# ZEISS LSM 510 UPRIGHT 2-PHOTON META OPERATING PROTOCOL

Foreword

The Zeiss LSM 510 upright two-photon 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 Chameleon pulsed infra-red (IR) laser, it is especially well suited for FRAP, FRET, deep tissue and live animal imaging 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 upright two-photon 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).
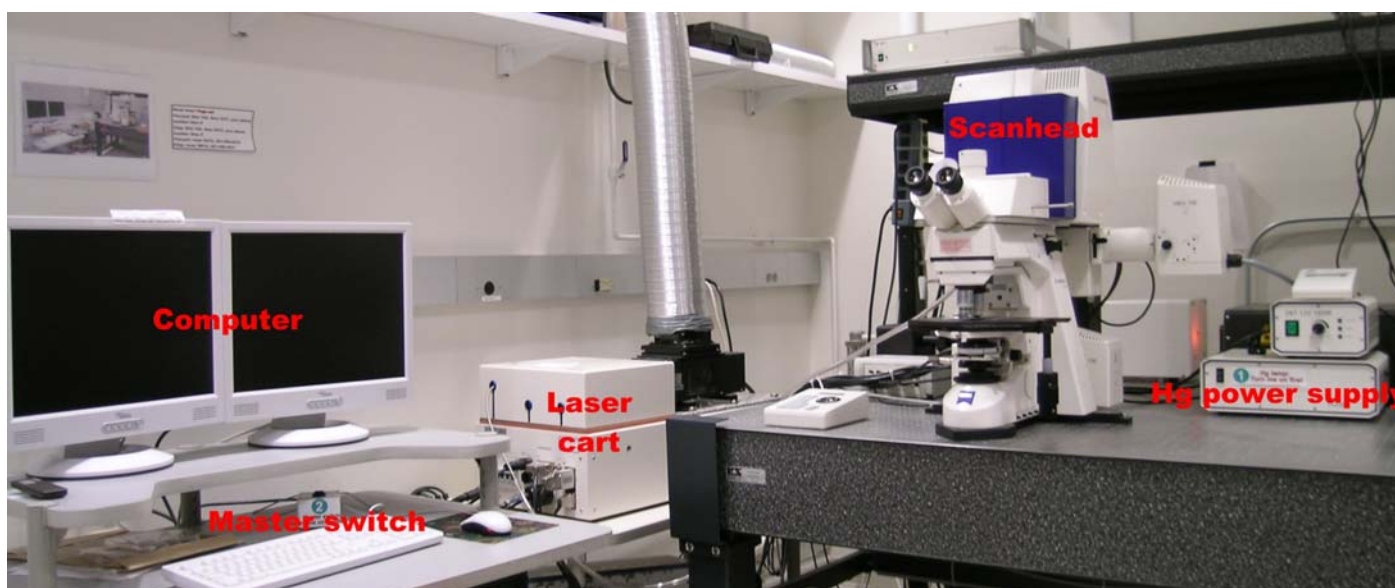
Overview

Before starting, locate and identify the different elements of the microscope:
- Computer monitors, CPU, keyboard and master switch 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 Chameleon laser head on the optical table.
- Chameleon control module on the shelve above the air table (not shown).

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) If you will be using the Chameleon laser, turn the key on its power supply from Standby to On. The Chameleon power supply is the large brown bow located to the right side of the shelve above the air table.
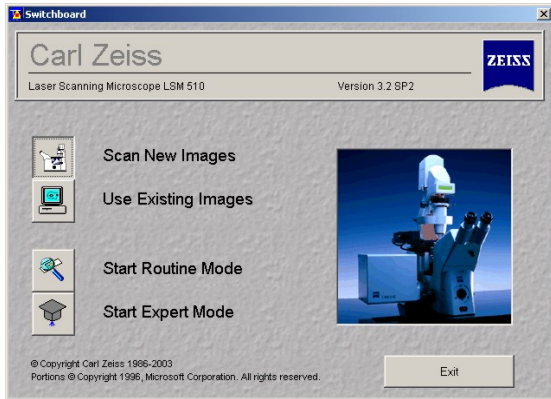
1.4) Turn on the computer monitors and log on the computer. If you are the first user of the day, reboot the computer. Double-click on the "LSM 510 Meta" icon on the desktop.

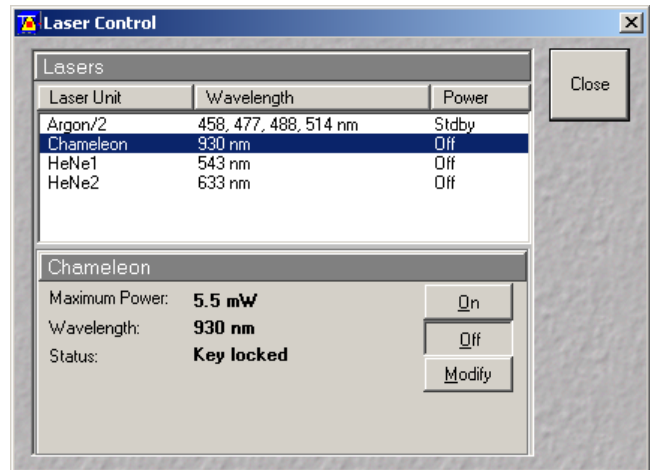1.5) On the LSM switchboard, select "Scan New Images" and "Start Expert Mode".

1.6) The main menu bar defaults to the "Acquire" subsection. Click on "Lasers" on the lower menu bar. Turn on the lasers required by your experiment: Argon (458, 488 and 514 nm), Chameleon (tunable range from 720 to 930 nm), Helium-Neon
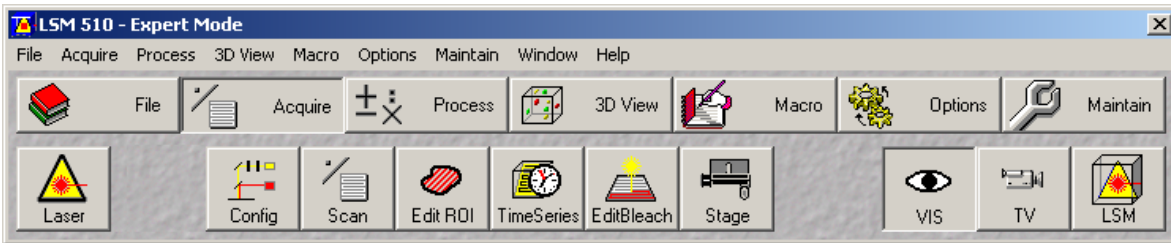
1 (543 nm) and 2 (633 nm).

     The argon laser has a standby mode and a slider for power setting. Put it on standby if not used for more then 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!

     The Chameleon laser operates in mode-locked mode and its output wavelength may be adjusted anywhere between 720 to 930 nm. Click on modify, then enter a new wavelength in the dialog box, and click Store. Do not try to adjust the AO frequency fine-tuning slider! The status area on the laser control panel will display "optimizing" for about 1 minutes. Do not scan until the status area show "Mode-locked". Never attempt to change wavelength while the laser is optimizing! "Key-locked" means the key on the power supply is still on Standby, turn it on On position.If not used for more than 30 minutes, put the Chameleon on standby by turning the key on its power supply!
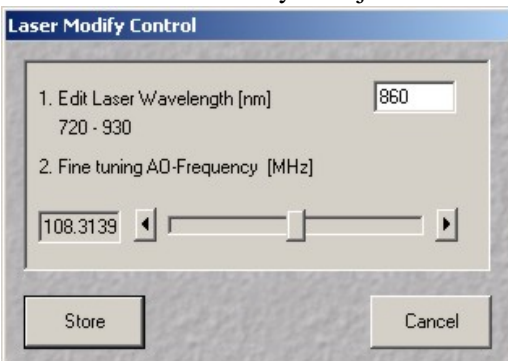
NB: not all experiments require the 4 lasers to be turned on. Do not

power a laser you will not use. Remember to put the argon and Chameleon lasers on stanby if not used for more than ½ hour!

2) Sample visual inspection

The Zeiss Axioskop 2 FS is a microscope stand for electro-physiology. As such, it incorporates very little in the way of automation. Almost everything on the microscope requires manual switching.
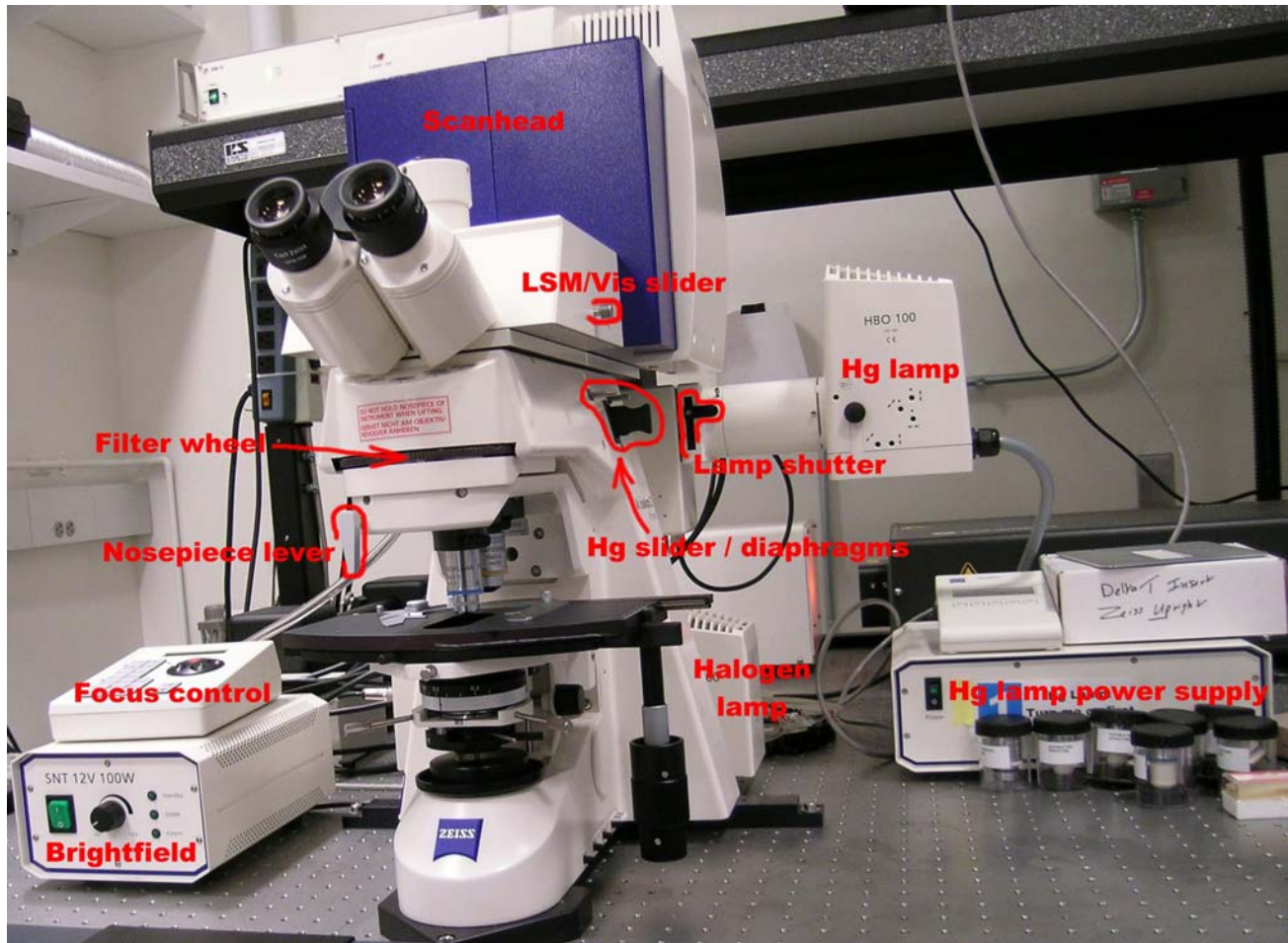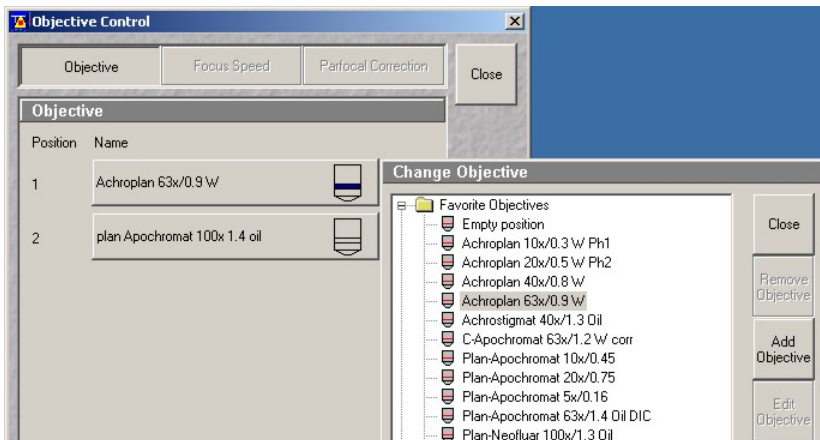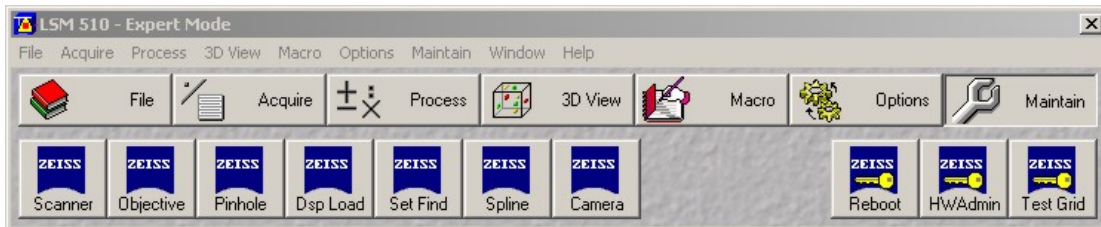

2.1) Objectives

The nosepiece on the Axioskop 2 FS is based on a manual-switching slider and accepts only two objectives at a time. The microscope was purchased with a set of water-immersion, long- working distance infra-red lenses designed for deep-tissue imaging with the Chameleon laser. However, all infinity-corrected Zeiss objectives may be used with the Axioskop 2 FS.

To replace an objective, move the nosepiece to its top position by pressing first Speed then the Up arrow on the focus drive keypad (see 2.2 below). Carefully unscrew the objective and store it in its container. Remove the new objective from its container and screw it on the nosepiece. Be careful not to drop the objective nor scratch its front lens! Do not leave objectives on the bench, always store them in their container! Empty positions on the nosepiece must always be plugged with an appropriate cap.

Once the proper objective is installed, changes must be made in software for proper confocal acquisition and image calibration settings. On the Main Menu Bar, click on Maintain, then on Objectives. Click on each position and select the appropriate objective from the list. Be certain to match not only the objective name and magnification (i.e. Achroplan 60x), but also the immersion media (oil, water), numerical aperture and working distance. These specifications are engraved on each objective barrel.

To switch from one objective to the other, move the nosepiece to its topmost position. Do not omit this step since some objectives are longer than others and there is a risk to break the specimen and damage the objective! Once the nosepiece is in its topmost position, turn the nosepiece lever 90 degrees counter-clockwise, slide the other objective in position and put the lever back in place. Proceed to focus as described below. If the lever does not rotate, it may be stuck halfway between the 2 positions. Try to pull or push, but do not force on the lever. Before acquiring images, the proper objective must be declared in the Scan Control window, as explained below in 3.3.

2.2) Focus controller

The Axioskop microscope stand does not have coarse and fine focus adjustment knob. Instead, one must use the focus control keypad. The High Speed and Low Speed keys toggle between fast and slow focus speed. Use the Up and Down arrows to move the nosepiece up and down. During scanning, the focus wheel may be used for very fine focus changes. The controller has many other functions currently unused. If you end up pressing by accident any other key than the one described above, simply press the Enter key to exit and return to focus mode.



2.3) 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 using the High-Speed focusing mode (you may need to use a flashlight). Then, switch to Low Speed and move the objective away from the specimen by pressing the Up arrow while looking through the eyepieces. Slowly moving the stage until some intensity fluctuation is perceived helps zeroing on the specimen.

When sliding inserts on the stage, always move the nosepiece to its upper position, raise the objectives with the nosepiece lever and leave it halfway between the two positions to prevent scratches on the front lens.

2.4) Brightfield

Unlike the LSM 510 inverted, switching between laser scanning and visual observation is entirely manual. Move the LSM / Vis slider on the side of the microscope to Vis. Make sure the reflector wheel is in the "1" position. Insert your specimen on the stage. Turn on the brightfield power supply and adjust intensity. If no light is visible, move the LSM / Vis slider once back and forth. This corrects a known issue due to the presence of a non-descanned detector on the microscope (see 5.3).

Set the microscope up for Kohler illumination as follows:
Open the field diaphragm by turning the protective disk covering the iris at the bottom of the microscope stand. Focus on your specimen using the focus controller as described above. 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. You may need to adjust the condenser numerical aperture for maximum sharpness. Open the field diaphragm until the edges reaches the limits of the visual field.. The microscope is now set up for optimum brightfield illumination.
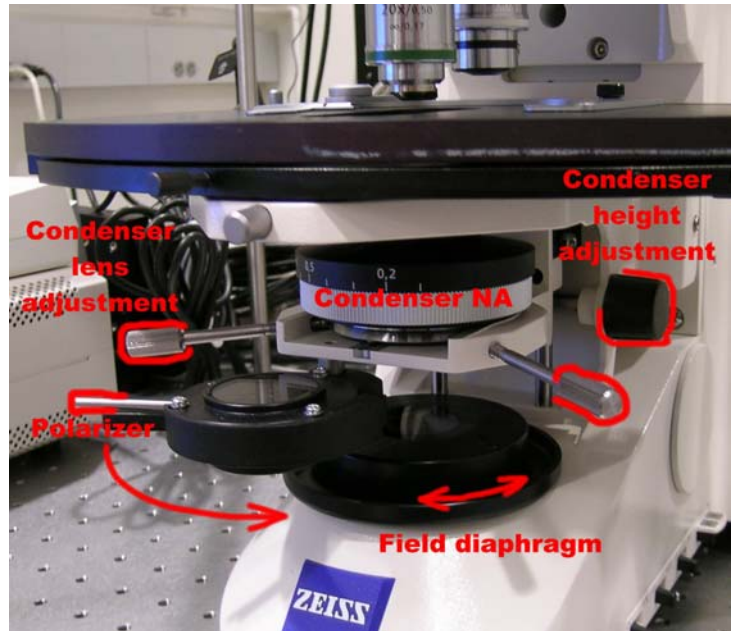
2.5) Differential Interference Contrast

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 upright, it is available with the 40x and 63x water immersion lenses.

Once Kohler illumination is achieved (see section 2.4), rotate the filter wheel to insert the analyzer in the optical path (the analyzer may need to be mounted in the filter wheel, tipically in the DAPI location). Locate and insert the proper Wollaston slider in the objective turret slit after removing the blank slider holder (contact staff to obtain DIC slider and analyzer cube). At the bottom of the condenser carrier, swing the polarizer in place and adjust it at zero degree for maximum extinction. Retardation may be adjusted by turning the adjusting screw on the DIC slider above the objective.
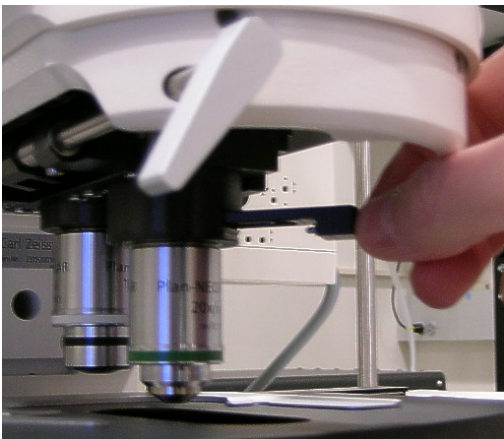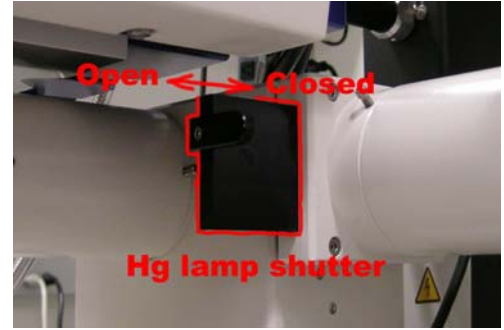
2.6) Widefield fluorescence

Move the LSM / Vis slider on the side of the microscope to Vis. Insert the appropriate reflector by turning the filter wheel. The positions are as follow: 1=empty (Laser scanning, brightfield, DIC), 2=FITC, 3=Rhodamin, 3=DAPI (DIC), 4=IR imaging with NDD. The mercury arc lamp is extremely bright. Attenuation is available through the field and aperture diaphragms near the lamp slider. Do not control the mercury lamp with the lamp slider located on the right side of the microscope, it is to remain open at all the time. Instead, use the lamp
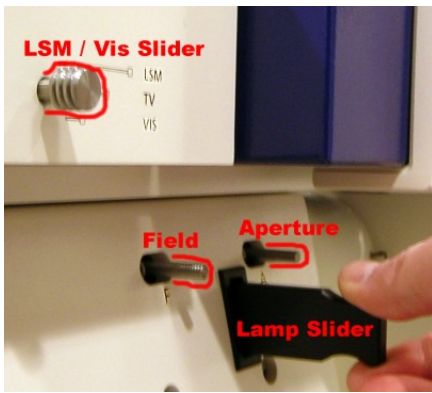
shutter located on the back of the microscope (see picture). The Hg lamp is in close proximity to highly sensitive Non-Descanned Detectors (NDDs). These detectors are controlled via the LSM / Vis slider. Moving the slider to the LSM position without closing the lamp shutter may expose the NDDs to enormous amounts of light coming from the Hg lamp, possibly damaging them. Never open the lamp shutter unless the LSM/Vis slider is on Vis!
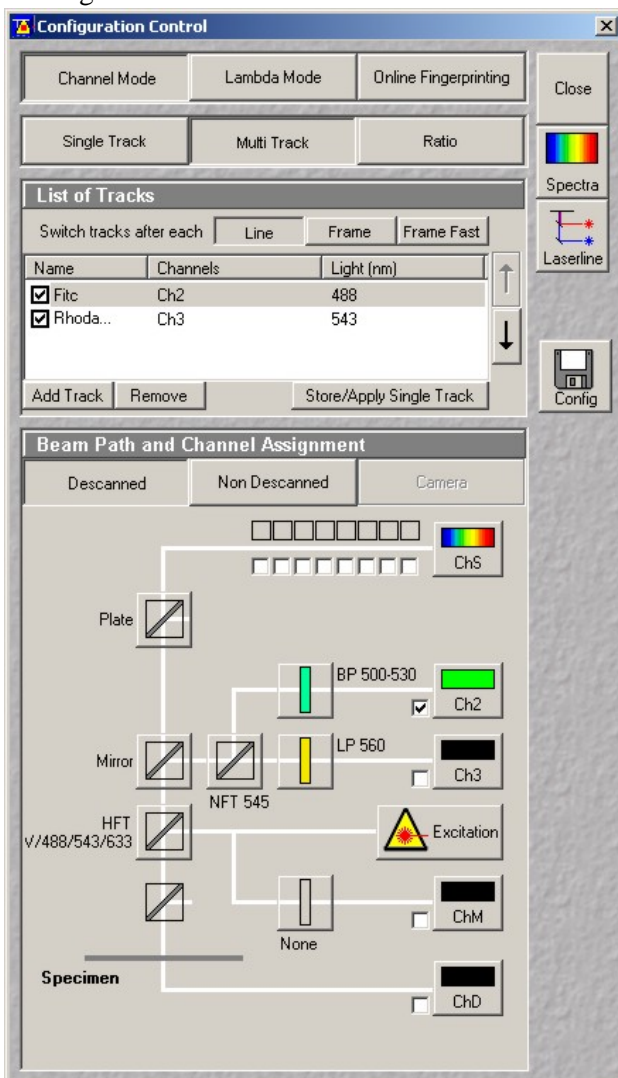
3) Acquiring images

3.1 Overview of the optics

Click the "Config" button on the sub 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 are currently available: an argon ion laser (458, 477, 488 and 514 nm), a Chameleon IR laser (tunable from 720 to 930 nm) and two helium-neon lasers (543 and 633 nm). Note the visible lasers are located on the laser cart and are fiber-coupled to one port of the scanhead. The pulsed IR laser is located on the back of the air table and is directly coupled to the other port of the scanhead.

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 so fast that it may be turned on or off from one pixel to the next. The Chameleon has its own dedicated Acousto-Opto Modulator (AOM).

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 photomultipliers 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 upright specifications for details (http://mic.nichd.nih.gov/zeiss_upright_spec.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.

A Non-Descanned Detector, located on the lamp port in the back of the microscope, is used for low-light imaging for deep-tissue excitation with the Chameleon laser. See section 5.3 for details.
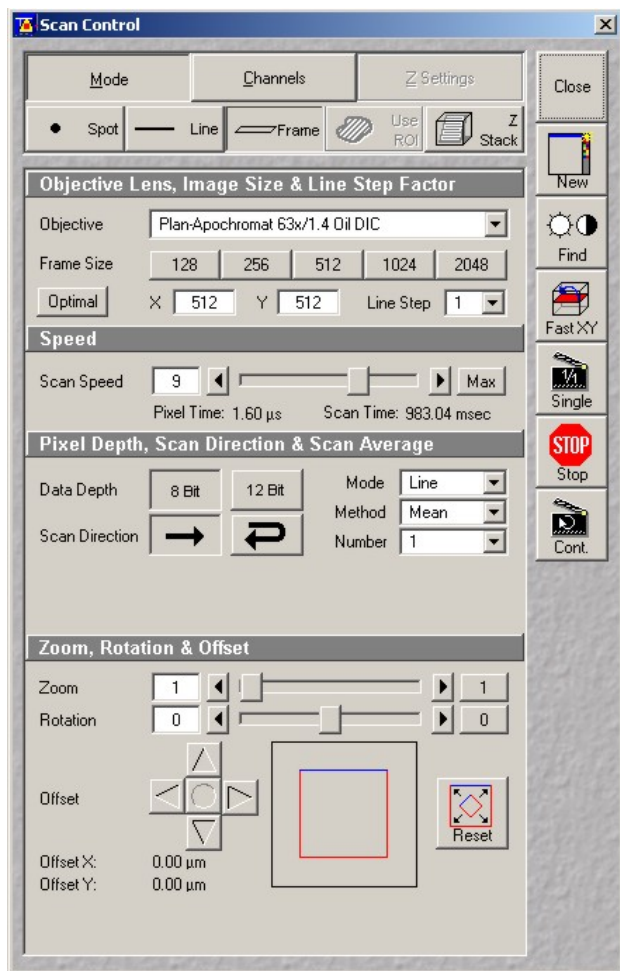
3.2) Configuring the instrument

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. 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 involves 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. However, this process adds considerable amount of time to the acquisition and may only be implemented as frame switching. If used extensively, it also generates 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 for ratiometric imaging experiments such as calcium imaging.

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 M.I.C. 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.
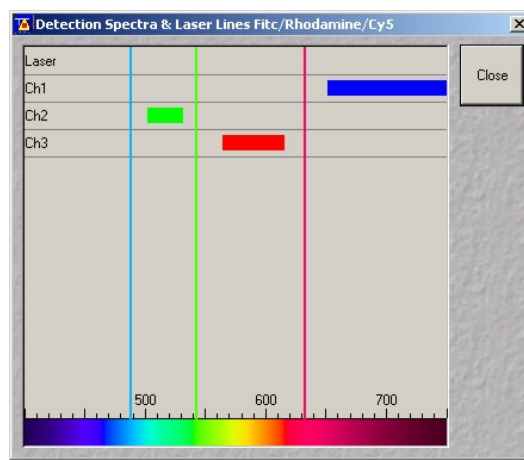
3.3) Acquiring an x-y image

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. For proper calibration and confocality, the objective selection must be set to whatever objective is currently in the working position (objectives must be switched manually and declared in software when exchanged on the nosepiece, see 2.1). 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 expanse of greater bleaching and longer scan times.

We recommend acquiring only 8-bit images (256 levels of gray) in unidirectional mode. Averaging (Mean) may be used to reduce noise if 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 Contol window, click on the Channels tab to view the settings for each detection channel. Each colored box shown 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.
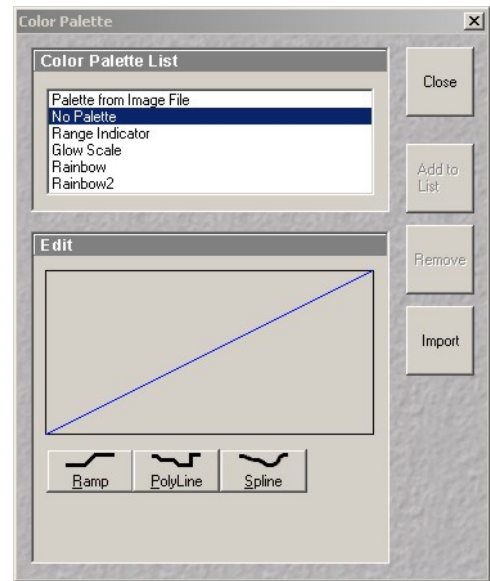
For optimum confocality, the pinhole diameter must be set to 1 Airy Unit. Note that opening slightly the pinhole does not significantly degrade confocality and may greatly improve light collection if the instrument is not perfectly aligned. The thickness of the optical section, calculated from the pinhole 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 gains. Gains in excess of the above values are a sure sign something is wrong with the instrument or with the
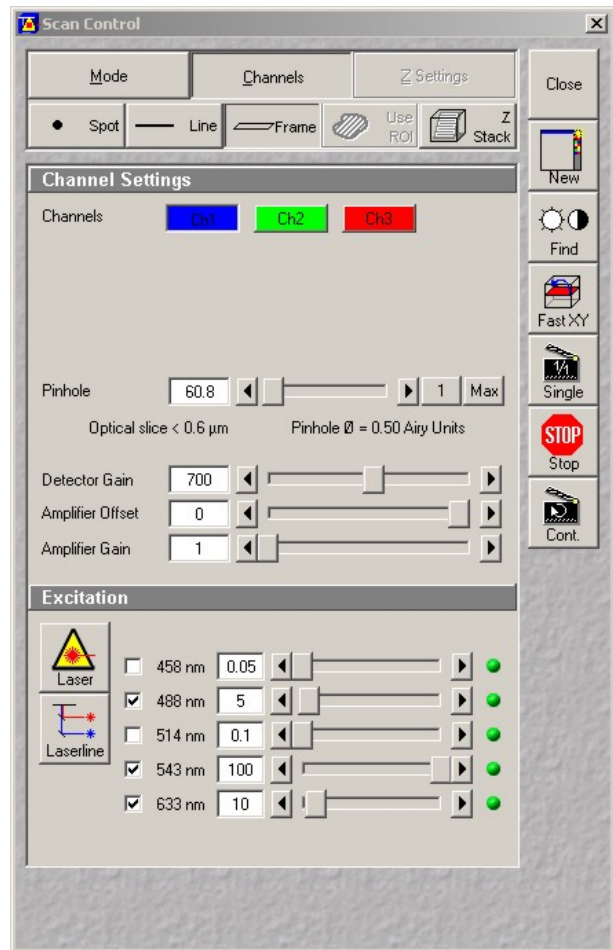
specimen. It should be remembered the mercury arc lamp on the Zeiss LSM 510 is extremely bright, and the intensity perceived during visual inspection is always greater than what may be expected during laser scanning. In other words, if the sample appears dim during visual inspection, there is little chance to acquire a good image.
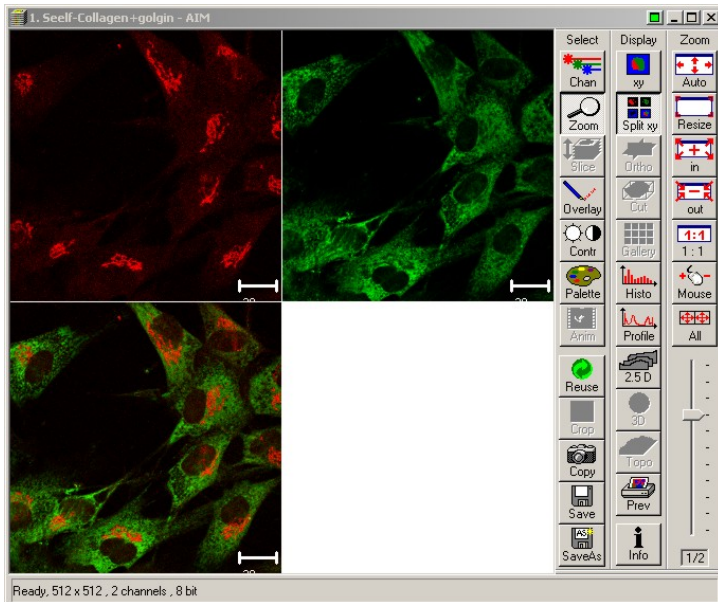
The Amplifier Offset may be used to adjust the black level. When high-quality images are desired, the range of intensity values must span the entire dynamic range available, i.e. from 0 to 255 for an 8-bit image ($2^8 = 256$). To do so, click on the Palette icon in the Image Window toolbar and select the Range Indicator palette. Adjust Detector Gain and Offset so that only a handful of pixels appear blue and red (under and over saturated, respectively). Avoid saturating more than a few pixels if you plan on analyzing 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 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 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 toobar (see 7.1).

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. See 5.3 for the non-descanned detector.
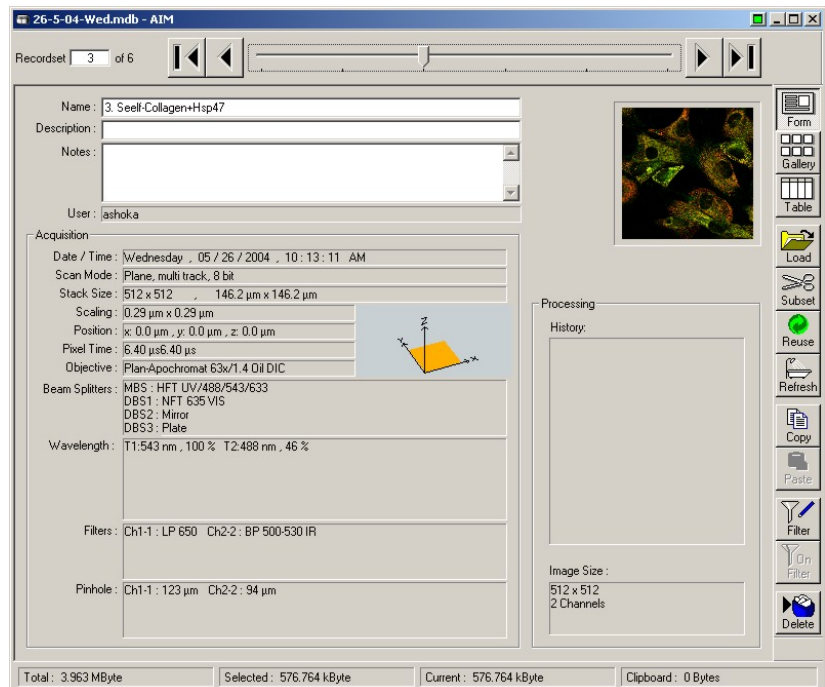
3.4) Acquiring a z-stack

In the Scan Control window, click on Z Stack to turn optical sectioning on. The Z Settings button at the top right 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, 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. For optimum consistency in multicolored stack, the optical section thickness on each channel must be identical. Since it increases at longer excitation wavelength, there is a choice between opening the pinholes on the shorter wavelength channels or closing them at longer wavelengths. This choice is made depending on the amount of light available from each channel and how much of it may be sacrificed by closing the pinhole without degrading image quality.

Once the confocal pinholes are set, click Start in the Scan Control window 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 upright microscope, the 40x and 63x water immersion lenses may be used for DIC. Refer to section 2.5 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 analyzer cube by rotating the filter wheel to "1". Start scanning with Fast XY and increase the gain until an image appears on the TLD pane. If necessary, adjust retardation on the Wollaston slider above the objective. Note that, unlike the LSM 510 inverted, switching from visual to laser-scanning DIC does not require rotation of the polarizer in the condenser. This is due to the different mount of the scanhead on the microscope stand (side port versus top port) and the accompanying change in polarization of the laser.

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.
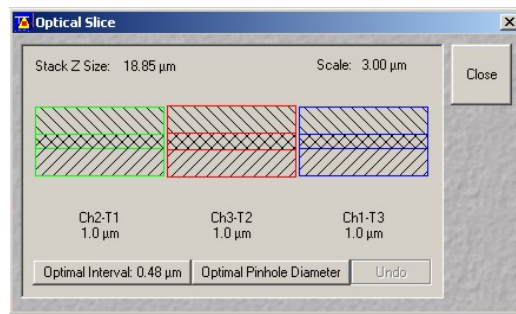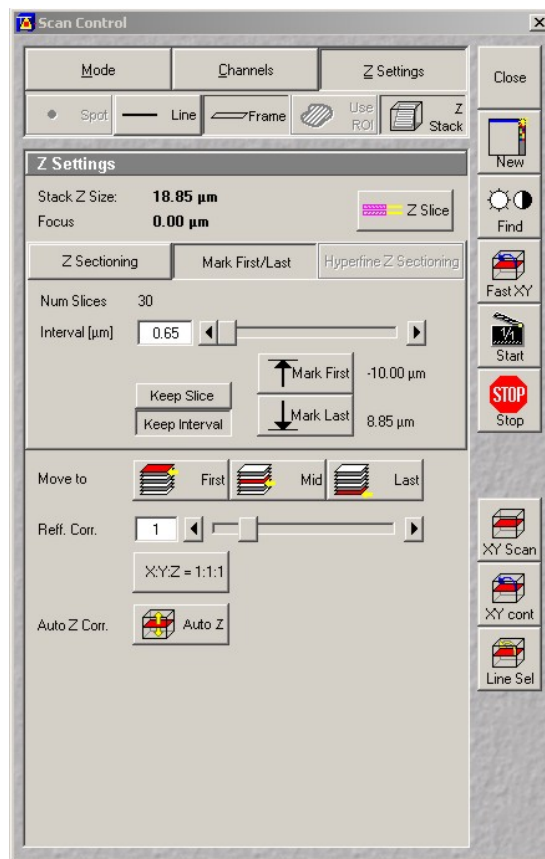
4) Spectral imaging

The Zeiss LSM 510 upright 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 (similar to an emission filter), but the Meta may also be used as a spectrometer to record spatially resolved emission spectra (lambda mode) or to resolve emission of overlapping dyes (emission fingerprinting). The Meta detector is particularly useful with 2-photon excitation. Dual-photon excitation spectra are broader than their single-photon counterpart and, in multi-labeling experiments, often result in simultaneous excitation.

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 may be defined, each grouping any number of contiguous elements. Turn a channel on by clicking on a 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 is twice as noisy as the PMTs due to the many electronics it contains. Sensitivity
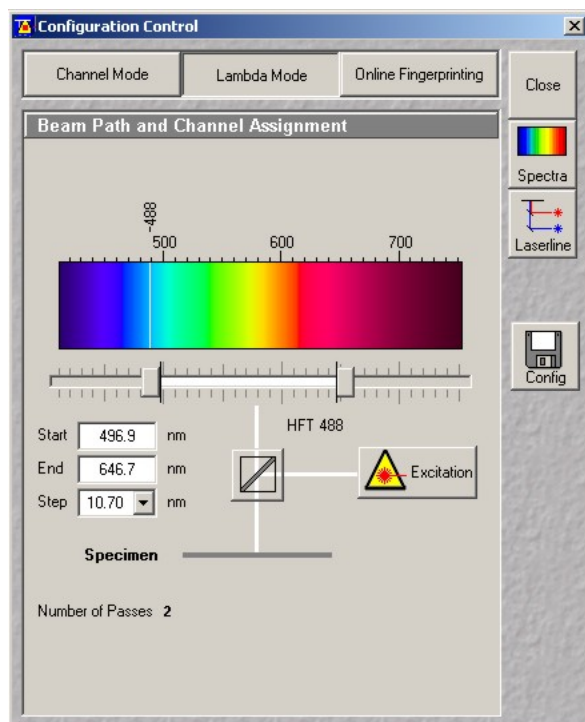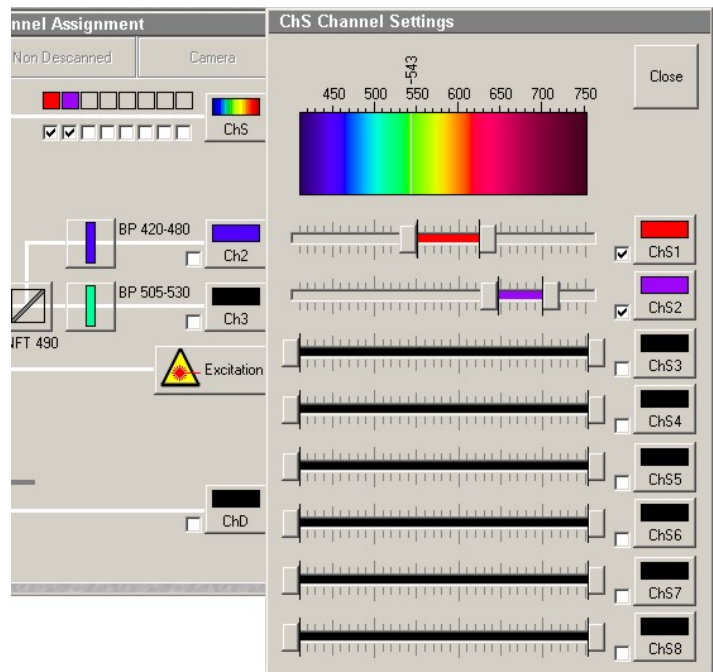
is also an issue when narrow spectral ranges are selected on the Meta channels.

Individual channels defined on the Meta do not have separate gain settings. A workaround is to adjust intensity through the amount of excitation power (laser transmission sliders at the bottom of the Scan Control window) or to trim part of the spectral range. However, when using the Meta for more than one color, the preferred alternative is to use a frame-switched, multi-track configuration. This setting offers independent gain and offset controls on each channel at the expense of additional scanning time. Note that, if the right emission filters are available on channel 2 or 3, we recommend to always choose 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 each detector in its array, that is one image for every 10 nm (10.7 nm to be accurate). To increase sensitivity, 2, 3 or 4 adjacent detectors may be pooled

together 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 the reduced light throughput. Since a
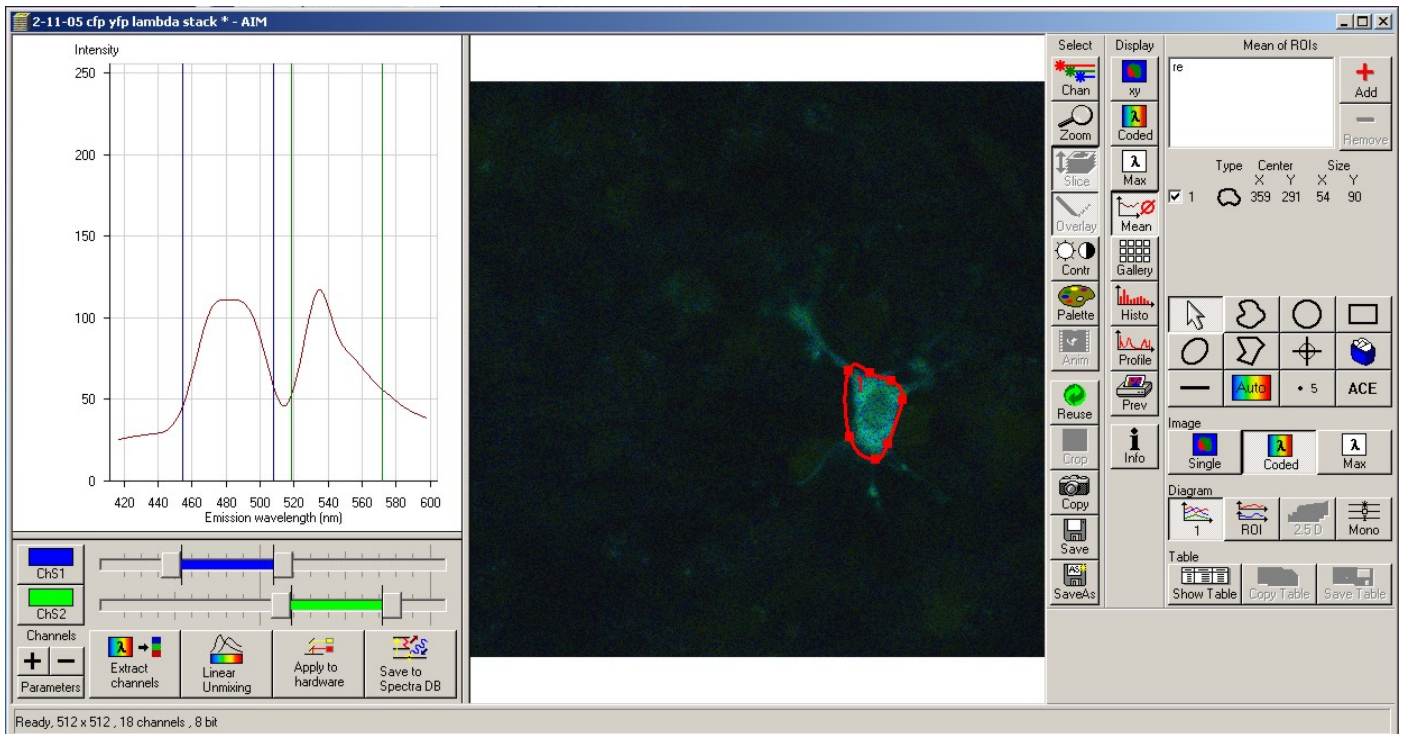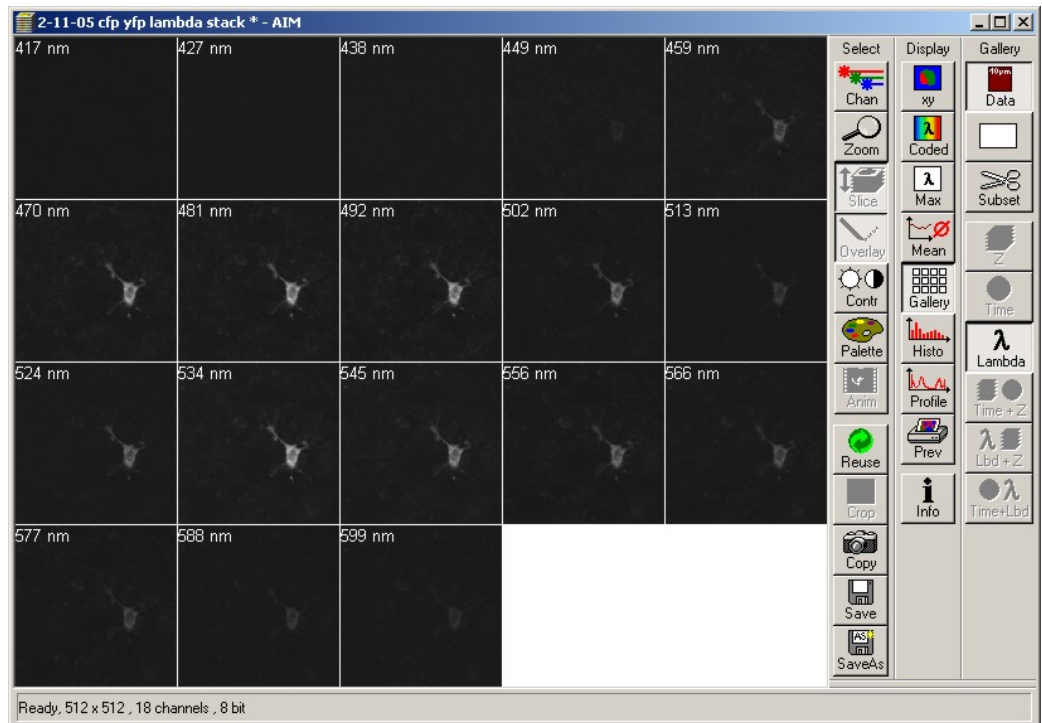
maximum of 8 images are recorded at once, more than a single pass is usually needed to complete the acquisition over large spectral ranges.

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 a new channel, adjust its range with the slider,

then click on Apply to Hardware to automatically configure the Meta detector to acquire in the defined range. Spectra may be saved in a common database for later use (online emission fingerprinting, see section 4.4 below) by clicking on Save to Spectra DB. The Extract Channels function 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 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 Fingerprinting in the Configuration Control window. Up to 8 previously acquired spectra may be called from the database by clicking on each button (RS1 to RS8). Adjust the spectral interval to scan with the slider located below the spectral graph. Note 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!

From this point on, acquire a normal xy image as described in section 3.3. The acquired image is deconvolved 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 highly overlapping dies.
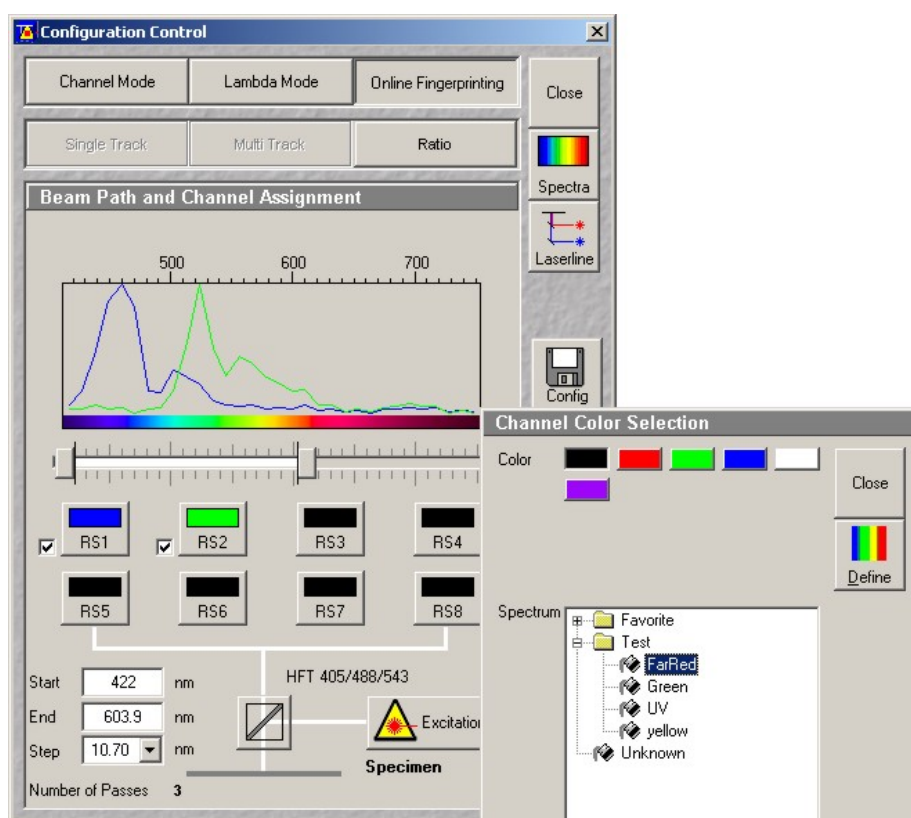
5) Two-Photon excitation

5.1) Principle

Conventional fluorescence involves the electronic transition of a fluorophore molecule to a high-energy state by absorption of a photon (see Jablonski diagram). Return to the ground state is accompanied by emission of a photon. Due to some energy loss during non-radiative transitions in the excited state, fluorescence emission is always at higher wavelength than the excitation (a photon's energy is inversely proportional to its wavelength).

Two-photon excitation involves the consecutive absorption of two photons, each providing half the energy required for the transition. Hence the excitation wavelengths are typically twice as long as their single-photon counterpart. Because of the extremely short life-time of the intermediate state ($\sim 10^{-17}$ sec.), the second photon must arrive shortly after absorption of the first one. In other words, two-photon excitation requires an enormously high photon density. Such intensities could hardly be sustained with continuously emitting lasers. Instead, infra-red sources pulsed around 80 to 100 MHz with a short duty cycle around 100 femtoseconds are used. During the pulse, the instantaneous photon density is high enough to promote dual-photon excitation. Unlike conventional laser, the emission of pulsed IR lasers can be tuned continuously in the 700 to 1000 nm range. Note that excitation with more than 2 photons is possible, which open the way to excite at lower wavelengths. However, the probability of successful excitation decreases with the number of photons required to induce the transition, which must be compensated by an even higher photon density.

The great advantage of infra-red over visible light is its lower absorption and scattering by tissues and biological materials, which greatly increases the depth at which an image may be acquired within a specimen. Moreover, two-photon excitation strongly reduces out-of-plane fluorescence, to the point that confocal emission pinholes are no longer necessary: since the laser intensity drops as the square of the distance from the focal plane, the probability of excitation decrease as the square of this distance in single-photon mode, but as the fourth power in dual-photon mode. Note that two-photon excitation does not improve z resolution, in part because the excitation wavelengths are twice as long as in visible. Increased penetration, lower scattering and reduced out-of-plane fluorescence make dual-photon excitation a method of choice for thick specimens such as tissue sections or live animal imaging.
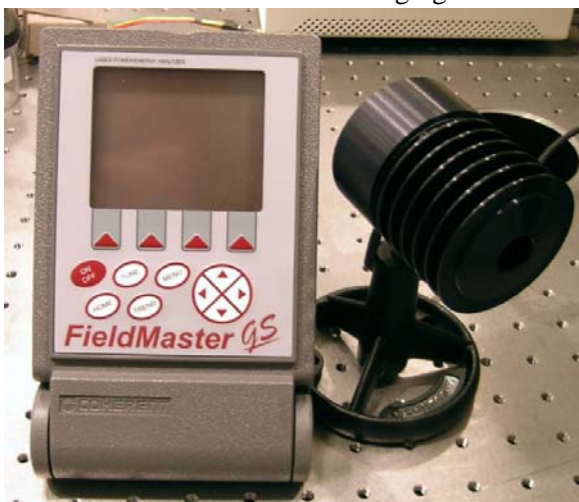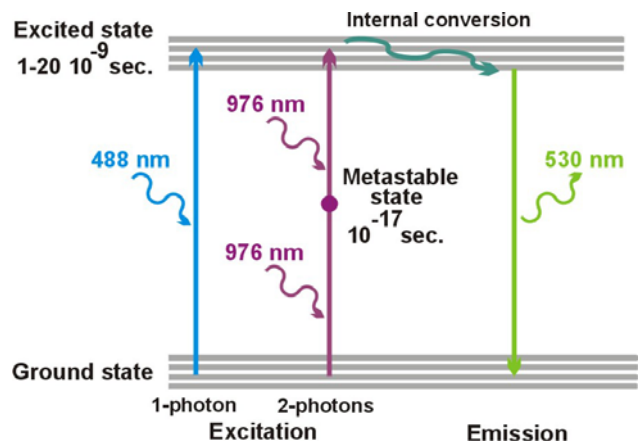
5.2) Chameleon laser

The Zeiss LSM 510 upright uses a multi-stage mode-locked laser, the Chameleon from Coherent. This latest generation of titanium : sapphire (Ti:Sa) pulsed IR laser has the advantage of automatic tuning and low maintenance. The available range is from 720 to 930 nm. The maximum power is around 1.7 Watts, but the output significantly decreases at lower and higher wavelengths. The power may be measured with the included meter and the IR cell. Some elements inside the Chameleon must be at a high temperature to operate. This laser is never switched off but rather put on standby with the key on its power supply. It has a dedicated Acousto-Opto Modulator for fast switching and attenuation.

Because an optical fiber would significantly degrade the pulse in width and intensity and affect the excitation performance, the Chameleon is directly coupled to the microscope's scanhead with mirrors. The alignment must be checked and corrected periodically. Beware the Chameleon is an extremely powerful, hence dangerous laser! For safety reasons, the beam is enclosed inside aluminum guides and the mirrors are protected with removable caps. Users should not attempt to align the laser on their own, the managing staff will perform this task.
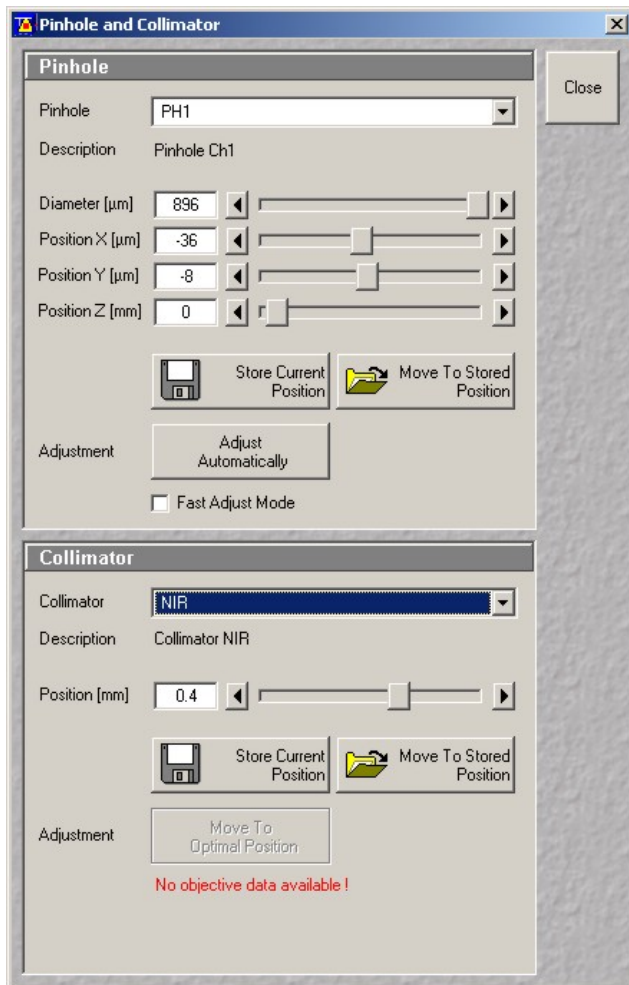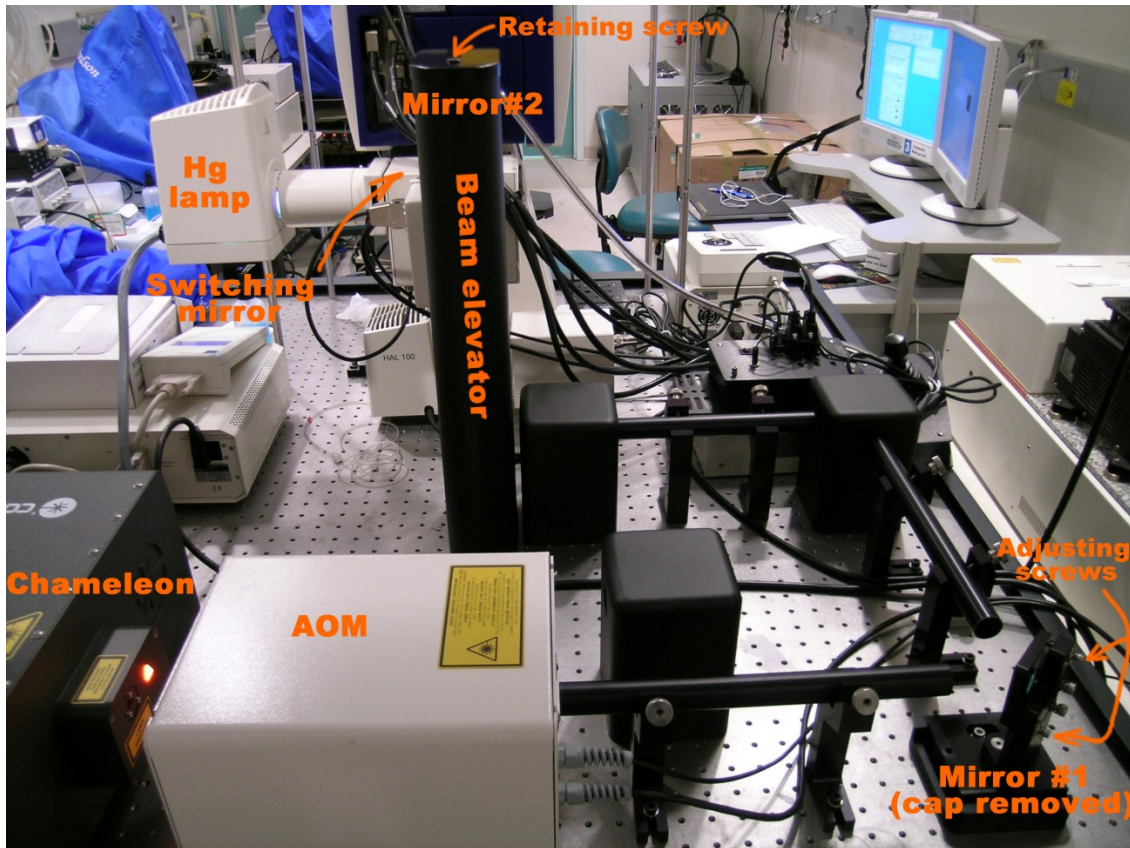
The first mirror after the Chameleon (#1) is adjusted for coarse alignment after pulling its casing straight upward. The mirror on top of the beam elevator (#2) must sometimes be used for fine alignment, in which case the enclosure around the beam elevator must be removed after unscrewing the top. While scanning, adjust the mirrors for maximum intensity in the IR channel. Return all casing and enclosure while scanning to ensure they do not affect optimum alignment. Note the aluminum guide between the scanhead and the beam elevator may sometimes come off and block the beam. In case of severe misalignment, it may be necessary to unscrew the objective, insert the IR power cell on the stage (after unscrewing its radiator fins) and search for the beam by reading on the power meter display.

Once proper alignment is achieved, adjust the IR collimation lens for each objective. This adjustment is again best handled by the staff. Set up a reflection method using the 488 and IR source on a corner of the Zeiss calibration grid. Lower the gains as much as possible, start scanning and adjust the gain on each channel. Click on Maintain on the main menu, then on Pinhole. On the bottom part of the Pinhole and Collimator window, select "NIR". While scanning, adjust the position slider for maximum brightness and sharpness. Pay special

attention to the co-registration of both images: the grid lines must be superimposed as well as possible. The IR collimator may also be adjusted coarsely on any dual-labeled specimen. Unfortunately, only a single position may be stored in software and this setting must be adjusted for each objective.

5.3) Non-Descanned detector

To take full advantage of the high penetration afforded by the infra-red laser, the LSM 510 upright is equipped with a special detector located on the mercury lamp port in the back of the microscope. Because the emitted light does not go through the scanning optics, this Non-Descanned Detector (NDD) is twice as sensitive as the detectors within the scanhead (also called "internals").

To use the NDD, close the mercury lamp shutter and click on Non Descanned in the beam path assignment in the Configuration Control window. Select an excitation line and an adequate emission filter. Filters for DAPI, Fitc and Rhodamin are currently installed. Rotate the microscope filter wheel to position 4, where a special dichroic transmitting IR but reflecting emitted visible light is installed. Because of this filter, the NDD cannot be used with visible excitation (although there is little point doing so) nor in combination with the internal detectors. Make sure the field and aperture diaphragms on the lamp port are fully opened (out).

Note the NDD has no confocal pinhole. In spite of this, the images recorded are fully confocal since two-photon excitation is by nature limited to the focal plane (remember the confocal pinholes on the internal detectors are always open with IR excitation).

Within the next few months, the microscope
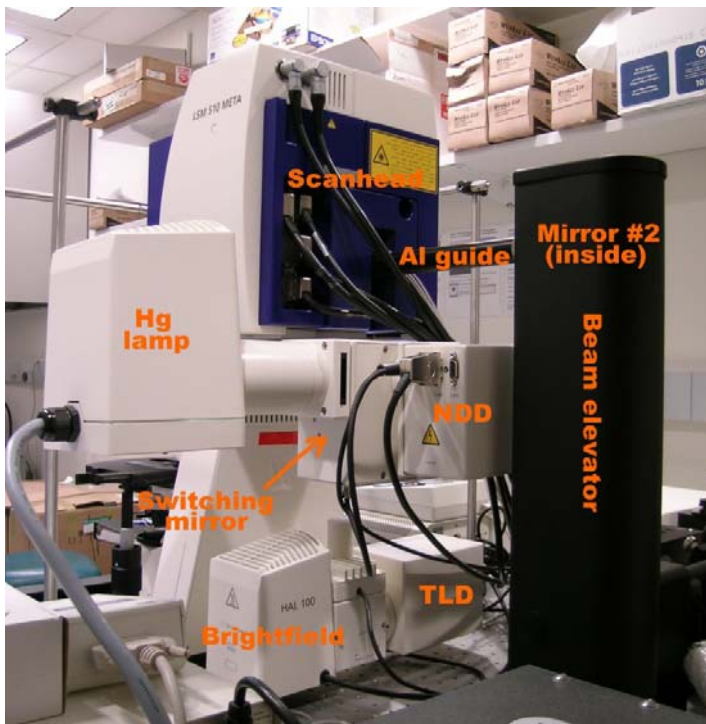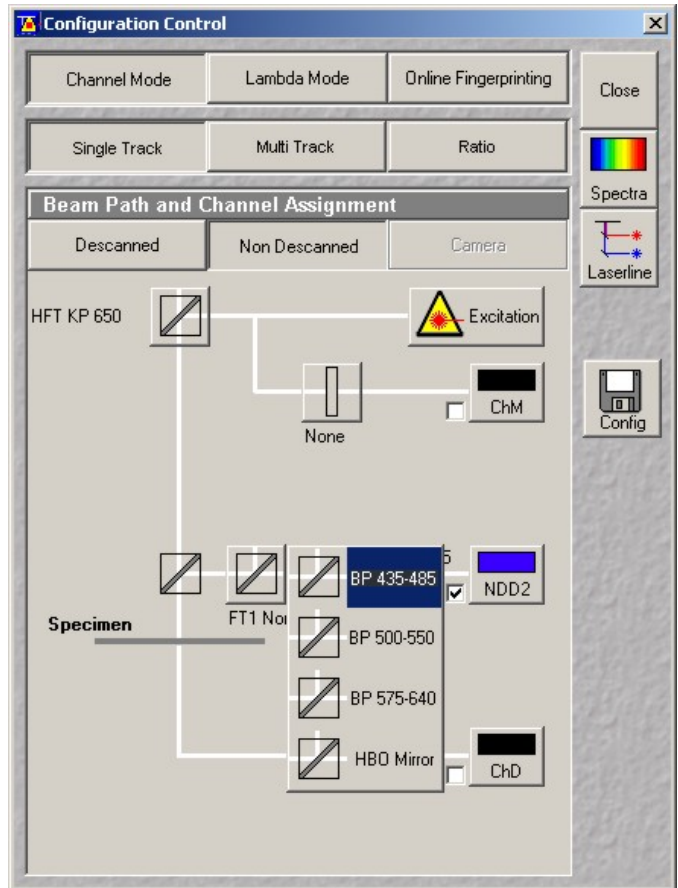
will be upgraded with a second NDD with its own filter set. Several dichroics will be added to break down and steer the fluorescence toward each detector. Since any change in wavelength in the Chameleon takes a significant amount of time, only simultaneous excitation will be available with IR / NDDs.

The high sensitivity of the NDD comes at a price: for optimum sensitivity, it is necessary to acquire images in absolute darkness. Almost any light source present in the room (overhead lights, computer monitors, equipment's LEDs, etc…) produces a noticeable signal and must be switched off or covered, especially when imaging at high gain. A removable black curtain must be installed around the microscope stand for NDD imaging.

5.4) Experimental considerations

The LSM 510 upright was purchased with a set of objectives specifically tailored to multiphoton imaging. These lenses typically have better transmission in the infrared, long working distance and large front lenses to prevent pulse dispersion. Plain objectives may also prove adequate for multiphoton imaging, albeit with a reduced performance.
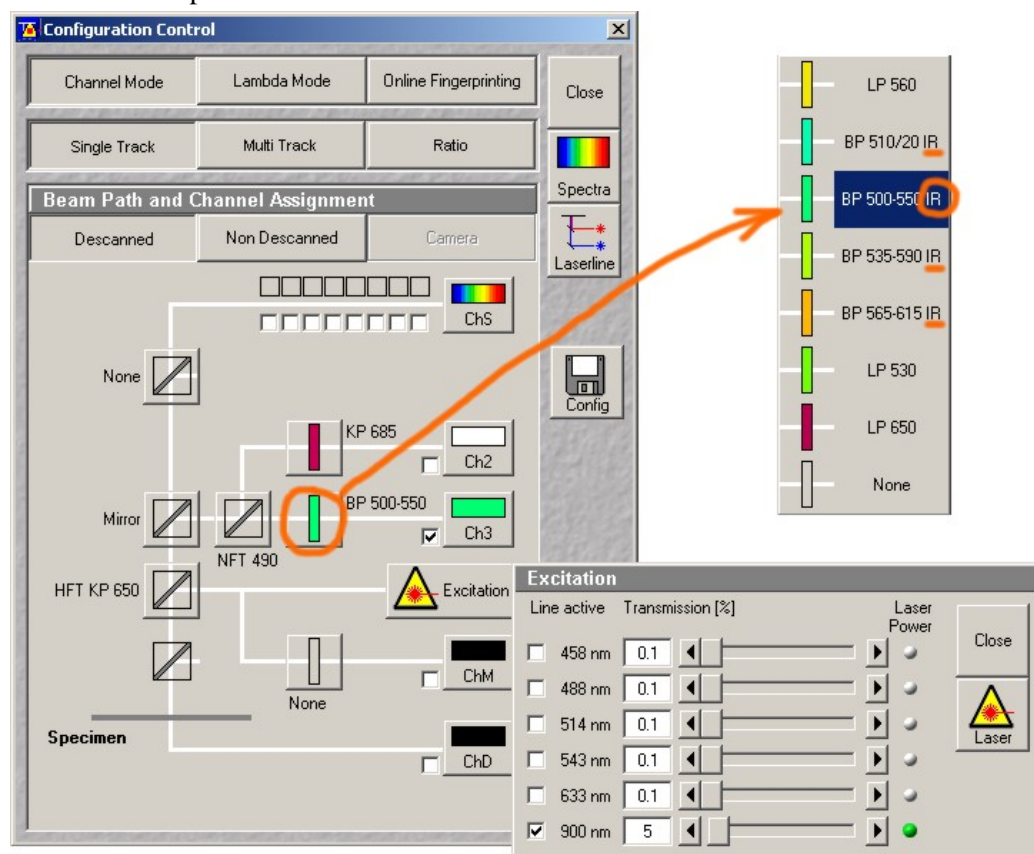
A typical configuration would include a low pass main dichroic such as KP650 (short pass 650 nm) if working in IR alone, or a dual wavelength KP 700/488 or KP700/543 which allow simultaneous excitation in the IR and green or IR and red, respectively. Always remember to select IR-blocking emission filters (BP 500-550 IR for example). For lack of an appropriate filter on the condenser, transmission imaging and DIC are not currently available in IR.

Before planning an experiment, check the multiphoton specifications of your fluorochrome, in particular its excitation spectra or at least its optimum excitation wavelength. Once on the microscope, this value may be established only by painful trials and errors. Most Alexa dyes have good two-photon excitation. DAPI may be excited around 790nm. CFP, GFP and YFP have fairly broad 2-photon absorption profiles and are best excited around 870-900 nm.

The heat generated by the absorption of IR photons by water may be very damaging given the Chameleon high output power. The presence of salt crystals or light-absorbing pigments such as melanin must be avoided at all cost. On a fixed specimen, formation of air bubbles is a sure indication that the sample is being cooked! Typically, less than 5% transmission is required for efficient excitation. Try optimizing the detector before using an excessive amount of power.

Blue/Cyan dyes
Alexa 350 (780 nm-800 nm)
Hoechst (780 nm-800 nm or 900-1100 nm)
DAPI (780-800 nm or 900 nm-1100 nm)
CFP (800 nm-900 nm)

Green dyes
Oregon Green (800 nm-860 nm)
Alexa 488 (800 nm-830 nm)
GFP (840 nm-900 nm)
BODIPY (900 nm-950 nm)
FITC (750 nm-800 nm)
DiO (780 nm-830 nm)

Yellow Dyes
YFP (890 nm-950 nm)

Orange dyes
DiA (800 nm-860 nm)

Red dyes
DiI (830-920 nm)
Rhodamine B (800 nm-860 nm)
Alexa 568 (780 nm-840 nm)

6) Other functions

6.1) 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 in 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 results 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.

6.2) ROI scanning

Since the LSM 510 is equipped with a fast-switching AOTF, it is possible to focus on custom
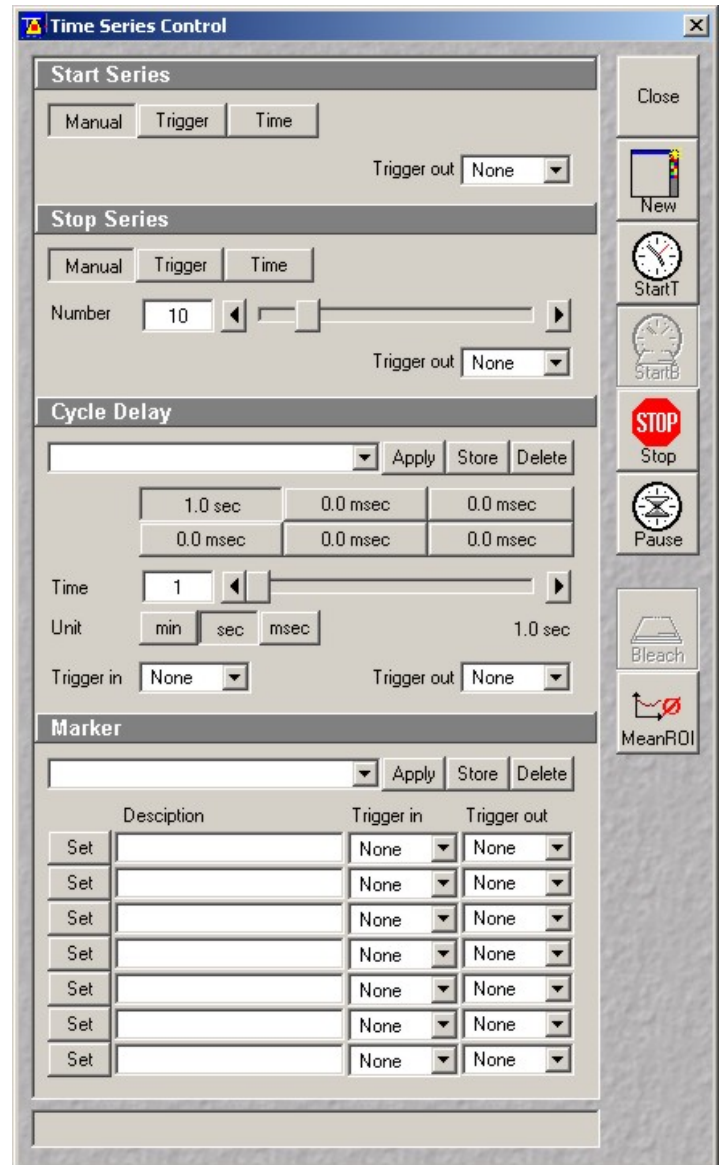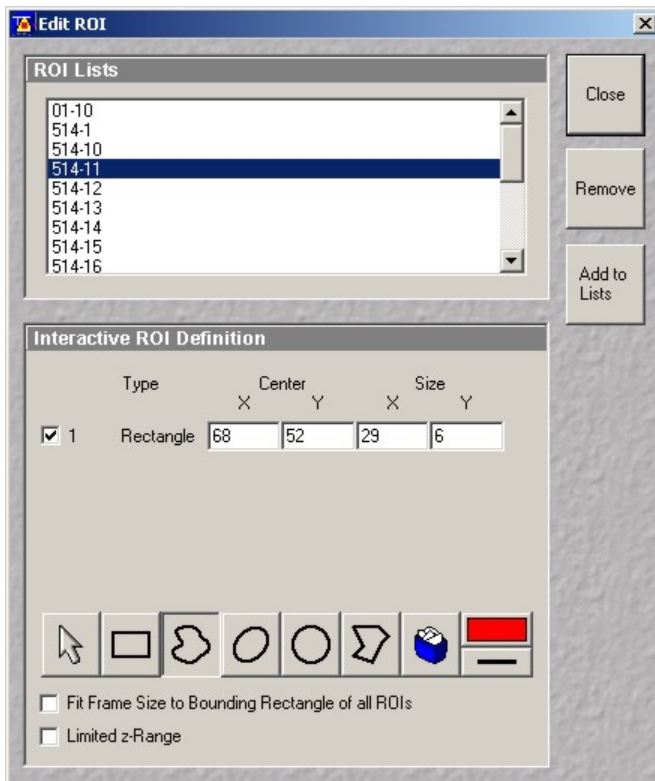
regions within the scanning area. Open the Edit ROI window by clicking on Edit ROI in the Acquire submenu on the main toolbar. Acquire an image by clicking on Single, and select part of the image using a tool from the Edit ROI window toolbar. Click on Use ROI in the scan control window menu. Only the selected ROI will be excited and measured. ROIs 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, rotation, reduced (or custom) resolution and higher scan speeds to decrease the scan time.

6.3) FRAP and Photoactivation

Fluorescence 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 488 and 514 nm lines and the Chameleon laser.

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 (roughly ½ recovery). 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 the delay between each (see 6.1). 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
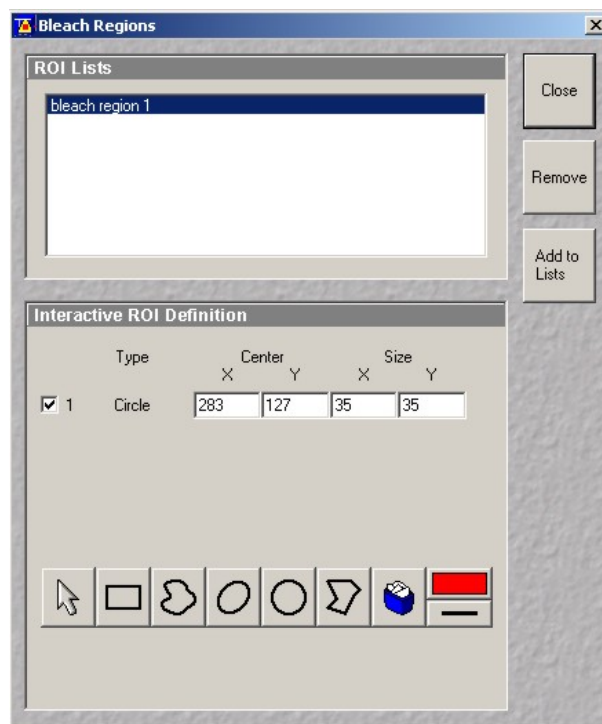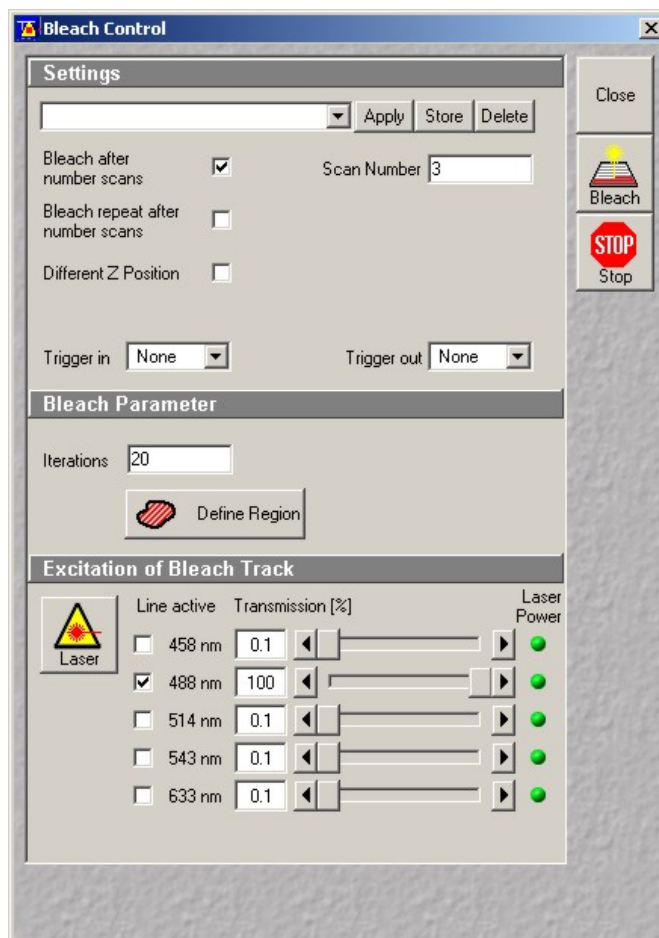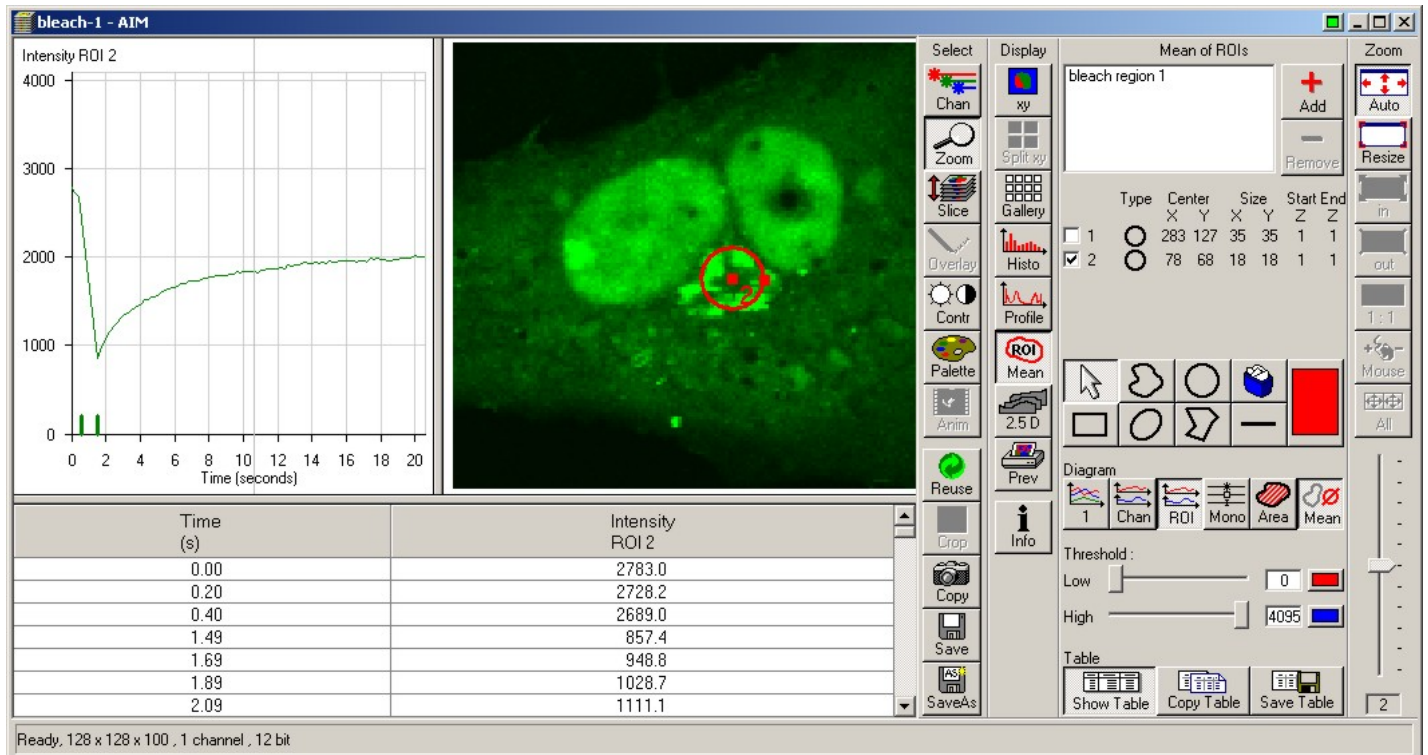
FRAP measurements, contact the M.I.C. staff.

6.4) 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
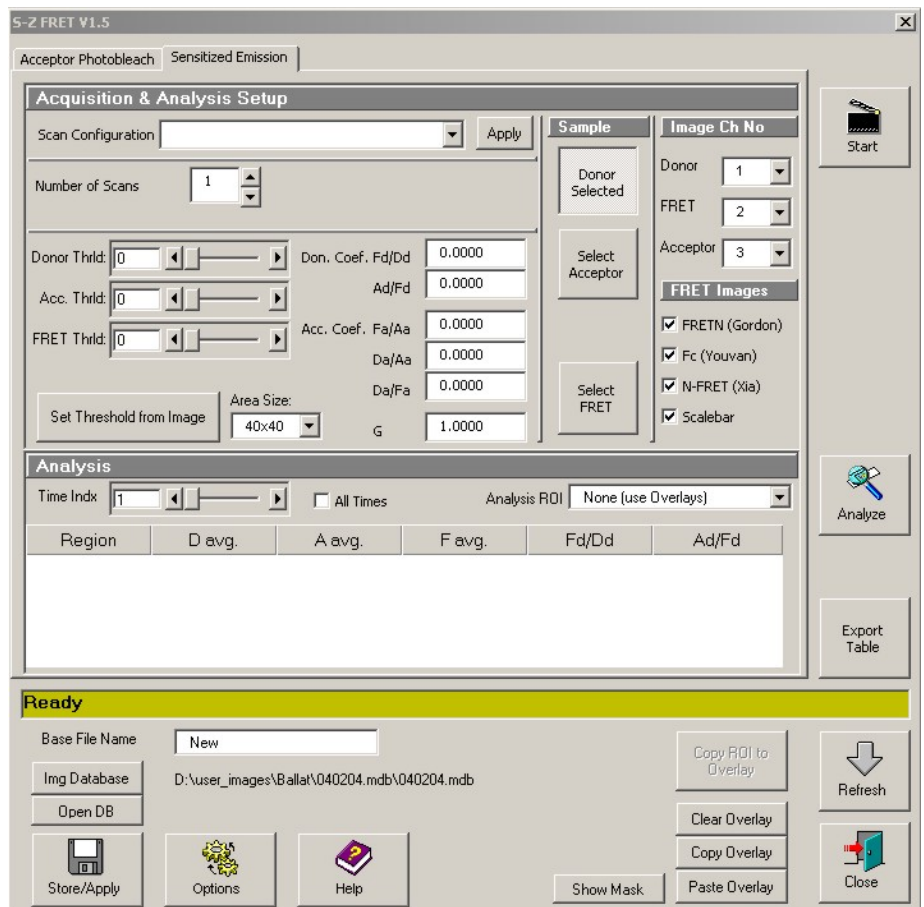
application before running experiments.

Zeiss has recently introduced a macro to perform FRET measurements on its LSM instruments. To correct for cross-excitation, bleed-through and background fluorescence, a FRET sensitized emission 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. Since two-photon excitation spectra are typically larger than their single-photon counterpart, the Chameleon laser does not improve CFP / YFP excitation resoulation. For CFP/YFP FRET, the best option is to use another instrument, the Zeiss LSM 510 inverted. This platform has a 405 nm line which specifically excite CFP but not YFP. Sensitized emission between Green and Red Fluorescent Protein has also been successfully tested with the 488 / 543 nm laser lines. Note that accurate FRET measurements are complex and should not be done without a thorough understanding of the method.

7) Post-acquisition image processing

The LSM 5 software includes an extensive collection of tools for image display and analysis. This sections 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 but has a less polished interface. 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 Improvision's Volocity for 3D reconstruction, real-time visualization and deconvolution. Contact us for details.

7.1) 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
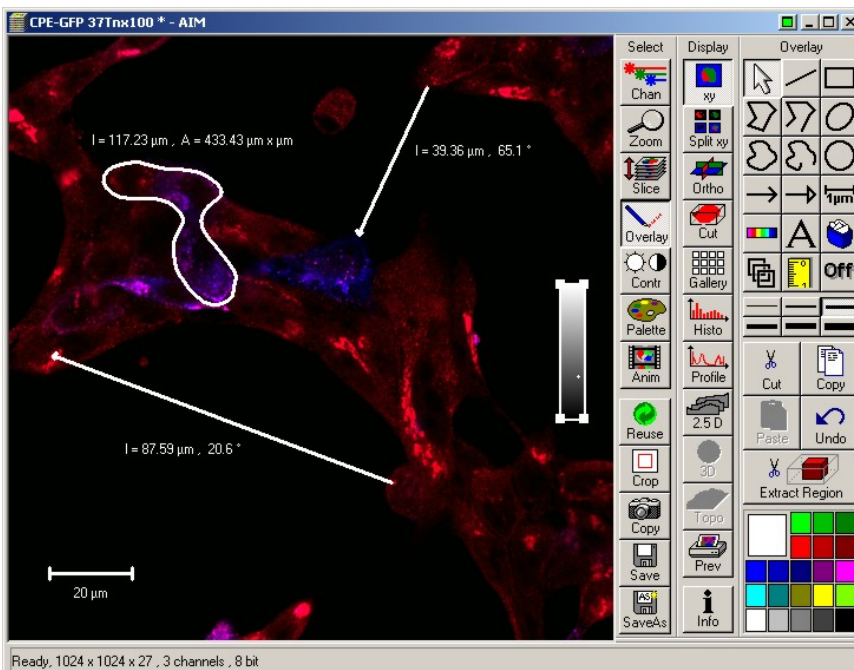
time, Z-position or wavelength (in a time serie, a Z-stack or a lambda stack, respectively), click on Gallery to show the Gallery toolbar, then click on Data.

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.

7.2) 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,
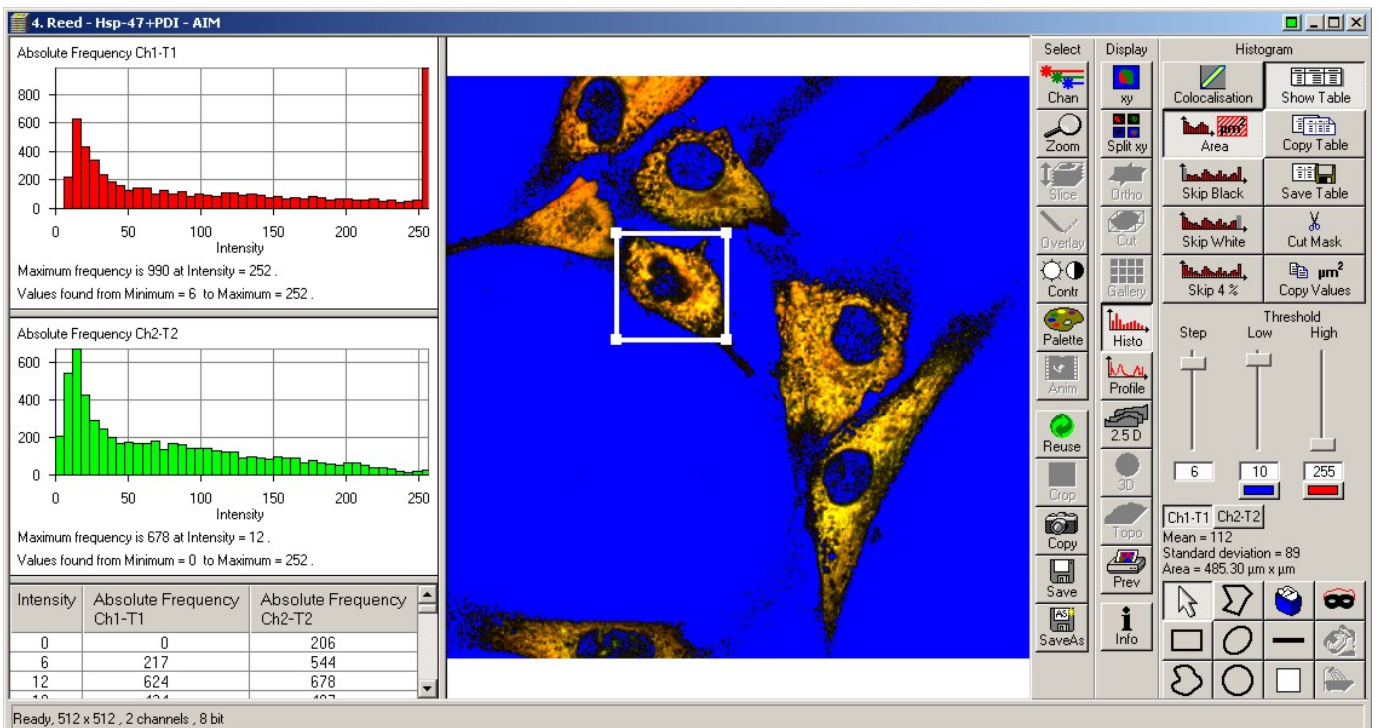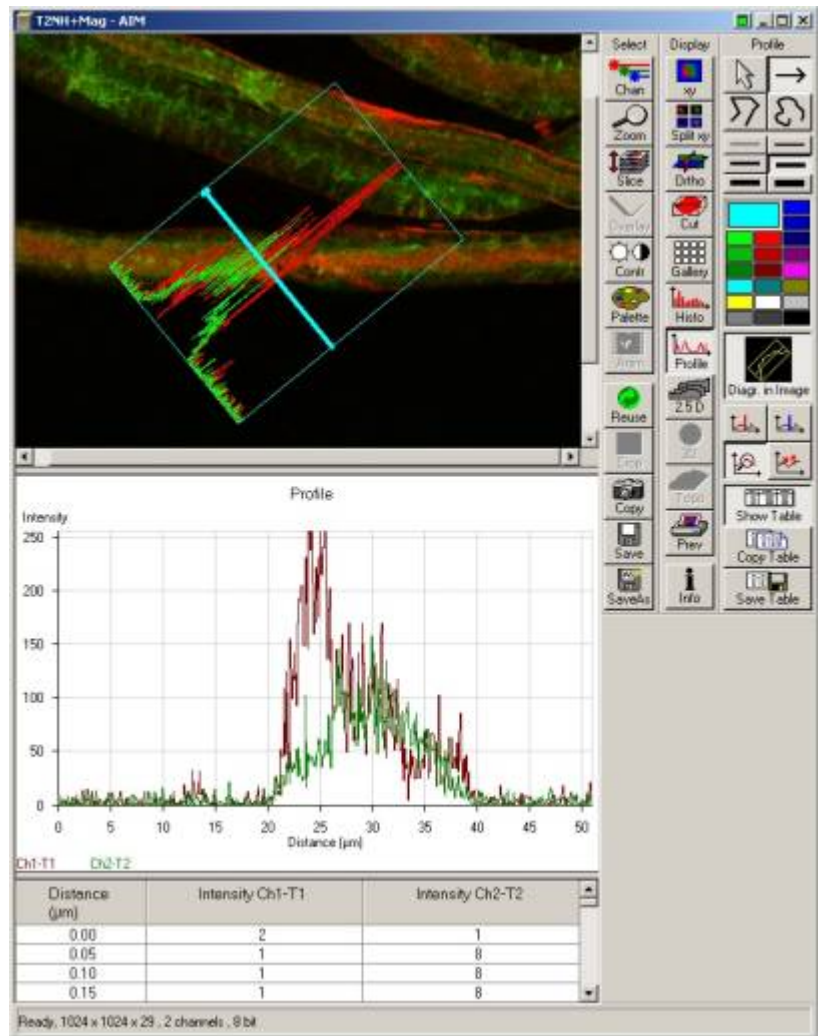
then on Save Data to export the results as a text file.

The Histo icon in the image window toolbar provides many intensity measurement functions. By default, intensity distributions 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 viewed and saved by clicking on Show Table and Save Data. Use the high and low threshold sliders to threshold the image for the
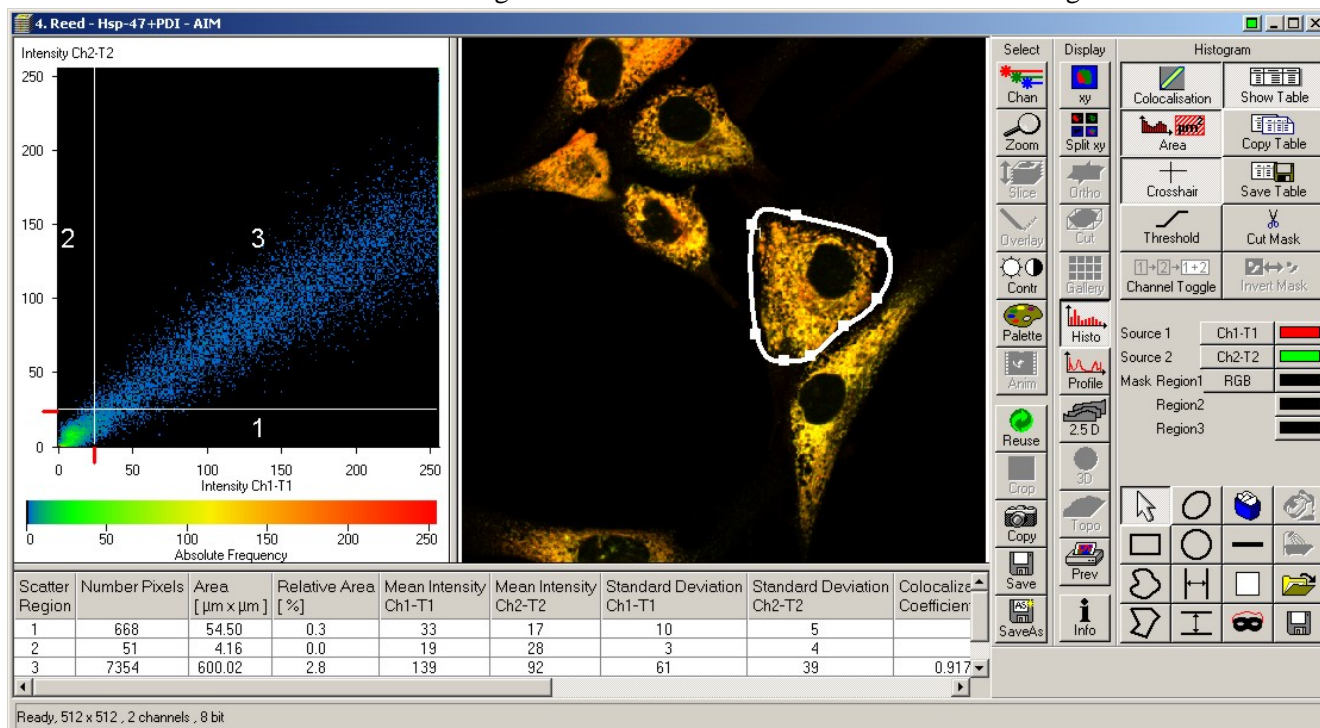
calculations.

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.

7.3) 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
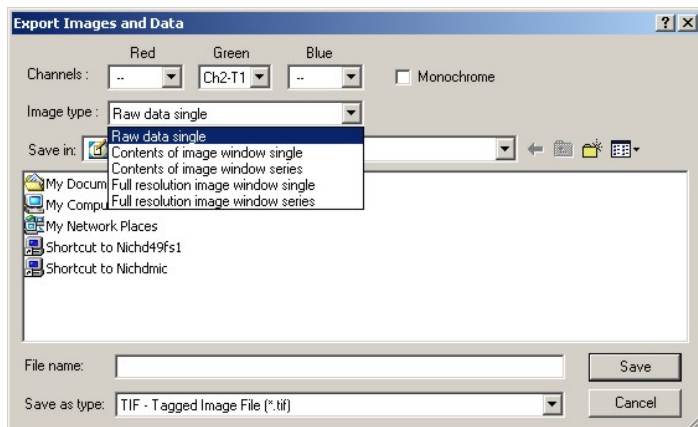
calculation of a single projection (maximum projection) or a series of projections with rotation about any of the X, Y and Z axis. The result may be put in motion through the Anim icon to emulate a 3D rendering. Simply select the axis of rotation, initial angle, number of projections and angle increment 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.
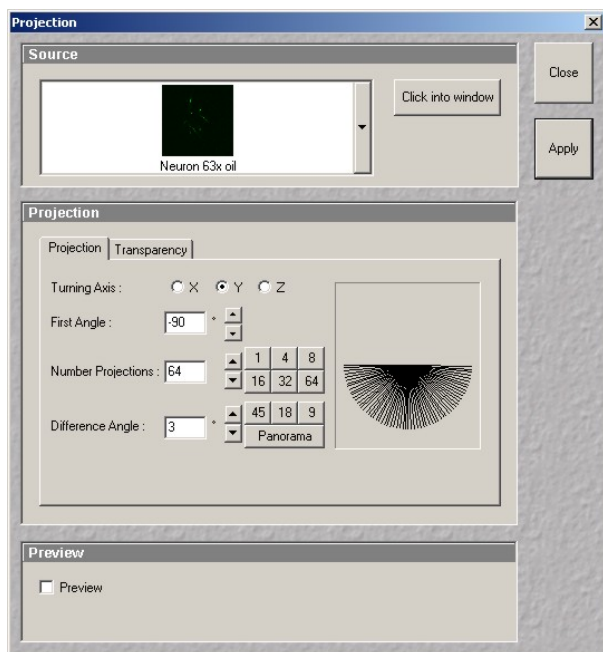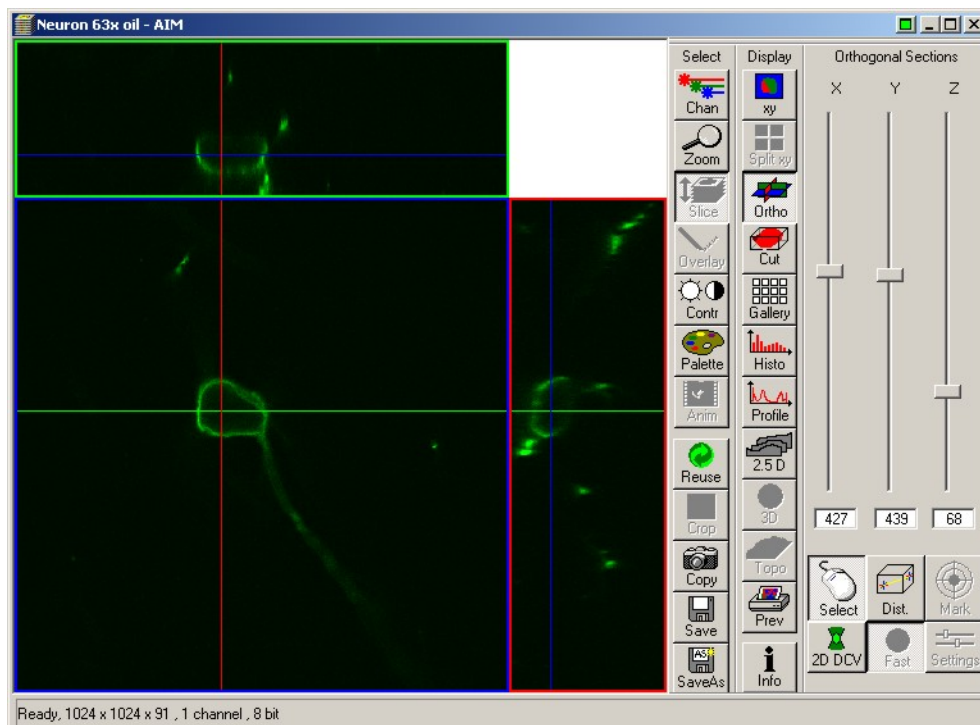
7.4) Exporting images

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 is color. It is crucial to understand the images recorded on the microscope are grayscale, not color. They appear in color only 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.
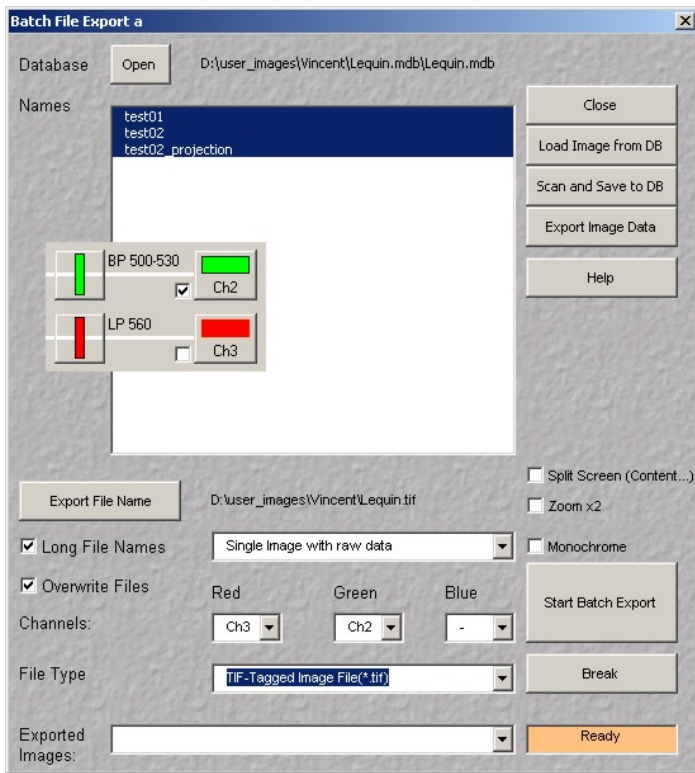
To export a large number of images from a database, we recommend using the Batch Export macro available from the Macro menu on the main menu toolbar. Click on Open to select a database, then fill each layer in the destination 24 bit color image with a channel (refer to the Configuration Control window 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.

7.5) Transferring images

The M.I.C. computers are on the network. If you have an account on "NICHD49FS1" server, double-click this icon on the desktop and login as "NIH\username" and your NICHD 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) where 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 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.

8) Turn off procedure

8.1) 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!

8.2) Clean all oil immersion objectives using lens cleaning paper only! Check dry objectives for oil smudges and clean them if required. Clean water immersion lenses by soaking the front lens onto distilled water for 5 minutes and wiping them with lens paper.

8.3) Move the nosepiece to its upper position with the high speed key and up arrow on the focus keypad.
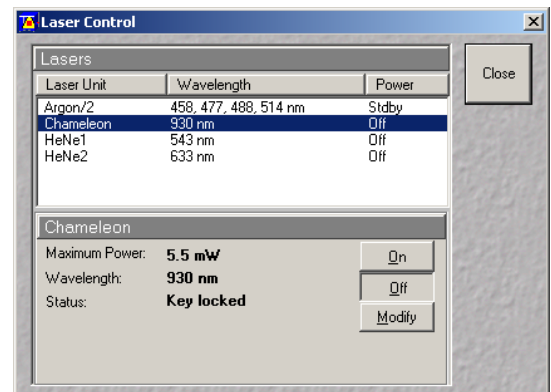
8.4) Quit the LSM software, log off Windows and turn both flat panels off.

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

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

8.7) Put the Chameleon laser on Standby using the key on its power supply.

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

9) 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). Is the LSM / Vis slider on Vis? When using a water lens, check for air bubbles trapped below the front lens.
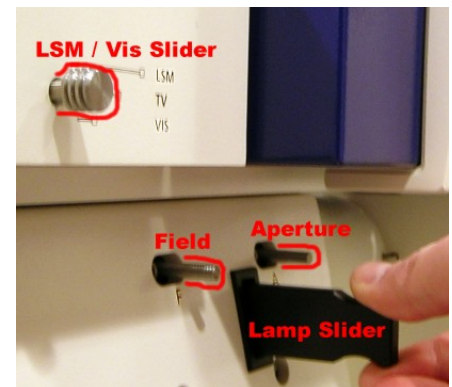
No image on scanning
Is there light escaping from the objective? If not, is the filter wheel on "1"? Is the LSM/Vis slider on LSM?
Is the laser turned on? Open the laser control panel.
When using a water lens, check for air bubbles trapped below the front lens.

No image on IR excitation
Is there light coming off the objective? Tune the Chameleon to near IR (720 nm), which is more visible than far IR. The beam path alignment or collimation may need to be adjusted.
Is the filter wheel on "1"?

No image / noisy image on NDD
NDDs may only be used with IR excitation. Is the filter wheel on position"5"? Are the field and aperture diaphragms on the lamp port open? Is the Hg lamp shutter closed? Turn off every light, dim monitors as much as possible.
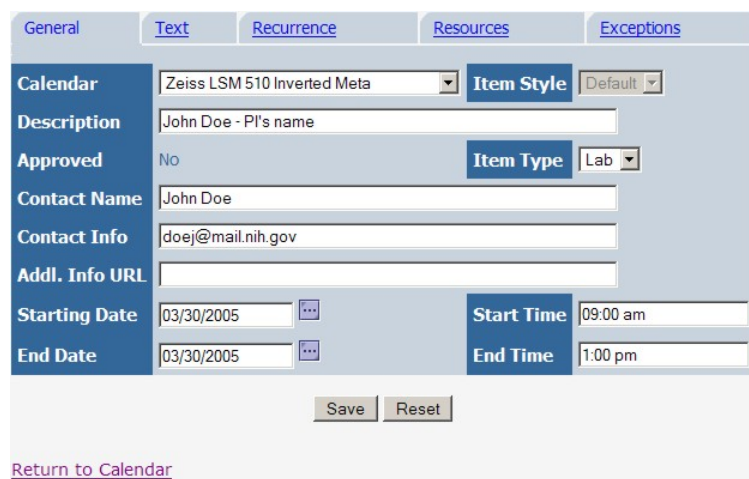
Calendar reservation

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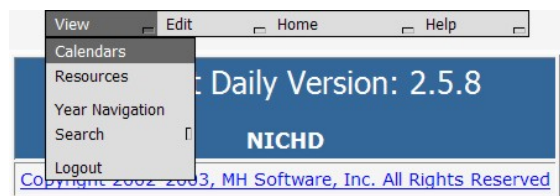
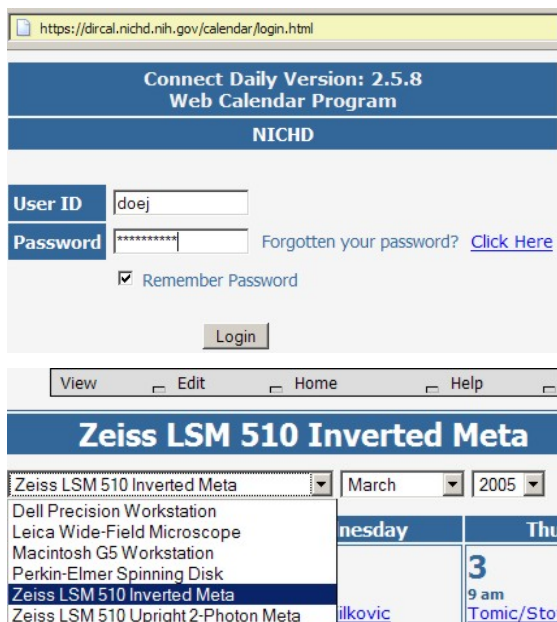3) Select a microscope from the drop-down menu.

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

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

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