Nikon A1Rsi
Laser Scanning Confocal Microscope
Information

• Location:
  – TCHRF (Building R) Room 3024
• Facility is open 24/7 to trained and badged users
• Book systems through [http://research.cchmc.org/mrbs](http://research.cchmc.org/mrbs)
  – Click on “confocal”
  – Click on “3024 Nikon A1Rsi inverted”
• Assistance on systems:
  – Mike’s desk is in 3006
  – Matt’s office is 3442
Technical Information

• Microscope:
  – The microscope is a Nikon Eclipse Ti inverted. It is a four-port, fully automated inverted microscope. It includes an motorized XY stage, high speed piezo Z stage stepper, and the following six objectives:
    • 4x Plan Apo
    • 10x Plan Apo λ
    • 20x Plan Apo VC DIC
    • 40x Apochromatic λS DIC- Water Immersion (Note: not Planar)
    • 60x Plan Apo IR DIC- Water Immersion
    • 100x Plan Apo TIRF DIC- Oil Immersion
  – X and Y movement is controlled via the joystick to the right of the scope. Z movement is controlled either by the knob on the right side of the joystick or by the large focus knobs on either side of the scope. Z-speed can be changed to course, fine, or extra-fine with selectors next to the focusing knobs.
  – Objectives and filters should be changed via the NIS-Elements software, do not use the buttons on the microscope.

• A1R Controller/Lasers:
  – There are four lasers on the system:
    • 405
    • Multiline Argon (457, 471, 488, 514)
    • 561
    • 647
  – The DU4 detector has four detection channels on photomultiplier tubes that can detect in the 400-820nm range. There is also a spectral detector on this system, a total internal reflection system, and a stochastic optical reconstruction microscopy system on this microscope.
Technical Information

• A1R Controller/Lasers (continued):
  – There are two types of scanners on the microscope: galvanometric and resonant. The galvanometric scans at ~1 frame per second at 512 x 512 pixels. Fast scanning is usually done with the resonant scanner, which oscillates at a continuous 7.8 kHz, and can scan up 30 frames per second at 512 x 512 pixels. Faster scanning can be accomplished with both scanners, under specific circumstances.
    • Max image size with the galvano scanner is 4096x4096.
    • Image size is limited to 512x512 with the resonant.
  – Confocality is achieved using the pinhole. This eliminates out-of-focus fluorescence and enables optical sectioning and eventual 3-D reconstruction of images. The pinhole on the Nikon A1R is variable from 12 to 256μm. Optical section thickness is dependent on the pinhole diameter, the wavelength of light used, and the numerical aperture (NA) of the objective.
Basic Laser Confocal Microscope Theory

• Terms:
  – **Eyepiece**: the portion of the microscope that you look through. These optics commonly have a magnification of 10x.
  – **Nosepiece**: The portion of the microscope that one or more objectives is mounted.
  – **Objective**: the objective is the set of optics that gathers light from the specimen being observed and focuses that light into a real image.
  – **Stage**: the stage is the location where the specimen being imaged is placed.
  – **Laser**: a device that emits coherent light arising from the stimulation of a gain medium. Lasers can be fixed-wavelength, or tuneable.
  – **PMT**: an acronym for photomultiplier tube. A PMT gathers photons and multiples the electric effect of them by inducing secondary electron emission across several voltage-positive dynode stages. The multiplicative effect can be on the order of $10^5$-$10^7$ electrons produced per single photon.
  – **Collimator**: a device that narrows and aligns non-parallel incident light.
  – **Dichroic Mirror**: meaning “two-colored,” a dichroic mirror will allow light of a certain range of wavelengths through, and reflect light of differing wavelengths.
  – **Filter**: Much like the dichroic mirror, a filter will only let a certain type of light through. They are organized into three major types: Bandpass (which transmit across a band of wavelengths), shortpass (which attenuate longer wavelength light), and longpass (which attenuate shorter wavelength light).
  – **Fluorophore**: A chemical compound that emits light upon excitation.
  – **Epifluorescence**: A type of fluorescence produced via reflected light, as opposed to transmitted light.
  – **Autofluorescence**: Natural fluorescence by certain biological structures where no fluorophore is added.
  – **Pinhole**: a variable-size iris (in our microscopes, anyway) that spatially limits incoming light. This is the mechanism that provides confocality, and eliminates out-of-focus fluorescence.
Basic Laser Confocal Microscope Theory

• Terms:
  
  – **Resolution**: the ability of an optical system to resolve two nearby objects separately. The higher the resolution of the system, the smaller the distance that can be accurately imaged. There are two primary types of resolution, and both are diffraction-limited: lateral resolution, and axial resolution. Lateral resolution is in the X-Y plane and axial resolution is in the Z plane. The resolution of the optical system is determined primarily by the NA of the objective being used. An objective with a smaller NA will have a lower resolution, and a higher NA is higher resolution.
  
  – **Airy Disk**: a diffraction pattern formed by a perfect lens at its spot of best focus. It is a series of concentric circles, as it is diffraction-limited. A lens with a larger NA will have a smaller central disk, thus giving greater resolving power.
  
  – **Rayleigh criterion**: this is the accepted criterion for minimum resolvable detail. If the central disk (maxima) of one Airy pattern intersects with the first minima of a second, then the two objects can be just resolved. If they are closer, then they are not resolved, and if they are farther apart, then they can be well resolved.
  
  – **Nyquist Theorem**: this establishes the minimal sampling rate necessary to be able to accurately reconstruct the object being imaged. The sampling rate is determined by the scan size and the scan area. These two parameters determine the pixel size. In order to properly sample, the sampling rate needs to be at least twice that of the original frequency. In this case, the pixel size should be at least half the size of the smallest object you want to resolve. Oversampling is acceptable, and is actually very common, but it will not add any additional information to the image.
Basic Laser Confocal Microscope Theory

- **Light Path**: In the Nikon A1 systems, the light follows a scan-descan path. The light leaves the lasers, enters the scan head, moves through the optical train to the sample, and excites it. The fluorescence is then collected in the objective and returned to the scan head. Since fluorescence is red-shifted, it can be re-directed via a dichroic mirror to exit out the rear optical output ports. This process (where the light is directed with the scan mirrors in both directions) is known as descanning. After that, the light moves to the A1 controller where it interacts with the PMTs and the signal is converted to an image in the computer.

![Diagram of Laser Confocal Scanning Unit with Linear and Resonant Galvanometer Hybrid Scanning System](image.png)

*Figure 1*
Basic Laser Confocal Microscope Theory

**Objective Specifications and markings:** choosing the right objective is critical to imaging success!

- **Manufacturer:** who made it
- **Magnification:** the ratio between apparent and actual size of the object being viewed. Manufacturers make objectives with mags varying from 0.5x to 250x.
- **Optical Corrections:** There are two primary optical corrections made in lenses: field correction and color correction.
  - Field correction is either **planar** (usually denoted as **Plan**), or it is curved (no correction printed on barrel)
  - Color correction comes in four flavors: monochromatic, **achromatic** (denoted as **Achromatic** or **Achromat**-two color corrected), **fluorite** (**Fluor**, **Fl**, **Fluar**-three color correction), and **apochromatic** (four colors corrected, labeled as **Apo**)
- **Numerical Aperture (NA):** the light acceptance angle which in turn determines the resolving power and depth of field of the objective
- **Tube length:** this is the length of the body of the microscope, between the objective and the eyepieces. Most modern objectives are corrected to infinity.
- **Cover glass thickness:** this can either be a single number, or a range if there is a correction collar. This is the optimal thickness of cover glass that can be used with this objective.
Basic Laser Confocal Microscope Theory

- Working distance (WD): this is the distance between the front lens of the objective and the cover glass when the specimen is in focus. Generally, higher mags = lower W, but long working distance (LWD) and extra/super-ultra-long working distance objectives (ELWD, SLWD, and ULWD, respectively) exist.

- Special properties: this will denote if this objective can be used for certain types of contrast, like Phase (denoted Ph) or differential interference (denoted DIC).

- Immersion medium: this will be printed on the objective barrel, or denoted by a colored ring.
  - Water is shown by a white ring, and **W** or **Water** is inscribed on the barrel.
  - Oil is a black ring, and **Oil** or **Oel** is printed.
  - Glycerol is denoted by an orange ring, and **Gly** is on the barrel.
  - Special immersion media, or multi-immersion objectives are given a red ring.

- Magnification Color Codes (for objectives found on our systems):
  - 4X: Red
  - 10X: Yellow
  - 20X: Green
  - 40X: Light Blue
  - 60X: Cobalt Blue
  - 100X: White
**Basic Laser Confocal Microscope Theory**

- **Aberrations and oddities:**
  - **Chromatic aberration:** an aberration where different wavelengths of light are not focused in the same plane (axial), or the different colors focus they focus at different positions in the same focal plane (transverse). This can manifest itself in several ways. Either each color will appear in a different plane, or as spots in the same plane. Objects can also appear to have colored edges (known as purple-fringing). This is corrected by placing two types of glass together, usually crown glass and fluorite., and adjusting them so that the light focuses in the same spot in the same plane. At our level, chromatic aberration can be mitigated by using an achromatic or apochromatic lens (depending on the desired number of colors corrected).

  - **Spherical aberration:** an aberration where the image appears blurred due to unexpected diffraction of light. This is far more common than chromatic aberration. A common cause is mixed media of differing refractive indices. Make sure you match refractive indices in your immersion media and mounting media as closely as possible. Do not allow for air gaps or bubbles! Another possible cause is using an objective with a curved field. If this is unavoidable, try imaging in the lens’ aplanatic point. This is a certain area in the center of a lens that will not exhibit any spherical aberration. A common solution to spherical aberration is to use a Plan lens.
Getting Started

1. Turn on the power strip on the left bottom side of the air table
2. Turn the key on the laser launch 90° clockwise to the 3-9 position
3. Go around the back of the left side of the air table, and press the button on the A1R controller.
Getting Started

4. Turn on the power to the 647nm laser using the switch on the right side of the box
5. Turn the key to the 3-9 position on the left side of the box
Getting Started

6. Log on to the computer, and double-click on the GUI (green arrow icon)
7. Set the desired 647nm laser power in mW (30 mW is acceptable for most applications), click “on” then “Activate”
8. Close the GUI
9. Open NIS-Elements
**Image Acquisition**

Your screen should have been set up during your initial training. If for some reason it was not, contact Mike or Matt.

Right now, we will focus on single-dimension image acquisition (ie a single picture in a single focal plane). There are four major elements used in single-dimensional image collection:

- Ti Pad
- A1plus Compact Gui
- OC (optical configurations) panel
- Scan Area
From here, you can change your objectives, light path, filter cubes, and adjust your Z-drive and lamp. You can also work with the Nikon Perfect Focus System. (PFS is not covered in this training)

Ideally, the only time you will use the Ti Pad is to change your objective. Everything else is controlled by the OC Panel.
This is a macro-driven list of all of the established optical configurations in this microscope. You can add more, this is only a default list of common settings.

The bottom portion controls the “eyes” portion of the scope. It will set the light path to the appropriate port, and place the correct filter in the light path. There are also several settings for TIRF microscopy.

The upper portion holds configurations for laser scanning. Each of the configurations shows what lasers are on, and some have special scanners and/or DIC/transmitted light on. All of this is customizable.
**A1plus Scan Area**

This dialogue allows you to change your scan size, type, and zoom. It also displays the current thickness of your optical section (confocality!), your pixel size, dwell time, and current resolution.

Scan Window: this is adjustable, so you can use either the window or the slider to adjust your zoom. Grab a corner, and resize it accordingly. After, you will be prompted to R-click to confirm, and the window will turn green again.

Scan size: can be changed here, but it is recommended to change it through the A1lus Compact GUI.

Pixel size, z-step, resolution, and section thickness are all found here.

Nyquist XY: sets the zoom to the highest resolving limit per the Nyquist Theorem. Anything higher will not result in a gain in information.
A1plus Compact GUI

Scan, Capture, Find:
Scan continually scans the image, it is a live view. Capture will take a snapshot of the current view. Find reduces the frame size and image quality to quickly acquire an area to scan. We do not train on the find function.

Scanner selector: the galvanometric scanners are slower, but can provide more variation on scan area and longer pixel dwell times. The resonant is much faster, but with a short pixel dwell time that may compromise resolution and image quality.

Eye port: not used on this system. Remove Interlock: this is a physical shutter that prevents lasing when the light path is sent to the eyes. It will turn red when it is on, and will need to be turned off when moving into confocal mode.

Unidirectional and bidirectional scanning (speed is gained, but resolution can be lost). If using bidirectional scanning, scan offset must be adjusted using the “…” button.

Pixel dwell time/frames per second time: determines how long the laser stays on one pixel. Longer times=more data and more laser. This can be good for areas of low signal, or very noisy areas. Be careful, however. Longer pixel dwell times can possibly lead to bleaching!

Scan size: this is the size, in pixels, of the area that you are scanning. The scan area is a square (in most cases), so this represents both the X and Y dimensions. More pixels=higher resolution (sort of- it depends on the definition of resolution. The size of structure you can resolve is limited by the objective’s NA. Image size gives you a larger raster, so the image can be displayed larger without loss of definition.
A1plus Compact GUI continued

**Normal/averaging/integration:** averaging scans multiple lines and divides subsequent scans into each other, potentially reducing noise at the expense of longer scan times. Integration sums scans together, which can also potentially reduce noise, but we don’t train on it.

**Channel Series:** When this is active, the system will scan with one laser at a time and collect image data on one PMT, minimizing crosstalk between channels. Scan time is longer, but channel separation can be greatly improved. Next to it is the channel order button, where you can determine the order of the channel series.

**Pinhole Adjustment slider/AU button:** the pinhole is important! It is what determines the size of the optical section. A smaller pinhole means a thinner optical section, and a larger pinhole gives more of a widefield-style of image (thicker slice). The AU button will optimize the size of the pinhole for maximum axial resolution based on the longest wavelength of light that you are using.

**Light path configuration:** this brings up the light path window. Please do not adjust any of these settings without consulting Mike or Matt.

**Laser Control Panel:** with this, you can turn lasers and detectors on and off and adjust your laser power, gain, and offset. We will discuss this in more detail in image acquisition.
Image Acquisition

Place your slide on the stage, coverslip down.

On your OC Panel, click the “eyes” function corresponding to one of your fluorophores.

Using the XY stage controller (to the right of the microscope) and the focus knobs on the microscope, find your sample. A good place to start is in a 1200-1400μm focal plane.

Once you have found your sample and focused, select “A1 Confocal Mode” on the OC panel.

Then, click on the correct optical configuration for your sample. (ie which lasers, detectors, and scanners do you want on?)
**Image Acquisition**

Click on “Remove Interlock,” and wait 3 seconds until it turns from red to subdued.

Click “Scan.” A new window will pop up, and you will see your sample. If the image looks blank, increase HV and adjust focus to visualize the sample.

At this point in time, you need to begin optimizing your image. Look at the top of the image window, and click on the saturation indicator icon.

This will highlight saturated pixels. It is a good idea to click the dropdown arrow next to it, and choose complementary colors.

Also, if it is not already selected, choose to split your channels.
Image Acquisition- Navigation

Part of the power inherent in this system is the ability to move around your specimen without having to continually switch between the eyepieces and the computer. There are three principal forms of XY navigation, and one primary means of Z navigation.

Clicking on this will turn the mouse pointer into a reticle that allows you to click and drag the image to reposition it. You can also right-click, and select “Move this Point to Center” to make a particular point of interest the center of your image. Lastly, you can use the XY controller joystick next to the microscope to pan around your image. Using the XY controller joystick can be cumbersome while scanning due to the delay in image acquisition.

Z navigation is accomplished using the mouse wheel. This is relatively fine movement, however. If you desire more coarse movement, you can use the focus knobs on the microscope or the Z-knob on the XY controller next to the microscope. There is a button that will change between Coarse, Fine, and Extra Fine focus.
This image, at first glance, looks okay. Looks can be deceiving, however. This image contains saturated pixels. With saturation, image data points are falling outside the dynamic range of the detectors (PMTs). Above this range, the radiance of the pixel cannot be quantified.
Turning on the **saturation indicator** displays the currently saturated pixels on each channel. It is helpful to change the pseudocoloring of saturated pixels to “complementary color” using the dropdown to the right of the pixel saturation indicator. The laser power and PMT gain can then be adjusted to minimize or eliminate saturated pixels.
Image Acquisition- Laser Power and Gain (HV)

There is a golden rule to know: **Use the lowest laser power necessary to form an acceptable image.**

It is generally a good idea to start with low laser power and increase the gain (HV) to brighten the image. Remember: the overall goal is to get an image with good signal-noise, not get a super bright image. At a certain point the gain becomes too high, and noise is unacceptable.

If you have maxed out your gain and you are still not getting a good image (and you’re in a good focal plane), then drop the gain down, and slightly increase your laser power. Increasing laser power should be done carefully, especially at high magnifications. More laser = more photons = increased possibility of bleaching or specimen damage. Once the laser power has been increased, try again increasing the gain until the image is satisfactory.

It is not recommended adjust the Offset slider, as this can clip low-intensity data. This low-intensity data can be fine-tuned in post-processing using LUTs.
Image Acquisition

When the PMT Gain and Laser Power are properly adjusted, you will get a good image, with no saturation.

Once you get a good image, click “Capture” on the A1plus Compact GUI to snap a picture of your scan area.
**Image Acquisition- Crosstalk**

Crosstalk, or spectral bleedthrough, occurs when the emission spectrum of one fluorophore crosses over into the emission spectra of another fluorophore. This will result in false signal on one or more of your channels. There are several ways to mitigate crosstalk, however. The first, and easiest, is to use the “Ch. Series” button on the A1plus Compact GUI. It will sequentially scan each channel, versus simultaneous scanning when it is off. The second way to reduce or eliminate crosstalk is through careful selection of your fluorescent dyes. Generally if there are more than two fluorophores without good spectral separation, Channel Series should be on. Another way to reduce crosstalk is via spectral detection or linear unmixing. This is rather unpractical, as it requires an entirely separate detection unit and is outside the scope of this presentation. The core does have a spectral detector. For more information on the spectral detector or virtual filter, contact Mike or Matt.

Note how bluer channels bleed into the redder channels; this is crosstalk.
**Image Acquisition - Multidimensional Acquisition**

Now that you are familiar with capturing a single image in a single focal plane, it is time to become familiarized with some of the other very powerful tools available in confocal microscopy. With the optical sectioning capability, we can construct very accurate 3D representations of our samples. Using the motorized XY stage, we can create large images of our entire specimen that can function as virtual “road maps,” negating the need to continually switch between the eyepieces and confocal modes. We can do time-lapse imaging. Under certain circumstances, it may be necessary to image the same sample with multiple optical configurations, which can all be automated. We can even combine these different modalities into a large, multidimensional experiment. All this is accomplished with the ND Acquisition Panel, seen below.

- **Time Lapse (Time):** sets parameters for a times experiment. Can set exposure length and exposure interval
- **Multipoint (XY):** Can set different points of the slide to be captured, or provide a point of reference for other modalities
- **Z-stack (Z):** Takes multiple images (optical sections) in the Z-plane which the software can stitch into a 3-D representation of the sample
- **Lambda (λ):** Allows for different optical configurations to be recalled
- **Large Image:** Captures multiple fields of view and composes them together into a large, continuous image
ND Acquisition- Time Lapse

Time Lapse allows for measurement across time. Multiple phases can be entered into the experiment. For each phase an interval (frequency of measurement) and a duration (length of measurement) needs to be chosen. After the correct parameters have been entered, click “Run now” to begin the experiment.
**ND Acquisition - Multipoint**

XY is the multipoint modality of the ND Acquisition Panel. It will store multiple XY (and Z, if Include Z is checked) points that can be used in other modalities. To add a point, simply click on the checkbox in the point name field. The fields will be populated with the center of the image that you are currently viewing. X and Y can be manually entered, as well. Clicking the little arrow next to the point name will update the point information to the spatial data of the center of your current field of view. Clicking “Run now” without any other modalities will take single images of each point in the XY tab.
**ND Acquisition- Large Image**

Large Image will stitch together multiple fields to create a large composite image. This is good for utilizing a higher-NA objective to get better resolution for a specimen that cannot fit into a single field of view. It is also excellent for creating a spatial road map of the entire area of interest, as XY data will be encoded into the final image.

**Large Image must be used in conjunction with another modality, such as XY or Time Lapse.** To use, choose either the number of fields in X and Y, or choose X and Y dimensions in mm. It is advisable to use the default 15% overlap for stitching. The software will account for this, and make any image adjustments as necessary. There is no need to account for the extra 15% when entering fields or mm. When the size is set and an additional modality is chosen, click “Run now” to begin the experiment.
ND Acquisition- Z-stack

There are three choices for Z-stacking. The first is define top and bottom with absolute position. In this, you choose the top and bottom of the stack. The reset button will reset all of your points. Simply navigate to the top of your stack, and click the “Top” button, and do the same for the “Bottom.” If you get them backwards, don’t worry. The software is smart that way. It will fix it for you. When you have chosen your points and the proper step size, click “Run now” to begin. Z-stack does not require and additional modality.

When you have built your Z-stack, you can double-click on each of the three points to check your stack. It is good to check your settings at the top, middle, and bottom to ensure nothing is amiss. For example, if you are using the Piezo Z-drive and your section is larger than 100 microns, it will change one of the sets points to limit the range.
I’m confused! What’s all this talk about step size, and why do I have a choice of Z devices?

Good question! Step size is important, because it is derived from the thickness of your current optical section. Nikon recommends a ~66% overlap between optical sections when constructing a Z-stack so it will stitch together nicely. It is acceptable to use less than that, but you never want your step size to be larger than your optical section. You lose data that way. There is a button next to the step size field that will automatically select the optimal step size.

There are several Z devices to choose from, too. They have different speeds and different ranges of motion. The Piezo Z is fast, but it is limited to 100μm in thickness. The normal Z drive is slower, but there is virtually no limit to the thickness of your Z section aside from the working distance of the lens (and the ability of light to penetrate your sample, but that’s another lesson!).
ND Acquisition- Z-stack symmetrical

The second choice in Z-stacking is symmetrical stacking. This will take a range above and below an absolute point. You need to specify the range in the range field, and the step size, just like before. Pressing the “Relative” button will keep your range, and allow you to change your absolute point as you mouse up and down. Pressing “Home” will make your current Z position the absolute point. When all parameters are set, press “Run now” to begin the experiment.
The third choice in Z-stacking is asymmetrical stacking. This will take a range above and below an absolute point, but in this instance you must specify how much above and below you would like the software to image. You need enter the distance above and below in the respective fields and the step size, just like before. Pressing the “Relative” button will keep your range, and allow you to change your absolute point as you mouse up and down. Pressing “Home” will make your current Z position the absolute point. When all parameters are set, press “Run now” to begin the experiment.
The lambda tab will recall saved optical configurations. Lambda scans are typically used in two situations:

1. When using the resonant scanner in bidirectional mode where “channel series” is unavailable.
2. When using fluorophores that require different primary dichroic mirrors. An example would be a sample expressing CFP and YFP that also contains DAPI and Alexa 647. CFP and YFP use a 457/514 dichroic while DAPI and Alexa 647 use the 405/488/561/640 dichroic mirror. A configuration can only have one dichroic mirror associated with it, necessitating a lambda scan to image this sample.
**Tying it all together**

The first question you should ask yourself is “what are the goals of my imaging?” The last thing you want to do is to spend most of your time fumbling around trying to find something, or poorly image something multiple times only to see your time run out. If you create a good plan in advance, then you can optimize your time on the system, and get the most bang for your buck.

Do you need to see the entire slide, or do you have a large mount? Don’t waste time trying to image the whole thing right off the bat with the Galvo. Switch to the resonant, and take a large image much faster. Find your area of interest, then switch to the Galvo scanner.

A large image at a low mag contains the same spatial information as one at high mag, but your fields are much larger. If you need to take a large image, maybe do it at a low mag, then choose a good ROI for higher mag.

**Higher magnification isn’t always better.** Remember, your resolution is driven by the numerical aperture of your objective lens. A 20X with a NA of 0.8 will resolve down to .37 microns at 488nm. A 40x with a 1.0 NA will resolve down to .29 microns at 488. Occasionally that eight nanometers may matter, but more often than not it will not hinder your imaging to be at a lower magnification. Axial (Z) resolution is also determined by your NA. It is generally (but not always) half of the lateral resolution. This is due to the point spread function of light. Without getting to physics-heavy, it goes to say that a higher NA=thinner optical section=less lower-intensity, out of focus light in the point spread function. **Tailor your objective to your imaging needs!**

Experience will be your best friend, but if you need assistance, we are always here to help.
**Image Size, Scan Zoom, and Photobleaching**

The image size (or scan format) is the number of X-Y pixels that the scanner is collecting in a single pass of the image. The smaller the scan format (ie 512), the larger the pixel, thus the lower the resolution of the image. A larger scan format (ie 1024) will result in a smaller pixel, and a higher resolution image. Higher scan formats take longer to acquire, however.

Confocal microscopes with galvanometric scanning mirrors are able to change the oscillation angle of the mirrors and scan a smaller area while maintaining the same number of pixels. This is called scan zoom. You cannot arbitrarily keep zooming in, though. At a certain point, oversampling will not add any more definition to the image. This is known as the Nyquist Limit (as discussed in Confocal Microscope Theory earlier on). Past the Nyquist Limit there is no additional data added to the image, and resolution cannot be increased any further.

Zooming and changing your image size can affect your pixel dwell time and the number of incident photons over the sampled area. Too much light can lead to photobleaching or possible photodamage of the specimen. This is to be avoided at all costs, as it is unrecoverable.
Before and after of severe photobleaching from the laser. Laser photobleaching is always square. If you see round areas of dimmed fluorescence, it is bleaching caused by the epifluorescent light source. Decrease your light power. It is recommended that you scan away from your area occasionally to check for signs of photobleaching, especially if you are working for long periods of time at high mag, high zooms, high power, or high pixel dwell times.
Changing Objectives and Immersion Media

There will be a need to change from a dry objective to an immersion objective, and that’s perfectly acceptable. There is a simple way to do this.

1. Capture your image at a lower magnification.
2. Change to the immersion objective using the Ti Pad. Do not use the buttons on the microscope!
3. Press the “Escape” button on the right side of the microscope.
4. Use the XY Joystick to expose the objective.
5. Apply the immersion media.
6. Right click on your image, and select a point to move to center.
7. Press “Refocus” on the right side of the microscope.

Sometimes, you will have to use your eyes to adjust the focus, but more often than not you will be very close to the same focal plane as before. The only small difference will be due to the changing refractive index of the immersion media.

**Once on immersion objective, DO NOT RETURN TO DRY OBJECTIVE WITHOUT PROPERLY CLEANING YOUR SLIDE**
**Shutting Down and Leaving the System**

First, remove your slide. Clean any immersion media off the objective using lens paper. Using the Ti pad, change to a lower magnification (10X or lower).

Manually lower the objective using the focus knobs to 500μm or lower.

Check the booking schedule at research.cchmc.org/mrbs. If someone is scheduled soon after you (within an hour or two) simply log off the computer and go about your day.

If no one is after you, or you are the last user of the day, shut down is the opposite of start up.

1. Log off the computer.
2. Turn the key on the 647nm laser, and wait one minute before turning the power off.
3. Depress the button on the A1 controller box.
4. Turn the key on the laser launch back to the 12-6 position.
5. Turn off the power strip on the left side of the microscope table.
A final note...

**SPIN YOUR ANTIBODIES!**

- really, really give them a good whirl
- vortex your primary, and spin it at max speed (12K rpm+ for at least 10 minutes)
- make your dilution using the supernatant, and spin it for at least 10 minutes at max speed
- apply that supernatant
- repeat for the secondary antibody