SOP Number 1

SOP Title Nikon A1R Laser Scanning Confocal Training and Operating SOP

	NAME	TITLE	DATE
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1. PURPOSE

This procedure outlines the steps to be used in training users for operating the Nikon A1R Laser Scanning Confocal Microscope system.

2. SCOPE

This procedure applies to all students, staff and researchers who use the Nikon A1R Laser Scanning Confocal Microscope system.

3. DEFINITIONS

The Nikon A1R Laser Scanning Confocal Microscope is used to eliminate diffracted and out of focus light from microscopic images by using a pinhole through which excitation light and emitted light passes. The system (now referred to as the Nikon confocal) includes lasers, scanning mirrors, Nikon Ti microscope with objective lenses, motorized stage, mercury arc lamp, HP computer, monitor and Nikon Elements 3.10 software for acquisition. The Nikon confocal is used primarily for imaging fluorescent dyes bound to biological samples.

4. RESPONSIBILITIES

4.1 It is the responsibility of the Microscopy Technician to organize regular training sessions for the safe use and upkeep of the Nikon confocal.

4.2 It is the responsibility of all Users to ensure they receive proper instrument and procedural training prior to using the Nikon confocal.

4.3 It is the responsibility of all Users to operate the Nikon confocal according to the procedures enclosed in this document.

4.4 It is the responsibility of all Users to immediately report any damage or malfunction of the Nikon confocal to the Microscopy Technician. Users are required to leave a note in the instrument log and to contact the Microscopy Technician, describing the problem and include your name and the date the problem occurred.

4.5 It is the responsibility of all Users to undertake a pre-use inspection of the instrument before operation.

4.6 It is the responsibility of all Users to undertake a post-use inspection of the Nikon confocal after operation making sure that the instrument is clean and ready for the next user

5. SPECIFIC PROCEDURE

5.1 Training overview

To begin, new users are questioned about their experience with microscopes, oil immersion lenses, fluorescent dyes, confocal microscopy, diffraction theory and digital imaging. After discussing diffraction, the need for the use of a pinhole for both

the excitation light source and any emitted light is made. A comparison of diffraction from a small fluorescent bead in an epifluorescent microscope to the same bead in a confocal microscope should be made. The basic principles of confocal microscopy can be made at this point as well as the advantages and disadvantages of epifluorescent microscopy versus confocal microscopy.

Next is a review of hardware. The specifications of the system can be pointed out at the same time. Powering up the system is also shown. The demonstration of oil immersion and focusing a sample is now done. An explanation of the concept of channels is made and then set up for different fluorescent dyes. Scanning images and quick capture of an image is demonstrated.

Look up tables are explained for adjusting contrast. Optimization of laser power and PMT gain is demonstrated for different channels. Setting up and saving a Z stack is shown. Overview of other available experiments can be done as needed.

Saved images can be viewed in several different modes such as maximum intensity projections or rotatable 3D. The training session is finished with instruction on how to clean up and power down.

5.2 Pretraining discussion.

Before training it is important to ascertain a new user's experience using microscopes. This includes use of fluorescent dyes, fluorescence microscopes, inverted vs. upright microscopes, digital detectors, acquisition software, image processing software, confocal systems and anything else that is relevant. As much as possible, training should be personalized for the new user.

A brief discussion of the properties of fluorescence may be necessary using diagrams online showing excitation and emission spectra of commonly used dyes (Invitrogen has a good spectraviewer website, http://www.lifetechnologies.com/ca/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html).

To understand the need for a confocal system, a brief discussion of diffraction is necessary. Diagrams describing diffraction from small fluorescent beads and the properties of Airy rings are useful. The diffraction from small fluorescent objects in samples to be imaged is what causes confusing out of focus light and blurry images.

Point out that a confocal uses a pinhole that is the same size or slightly smaller than the first Airy ring (Figure 1, arrow), thus eliminating diffraction in the XY plane. Both the exciting and emitting light must pass through a pinhole in order to achieve confocal imaging.

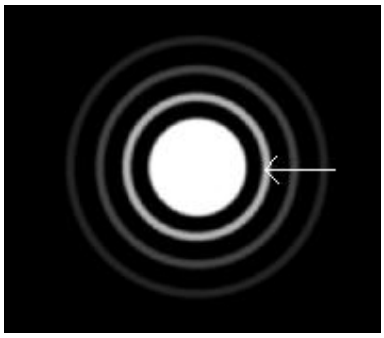


Figure 1. Diffraction pattern of a small sphere. The arrow points to the first Airy ring.

While proper use of a pinhole eliminates most of the diffraction in the XY plane, there is significant diffraction left in the Z axis as shown below by the lateral view of a confocal image (Figure 2).

Figure 2. Lateral view of the diffraction pattern of a small fluorescent bead.

The use of immersion oil to match the refractive index of the objective and the coverslip (the coverslip must be #1.5 thickness) allows for better optics, or less bending of light, and better imaging. The problem with inverted microscopes is that immersion oil can easlily flow down the outside of a lens finding its way into the lens or the microscope itself causing extensivedamage Lenses are the one thing on the whole confocal system that we manipulate physically by controlling the focus and can be made to physically contact something else. They are also very expensive.

It is important to stress the use of minimal amounts of immersion oil to prevent damage to lenses. The amount of oil used must be demonstrated later when first using a sample slide. Approximately 10-20ul is enough. When the slide and coverslip are turned upside down with the drop of oil on the coverslip, the drop of oil hangs down and the objective easily makes an interface with this amount of oil and the interface will be maintained when moving the slide around.

It is important to stress that when a user has multiple slides, excess oil must be cleaned from around the edges of the objective preventing oil from flowing down the side of the lens and into the barrel of the lens. A kimwipe can be used for this without touching the glass lens (see arrow in Figure 3).

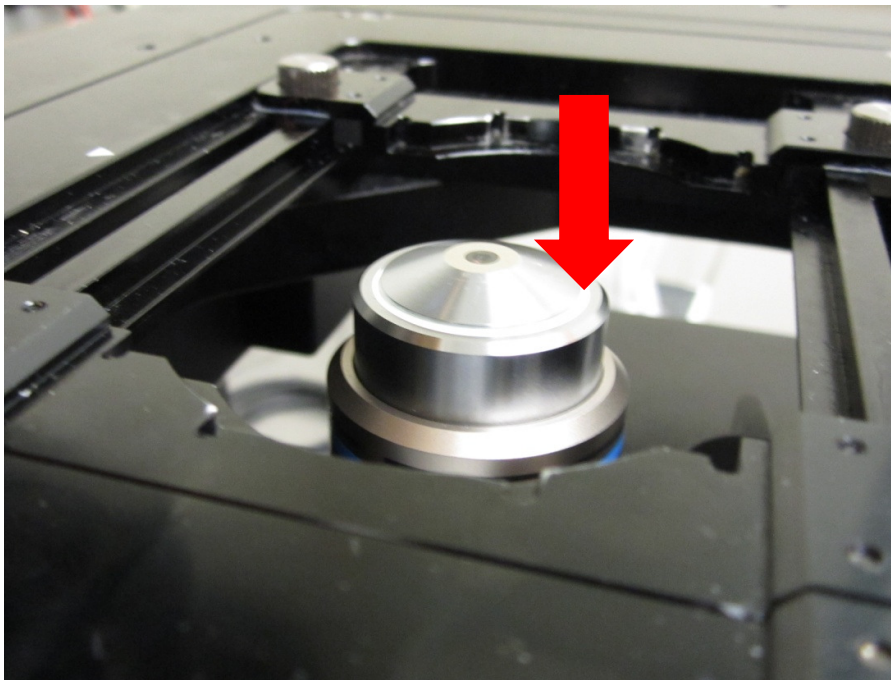


Figure 3. The area around oil immersion lenses that must be cleaned between samples.

Lens paper and/or cotton tipped swabs with small amounts of isopropanol (all available in the room, Figure 4) must be used to clean lenses after use. The cleaning procedure will be demonstrated. Excess oil is removed with lens paper first. Isopropanol is dropped on lens paper or swab and used to gently clean the lens. A kimwipe is used to check for any excess oil on the outside of the lens. Do not remove the lens from the microscope.

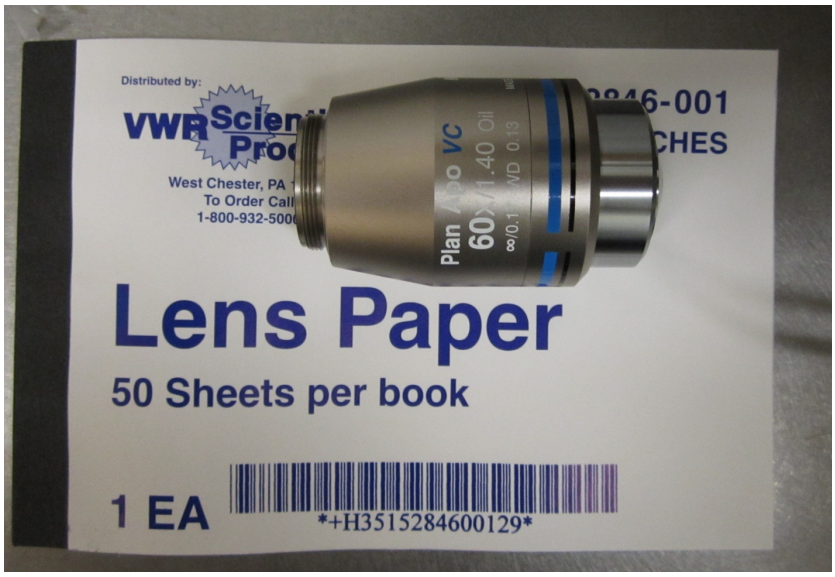


Figure 4. Lens paper to be used for cleaning lenses.

5.3 Purpose of Confocal Microscopy

The purpose of a confocal microscope is to eliminate diffraction and out of focus light by using a point source of excitation light and collecting emitted light through a pinhole. The effect of using the pinhole also causes a large reduction in the depth of the plane of focus necessitating the collection of a series of images in different focal planes (also called a Z stack). The Z stack can then be projected and viewed as a single image. The effect of reducing the depth of focus usually results in less light or lower intensity signal requiring longer exposure times and slower imaging. Thus, confocal microscopy usually takes longer than conventional wide field or epifluorescent microscopy which illuminates a large field and collects all the light over a large depth of focus and can give a more intense signal. For some applications, such as already shallow signal or samples that have all the fluorescence in one narrow plane with high signal to noise ratio, confocal microscopy, which is expensive and slower, may not be necessary.

5.4 Instrument Specifications

Lasers:

L1 is a solid state 638nm laser

L2 is a solid state 405nm laser

L3 is a Ar gas laser with 457nm, 477nm, 488nm, and 514nm laser lines

L4 is a solid state 561nm laser

There are four Photomultiplier tubes (PMTs) for fluorescent detection. Each PMT has different filter sets for imaging blue, green, orange and red emitting dyes. Table 1 is a list of available filter sets and examples of which laser lines are used with each filter set and which fluors can be detected with each PMT. These are also easily viewed in the confocal settings of the acquisition software.

Minimum Pixel Set: 32X32

Maximum Pixel Set: 4096X4096

Maximum Frame Rate: 420 Frames per Second (512X32 pixels)

Galvano scanner and Resonant scanner

Table 1

PMT	barrier filter	dichroic	laser line	dye
1	450/50	480DCLP	405	DAPI plus longer wavelengths
1	485/30	505DCLP	457	CFP plus longer wavelengths
2	525/50	560DCLP	488	GFP or YFP plus longer wavelengths
3	585/30	600DCLP	561	DsRed/tomato mCherry plus longer wavelengths
3 and 4	605/75 650 LP	640DCLP	561 and 640	any red any far red
4	525LP	none	514	YFP (no red)
4	575LP	none	561	any red

There are several primary dichroic mirrors allowing for the use of different combinations of lasers and detectors. They are listed below.

405/488

405/488/561

405/488/561/640

457/514

405/488/543/640

BS 20/80

405/488/543

457

The combination of laser lines, primary dichroic mirrors, filter sets and PMTs allows for imaging a wide range of fluorophores. A subset of those available is listed below.

DAPI, Hoechst33258, CFP, GFP, FITC, Cy2, AF488, YFP, Cy3, rhodamine, RFP, mCherry, AF555, AF 568, AF595, Texas Red, Cy5, AF638, PlumFP. Please note that combinations of fluorophores that can be imaged at the same time are limited by combinations of lines available on the primary dichroic mirror.

There is also a transmitted light detector which can be used to collect brightfield or DIC images simultaneously with the four PMTs.

There is a spectral detector with 32 available channels and small, medium, and large ranges of detectable spectra settings. This detector cannot be used in conjunction with the 4 standard PMTs. This detector can be used to collect lambda stacks (images of different emission ranges) and to perform spectral separation.

There is a motorized stage and a piezo focus drive.

There is a mercury arc lamp for viewing fluorescent dyes with the eyepiece.

The microscope has several lenses:

10X dry,

20X multi-immersion 0.8NA,

40X oil 1.3NA,

60X water 1.2NA,

60X oil 1.4NA.

The acquisition software is Nikon Elements 4.2 which runs on a Windows 7 Pro computer.

5.5 Hardware Review

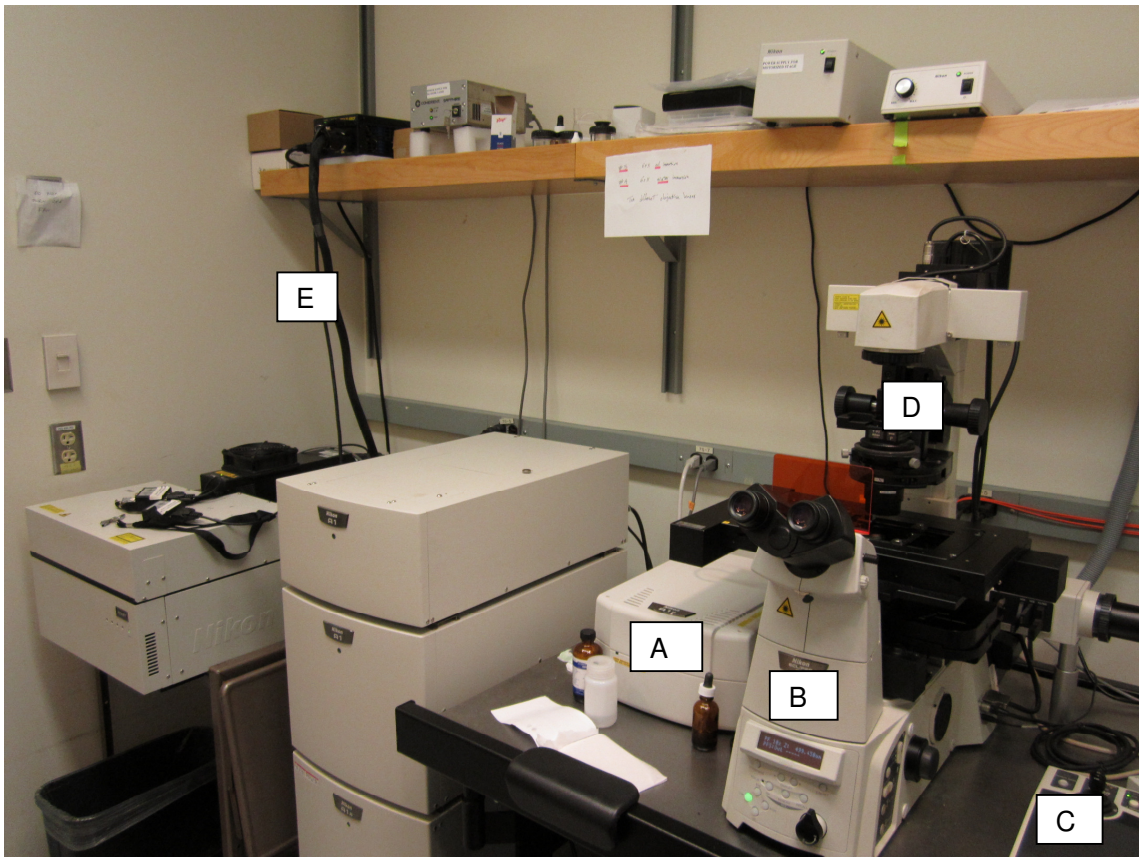


Figure 5. Scan head (A), Ti Eclipse Microscope (B), Motorized Stage controller (C) ,Condenser (D) and Lasers (above and below E).

5.5.1 A1R-si Scan Head

The scan head contains both the standard Galvano scanner and the fast Resonant scanner plus the primary dichroic mirrors, Figure 5 A.

5.5.2 Ti-E Microscope, Figure 5B

Figure 6. The front controls of the microscope.

5.5.3 Joystick for motorized stage and focus drive (Figure 7). Twist the joystick to change speeds.

Figure 7. Joystick controller for motorized stage.

5.5.4 The Intensilight arc lamp is used only for viewing fluorescence through the eyepiece. The top button opens and closes the shutter for the lamp, the bottom two buttons control intensity with most intensity on the right settings and least intensity on the left (Figure 8).

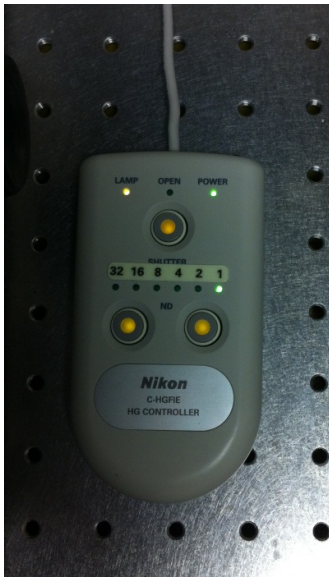


Figure 8. Controller for the Intensilight Arc lamp.

5.5.5 Controls on the left side of the microscope (Figure 9)

The on/off switch (Figure 9A) shown below is for transmitted light to the eyepiece and will be on when the microscope is powered up. This must be manually turned off before confocal imaging. The intensity of this lamp is controlled by knob (Figure 9B). The objective lens can be changed with the (Figure 9C) switch or by the software (for training purposes, please use the software). There is a focus control knob (Figure 9D) on the left and and focus change switch (Figure 9E).

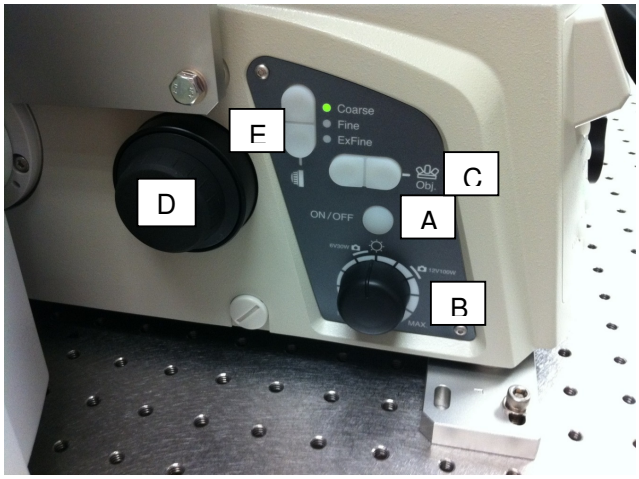


Figure 9. Controls on the left side of the microscope.

5.5.6 Controls on the right side of the microscope (Figure 10).

The right side of the microscope also has a focus knob (Figure 10A) and a focus speed switch (Figure 10B). There is a switch for changing filter cubes (Figure 10C) (for training purposes please use the software for this). The focus drive can be lowered and deactivated with the escape button (Figure 10D) for changing samples, and, returned to the previous focus with the Refocus button (Figure 10E). PLEASE NOTE, only use the Escape and Refocus when using the exact same type of slides and mounted samples.

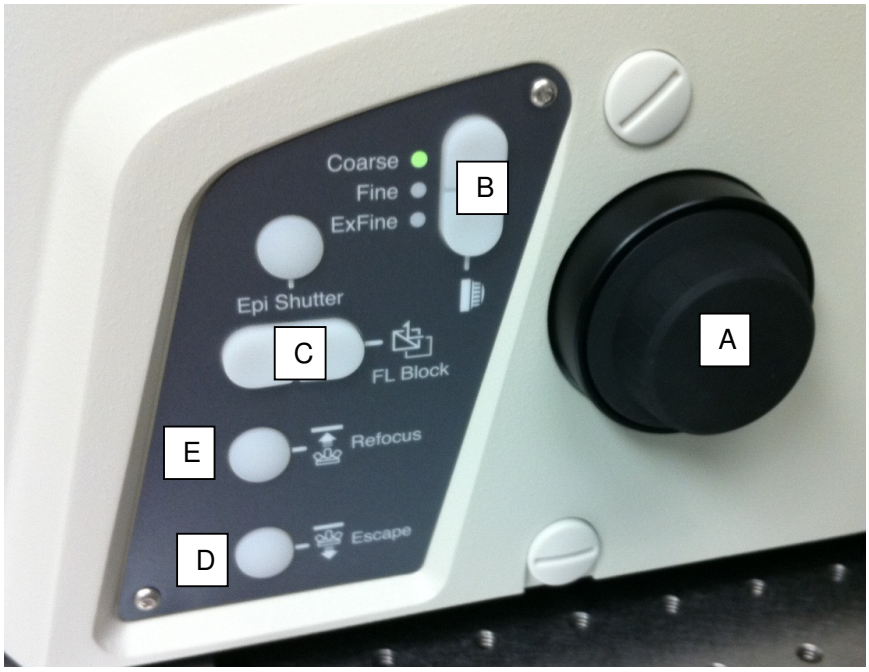


Figure 10. Controls on the right side of the microscope.

5.5.7 The Confocal Keypad (Figure 11) can be used to adjust laser settings and gain of PMTs during confocal scanning. Channel Select will allow you to switch between wavelength for adjustment and the software will show a small arrow next to laser being adjusted, i.e. 405nm. The Scan Speed and Zoom can also be changed with the Confocal Keypad.

Figure 11. Remote controls for Laser Power, PMT gain, Scan Speed, zoom and Channel Select.

5.6 Power up

Please note that not all lasers need to be powered up. Also note that the computer must be powered up last and the microscope must be powered up second last.

5.6.1 Lasers

Argon (457/471/488/514) Laser switch (Figure 12A). Flip the on/off main switch and turn the key clockwise.

Figure 12. Power sources for the Ar gas laser (A) and the solid state 561nm laser (B)

561nm Laser key switch (Figure 12B). Turn the key clockwise.

05nm Laser, insert and turn the key clockwise (Figure 13).

640nm Laser, insert and turn the key clockwise (Figure 13).

Figure 13. 405nm and 638nm solid state laser power switches.

5.6.2 A1 Confocal Controller, push the button on left side (Figure14).

Figure 14. Power switch for main confocal controller.

5.6.3 Power Supply for Motorized Stage (Figure 15).

5.6.4 Power Supply for Halogen bulb (Figure 15)

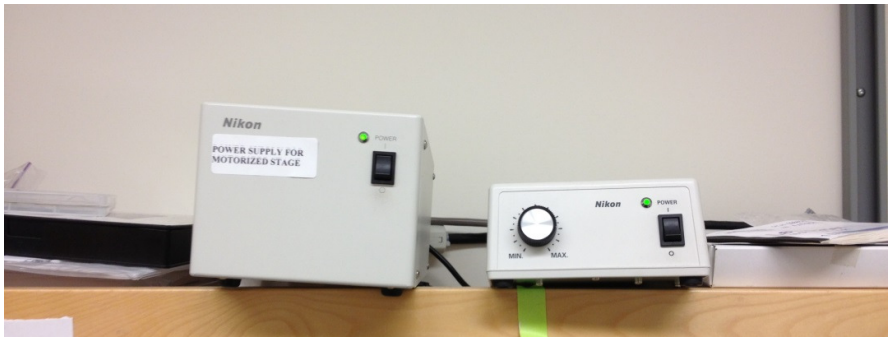


Figure 15. Power supplies for the Motorized stage on the left and Halogen bulb on the right.

5.6.5 The Intensilight Fluorescence Light Source is on the floor beside the antivibration table. The power switch is on the front bottom right (Figure 16).

Figrue 16. Intensilight Arc lamp and power source.

5.6.6 The microscope power-switch is on back right of microscope (Figure 17).

Figure 17. Power switch for the Ti Eclipse microscope

Upon power up, the Ti-E Microscope centers the motorized stage, places the focus drive at ~501um from the bottom of the range, and changes the light path to the eyepiece as shown in (Figure 6). It is recommended that for the purpose of training, the controls shown below are left as is; the Nikon Elements software controls everything that needs to be changed when required.

5.6.7 Turn on the computer workstation and Monitor (Figure 18)

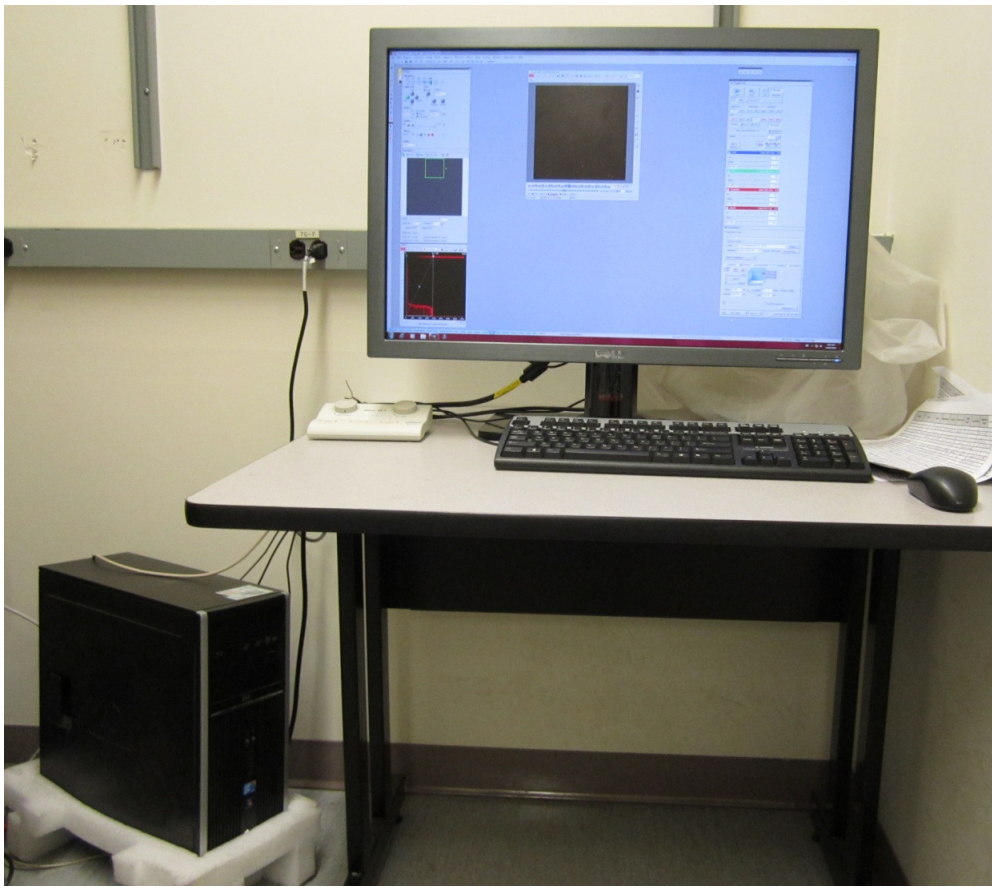


Figure 18. HP workstation computer and monitor.

5.6.8 Launch the Nikon Elements C 4.2 software.

5.7 Using oil immersion and focusing a sample

Oil must be used sparingly. A very small drop, 10-20ul, placed on the coverslip is enough to make a proper interface between the sample and an oil immersion lens.

5.7.1 To see fluorescent samples with the eyepiece on the microscope:

Make sure to turn on the Intensilight fluorescence light source (Figure 16). Place your sample on the stage and choose an objective. Select “EYE” from front panel (Figure 6, this is the default setting on startup). On the right microscope panel (Figure 10), rotate “FL Block” to desired fluorescent filter. Open the fluorescent shutter on the remote control for the Intensilight arc lamp (Figure 8).

5.7.2 Kohler Illumination. The condenser (Figure 19) is used to focus light on the sample in transmitted light optics. If transmitted light optics are not needed, this subsection can be skipped. The various parts of the condenser important for Brightfield or DIC optics are: (Figure 19A) The Field Aperture, (Figure 19B) The condenser focus knob,

(Figure 19C) The condenser turret (set to 'A' for brightfield and 'DICN2' for DIC optics), (Figure 19D) Centering screws, (Figure 19E) Polarizer.

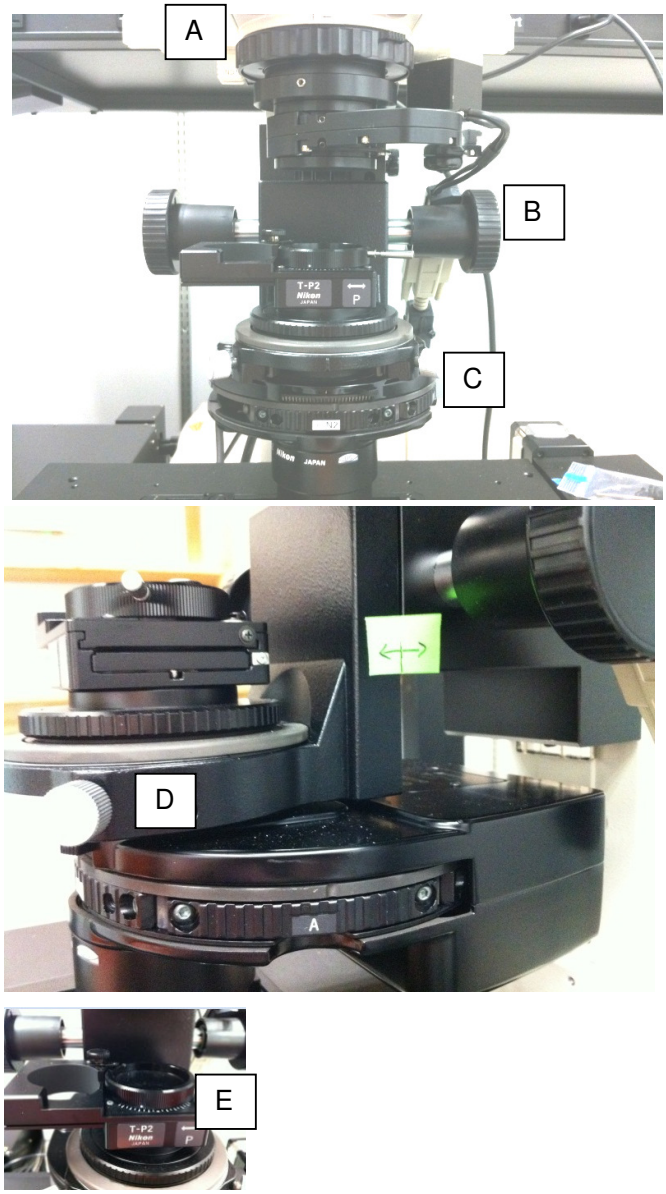


Figure 19. Different views of the the condenser.

To achieve proper illumination of the samples (Kohler Illumination), several adjustments must be made. Use your 10x objective to start and put the condenser turret (Figure 19C) in the A position. Maker sure the light path is on the eyepiece (Figure 6) and select DIC from the list of optical configurations at the top of the Nikon Elements software (Figure 21).

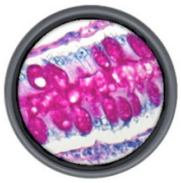
5.7.2.1 Focus on a sample

Close down your field aperture (Figure 19A)

Focus your condenser (adjust condenser height with (Figure 19B))

Center your condenser with the centering screws (Figure 19D)

Open field aperture (Figure 19A)



Please note that condenser focus will need to be adjusted for different objective lenses

5.8 Software

The software is Nikon Elements C version 4.2 (Figure 21). Most dialogs can be found under the View tab (Acquisition/Analysis/Visualization Controls) in Elements. As a shortcut these can also be found by right clicking in the open space of Elements. Elements obeys most Windows-based functions, shift/control and Alt are used often; right-clicks are used to display options/properties. Elements also uses “drag and drop” for overlaying windows and datasets

5.8.1 Microscope Control

The Ti Pad (Figure 20) can control almost anything on the microscope from within the software. The Ti Pad state shown in Figure 20 is the default state at startup if no changes have been made on the microscope. Each button is clickable (i.e., click on 20x to move to the 20x objective). The Light Path is dependent on the type of imaging you want to do:

E100: 100% to the eyepieces

L100: confocal

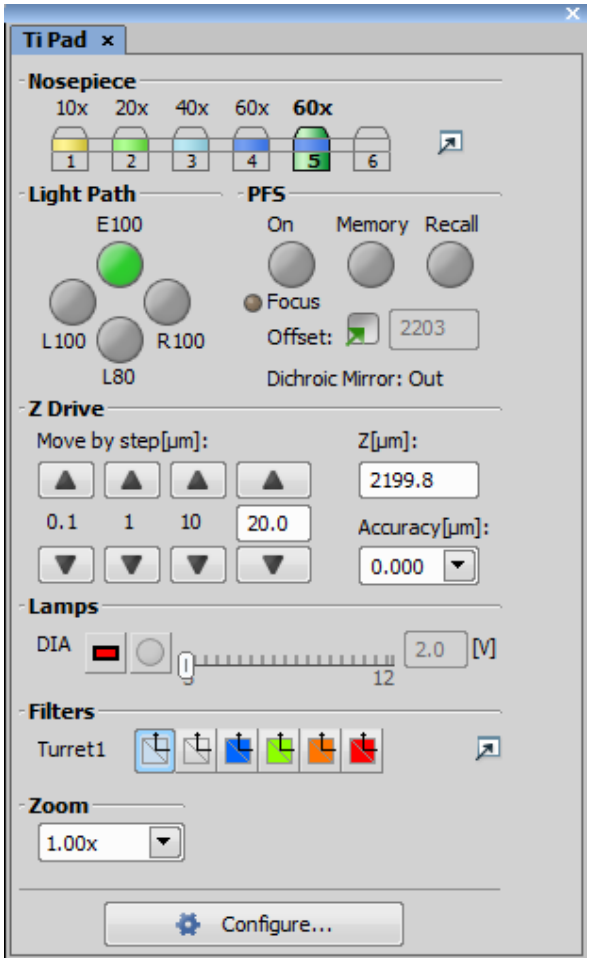


Figure 20. The Ti Pad dialogue box.

5.8.2 A typical layout for all the necessary controls and dialogue boxes is shown in Figure 21.

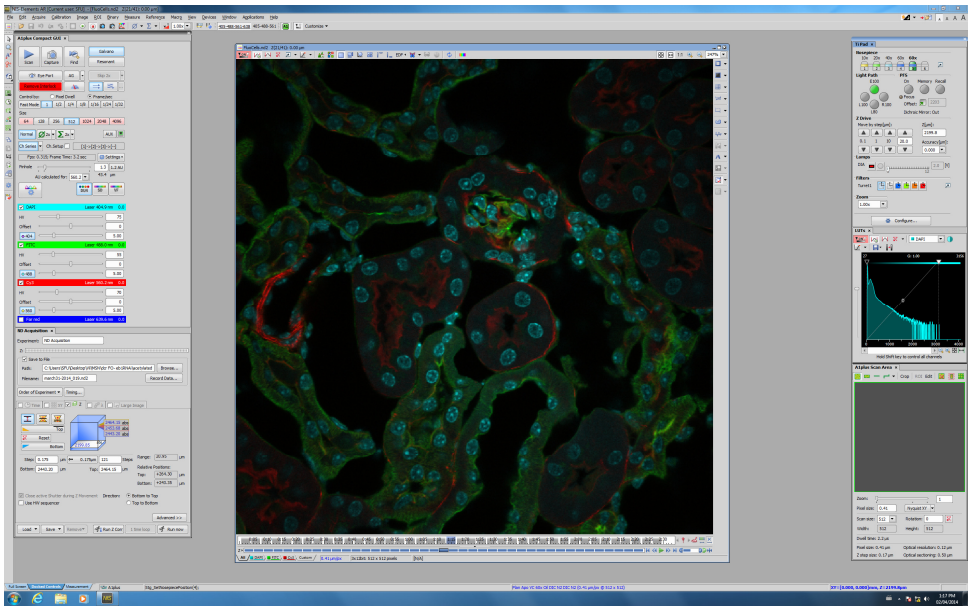


Figure 21. Layout of the all the controls and dialogue boxes of Elements C 4.2

5.8.3 Confocal Acquisition: Quick Start

The A1plus Compact GUI (Figure 22) is a wizard that will walk you through acquiring a confocal image.

Make sure you have previewed your slide through the eyepieces and focused on an area of interest. Click on the L100 lightpath button of the Ti Pad (Figure 20), click the empty position for the filters turret, close the intensilight shutter if used and turn off the halogen bulb. Click on “Remove Interlock” in the GUI (will change from red to grey, Figure 22). Choose Galvano for standard imaging. Click the ‘405-488-561-640’ optical configuration button (the top of Figure 21) and select or deselect the DAPI, FITC, Cy3, Qdot channels as desired. Set the HV-Gain at 90 to start. Set the offset-background adjustment at 0. Set the laser power for each channel at 5% to start. Start with a scan size of 512x512 and scan speed 1 frame/sec. Select “Normal” and “Ch Series”. Select 1.0 AU for Pinhole. Start “SCAN” and a live scan window will appear. Adjust settings if needed.

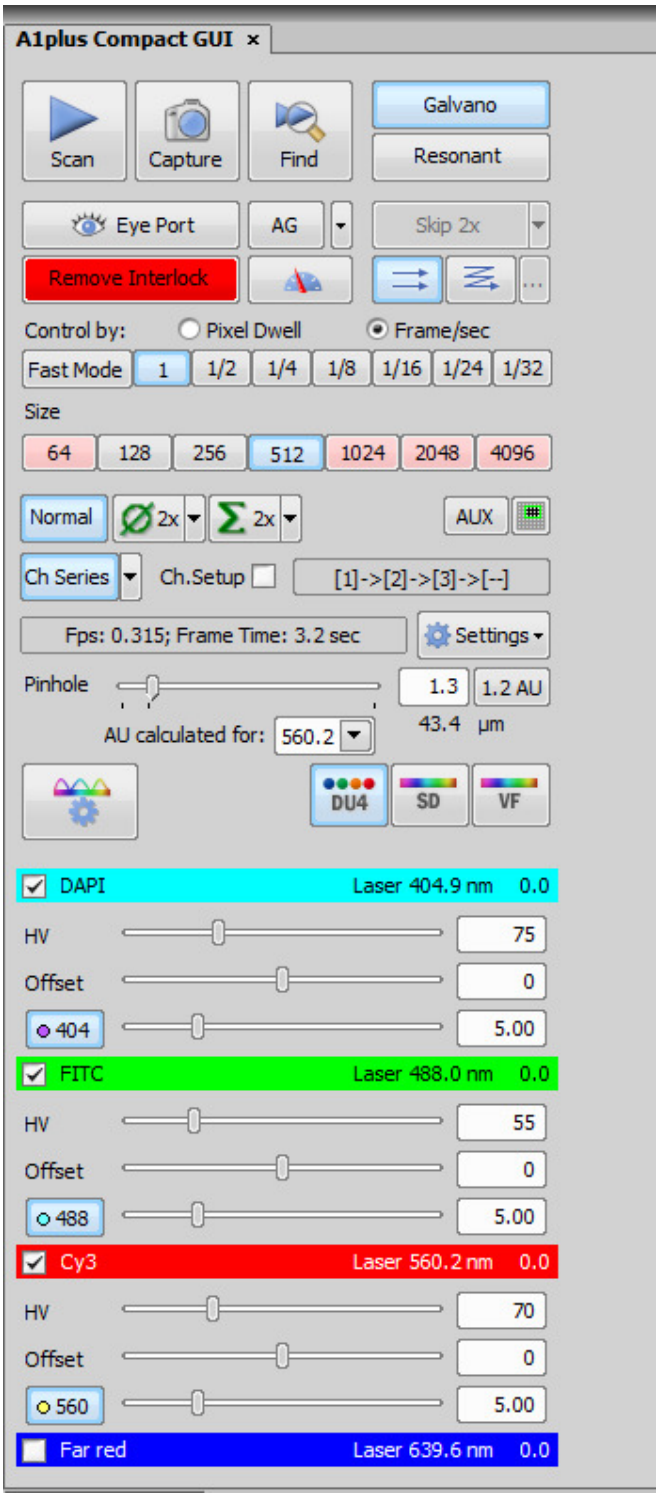


Figure 22. A1Plus Compact GUI for controlling confocal imaging.

5.8.4 To view the channel setup, use the “Setting”  button on the A1 Simple GUI (Figure 22)

A new dialogue box appears (Figure 23) which is very useful for demonstrating the light path and all the elements of a “channel” which are laser line (A), primary dichroic mirror (B), filter block (C) and PMT (D).

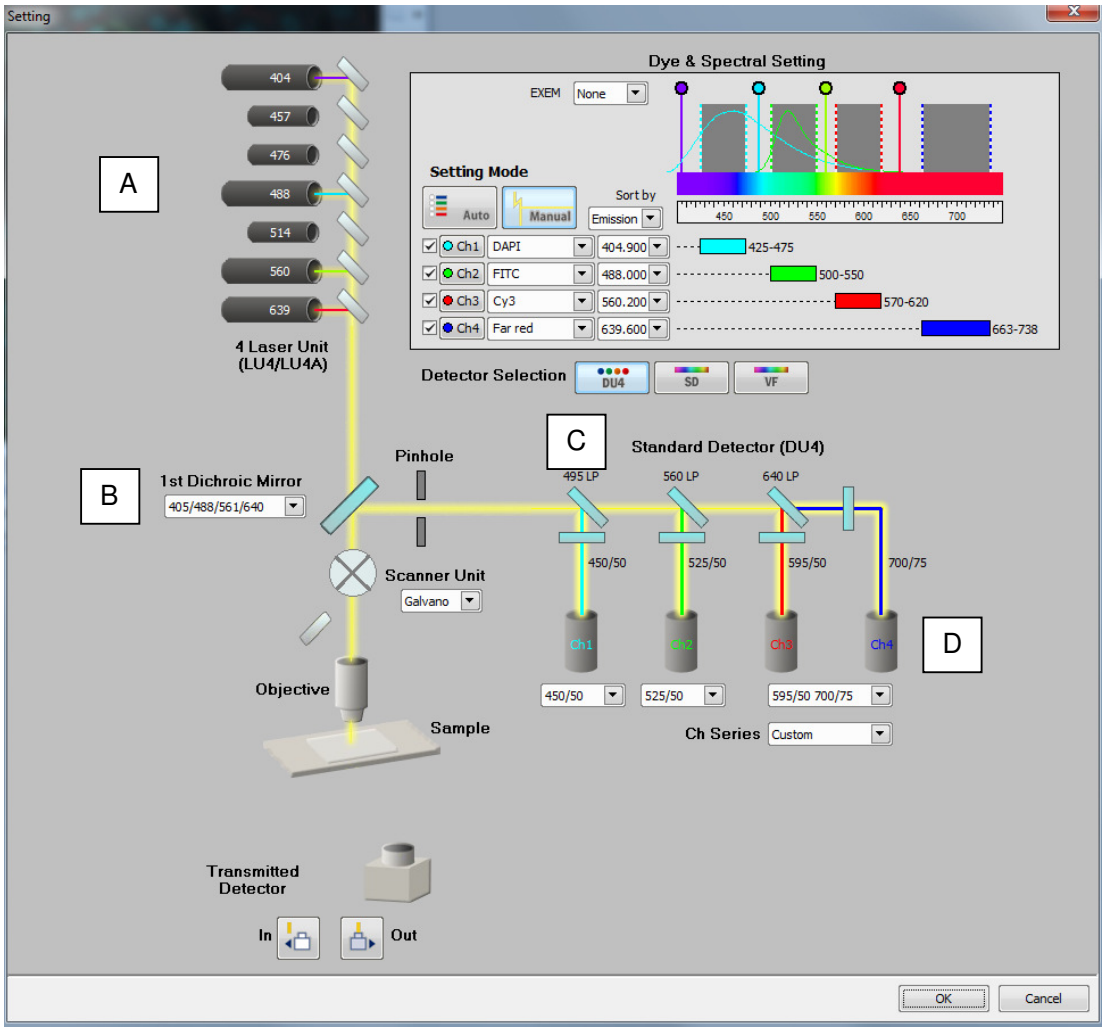


Figure 23. The Channel Settings dialogue box.

5.8.5 Look up tables (LUTS, Figure 24)

The LUTs menu can be used for brightness/contrast adjustment (using triangular sliders) as well as for showing a saturation indicator (A) which can be used to show what pixels are saturated. Use the drop down arrow to select “complementary color”

There is a slider (B) on the left which makes the Y axis logarithmic when used. Also, the button labelled “keep auto scale all” (C) will make the image auto contrasted all the time.

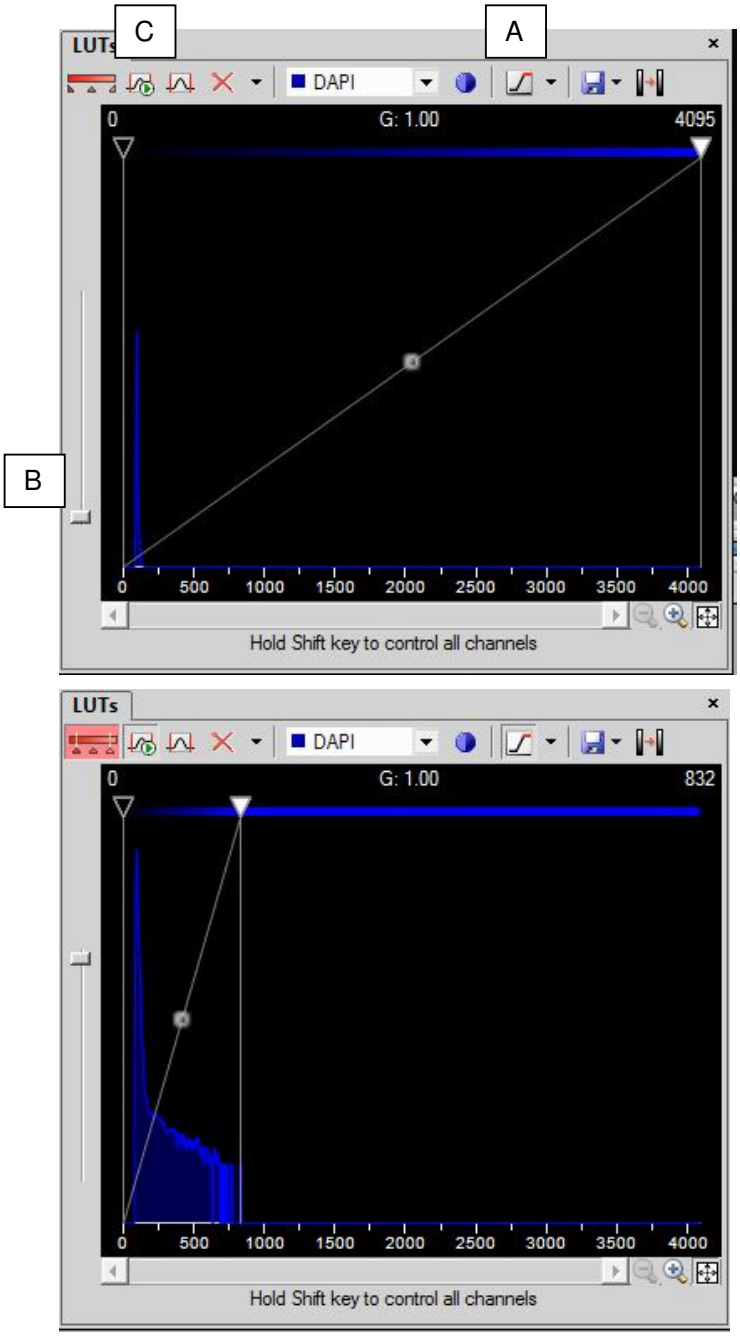


Figure 24. Look Up Tables (LUTs) for adjusting contrast. Saturation toggle switch (A), keep auto scale on switch (B), and make Y axis logarithmic (C).

5.8.6 Scan Area, Zoom and Resolution Improvements (Figure 25)

Locate the “A1 Scan Area” dialog. You can click and drag the green borders of the image to zoom into an area of interest. The pixel size will decrease and keep the image size the same. Right click with the mouse or press enter to accept the change.

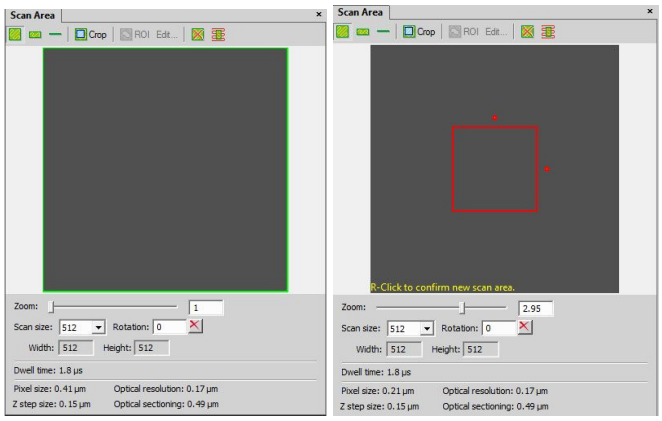


Figure 25

5.8.7 ND Experiments (Figure 26)

Experiments can be run on in a time lapse or continuous movie mode, , stage positions for collecting different positions on a slice., Z-stacks, wavelength switching for viewing image intensities and different wavelength emissions and/or image stitching for viewing several overlapping images to make one large image..

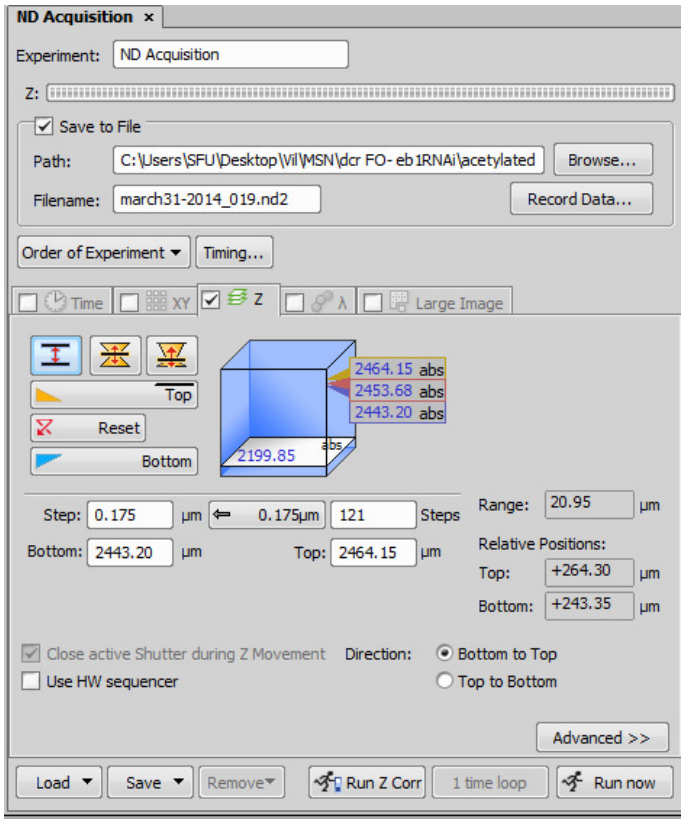


Figure 26. The ND Acquisition dialogue box for running different experiments.

5.8.7.1 Z-Stacking/Optical Sectioning

There are three methods for setting the top and bottom limits for optical sectioning.

Manually set top and bottom. Choose a middle plane (Figure 27) and click “reset”, go to a live view and focus towards the bottom (click bottom) then focus to top (click top). Set your step size (distance between each plane) and/or the number of steps you want (image total).

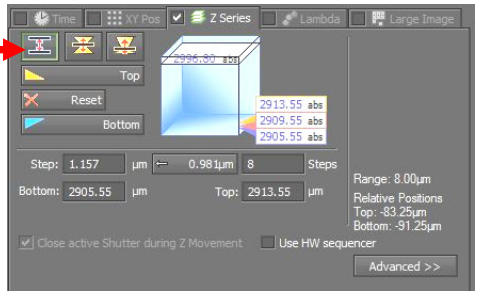


Figure 27. Setting the top and bottom of a Z stack manually.

Setting a symmetric top and bottom from a middle point of focus (Figure 28). Choose a middle plane and click “home” then input a distance range (equal from top to bottom).

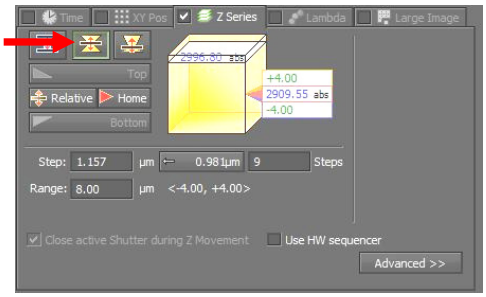


Figure 28. Setting top and bottom of a stack as a certain distance from a middle point.

Setting a asymmetric top and bottom from a middle point of focus (Figure 29). Choose a plane somewhere in the sample and click “home” then input a staggered range above and below.

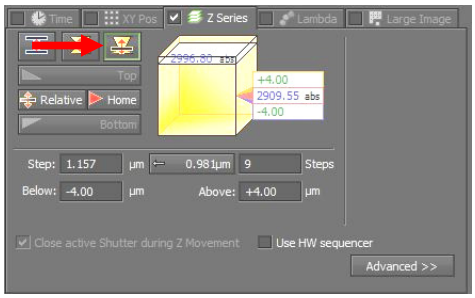


Figure 29 Setting an asymmetric top and bottom based on a point of focus.

Other experiments that can be run using the ND Acquistion function are:

5.8.7.2 Time Lapse

Taking images over time. This can be done in conjuction with other experiments in combination Please see page 4 of the Appendix.

5.8.7.3 X Y Stage positions

Taking images at preset positions on a slide. This can be done in conjuction with other experiments. Please see page 5 of the Appendix.

5.8.7.4 Lambda-wavelength

You can add automated wavelength switching to an experiment by checking off the checkbox for “Lambda”. This allows imaging at different ranges of emission wavelengths Please see page 6 of the Appendix

5.8.7.5 Image Stitching

You can add image stitching to an experiment by checking off the checkbox for “Large Image”. Please see page 7 of the Appendix

5.8.7.6 Spectral Acquisition

The Spectral detector is an array of detectors which can be used to image a large range of emitted wavelengths including fluorophores with large overlap in their emission curves or for the characterization of new and previously unused fluorophores or for fluorphores for which we don’t have specific filters. Spectral detection can be great for increasing the # of fluorophores you can use in a single experiment; it can also be used to subtract autofluorescence. To set up and use the spectral detector, please see pages 8-9 of the Appendix.

5.8.7.7 Spectral Unmixing

Spectral Images can be unmixed a few different ways. The overlap between two or more fluorophores is calculated based on control images and the overlap is discarded. This allows the user to view fluorophores with overlapping emission curves. Please see pages 10-11 of the Appendix.

5.8.8 FRAP (Fluorescence Recovery After Photobleaching)

FRAP and simultaneous imaging with FRAP or photoactivation can be performed using the two scanners, Galvano and Resonant. Please see pages 13-19 of the Aappendix.

5.8.8.1 Analysis

Analysis of FRAP experiments can be done in Elements. Please see page 20 of the Appendix.

5.9 Post Acquisition Image Adjustments

5.9.1 Scale Bars (Figure 30).

A Scale Bar can be added at any time from the right side image window's toolbar. Once positioned where you choose, you can right click on it to change its properties or to "burn it" into the image.

*it is recommended to save an original image before burning a scalebar into an image, save the second as a copy.

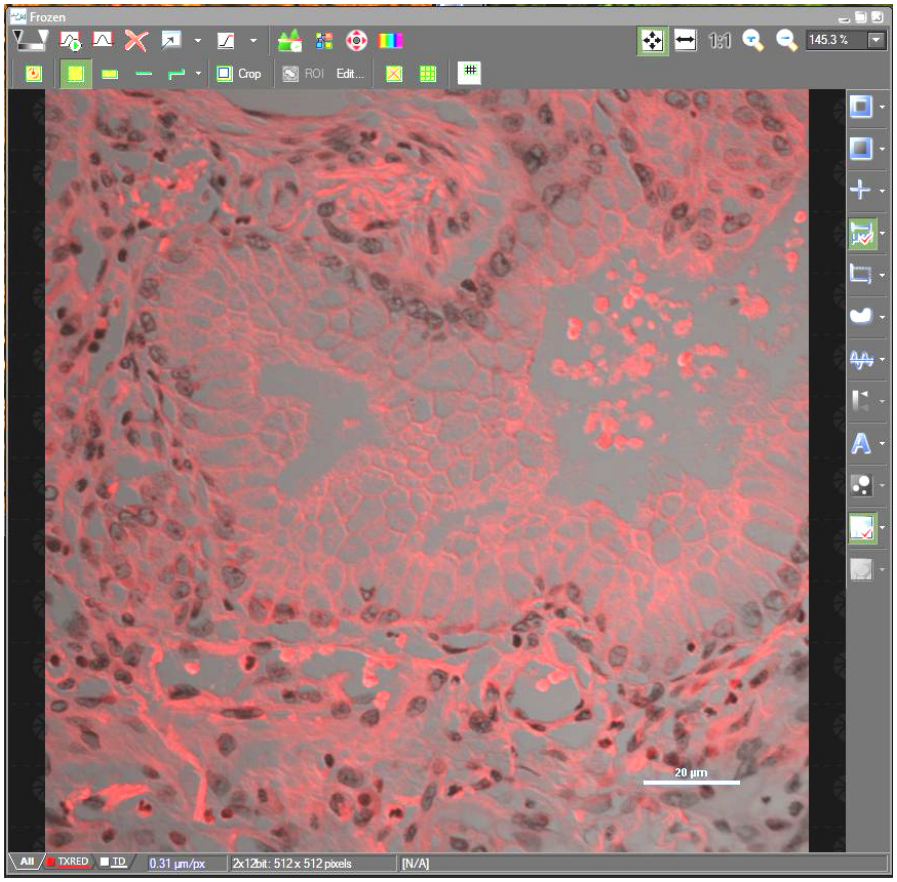


Figure 30. Setting Scale bars.

5.9.2 Data can be visualized several different ways including:

2D, sliced, tiled, 3D, projections and movies can be made of time lapses or rotating 3D images. Please see pages 23-24 of the Appendix.

5.10 Clean up and Power Down

It is extremely important to clean excess oil off the oil immersion lenses in between slides. Oil will flow down the outside of the objective slowly when new slides with more oil are placed on the stage. Using a piece of lens paper, wipe all oil off the top

and outside of the objective lens. When finished imaging, repeat this. Then, using the dropper supplied, add one drop of isopropanol to a piece of lens paper and wipe the top and sides of the objective again, then dry with lens paper.

The power down procedure is the reverse of the power up. Start with software, computer, microscope, intensilight lamp, halogen light source, motorized stage, main confocal controller and then any lasers that were used.

5.11 Signature Page

Please read the SOP, particularly section 4 (Responsibilities) and attached Policies for Cleaning and Inspecting lenses, Data Storage, and Power Down. Then sign below.

Data Storage Policy

The confocal acquisition computers are not to be used for data storage. Backup your images at the end of each session and then delete from the acquisition computer hard drive at the soonest possible opportunity. Images not deleted from the acquisition computers can be deleted without notice at any time if needed to create free hard drive space.

Lens Cleaning Policy

It is the responsibility of every user to inspect the objective lenses and the rest of the microscope and anti-vibration table for oil or other waste. Any problem needs to be noted on the log and the Microscopy Technician notified immediately. Failure to report problems could result in the most recent user on the log to be held responsible.

It is the responsibility of every user to check and see if they are the last user of the day. This must be done using the Nikon Confocal calendar on the MBB Facilities web site. Failure to power down the Nikon Confocal overnight will result in disciplinary action

Actions for not properly cleaning lenses and failure to power down:

1st offence: Verbal discussion

2nd offence: Email user and their supervisor plus the Microscopy Oversight Committee and the Chair of MBB

3rd offence: User's supervisor is charged $100 and the user is notified that they can potentially be banned from use.

4th offence: The user is banned from using the confocal until further notice.

I,_____, have read the SOP and attached policies and agree to them.

Date:

Signature:

6. CHANGE HISTORY

SOP no.	Effective Date	Significant Changes	Previous SOP no.

7. APPENDIX

Please see attached PDF file.