

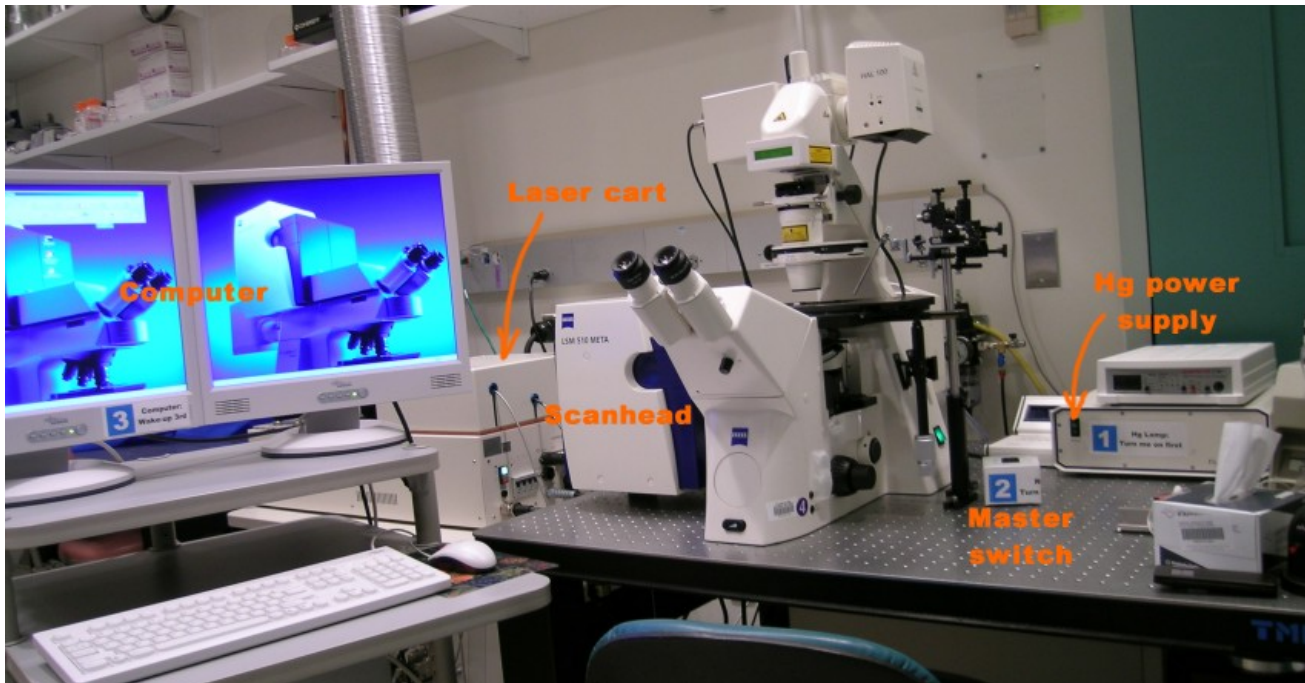
ZEISS LSM 510 INVERTED META OPERATING PROTOCOL

Foreword

The Zeiss LSM 510 inverted confocal microscope is a state-of-the-art laser scanning instrument owned by the NICHD Microscopy & Imaging Core (M.I.C.). Equipped with a fast-switching Acousto Optic Tunable Filter (AOTF) and a 405 nm laser diode, it is especially well suited for FRAP, FRET, photoactivation and photoconversion of fluorescent proteins. Spectral imaging and on-line emission fingerprinting are supported via a Meta attachment on the scanhead.

To access this instrument, users should first contact the M.I.C. personnel to discuss what equipment and methodology are best suited to their research goals. After completing and signing a form outlining the acceptable rules of use, they will be provided with access to our on-line reservation system, card key access to the facility and accounts on our computers and file server. Training is required before using any of the facility's equipment, and must be scheduled in advance of planned use. Refer to the M.I.C web site (<http://mic.nichd.nih.gov>) for details and contact information.

This manual covers only basic operations of the LSM 510 inverted microscope and software. For a more in-depth coverage, refer to the Zeiss documentation available from our web site (in particular, http://mic.nichd.nih.gov/lsm_doc/pdf/Operation_In_Expert_Mode.pdf).



Overview

Before starting, locate and identify the different elements of the microscope:

- Computer monitors, CPU and keyboard on the computer cart.
- Laser module and electronic interface on laser cart to the left of the optical table.
- Scanhead / microscope assembly, Hg lamp control module and master switch on the optical table.

1) Turn on procedure

1.1) Turn on the mercury arc lamp by pressing the on/off switch on the lamp power supply. Wait 120 seconds until the countdown on the lamp control module is finished.

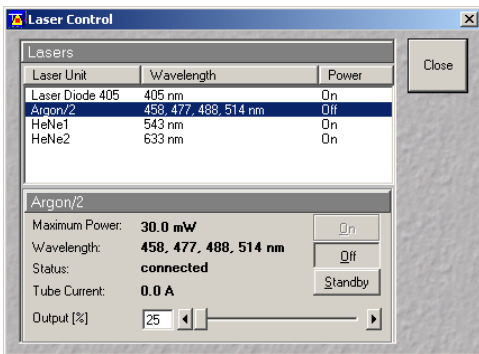
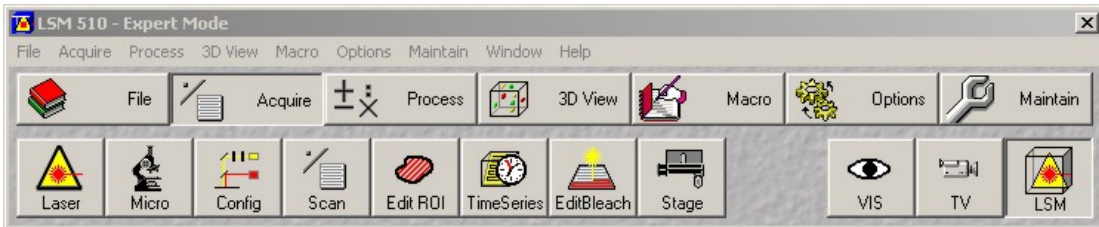




1.2) Turn the system on (lasers, electronic interface box, scanhead and microscope) with the master switch located on the air table.

1.3) Turn on the computer monitors and log on the computer. If you are the first user of the day, reboot the computer. Double-click on the “LSM 510 Meta” icon on the desktop.

1.4) On the LSM switchboard, select “Scan New Images” and “Start Expert Mode”.



1.5) The main menu bar defaults to the “Acquire” subsection. Click on “Lasers” on the lower menu. Turn on the lasers required by your experiment: Diode (405 nm), Argon (458, 488 and 514 nm), Helium-Neon 1 (543 nm) and 2 (633 nm). The argon laser has a standby mode and a slider for power setting. Put it on standby if not used for more than 30 minutes. Do not increase output power except for photobleaching, and then do so at the last moment. Do not run at high power longer than require. Never exceed a tube current of 8 amps!

NB: not all experiments require the 3 lasers to be turned on. Do not power a laser you will not use.

2) Sample visual inspection

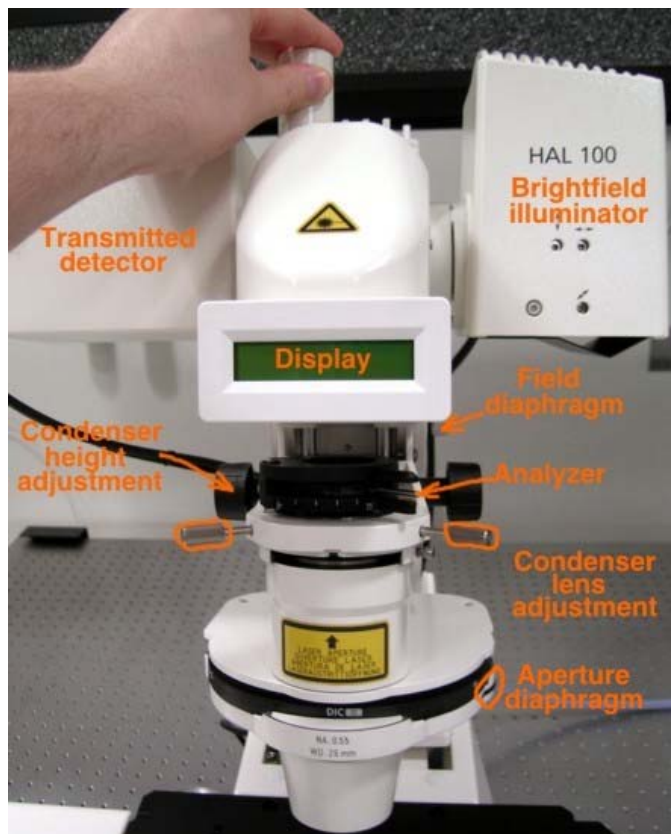


The Zeiss Axiovert 200 M is a fully motorized microscope. The instrument configuration may be reviewed and changed entirely in software, but we recommend setting up the microscope manually. Objective and reflector may be selected via the function keys located at the bottom right of the microscope stand. The lens, reflector and brightfield intensity status are always visible on the microscope LCD display. For safety reasons, the laser beam is blocked from entering the eyepieces. To switch between visual inspection and laser scanning, press the “Vis” or “LSM” key on the “Acquire” sub-menu. Remember to click on “Vis” or no image will show in the oculars.

2.1) Sound practices in focus adjustment

Finding the focal plane may be difficult, especially with a high-magnification objective on a dim sample. To avoid driving the objective through the specimen, move the objective as closely as possible to the slide (you may need to use a flashlight). With an immersion objective, the distance between the objective and specimen may be assessed by the size of the oil meniscus. Note which way the focus knob rotates to move the objective up (“thumbs up” on the Zeiss Axiovert 200 microscope). Then, move the objective away from the specimen (“thumbs down”) while looking through the eyepieces. Slowly moving the stage until some intensity fluctuation is perceived help zeroing on the specimen. Once the specimen is in focus, switching to another objective will preserve (approximate) focus.

To prevent scratches on the front lens, remember to move the objective turret to its lowest position before swapping stage inserts or at the end of your experiment.



up for optimum brightfield illumination.

2.3) Differential Interference Contrast

Differential Interference Contrast (DIC) is the contrasting method of choice for high-quality transmission imaging. DIC requires special objectives matched with a Wollaston prism. On the Zeiss LSM 510 inverted, it is available with the 40x, 63x and 100x oil immersion lenses.

Once Kohler illumination is achieved (see section 2.2), locate the polarizer slider below the objective turret and push it in place. Insert the analyzer on the condenser assembly and adjust it at zero degree for maximum extinction. Rotate the aperture carousel on the condenser to “DIC III”. Retardation may be adjusted by turning the adjusting screw on the Wollaston prism below the objective. Accessing the Wollaston may be difficult due to the silicon washers and rubber skirt protecting the objective carousel from oil and water infiltrations.

2.2) Brightfield

Click on “Vis” on the “Acquire” submenu, then press the contact to turn on the halogen lamp on the microscope. You may need to adjust intensity with the two-way switch at the bottom front of the microscope stand. Check the display to make sure no reflector is in place.

Insert your specimen on the stage. The condenser column may be tilted back for easier access. Note: do not tilt the column by grabbing the display, always use the metal knob on top.

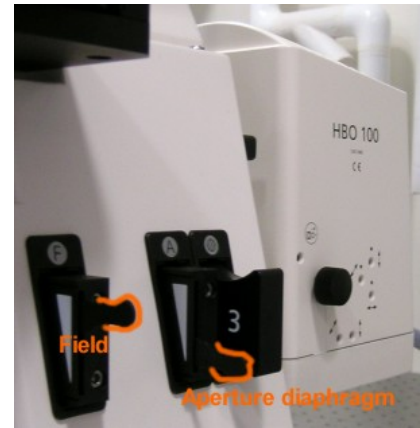
Set the microscope up for Kohler illumination as follows: Make sure the setting ring on the condenser turret is on “H”. Open the field diaphragm completely by pulling the lever to the front. Open the aperture diaphragm by turning the wheel on the condenser toward the front. Focus on your specimen using the coarse / fine focus wheel located on each side at the bottom of the microscope. Close the field diaphragm until its edge appears in the field of vision. Focus the edges by moving the condenser up and down. Center the field diaphragm with the centering screws, and open the field diaphragm until the edges reaches the limits of the visual field. The microscope is now set



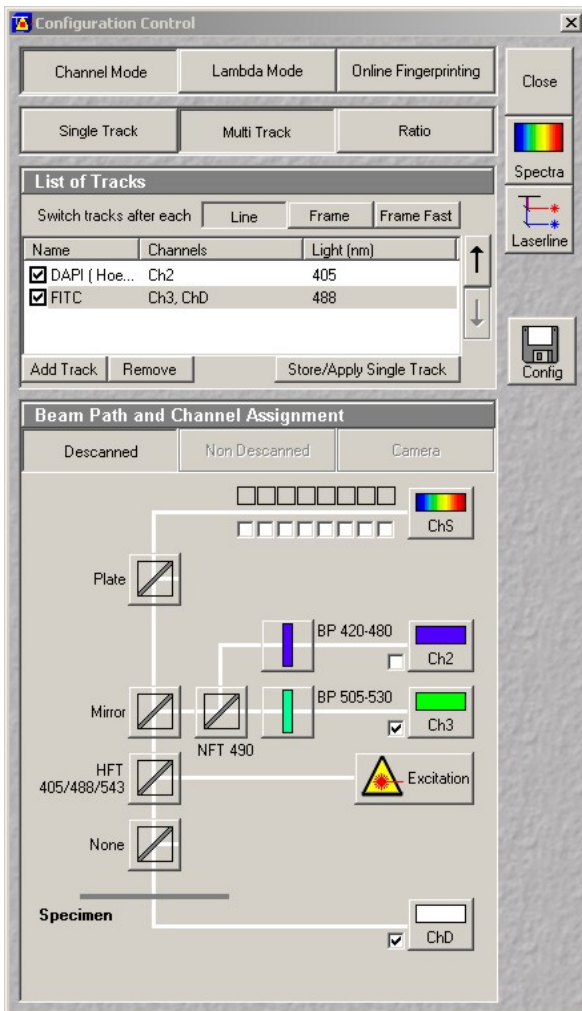
Be careful not to disturb these essential protections.

2.4 Widefield fluorescence

Click on “Vis” on the “Acquire” submenu, then press the contact on the microscope stand to turn on fluorescence. Insert the appropriate reflector using the two contacts on the right side of the microscope and referring to the LCD display. Filter cubes for DAPI, FITC, Rhodamin and Cy5 are available. The mercury arc lamp is extremely bright. Attenuation is available through the field and aperture diaphragms at the bottom right of the microscope.



3) Acquiring images



3.1 Overview of the optics

Click the “Config” button on the main menu toolbar. A diagram of the microscope optics appears in the Configuration Control window. The components in each location may be selected from a list appearing when clicking on individual icons.

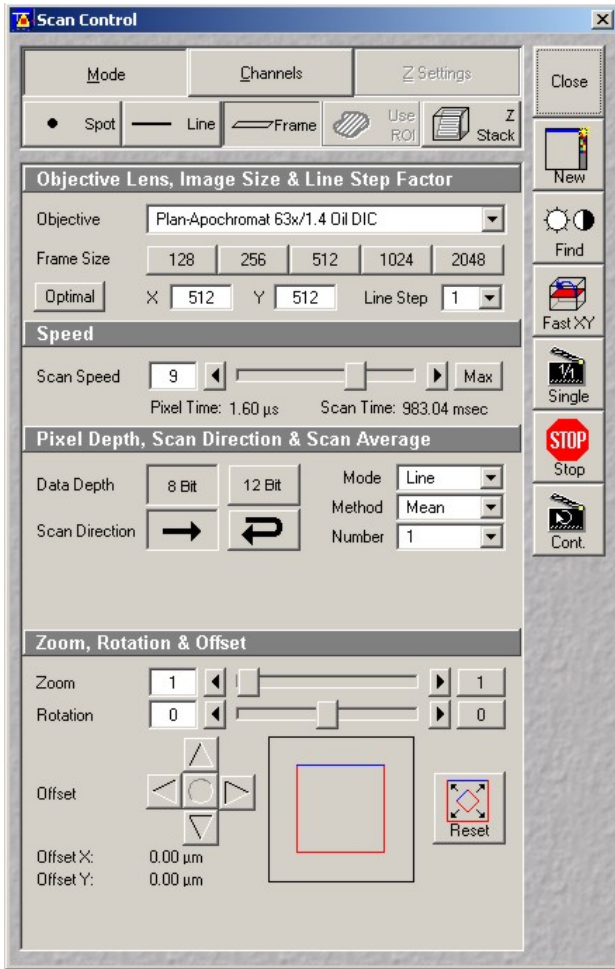
4 lasers in the visible range are currently available: a diode laser (405 nm), an argon ion laser (458, 477, 488 and 514 nm) and two helium-neon lasers (543 and 633 nm). While older confocal microscopes used a combination of filters and neutral density attenuators to select laser line and intensity, the LSM 510 is equipped with an Acousto-Opto Tunable Filter (AOTF). This device allows line switching and attenuation fast enough so that it may be turned on or off from one pixel to the next. A number of main dichroics are available for single or multi-line excitation. Two sets of secondary dichroics split the emitted fluorescence at various wavelengths and stir it toward one of the three available detectors: two photomultiplier tubes (PMTs) and the Meta detector. Each photomultiplier has a set of emission filters. The Meta detector is a spectral detector and is in itself a configurable emission filter (see 4.1 below). The number of possible configurations is dictated by the available secondary dichroics and emission filters. PMT number 2 (Channel 2) is used mostly for the lower emission range (blue / green fluorescence), while PMT #3 (Channel 3) is geared toward green / red fluorescence. Refer to the LSM 510 inverted specifications for details (http://mic.nichd.nih.gov/zeiss_specifications.htm).

A transmitted detector is also available. This device may be used in particular for DIC imaging and, due to its nature, has no emission filters.

3.2) Configuring the instrument

By default, Channel Mode is selected in the configuration control window. Three tabs are visible below: Single Track, Multi Track and Ratio.

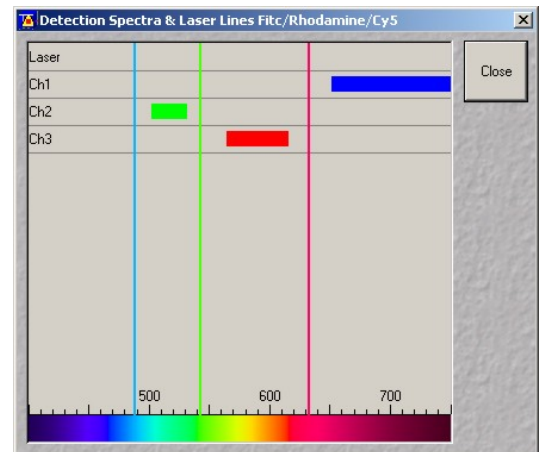
Single Track may be used either for single-line excitation, or, in the case of multilabelling experiments, for simultaneous multi-line excitation. Note that simultaneous excitation opens the door to potential bleed-through artifacts and should be avoided.



MultiTracks, or sequential excitation, minimizes cross-talk by acquiring sequential images each at one excitation wavelength. Switching of the excitation line is accomplished with the fast AOTF and may or may not involve mechanical switching of the optic. If the optic remains the same between the different configurations, switching may be accomplished on a line-by-line basis. Swapping main dichroics and filters is slow and, when required, may only be implemented between each frame. Each method has drawbacks. Line switching is extremely fast but the optical configuration is a compromise to accommodate the requirements of each fluorophore, i.e. a lower-performance multi-line dichroic and band-pass emission filters must be used. Higher performance may be achieved by using different optics on each channel, but this process adds a considerable amount of time to the acquisition and may only be implemented as frame switching. If used extensively, it also generates a lot of tear and wear on the microscope and scanhead.

The third tab in the Configuration Control window is Ratio. When turned on, channel ratio functionality with coefficients appears at the bottom of the Configuration Control window. This feature allows on-the-fly display of ratiometric imaging such as calcium imaging with indo.

A large number of pre-set and tested configurations are readily available, both in single and multi-tracks modes: click the “Config” icon



to the right of the Configuration Control window, choose a configuration from the list and click Apply. Spectra opens a window with a visual display of the excitation lines and emission window for each channel.

While users may create their own custom configurations, we do not recommend they do so. Besides the time and effort involved in understanding the optic in details, custom configurations require specific tuning of the instrument (such as pinhole alignment) that is best handled by the staff. User-created configurations also open the door to potential data quality and instrument welfare issues. If your needs are not addressed by a canned configuration, contact the M.I.C. staff for help.

3.3) Acquiring an x-y image

Once a configuration is applied, bring up the Scan Control window by clicking on the Scan icon on the Acquire sub-menu. At the top are three tabs: Mode, Channels and Z Settings. Z Settings is disabled until Z Stack (right below) is enabled. By default, frame scanning is selected, but users have the option of scanning a point, a line or a custom Region Of Interest (ROI).

Click on Mode to view the scanning parameters. The objective selection allows to swap objectives in software, but we recommend not to use this option. If a different objective is needed, switch to visual observation (Vis), select the objective manually on the microscope stand and adjust focus. Square or custom image resolutions (number of pixels in x and y) are available in Frame Size.

Depending on the objective and image resolution, the software determines a default Scan Speed, which may be reduced to improve the signal at the expense of greater bleaching and longer scan time.

We recommend acquiring only 8-bit images (256 levels of gray) in unidirectional mode. Averaging (Mean) may be used to reduce noise if a high gain is required to obtain an image. Accumulating (sum) will equally increase signal and noise. Averaging and accumulating may be done on a line or frame basis.

The zoom feature is a real optical zoom. Instead of scanning the entire field of view, the scanner will focus on a custom area with the same pixel resolution. Remember the resolution of a light microscope is limited to 0.2 micrometer. With high magnification objectives and high image resolutions (1024 x 1024 pixels and beyond), using the zoom function results in pixel size much lower than 0.2 micrometer, i.e. empty magnification. The actual pixel size may be checked after an image is acquired by clicking the Info button on the image window toolbar

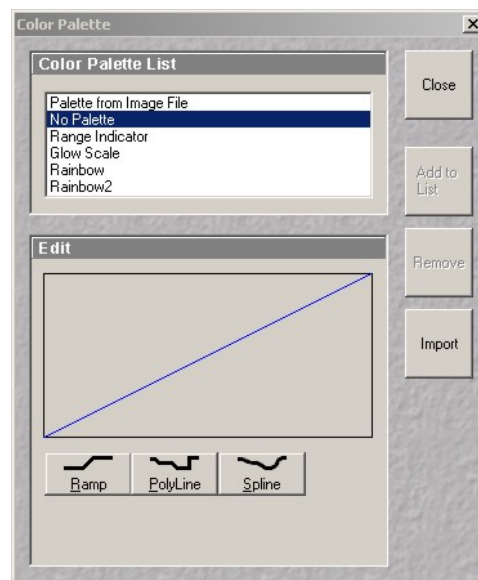
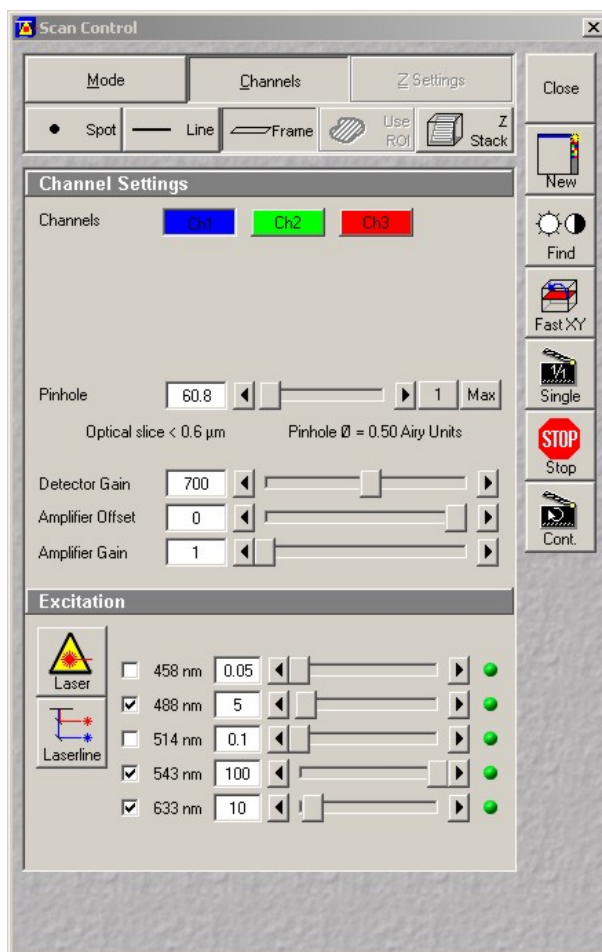
Back to the top of the Scan Control window, click on the Channels tab to view the settings for each detection channel. Each colored box represents a channel. Boxes on top of each other indicate sequential tracks (sequential excitation), while boxes on the same row represent simultaneous excitation (single track). Combinations are possible.

Click on a color box to reveal the acquisition parameters: Pinhole diameter, Detector Gain, Amplifier Offset, Amplifier Gain, Excitation line(s) and Transmission may all be adjusted for each channel.

For optimum confocality, the pinhole diameter must be set to 1 Airy Unit. Note that opening slightly the pinhole does not significantly degrade confocality and may greatly improve light collection if the instrument is not perfectly aligned. The thickness of the optical section, calculated from the pinhole size, the objective characteristics and the excitation wavelength, is shown below the pinhole diameter input box. The resolution of a light microscope gets worse at higher wavelengths. In multi-colored stacks, adjust pinhole openings on each channel so that the optical slice thicknesses remains the same. Detector Gain represents the excitation voltage applied to the photomultiplier tubes or to the Meta detector. To prevent damage, remember to set it at the minimum value before scanning.

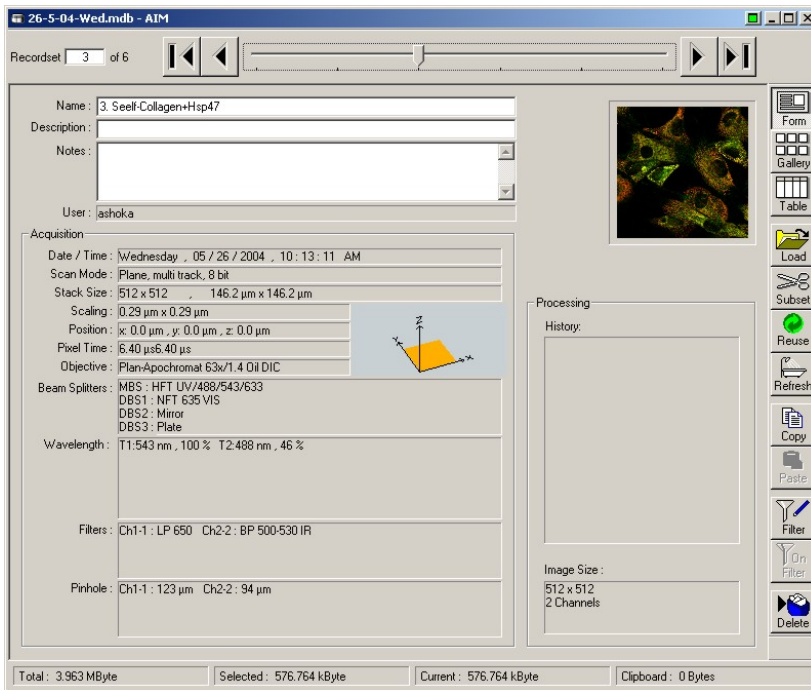
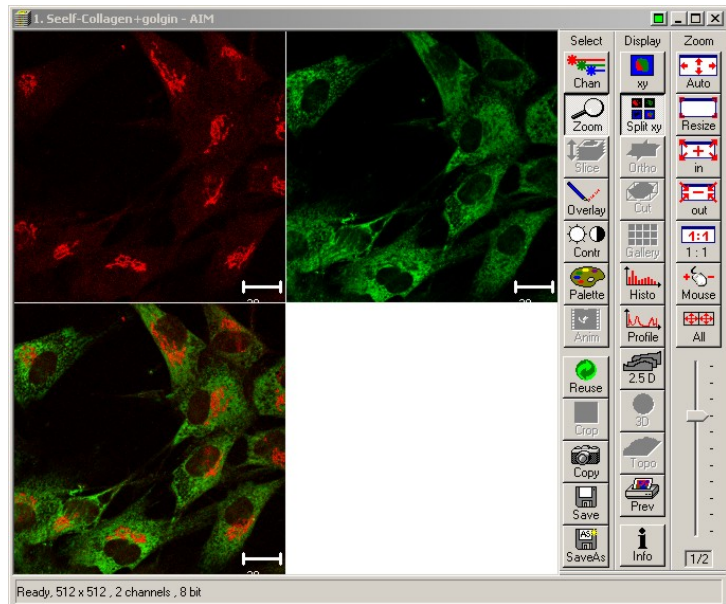
Click Fast XY for a preview of the image. Fast XY scans at maximum speed with a resolution of 512 x 512 pixels, irrespective of the settings entered in the Scan Control window. While scanning, slowly increase Detector Gain on each channel until an image is visible. To minimize photobleaching, do not scan longer than necessary. Gains above 850 volts on the PMTs and 700V on the Meta generate high levels of noise, some of which may be reduced by averaging. A frequent error with the confocal microscope is to use excessive detector gain. Gains in excess of the above values are a sure sign something is wrong with the instrument or with the specimen. It should be remembered the mercury arc lamp on the Zeiss LSM 510 is extremely bright, and the intensity perceived during visual inspection is always greater than what may be expected during laser scanning. In other words, if the sample appears dim during visual inspection, there is little chance to acquire a good image.

The Amplifier Offset may be used to adjust the black level.



When high-quality images are desired, the range of intensity values must span the entire dynamic range available, i.e. from 0 to 255 for an 8-bit image ($2^8 = 256$). To do so, click on the Palette icon in the Image Window toolbar and select the Range Indicator palette. Adjust Detector Gain and Offset so that a handful of pixels appear blue and red (under and over saturated, respectively). Avoid saturating more than a few pixels if you plan to analyze the image later. Click on “Single” to acquire an image at the resolution and scan speed defined in the Mode panel.

In the image window, you may toggle between single and multi-pane view with the XY and Split XY icon in the toolbar. Click on Zoom to display the zoom toolbar, where Auto fits the



images), name your database then give a name to your current experiment. Since the exact configuration is saved, the instrument may be later reset exactly the way it was by clicking Reuse. Images may be annotated by clicking on Overlay and using any item from the overlay toolbar.

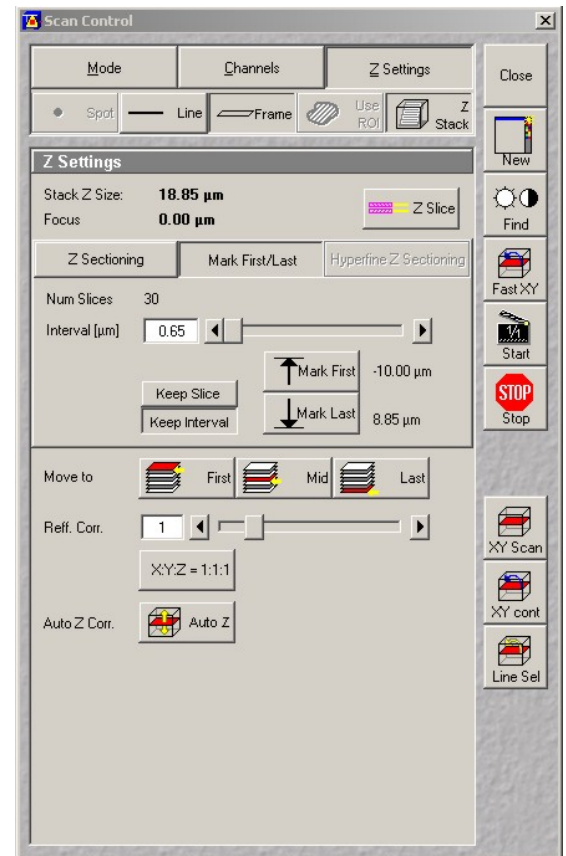
Besides two PMT detectors (channel 2 and 3), a Meta detector is available. Configuration and use of this powerful option are explained in chapter 4.

3.4) Acquiring a z-stack

In the Scan Control window, click on Z Stack to turn optical sectioning on. The Z Settings button above becomes active. Select it, then click on Mark First / Last in the Z Settings window. Start scanning with Fast XY, then manually change focus until reaching the top or bottom of your specimen. Click Mark First,

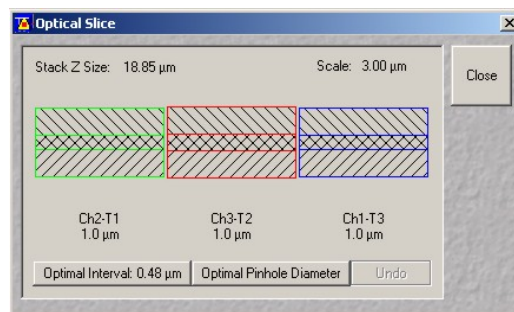
image to the window and the Mouse icon allows zooming in and out with the mouse left and right button, respectively.

Click on SaveAs to save the image. By default, the LSM software saves images with an “.lsm” file extension and output the acquisition parameters (microscope configuration, laser line, attenuation...) in a separate database file. Click on Create New MDB (database), browse to your folder under D:\user



then proceed the other way and click Mark Last. The total specimen thickness is shown and is sampled by specifying either a number of slices or a distance increment between slices. For maximum resolution, we recommend between 20 to 40% overlap between consecutive slices. Click on Z Slices for fine-tuning.

In the Scan Control window, click Start to begin acquisition of a Z stack, or XY for a single slice at high resolution. Select Gallery from the image window toolbar to display sequential images in the stack (composite image only, individual channels may be turned on and off with Chan and selecting None). Once the stack is recorded, click on Slice to display a slider and browse through the stack, or Animate to start an animation.



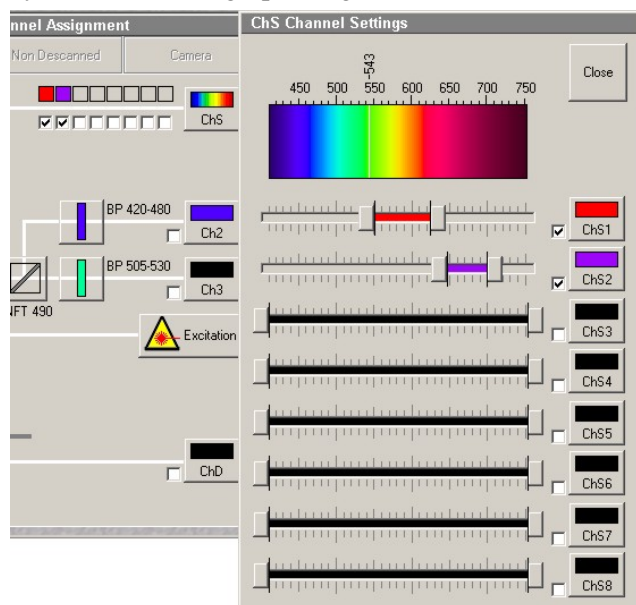
3.5) Acquiring transmitted images

In parallel to fluorescence detection, the transmitted part of each laser beam may be collected to obtain a transmitted image. Differential Interference Contrast (DIC) offers the best performance in transmitted imaging. On the LSM 510 inverted microscope, the 40x, 63x and 100x oil objectives may be used for DIC. Refer to section 2.3 for setting up visual DIC, then turn on the transmitted detector by selecting the TLD box at the bottom of the Channel Mode pane in the Configuration Control window. The transmitted detector is exposed to the full power of the excitation laser beam. Make sure the gain is turned down at minimum level before scanning! Remove the bottom polarizer and rotate the polarizer in the condenser assembly by 90 degrees. Start scanning with Fast XY and increase the gain until an image appears on the TLD pane. If necessary, adjust retardation with the Wollaston prism below the objective.

Once properly setup, the transmitted detector may be used as any other detector to acquire flat images, Z-stacks or time series. In the Channels pane of the Scan Control window, the TLD channel appears on the same row as the detector whose excitation line is used. Since there is no emission pinhole, transmitted images are not strictly confocal and may not perfectly register in the vertical (Z) direction with images acquired from the PMTs or the Meta detector.

4) Spectral imaging

The Zeiss LSM 510 inverted is equipped with a Meta attachment. The Meta is a wavelength-dispersive element spreading the emitted light toward an array of 32 detectors, each with a 10nm-wide window. Elements may be combined into a custom spectral range (akin to an emission filter), but the Meta may also be used as a spectrometer to record spatially resolved emission spectra (lambda mode) or resolve emission of overlapping dyes (emission fingerprinting).



Important note on using the Meta detector: The user must be very careful to avoid scanning near or across an active laser line: while each laser line is blocked inside the Meta with a pin, scattered light is visible on neighboring channels, to an extent depending on each laser and on the proper Meta calibration. At high gain, such scattered light blind the detector, potentially damaging it.

4.1) Meta channel imaging

Refer to section 3.3 for instructions on acquiring XY images. On the Configuration Control window, the middle and bottom detectors (Channel 2 and 3, respectively) are standard photomultiplier tubes outfitted with emission filters. The top detector (channel 1 or ChS) is the Meta detector. Click on the ChS icon to open the Meta settings panel. Up to 8 channels, each grouping

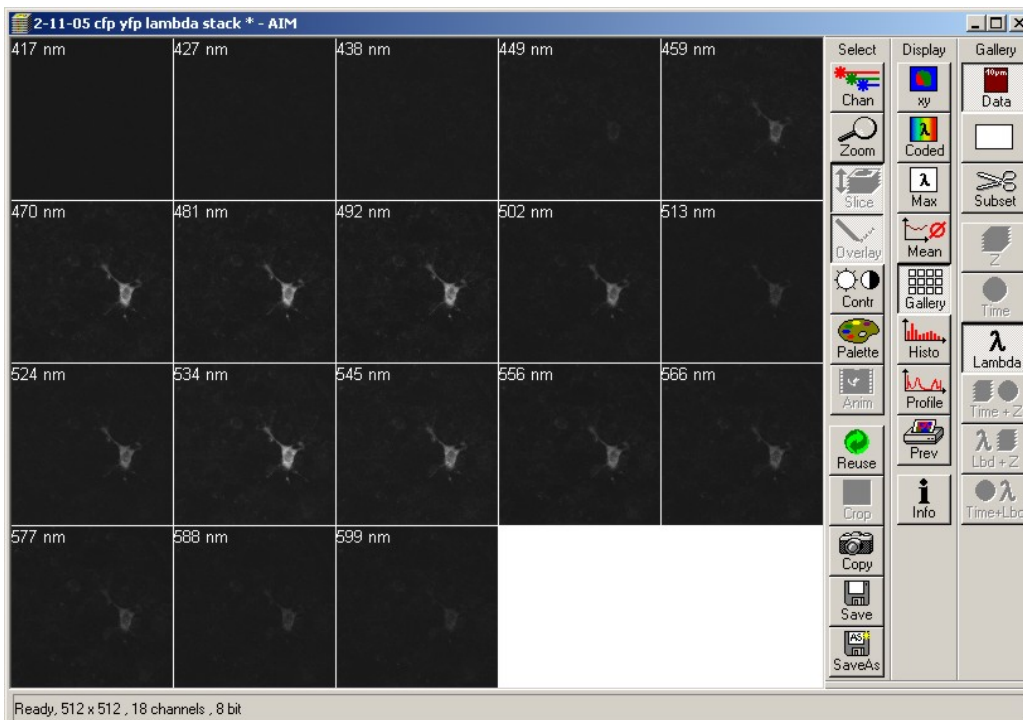
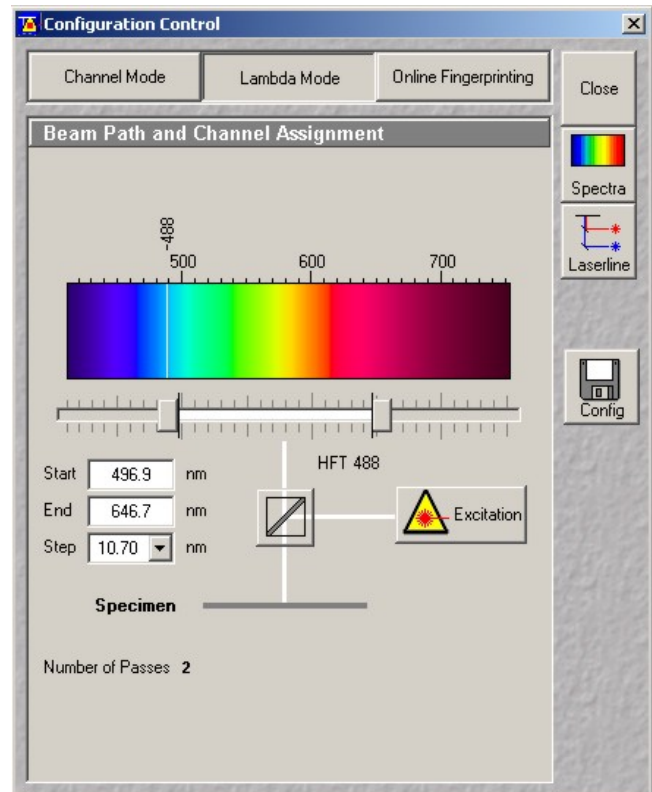
any number of contiguous elements, may be defined. Turn a channel on by clicking on its checkbox and select a spectral range with the slider. No overlap between channels is allowed.

Everything else being equal, the Meta detector and the PMTs have similar sensitivities except in the red where PMTs have an edge. However, the Meta detector is twice as noisy as the PMTs due to the electronics it contains. Sensitivity is also an issue when narrow spectral ranges are selected on the Meta channels. Individual channels defined on the Meta do not have separate gain settings, but intensity may still be adjusted independently either through the amount of excitation power (transmission sliders at the bottom of the Scan Control window) or by cutting part of the spectral range. However, when using the Meta for more than one color, the preferred alternative is to use a frame-switch, multi-track configuration. This setting offers independent gain and offset controls on each channel at the expense of additional scanning time. If the right emission filters are available on channel 2 or 3, we recommend to always use the PMTs instead of the Meta.

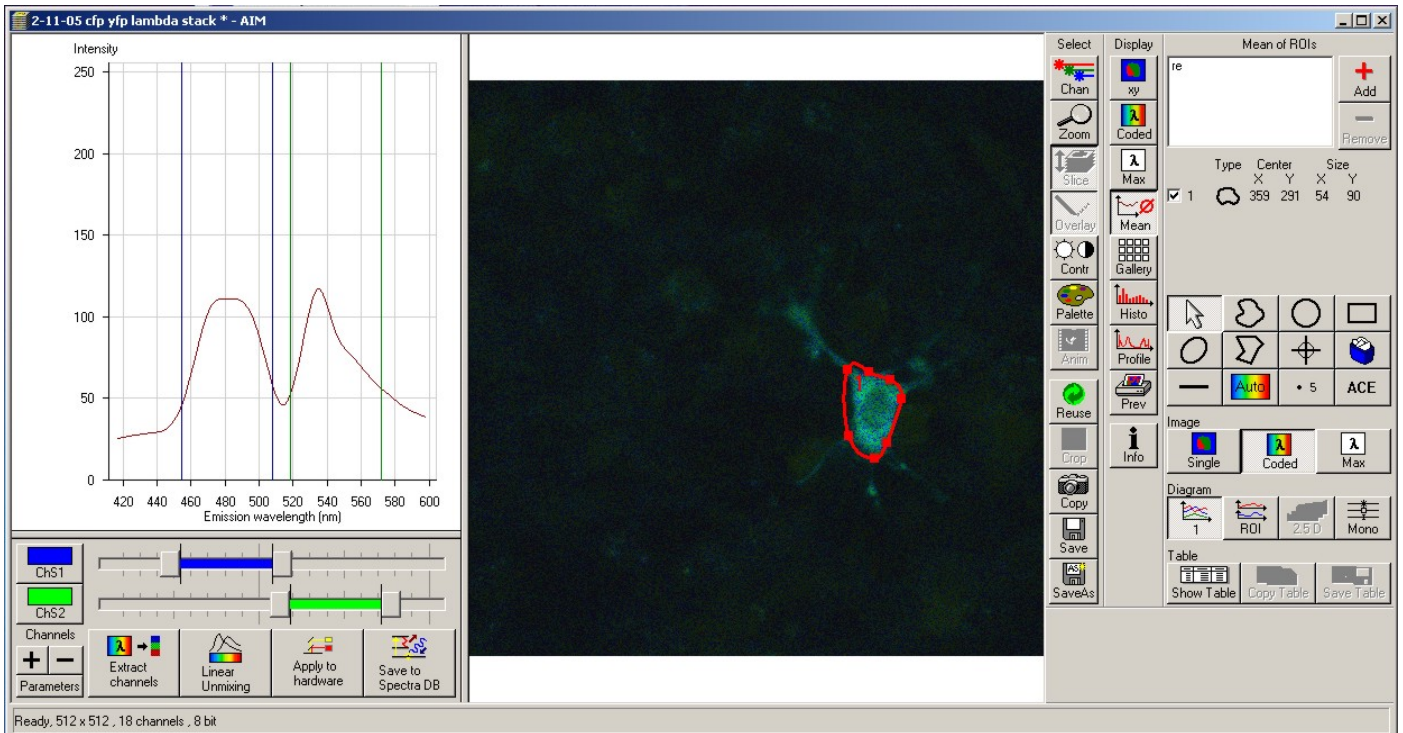
4.2) Lambda mode

Click on Lambda Mode in the Configuration Control window. In this modality, the Meta acquires an image from every detector in its array, that is one image for every 10 nm (10.7 nm to be accurate). 2, 3 or 4 adjacent detectors may be pooled together to increase sensitivity by using the Step function, resulting in an image every 21.4, 32.1 or 42.8 nm. After selecting a spectral range on the slider, click on Excitation to select one or more laser lines, then select an appropriate main dichroic.

Click Fast XY in the Scan control window and adjust gain and offset as when acquiring a normal image. The emission pinhole should be left wide open to account for reduced sensitivity. Since a maximum of 8 images are



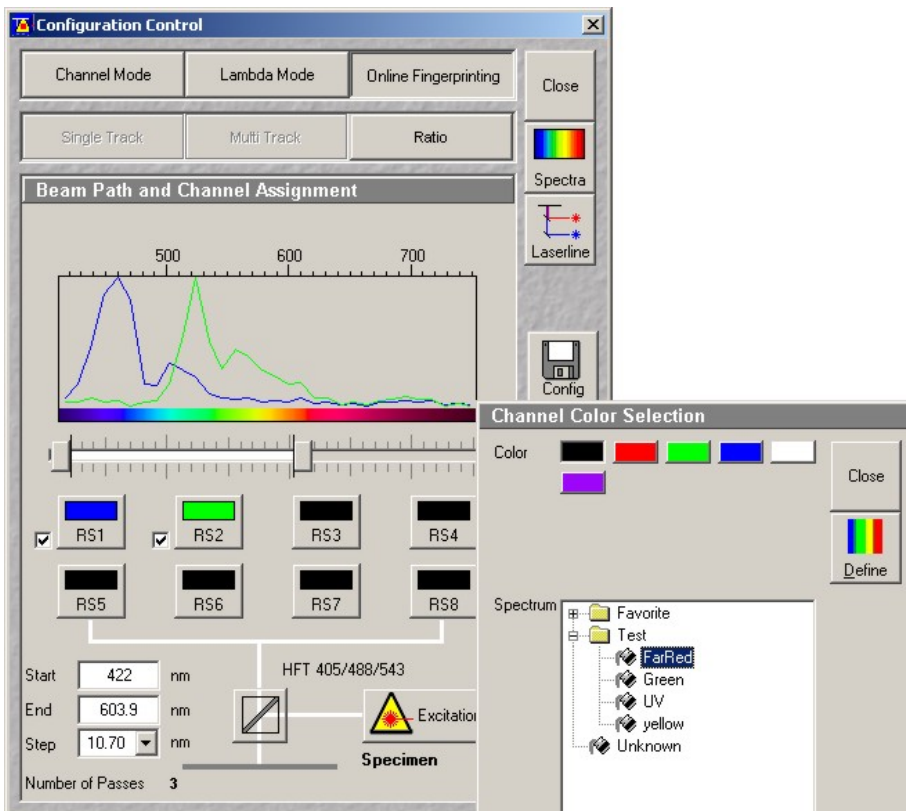
recorded at once, more than a single pass is usually needed to complete the acquisition over large spectral ranges. In analogy to a z-stack, a sequence of images acquired at different wavelengths is named a lambda stack. Click on Data in the image window toolbar to display the wavelength on each image. To view the emission spectra, click on Mean, select an ROI from the tool palette and highlight part of the image. The corresponding spectra is shown in the graph to



the left. Click on Show Table, then on Save Table to view the actual values and save them as a text file, respectively.

Spectral information may be used in many ways. Custom channels may be defined on the Meta with the sliders below the emission spectra: click on the + sign to create an extra channel, adjust its range with the sliders, then click on Apply to Hardware to configure the Meta detector. Spectra may be saved in a common database for later use (online emission fingerprinting, see 4.3 below) by clicking on Save to Spectra DB. Extract Channels separates the spectra into single-peak components. Linear Unmixing performs a task similar to

Automatic Component Extraction (ACE, located at the bottom right in the ROI toolbar): each deconvolve the image using reference spectra originating from different locations in the image. We strongly recommend not to use these two functions unless there is strong evidence the fluorophores are spatially separated (for example, beads with a different dye on each or a mixed population of cells expressing CFP alone and GFP alone). In most situations, rigorous image spectral deconvolution requires the emission spectra of individual components measured on their own.



4.3) Online emission fingerprinting

Emission fingerprinting uses the Meta detector to decompose the lambda stack

according to 2 or more reference spectra. To obtain meaningful results, it is imperative to first record the individual spectra of each fluorophore alone (using the Lambda mode as explained in section 4.2). Click on Save to Spectral DB to save each spectra.

When ready to scan the actual specimen, click on Online Emission Fingerprinting in the Configuration Control window. Up to 8 previously acquired spectra may be called from the database by clicking on each RS1 to RS8 button. Adjust the spectral interval to scan with the slider located below the spectral graph. The conditions under which each spectra was acquired must be identical and must match the final scan. In particular, objective and pinhole opening must remain the same. It is not recommended to scan near or across an excitation line.

From this point on, acquire a normal xy image as described in section 3.3. The acquired image is deconvoluted on the fly. The image window displays an image for each reference spectra plus a composite image. The processed image may be very different from the real one, in particular if there is much overlap between the different emissions.

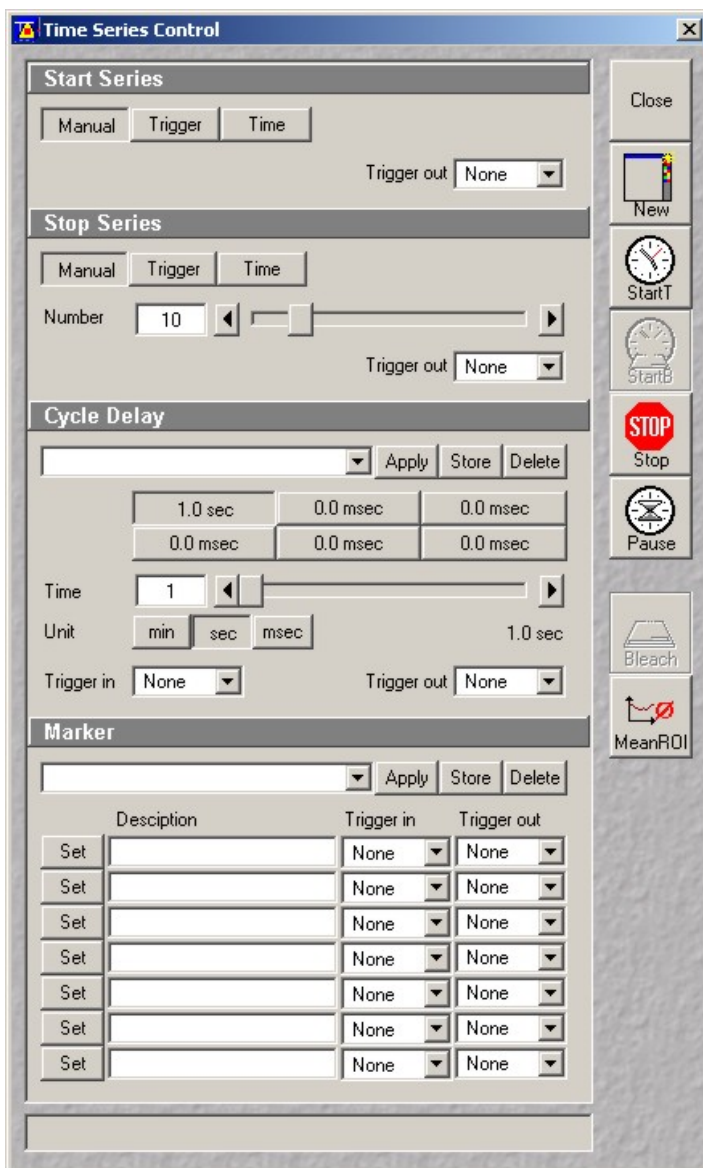
There have been some concerns about the statistical reliability of spectral image deconvolution on the Zeiss platform. Such considerations are beyond the scope of this writing, but as a general rule, it is unwise to push deconvolution to its limits by using extremely overlapping dies. Used in moderation, online fingerprinting is a powerful tool.

5) Other functions

5.1) Time series

To acquire a series of images at specific time intervals, adjust first the gain, offset, scanning area and resolution in the Scan Control window to obtain a satisfactory image (see section 3.3). Click on Time Series on the Acquire submenu to open the time serie window. Until the microscope is outfitted with an automated valve controller, Start and Stop Series must be left on Manual. Under Stop Series, enter the number of images to acquire in the Number box. Select a delay between acquisitions in Cycle Delay, keeping in mind the scan time adds up to the delay. For example, a delay of 1 sec with a scan time of 0.1 sec result in an image every 1.1 sec. For maximum speed (no delay), enter 0 msec.

Click on StartT to begin acquisition. Click on Gallery in the image window toolbar to display the complete series of images, click on Data to show the time on each image. Once recording is complete, click on Overlay and draw a region in the image to plot its intensity versus time. Several regions may be declared at once. Click on Show Table and Save Table to display and save the results as a text file, respectively.



5.2) ROI scanning

Since the LSM 510 is equipped with a fast-switching AOTF, it is possible to focus on custom regions within the scanning area. Open the Edit ROI window by clicking on Edit ROI in the Acquire submenu on the main toolbar. Acquire an image by clicking on Single, and select part of the image using a tool from the Edit ROI window toolbar. Click on Use ROI in the scan control window menu. Only the selected ROI will be excited and measured. ROI are saved with the experiment and may be recalled for later analysis.

Important: do not select an ROI on a Fast XY image! The ROI size and position is referenced to the image resolution. Unless you also specified a 512 x 512 pixel resolution in the scan control window, the ROI will move accordingly and will be located elsewhere in the image, sometimes beyond the edges.

ROI scanning does not modify the scanned area but rather turn the laser on and off with the AOTF. Hence, the overall scanning speed is unchanged. If time is an issue, use a combination of zoom, reduced (or custom) resolution and higher scan speeds to decrease the scan time.

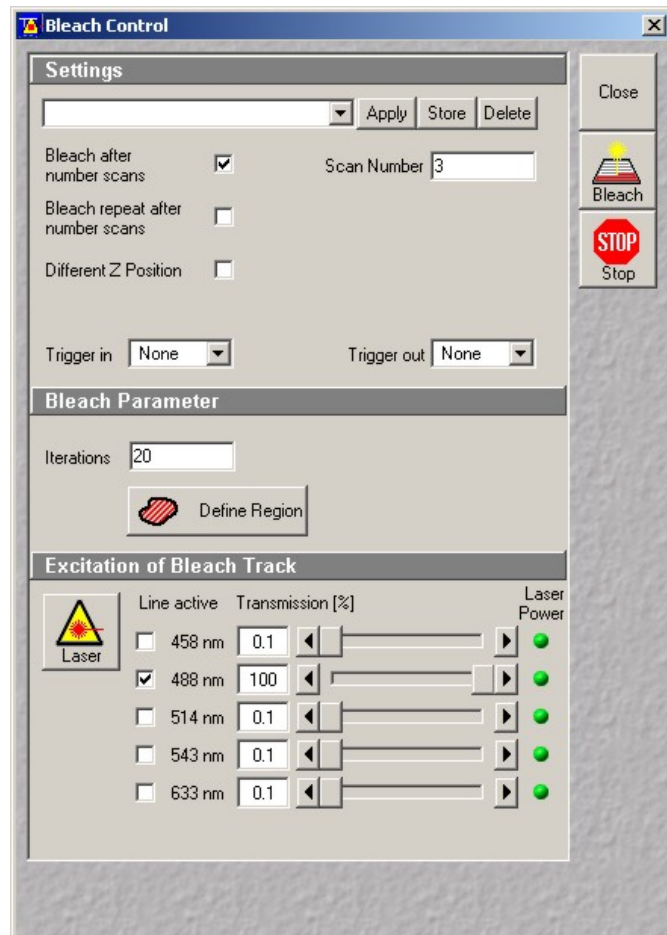
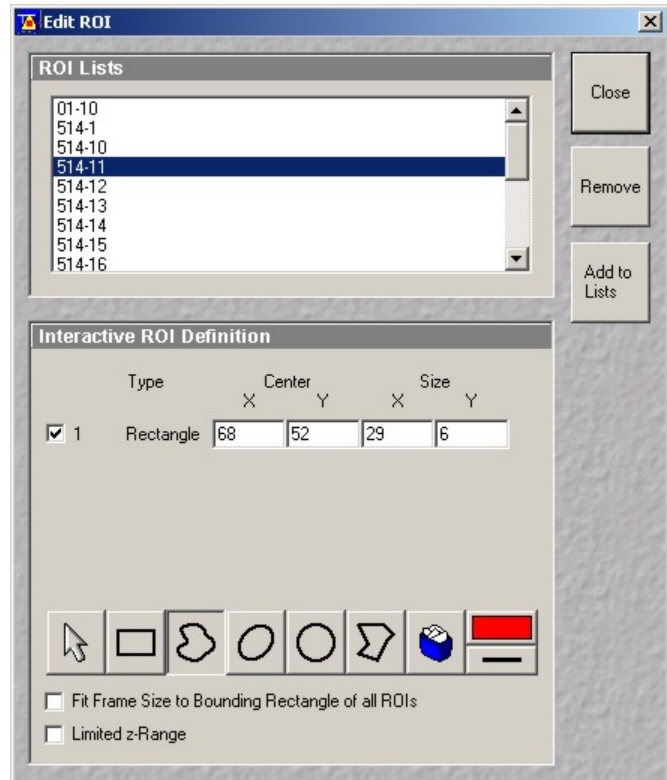
5.3) FRAP and Photoactivation

Flourescence Recovery After

Photobleaching (FRAP) is implemented as a combination of time serie and a custom ROI repeatedly scanned at maximum laser intensity for bleaching. Since high laser power is required to photo-degrade a dye, successful bleaching may only be obtained with the 405, 488 and 514 nm lines.

Adjust the scanning parameters to obtain a good image of your specimen. When bleaching with the 488 or 514 nm lines, the argon laser output may be increased in the laser control window, taking care not to exceed a tube current of 8 amps. For fast-recovering samples, the scan time may be reduced by zooming and reducing the image resolution. Scan an image at its actual resolution by clicking on Single in the Scan Control window. Click on Edit Bleach in the Acquire submenu. Check Bleach After Number Scans and enter the number of prebleach scans desired (usually 3). There is also the option to bleach repeatedly after any number of scans, or to bleach at different z levels.

Under Bleach Parameters, enter the number of bleaching scans. A single scan at full power is not enough for optimum photobleaching (at least 30 to 50% intensity reduction) and this setting must be

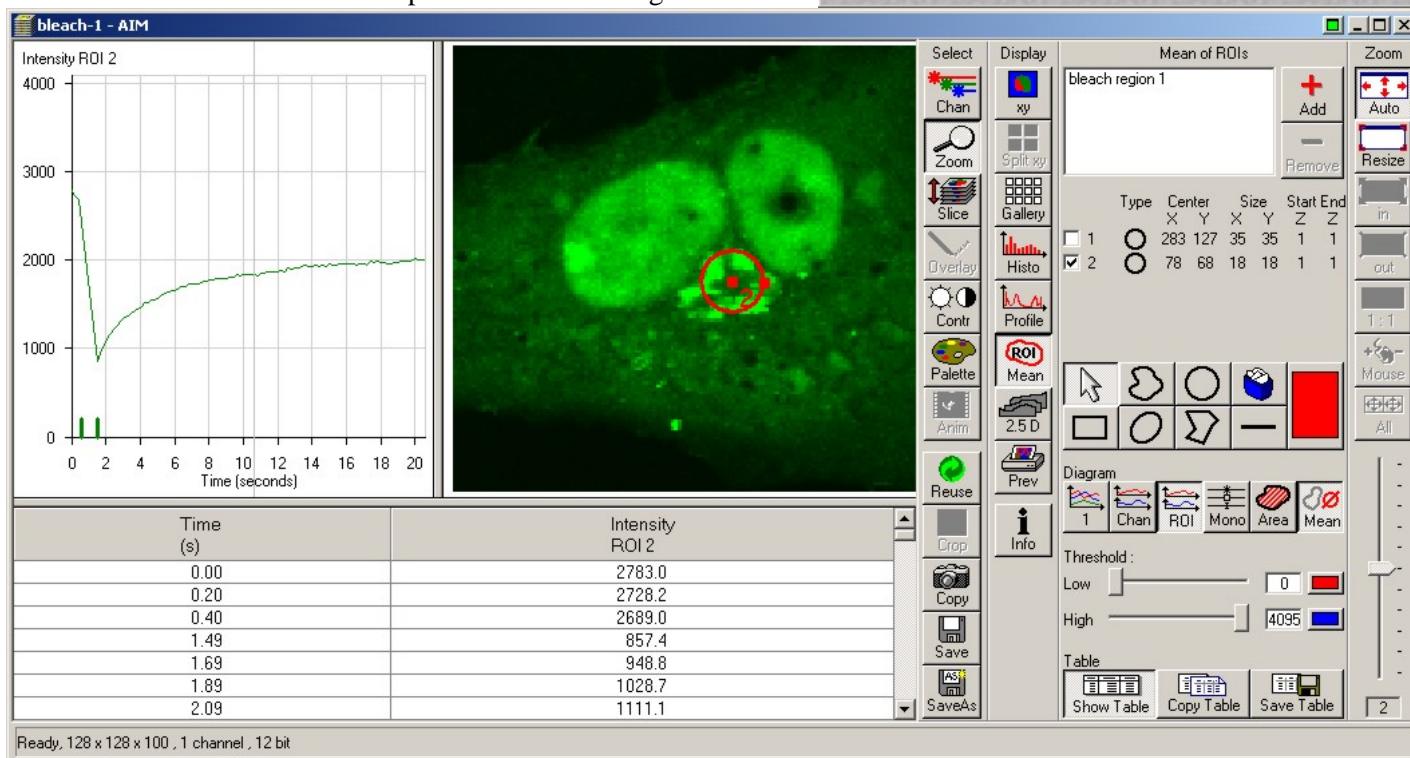
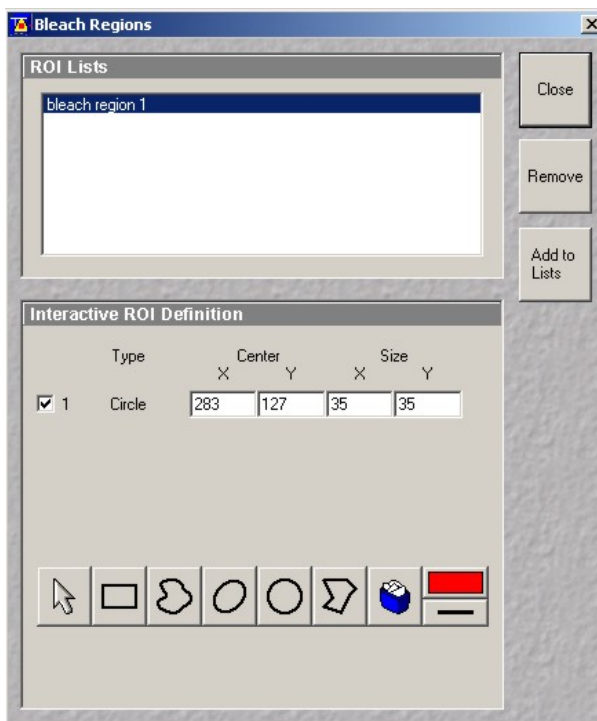


determined by trial and errors. Note the bleaching time should not exceed 10% of the characteristic recovery time. Select the laser line used for bleaching and increase its transmission to 100%.

Click on Define Region to open the Edit ROI (now called Edit Bleach) window. Select a bleaching ROI as described in section 5.2, making sure the resolution of the image is the same as the scanning resolution (do not use a fast xy image!). It is possible to use multiple ROIs.

Open the Time Series Control window and enter the total number of images and delay between each (see 5.1). Click on StartB to start the bleaching experiment. Click on Gallery to display the image serie. Once recording is complete, click on ROI and select the bleached ROI from the list to view the corresponding recovery curve. The Show table and Save Table functions may be used to export the results.

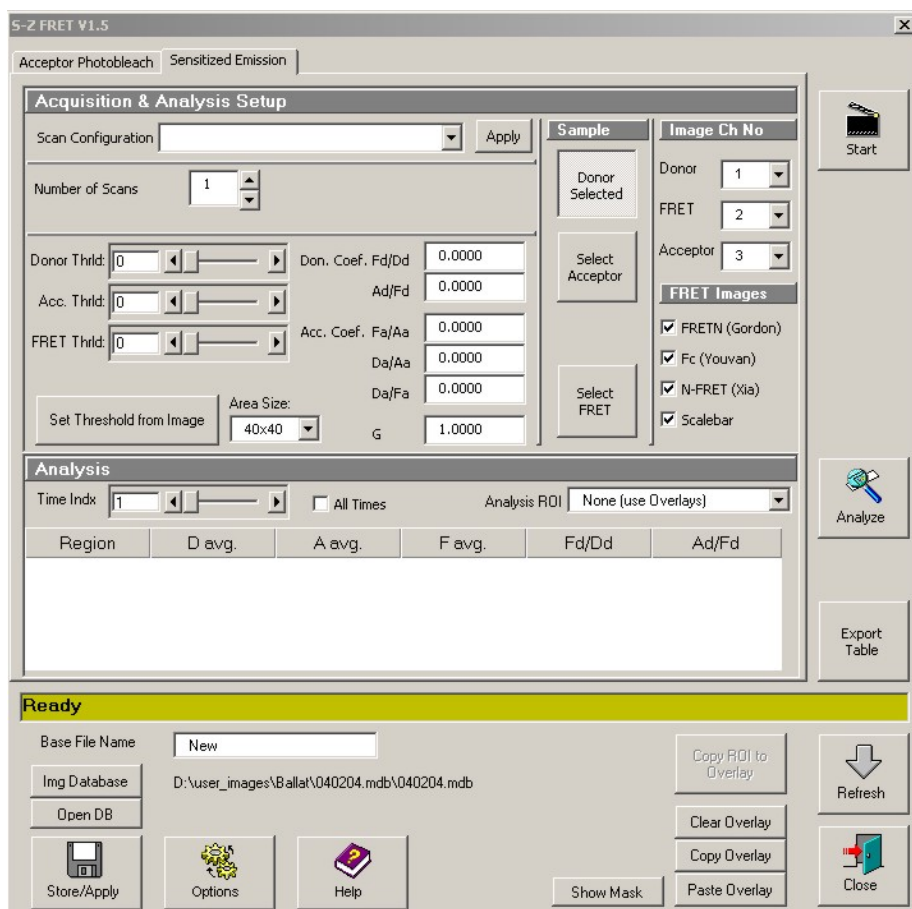
Because of the peculiar nature of FRAP on a confocal microscope and the complex underlying diffusion processes (for example diffusion in the Golgi), it is difficult to quantitate the recovery and calculate an accurate diffusion coefficient and mobile fraction. One exception is with low magnification /



low numerical aperture lenses on flat specimens, or when measuring diffusion in a flat object such as plasma membranes. If you are interested in quantitative FRAP measurements, contact the M.I.C. staff.

5.4 FRET

Fluorescence (Foester) Resonance Energy Transfer (FRET) is a non-radiative transfer of energy between a donor and an acceptor molecule. FRET pairs have extensive overlap between the emission spectra of the donor and the excitation spectra of the acceptor. FRET is most frequently measured as an increase in donor emission when the acceptor is removed (acceptor photobleaching) or as fluorescence emission of the acceptor when exciting the donor (sensitized emission). The later is more sensitive than the former, but also more prone to false positive results. Both molecules must be in close spatial proximity, between 2 to 20 nm.



With the development of Green Fluorescent Protein (GFP) and its mutants, FRET has become a popular technique to assess intermolecular interactions or conformational changes in recombinant proteins. However, FRET should not be used without a thorough understanding of its underlying principles and pitfalls. If you are not familiar with FRET, we recommend acquiring a basic knowledge of this application before running experiments.

Zeiss has recently introduced a macro to perform FRET measurements on its LSM instrument. To correct for cross-excitation, bleed-through and background fluorescence, a sensitized emission FRET experiment consists of 3 series of 3 images: acceptor emission with the acceptor excitation, donor emission with the acceptor excitation (FRET channel), and donor emission with the donor

excitation, each collected with the donor alone, the acceptor alone and the donor/acceptor pair. This series of nine images may be fed directly to the sensitized emission macro for calculation of FRET coefficients. Acceptor photobleaching is also available. Note that beyond the most common artifacts (bleed-through, cross-excitation and background fluorescence), further corrections may be required for greater FRET accuracy.

One of the most commonly used FRET pair is the Cyan and Yellow Fluorescent Proteins. When exciting CFP at 458 nm, cross-talk between channels is severe. The 405 nm line available on the LSM 510 inverted does not excite YFP and is a perfect choice for CFP/YFP FRET. Sensitized emission between Green and Red Fluorescent Protein has also been successfully tested. In spite of this, accurate FRET measurements are complex and should not be done without a thorough understanding of the method.

6) Post-acquisition image processing

The LSM 5 software includes an extensive collection of tools for image display and analysis. This section covers the basic functionalities of the LSM software. For an in-depth description, refer to chapter 5.13 of the LSM documentation available from our web site. The free version of the software, LSM Image Browser, may be used to visualize and export images but lacks all measurement functionality. Another free software package, ImageJ, offers excellent image analysis functions. Both ImageJ and the LSM image browser are available from the M.I.C. staff on request.

For advanced image analysis and quantitation, the facility offers several applications running on dedicated workstations: Universal Imaging's Metamorph for image analysis, and Improvion's Volocity for 3D reconstruction, real-time visualization and deconvolution. Contact us for details.

6.1) Image annotations

To add text annotations, scalebars, intensity ramp and distance measurements to an image, click on Overlay in the image window toolbar. Select a tool from the overlay palette and drop it on the image. Each annotation is saved independently from the original image and may be erased or edited later. To display the time, Z-position or wavelength (in a time serie, a Z-stack or a lambda stack, respectively), click on Gallery to show the Gallery toolbar, then click on Data.

Brightness, contrast and palette settings may also be used to enhance specific features in an image. Click on the Contr icon in the image window toolbar. You may adjust each channel individually or influence all channels at the same time by choosing All on the bottom toolbar. To change the palette used to display the image, click on Palette and select a palette from the list. Range Indicator is commonly used to check for under- and over-exposed pixels during acquisition. Each channel may be turned on or off independently by clicking Chan in the image window toolbar and selecting any color available or Off. Click on Color to add a custom color to the palette (a custom color may also be assigned to each channel during acquisition by clicking on each channel icon in the Configuration window, then selecting Define). Brightness, contrast and palette settings are global and affect every image in a stack, but do not modify the original image unless it is saved again.

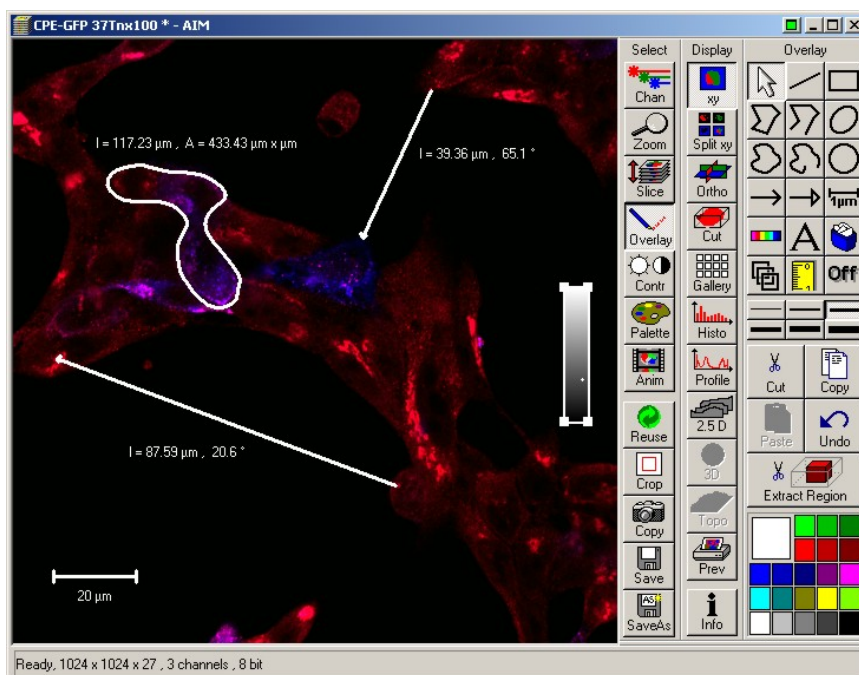
Several options are available to visualize images in a stack, regardless of its nature (time, Z or Lambda series). Gallery mode displays an overview of the entire stack laid flat. For full resolution, click on XY in the image window toolbar and select Slice to show a slider for browsing through the stack. To animate the stack, click on Anim to bring up the Animate control panel.

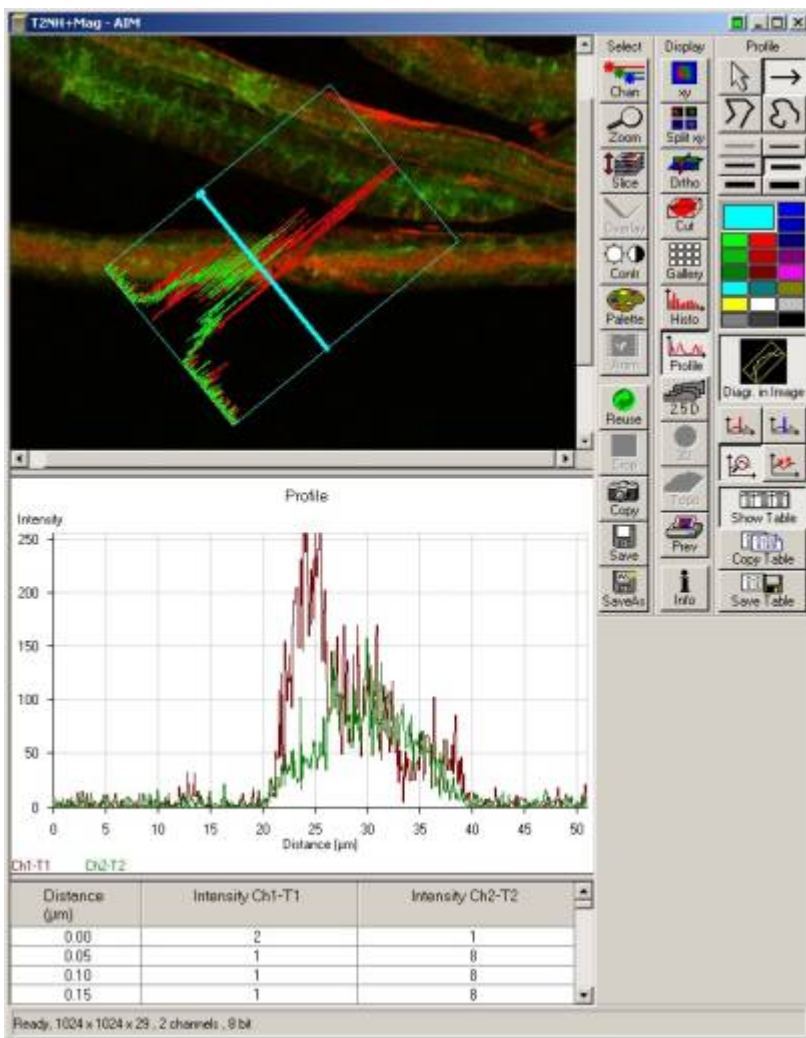
6.2) Intensity measurements - Colocalization

To display intensity across a line, click on Profile in the image window toolbar. Select a line thickness (1 to 6 pixels) from the menu to the right and drop it on the image to show the intensity profile plot. If more than 1 channel is present, a profile is shown for each channel. Click on Show Table to view the numerical values, then on Save Data to export the results as a text file.

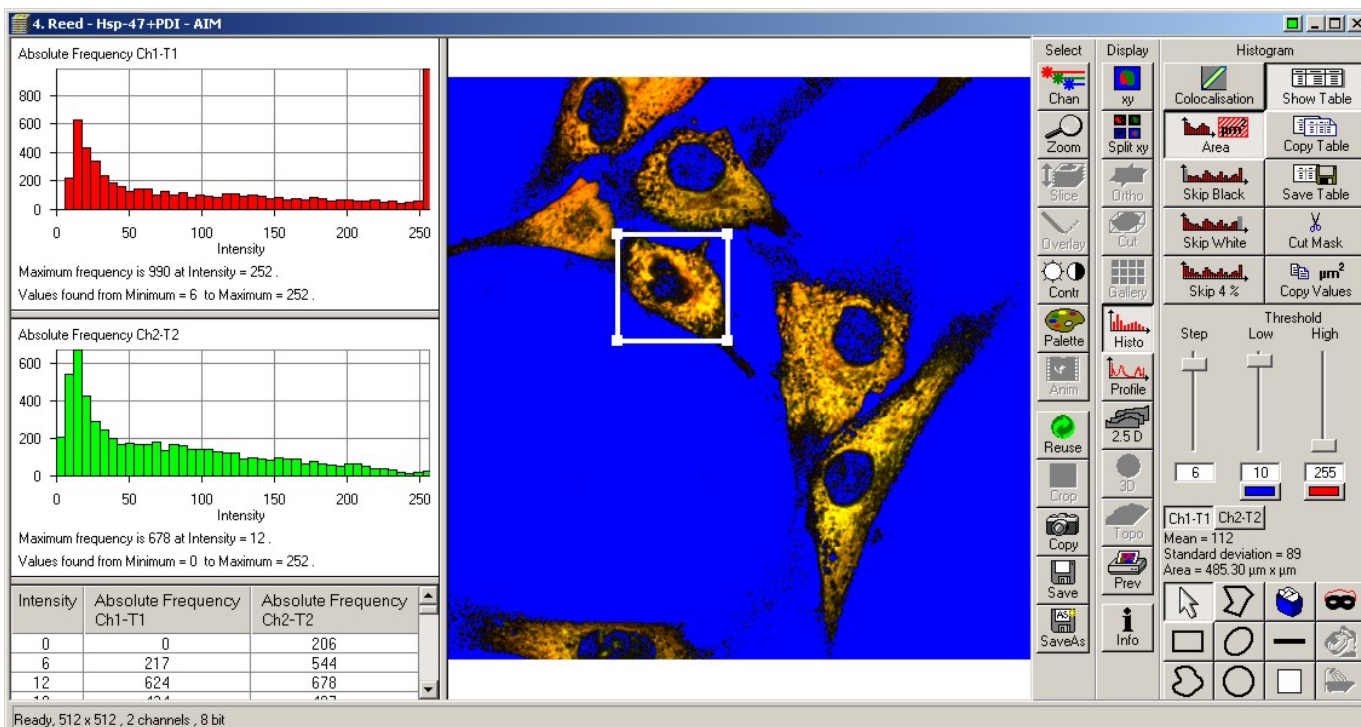
The Histo icon in the image window toolbar provides many intensity measurement functions. By default, intensity distribution for each channels are shown. Click the Area icon. Select a shape from the toolbar at the bottom and highlight a region in the image. The surface area, average intensity and standard deviation are shown above the tool palette (lower right). For an image series, the results may be view and saved by clicking on Show Table and Save Data. Use the high and low threshold sliders to threshold the image for the calculations.

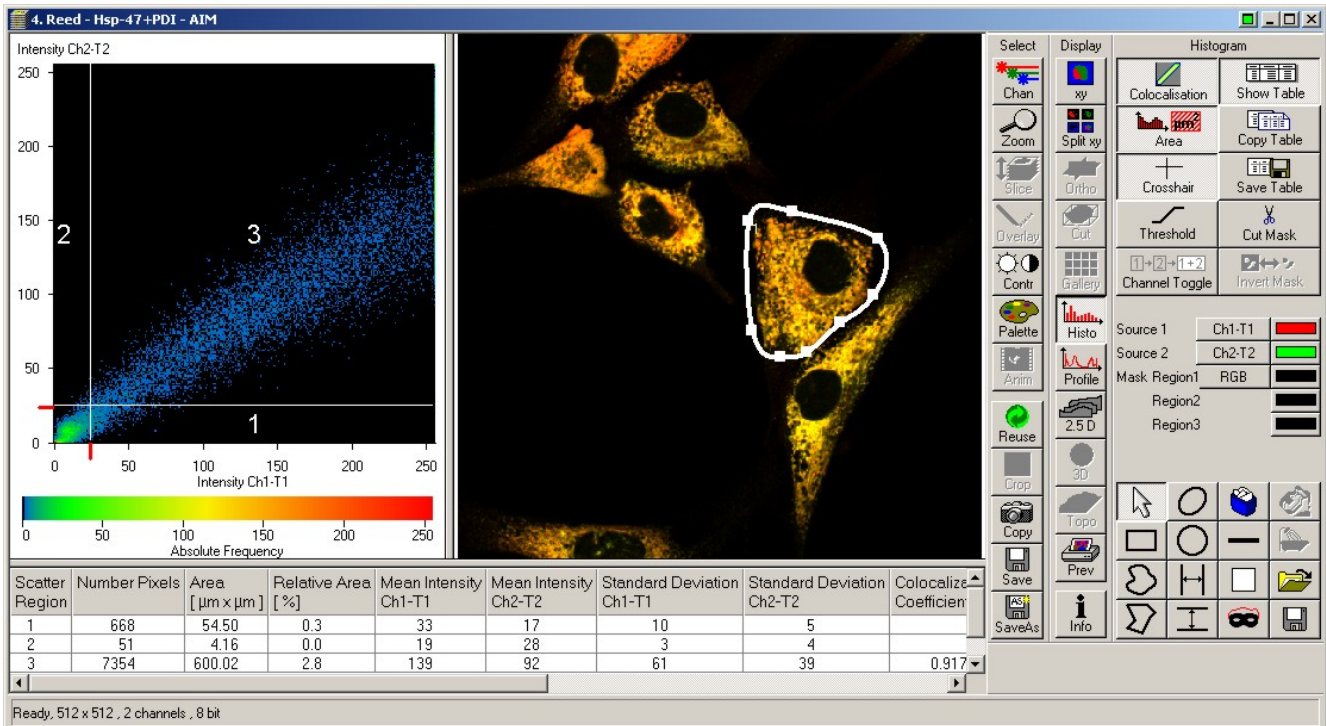
Another feature frequently used is the colocalisation function. This tool analyzes the correlation between 2 channels by plotting the intensity on one channel versus the other channel for each pixel pair (i.e. identically located pixel on each channel). A perfect correlation (for example using twice the same image) translates on such scattergram as a perfect diagonal line. Realistically, the correlation between 2 channels is seen as an elongated cluster of points with a slope depending on the global intensity ratio between the 2 channels. This analysis requires first removal of low-intensity pixels, i.e. to threshold the image. Click on Crosshair to show the threshold values on the scattergram. Click on Show Table to display colocalization coefficients. Only pixels in region 3 of the crosshair are analyzed. It is also possible to select part of the image with one of the shape tools and restrain the analysis to that region. There are many ways to quantitate colocalization. We





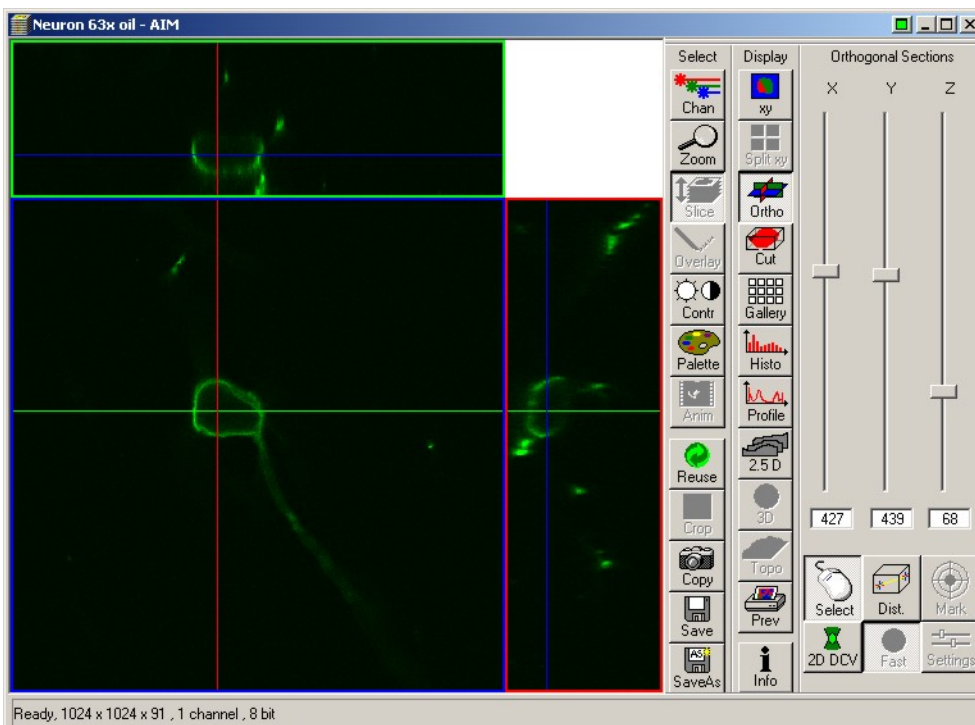
recommend the correlation coefficient value as a fair quantitative measurement. Since colocalization analysis involves setting thresholds on each channel, it is, by nature, a relative measurement. Colocalization values may only be compared between specimen imaged and analyzed with exactly the same parameters and threshold values.





6.3) 3D rendering

The LSM software offers a number of visualization options for image stacks. Click on Ortho in the image window toolbar to activate the orthogonal view. In this mode, the XY plane displays a crosshair. The corresponding ZX and ZY sections are shown on the top and right, respectively. When the crosshair is moved, the top and right images are updated. Similarly, the top and right images have crosshairs controlling two of the three planes. X, Y and Z level may also be adjusted with the sliders to the right. 3D Distance measurements are possible by clicking on the Dist icon. Note the Cut icon allows for oblique sectioning through any slope in the volume.



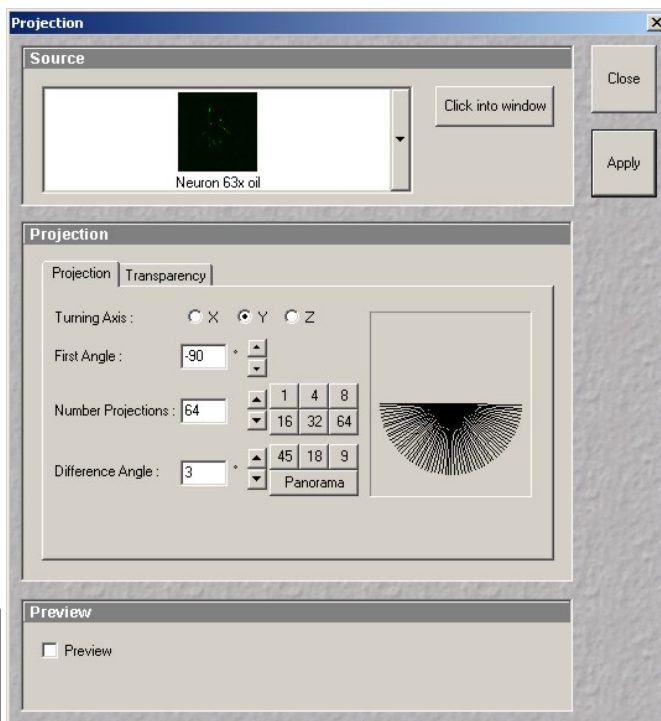
2.5D allows to display the two-dimensional intensity distribution of an image in a pseudo 3-D mode. Additional rendering options are available through the 3Dview menu from the main menu. Projection allows calculation of a single projection (maximum projection) or a series of projections with rotation about any of the X, Y and Z axis. The result may be put in motion through the Anim icon to emulate a 3D rendering. Simply select the axis of rotation, initial angle, number of projections and angle increment and click on

Apply. Advanced projection modes may be selected in the Transparency tab. The resulting image sequence is independent from the source images and may be saved independently.

6.4 Exporting images

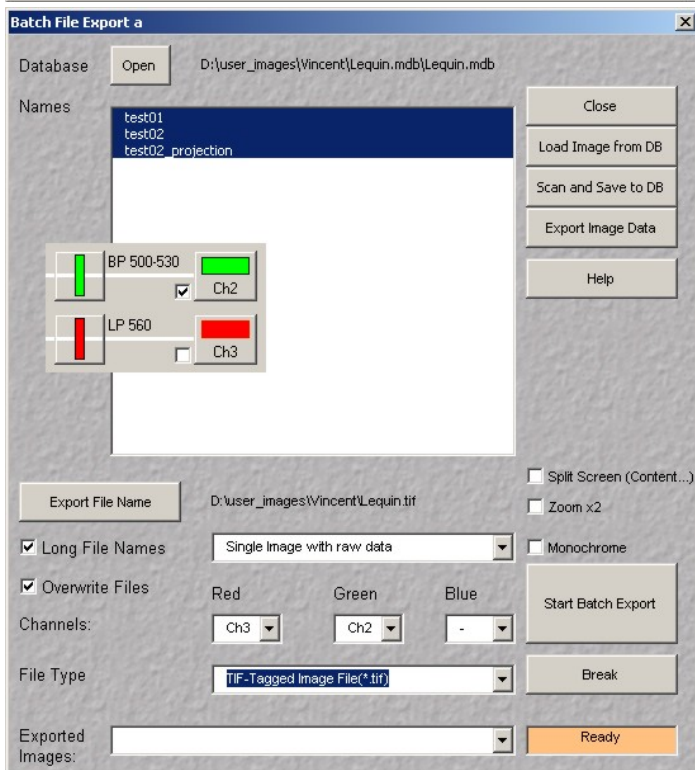
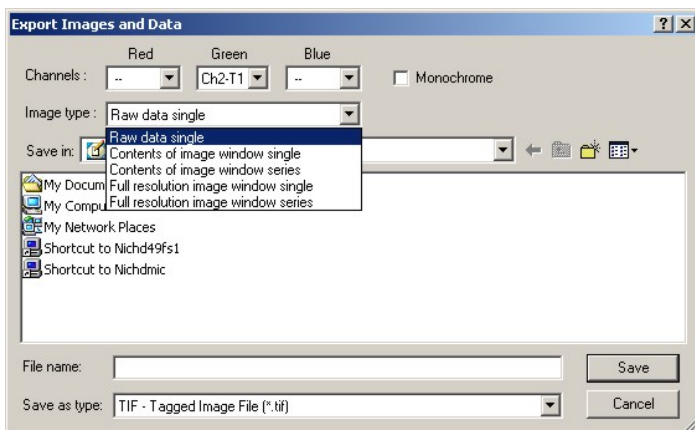
Images acquired on the LSM 510 are saved with an “lsm” extension as a proprietary tiff file format with a custom header. With the exception of specific software (LSM software, Metamorph, ImageJ and Volocity), these files cannot be open with standard bitmap-editing programs (Corel Photopaint or Adobe Photoshop).

To convert individual images, select Export from the File main menu toolbar. Two options are available: a monochrome image or a color one. The default setting is color. It is crucial to understand the images recorded on the microscope are grayscale, not color images. They



appear in color because a pseudo-color scheme is applied (i.e. a black to –say- green instead of black to white color ramp). To export as an 8-bit grayscale image, check the Monochrome box and select a channel. Color images are created by sending each channel onto one of the three layers of a 24-bit RGB color image. Hence it is only possible to export using any of these 3 colors (red, green, blue). Select the channel to fit onto each layer, then select an image type mode from the list (most often raw data single). Enter a filename, a destination folder and click Save. A composite image is created by exporting several individual channels onto the same color image.

To export a large number of images from a database, we recommend using the Batch Export macro available from the Macro menu on the main menu toolbar. Click on Open to select a database, then fill each layer in the destination 24 bit color image with a channel (refer to the Configuration Control window to determine which channel is what: load your image and click on Reuse to restore the original configuration). Click on Export File Name and select a destination and a generic file name. Always use a different folder to avoid overwriting the original images! The macro appends each image name after the filename specified. For example, naming the series “red” results in a series of files named red_image1.tif, red_image2.tif, etc... and makes things easier later. Click Start Batch Export to start. Repeat the process as many times as required, usually once for



each channel then once more for a composite image.

6.5) Transferring images

The M.I.C. computers are on the network. If you have an account on “NICHHD49FS1” server, double click this icon on the desktop and login as “NIH\username” and your NICHHD domain (email) password. Browse to your folder, open Windows explorer and drag your files onto your network folder. Alternatively, the facility owns a 10 TB file server (nichdmic) on which you may obtain an account with an initial quota of 20 GB storage space. Click the nichdmic icon on the desktop. If you used the same username and password on the microscope and on our server, the process is transparent.

To connect remotely to our server, from a PC: 1) Click on "Start", 2) Select "Run", 3) Enter "\\nichdmic\username" where "username" is your user name on the server, 4) If prompted, enter your username again then your password. Do NOT prefix your username with "NIH\". You may run into trouble if your computer logs onto the NIH domain by default and remember your username as “NIH\username”. In that case, use your full name (John Doe) to login onto nichdmic.

From a Macintosh: 1) In Finder, click on "Go", 2) Select "Connect to server", 3) Enter "afp://nichdmic.nichd.nih.gov/username" where "username" is your user name on the server, 4) When prompted, enter your password.

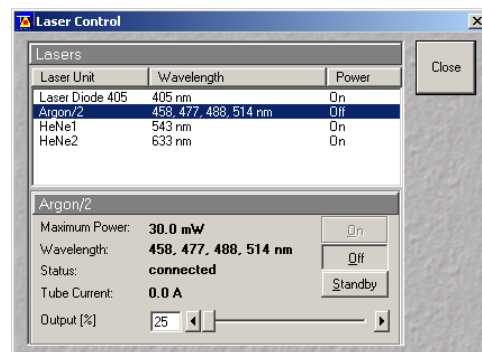
The facility also offers data CD and DVD burning upon request.

7) Turn off procedure

7.1) After saving the acquired images, open the “Lasers” control panel and turn all lasers off . Wait 5 minutes until the cooling fan on top of the Argon laser stops spinning!

7.2) Clean all oil immersion objectives using lens cleaning paper only! Check dry objectives for oil smudges and clean them if required.

7.3) Move the objective turret to its lowest point with the coarse focus knob.

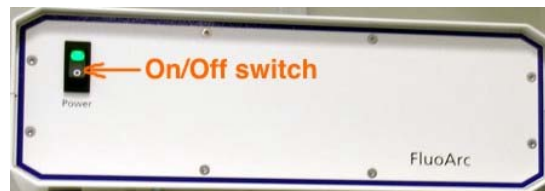


7.4) Quit the LSM software, log off Windows and turn both flat panels off.

7.5) Shut down the system using the master switch on the computer cart

7.6) Turn the Hg lamp off with the switch on the lamp power supply.

7.7) Fill up the logbook, clean all work areas.



8) Troubleshooting

Error messages at startup

If the microscope stand or another part of the instrument was turned off using its own on/off switch, the master switch will not turn it on. Contact the M.I.C. staff for help.

No image in widefield fluorescence

Is the mercury lamp on? Check for heat and light escaping from the lamp housing. Try opening the aperture and field diaphragms on the lamp port (back of the microscope, right side). Remember to click on "Vis" on the main menu toolbar.

No image on scanning

Is there light escaping from the objective? If not, is the condenser assembly tilted backward? Put it back upright. For safety reason, the laser beam is blocked when the condenser is tilted back.

Is the laser turned on? Open the laser control panel.

Is the right objective shown on the microscope's LCD display? Loading a method sometimes triggers an objective change with possible loss of focus, insufficient immersion oil, etc... Check for proper focus by visual inspection.



Calendar reservation

1) Got to our website (<http://mic.nichd.nih.gov>) and click on Microscope Sign Up, or go directly to <http://dircal.nichd.nih.gov>. Log in with the username and password provided by the managing staff.

2) Click on View and select Calendars.



3) Select a microscope from the drop-down menu.

4) Click on the date number of the reservation to open the reservation page. Fill all the fields as shown:

Description: your first name - PI name – live (if live

A screenshot of the reservation form. The form has tabs for 'General', 'Text', 'Recurrence', 'Resources', and 'Exceptions'. The 'Text' tab is active. Fields include: 'Calendar' (Zeiss LSM 510 Inverted Meta), 'Description' (John Doe - PI's name), 'Approved' (No), 'Contact Name' (John Doe), 'Contact Info' (doej@mail.nih.gov), 'Addl. Info URL' (empty), 'Starting Date' (03/30/2005), 'End Date' (03/30/2005), 'Start Time' (09:00 am), and 'End Time' (1:00 pm). There are 'Save' and 'Reset' buttons at the bottom. A 'Return to Calendar' link is also present.

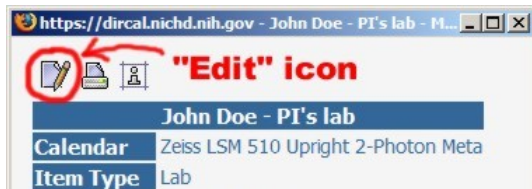
experiment).

Start / End date: enter date in the format MM/DD/YYYY or use the date picker.

Start / End time: enter time as “hour : minutes am/am”. Do not use 24-hour time (18:00 instead of 6:00 pm). Do not use anything else than a colon between hours and minutes!

Click on Save to finalize your reservation.

5) To cancel a reservation, repeat steps 1-3, click on the reservation and click on the Edit icon. Click Cancel at the bottom.



Other issues:

Immediately contact the M.I.C. staff for assistance:

Vincent Schram: room 5A10, 301-496-6419

Pager: dial 102, 11327, your phone number.

Chip Dye: room 5W14, 301-496-3627

Pager: dial 102, 11568, your phone number.

This manual written by Vincent Schram, Ph.D., March 2005. Use, duplication and distribution outside of NICHD is not authorized.

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