Western
Biotron Experimental
Climate Change Research

Instructions for Nikon Ti2E System with Spinning Disk Confocal

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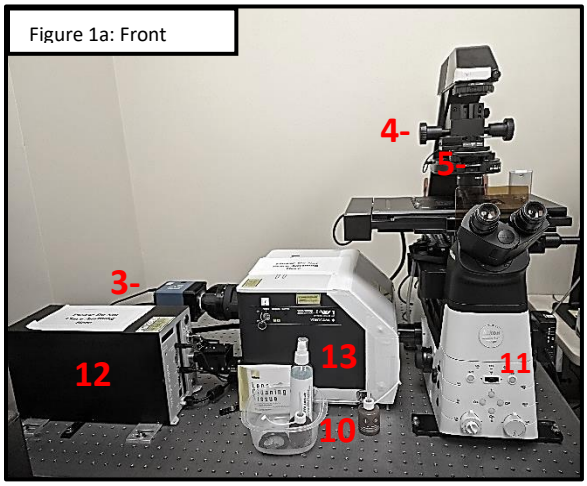
MICROSCOPE PARTS:



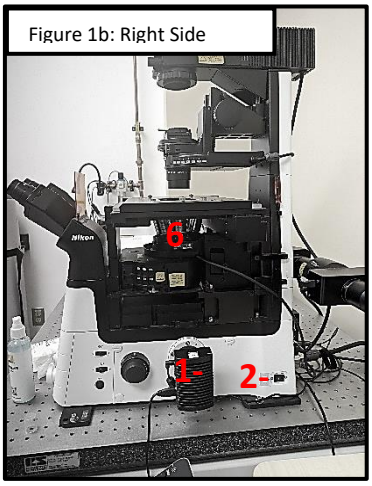


Figure 1: Microscope Parts & Layout

1. Brightfield camera (Fig 1b-1)
2. Microscope Power (Fig 1b-2)
3. Fluor. camera (Fig 1a-3)
4. Condenser focus (Fig 1a-4)
5. Field Diaphragm (Fig 1a-5)
6. Objectives (Fig 1b-6)
7. Joystick XY Control (Fig 1c-7)
8. Focus Control (Fig 1c-8)
9. Objective selection buttons (Fig 1c-9)
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11. Escape Button (Fig 1a-11)
12. Ziva Laser Engine (Fig 1a-12)
13. CSU-W1 Scan Head (Fig 1a-13)
14. CSU-W1 Control Box (Fig 1d-14)
15. Main power switch (Fig 1d-15)
16. LED Excitation (WF) (Fig 1d-16)
17. UPC (Do not touch) (Fig 1d-17)

GLOSSARY:

BF	Brightfield
DIC	Differential Interference Contrast
WF	Widefield Fluorescence
PFS	Perfect Focus System
LUT	Look Up Tables (Display adjustments)
NIS	Nikon Imaging Systems
BD	Batch Deconvolution
LED	Lamps for Widefield imaging
ms	Milliseconds
OC	Optical Configurations- channels
.nd2	Nikon Dimensions file format
OME	Open Microscopy Environment format
Tiff	Tagged image file format
Jpeg	Joint photographic experts group format

START UP SYSTEM:

1. Sign in on the Calendar systems: Name, Supervisor, Start time (BOTH online AND on the paper calendar at the workstation).
2. Turn on computer if not already on.
3. (Switch 1) Turn on Nikon main power (Fig 2-1).
4. (Switch 2) Turn on microscope power switch within 60 seconds (Fig 2-2).
5. After this, you may turn on power switches in any order. They are numbered as follows:
6. (Switch 3 and Key 4) Aura LED Light Engine below table. Switch turns green, turn the key clockwise. (Fig 2-3, 2-4).

Figure 2: Turn on Microscope Stand & Illumination

7. (Switch #5 and Key #6) Ziva Laser Light Engine on tabletop. Switch turns green, turn the key clockwise (Fig 2-5, 2-6).
8. (Key #7) CSU-W1 Scan head (Fig 3-7).
9. (Switch #8) Fluorescent Camera: <u>very tiny</u> metal toggle below the power cable-switch moves towards you/right for "on" (Fig 3-8).
10. (Switch #9) Brightfield Camera on right side of microscope-press and be sure green light comes on (Fig 3-9).

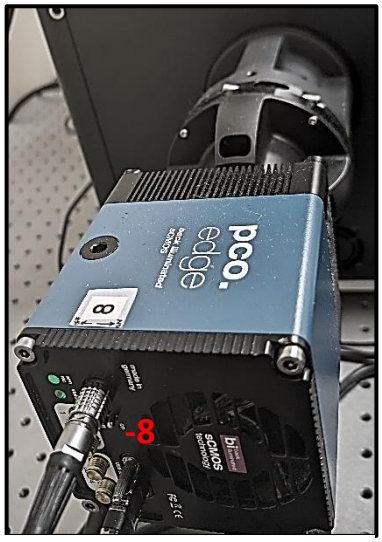
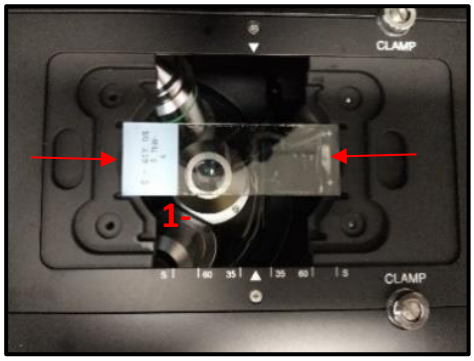


Figure 3: Scanners and Cameras

Loading Samples

Figure 4: Universal Stage Holder



1. Ensure the objective (Fig 4-1) is on 5x-10x magnification to start, by engaging buttons 1 or 2 on joystick pad (Fig 1b-6; Fig 1d-9) or changing it in the software.
2. If you MUST load samples directly on oil lenses, ensure the stand is in the "Escape" position (see p.7) (Fig 1a-11) to avoid damaging lenses. (Button on front of stand will light up green in "Escape" position). See staff to learn to apply & clean oil properly.
3. Tilt top part of microscope backwards before inserting slide into universal stage holder (Fig 4-1).
4. Ensure slide is facing coverslip down, completely flat, is sitting recessed on lower "shelf" within the holder, and holders wrap around slide edges. This stage can also be adjusted for 35mm or 60mm coverslip bottom cell culture dishes by moving the sliding holder in direction of arrows.
5. **Please see staff if you require the 96 well plate or live cell stages, as adjustments must be made in the software and setup.**

Starting the Software:

Figure 5: Check before turning on software

1. BEFORE launching software check:
   a. BOTH Light Engines show 2 lines of yellow writing (Fig 5-1).
   b. Joystick Control Pad shows lit display and you can read magnification (Fig 5-2).
   c. Use a low magnification (Fig 5-3: Buttons L-R are 5x-100x).
2. Launch NIS software (Fig 5-4). (Not NIS-BD (Fig 5-5)).
3. Username is "NIKON User" with no password.
4. Select "**Acquisition mode"**, click "OK".
5. If you get an error here:
   - Try toggling both cameras off and on and clicking "Try Again".
   - Check that you followed steps 1 a & b, and all 9 switches/keys were turned on properly.
   - Try restarting all hardware. **Wait 2 minutes** before restarting the main power switch#1.
   - Get staff help if this still does not work.

Software Layout:

1. Shortcuts Panel.
2. ND Panel for multidimensional Acquisition.
3. Main Image Window with visualization controls top, right and bottom.
4. Open Image Thumbnails.
5. Main Acquisition Panel includes:
    a. Camera controls.
    b. T12 Pad Microscope Controls.
    c. Illumination intensity controls.
6. Look Up Table Controls (visual/display contrast adjustments).

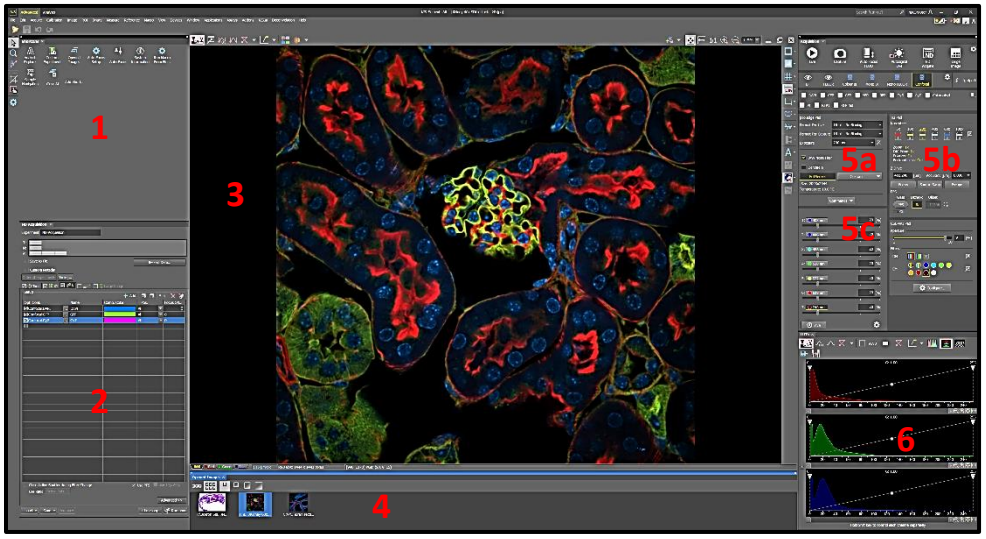


Figure 6: Software Layout

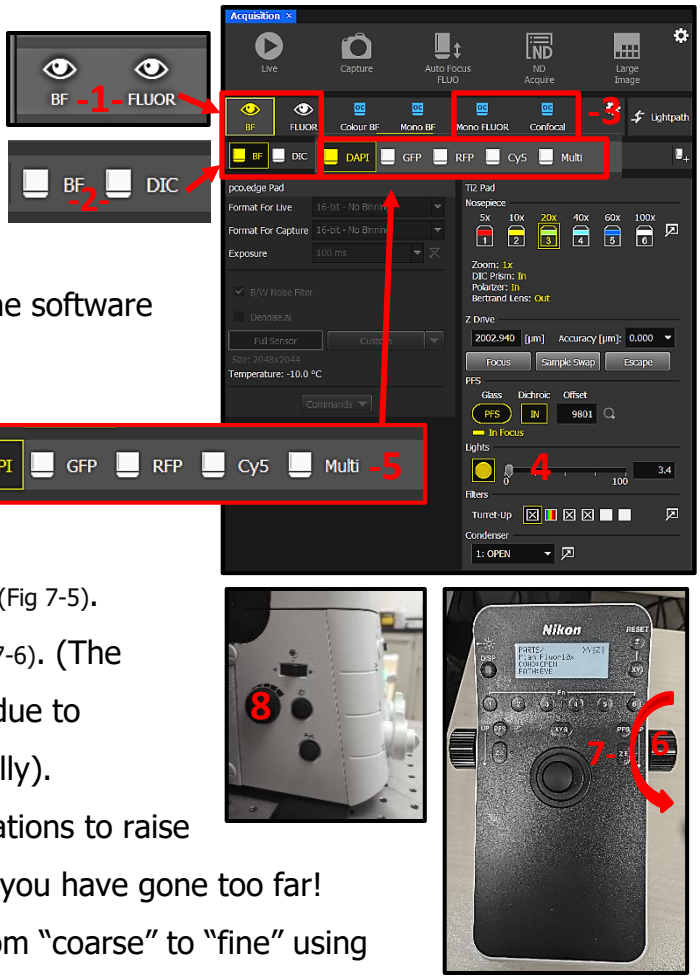
GETTING STARTED:

1. Select the visualization mode: Eyepiece viewing modes (Fig 7-1) have an eye symbol.

2. Viewing by eye may not be necessary due to onboard automated focusing controls- you can go straight to "focus using software" and skip the manual focusing next page if desired.
3. "Color BF" is for stained histology slides using the color camera. Brightfield (BF) and coloured Differential Interference Contrast (DIC) choices are available (Fig 7-2).
4. "Mono BF" is used for BF and DIC modes in greyscale. These modes are compatible with fluorescent imaging as they use the same monochrome camera (Fig 7-2).
5. Using the transmitted light path (BF or DIC) requires daily set up of the lens alignment for the specific lens, or you will experience poor sharpness and/or shadows. Refer to Appendix I.
6. "Mono Fluor" and "Confocal" options are for fluorescence imaging (Fig 7-3). Begin on "Mono Fluor" first to find focus-confocal is more difficult to set up.

To Focus by Eye:

Figure 7: Focus by Eye, Control Pad



1. Engage the Brightfield "BF" or "Fluor" options for eyepiece viewing (Fig 7-1).
2. **For Eyepiece BF:** Select from "BF" or "DIC" (Fig 7-2). Adjust the comfort level of the transmitted lamp intensity using the dial to the left of the microscope stand (Fig 7-8) or in the software Ti2 Pad (Fig 7-4).
7. **For Eyepiece Fluorescence:** choose DAPI, GFP, RFP, (Cy5 has poor visibility by eye), or multi wavelength, which allows you to see most signals simultaneously (Fig 7-5).
3. **Focus** using the dial on the joystick panel (Fig 7-6). (The focus knob on the stand is not recommended due to buttons there which you can engage accidentally).
4. Turn the focus knob anti-clockwise several rotations to raise the objective towards the sample. *If it beeps you have gone too far!
5. **For oil lenses:** reduce the Z-control speed from "coarse" to "fine" using the Z-push button (Fig 7-7). Please get training in proper use of oil from staff or you can damage lenses!
6. **Within Software**: Once you move to working within the software, focus is done using the mouse wheel, and moving the stage is done by activating the directional arrow  icon above the main image window (Fig 6-3) and holding down the left mouse button.
7. **Protect from bleaching:** be sure to disengage the lamp by toggling the live camera on and off (Fig 8-2) in the software to close the shutter and stop the illumination of your sample.

To Focus Using the Software (Fluorescence described-see BF exposure setup if needed):

Choose "Mono Fluor" (Fig 8-1) instead of confocal to find your focus, and try a non-oil lens first.

1. Launch the "Live" camera button (Fig 8-2). This will display "Freeze" when it is active (Fig 8-3).
2. In the Ti2 Pad, engage the "Focus" button below the objective controls (Fig 8-4). This will bring the lens close to focal plane for slides.
3. Engage one of the optical configurations (Fig 8-5). Choose DAPI, GFP, RFP, Cy5, Cy7, chlorophyll, or use reflected mode for opaque surfaces.
4. For proper exposures, refer here for fluorescence, or here for BF: Adjust parameters so you can see some light. Try the camera exposure (Fig 8-6) at ~100 ms for bright samples, or ~500 ms for dim samples. Try the fluorescent light sources (Fig 8-7) at 20%- 40% max.
5. If you still can't see: temporarily use the auto dynamic range icon (Fig 8-8) in the LUT panel. Reset this once in focus! (Fig 8-9)
6. Once blurry objects appear, manually focus using the mouse wheel, or use the "Autofocus" button (Fig 8-10, top). Autofocus takes 1-2 min. to find the focus for you. TIP: Hold down the shift key to set the mouse to coarse focus, or the autofocus to longer range.
7. Once focused, freeze the image (Fig 8-3) to stop the light excitation and prevent bleaching.

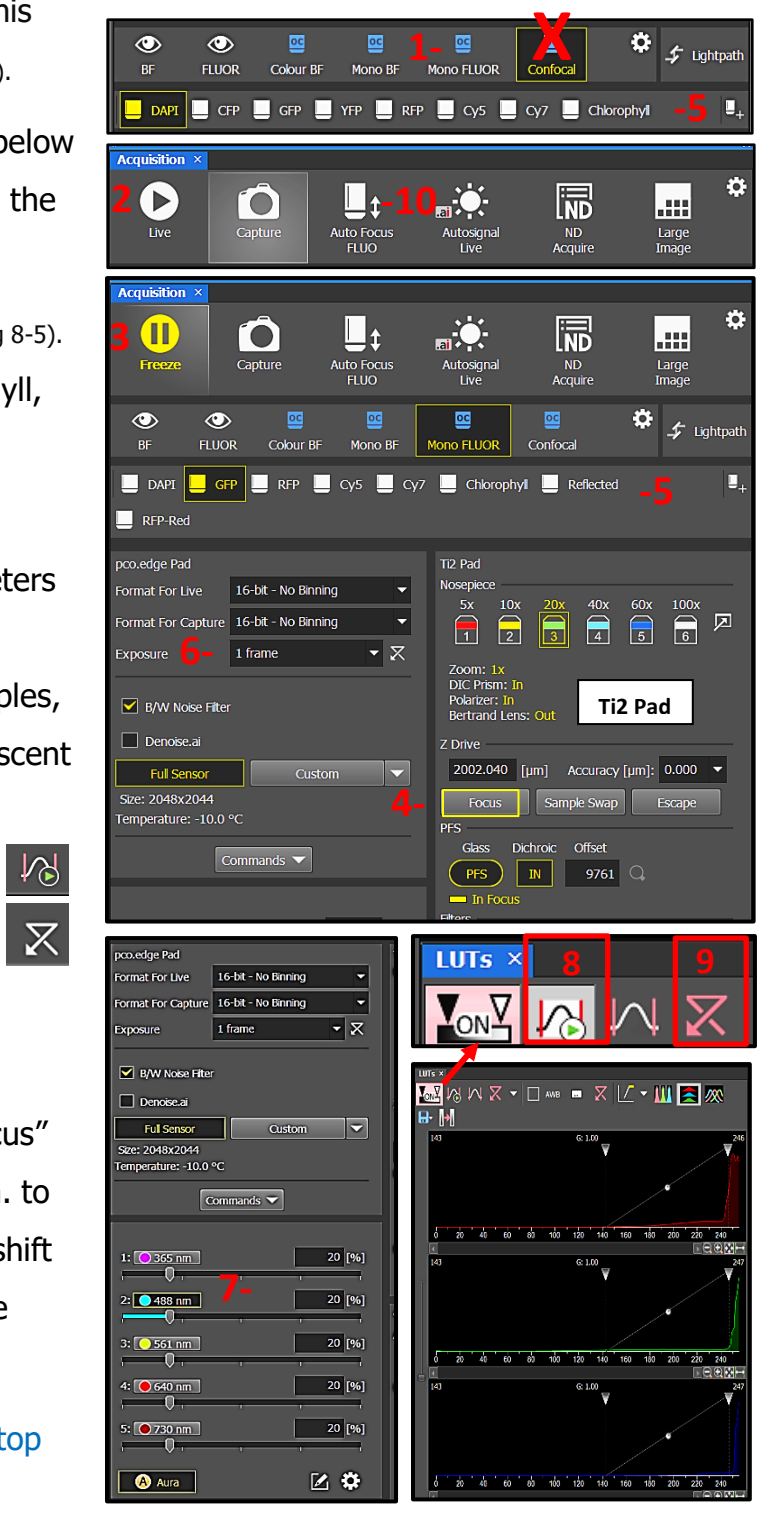


Figure 8: Using Software to Focus and see

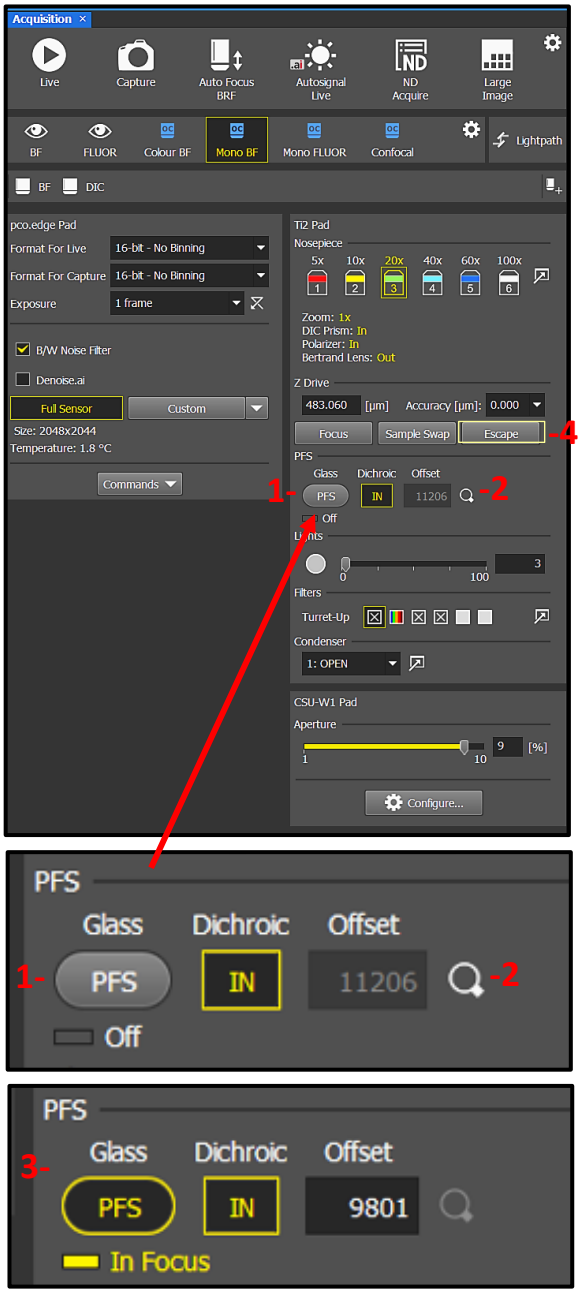
8. Switch to confocal now if desired and proceed to **optimize each separate optical configuration (OC)** needed if you plan to do multichannel imaging.

Using the Perfect Focus System to Maintain Focus:

The Perfect Focus system (PFS) uses an angled laser to monitor the coverslip position, instead of the fixed z-position of the lens. From the coverslip, an "offset distance" that achieves perfect focus is set, so that the real sample plane stays in focus during challenging focus conditions like stage movement, sample tilt, or temperature-based focus drift in live cell experiments.

- **When to use it:** Great for live cell imaging, stitching and tiling, and samples that are uneven; or anytime you need to drive the stage around and maintain constant focus.
- **When not to use it:** Not if the refractive index matches the glass (oil immersion + permanent mounting media). Not with the bare surface 50x lens. Not with Cy7 alone as it uses near-IR to focus. Not for very tiny or sparse samples.
- **Be careful:** Not to engage it accidentally with buttons on the stand, joystick or in ND analysis!

Figure 9: Using Perfect Focus and Escape



Set up and Operate PFS:

1. Ensure PFS is off (not yellow) to begin (Fig 9-1).
2. Focus on your sample.
3. Click the magnifying glass icon right of PFS to use this plane as the reference point (Fig 9-2).
4. Wait for the PFS to find the correct offset.
5. The PFS will automatically turn on/yellow (Fig 9-3) and the PFS button on the stand will be lit.

Using the "Escape" Function:

- "Escape" (Fig 9-4) should be used to lower the lens temporarily to change samples or add oil.
- When in use, the focus values in the bottom right of the task bar turn from blue to orange.
- When Escape is active you will NOT be able to focus!

SETTING UP PROPER EXPOSURE (Widefield/Confocal)

The brightness of your sample comes from: 1) Quality of staining/expression, 2) Proper Focus, 3) Camera Exposure, 4) Lamp or Laser intensity, and 5) Post-capture display adjustments in the Look Up Table (LUT). For good image/data quality, and to enable image deconvolution algorithms: ***always prevent saturation.***

1. Focus in "Mono Fluor" mode. Switch to "Confocal" Mode only after you focus.
2. Use the saturation indicator (Fig 10-1, above image).
3. Using dropdown arrow, set saturation to "complementary" to visualize overexposure (Fig 10-2).
4. **Camera exposure**: Try 100 ms -500 ms to start (Fig 10-3). It's slow to focus above ~500 ms
5. **Lamp/laser intensity:** Start at 20%, don't exceed 40% (Fig 10-4).
6. Exposure + lamp/laser level sets the "real" photon capture and intensity values.
7. Long exposures and/or high intensity cause bleaching and phototoxicity!

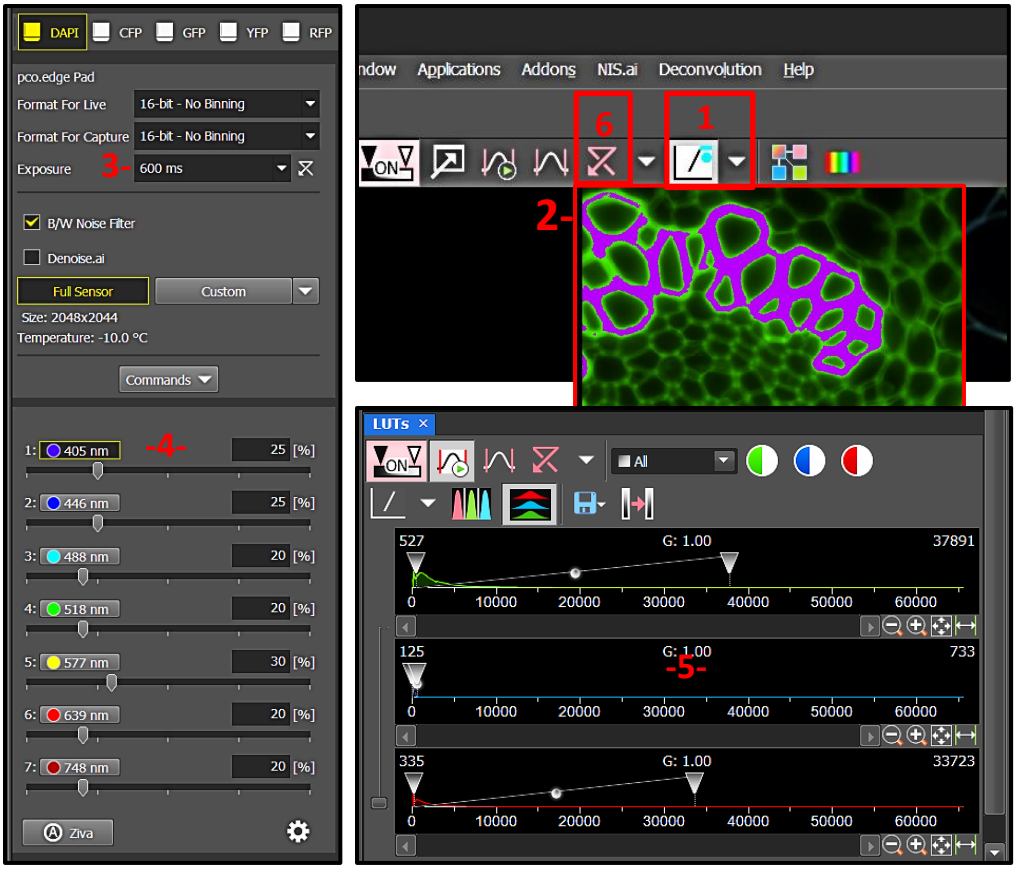


Figure 10: Proper Exposure Setup

8. **LUT:** The settings in the look up table, or "LUT" (Fig 10-5) only affect how you see the image display and aren't a "real" part of your image. See Appendix III to use these properly. ONLY adjust LUTs if you can't see at the suggested maximums (~1 sec and ~35-40%). LUT enhancement IS needed for weak signals or live sample imaging to preserve signals.
9. Reset the LUT (Fig 8-9 or 10-6) before final capture setup, or new channels and samples will display inaccurately!

SETTING UP PROPER EXPOSURE (Brightfield/DIC)

The brightness, sharpness and color correction of your sample consists of 5 parts: 1) Proper (Köhler) alignment of the lenses, 2) lamp intensity, 3) camera exposure, and 4) white balance all affect the real image. 5) The LUT (Look Up Table) histogram is used to adjust the DISPLAY contrast post capture.

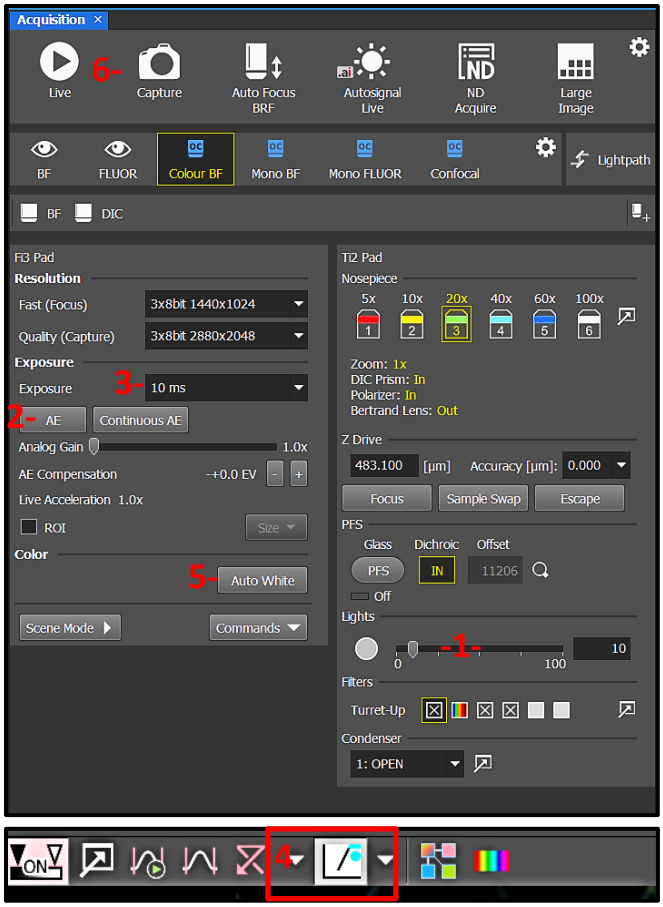


Figure 11: Setting Brightfield Illumination

1. Before beginning, follow the Köhler illumination setup process. (See staff/refer to Appendix I).The flatness of field and sharpness of imaging relies on proper alignment of the microscope.

2. Engage the Auto Exposure "AE" button (Fig 11-2). The lamp intensity (Fig 11-1) and the camera exposure (Fig 11-3) work together create the "real" intensity values that will be saved. Adjust the exposure (Fig 11-3) if needed.

3. Activate the saturation indicator above the LUT or image panel (Fig 11-4) and choose a contrasting color in the dropdown menu. This will display any over exposure in the image. Avoid image saturation within the tissue area (the white space can tolerate some). Oversaturating leads to poor image quality, incorrect data, and an inability to apply many image processing algorithms.

4. The white balance correction sets the colors accurately in your image. Move the stage to an empty part of the image (pure white area) before engaging the "Auto White" button (Fig 11-5).

5. The settings in the look up table, or "LUT" (Fig 10-5 & 11-4) ONLY affect how you see the image onscreen. It applies POST CAPTURE changes to contrast and brightness that are not a "real" part of your image. See the sections on proper use of this on Appendix III-page 20.

6. For simple imaging, click "Capture" (Fig 11-6) next to the "Live" button on Acquisition Panel, or use the ND Acquisition panel for large image stitching, Z-stacks, etc.

USE OF THE ND MULTI DIMENSIONAL ACQUISITION PANEL:

The ND acquisition panel is used for the following imaging dimensions:

- Multichannel imaging
- Z-Stacks
- Multi Point Imaging
- Large Image tiling and stitching
- Time Series

NOTE: ND acquisitions are checkbox activated. Any imaging dimension you have checked in the yellow outline (Fig 12-3) will run when "Run Now" is selected (Fig 12-6).

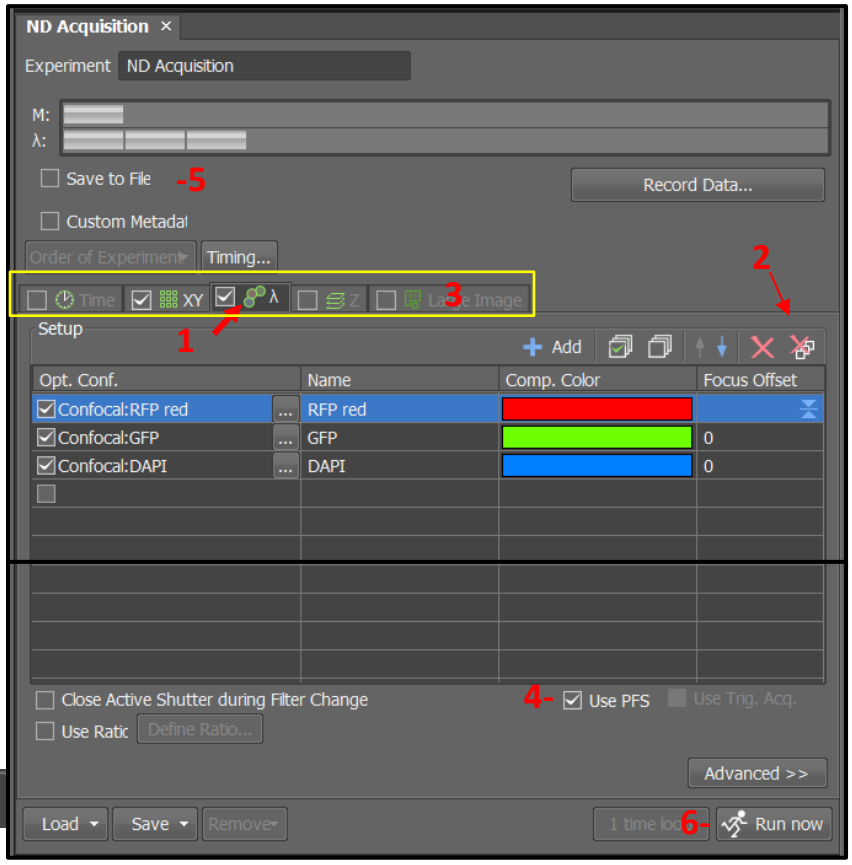


Figure 12: Multichannel Acquisition

Multichannel Imaging:

1. Set up each channel's camera and lamp/laser intensity individually on the main Acquisition panel as directed on p.8-9.
2. Go to the ND Acquisition panel and navigate to the **λ** controls  (Fig 12-1). Check the box.
3. Delete the previous user's settings by pressing the multi X (Fig 12-2).
4. Add the appropriate OCs for your imaging. Combine any settings that use the same camera, such as Mono DIC +Widefield +Confocal settings. Do not combine the color camera and monochrome camera settings or the images will not overlay. *Watch for Confocal versus Widefield options for the same dyes-choose correctly!
5. The software will harvest the exposure and illumination settings into the ND acquisition setup.
6. Beware of checkboxes for other ND functions or they will all try to run! (Fig 12-3, yellow box area).
7. Decide whether you are using the PFS system for perfect focus. Activate it at the bottom only if it has been set up and you wish to use it during imaging (Fig 12-4).
8. Check the "Save to File" (Fig 12-5) to be sure it is not autosaving to a folder. Read more here.
9. Click "Run now" to collect the image set (Fig 12-6).

Setting up Z-Stacks Using Top and Bottom Mode:

1. Ensure the "Z-Stack" checkbox is checked to activate it (Fig 13-1).

2. Select the "Top-Bottom" mode icon (Fig 13-2).
3. Reset the previous user's settings (Fig 13-3).
4. Using the mouse wheel, scroll forward/away from you (clockwise viewed from right) to focus to the "Top" of the stack.
5. Tip: use a channel with signal throughout the cell and stop when things are <u>just</u> getting blurry or dim-don't go too far.
6. Set the "Top" Z-height (Fig 13-4).
7. Using the mouse wheel, scroll backwards/towards you (anti-clockwise viewed from right) to focus to the "Bottom" position.

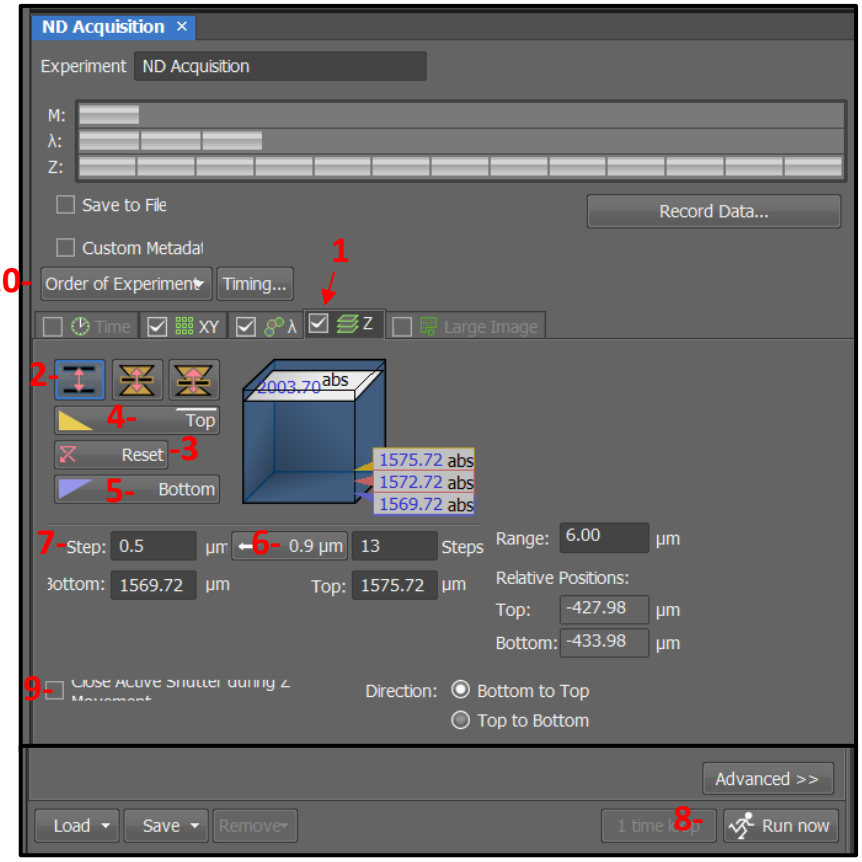


Figure 13: Z-Stack Acquisition-Top and Bottom

8. Set the "Bottom" Z-height (Fig 13-5).
9. **For optimal resolution/deconvolution/3d volume:** engage the z-stack optimization button (Fig 13-6) to set the number of slices needed to avoid any volume loss. (For confocal deconvolution-type in the step size (Fig 13-7) appropriate for magnification (see Appendix II).
10. BOTH the wavelength λ selection and Z-stack selection must be checked/ activated.
11. Click "Run Now" (Fig 13-8).

Order of Operations and Optimizing Speed:

- Leaving "Close Active Shutter during Z" <u>unchecked</u> will speed acquisition (Fig 13-9).
- The order of the ND Acquisition panels affects the order of operations, proceeding <u>right to left</u>. Drag the panels to set the order, or select "order of operations" (Fig 13-10) to adjust it. The <u>channel order</u> operates top to bottom as listed in the multichannel setup (Fig 12).

- In the examples shown (Fig 12 &13): Z on right takes precedence over λ (Fig 13). Proceeding top to bottom in the multichannel (Fig 12), all the RFP layers are collected first, then all the GFP layers, then the DAPI layers. This order takes less time. It is good for fixed samples, but not good for active samples as the channel locations could shift.
- If you move the Z-stack panel LEFT of the multichannel λ or set the "order of experiment" as shown (fig 14): at each layer, RFP/GFP/DAPI will be collected, THEN it will move to the next layer. This takes more time, but co-localization is more accurate if samples move.

Figure 14: Order of Operations

Setting up Z-Stacks Using Center Mode:

1. Ensure the "Z-Stack" checkbox is checked to activate it (Fig 15-1).

2. Select the "Center Mode" option for the Z-stack (Fig 15-2).
3. Adjust the focus to the center of your image (be sure to set up the PFS properly if you will be using that-see page 7).
4. Engage the "Relative" icon to set the middle position (Fig 15-3).
5. There are 2 approaches:
6. FOR OPTIMAL RESOLUTION: Type in the range in um that you want to span (Fig 15-4) and click the optimal spacing button (Fig 15-5) to set the correct number of slices so no image data is lost. (See Appendix II for confocal).
7. FOR SPEED: Type in the range in um you want to span (Fig 15-4), then type in the number of slices you want (Fig 15-6). NOTE: Subsampling causes errors in deconvolution and rendering.
8. Click "Run Now" (Fig 13-8).

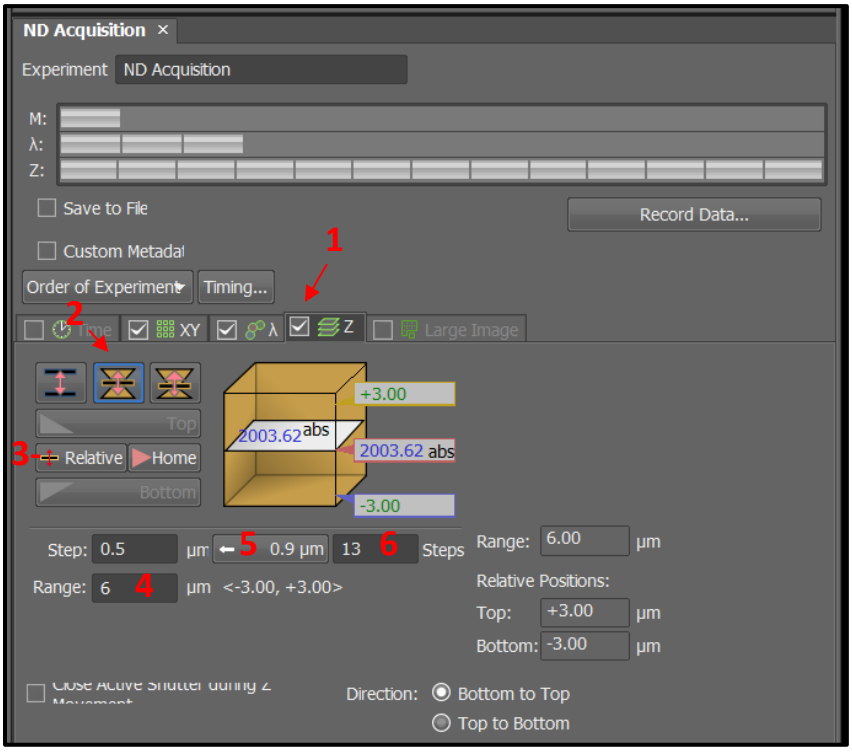


Figure 15: Z-Stack Acquisition-Center Mode

Large Image Stitching (Simple):

1. Activate the "Large Image" Checkbox (Fig 16-1).
2. Select the X and Y scan area dimensions desired (Fig 16-2) and focus on the sample. Imaging will proceed with the current stage position assumed as the center.
3. Select the desired overlap (Fig 16-3). 10-15% works well for most applications. If objects are sparse you may need more overlap.
4. Select the stitching mode (Fig 16-4). Blending works well for most applications. If you have problems with the result, try "Optimal Path". You can also choose "None" and do that step later in image processing.
5. "Image Registration" (Fig 16-5) can be used to correct misalignment.

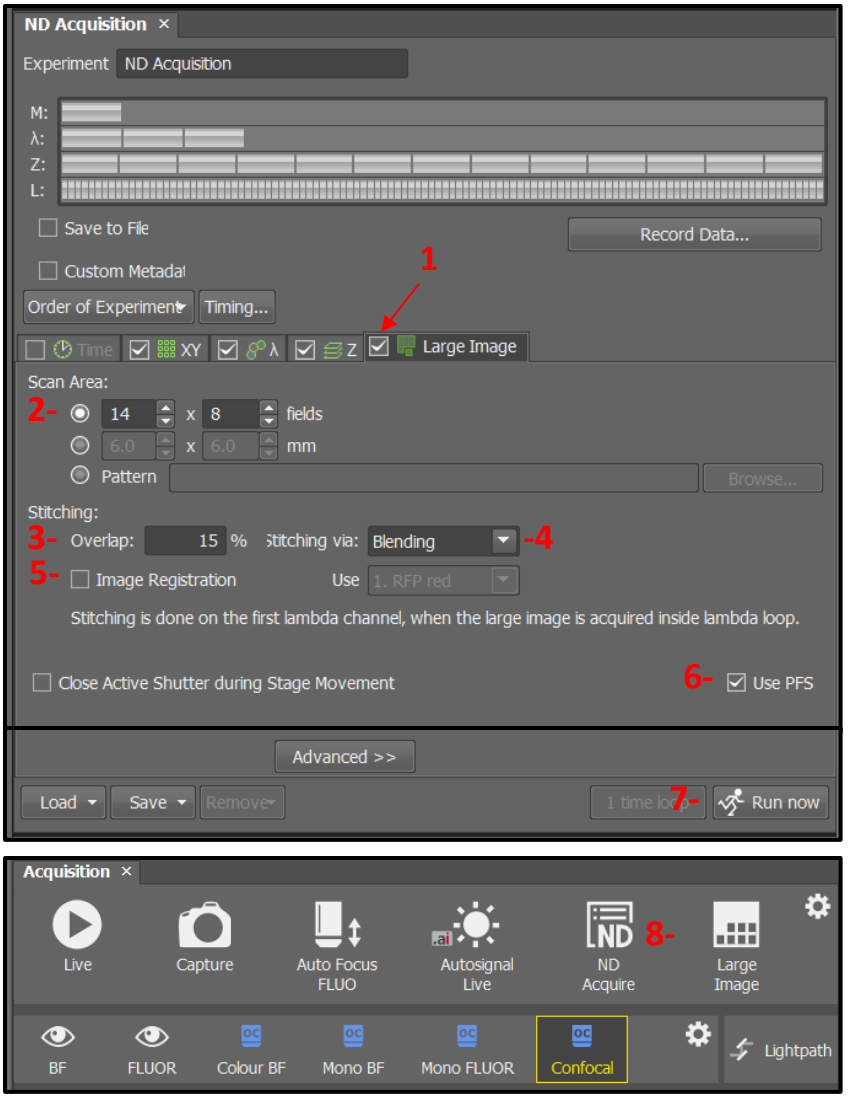


Figure 16: Tiling and Stitching

6. Using the perfect focus for the stitch (Fig 16-6) is recommended. See page 7 to set this up properly.
7. Click "Run Now" (Fig 16-7).
8. Advanced stitching setups are recommended for whole tissues. Please see staff for training.
9. Advanced "Large Image" stitching is accessed through the top of the main Acquisition Panel (screen top right) (Fig 16-8).

XY Multipoint Imaging:

1. Clear the previous users' locations by clicking on the multi-X (Fig 17-1).
2. Add Multipoint locations from the onscreen image by pressing the spacebar OR right-click on a specific location in an image and choose "Add location to Multipoint."
3. TIP: Image whole tissues or coverslips at low mag or with image stitching, then set locations from within the image for high mag capture. Use Advanced (Fig 17-2) settings to set up chambers & well plates.

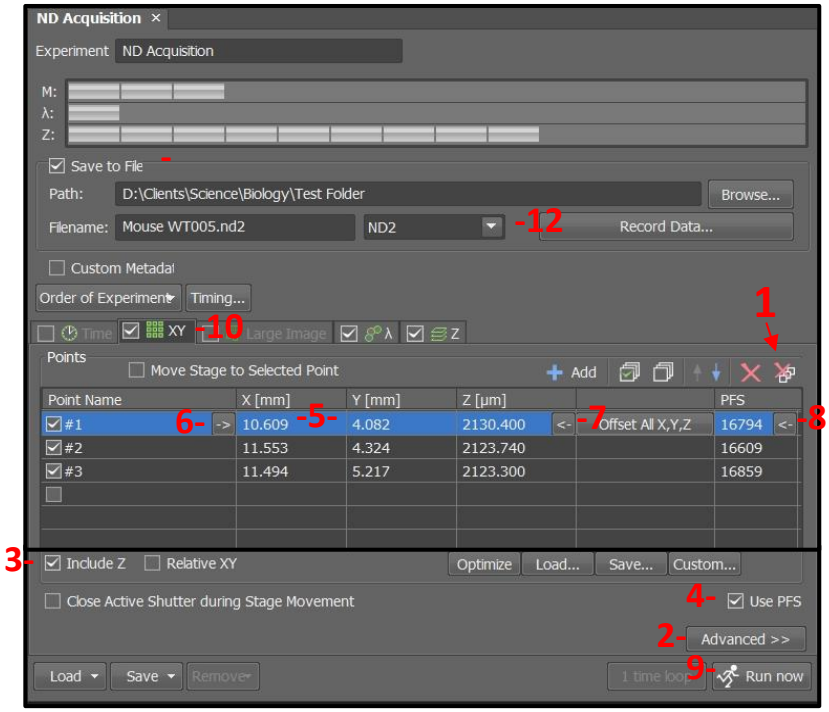
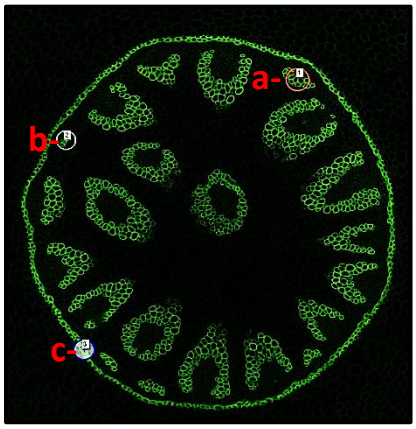
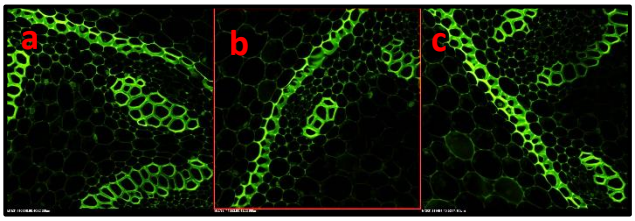


Figure 17: XY Multipoint Imaging

4. **Choose a focus strategy:** either use "Include Z" (Fig 17-3) OR "Use PFS" (Fig 17-4) strategy to set focus per location, not both.
5. In this example, 3 locations (Fig 17-a, b, c) were selected on a 5x pre-scan, added to the XY panel, then the magnification was increased to 20x, and the PFS for that lens was set (see p.7.)



6. **To switch to an oil lens instead:** activate the "Escape" button in the Ti2 panel, lower the stage and drive to a position where you can safely add oil. Return to the coverslip, deactivate "Escape".



7. Double click under the X or Y column (Fig 17-5) in the to drive the stage to the desired location.
8. Center and refocus (for "include Z" strategy) or re set the PFS for this lens (to "Use PFS").
9. Use the white arrows next to each column to update the XY location (Fig 17-6), Z-focus height (Fig 17-7), or PFS offset (Fig 17-8) for each XY location.
10. Click "Run Now" (Fig 17-9). If the XY checkbox is active (Fig 17-10), the locations will be imaged in sequence. If it is not checked, only the current location will be imaged.

Time Series /Time Lapse Imaging:

1. Begin by activating the ND Time checkbox (Fig 18-1).
2. Use multi X to clear previous (Fig 18-2).
3. Each time series definition is a "Phase" (Fig 18-3), and a time series setup can have more than one phase.
4. The "Interval" between rounds of "movie" imaging is used to define a delay between rounds of filming (Fig 18-4), or "no delay" can be chosen if you just want the camera to keep filming as fast as possible between phases.
5. The "Duration" (Fig 18-5) defines how long you want the filming to continue, while the "Loops" (Fig 18-6) defines how many individual image still frames you want. Click on one header or the other to set the flag (Fig 18-6), which determines which option takes priority.

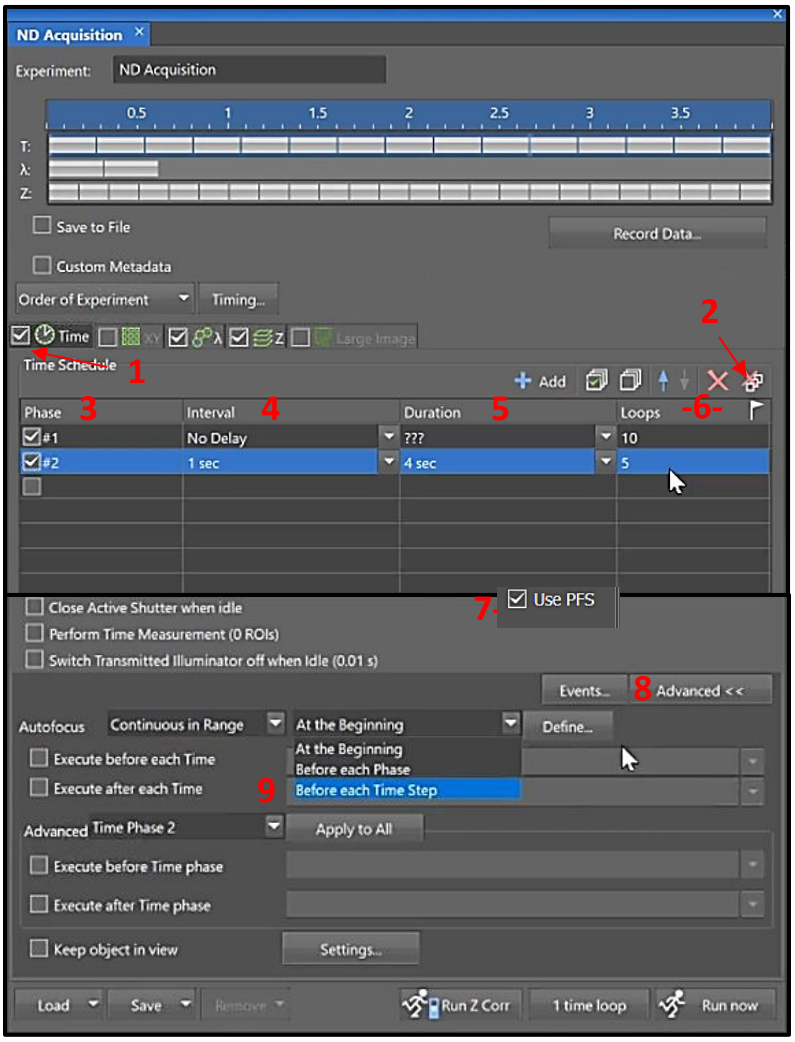


Figure 18: Time Series Setup

6. In the example above, 10 consecutive images would be captured with no delay between them to start. Following that, there would be a 1 second delay, and then filming would continue until 5 images were captured. If the flag were moved to the "Duration" column by clicking there, in the second phase, filming would instead continue for 4 seconds, and the number of images captured in that time would depend on the image exposure time.
7. Choose a Focus Strategy before running. "Use PFS" (Fig 18-7) is recommended, as it corrects for focus drift inherent in long term imaging.
8. You can also open the "Advanced" (Fig 18-8) settings and choose the "autofocus before each time step" (Fig 18-9) method of imaging. This must be optimized-see staff.

Saving Your Files: (see Appendix IV for Tiff Exports)

- All files must be stored on Drive D under your faculty/department/supervisor/your name unless staff indicate otherwise. **Files left on the c-drive or desktop will be discarded!!**
- **Always** save your files as .nd2 format, and keep this full featured file as a backup:
  - Nd2 files contain vital metadata about your image capture for publication.
  - They include scaling information for measurements.
  - They store colour and contrast information from the Lookup Table* (LUT) , but do not apply it to the underlying data, so analysis stays accurate.
  - They hold all the dimensions of a multidimensional experiment in one file, such as all the color channels, time points, Z-layers etc.
- Exports to tiff, jpeg or other file formats can be done in batch mode when you finish imaging.
- You can download a free version of the NIS Viewer for simple viewing and tiff exports: https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
- Our licensed software must be used for batch exporting and full features
- See Appendix V for tiff export instructions.

Quick jpeg or tiffs for Powerpoint and Email:

- Press the "X" key on the keyboard while viewing any image or projection. A screenshot of what you see is instantly created; including any annotations, scale bars, or brightness adjustments
- This can be quickly saved as a jpeg for use in talks and emails
- NOTE: DO NOT use these files for your final thesis, publications, or image analysis due to lower detail resolution.
- Clicking Shift-Z will provide a higher resolution screenshot, but this may be altered from the original. Always keep the .nd2 files. See Appendix V for details on tiff file exports.

Automatic Saving*

"Save to File" (Fig 17-11) is recommended for large image sets (tiling, multipoint, time series).

- Activate the "Save to File" checkbox and ensure "ND2" is set as the format (Fig 17-12).
- Navigate to the folder you wish to use for your images.
- Give the files a base name. Incremental numbers will be added as you image.
- **PLEASE TURN THIS OFF WHEN FINISHED or all future files will save there!**

SHUTTING DOWN

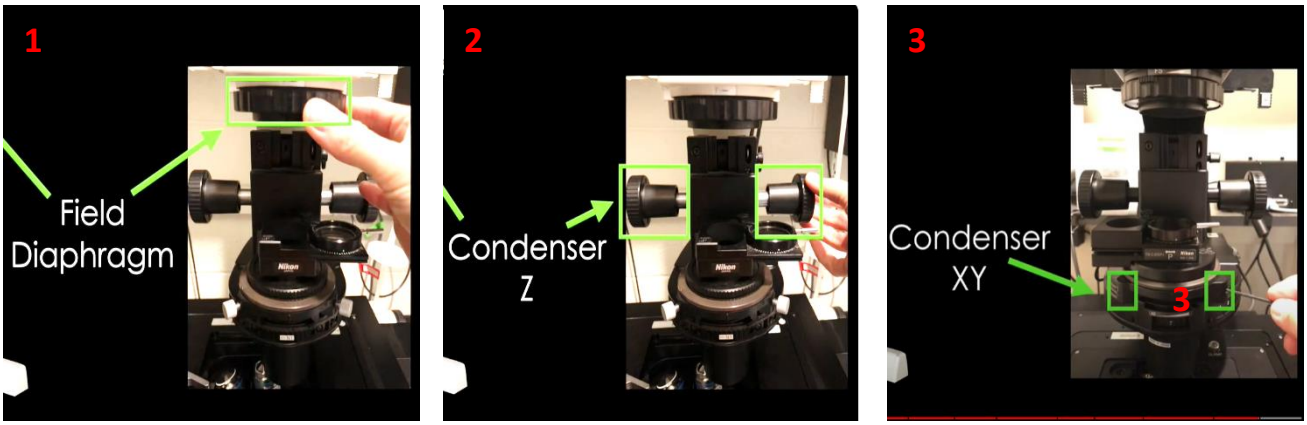
1. Before quitting, check the calendar to be sure no one is coming right after you.
2. The lasers take 10 minutes to warm up and should be left on if another user needs them within 30 min.
3. Be sure you have saved all of your files with the most recent changes.
4. Close the software.
5. Click "Close All" in the shortcuts panel (Fig 6-1) to close images you do not wish to save, or "Cancel" to go back and check on any you are unsure of.
6. Clean any oil you have used from the lenses using the dry lens paper. Be sure to also check the sides and the ring around the edge. Oil residue can damage the $$$ lenses!
7. The order of shut down of the switches does not matter EXCEPT that you need to shut off switch #1 last. Be sure that all 9 switches are shut off.
8. Cover the microscope with the dust cover if you are the last person in the day or after 4 PM.
9. Sign out on the paper log sheet.
10. **Leave the computer on!!**
11. Turn off any room lights you were using.

NOTE:

- **Transfer your files using your OneDrive account only:**
  - USB devices are not permitted except by special arrangement due to malware risks.
  - If a USB device must be used, it has to be scanned by staff at every visit BEFORE you insert it!!
  - Failure to follow these rules may result in lost privileges

APPENDIX I: SETTING UP KÖHLER ILLUMINATION:

Köhler illumination ensures that BOTH the objective and condenser lenses are focused and aligned at the sample plane to enhance sharpness and avoid shadows in the image.



Microscope Parts:

1. Field Diaphragm (Fig 19-1).
2. Condenser focus knobs (Fig 19-2).
3. Condenser XY centering & Orange Allen keys (Fig 19-3).

Follow These Steps:

- Start on 10x magnification (in case the alignment is very far off) and focus on your sample first using bright field mode (BF)
- Close the field diaphragm (Fig 19-1) all the way to the left. You may see a blurry, off center circle of light (Fig 19-4) or a sharp octagon (Fig 19-5) depending on the condenser focus.
- Use the condenser knobs (Fig 19-2) to focus until you see a sharp octagon (Fig 19-5).
- Use the orange Allen keys to center the Condenser so the octagon moves to the middle of the field (Fig 19-6).

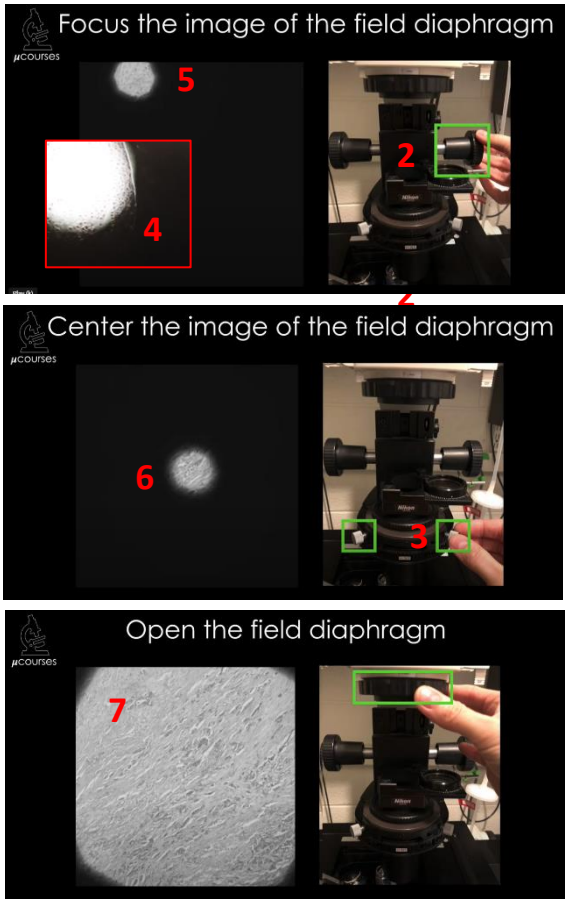
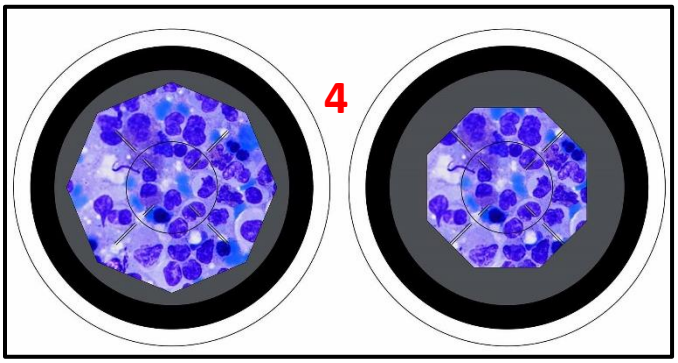


Figure 19: Köhler Illumination

- Open the field diaphragm until the edge of the bright octagon JUST leaves the field of view- a little more open that shown here (Fig 19-7).
- Move to the lens you wish to use for imaging and repeat these steps.

- Now remove one eyepiece and look down the scope with your OPPOSITE eye only (Fig 20-1).
- Set to Eyepiece-BF. Turn down the illumination for comfort on the left side of the stand (Fig 20-2).
- Close the aperture diaphragm (Fig 20-3) until you see the octagon filling about 60-90% of the visible light (Fig 20-4).



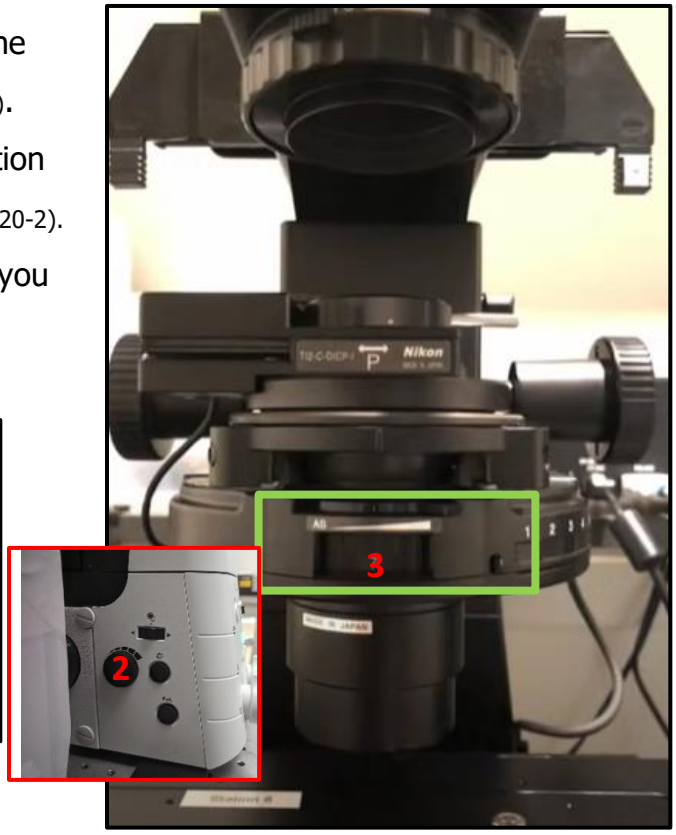


Figure 20: Köhler Illumination Aperture Diaphragm Adjustment

APPENDIX II: RECOMMENDED Z STEPS FOR SPINNING DISK

Wavelength	40x Lens	60x Lens	100x Lens
Default ND Z-Step size	0.6 um	0.3 um	0.2 um
Recommended	**0.3 um**	**0.2 um**	**0.18 um**
DAPI	<0.294 um (XY subsample)	<0.216 um	<0.188 um
CFP	<0.320 um	<0.235 um	No Error (0.2 um OK)
GFP	<0.335 um	<0.261 um	No Error (0.2 um OK)
YFP	<0.374 um	<0.275 um	No Error (0.2 um OK)
RFP	<0.432 um	<0.317 um	No Error (0.2 um OK)
CY5	<0.465 um	<0.342 um	No Error (0.2 um OK)
CY7			

APPENDIX III: USING LOOK UP TABLES

Good images have a wide dynamic range when viewed in the LUT panel histogram (Fig 21-1). Images with poor signals and/or sparse signal will have a short, sharp peaks (Fig 21-2), which may indicate poor sample preparation and/or poor excitation/emission matches.

1. Engage the histogram stretch button (Fig 21-3)  to see your dynamic range in more detail after capture. Aim for wide ranges of signal.
2. For more brightness, drag settings in from right towards center (Fig 21-4).
3. For improving black level/contrast, drag settings in from left towards center (Fig 21-5). (Typically in the depression of the bimodal histogram is best for black levels).
4. Adjust gamma (Fig 21-6) to set midrange only if needed- CAUTION Gamma is not linear!

Controls:

- LUT On/off (Fig 21-7).
- Auto/dynamic LUT (Fig 21-8) keeps things visible in a range of conditions such as during focus.
- Static LUT (Fig 21-9) –used for consistent settings.
- Reset LUT to full range (Fig 21-10).
- Saturation Indicator (Fig 21-11).
- Horizontal/vertical view (Fig 21-12).
- Save or Copy/paste options to create consistent settings for figure panels (Fig 21-13). It is unethical to alter comparative images differently!
- Apply LUT (Fig 21-14) will permanently alter the image data and is not recommended!

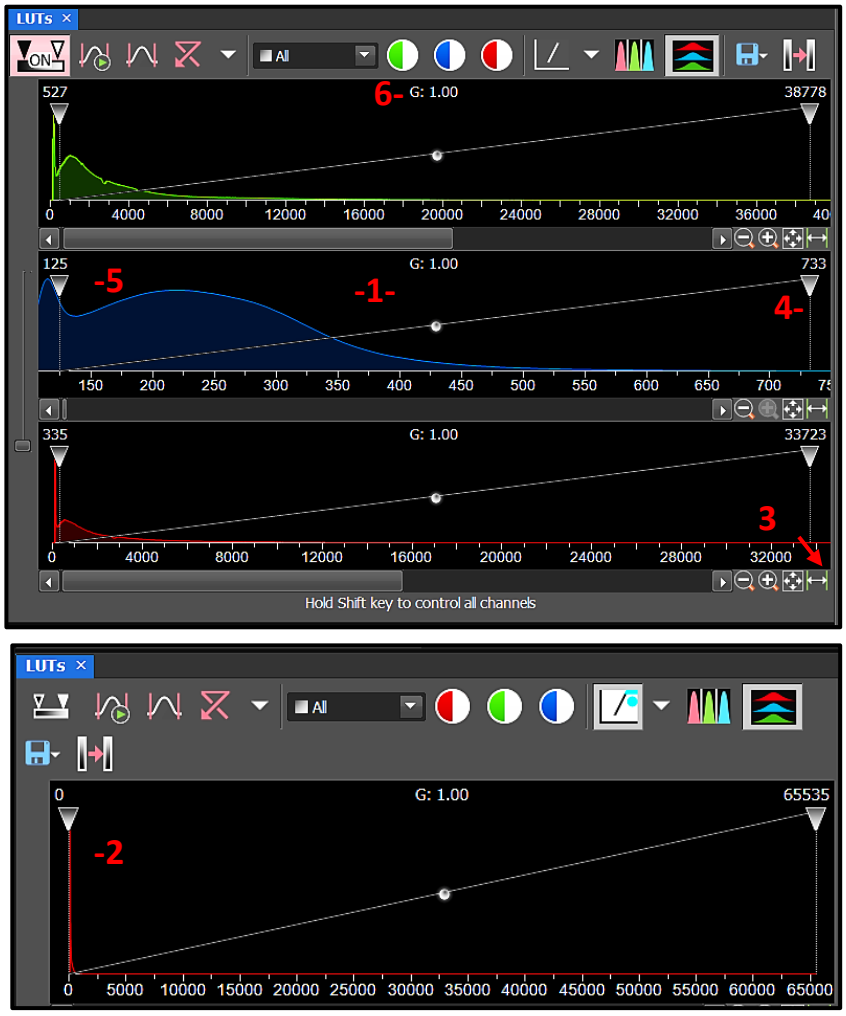


Figure 21: Using Look Up Tables (LUTs)

APPENDIX IV: ADDING SCALE BARS

Adding Temporary Scale Bars in Display:

- The scale bar toggle-on switch is located right of the main image panel (Fig 22-1).
- Each magnification is pre-set for a specific colour and length.
- To change the look, right click on the bar and select "Scale Bar Properties".
    - Change the bar, color, or size on the scale tab (Fig 22-2).
    - Change the font (Fig 22-3).
    - To change the size, deselect the "Automatically adjust size" (Fig 22-4), and the selection of sizes will open.
- This is a VISUAL DISPLAY bar only…it will not come through as a permanent, exported feature in the image.

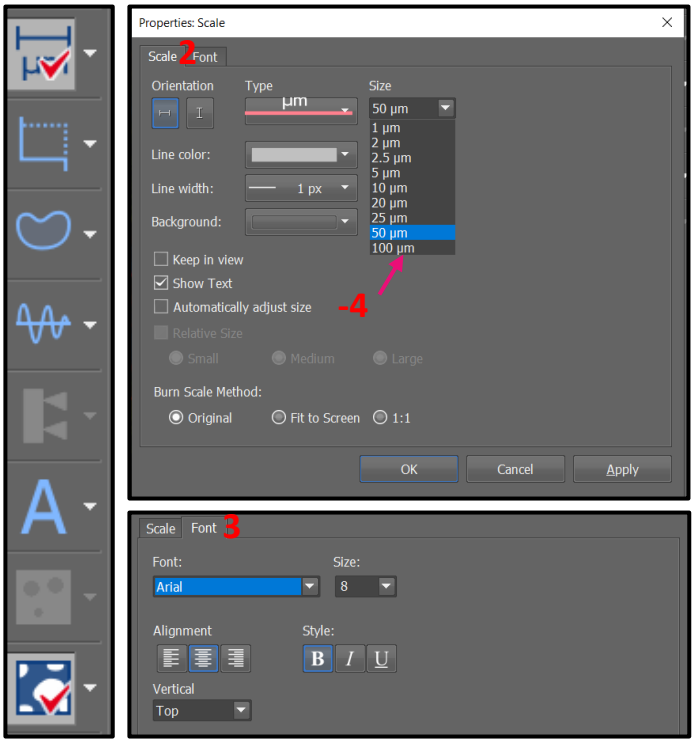


Figure 22: Scale Bars

Adding Permanent Scale Bars:

There are 2 options:

1. "Burn" the bar as you see it into the image, which changes your multichannel image to an RGB image and alters your intensity values. (You will get a pop up warning). To do this, use the dropdown arrow (Fig 21-1) and select "Burn in Scale Bar".
    a. Use this for publications only. Save this altered file with a new name for later export and DO NOT use it for intensity analysis.
    b. You may need to crop each channel and do this individually on each-see staff.
2. Use the Automatic "Burn Scale" checkbox feature in the "Save/Export to Tiff" options described in **Appendix V**. This may be a different font, color, size or location than what you see onscreen in the display.

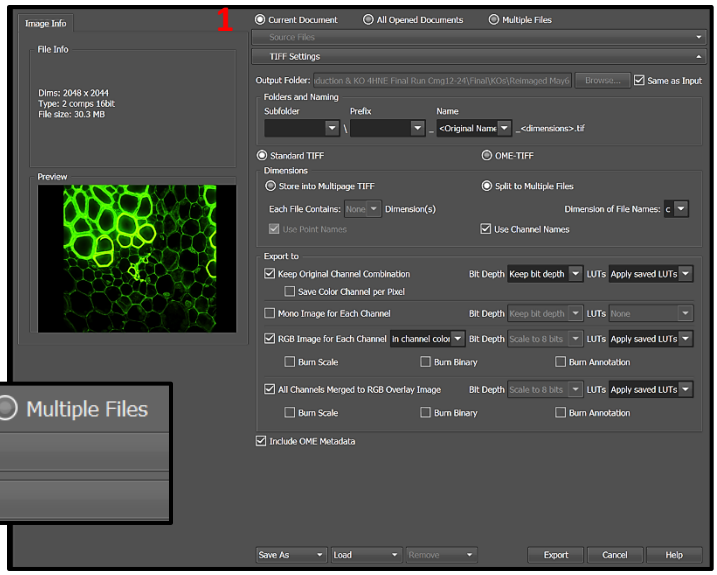
APPENDIX V: EXPORTING TIFF FILES

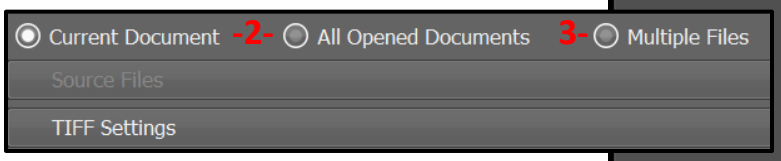
Understand & Think About Your Saving and Export Goals First:

- Do you really need a tiff (or jpeg) image?
    - **Tiff exports** are normally only used for publications and in thesis documents, but are "non-lossy" and thus bettter than jpeg for longterm image integrity.
    - **jpegs** are better for talks and email as they are compressed and load fast. However jpegs are "lossy", meaning they can lose image data over muitple re-saves.
    - **Nikon .nd2 files and OME tiff** exports include metadata: For these formats, multiple image dimensions store within single files that most analytical software can understand.
    - **Software at Integrated Microscopy:** .nd2 and OME tiff formats can be used directly in Nikon GA3, ImageJ/Fiji, Image Pro and Imaris software.
- Multidimensional files like Z stacks, time series, multipoint and unstitched large images:
    - Can consist of dozens-hundreds of individual image panels. Do you want all of these as individual tiffs?
    - You may want to create 3D projections, extended focus images, or crop out single planes or time points.
    - From these alterations-you can also either take screenshots as described on p.16, or save them as new .nd2 files BEFORE exporting.
- Large image scans:
    - May need to be downsized before export, as tiffs are size restricted (see staff).
- Choose Correct Bit Depth:
    - The mono camera captures in 16 bits (65,535 levels of intensity), useful for analysis.
    - PPT, Word, and web software can only read 8 bit images (256 intensity levels). Humans only see about 50-100 levels by eye.
    - For Image Analysis: Use the .nd2 files directly, otherwise OME tiffs are best. For regular tiff, exports should include the full 16 bit dynamic range with no scale bars, which can interfere with object recognition. Use mono image choices (not RGB) for this.
    - Images for publications and talks: should be converted to 8 bit, typically with saved LUTs applied, with or without the scale bars burned in (see section on scale bars)
- Use LUTs Carefully: Ensure consistency with the use of LUTs for scientific integrity-see staff.
    - See Appendix III for instructions on using LUTs correctly.
    - Use Copy/Paste, or Save/Load the settings across images for use in the same figure panel, and apply them when you export. Otherwise you are misrepresenting the signals.

Using the Save/Export to Tiff Functions (Single or Batch Exports):

- From the file menu, select "Save/Export to Tiff" to open the popup window shown
- At the top (Fig 23-1, 2), choose from exporting:
  - the "Current Document".
  - "All Opened Documents".
  - "Multiple Files" contained in a folder with or without subfolders (to batch export).





Batch export mutiple stored files:

- Select "Multiple Files" (Fig 23-3).
- Browse to folder location (Fig 23-4).
- Choose to"Include Subfolders" if required (Fig 23-5). This will load all images in both the master and subfolders.

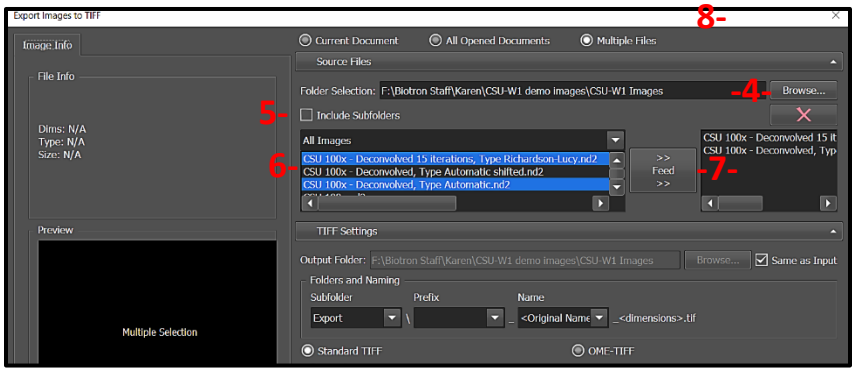


Figure 23: Save/Export to Tiff-Selecting Files for Export

- Select indivdual files from the list, or click CTRL-A to select them all (Fig 23-6).
- Click "Feed" (Fig 23-7) to add them to the active list for export.
- Select "Export" (Fig 23-8) only after you have also set up the naming and folder arrangements as described next.

Setting Storage Location, File Type, and Advanced Naming:

Where to store the exported images:

- "Same as Input" (Fig 241) to store the tiffs in the same folder as the orginal .nd2 image.
- "Browse" will allow you to designate an alternate folder location (Fig 24-2).

Tiffs in lists vs. in subfolders:

- Leaving the subfolder blank means exported tiffs will all appear in one great list (Fig 24-3).
- The other options will create a subfolder named in that format, containing only the tiffs exported from each .nd2 file.

Separate tiffs vs Multipage Tiffs:

- "Split to Multiple Files" is the correct choice to get each of the channels or dimensions as separate files (Fig 24-4).
- "Store into Multipage Tiff" is not typically used. Export as an OME tiff if you want multidimensional tiff files.

Advanced Naming Options (optional):

- To add dimensional names to the file name:
  - Activate the checkboxes for channel or point names (Fig 24-5, 6).
  - Define the image dimensions you have (Fig 24-7).
  - Define the naming order if desired (Fig 24-8).
- To add a Different Name, Date or Prefix:
  - Use the dropdown options for the subfolder, prefix and name (Fig 24-9).

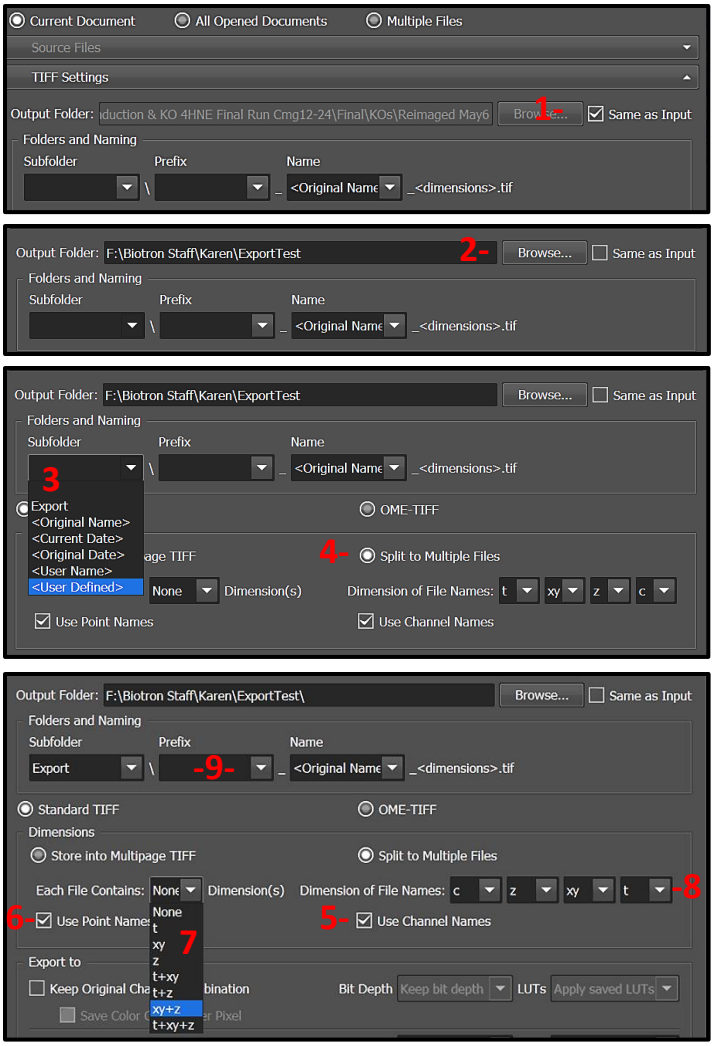


Figure 24: Save/Export to Tiff-Storage and Naming

Typical Export Choices for Multichannel Fluorescent Images:

For Publications and Talks:

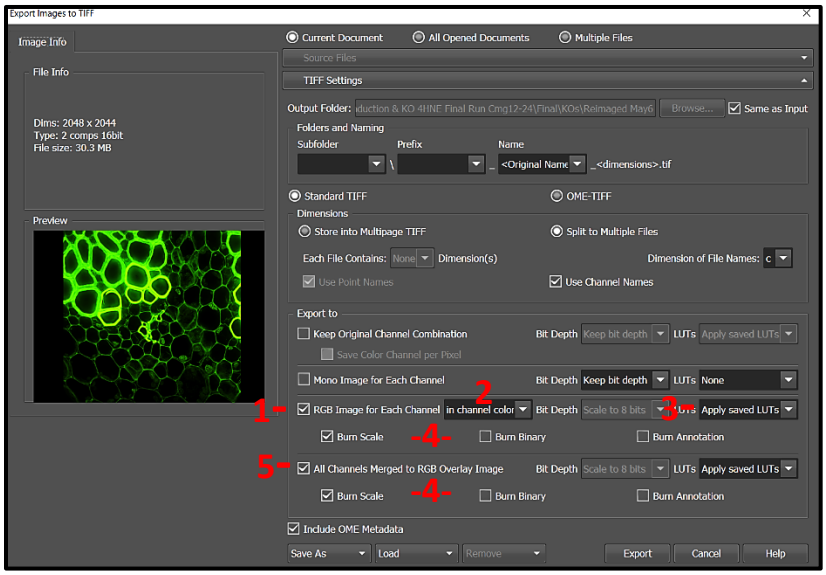


Figure 25: Fluorescent Exports-Publication

1. Choose "RGB Image for Each Channel" (Fig 25-1) and/or "All Channels Merged to RGB Overlay Image" (Fig 25-5).
2. Select "In Channel Color" (Fig 25-2). Bit Depth is automatically 8 bit, as needed for publication.
3. "Apply Saved LUTs" to include contrast adustments (Fig 25-3).
4. Decide whether to "Burn Scale" bar* (Fig 25-4), overlays or other annotations.

- *See p.19 to decide if this scale bar option is appropriate. If you already burned in a scale bar on the original image, this would create an overlaid duplicate. This one is an auto default-you may not like its appearance or location.

For Image Analysis:

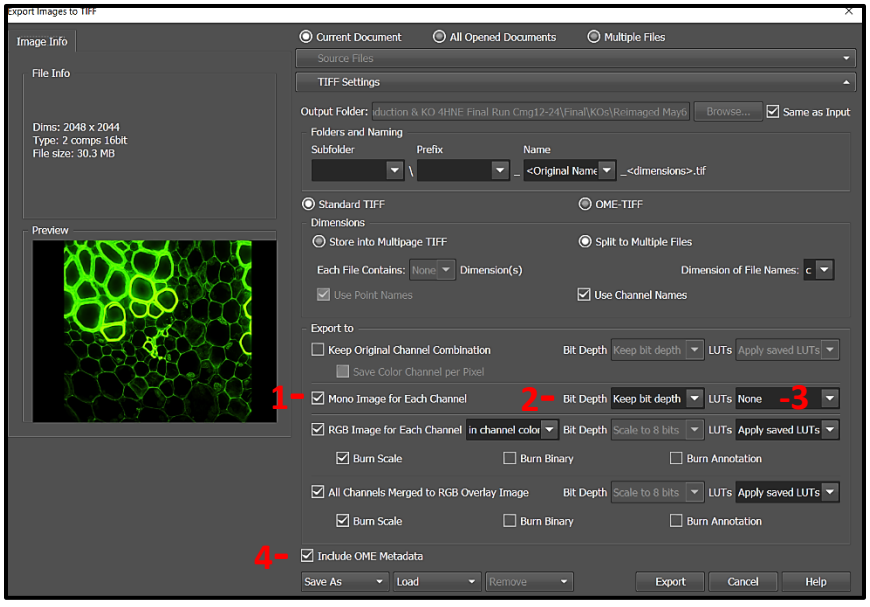


Figure 26: Fluorescent Exports-Analysis

1. Most analysis at Biotron can be done directly on fluorescent .nd2 files.
2. For other software types, follow the directions for OME tiff export on p.26 in the Brightfield example
3. For plain tiff, export as follows:
4. Select the "Mono Image for Each Channel" (Fig 26-1).
5. Select "Keep Bit Depth" (Fig 25-2) to maintain the 16 bit intensity range for better data.
6. Select "LUT-None" (Fig 26-3) to avoid altering your data on output with contrast adjustments.
7. Select "Include OME Metadata" (Fig 26-4). This will add some scaling information that can be read by many 3rd party analysis systems to help you avoid needing to calibrate the scale.

Export Choices for Brightfield/Colour Camera Images:

For Publications and Talks:

1. Choose "Keep Original Channel Combination" (Fig 27-1).
2. Scale Bit Depth to 8 bit (Fig 27-2).
3. "Apply Saved LUTs" to apply contrast adjustments (Fig 27-3). See p.19 for notes on using LUTs correctly.
4. "Save Color Channel per Pixel" improves tiff compatbility with multiple software types (Fig 27-4).

For Image Analysis:

- Choose OME Tiff Option (Fig 27-5).
- OME means "Open Microscopy Environment". It preserves multiple dimensions, bit depth, colour and scaling metadata.
- OME files can be used across multiple software platforms.

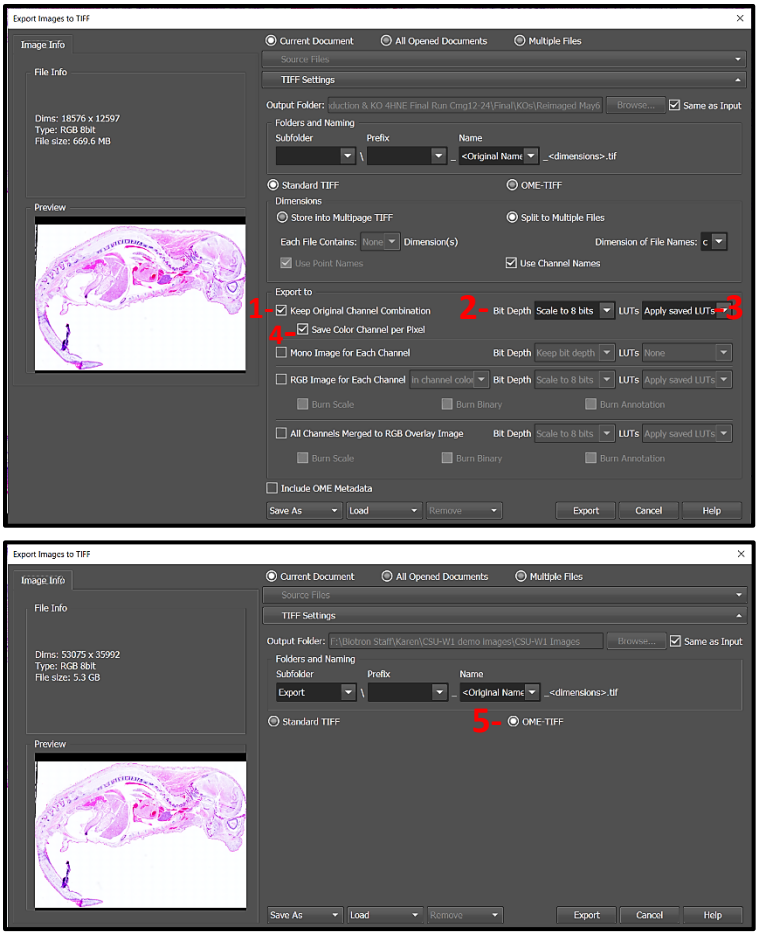
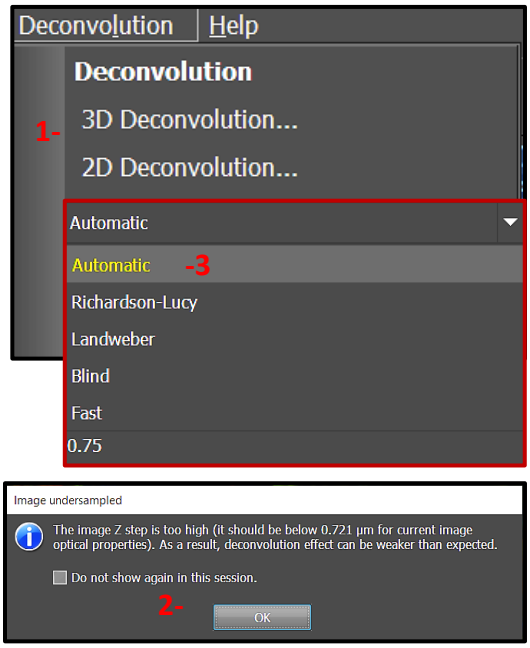


Figure 27: Brightfield/Colour Camera Exports

APPENDIX VI: DECONVOLUTION AND NIKON AI IMAGE PROCESSING

Deconvolution in NIS Elements:



- Deconvolution uses algorithms based on image optics to reassign out of focus, hazy light back to its point source, creating higher spatial resolution.
- Nikon offers a variety of algorithms and options that you can read about in the help menu. Here we illustrate our 2 easy favourites only.
- Launch either 2D or 3D Decon from the Deconvolution menu (Fig 28-1) as appropriate.
- Deconvolution is not ideal for low magnifications: it shows an error message (Fig 28-2) below 40x, or if you have undersampled confocal images in your Z-Stack setup. See Appendix II. You can still deconvolve, but it may be less effective.
- Select the algorithm you prefer (Fig 28-3) See staff or the help menu. The metada populates itself.

Automatic Deconvolution:

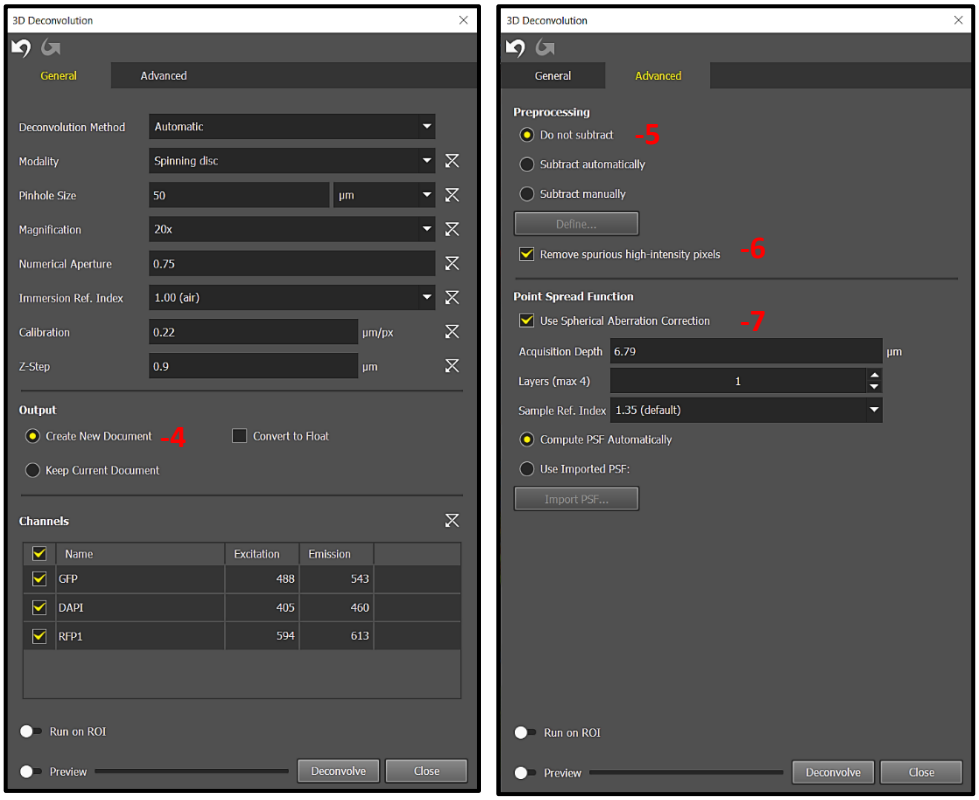


Figure 28: Automatic Deconvolution

- Choose "Create New Document" (Fig 28-4).
- Decide whether to preview or not.
- In "Advanced", do not use background subtraction (Fig 28-5) unless you understand the effects on your analysis data.
- "Remove spurious high intensity pixels" (Fig 28-6) and "Use Spherical Aberration Correction" (Fig 28-7) are useful additions for confocal images. See the help menu for definitions.

Richardson-Lucy Deconvolution:

- This is an iterative, self-correcting algorithm, so you need to set your iteration preferences (Fig 28-1).
- Typically 20-30 iterations (Fig 29-1) with "Auto Stopping" activated (Fig 29-2) is a good start.
- Follow the other rules on p.27 for the rest of the setup.

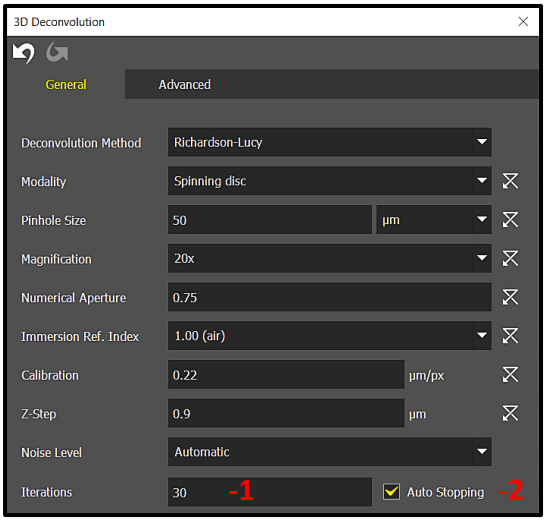


Figure 29: Richardson-Lucy Deconvolution

Batch Deconvolution (BD):

1. Once you have established the best settings and algorithm for your data set, you can set up batch deconvolution.
2. If Batch Deconvolution software is already running- please do not close it or remove any files. Just add your own files and settings.
3. BD runs in the background until all the files have processed. Others can use the system while it runs.
4. Begin by adding single files (Fig 30-1) or whole folders (Fig 30-2) of images.
5. Previously deconvolved images will be detected as unsuitable and not processed (Fig 30-3).
6. Suitable files appear in white (Fig 30-4).
7. Files with problems will be flagged in red (Fig 30-5). This is usually from "Invalid definitions" (metadata) for the algorithms or corrupted file saving. Often these are tiff or jpeg files in the folder. Try renaming corrupted files.

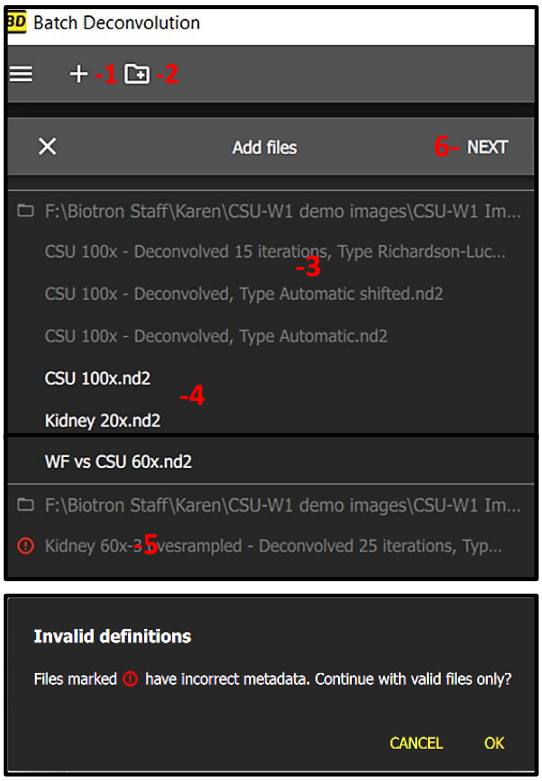
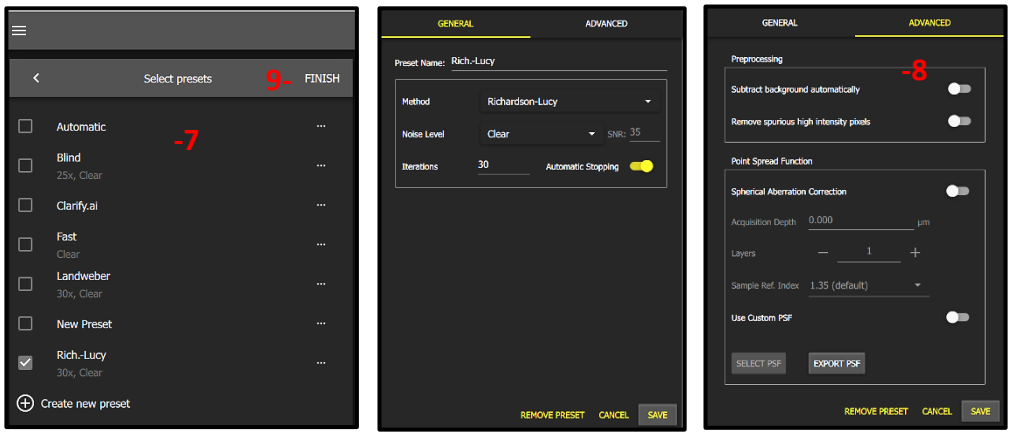


Figure 30: Batch Deconvolution

8. Click "Next" (Fig 30-6) to set up algorithms (Fig30-7) & settings (Fig 30-8).
9. Click "Finish" (Fig 30-9) to finalize and activate. Leave software running.



Using AI Image Processing (Clarify, Denoise, Restore)

NIS Elements' AI algorithms are used primarily to make images look sharp and "pretty" for publication and communication. They are not uniform in their treatment of each area of the image for analysis. The resulting images CAN be used for object recognition such as finding & counting cellular outlines, but they should not be used for intensity-based analysis.

Clarify AI

Clarify is particularly useful for low magnification images (20x and under). It is not recommended for spinning disk images-use Decon instead.

- Launch "Clarify.ai" from the AI menu next to the deconvolution.
- The metadata should populate automatically as shown, but you may need to select WIDEFIELD modality.
- Select "Create New Document" (Fig 31-1) to avoid modifying the original.
- Select the channels (Fig 31-2) to Clarify-do not clarify DIC or brightfield images
- Choose whether to "Denoise" (Fig 31-3) or not.
- Click OK" (Fig 30-4) to create new document
- NOTE that these algorithms auto-name the file and auto-set the LUT for you-adjust that as needed when finished.

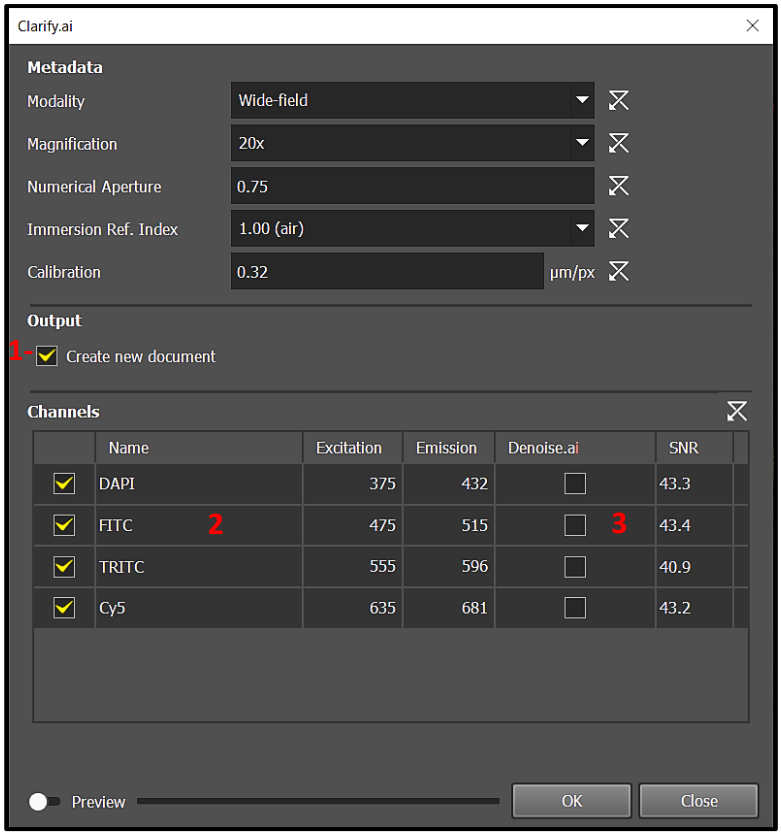


Figure 31: Clarify.ai

Denoise AI, Restore AI

As we use these infrequently, see staff if you need help with them.

- "Denoising" cleans up detector and camera shot noise (specular appearance), and is very useful for point scanning confocal images, or weaker images from our system.
    - Often we apply "denoise" within the clarify algorithm or in GA3 analysis workflows

- "Restore" is a whole image clean-up. It will choose on its own where to apply clarify, denoise, background subtraction, or deconvolution to various parts of the image to make it as pretty as possible.
    - It is HIGHLY non-uniform in its approach, and very slow.