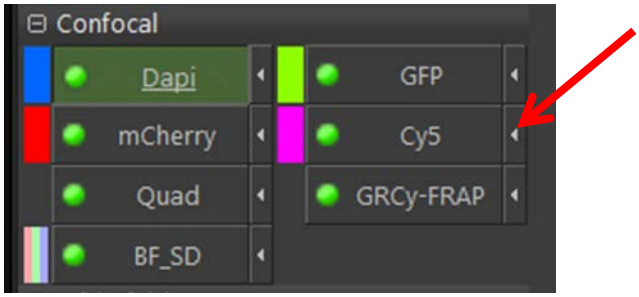
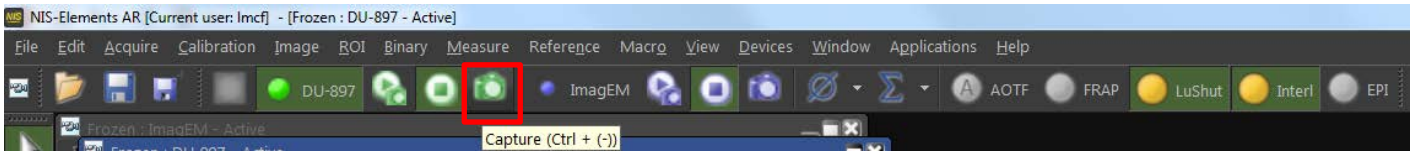


5) To capture a single plane image in one color, click the snap button for the DU-897 camera (shortcut: Ctrl- to snap a single image).

6) To capture multiple timepoints, XY positions, Z positions, wavelengths, colors, or a montage, see section on ND Acquisition set up.

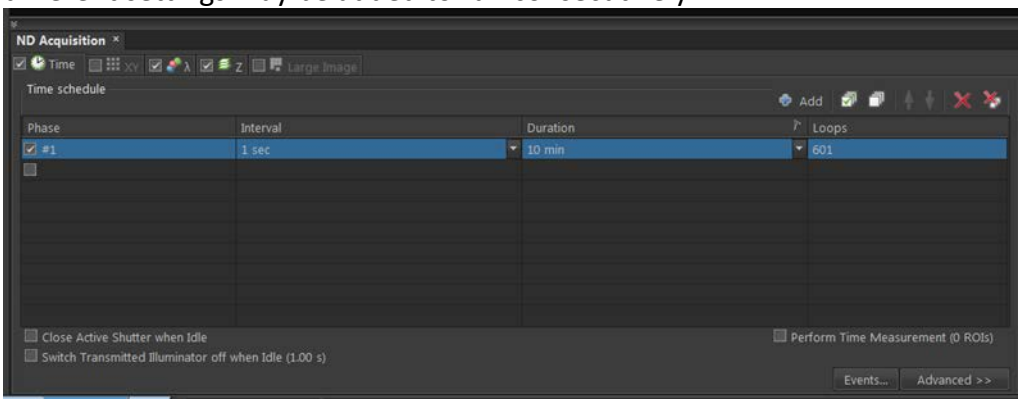


Setting up and Acquiring an ND Acquisition:

After configuring the OCs to your liking for all colors you want to image, set up the ND Acquisition. At the bottom of the screen is the ND Acquisition window that is used to capture images in multiple dimensions.

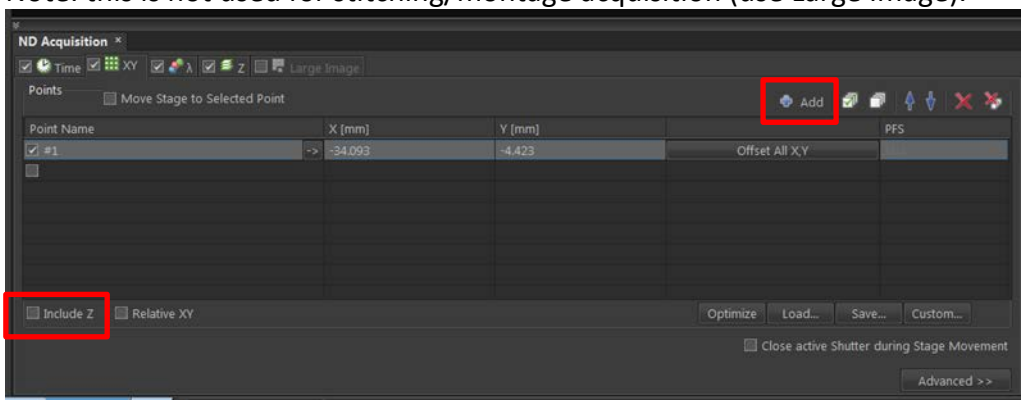
Options (uncheck those you don't want):

1) Time – choose the interval and duration of the desired time course. Multiple phases with different settings may be added to run consecutively.

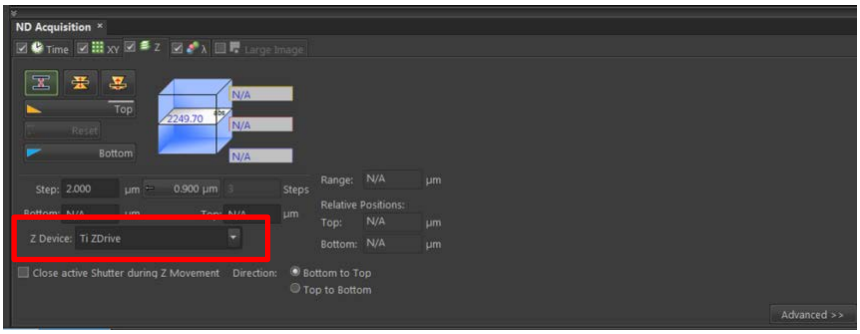


2) XY positions – Once the sample is positioned correctly, click "+Add" button. Check the "Include Z" box to also lock in the Z position. Find a different field of view, and click "+Add" button to add. Continue until all desired positions are locked in. Check the "Include Z" option to also lock in Z coordinate.

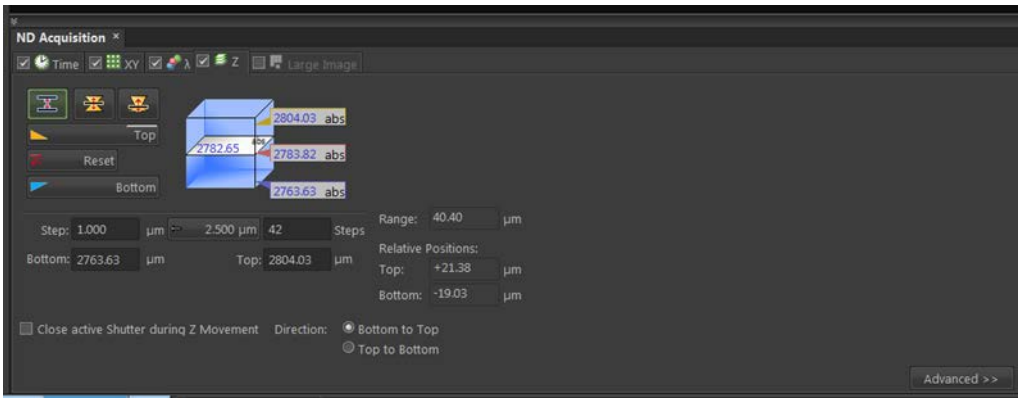
➔ Note: this is not used for stitching/montage acquisition (use Large Image).



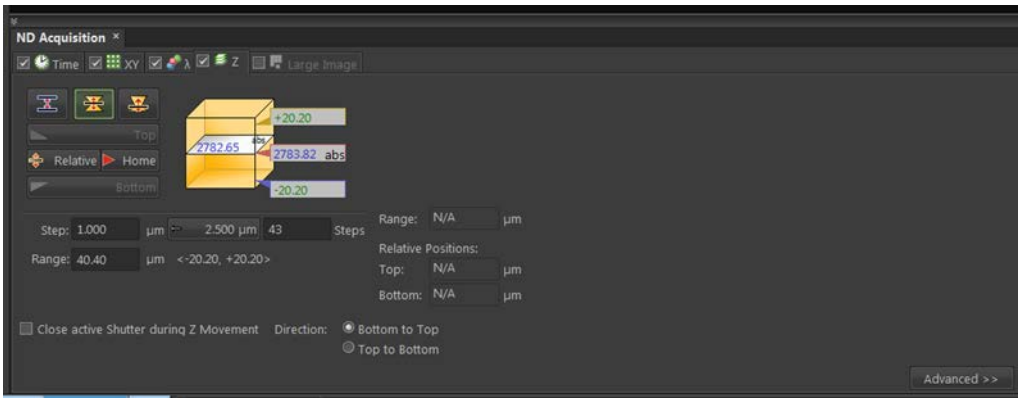
3) Z – **NOTE – VERY IMPORTANT –** The Z Drive must be the Ti ZDrive. You should normally not see this option at all. If this is visible, please let Jolien know.



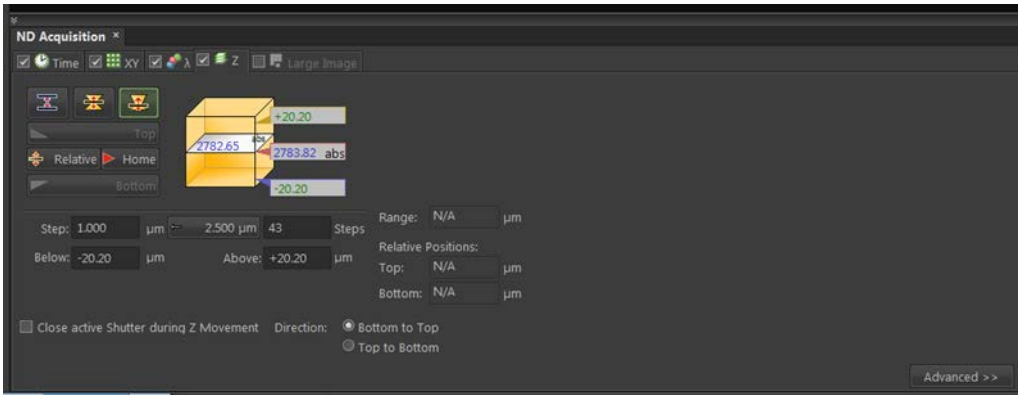
a. Z- Set top and bottom – With the image window live, adjust focus knob to go to one extreme, click Top, go to other extreme, click Bottom. This will set the range to acquire. Then set the step size or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For faster acquisition, uncheck the "Close active Shutter…" option.



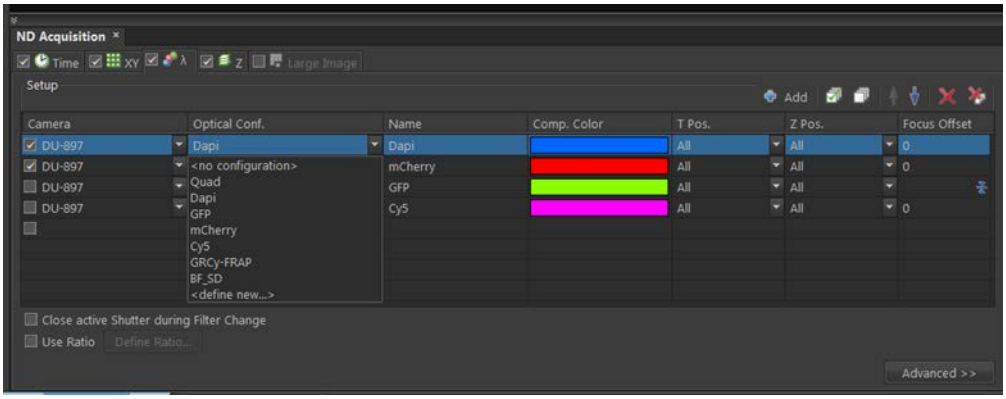
b. Z – Range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. Then set the step size, total range, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For faster acquisition, uncheck the "Close active Shutter…" option.

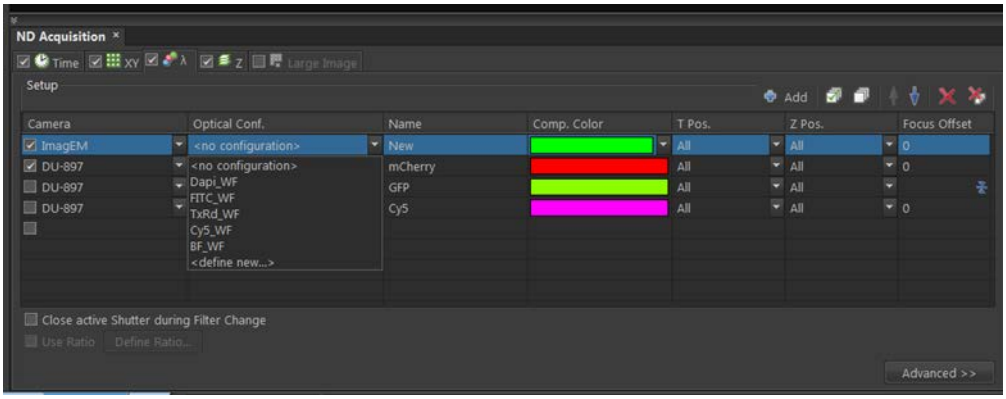


c. Z – Asymmetric range around center – With the sample focused in the center plane, click the "Home" button to set the center home position. Then set the step size, range to acquire above, range to acquire below, and/or number of steps. The software will tell you what the optimal Z step is for the objective selected. Click on this button to use the suggested optimal step size. For faster acquisition, uncheck the "Close active Shutter…" option.

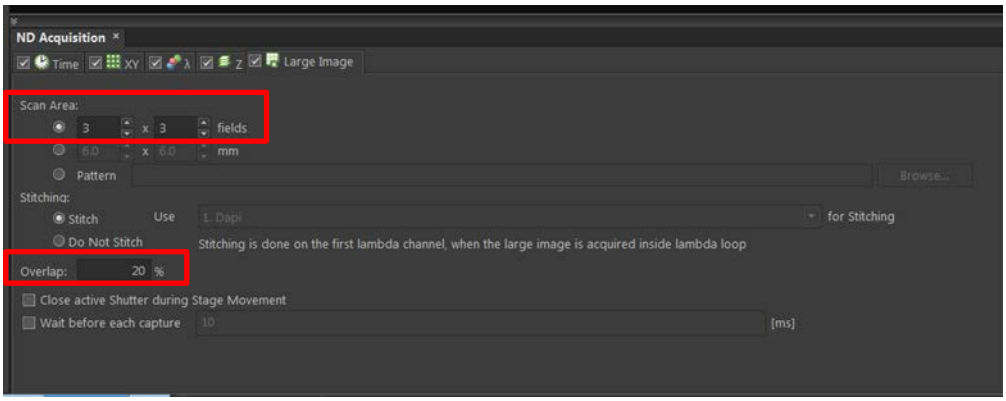


4) <u>Lambda</u> – Select the appropriate camera – DU-897 for confocal and ImagEM for widefield. Then select the desired optical configuration (OC) from the drop down. Select if you want to acquire this wavelength at all Z positions or just a subset of the total planes. You should already have dialed in settings for these OCs.

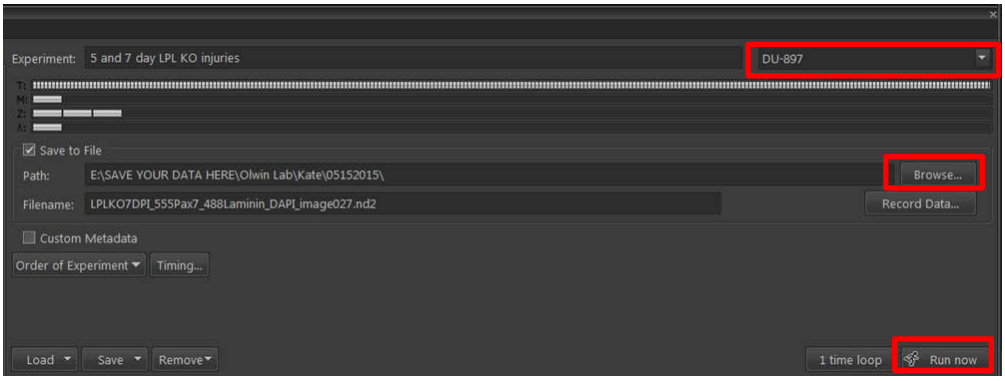




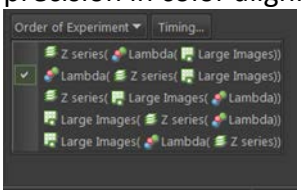
5) Large Image – Use this option to generate montages, with or without stitching. Position your sample in the middle of the region you wish to stitch. Set the number of fields of view you wish to include and check or uncheck Stitch. Select the percent overlap (typically 10-20%). If you do not select stitching, the image can be stitched after acquisition by another program like ImageJ/FIJI. Stitching works best for samples with sufficient detail overlapping the multiple regions to permit the software to determine how to align the images.



6) Right side – Name your experiment. Choose the camera you are using from the drop down. Check or uncheck save to file. If you check save to file, select the correct file path and file name. File path must go to the E:\ drive (NOT C:\).



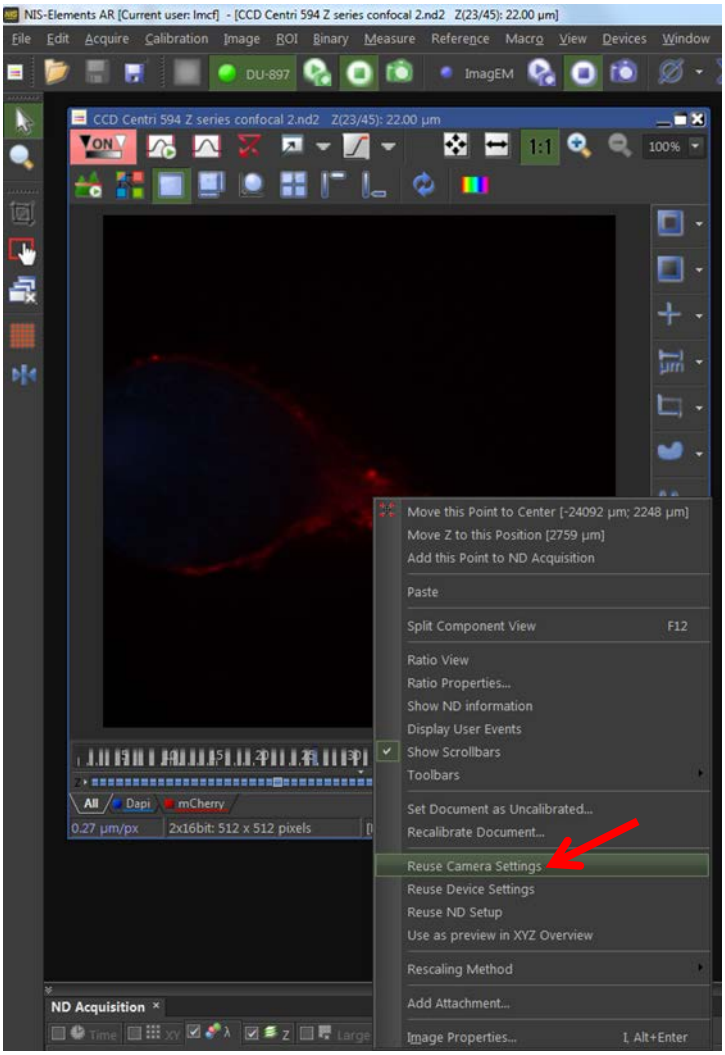
7) Order of Experiment – Choose the order in which images should be acquired. Read from right to left, the stuff inside the parenthesis happens first. As shown below, it will be fastest to acquire all montage images at a single z position, then all z positions, and then switch color. You will not be able to interact with the image window during acquisition. For greater precision in color alignment, take each color before moving X, Y, or Z.



8) When you're ready, click "Run now" to acquire.

Reusing previous settings:

Open a previously saved image, right click anywhere on the actual image, and from the list of options choose “Reuse camera settings,” “Reuse device settings,” and/or “Reuse ND Setup.”

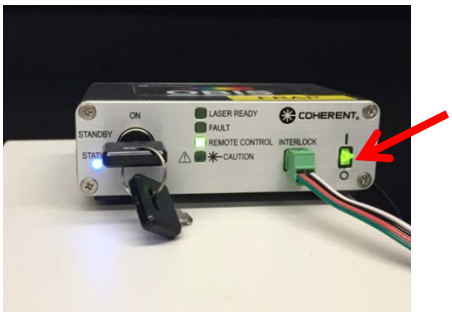


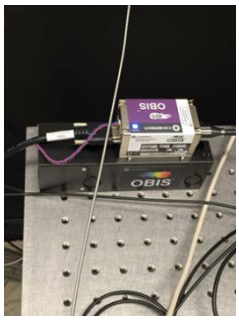
FRAP/Photoactivation Using the 405 nm Coherent OBIS Laser:

- ➔ **FRAP imaging can only be done to the confocal camera and through a multi-band emission filter.** Multi-color imaging may lead to channel bleedthrough, so be sure to do the proper control imaging.
- ➔ **FRAP can only be done with imaging of 488 nm, 561 nm, 640nm. No 405 nm since the FRAP laser used for photobleaching or photoconversion is 405 nm light.**

1) Turn on microscope as normal.

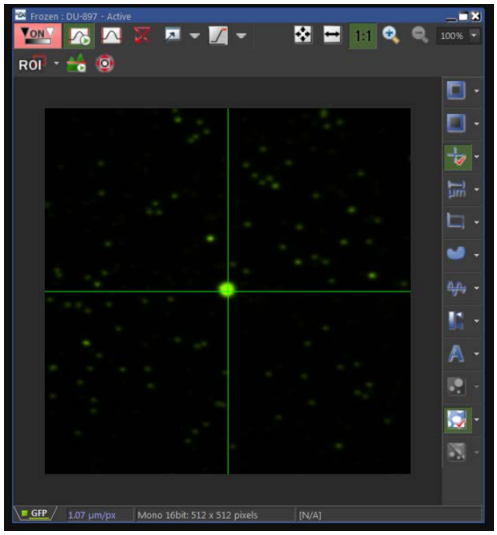
2) Turn on FRAP laser switch (do not turn the key yet).



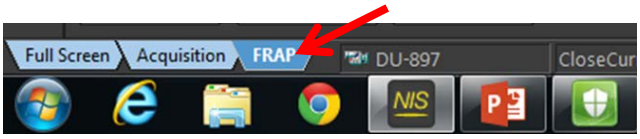


3) Find and focus on your sample as normal.

4) Turn on crosshairs and position region of interest in center of crosshairs (middle of image). Easy way to do this – right click on position of interest and select “Move this position to center.”

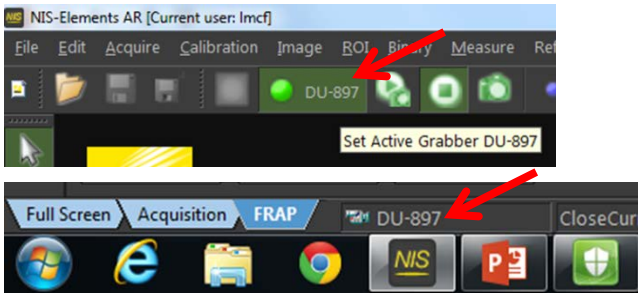


5) Switch to FRAP tab at the bottom of the screen.

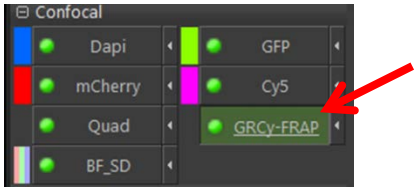


6) Turn the key on the FRAP laser box. From this point forward, no imaging through the oculars. If you do switch back to oculars, this will trip the FRAP laser interlock. To reset the interlock you will need to turn the key to Standby and then back to On.

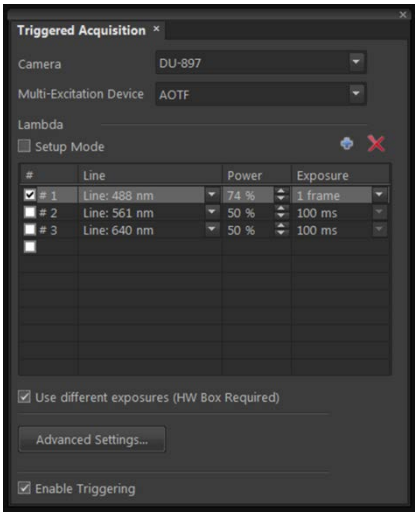
7) Set the active grabber to DU-897 by clicking on the camera name at the top (or just make sure it is already set to DU-897).



8) All FRAP imaging will be done using the GRCy5-FRAP OC. This OC uses the quad pass emission filter.



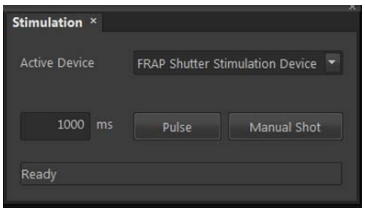
9) Use the Triggered Acquisition panel to select the laser line(s), laser power(s), and exposure time(s). You can't use 405 nm. The readout speed and gain on the DU-897 Camera Settings is the same for all channels. Once you've selected the proper channels and settings, click on the white arrow next to the GRCy-FRAP OC to lock these in.



Three options:

Manual:

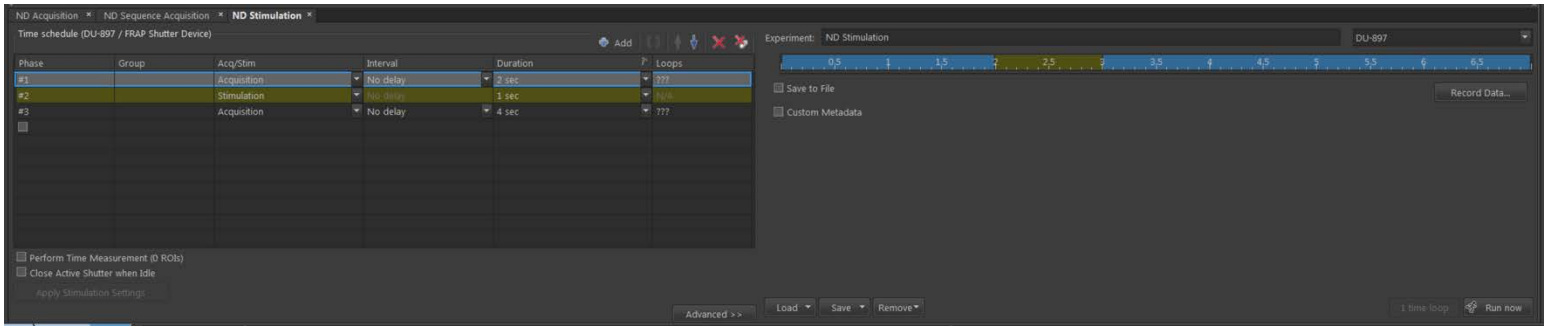
➔ On Stimulation Pad, with the active device set at the FRAP Shutter, use either Pulse and type in a pulse length in milliseconds or Manual Shot which will expose for as long as you hold down the button.



ND Stimulation:

➔ Just images the single focal plane (no Z stacks) and uses the lambda as set up in the Triggered Acquisition/GRCy-FRAP OC.

➔ In the ND Stimulation tab at the bottom, set up the action schedule you would like to image. Typically this consists of #1 Acquisition (pre-bleach), #2 Stimulation (bleach), and #3 Acquisition (post-bleach). Choose "Save to File" if desired and click "Run now" at the bottom right.



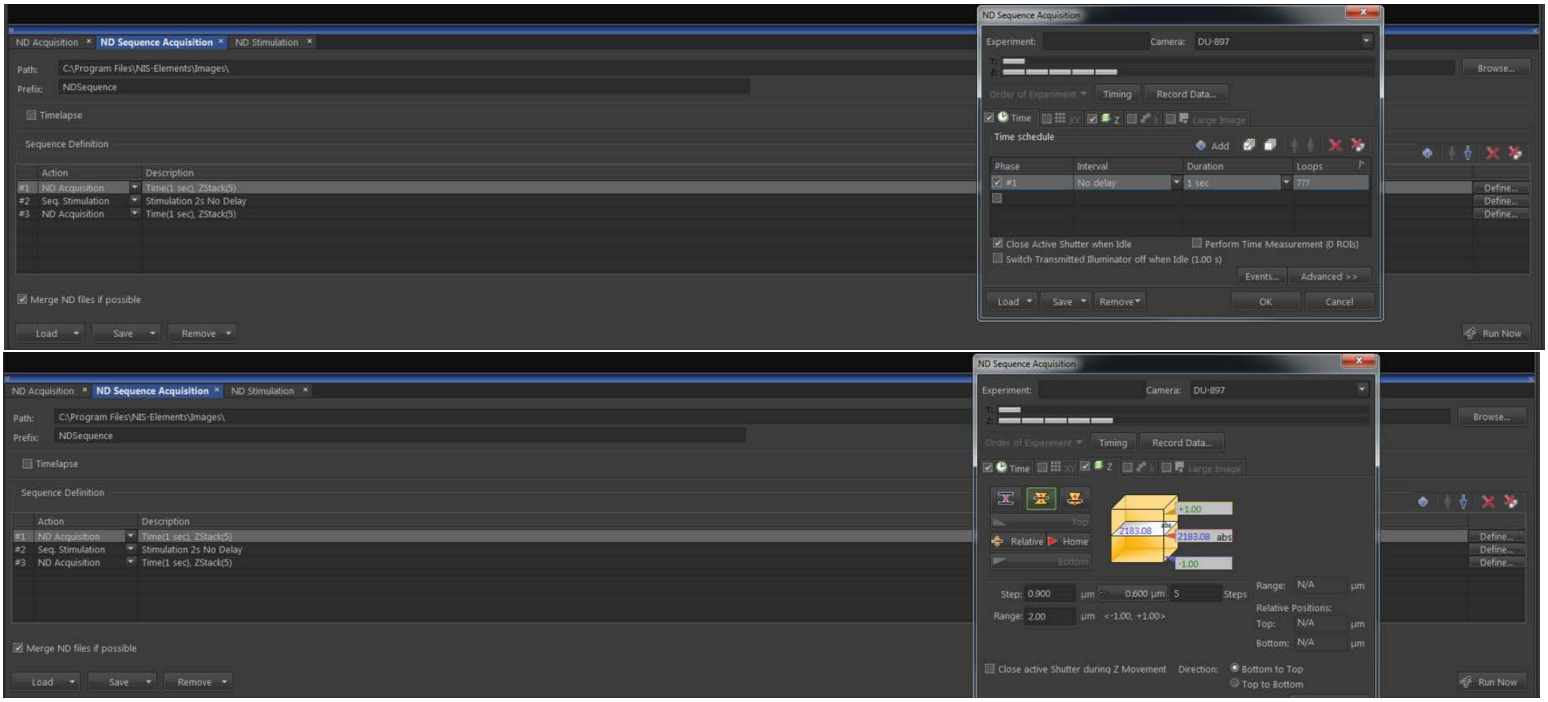
ND Sequence Acquisition:

➔ You can set up Z series & time points to take during acquisition using this mode. Probably the most useful option.

➔ Choose a file save path and file name. This option automatically saves – you must select a file path.

➔ In the ND Sequence Acquisition tab at the bottom, set up the three imaging actions #1 ND Acquisition (pre-bleach), #2 Seq. Stimulation (bleach), and #3 ND Acquisition (post-bleach).

➔ For each action, define the parameters by clicking “Define…” on the far right. This will bring up a window that looks the same as the normal ND Acquisition window. Set up time (how long and how often do you want to acquire images?) and Z range. No lambda (that’s done through the Triggered Acquisition panel). Remember that the microscope needs enough time to acquire the Z stack, and if you set the time interval shorter than that required time, it will just run without delay.



➔ Check on the “Merge ND files if possible.” This will combine the pre- and post-bleach acquisitions, assuming you used the same time and Z conditions for both.

➔ All set? Click “Run now” in bottom right.

Acquiring Triggered Acquisitions:

Triggered acquisitions are useful for high-speed imaging, especially in multiple colors. This allows fine control of the laser exposure and the camera readout to minimize over-/under-lap.

Triggered acquisitions can only be done in confocal mode. Note – since this uses a quad emission filter, it is possible to have bleed-through from one channel to the other. Be sure to do proper controls (imaging in single pass OC mode) to make sure what you see is not due to bleed-through/ cross-talk.

1) Find and focus on your sample as normal.

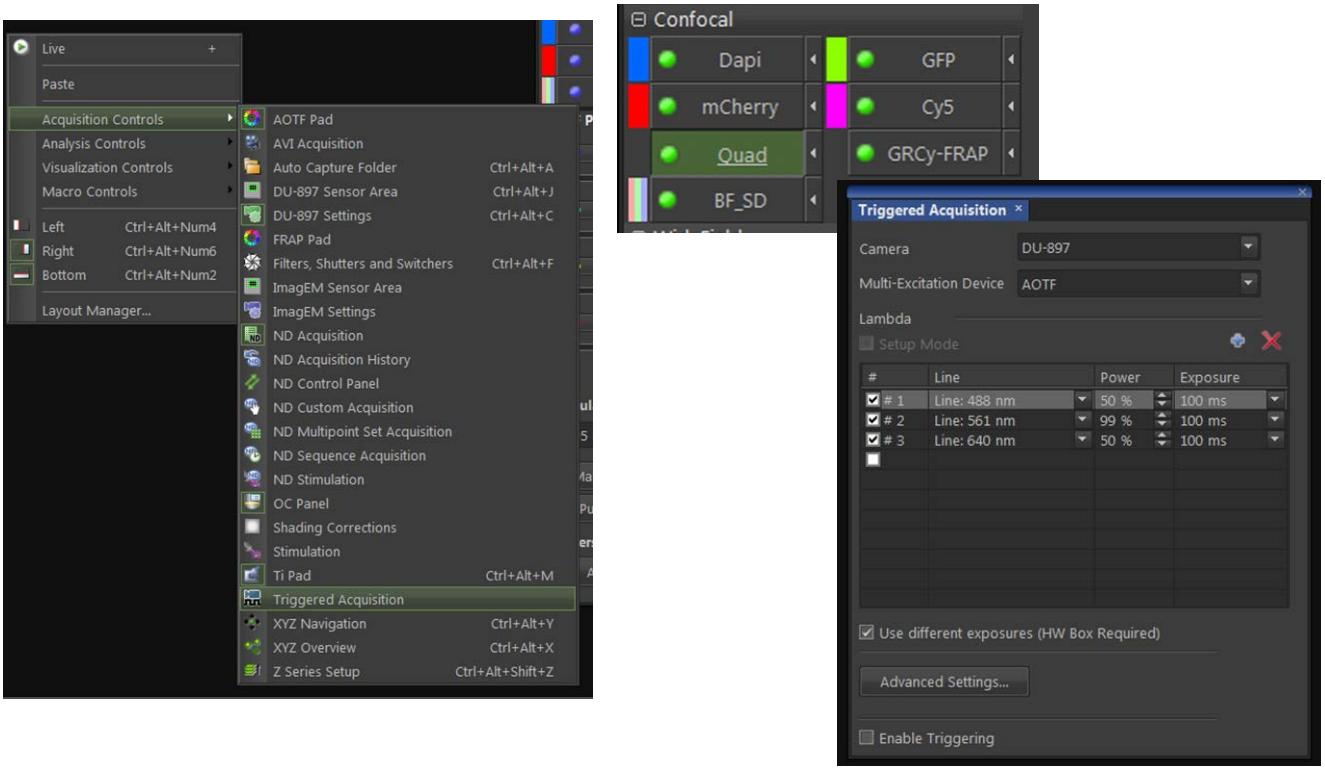
2) Bring up the Triggered Acquisition GUI. Right click on black background, select Acquisition controls, and select Triggered Acquisition from near the bottom of the list.

3) Select the Quad OC from the confocal panel.

4) Select the laser colors, power and exposure time. Gain remains the same for all channels (from DU-897 camera controls).

5) After setting laser lines, power and exposure time, click on white arrow next to the Quad OC button to lock in colors and settings.

6) In the ND Acquisition window, do not select the lambda tab. Select other parameters (time, Z series, etc) as desired. Click run now.



Perfect Focus System (PFS):

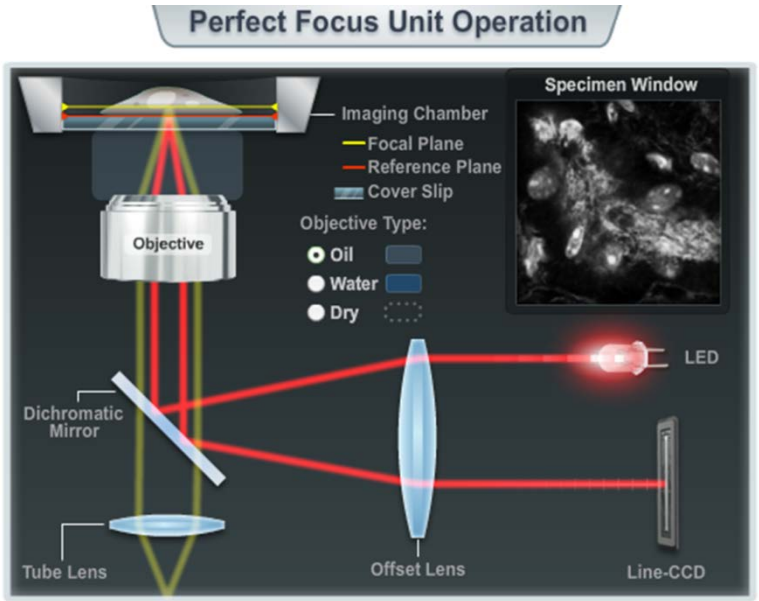
Nikon's Perfect Focus System (PFS) is a very effective method of maintaining sample focus for timelapse experiments or across uneven coverslips. PFS uses a far red LED light to find the sample/glass interface. To use the Perfect Focus:

1) The sample must be close to focused and the green light next to the PFS ON button must be lit up. If it is not on, the PFS cannot find the appropriate interface. This can be the result of a very out of focus sample or not enough difference in refractive index.

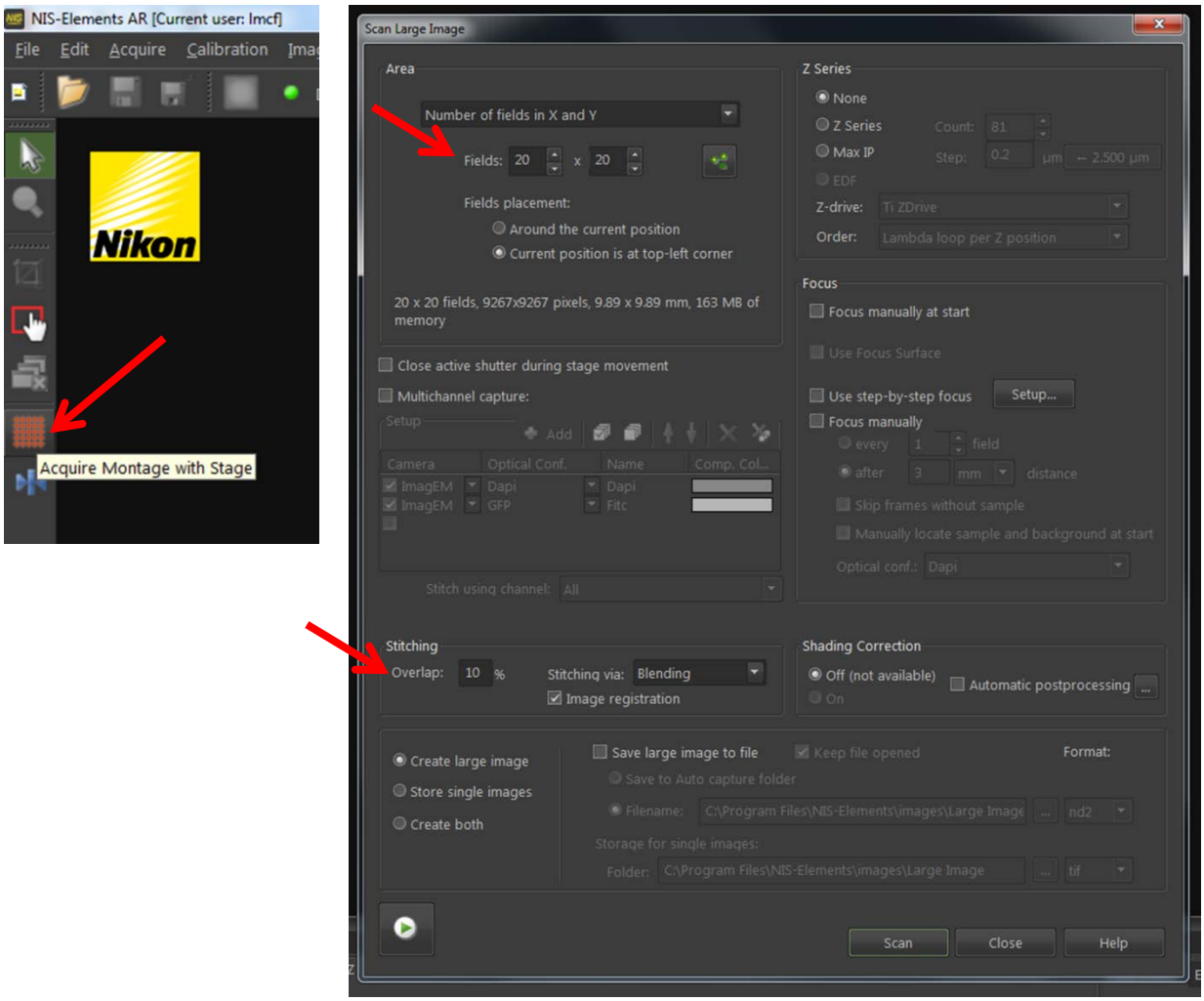
2) Turn on the PFS by pressing the left-most ON button on the front of the microscope. The sample will now go out of focus (the microscope moves to the PFS interface reference plane).

3) The user can then set the "PFS off set" using the PFS focus knob. The knob can be toggled between coarse and fine using the blue button. The normal focus knobs will not function as long as PFS is on.

4) Z-series ranges, etc can be set using the PFS focus knob, just as you would normally use the focus knob.



Scan Large Image:



This is a useful alternative to using the ND Acquisition montage feature. This can be beneficial to scan a coverslip at low magnification, and then focus in on cells of interest at higher magnification.

1) Get all OC settings dialed in as described above (for confocal or widefield).

2) Click the shortcut on the left toolbar (square of small red squares).

3) Choose appropriate settings in the “Scan Large Image” window. This window will let you set the current position as either the top left or center (not an option in ND Acquisition). You can choose multiple colors. Stitching overlap will likely be 10-20%.

4) As long as you do not remove your sample from the stage, the coordinates are remembered. You can then right click on the large image to move that cell to the center at higher magnification for subsequent imaging.

When you're finished:

1) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!

2) Leave the microscope on 10x objective for next user.

3) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Server.

4) Close NIS Elements.

5) Log use in Excel sheet, save and close.

➔ **If someone is signed up immediately (Check the MCDBCal!):**

6) Log off Windows.

➔ **If someone is signed up within the next two hours (Check the MCDBCal!):**

6) Switch OFF all laser lines by pressing colored buttons.

7) Log off Windows.

➔ **If no one is signed up within the next two hours or you are the last of the day:**

6) Shut down system in reverse numerical order. (Start → Shut down PC, turn keys #4 and #3 to off position, pick up remote and **aim** to turn off FRAP, #2 , #1.)

7) Cover the microscope taking care not to put the cover over the cameras or liquid light guide on the back right of the microscope.

APPENDIX A – Saving to your lab's folder on Collie (MCDB Dept Server):

➔ **Questions regarding MCDB Server should be directed to Erik Hedl.**

1) Map the network drive. Right click on Computer icon on desktop or Computer in Start menu. Click on "Map network drive…"

2) Drive letter should be Z: and the folder should be \\collie.int.colorado.edu\<your lab name> (for example \\collie.int.colorado.edu\OlwinLab).
   If your login for Collie is not your IdentiKey, check the box "connect using different credentials."

3) Click finish.

4) If you selected "connect using different credentials," enter your username and password.

5) Click Ok and the drive should now appear. Copy over your files. If you have a lot of files, be sure to allow time for the transfer. Under ideal conditions you will be able to copy close to 42 gigabytes per hour, don't count on ideal conditions. Also, it is safer to copy (not move) your files, then delete after the copy has completed.

➔ You cannot, must not, should not reserve time on the microscopes in MCDB Cal to copy data – reservations are for imaging only.

➔ The LMCF PCs are not long-term data storage places and we are not responsible for lost data.

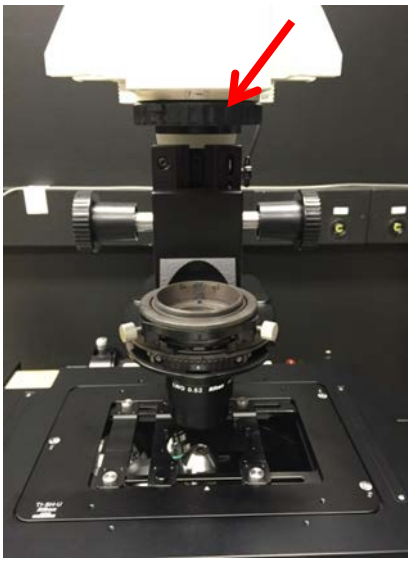
➔ Do not forget to always safely store and backup your raw data – this represents the ground truth of what you acquired that day and has all the associated metadata. Many journals are now requiring that you submit raw data with your manuscript.

APPENDIX B – Kohler Alignment for Brightfield Imaging:

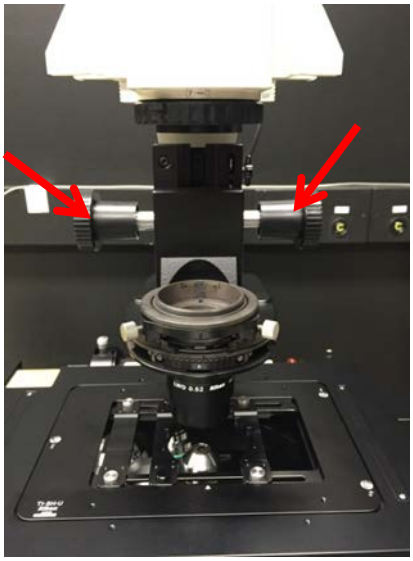
Kohler alignment of the microscope provides optimal performance and contrast of the brightfield diascopic illumination by providing the most uniform illumination of the sample.

1) Focus on sample using Brightfield OC

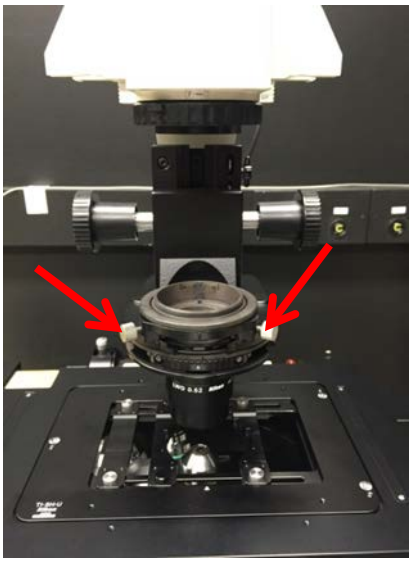
2) Close field diaphragm aperture by rotating as far as possible counter-clockwise.



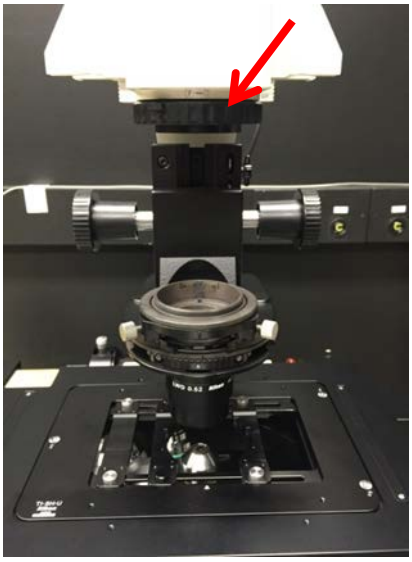
3) Adjust focus of field diaphragm by changing condenser height until the octagon generated by the field diaphragm aperture is in focus at the sample.



4) Adjust positioning screws to center field diaphragm aperture octagon

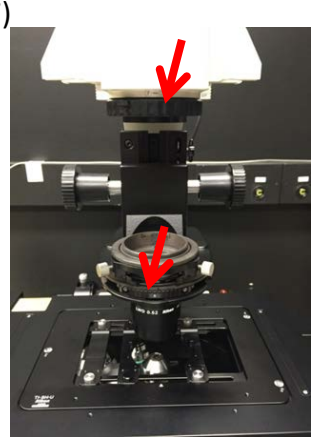


5) Open field diaphragm aperture



6) To adjust brightfield image: adjust the opening size of the field diaphragm and/or iris diaphragm, adjust the intensity of DIA lamp on the Ti pad, and/or adjust the camera settings.

7)



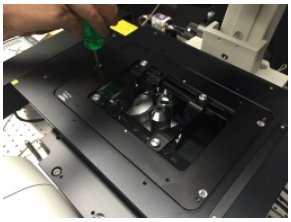
APPENDIX C – Stage Lift and Piezo Z Use:

➔ **Use of the piezo absolutely requires prior authorization from Jolien.**

To Raise the Stage and use the Piezo:

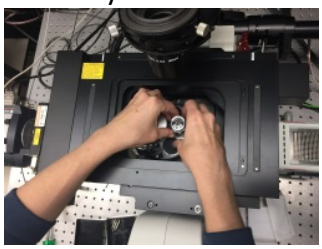
1) Make sure Elements software is not open.

2) Unscrew the three clear plastic screws.



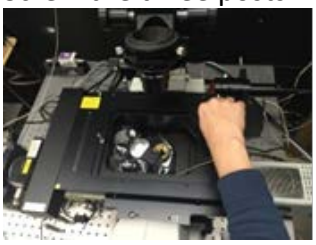
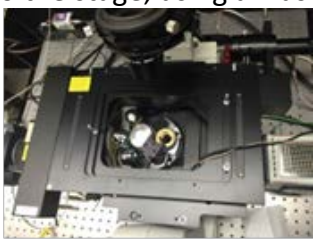
3) Lift out the stage insert.


4) Carefully unscrew the objective and place it in a very safe place.



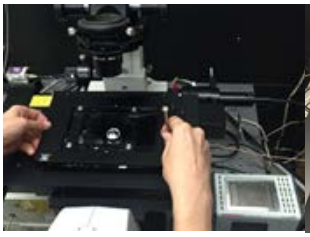
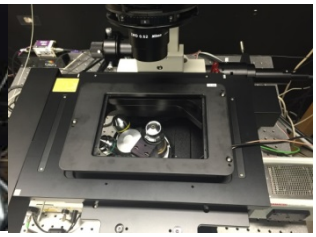
5) Screw the piezo insert into the objective spot. The wires go over not under the stage. Go from ~11 o'clock to ~7 o'clock, clockwise. Make sure the wires are not tight at the base of the piezo unit.


6) Screw the three posts into the stage, using a washer as a spacer for each one.

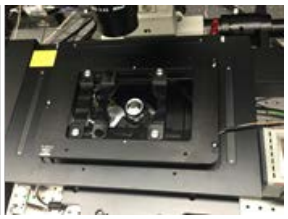
7) Carefully screw the objective into the piezo unit. Do not overtighten.


8) Place the stage insert on top of the three posts. Don't let it slip and fall onto the objectives. Use the correct insert for your application (slides, multiwell plates, or holding the TokaiHit incubation chamber.

9) Screw in the three small screws.



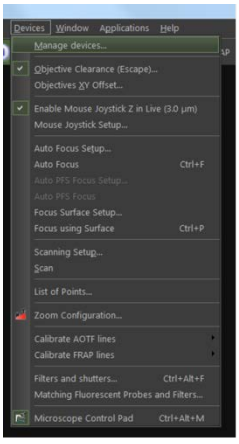


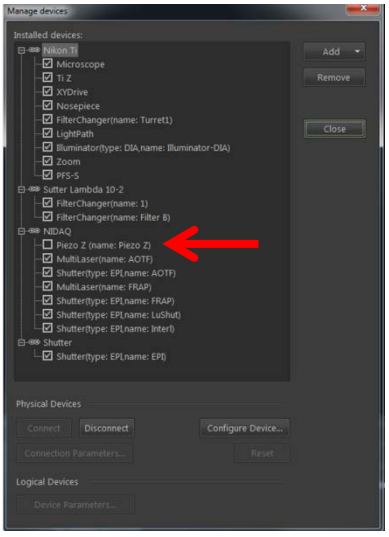
10) Turn on the system and the piezo unit (blue/grey box, power switch is in back left).

11) With focus on coarse, turn knob to get to ~7500 on the front display (or just touching the oil of the 100x lens).

12) Log into PC and open software.

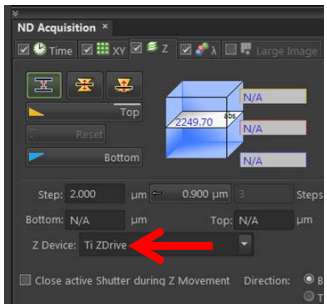
13) Enable the Piezo Z in Devices → Manage Devices.





14) Place sample on stage, adjust focus, image.

15) Don't forget to use Piezo as your Z drive (instead of Ti-E Drive).



Done (reverse of start-up steps):

1) Disable Piezo Z in Devices → Manage Devices.
2) Remove sample, clean oil off of objective.
3) Close NIS Elements software.
4) With focus on coarse, turn the knob to get to ~1200 on the front display. Please don't use Escape button.
5) Turn off Piezo unit (blue/grey box, power switch is in back left).
6) Unscrew three small screws. Place in a secure box for storage.
7) Lift off stage insert.
8) Remove three posts and washers from stage. If the bottom screw gets stuck, use the small flathead screwdriver in the storage box.
9) Carefully unscrew objective from piezo unit and place it in a very safe place.
10) Unscrew piezo unit counter-clockwise and lift out. Place back on top of laser unit for storage.
11) Carefully screw the objective back into place.
12) Place the slide holder stage insert back on the stage.
13) Screw in the three clear plastic screws. Don't over-tighten.

Follow normal procedure for turning off or leaving microscope in standby, based on sign up calendar.

APPENDIX D – TokaiHit Incubation chamber for live cell imaging:

- ➔ **Use of the live cell chamber absolutely requires prior authorization from Jolien.**

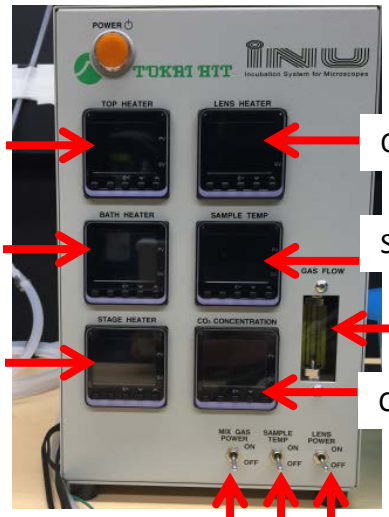
- ➔ **Each such user must have passed appropriate training for handling of the live cells through their laboratory's (PI's) approved IBC protocol.**

Starting the system:

- Install proper stage insert on microscope.
- Remove the chamber from storage box.
- Install proper insert for your sample type – slide/chamber slide or 35 mm dish.
- Make sure all three switches at the bottom of the Tokai Hit unit are off.
- Ensure that the water tube is closed.
- Remove lid and add di H_2O to the chamber to fill the bottom but not to the gas inlet valve.
- Insert a dummy dish or slide.
- Turn on main power switch.
- Turn on appropriate switches at the bottom of the unit – Lens Power (for objective heater), Sample Temp (for sample temperature probe), Mix Gas Power (if using CO_2).
- Confirm the unit is set to appropriate temperatures.
- Turn on CO_2 cylinder. Ensure that flow coming out of cylinder is between 14.5 psi and 21.7 psi.
- Let unit sit for 30 min to reach equilibrium.
- Carefully place on stage and insert sample.
- Image.
- For long-term imaging, you may need to use a syringe to add water to the unit.

Done:

- Remove sample.
- Carefully remove chamber from microscope stage. If you were using a high power objective, switch to 10x.
- Turn off CO_2 cylinder.
- Turn off all switches at the bottom of the unit.
- Turn off main power switch.
- Use syringe to remove water from chamber.
- Wipe inside of chamber with EtOH.
- Let sit on bench to cool.
- Once cooled, carefully return to storage box.
- Remember to re-install slide stage insert on microscope.



Top lid of chamber heater setting

Objective lens heater setting

Chamber water bath heater setting

Sample probe readout

CO_2 gas flow

Stage heater setting

CO_2 concentration setting

CO_2 Mixer ON/OFF

Objective lens heater ON/OFF

Sample temp probe ON/OFF

Sample chamber (with slide insert) and objective heater

CO_2 gas flow – do not fill water above this level!

CO2 cylinder – When on, flow should be between 14.5 psi and 21.7 psi

APPENDIX E – Factors that Affect Quality of Digital Images:

This microscope is equipped two EM-CCD cameras (=electron multiplying charge-coupled device). As a result, this camera has several nifty features that can help improve image acquisition or can lead to problematic image acquisition.

The goal of image acquisition is to acquire a quantitative image that is as close to the fundamental limits of resolution as you can achieve. This requires good signal to noise ratio (SNR). You want your signal to be visible above the background, which is affected by the noise level. Noise causes variations in intensity values and can come from a variety of sources and include: Poisson noise (shot noise, due to photon flux, less impact as signal increases), Read noise (errors as camera chip is read, independent of exposure time), and Dark noise (heat causes spurious electrons to pop, builds with exposure time). If your signal of interest is close to the background level and you have high noise, you will not be able to accurately resolve your signal within the noise variations.

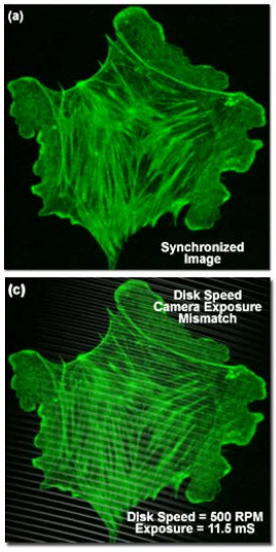
To increase signal: use a #1.5 coverslip, clean coverslip and slide, mount as close to coverslip as possible, use camera binning (but this leads to larger pixels/less resolution)

To decrease noise: use EMCCD mode only when signal is limiting

To decrease background: clean coverslips, optimize sample labelling protocol, dark room, close field diaphragm

1) Exposure time

The exposure time should be as fast/short as possible to obtain a good SNR image. However, you may sometimes notice artifacts resulting from mismatch between exposure time and disk rotation speed. Faster exposure times can lead to streaking artifacts across the image (see image for example). Longer exposure times will lead to increased photobleaching, and if you want to acquire a thick Z-series (many planes) or are imaging live cells, don't set exposure time too high. For live cells, photobleaching and phototoxicity are more important considerations and exposure times should be carefully considered.



2) Bit Depth

Bit depth refers to the number of grey levels the image contains (2^X grey levels), for example 8 bit images have 256 grey levels and 14 bit images have 16,384 grey levels. Increasing grey levels will increase file size but allow for increasing accuracy in intensity values. If your experiment includes very precise quantification of intensity values, then consider using higher bit depth. If your structure of interest only covers a very small range of the total intensity distribution, consider using a higher bit depth to obtain more information about that structure. The tradeoff to using a higher bit depth is that you will have to

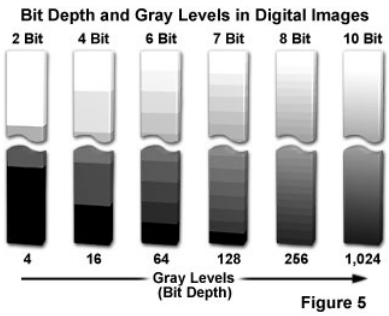


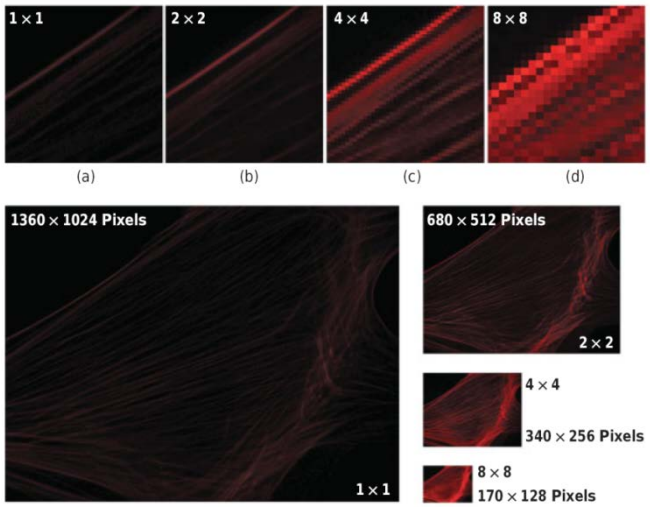
Figure 5

increase the exposure time, bin the image, or increase the gain - each of which will have its own consequences. In general, 12-14 bit is plenty.

NIS Elements by default will not autoscale the first image you acquire. Click the continuous autoscale button to turn autoscale on or click the single autoscale button to just adjust it once. It is imperative that your image not contain under- or over-saturated pixels since you will lose information regarding intensity distributions or subtleties in structure.

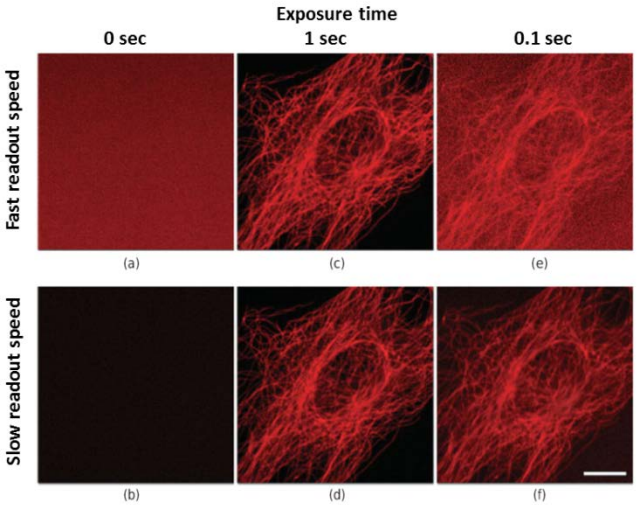
3) <u>Binning</u>

Binning is the combining together or pooling or adjacent pixels into a single, larger pixel. For example, 2x2 binning will combine a total of 4 pixels (2 per side) into a single bigger square pixel. This has the benefit of producing a brighter pixel (better SNR) at the same exposure time at the cost of reducing resolution. For live samples binning can be useful to allow for acquisition of a greater number of images (Z-series or timelapse) with less photobleaching and phototoxicity. The two EM-CCDs on this system already have quite large pixels, and it is not a good idea to bin unless you have a specific need.



4) <u>Readout Speed</u>

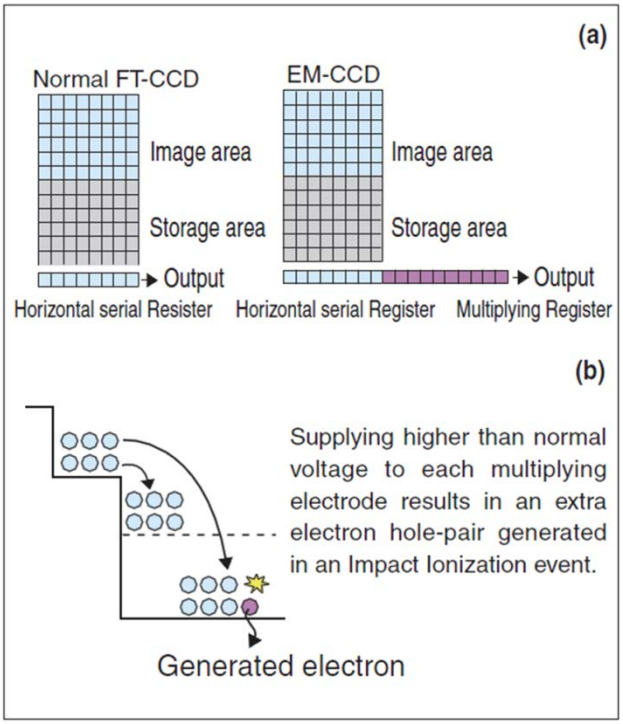
These cameras can be operated in EM-CCD mode with faster readout speeds or as a conventional CCD with slower readout speeds. In general, **<u>increasing readout speed will also increase noise/decrease SNR</u>**. Faster readout speeds should be used for live specimens in which you want to capture rapid dynamic processes.



5) <u>EM Gain</u>

When operated in EMCCD mode, you can adjust the amount of electron multiplication (EM) gain applied to your image. This is accomplished by running the output electrons through a multiplication register and applying a high voltage which at a low frequency will result in an "impact ionization event" that duplicates the electron and increases the image intensity value. This can be very useful for low light/dim samples or samples very prone to photobleaching/

phototoxicity that cannot tolerate higher exposure times. However, **increasing the gain will reduce SNR** since the background is also subjected to EM gain, blurring the intensity differences between objects (since the standard deviation of intensity values is increased). If your experiment requires rigorous quantification of actual intensity measurements (e.g., determine absolute number of proteins in a complex) or if you are utilizing intensity to assess levels of protein (e.g., compare expression in wild type vs. mutant), be very careful in application of gain and be very consistent. (Reminder – you can open a previously acquired image, right click, and select "Reuse Camera Settings" and "Reuse Device Settings" to ensure consistent settings from day to day.)



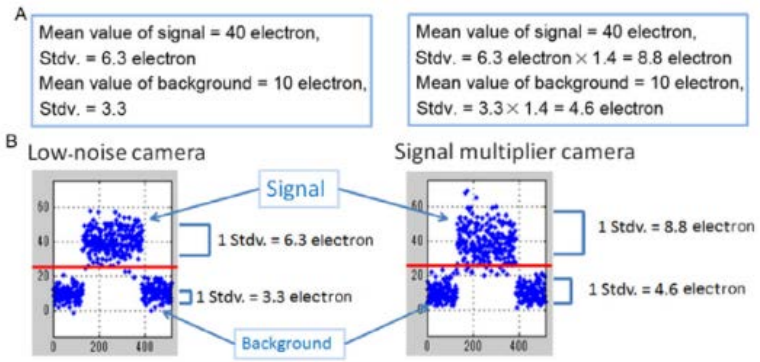


FIGURE 11.9

(A) Signal Multiplication noise Increases Stdv. of a signal by a factor of 1.4. (B) Increased Signal Stdv. in an image blurs intensity differences between objects.