

University of Minnesota Nano Center Standard Operating Procedure

Equipment Name: Confocal Microscope

Model: Nikon Eclipse Ti/Nikon C2

Location: PAN 181

Revision Number: 3

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A. Introduction

- 1. Tool Description.** The Nikon C2 Confocal is a multi-purpose microscope system designed to function as a) a high quality manual inverted microscope, b) a fluorescence-enabled microscope, and c) a confocal microscope system, depending on user preference.
- 2. Tool Components.** The Nikon Confocal system is built on an advanced Nikon Eclipse Ti light microscope with an inverted geometry, making it ideal for examination of biological specimens suspended in liquids or adhered to coverslips. The microscope is equipped with an LED white light source, light source filters, and a polarizer for observing with transmitted light. It has five objectives mounted in a motorized nosepiece, giving magnification of 40x to 600x. Phase contrast microscopy may be used with all objectives to see more detail in transparent samples. The Nikon Eclipse Ti microscope is mounted on an air table to minimize vibrations.

The Eclipse Ti serves as the base for two microscopy systems: wide-field fluorescence and confocal microscopy. *Fluorescence studies* are enabled by a Sola white light engine, a set of four filter cubes mounted in a motorized filter wheel, and a Zyla 5 megapixel monochrome digital camera. (If color images are desired, please use the Leica DMI 3000B microscope, also located in room 181.) After adding specific fluorescing chemicals (fluorophores) to a sample, users can illuminate their sample with selected excitation wavelengths, causing the fluorophores to emit light at a characteristic wavelength. The microscope's filters allow only the narrow emission band to pass on to the eyepiece or camera, filtering out the excitation light and revealing the emission of the fluorophores against a dark background. Images can be collected with the Zyla camera at each emission wavelength, and can be stacked to produce a colored image.

Confocal microscopy allows sharp imaging of samples with more relief and depth than can be focused using conventional microscopy. Confocal capability is enabled by a) a point light source, consisting of a set of four lasers at 405, 488, 561, and 640 nm, which is scanned over the image plane; b) a confocal imager, which is mounted on the Eclipse's left camera port; and c) the Nikon C2si confocal controller, which manages the scanning and image reconstruction functions. For a fuller description of confocal microscopy, please see the references below.

Both the fluorescent and confocal functions are controlled using the Nikon Elements software. The operator uses Elements to select excitation wavelengths, fluorescence filters, and illumination intensity, and to collect, edit, and store images taken with either the the Zyla camera. All confocal imaging parameters are likewise set within Elements.

- 3. Safety.** Laser light is used for the confocal function. The shortest wavelength laser emits at

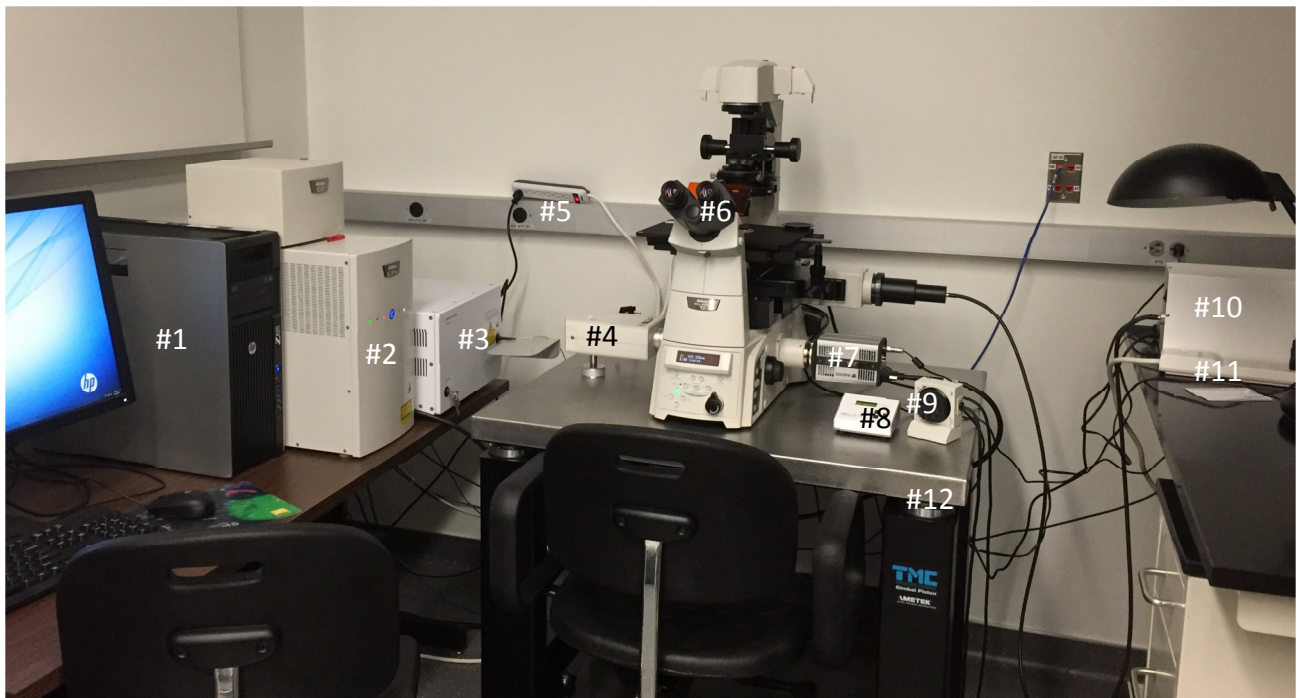
405nm, which presents a UV radiation hazard. DO NOT LOOK into the area on the stage illuminated by the 405 and 488nm laser sources.

4. Restrictions/requirements.

- a. The Nikon system for wide field fluorescence or confocal microscopy is optimal when the room darkening curtains are shut. Be sure the curtains are closed to prevent room light from entering.
- b. The Ti microscope is mounted on an air table set to dampen vibration. It is not a rigid support, so avoid leaning on it or resting your elbows on the table, as this will cause the table to shift position.

5. **Required Facilities.** The air table on which the microscope is mounted requires clean dry air at a pressure of at least 20 psi. The system requires 120V AC power and a connection to a Windows-based computer.

6. Parts.



#1. Computer

#2. C2si controller

#3. Agilent laser box

#4. Confocal system camera

#5. Power strip 2

#6. Eclipse Ti microscope

#7. Zyla camera

#8. CoolLED light source control pad

#9. Focus wheel

#10. Sola light source

#11. Power strip 1

#12. Air table

B. Start-up Procedure

1. Begin a new entry on the tool log sheet. List your name, the date, and the type of sample you are imaging (e.g., bacteria, mammalian cells, tissue sample, etc.). At the end of your session, add to the log a note on any issues that you may have encountered; if none, put “OK”.
2. Rouse the Nikon Confocal computer if it has gone to sleep by striking any key. Do a software restart of the computer (this is necessary to reload the camera drivers).
3. After restarting, log onto the computer using your x500 user name and password.
4. Hardware power up procedure—must be done in order (see picture on p. 2 for locations of items)
 - a. Turn on power strip #1, marked “Belkin” (strip is located on table to the right of the microscope). This energizes the Eclipse Ti microscope, Zyla camera, and Sola light source. Leave the Sola light source manual override switch in the off position (the light in the front should be off). (If you plan to do confocal imaging, continue with steps b-d. If you are not using the confocal function, go to step 6).
 - b. Turn on the C2si controller by pressing the front button on the C2 case.
 - c. Turn on power strip #2. This energizes the Agilent laser control system. (sitting on cord strip on wall)
 - d. Ensure that the keyed switch on the Agilent box is in the “on” position (horizontal). Select one or more of the laser wavelengths you plan to use, and turn these on by pressing the appropriate button on the front of the laser control system. Hold the button for 1-2 seconds until it lights up.
5. Open the Nikon operating software, NIS Elements, by double clicking its icon on the computer desktop. User guides to the Elements software are found on the computer in the “Documents\Public Documents” folder.
6. When Elements starts up, you will be prompted to select a camera input, either “ANDOR Neo/Zyla”, “Nikon Confocal”, or “multi-camera.” If you plan on using bright field, phase contrast, or wide-field fluorescence microscopy, select the ANDOR Neo/Zyla option. If you plan to do confocal microscope imaging, select the Nikon Confocal imager. If you need both cameras, select the multi-camera option.

C. Operation: Manual Microscopy

For any observation method, you will need to initially find and focus on the area of interest, which is usually done using the microscope in the manual bright field or phase contrast modes.

1. If your sample is on a standard 1 x 3” microscope slide, place it in the holder provided. If you prefer, you can also use sample holders with circular apertures.
2. Check the sliding filters on the back of the Ti to ensure that the light path is clear.
3. Turn on the CoolLED Light source using the CoolLED control pad and adjust intensity.
4. Select a low power objective (4 or 10x) using the motorized nosepiece control, on the left side of the microscope base.
5. Position your slide by adjusting the stage control, located on the right side of the microscope stage. It is best to place an edge of a coverslip or obvious part of the specimen in the illuminated field, giving you a visible feature to focus on. For best optics, put the coverslip down towards the objective lenses.

6. Check to ensure that the light path is going through the eyepiece:
 - a. The light path indicator on the front of the microscope base should be at the “Eye” position.
 - b. The mechanical shutter knob near the right eyepiece should be at fully inserted position.
7. Look through the eyepiece and move the stage until you see a slide edge. Use the manual focus knobs on either side of the microscope to bring the top of the edge into focus (or, if you have inverted your slide, the bottom edge). Note that you can set the focusing to be coarse, fine, or extra fine by using focus selectors, located on either side of the microscope base.
8. You can optimize the light level using either the CoolLED light intensity (on the remote pad to the right of the microscope) or by adding filters to change light intensity and color. The filters are
 - a. ND: Neutral density, for lowering light intensity with no color change
 - b. GIF: green interference filter
 - c. D: provides a simulated daylight color balance
 - d. NCB: provides a color balance preferred for video applications
9. For simple bright-field microscopy, set the phase ring selector (located in the light source condenser) to position A.
10. To use phase microscopy, swivel the appropriate phase filter into the light path. Each of the objectives has a matched phase ring.
 - a. 4x objective: use the PhL phase ring.
 - b. 10x, 20x, 40x objectives: use the Ph1 phase ring.
11. If your image appears unnaturally colored (blue, green, or red), you are looking through a filter cube. On the front panel display of the Ti Eclipse, a display setting indicates the type of filter cube in the light path. Installed filters are labeled DAPI, GFP, and Texas Red, with two open positions in the filter turret. Use the “FL Block” control on the right side of the microscope to switch the filter turret to an open position.

D. Operation: Fluorescence Microscopy

Once you have found the region of interest on your sample, you can use either the fluorescence or confocal imaging modes. The fluorescence mode collects sample images from the entire field of view at once, and is thus called “wide-field” or “epifluorescence.” This is to distinguish it from confocal imaging, which also uses sample fluorescence but assembles the image in a pixel-by-pixel process. You may observe fluorescence using either the eyepieces or the camera.

Light source: The Sola light engine supplies broad spectrum white light to the Nikon’s rear illumination port. It may be turned on using either its front panel switch or using the Elements software (more on that below). There are several filters located in the rear illumination port which slide in and out of the Sola light path. Begin your work with all the filters in the out position.

Note that the Cool LED light source should be turned off while in epifluorescence mode. This can be turned on and off using the Cool LED remote to the right of the microscope. The Cool LED source needs to be turned back on for brightfield or phase contrast.

Light Filters. The Ti is equipped with a set of four filter cubes mounted in a motorized filter turret. These filters have a dual purpose: 1) they pass a section of the white light spectrum to the sample to

illuminate it with a narrow range of excitation wavelengths, and 2) allow only specific emission bands to pass back to the eyepiece or camera, filtering out the excitation light and revealing the emission of fluorescence in the sample. The Nikon presently has the following filter cubes installed.

Filter Cube Label	Excitation Wavelength, nm	Emission Wavelength, nm
DAPI	340-380nm	435-485nm
GFP/FITC	465-495nm	515-555nm
Texas Red	540nm	605nm

Viewing epifluorescence through the eyepieces:

1. For viewing by eye, ensure that the light path control on the Ti's front panel is directed to "EYE."
2. Turn off the Cool LED light source using the Cool LED remote pad.
3. Confirm the correct filter cube is in position (change filters using the "FL Block" control on the right side of the microscope). Ensure that the curtains are fully closed for best images.
4. View the specimen through the eyepieces and adjust intensity as needed. Note that most specimens will photo-bleach with extensive exposure to excitation light, so try to minimize the light intensity and the amount of time the light hits your specimen.

Using Elements software for epifluorescence:

1. To view and collect images using the Zyla camera, select this camera when starting the *Nikon Elements* software. When this camera is selected, *Elements* will open with the control panels relevant to epifluorescence.
2. Also, set the light path on the microscope to R100 (100% of light to right camera port).
3. *Elements* software controls
 - a. *Magnification* (4x, 20x, etc.): When you click on one of these "Optical Configuration" (OC) buttons, the software automatically changes the settings on the microscope and in the software.
 - b. *Channel* (Texas Red, GFP, DAPI, and Brightfield or DIC): These "buttons" are also OCs that switch the filter cubes on the microscope (as opposed to the user manually switching the cubes using the "FL Block" control on the microscope).
 - c. *View mode*: view with eyes, view with camera, or view with confocal (this changes the light path to camera or to eyes, but it does not show a live image).
 - d. *Sola Pad*: this window allows the user to adjust intensity of light for epifluorescence illumination.
 - e. *Camera settings (Zyla settings)*: this window is for image acquisition and controls camera resolution, exposure level, and capture speed.
4. To show a live image, hit the green arrow button in the top menu bar.
 - a. Hit auto exposure in the *Zyla Settings* window to get a good start for an exposure.
 - b. At first, leave the Dynamic Range set to 12 bit and the Gain set to 1. If the auto exposure time is too long, bump the gain up to 4.
 - c. The other *Zyla settings* will not be changed in normal use.
 - d. In the "Live" window, click the "x-y graph" button to show areas that are over exposed. This button can be clicked on or off.

- e. Turn down the Sola light level until you no longer have over-exposed regions.
 - f. Adjust zoom to see the image in more detail.
5. Once the settings look good, hit the camera button to take a picture. File -> Save as -> and save it as a .nd2 so all the information is saved. Repeat this process to acquire images using other channels.
 6. To merge channels, click on the “GFP” channel tab at bottom left of picture window and drag into the other channel that is open. This now gives you three tabs to choose from, GFP, Texas Red, and All (merged). This merged file can also be saved (versus individual channel files).
 7. Once both or all channels look decent for brightness, you can set up an experiment in *ND Acquisition* on the left side of the software to speed image acquisition of multiple channels. In the ND acquisition tab, click the Lambda button and add the channels you want to use to take pictures. Then, hit “Run now” and it will take the pictures and open all of them in a new window. This way, you don’t need to drag the channels into one window. (see detailed instructions below).
 8. If you then change the preset settings from what you had in the experiment, a red exclamation point appears next to the channel setting OC button. If you then right click in the exclamation point, you can either “Assign current settings,” or “Show differences.” If you don’t want to permanently (for all users) change the setting, just click on the channel and it will not save this change permanently.

E. Operation: Confocal Imaging System

A confocal imaging system is particularly advantageous due to its ability to collect a shallow depth of field, eliminate out-of-focus light, and collect optical sections through thick specimens. One of the most common applications of confocal microscopy in biomedical sciences is taking images of living cells or tissues labeled with fluorescent probes.

Confocal imaging differs from wide-field fluorescence/epifluorescence microscopy. In wide-field fluorescence, the entire specimen is exposed to light from the Sola light source and the image can be viewed by eye or by camera. With confocal, a laser light is focused through a pinhole and then scanned across a single focal plane of the specimen. Each plane collected by the confocal system is called an optical section. These optical sections can then be “stacked” by the computer software to create a 3-dimensional rendering of the specimen.

The confocal imaging system can only be operated and viewed using the *Nikon Elements* software. Many of the basic operations are the same as in epifluorescence operation. Also, a general hint is that the software makes extensive use of right click and long click to access settings, so start there when setting up your scans. An “X” symbol in any menu will reset values to the appropriate default.

To view and collect images using the confocal system:

1. Select Confocal when starting the *Nikon Elements* software. When this camera is selected, *Elements* will open with the control panels relevant to the confocal.
2. Set the light path on the microscope to L100 (100% of light to left camera port).
3. Select an appropriate Optical Configuration (OC) for your needs (405-488-561 for example).

Every OC has one or more channels (laser/filters/detector combination), so check the channels you will use, and include TD if desired (TD will use the longest laser wavelength first when pictures are taken in Series).

4. Settings:
 - a. Start with a full field of view (FOV) (no zoom, no cropping) by hitting the red X icon in the C2plus Scan Area window.
 - b. Select Pixel Dwell Time (1.9) in a medium size frame (512).
 - c. Unselect Averaging and Sequential scanning of the signal.
 - d. Frame size (64 to 2048 pixels), dwell time (1.9-44.2 μ s), scan directionality (uni- or bi-), and image averaging determines the speed of acquisition in frames per second.
5. Press Scan, and the confocal image appears in the live window.
6. Adjustments
 - a. Search for your field in Z using the focus remote pad or scrolling the mouse over the active acquisition window (forward is away from the sample or towards the top of the stack, backwards deep into the sample towards the bottom of the stack).
 - b. Use the sliders in each channel to adjust laser intensity, detector HV (sweet spot 80-120, noisy > 150; a good start is obtained using the autogain button, AG).
 - c. If there is cross talk between the channels (DAPI bleeding into the 488 channel for example), select Sequence Scanning. This will do sequential scanning of each channel.
 - d. Adjust the frame Size and Zoom to your resolution needs in the C2plus Easy GUI using your brightest channel, keeping in mind Nyquist resolution requirements. Crop out undesired field areas.
 - e. You may need to choose Averaging to obtain the appropriate S/N ratio and reduce noise to acceptable levels. Average lines (2x-32x), this decreases noise in the image at a cost of acquisition speed.
7. Press Capture by hitting the camera button in the top menu bar. The single optical section image is acquired but not saved. To save, go to File -> Save as -> and save the image as a .nd2 file so all the microscope setting information is saved along with the image. Repeat this process to acquire images using other channels.

F. ND acquisition

Acquiring more than one optical section, channel, field, or time requires the use of ND (Nth Dimension) acquisition software. This software also allows concatenation of several OCs that can be applied to the same or different fields. If a simple time lapse or Z-series is desired there is no need to use ND Acquisition. All of these acquisition options can be combined to generate exceedingly complex protocols.

Order of Experiment: determines the order in which the different modes are implemented.

Timing: provides an estimate of total acquisition time.

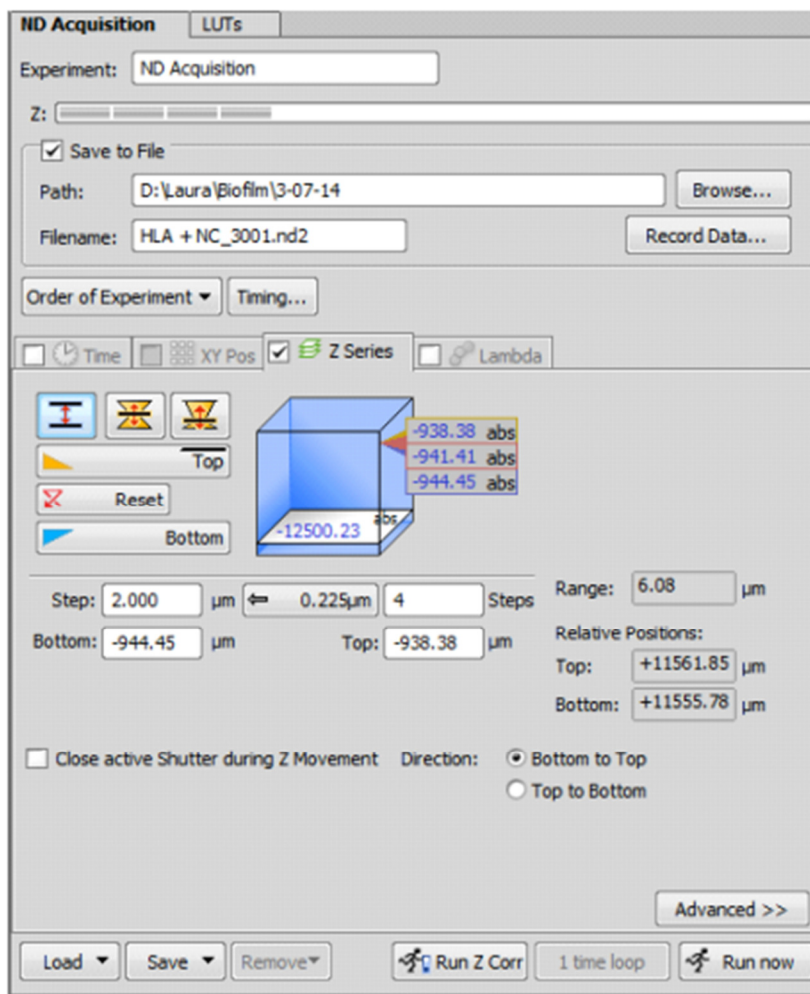
Save Options: Check Save to File box so acquired images will be automatically saved. Update the folder to which files will be saved in Path. Enter a filename that will be used as prefix for all acquired files.

Time: allows time lapse image acquisition. Each **Phase** can have its own Interval (time between images), **Duration** (total phase time) and **Loops** (recycling the duration and interval n times).

Z: allows imaging different optical sections of a FOV. To define your range you can select the Top and Bottom of your stack. Range gives the number of sections. Select the spacing between the sections using Step. Check the box for Close Active Shutter during Z Movement.

Lambda: allows combining different channels to sequentially image the same FOV.

Click Run now to perform the Acquisition.



F. Tool Shutdown

When your work is complete,

1. Save any open images that you wish to keep, and then close the Elements software
2. Shut down the hardware in reverse order from the start-up procedure: turn off the laser(s), shut down the Agilent laser control box, turn off the C2 confocal power switch, then turn off the Ti power strip.
3. Log out of the Nikon Confocal computer. DO NOT turn off the computer, since the on/off switch does not always work. Instead, leave the computer on and power down the monitor.

G. Resources

For a summary of imaging techniques:

<http://zeiss-campus.magnet.fsu.edu/articles/livecellimaging/techniques.html>.

<http://www.microscopyu.com>.

For more information on *Nikon Elements*:

<http://www.nikoninstruments.com/Products/Software/NIS-Elements-Basic-Research>