Tips for Proper Microscope Use:

1. Clean the objective **before** and after you use it.
2. For a given slide, clean the oil off the coverslip before switching from an oil to an air objective.
3. Use just enough oil to cover the glass of the objective. Excess oil can seep into the system and cause major damage.
4. When using an oil objective and switching to a new slide:
   A. Press ‘Escape’ to escape the objective.
   B. Remove the current slide. (Add more oil to the objective if needed)
   C. Add the new slide.
   D. Press ‘refocus.’ The objective will automatically return to the previous focus position.

![Microscope controls]

5. If the objective is ‘Escaped’ when you arrive, be sure to press ‘refocus’ and then focus down manually before adding your sample. Otherwise, the objective might crash into your sample.
6. If handling DIC sliders, please wear gloves.
7. Always be aware of the position of the objective relative to the stage. Bumping the objective into the stage can instantly cause major damage to the objective or to the stage.
8. Do not use 8-well chambered cover-glass dishes, because the wells are too small. You will not be able image in the end wells and you will not be able to collect a DIC image in any well.
9. Do not save data anywhere on the C: drive, as it will be immediately deleted.

Thank you for following these guidelines! If you are ever unsure what to do, please ask any Microscopy Core staff for help any time!
Quick start guide for confocal with Nikon Elements software

1. If device is off: Power on the device by turning on the orange labeled switches in ascending order and log in with your level 1 password.

2. Open NIS-elements shortcut from the desktop. Click the icon WITHOUT the ‘A’.

3. Click the restore layout and restore OC buttons on the far left toolbar to reset settings to default.

4. Select the appropriate optical configuration (OC). For example:

<table>
<thead>
<tr>
<th>Table 1: List and Brief description of Optical Configurations (OCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
</tr>
<tr>
<td>A1 DIC x*</td>
</tr>
<tr>
<td>A1 Spectral</td>
</tr>
<tr>
<td>A1 Spectral DIC x*</td>
</tr>
</tbody>
</table>

*x represents the laser that will be used to make the transmitted light image.

5. Choose the appropriate objective (see Tips for Choosing the Right Objective on pg. 4).

6. Check if the objective is ‘Escaped.’ If so, reset the escape (see Tips for Safely and Quickly Focusing on Your Sample on pg. 6 step 1).

7. Clean the objective (see Cleaning Objectives on pg. 5).

8. Check for a correction collar. All numbered collars set to ‘17,’ or the multi-immersions to the immersion media you are using.

9. Add immersion droplet (as little as necessary, see Tips for Proper Microscope Use: on pg. 1 step 3).

10. Put your sample on the stage (see Tips for Safely and Quickly Focusing on Your Sample on pg. 6).
11. To look into the microscope with your eye, click the ‘Eye Port’ button (highlighted when pressed).

12. Select the filter cube. DAPI, circled in red below, is a reliable fluorescence for finding cells/tissue.

13. Center the sample (see Tips for Safely and Quickly Focusing on Your Sample on pg. 6).

14. Focus onto the sample (see Tips for Safely and Quickly Focusing on Your Sample on pg. 6).

15. If doing DIC, align the condenser (see Aligning the Condenser for DIC on pg. 7).

16. To collect an image on the computer screen, click on the ‘Eye Port’ button again (see step 11).

17. OPTIONAL #1: If you need to re-use the same camera settings from a past image:
   A. Open that image.
   B. Right click on the image.
   C. Select ‘reuse camera settings.’
   D. Skip to step 21.

18. OPTIONAL #2: If using spectral detection (this is rare), change the light path appropriately (see Spectral Imaging on the A1si on pg. 30).

19. Select the channels you want to use by clicking the checkbox of each channel as needed.

20. Select the appropriate pin hole size. Setting the size to 1.2 ‘AU’ is a reasonable starting point.

21. Click ‘scan’ to focus the confocal.

22. Adjust confocal parameters, starting with resolution settings, for good image quality (see Tips for Good Quality Imaging on pg. 9).

23. Take the appropriate snap-shot, time series, z-stack, or large image (see Time Series Acquisition on pg. 15, Z-Stacking on pg. 17, or Large Image Acquisition on pg. 23).

24. Save image in an approved location (see Tips for Proper Microscope Use: on pg. 1).

25. When done, remember to move the objective to 520 µm, clean the objective, and log off.
**Tips for Choosing the Right Objective**

There are four main properties used to determine the best objective for your purpose.

A. The refractive index (RI) of your mounting media. Generally, the RI of the objective immersion used should match the RI of the sample.

B. The depth you need to image, which may be limited by the working distance (WD) of the objective.

C. The resolution and light gather capability that you need, which is set by the objective’s numerical aperture (NA).

D. The size of the field of view you need, which is determined by the objective’s magnification.

**Match Refractive Index of Mounting Media and Immersion Media**

1. Observe how your sample is mounted (i.e. how your sample and coverslip are attached).

   A. If mounted on a glass slide, check the index of refraction of the solution between the coverslip and the glass slide.

   **Example:** This can be water or buffer, glycerol, or a commercially available mount like ProLong Gold. This is called the ‘mounting media.’

   B. If your sample is placed into a cover-slip bottomed culture dish or chambered plate with buffer, then your sample is submerged in water, and water is the mounting media.

2. Use an objective for which the immersion media’s refractive index matches the mounting media’s.

**Choose the Right Working distance (WD) for the Depth You Need**

3. WD is how far you can, in principal, image into your tissue, before the objective hits the glass coverslip. Choose an objective with a WD that will reach your target regions.

   **Note:** Light scatter and aberrations may make image quality very poor before the working distance is reached. If this occurs, consider tissue clearing protocols.

**Choose the Right Numerical Aperture (NA) for Your Resolution Needs**

NA describes the cone of light coming from the objective and determines how much detail can be visualized. On a confocal, the larger the NA, the better the xy resolution and optical sectioning.

4. For high detail, use NA > 1.1

   **Example:** If looking for intracellular structures or intracellular expression localization.

5. For low detail, you can use NA < 1.0 or lower.

   **Example:** Counting cells or identifying cells with different dyes.

**Choose the Right Magnification for the Appropriate Field of View**

6. Low magnification gives a larger field of view. Use Low magnification objectives for projects requiring tiling of whole slices of tissue.
Cleaning Objectives

This is a broad overview of best practices while cleaning objectives. Finger oil or Nikon oil left on the objective can lead to poor image quality for you or any user following you.

1. Keep the lens paper sheets in the book clean from finger oil and dust:
   
   **Example:** Use the center of a sheet for cleaning, and the outer edges for holding.

   ![Just cleaning](image)

2. Hold the paper firmly so that you can scrub the objective using your finger without a fear of the paper slipping.
   
   **Example:** Wrapping the paper around a finger is a way to do this.

3. Using an unused, clean spot, press the paper firmly to the center of the objective lens and scrub in a spiral. Look at the paper after a spiral scrub and note the amount of oil on the spot.
   
   **Note:** This pushes grime outward, while never allowing it to retreat back to the center.

![Spiral scrub](image)

4. Repeat steps 1-3 until two successive spots are dry.

5. If oil persists after 6 or more spots, then:
   
   A. Follow steps 1-2 with a new, unused lens paper sheet.
   B. Apply some ethanol on the spot on the lens paper you will use to scrub the objective.
   C. Follow step 3.
   D. Clean once more with a dry, unused spot on the lens paper.
Tips for Safely and Quickly Focusing on Your Sample

1. First make sure the objective isn’t escaped.
   A. To check, move the focus knob and see if the height value on the microscope changes. If it does, you are good to go, if it doesn’t, then:
   B. With NO sample on the stage, press the ‘refocus’ button on the microscope. The objective will move up.
   C. Use the focus knob to bring the objective to a ~500-microns height.

2. Put oil/water/glycerol on the objective, depending on your sample and the objective (remember to check for a correction collar).

3. Put your sample on the stage, coverslip down.

4. Use the stage control joystick to position the sample directly above the objective.
   
   Note: You can do this by looking at the objective from directly above and moving the sample to be directly between your eye and the objective lens.

5. If using an immersion: move the objective up until the immersion droplet touches the coverslip.

6. While the microscope is in eyepiece mode and the light off, look through the eye-piece and turn on the fluorescence light source on.

7. IF USING OIL: Look through the eyepiece and press escape.
   A. If the sample comes into focus briefly during the objective’s decent, then press ‘refocus’ and move the objective down at moderate speed in coarse mode until the sample is in focus. Skip to step 9.
   B. If the sample is always out of focus during the objective’s decent, then press ‘refocus’ and move the objective up at moderate speed in coarse mode until the sample is in focus. Skip to step 9.

8. Move the objective with the focus knob at moderate speed (~1 turn for ~5 s) in coarse mode until the sample is in focus.

9. You can now click ‘eye-port’ and continue to image with confocal.
Aligning the Condenser for DIC

1. Start with your sample in focus.

2. Close the field stop. This is an aperture located just above the condenser, labeled with an ‘F’.

3. While looking at your sample, adjust the height of the condenser until you can see a focused octagon.

4. Use the two centering screws to place the hexagon in the center of the field of view.

5. Open the field stop just enough to completely lose sight of the octagon.

6. Check to see if the DIC slider is under the objective you are using.

   **Note:** Use gloves while handling DIC sliders.

7. Rotate the wheel on the condenser until it shows ‘N2’ or ‘N1,’ corresponding to the appropriate objective.
8. Repeat all these steps if switching to a new objective. Otherwise, the condenser is aligned for the duration of the session.
Tips for Good Quality Imaging

Choosing Resolution Settings

1. Decide how much detail you need. More resolution almost always costs time, and you need to decide the right balance.
   
   A. Use pixels of 0.5 microns if counting cells or identifying cells with dyes.
   B. Use pixels half the size of the ‘Optical Resolution’ if you are looking at intracellular-expression localization or at sub-micron details. ‘Optical Resolution’ is reported in the ‘A1 Scan Area’ menu.

2. To change the pixel size (Blue circle), change the zoom in the ‘A1 scan area’ menu (Yellow circle), or change the number of pixels in your image (Red circle).
Changing Intensity Settings: Multichannel Imaging

**Note:** This section is for changing intensity settings for the normal imaging. For changing intensity settings for spectral imaging, skip to Changing Intensity Spectral Imaging on pg. 12.

3. While changing intensity settings, keep the resolution settings from Choosing Resolution Settings on pg. 9.

4. While changing intensity settings, make sure you are looking at a region that is bright with all the dyes you’ll be monitoring in the experiment. This will allow you to use the same settings for the whole experiment.

5. After every full image capture, the Elements software reports a Look Up Table (LUT) in the LUT window. Each channel has its own histogram.

6. If not open, right-click on the background → visualization controls → LUTs to open it.

7. Move the slider at the left of the LUT window all the way up.

8. The grey curve is a histogram that shows frequency vs. pixel intensity. Below is an example of an LUT for a good quality image:

A. Always have a gap between “0” and your noise peak (Blue circle).
B. Always have a ~2000 unit gap between the brightest pixel of your sample and 4095 intensity level (Red circle).

**Note:** Sometimes there is a bright piece of junk in your sample. If these pixels are not important to you, you can allow them to fall within or above the ~2000 unit gap without sacrificing image quality. Just remember to ignore those pixels during analysis.
9. Below is an example of an LUT for a bad quality image:

A. In the blue circle, pixels too dim to detect. They are called ‘undersaturated pixels.’
B. In the red circle, pixels are too bright to be detected properly. They are called ‘oversaturated pixels.’
C. OPTIONAL: use the ‘saturation tool’ to see which pixels on the image are saturated or undersaturated. You can use the drop-down arrow to choose the color or each category of pixels.

10. Change the offset for each channel until there is a gap between the noise peak and 0 intensity. To do this, change the offset of the appropriate channel and then take a new image.

   **Note:** Do not use the offset to cut off the noise peak. You often need the noise pixels to measure signal to noise. You can always image the noise, and then suppress them in the display later (see

11. While looking at an image taken at a bright part of your sample, make sure the brightest pixel/pixels are falling at 2000-2500 on the x-axis. To do this:

   A. Find a bright part of your sample. Either use the eye-piece mode, or use ‘scan’ in confocal mode.
   B. Take a snapshot.
   C. Change either Pixel dwell, ‘HV’ (Gain), or laser power to adjust pixel intensity. For more information on the trade-offs of these settings, see *Pixel Intensity Settings* on pg. 13.
   D. Take another snapshot.
   E. Repeat until the LUT for each channel’s brightest pixel is between 2000 and 2500 on the x-axis.

   **Note:** If there is a bright part of your sample that is not important (like a piece of junk), then you can safely allow those pixels to saturate without losing any image quality in the part of the image you care about.
Changing Intensity Spectral Imaging

3. While changing intensity settings, keep the resolution settings from Choosing Resolution Settings on pg. 9.

4. While changing intensity settings, make sure you are looking at a region that is bright with all the dyes you’ll be monitoring in the experiment. This will allow you to use the same settings for the whole experiment.

5. After every full image, the Elements software reports a spectrum in a window called ‘Look Up Tables (LUTs).’

6. OPTIONAL: Move the slider at the left of the LUT window all the way up to exaggerate low intensity wavelengths.

7. Adjust pixel intensity settings to make sure the white line reaches about halfway up the y-axis. To do this, change intensity settings and take a new image (see Pixel Intensity Settings on pg. 13 for a description of the intensity settings’ tradeoffs).

8. Adjust the brightness and contrast sliders in the ‘LUTs’ window as desired to change the display.

   Note: This only changes the display. You need to take a new image to see the impact of any changes in intensity due to intensity settings changes.
Pixel Intensity Settings

There are three settings that are used to adjust pixel intensity. Each has their own advantages and disadvantages. Below are the settings and the effects of changing those settings.

1. Pixel dwell (reported as µs per pixel). Higher pixel dwell results in:
   A. Brighter pixels.
   B. Less pixel to pixel fluctuation (less noisy images).
   C. More rapid photobleaching.
   D. Longer acquisition times.

2. ‘HV’ or gain. Higher gain results in:
   A. Higher intensity pixels.
   B. Noise amplification.

   Note: A channel either has an ‘HV’ or an ‘HV(GaAsP’ detector.

3. Laser power (reported in percent laser power). Higher laser power results in:
   A. Brighter pixels.
   B. Improved signal to noise.
   C. More photobleaching.

   Note: there is a gain and a laser power setting for each channel, but the pixel dwell affects all the channels at once. Use laser power and gain for fine tuning individual channels.

Table 2: Summary of the Effects of Increasing Each Intensity Setting

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Channels that will Brighten</th>
<th>Shot Noise</th>
<th>Photobleaching</th>
<th>Common Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pixel Dwell</td>
<td>More time</td>
<td>All channels</td>
<td>Less</td>
<td>Faster</td>
<td>2-12 µs</td>
</tr>
<tr>
<td>Laser power</td>
<td>No change</td>
<td>A single channel</td>
<td>Less</td>
<td>Faster</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Gain (HV)</td>
<td>No change</td>
<td>A single channel</td>
<td>No change</td>
<td>No change</td>
<td>60-80;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RULE: &lt;110</td>
</tr>
<tr>
<td>Gain (HV Gasp)</td>
<td>No change</td>
<td>A single channel</td>
<td>No change</td>
<td>No change</td>
<td>1-25; RULE: &lt;50</td>
</tr>
</tbody>
</table>
Some Tips

1. If your signal in one channel is already too bright, then the best place to start to reduce the gain.
2. If your signal is far too dim, the best place to start is by increasing the gain 5 or 10 at a time.
3. Since Pixel Dwell effects all channels at once, it is better to choose the right pixel dwell first considering your tolerance for pixel to pixel fluctuations, acceptable photobleaching, and intensity.
4. Pixel dwells between 4.5 and 12 μs are the most popular, considering the low pixel to pixel fluctuations and relatively short acquisition times.

Adjusting the Brightness and Contrast Sliders in the LUT Window for Better Display

This is for making an image easier to look at and interpret with your eyes. It won’t alter the data or effect data acquisition in any way.

1. Click and drag the ‘brightness slider’ on the LUT to make the image look brighter.
2. Click and drag the ‘contrast slider’ on the LUT to suppress the noise in the display.
**Time Series Acquisition**

This is for taking single images, large images, z-stacks, in any combination at specific time intervals (See [Z-Stacking](#) on pg. 17 or [Large Image Acquisition](#) on pg. 23).

1. Open ND acquisition menu, if not already open. If not open, click the ‘ND’ button on the far left panel.

![ND button](#)

2. Select the ‘time series’ tab.

![Time series tab](#)

3. Check the checkbox on the time series tab.

4. Either uncheck the ‘save file’ checkbox at the top, or browse to find an approved save location.

![Save to File](#)

5. Select the appropriate interval. This is the interval between the start times of each loop.

![Time Schedule](#)

**Note:** Make sure the interval is larger than the time it takes to carry out a full loop. For instance, make sure the interval is 3 min or longer for a 3 min z-stack. You will end up with the appropriate z-stack, but with a different interval than you designated.

6. Select the time-series duration.
7. Check the checkboxes for z-stack and/or large image in their respective tabs. Every time loop will contain all the checked options.

   **Example:** If z-stack and large image tabs are ‘checked,’ then each loop will contain a large, z-stacked, image. (see *Z-Stacking* on pg. 17 and *Large Image Acquisition* on pg. 23).

8. For long acquisitions, setup perfect focus (see *Perfect Focus Setup* on pg. 28).
Z-Stacking

This is for taking a z-stack for a region in your image. You can do a z-stack, large image, and time-series at the same time (see Time Series Acquisition on pg. 15 and/or Large Image Acquisition on pg. 23).

1. Open ND acquisition menu, if not already open. If not open, click the ‘ND’ button on the far left panel.
2. Select the ‘z-stack’ tab.
3. Check the checkbox on the z-stack tab.
4. Either uncheck the ‘save file’ checkbox at the top, or brows to find an approved save location.
5. Choose the appropriate z-stack mode
6. Follow the instructions for the appropriate mode (see, Top-Bottom Mode or Center Mode).

Top-Bottom Mode

This mode is most useful for tissue slices.

1. Top-bottom mode button is the left-most of the three options. Select it.
2. Input the ‘step’ size. This should be the optical sectioning value from ‘A1 scan area’ divided by 2.
3. Change the microscope focus speed to fine or extra-fine.

4. Click ‘scan’ to see a live image. You may want to temporarily speed up acquisition here by changing the resolution.

5. Turn the focus knob until focused on the top of the desired z-stack.

6. Click the ‘top’ button.

7. Make sure the number on the scope now reads exactly the same value as the top tab of the z-stack.

8. Turn the focus knob until focused on the bottom of the desired z-stack.

9. Click the ‘bottom’ button.

10. Make sure the number on the microscope reads the same value as the bottom tab of the z-stack.
11. Check the ‘range’ on the right of the ND window and make sure it makes sense.

12. Click ‘run now.’ For z-stack visualization, see Z-Stack Visualization on pg. 21.

Center Mode

Most useful for z-stacks of small objects of a known size or size range.

1. Center Mode is the middle of the three options. Select it.

2. Input the ‘step’ size. This should be the optical sectioning value divided by 2.

3. While focused on the center of your object, input the range.

   Note: There are two places for ‘range.’ Use the leftmost one while setting the range manually. The rightmost one is for calculating the range automatically in the other z-stack setting modes.
4. Click the ‘home’ button.

5. Make sure the number on the microscope reads exactly the same value as the middle of the z-stack.

6. If doing a large image at the same time as a z-stack, click the ‘relative’ button, and proceed to Large Image Acquisition on pg. 23.

7. Click ‘run now.’ For z-stack visualization, see Z-Stack Visualization on pg. 21.
Z-Stack Visualization

There are three main methods to visualizing a z-stack. This section explains how to quickly do them and what they show.

Volume View

This view is for seeing the surface of your sample in the z-stack. It is not good for seeing the inside of a thick piece of tissue.

1. Click the ‘volume view’ at the top of the window of your z-stack.

2. Click and drag to rotate the view and the mouse scroll wheel to zoom in and out of the representation.

3. You can change the brightness and contrast slider in the LUT window just like in standard images.

   Note: It takes a few seconds to render changes to the brightness and contrast sliders in the LUT window.

Orthogonal Slices

This view is for seeing cross-sections of your sample. The position of the orange lines represents the place the software is ‘cutting’ your sample to see the detail of the cross-section.

1. Click the ‘orthogonal slices’ view at the top of the window of your z-stack.

2. Move the cross-hairs to where you wish to see the cross-section of the sample.
3. The vertical orange line is the position of the cross section displayed at the right of the z-stack in this window.

4. The horizontal line is the position of the cross section displayed at the bottom of the z-stack in this window.

   **Note:** The cross-section image is to scale, so if the z-stack is also a large image, you may need to zoom in to see the cross-sections. **Example:** With a 1 mm by 1 mm large image with a 5 µm z-stack, the orthogonal cross-sections will be a small, nearly invisible ribbon at the right and bottom of the orthogonal slices representation.

**Maximum Intensity Projection**

This representation is good for finding small objects in the potentially large z-stack. It will give a 2D image that only shows the brightest pixels through the depth of the z-stack. Particularly good for locating the x-y positions of a network of nerve processes or small spherical objects in a large volume.

1. Click the ‘maximum intensity projection’ view at the top of the window of your z-stack.

2. You can change the brightness and contrast slider in the LUT window just like in standard images.

3. You can have the z-stack next to the maximum intensity projection, and use the mouse scroll wheel to navigate the z-stack while looking at the maximum intensity projection.

   **Note:** The maximum intensity projection only shows the x-y position of an object. It cannot show if there is overlap between two similar objects. For that, you need to navigate in the z-stack to look for overlaps.
Large Image Acquisition

This is for taking a series of images to automatically make a larger image. You can do a large image and z-stack and/or time series at the same time.

Note: If doing a z-stack and a large image at the same time, set up the z-stack parameters using center mode first (see Z-Stacking on pg. 17 and Center Mode on pg. 19).

1. Open ‘ND acquisition’ menu, if not already open. If not open, click the ‘ND’ button on the far left panel.
2. Go to the ‘Large Image’ tab in the ‘ND acquisition’ window.
3. Check the box in the ‘large image’ tab.
4. Check the box in the ‘time series tab’ (this is a way to preclude a glitch).
5. Open the ‘xyz overview’ window (right click on the background → acquisition controls → xyz overview).
6. Click on the ‘ND overview’ tab.
7. Use the mouse scroll-wheel to zoom in to the cross-hairs.

8. You may need to remove a large image area from a previous user. If so:
   A. Right click on the grey area.
   B. Select ‘clear preview.’

9. Remove all prior saved points. The button will be greyed out if there are none.

10. Go to the focal surface tab.

11. Remove all prior saved points. The button will be greyed out if there are none.

12. Go back to the ‘ND overview’ tab.

13. Click ‘scan’ to see a live image.

14. Move the stage until you find a field of view you want to include in the large image.
   
   **Optional:** you can also change the focus. This is useful for a large image where the sample isn’t flat.

15. Click the ‘add’ button to save this position.
16. Go back to the ‘Focal surface’ tab.

17. Click the ‘add’ button here too.

18. Repeat steps 12-17 until all the important regions are selected.

19. Go to the ‘ND overview’ tab.

20. Right click on the grey area and select ‘define area.’

21. Draw a tight rectangular area around your points by clicking and dragging on the grey area.

22. Right click the resulting rectangle→scan large image.

**Note:** A new window pops up. Don’t press ‘enter’ until all the right settings are selected.
23. Select the objective that you are currently using for both drop down lists at the top right.

Note: If you don’t select the current objective for both menus, the objectives can unintentionally switch mid experiment.

24. Make sure the selected region reads ‘large image from xyz overview.’

25. Select ‘create large image.’

26. Uncheck ‘save file automatically,’ or brows for an approved save location.

27. Change the overlap to 10 % (for typical samples).
28. Check ‘use focal surface.’

29. If just doing a large image, select ‘none’ at the top left of the window.

30. If doing a large image and z-stack together, select ‘z stack.’

   **Note:** If doing the z-stack and large image together, make sure you already set up a z-stack in center mode (see *Center Mode* on pg. 19). If the ‘relative’ button in the z-stack tab is selected correctly, the large image window will use the values there for z-stack parameters.

31. The large image window should use the values in the z-stack tab of the ‘ND acquisition window.’ So you can ignore the options in this large image window.

32. Click ‘scan’ in the ‘scan large image’ window.
Perfect Focus Setup (PFS)

This is best for keeping the objective focused during long time-series. It uses an extra laser to determine the height of the coverslip precisely, and automatically corrects focal fluctuations in real time.

Note: The A1hs has a different PFS setup than the A1si and A1ss.

A1Hs PFS Setup

1. Right before starting a time series acquisition, click the ‘offset’ button.

2. Now the PFS focus is the same as the current focus.

3. Press the ‘PFS on’ button.

4. PFS is on. Now the focus knob can no longer change the focus, to do so use the PFS focus knob.

A1ss and A1si PFS Setup

1. Manually pull the PFS lever to the ‘IN’ position to turn on PFS. The lever is under the stage.
2. Right before starting a time series acquisition, click the ‘offset’ button.

3. Now the PFS focus is the same as the current focus.

4. PFS is now on. Now the focus knob can no longer change the focus, to do so use the PFS focus knob.
Spectral Imaging on the A1si

This technique is good for directly measuring the whole emission spectra of a dye in samples and for differentiating between the signal of dyes with similar colors (emission spectra).

1. Start an imaging session as normal, but choose one of the spectral QC’s (see Quick start guide for confocal with Nikon Elements software on pg. 2, Steps 1-16.

2. Click the ‘light path’ button to open the ‘light path’ menu.

3. Check the excitation spectra of your dyes for deciding on the lasers needed.

4. In the list of channels, select the laser or lasers you are using. Check one channel per laser.

5. Change the dichroic to 20/80 BS.

6. Check the emission spectra of all your dyes to identify all the wavelengths you need to probe.

7. Extend the wavelength bar until it includes all relevant emission wavelengths.
**Note:** Starting wavelength should be at least 15 nm greater than your shortest wavelength laser.

8. In the dropdown menu for ‘resolution,’ select ‘10 nm.’

9. Note the number of bins, which the software calculates automatically.

   **Note:** Make sure that the number of bins is equal to or greater than the number of dyes. This is the minimum number of bins where spectral unmixing can separate all the dyes.

   Select the ‘binning’ tab.

10. Select the appropriate binning level. 2 bin will merge the 10 nm bins into 20 nm bins.

   **Note:** Use binning to increase the signal for each bin. I recommend binning 2.

12. Image using the steps 17-24 from the *Quick start guide for confocal with Nikon Elements software* on pg. 2.

13. OPTIONAL: If doing linear spectral unmixing (see *Linear Spectral Unmixing* on pg. 33).
Linear Spectral Unmixing

This technique is good for better separating the signal of one dye from another when the dyes’ spectra are close.

1. Prepare samples with at least:
   A. A positive control for each dye,
   B. The experimental case that has a mixture of all the dyes in one sample,
   C. and appropriate negative controls (unstained).

2. Image each control following the steps 1-13 in *Spectral Imaging on the A1si* on pg. 30.

3. Draw a region of interest (ROI) that has strong signal for one positive control with only one dye. The ROI tool is found on the right panel for any image.

4. At the top of the software window, select image→spectral unmixing settings. The following window opens.
5. In the window drop down, select ‘ROI.’

6. Click ‘add region.’

7. Choose a color for that dye.

8. Look at the spectra of the ROI, and make sure the signal is much stronger than the noise in the expected emission spectra.
9. Repeat steps 3-8 for each and every positive control with one dye.

   **Note:** If you are spectral unmixing with the same dye in a similar tissue and same sample prep in the future, you can reuse the positive control images, but remember that the resulting unmixing is relative to the amount of dye in the positive control.

10. Take an image of a sample you wish to unmix.

    **Example:** The positive control with all four dyes at once is a good sample for testing the effectiveness of the unmixing.


12. Click ‘unmix.’

13. The resulting image shows different channel tabs for each of the dyes.

14. Save in an approved manner.

15. You can repeat steps 10-14 with any additional samples you in which you used the same:

    A. Light path settings.
    B. The same dyes.

16. When done, remember to clean the objective, log off, and manually move the objective to ~500 µm.