

ZEISS LIVE DUOSCAN OPERATING PROTOCOL

Foreword

The Zeiss Live DuoScan system was purchased by the Institute during the Fall of 2006 and installed in May 2007. This instrument incorporates the latest advance in high-speed, high sensitivity confocal imaging. The main difference between the new Live scanhead and a regular LSM 510 point scanner is the adoption of a line scanning mechanism. Instead of scanning the specimen with a diffraction-limited spot, the Live uses a horizontal line. The detection of an entire line of pixels is made by a linear CCD of 512 elements. The longer pixel dwell time in a line versus a point scanner is the reason why the Live is more sensitive (and/or faster) than conventional point scanners at similar laser exposure. However, this increased sensitivity comes at a cost of some limitations: 1) Since the instrument is specifically designed to prevent bleaching, FRAP or photoactivation of custom regions requires a separate point scanner (DuoScan unit). 2) A line scanner has only limited option for zoom and rotation of the image field. The table below shows the main differences between the Live scanhead and conventional LSM 510 point scanners:

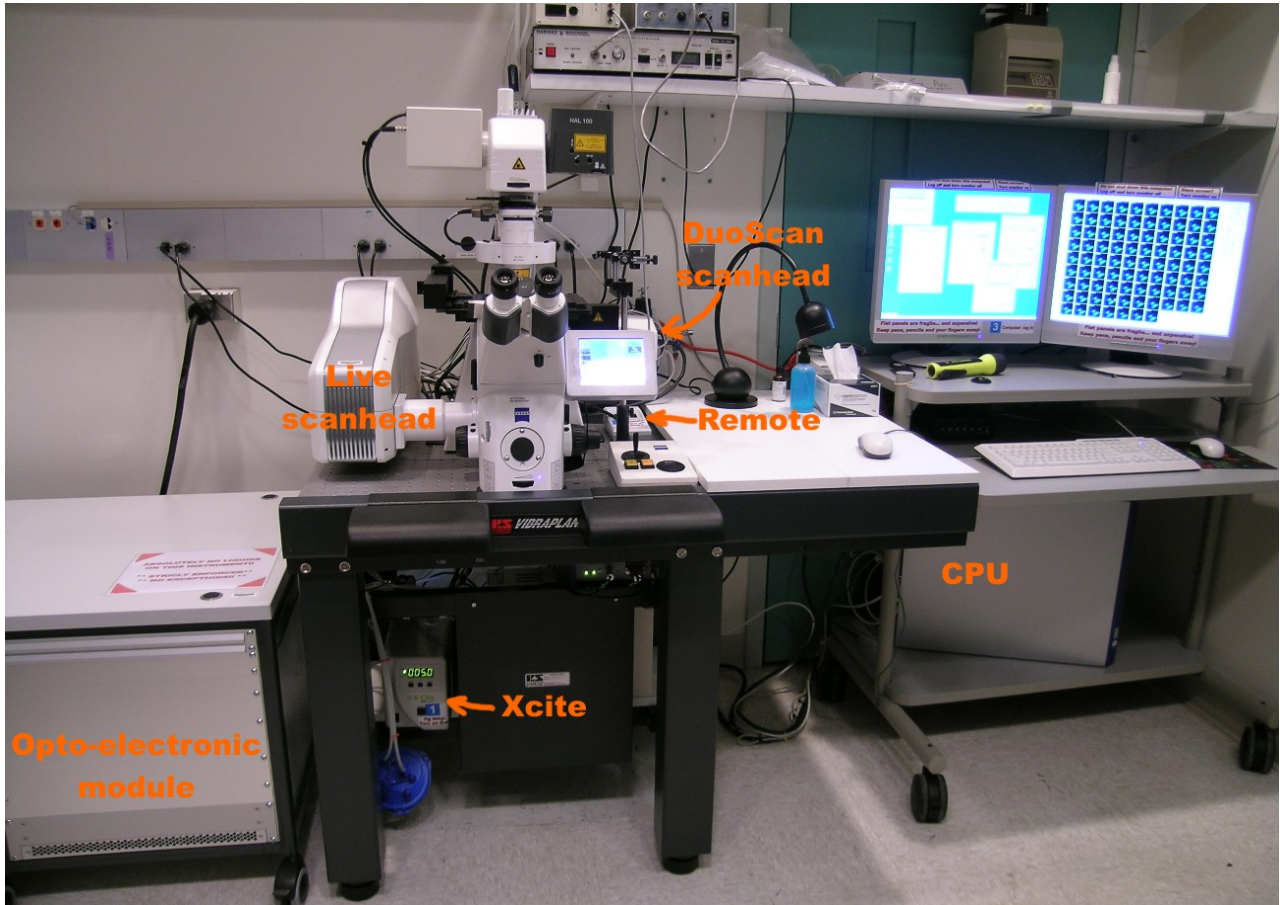
	LSM 510	Live DuoScan
Principle	Point scanner	Line Scanner
Best for	High resolution	High speed/sensitivity
Main dichroic	Dichroic mirrors	Achrogate
Detector	PMTs	Linear CCDs
Confocal aperture	Pinhole	Slit
Focus	Microscope	Microscope + Piezo
Frame rotation	Yes	No
Excitation power vs. zoom	Increases	No change
Signal vs. zoom	No change	Decreases
Bleaching vs. zoom	Increases	No change
Photobleaching with:	Imaging scanhead	DuoScan unit

The Live DuoScan is equipped with solid-state lasers (405, 489, 561 and 647 nm), a fast-switching Acousto Opto Tunable Filter (AOTF), the new Axio Observer Z1 microscope and other improvement in optics. Although the instrument has only two detectors, dual-bandpass filters allow for up to 4-channel detection. High-speed volume acquisition is possible with a piezo objective stage. FRAP, FRET, and photo-activation of fluorescent proteins are fully supported. Temperature control, heated perfusion and solution change are available, and the microscope will be equipped with an environmental chamber in the next few months.

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. The Live DuoScan platform is significantly more complex to use than a regular LSM 510 and requires a period of training even for users already proficient with LSM 510 instruments. 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 Live DuoScan. For a more in-depth coverage, refer to the Zeiss documentation available from our web site (http://mic.nichd.nih.gov/lsm_doc/pdf/Operation_In_Expert_Mode.pdf). **UPDATE**

Overview



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

- Computer monitors, CPU and keyboard on the computer cart, remote (master switch) on the air table.
- Opto-electronic module on the left of the air table.
- Scanheads / microscope / stage controller on the optical table, Xcite light source below the table.
- Perfusion / temperature control hardware controller on the shelf above the air table.



1) Turn on procedure

1.1) Turn on the Xcite light source by pressing the on/off switch on the controller.

1.2) Turn the system on (lasers, electronic interface box, scanhead and microscope) by pressing both switches on the remote located on the air table.

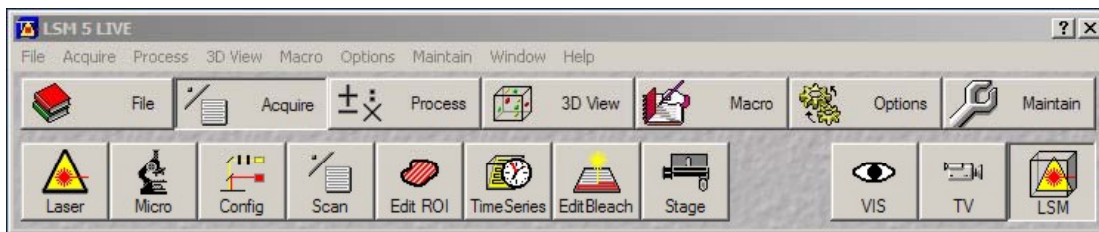
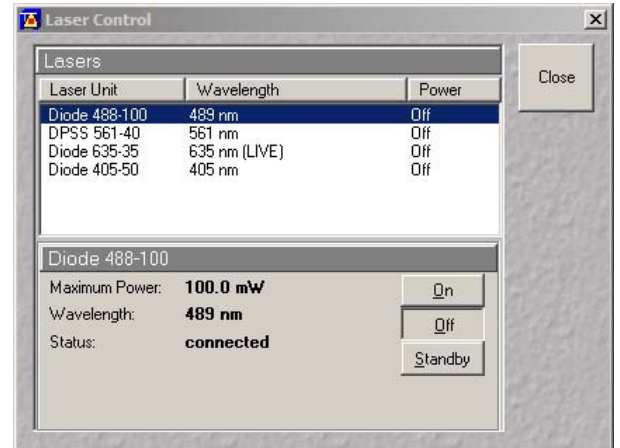
1.3) Turn on the CPU and monitors. Aug 07: due to driver issues, it is not currently possible to let the computer run



when the microscope is shut down. If the computer is already running, it may become very unresponsive and / or crash when the instrument is turned on. Log in using the password and username provided by the staff.



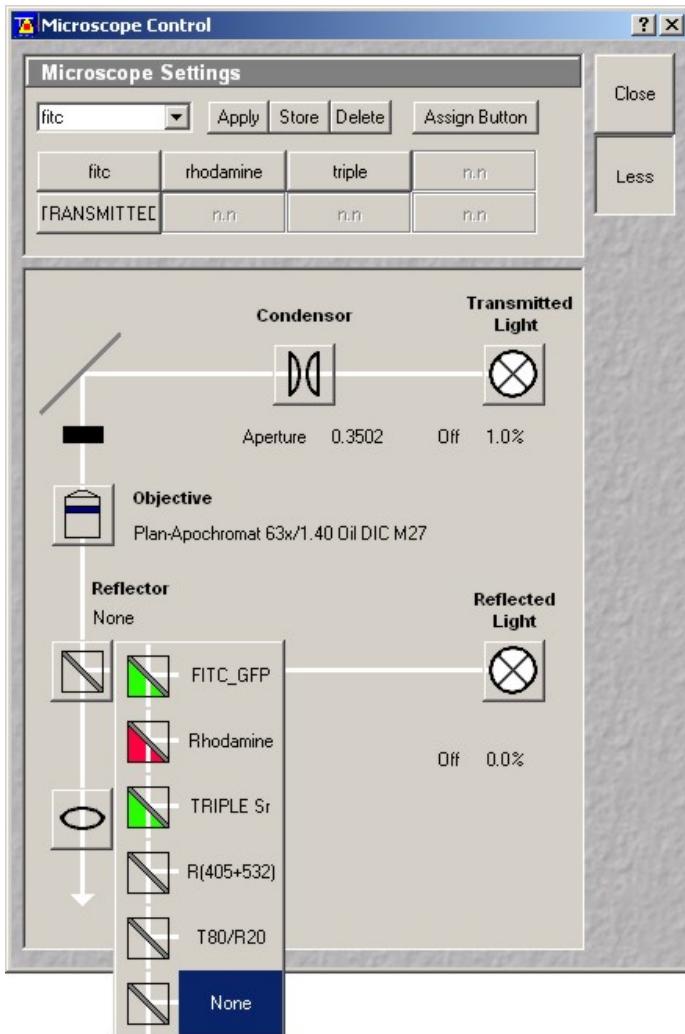
1.4) Double-click on the Live DuoScan icon on the desktop. On the LSM switchboard, select Scan New Images and Start.



1.5) The main menu defaults to the Acquire subsection. Click on Lasers on the lower menu bar. Turn on only the lasers required by your experiment. Most lasers have a standby mode, put them on standby when not used for more than 30 minutes.

2) Sample visual inspection

The Zeiss Observer Z1 is a fully motorized microscope controlled either via a touch-screen or with buttons on the stand. The functionality of the buttons has been changed compared to the Axiovert 200M, which render their use impractical. The best option to control the microscope is the Microscope control panel on the LSM software (click on Micro under Acquire). Alternatively, the touch-screen on the microscope can be used to access some functions. As a safety measure, all light entering the eyepieces is blocked by an internal shutter. Always remember to click on Vis in the Acquire menu when switching to visible mode.



2.1) Stage

The Live DuoScan is equipped with a motorized x-y stage controlled via a remote. Use

the joystick for coarse adjustment, then click either the X or Y button and use the wheel for fine motion. The stage may also be controlled in software by clicking on Stage under acquire. Multiple-location acquisition is supported using the MultiTime macro. Refer to the Zeiss documentation for proper instruction on the MultiTime macro functionalities.

2.2) Focus controller

The Axio Observer has a conventional fine and coarse focus knob on each side to control the focus drive. One position in the nosepiece is also equipped with a piezo focus drive controlled in software and used for high-speed volume collection.

2.3) Sound practices in focus adjustment

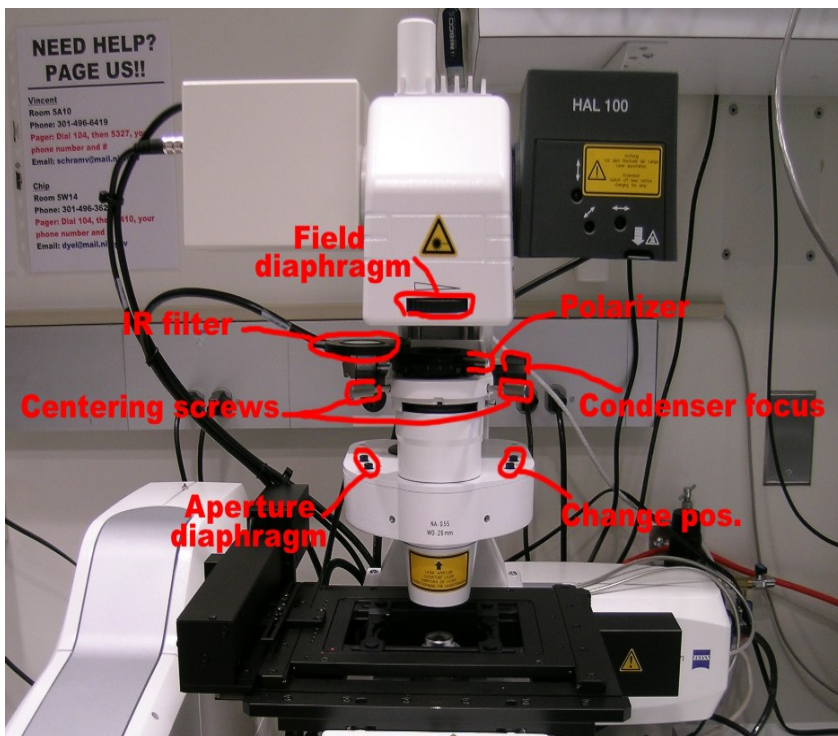
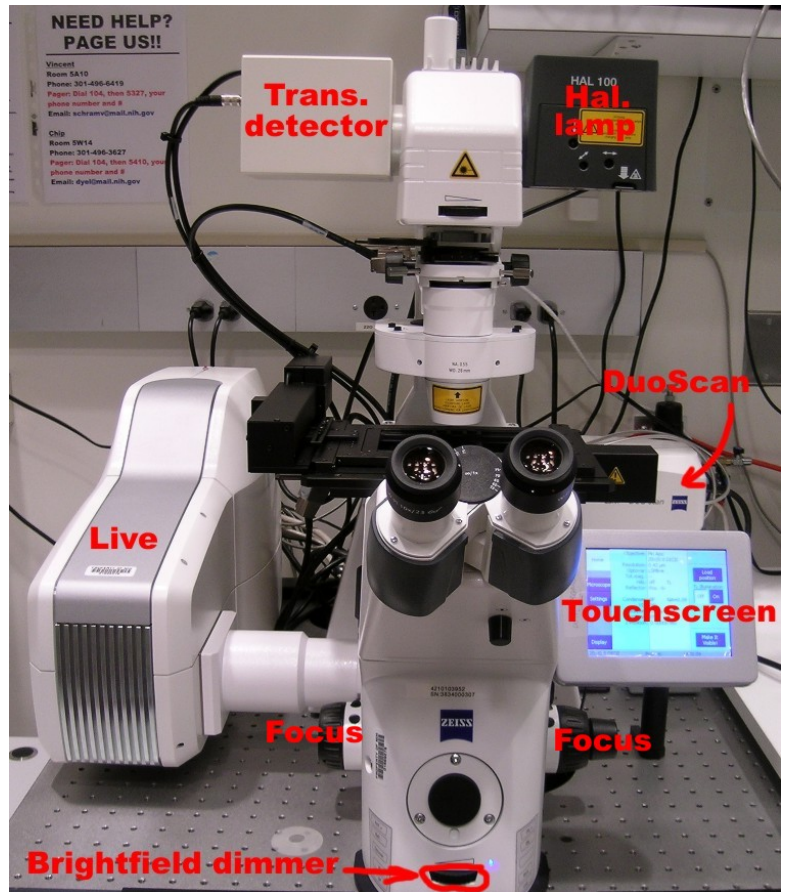
Finding the focal plane is sometimes difficult, especially with a high-magnification objective on a dim sample. To avoid driving the objective through the specimen, move the objective as close as possible to the

coverslip using the coarse focus knob (check the size of the oil meniscus when using an oil objective, you may need to use a flashlight). Then, slowly move the objective away from the specimen with the fine focus knob while looking in the eyepieces. Slowly moving the stage back and forth until some intensity fluctuation is perceived helps zeroing on the specimen. Remember to move the nosepiece to its lowest position when installing or removing stage inserts to avoid damaging the objective!

2.4) Brightfield

Switching between laser scanning and visual observation is entirely automated. Click on the Vis icon on the menu below Acquire, then Micro to the right. Select Transmitted and adjust the intensity of the halogen lamp by using either the dimmer at the front bottom of the microscope or the slider after clicking on the Brightfield icon in the microscope control panel window.

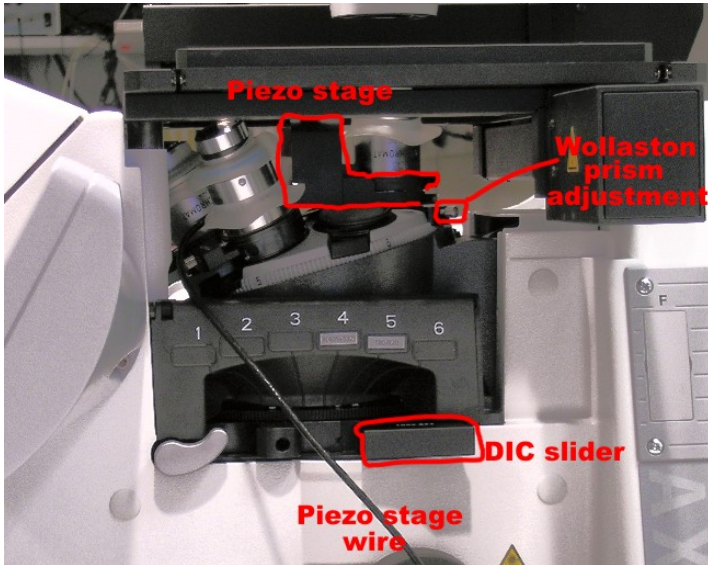
Set the microscope up for Kohler illumination as follows: Open the field diaphragm fully by turning the wheel on top of the condenser. Focus on the specimen as described above. Fully close the field diaphragm and adjust the condenser focus until the edges of the diaphragm are in sharp focus. Center the field diaphragm with the centering screws. You may need to adjust the condenser's 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 (Kohler) illumination.



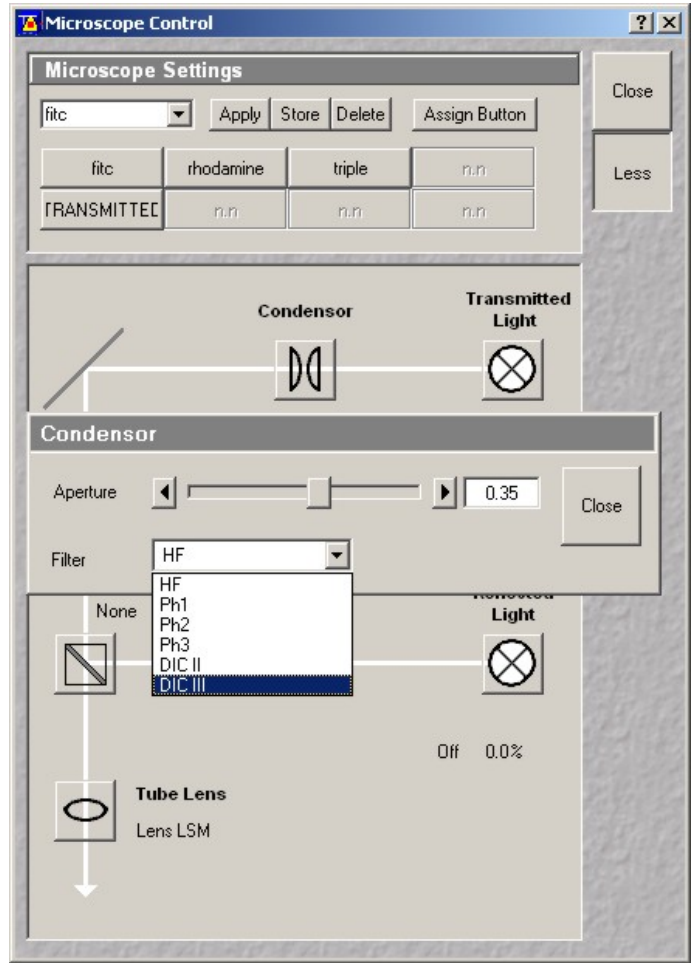
2.5) Differential Interference Contrast

Differential Interference Contrast (DIC) is the method of choice for high-quality transmission imaging. DIC requires special objectives matched with specific Wollaston slider. All objectives on the Live DuoScan (10 and 20x air, 40 and 63x oil) are DIC-grade lenses outfitted with Wollaston prisms.

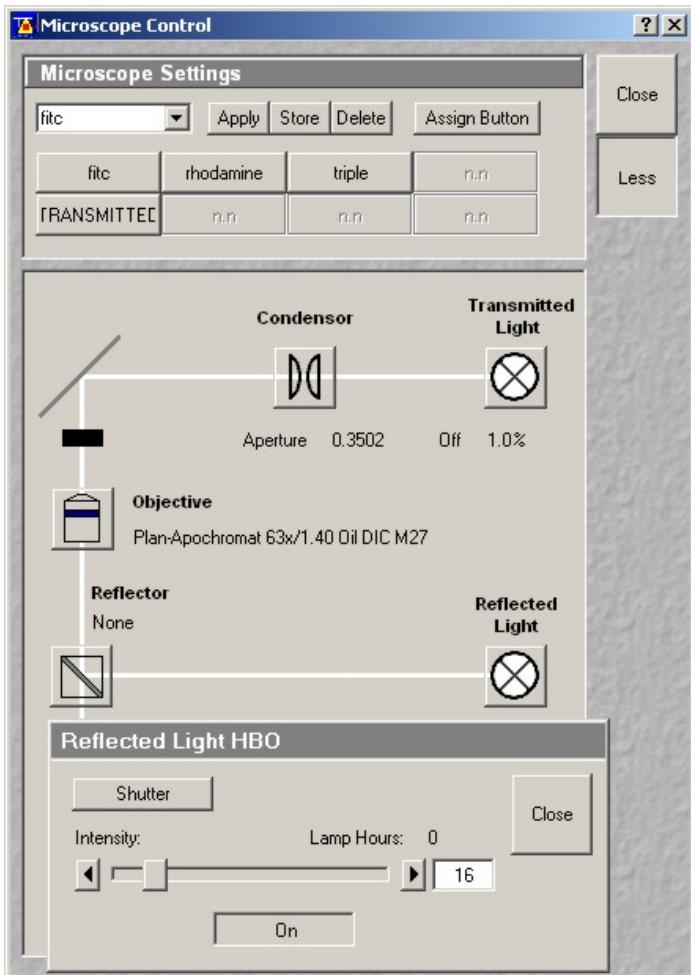
Once Kohler illumination is achieved (see section 2.4), insert either the DIC II or DIC III prism in the condenser filter wheel (DIC II for 10 and 20x, DIC III for 40 and 63x objectives) using either the touchscreen (Microscope -> settings -> transmission path) or the microscope control panel in the LSM software (click on Condenser, then select from the Filter list). Insert the polarizer filter at the top of the condenser column. Be careful not to insert the IR filter located on the



same mount than the polarizer. Insert the polarizer below the objective turret by sliding out the slider (Aug 07: the Core owns a single slider-mounted polarizer shared between the LSM 510 inverted and the Live DuoScan platforms. An alternative is to swap one of the filter cube in the microscope filter wheel with a cube-mounted polarizer. Contact Staff for help). Adjust the angle of the polarizer on top of the condenser for maximum



extinction. Retardation may be adjusted by turning the adjusting screw on the Wollaston slider below the objective.



2.6) Widefield fluorescence

The instrument is equipped with 3 fluorescence filter cubes: FITC, Rhodamine and triple bandpass (DAPI, FITC, Rhodamine). Other filters cubes are available, contact Staff for details. The Live DuoScan does not use a conventional mercury arc lamp but a new long-life arc lamp, the Xcite. The lamp itself is contained within the module below the air table and fiber-coupled to the side of the lamp port in the back of the microscope (the DuoScan scanhead is on the direct path on the lamp port). The Xcite controls light intensity via a diaphragm located within the controller. Only a limited number of intensity settings are available. The Xcite source is brighter than a conventional Hg lamp, and even the lowest setting (12% transmission) may cause bleaching during visual observation. Because of the DuoScan module, it is not possible to insert an aperture diaphragm slider on the lamp port. To reduce intensity, the only option is to use custom fluorescence cubes with either narrow-pass exciters or extra neutral-density filters on the excitation path.

Another shortcoming of the Xcite module is the impossibility to shutter the source from the microscope. The Xcite has to be turned on and off in software. An alternative option is to rotate the filter wheel to the open position with the touchscreen. To control wide-field fluorescence, click on the FITC, RHOD or Triple icon at the top of the microscope control panel window. To turn the Xcite off, click on the Reflected light icon and click on the Shutter or On button.

3) Acquiring images: Live scanhead

3.1 Overview of the optics

Click the Config button on the Acquire sub menu. 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, all high-power solid-state, are available: 405 diode, 489 diode, 561 DPSS and 647 nm diode laser. The lasers are located on a small optical bench inside the opto-electronic console. All laser delivers more power than conventional gas lasers on older LSM 510 microscopes. On the other hand, that power is spread over a line rather than concentrated on a diffraction-limited spot. Typical transmission settings are:

Wavelength (nm)	Total power (mW)	Transmission setting
405	50	5-20%
489	100	2-10%
561	40	8-25%
635	27	15-60%

A total of 4 optical fibers is connecting the console to the microscope: visible (489, 561 and 635 nm) and 405 nm are split and each is sent onto two separate fibers ending either in the Live or DuoScan scanning unit. Each fiber has its own fast-switching Acousto-Opto Tunable Filter (AOTF) for laser line selection and attenuation.

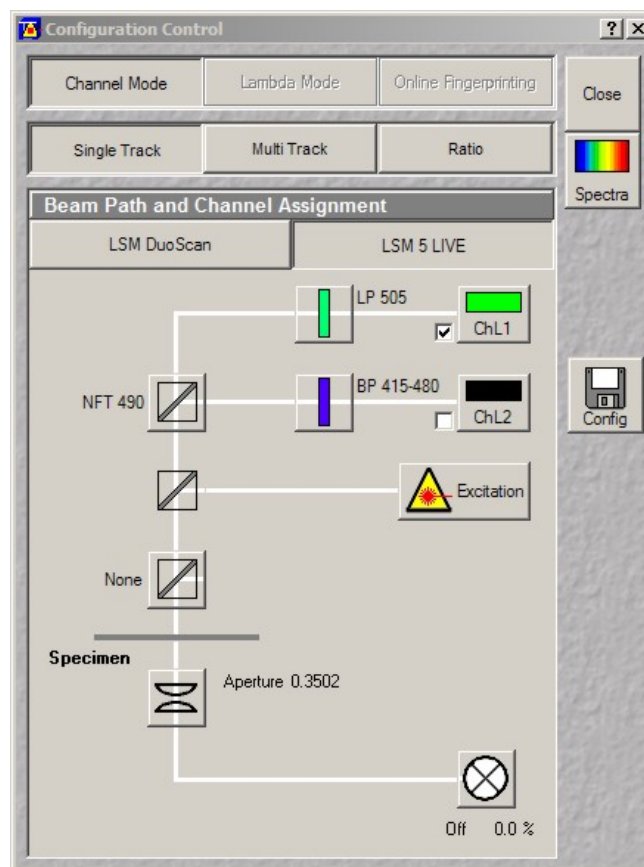
There is no main dichroic to choose from. The main dichroic on the Live is a wavelength-independent line mirror on a glass plate reflecting the linearly-shaped laser beam from the Live scanhead (Achromate). A set of secondary dichroic split the emitted fluorescence according to color and stir it toward one of the two available detectors. The Live use high quantum-yield linear CCDs with 512 pixels for detection. Each detector has a set of emission filters, some of which are double-bandpass filters allowing for rapid (AOTF switched) multichannel detection using a single detector. The number of possible configurations is determined by the available secondary dichroics and emission filters. Channel 2 is used mostly for the lower emission range (blue / green fluorescence), while channel 1 is geared toward green / red fluorescence. Refer to the Live DuoScan specifications for details (<http://mic.nichd.nih.gov/URL>) **UPDATE**.

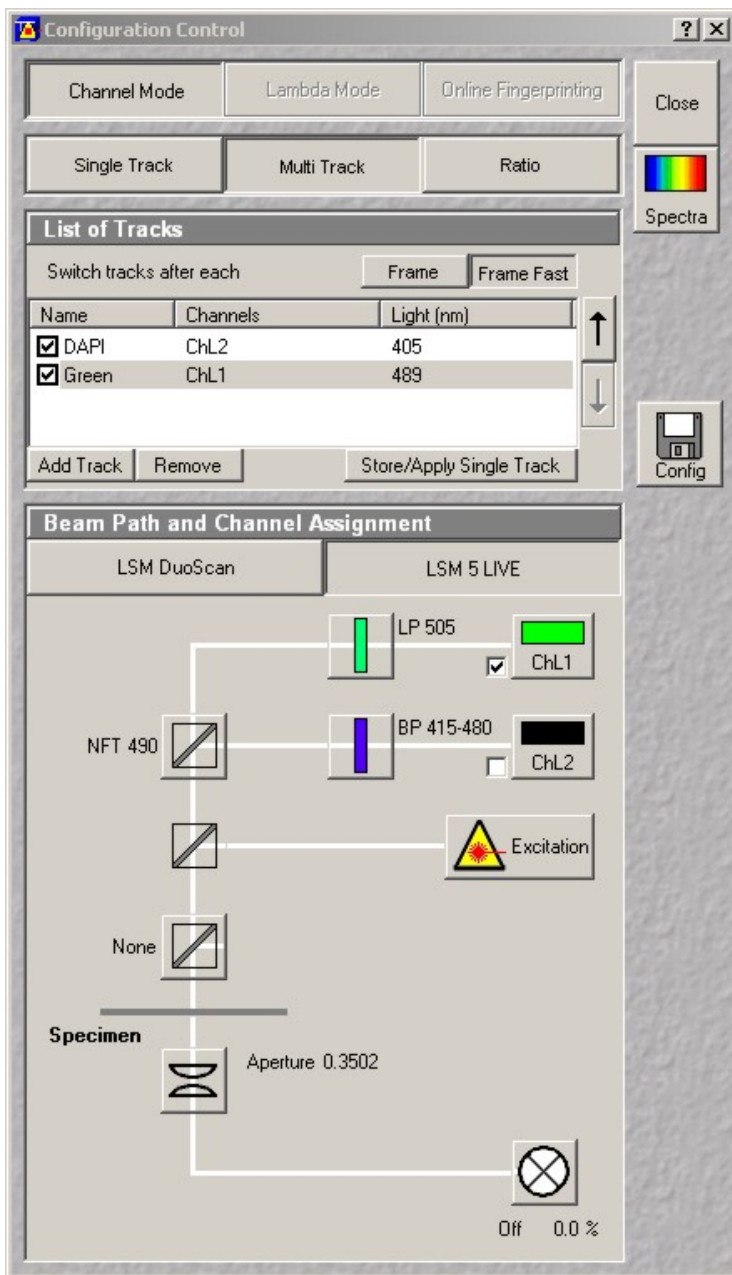
A transmitted detector, located to the left side on top of the condenser column, is also available. This device is used for DIC imaging with the DuoScan acting as a light source.

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.





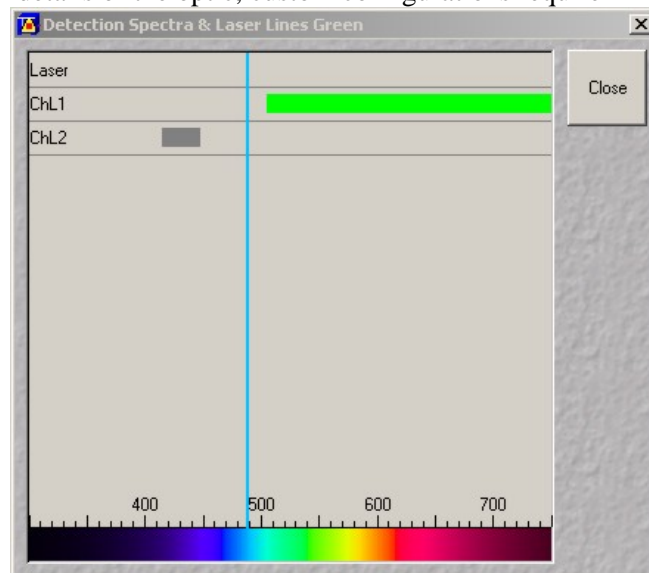
specific tuning of the instrument (such as pinhole alignment) which is best handled by the M.I.C. staff. User-created configurations also open the door to potential data quality and instrument welfare issues. If your needs are not addressed by a canned configuration, contact the M.I.C. staff for help.

MultiTracks, or sequential excitation, minimizes cross-talk by acquiring sequential images at each excitation wavelengths. On a regular LSM, channel switching in multitracks can be done on a line or frame basis, and the frame mode may include a change in optics between each frame. On the Live system, line switching is not available. Furthermore, since the Live is a platform designed for fast acquisition, it is strongly recommended to keep the same optic between different channels. Note that in Fast Frame mode, the software does not wait for a feedback from individual filter wheels before switching channels. In the exceptional circumstances where a change in optics is required, do not use Fast Frame or the instrument will crash badly!

The third tab in the Configuration Control window Channel Mode 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 ratio images for ratiometric experiments such as calcium imaging.

A large number of pre-set 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.

We strongly recommend against users creating their own configurations. Besides the time and effort involved in understanding the details of the spectra, custom configurations require



3.3) Acquiring an x-y image

Once the instrument is configured, bring the Scan Control window up 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 turned on. By default, frame scanning is selected, but ROI scanning is also available once a specific Region Of Interest has been selected (rectangle only).

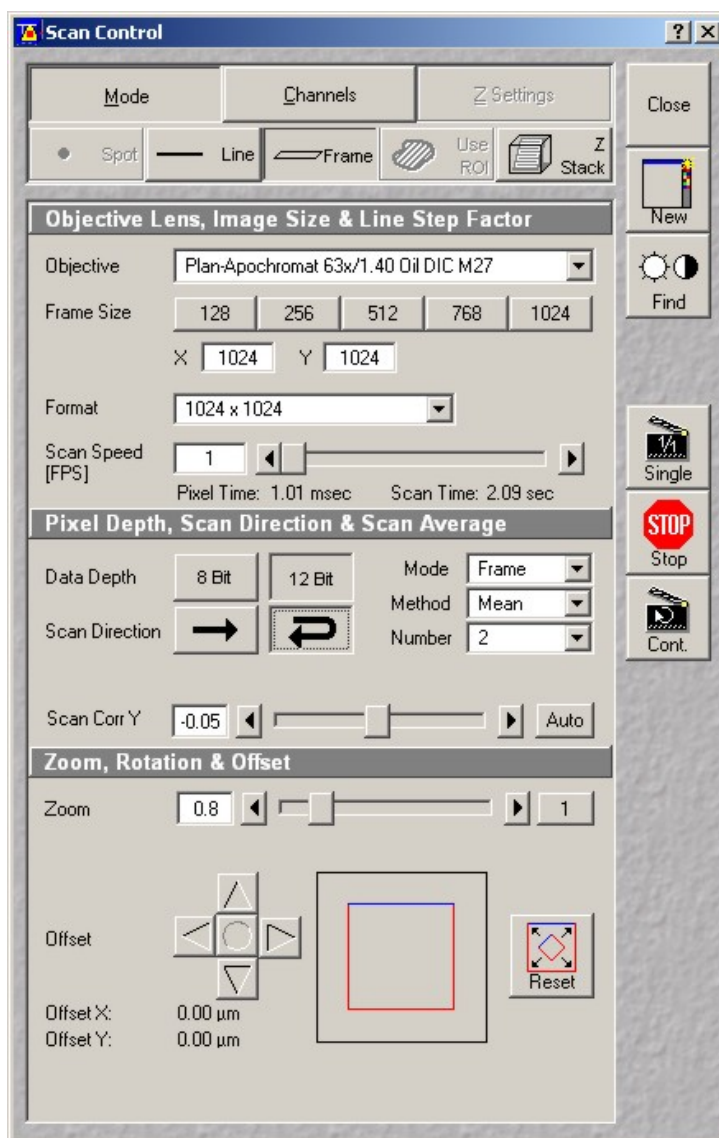
Click on Mode to view the scanning parameters. Due to the nature of line scanning, options for custom scanning are more restrained than on a conventional LSM 510. No frame rotation is available. Only cropping in Y improves scan speed. Cropping in x is possible, but does not change acquisition speed. Zooming is done exclusively on the detection side by inserting a smaller confocal aperture. There is no zoom optics for the excitation laser. Since the exposure remains constant per surface area, zooming on a Live system strongly reduce the signal intensity. Last, acquiring images with an horizontal resolution higher than 512 pixels (the resolution of the linear CCD detector) is possible and implemented by multiple scans with a sub-pixel shift on the detection path (i.e. 2 scans for 1,024 pixels, 3 scans for 1,536 pixels).

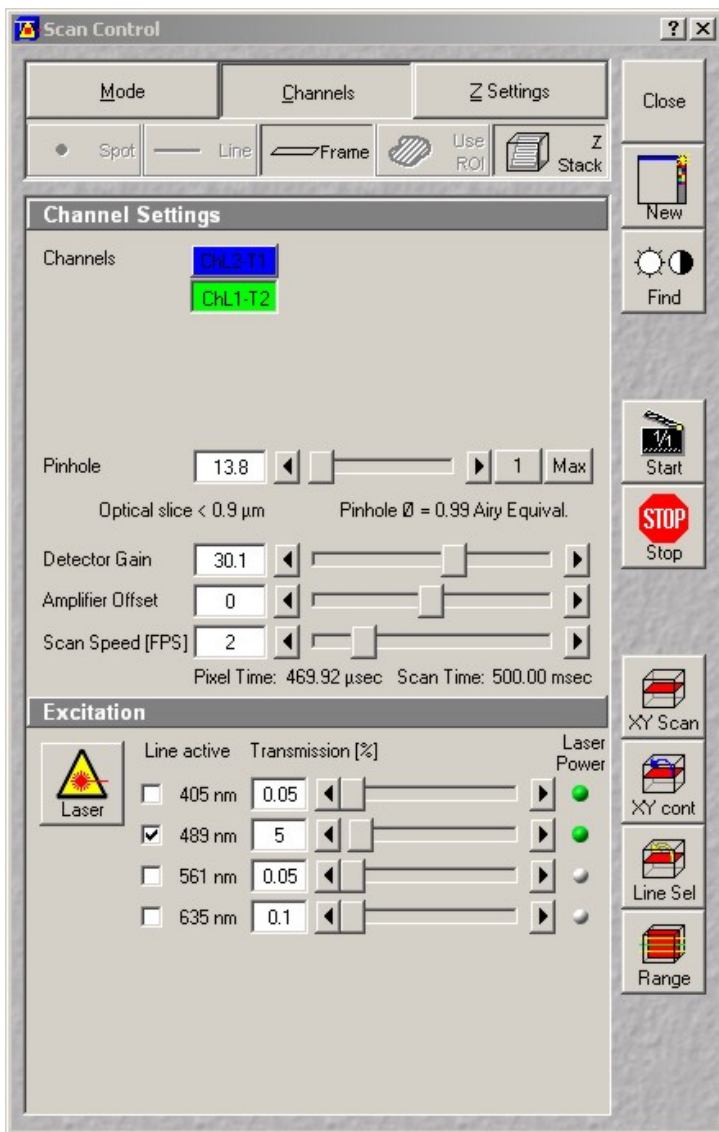
The scan speed, defined in frames per seconds (FPS), is adjusted on the Channel pane rather than here. Unlike a point scanner, bi-directional scanning refers to full images rather than lines. In bi-directional mode, an image is recorded on both the upward and downward sweep of the laser line. Bidirectional scanning introduces

a shift between odd and even images, which is corrected either manually by trials and errors or automatically by clicking on the Auto compensation button. Bi-directional scanning has only a modest influence on the scanning speed. 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. It is strongly recommended to reduce the scan speed rather than resort to averaging.

As explained above, zooming strongly reduce signal intensity because the excitation power does not increase with zooming. Zoom factors are more limited than on an LSM 510, but users still should remember that a light microscope is physically limited to an in-plane resolution of 0.1 to 0.2 μm (click on the Info button on the image window to view the pixel size).

Click on the Channels tab on top of the Scan Control window 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), boxes on the same row represent simultaneous excitation (single track). Combinations are possible. Click on a box to reveal the acquisition parameters. Confocal aperture, Gain, Offset, Amplifier Gain, Frame Rate, Excitation line(s) and Transmission must be adjusted for each channel. An important difference between the Live and point scanner units is that the confocal pinhole (diaphragm) is replaced with a confocal slit. Since continuously-opening slits are difficult to manufacture, the Live is using a slide with a dozen different aperture slits on it. As a consequence, only a discrete number of confocal apertures are available. For optimum confocality, the confocal slit must be set as close as possible to 1 Airy Unit. The thickness of the





scanner, it is more important than ever to avoid unnecessary scanning to minimize photobleaching, especially in Continuous scan mode.

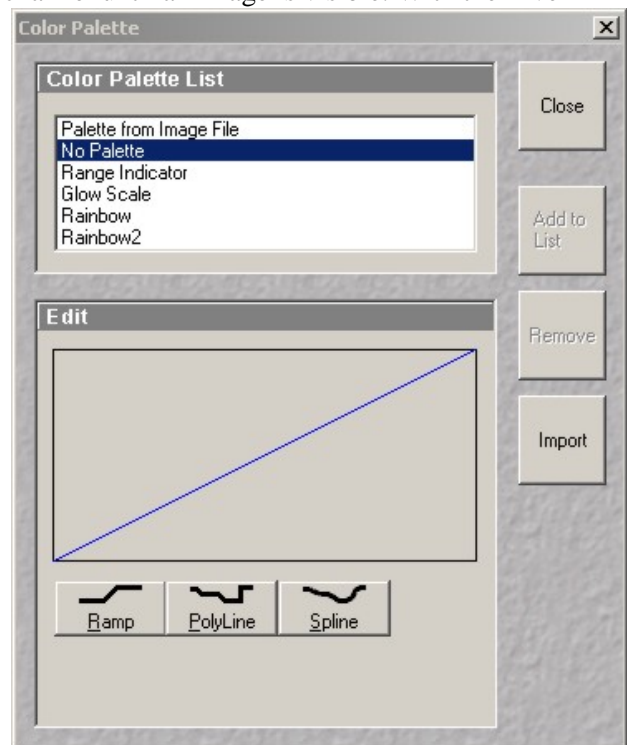
The Offset slider is used to adjust the black level. For optimum image quality, the intensity values must span the entire dynamic range available, i.e. from 0 to 255 for an 8-bit image ($2^8 = 256$). To adjust the black and white values, click on the Palette icon in the Image Window toolbar and select 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 especially if you plan to quantitate the image later. Due to the high dynamic range of CCD detectors, acquiring in 12-bit mode is more efficient at revealing subtle changes in intensity than on a regular LSM 510.

optical section, calculated from the aperture size, the objective characteristics and the excitation wavelength, is shown below the adjustment slider.

For detectors, the Live is using high quantum-yield 512-pixels linear Charge Coupled Devices (CCDs) instead of Photomultiplier Tubes (PMTs). CCDs have a higher dynamic range (detectable light intensity) than PMTs but increasing the gain on a CCD does not improve the sensitivity as dramatically as on a PMT. The gain values are between 0 to 50. Although high gains generate far less detection noise than on a PMT, it is recommended to keep the gain below 35-40 for optimum image quality. Always remember to reduce the gain before scanning a new sample to avoid over-exposing the detector!

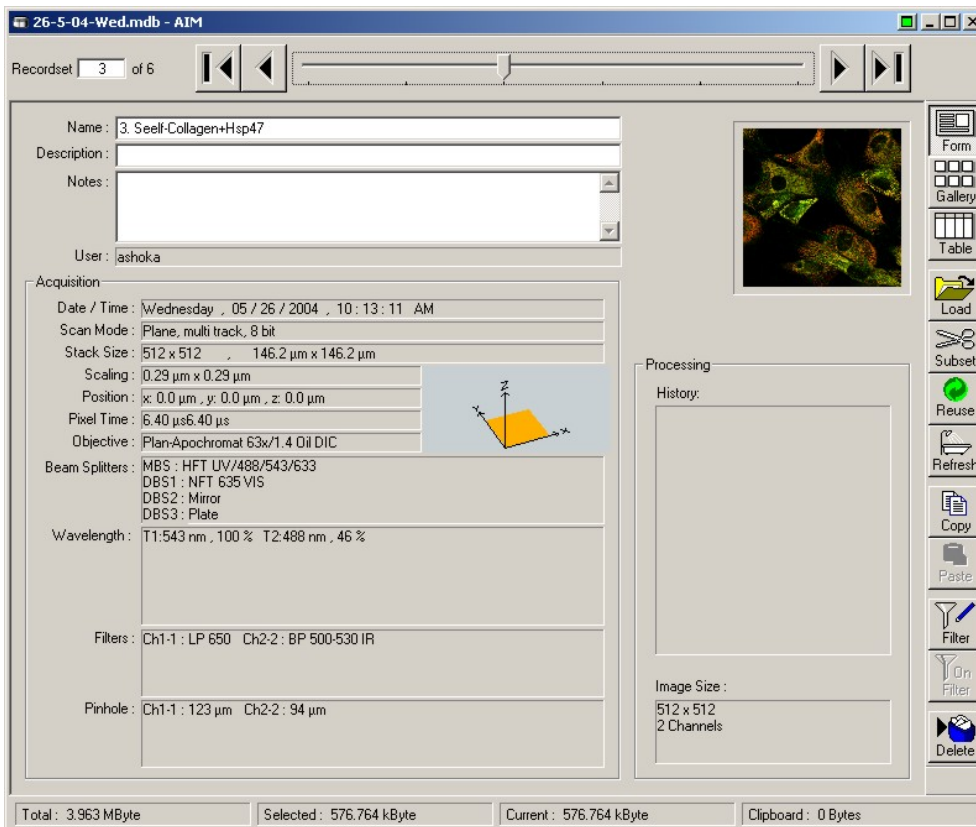
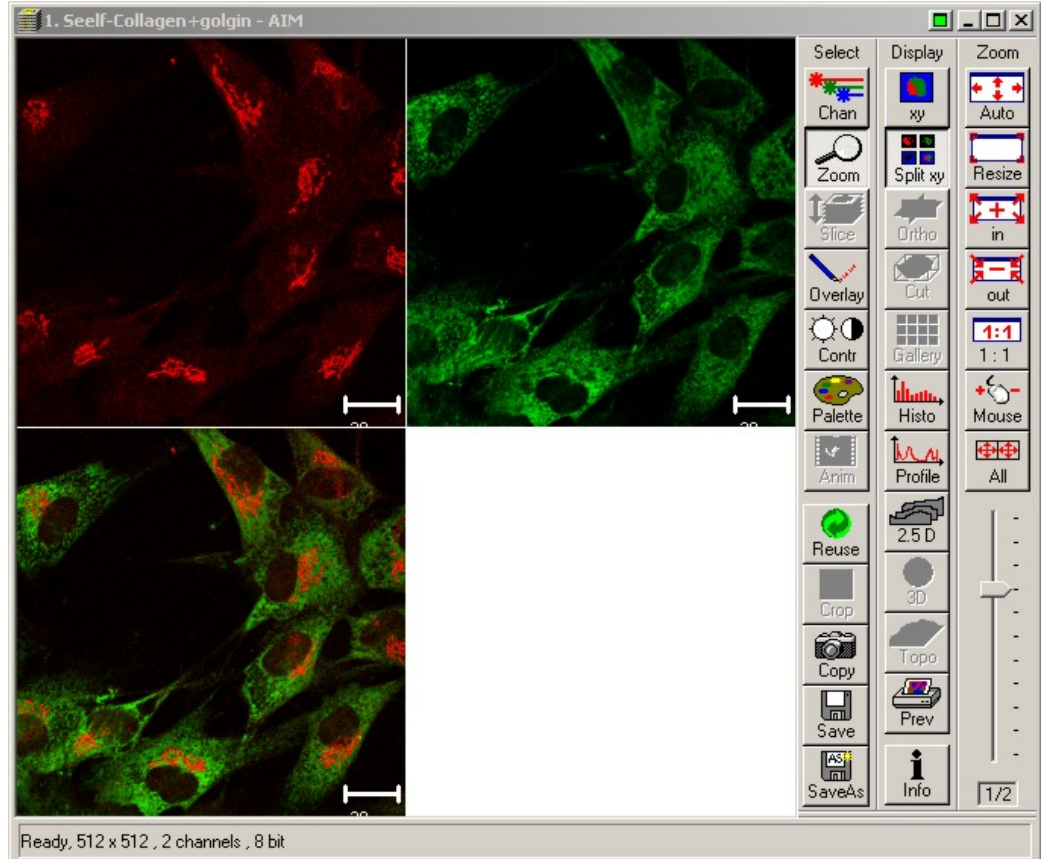
The scan speed, in Frames Per Second, has a tremendous influence on signal intensity, hence on image quality and amount of bleaching. Lower scan speeds produce higher signal (better image) and allow for lower laser exposure. The default FPS is always very high, between 15 to 30 FPS, and the user should evaluate whether such a high scan speed is truly required.

The Fast XY button is no longer present. To obtain an image, start scanning using either the Single or the Continuous button (if excessive bleaching is observed, set up a time serie with an interval between consecutive images, see section 5.1). Slowly increase Detector Gain on each channel until an image is visible. With the Live



In the image window, 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. The LSM software uses a proprietary file format (*.lsm) based on the tiff standard with the acquisition parameters saved in the header. Images are saved in a database, special folders with a ".lsm" extension. Inside this folder are also created small files with the list of available images in the folder. Click on Create New MDB (database) and browse to your folder under D:\user images\. To prevent accidental file losses, it is recommended to create a new database for each imaging session. Give the new database a name, and enter a name for the current image. Since the

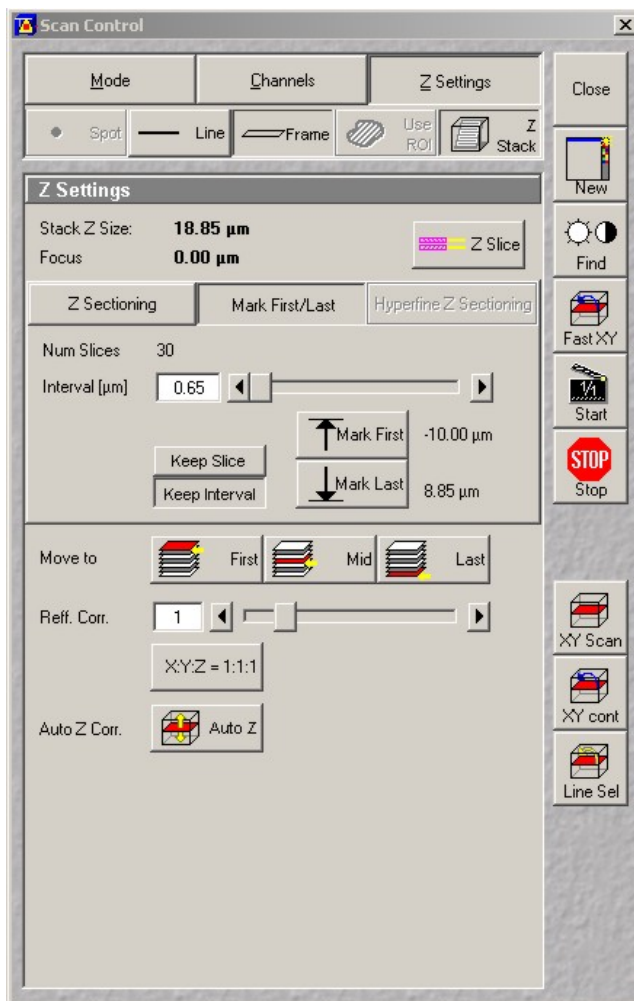


entire instrument configuration is saved, it is possible to later re-open the image and click on the Reuse button in the image window toolbar to reset the microscope exactly the way it was at the time the image was acquired.

3.4 Acquiring a z-stack

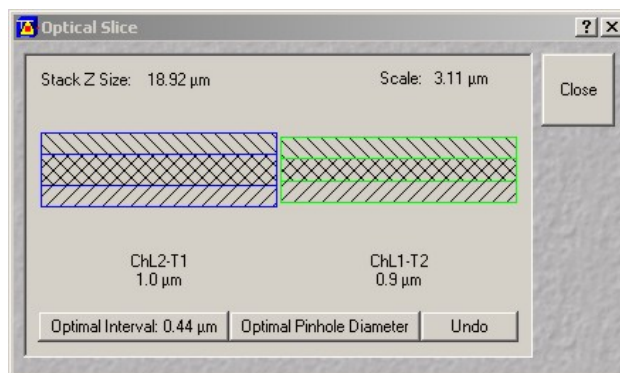
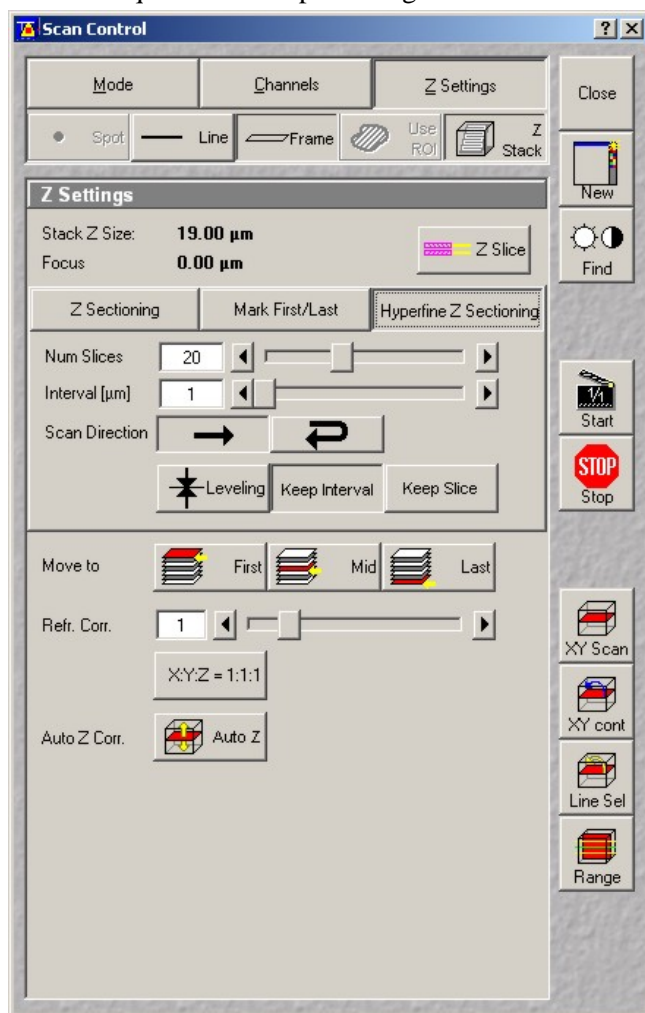
In the Scan Control window, click on Z Stack to turn optical sectioning on. The Z Settings button at the top right becomes active. Click on it, then click on Mark First / Last in the Z Settings window. Start scanning, then change focus (either manually or with the Stage control panel) until reaching 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. The next step is to decide on a spacing between consecutive slices. As a rule of thumb, between 20 to 50% overlap is required for optimum resolution. Click on Z Slices for fine-tuning, then on Optimum for 50% overlap. When acquiring multi-color stacks, the optical slice thickness on each channel should be identical. However, only a limited number of confocal apertures are available on the Live and it may not be possible to perfectly match the slice thickness of each channel.

To maximize the potential of this high-speed confocal instrument, the software controls a piezo-actuated objective adapter for rapid focus changes during volume acquisition. The piezo stage is much faster than



the microscope focus drive and reduces volume acquisition times by as much as 70%, but is only available with the 63x lens. Click on the Hyperfine Sectioning Tab to access the piezo settings. The upper and lower position and slice spacing entered under the Mark First Last tab are transferred to the Hyperfine pane. As soon as the Hyperfine pane is selected, the LSM software controls focus via the piezo stage. The piezo stage has a much smaller travel range (approx. 100 μm) than the AxioObserver focus drive. Click on Leveling to home the piezo stage and ensure maximum piezo focusing range up and down.

Click Start in the Scan Control window to



begin acquisition of a Z stack. 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 by clicking on Chan and selecting None). Once the stack is recorded, click on Slice to display a slider and browse through the stack, or click on Animate to start an animation.

3.5) Acquiring transmitted images

On a point scanner, transmission imaging is implemented by collecting the laser beam coming through the specimen via the condenser and sending it to a transmitted detector. The transmitted detector is usually a PMT without a confocal pinhole. On a line scanner, such detector cannot discriminate individual pixels in a line. On the Live DuoScan system, transmitted images are acquired in one of the two following ways:

1) DuoScan / transmitted detector (PMT): this modality uses the DuoScan as a light source and the transmitted detector for imaging. Set up visible DIC as described in section 2.5. Remove the polarizer slider below the filter cube turret (the lasers are already polarized). In the Multitrack configuration window, add a new track and click on the LSM DuoScan tab. Click on excitation and select a laser line and a –low- level of transmission. Insert the T80/R20 beam combiner in the filter cube turret, click on the transmitted detector (ChD) to turn it on. Go to the channel pane in the Scan Control window and make sure the gain on ChD is set to the minimum value. The transmitted detector is exposed to the direct laser beam and could be damaged if overexposed! Start scanning and adjust the gain and offset on the transmitted detector. Adjust the polarizer in the condenser arm for maximum extinction. This implementation of DIC is much slower than the Live scanhead and cannot be done concurrently with imaging. Due to different scan fields of the Live and DuoScan units, the transmitted image often does not match the fluorescence image. Since the DuoScan field is not adjustable, set the zoom on the Live scanhead for best superposition (around zoom 0.7). You may need to use the Image Match macro (see section 4.3) for exact registration of both images.

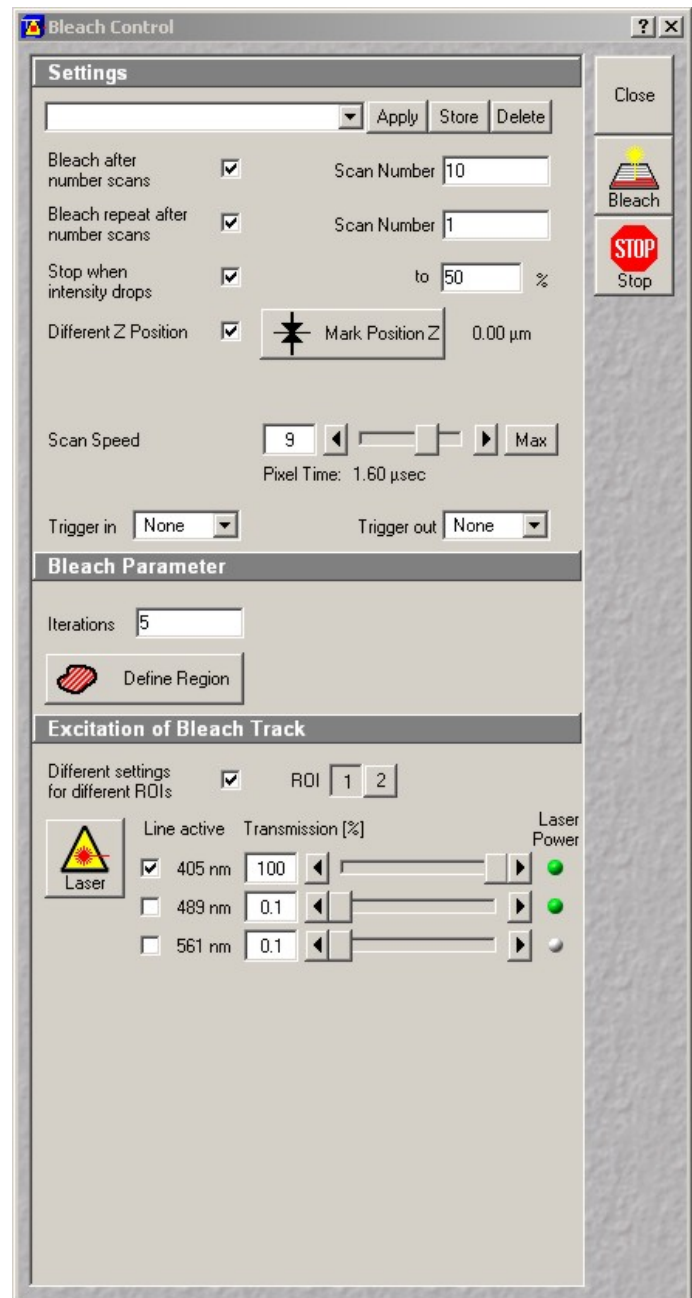
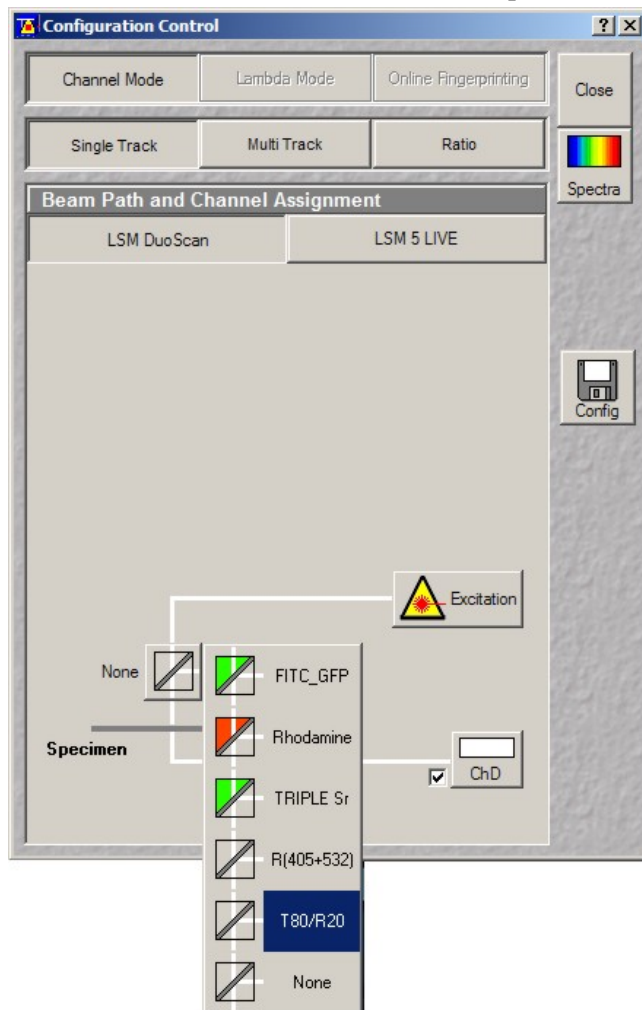
2) Halogen lamp / Live scanhead detector: this implementation uses the infra-red spectra of the halogen lamp as a light source and channel #1 in the Live scanhead as the detector. In theory, this implementation allows for high-speed DIC in combination with fluorescence imaging. After configuring the instrument for visible DIC (section 2.5), insert the IR filter on the condenser. Fully open the condenser aperture for maximum light throughput. Remove the polarizer slider below the filter wheel turret. Click on the LSM 5 Live tab in the Configuration Control window, click on the emission filter button on Channel 1 and select either BP 665-750 IR 0° or BP 665-750 IR 90°. Use a confocal aperture for optimum image quality. With the analyzer now part of the emission filter, you may need to adjust the polarizer in the condenser arm for maximum extinction. When recording a fluorescence image in parallel, (blue or green only), insert the NFT 635 and insert an appropriate emission filter before Ch2. Click on the Halogen lamp icon at the bottom left and set it between 50 to 100%. It may seem dangerous to expose the sensitive Live detector to such a high light intensity, but most of the lamp power is cut by the IR filter, and the crossed polarizers and confocal slit further reduce the light intensity. Start scanning, adjust the gain and offset on Ch1 and check the polarizer in the condenser for maximum extinction. Aug 07: due to insufficient light intensity, this implementation of DIC is not a viable alternative to the first option above. Zeiss recommends using a condenser with a minimum numerical aperture of 0.8. The current condenser on the Live DuoScan is NA 0.5.

4) Photobleaching: DuoScan

4.1) Description

A major limitation of the Live scanhead is its poor performance for photobleaching experiments. By design, the Live uses very low level of excitation light and produces little bleaching in the specimen. Since there is no zooming optic on the excitation side, zooming does not increase bleaching efficiency (zooming is an efficient way to increase bleaching on a point scanner). Furthermore, there is no provision to bleach a custom Region Of Interest (ROI) other than horizontal stripes.

To compensate for these limitations, the Live DuoScan is outfitted with an auxiliary point scanner on the lamp port (DuoScan head, sometimes called Exciter). This scanning unit has no detectors and is intended exclusively for FRAP or photoactivation of custom ROIs. Visible and near-UV light is fed from the opto-electronic module to the DuoScan via two fibers. The DuoScan is receiving roughly 50% of the total light emitted by the lasers. The 561 nm laser delivers enough power to bleach red dyes and RFP constructs, but the 635 nm laser is not available on the DuoScan. The laser beams from the DuoScan and the Live scanheads are combined in the microscope with permanently-mounted filters in the fluorescence filter wheel. Two filters are currently available, the T80/R20 and the R(405+532). The double dichroic R(405+532) can be used only with the 405 laser line. The T80/R20 splitter transmits 80% of the light to and from the Live scanner while reflecting 20% from the DuoScan head, resulting in 20% light loss on the imaging side and 80% loss on the excitation (DuoScan) path. These

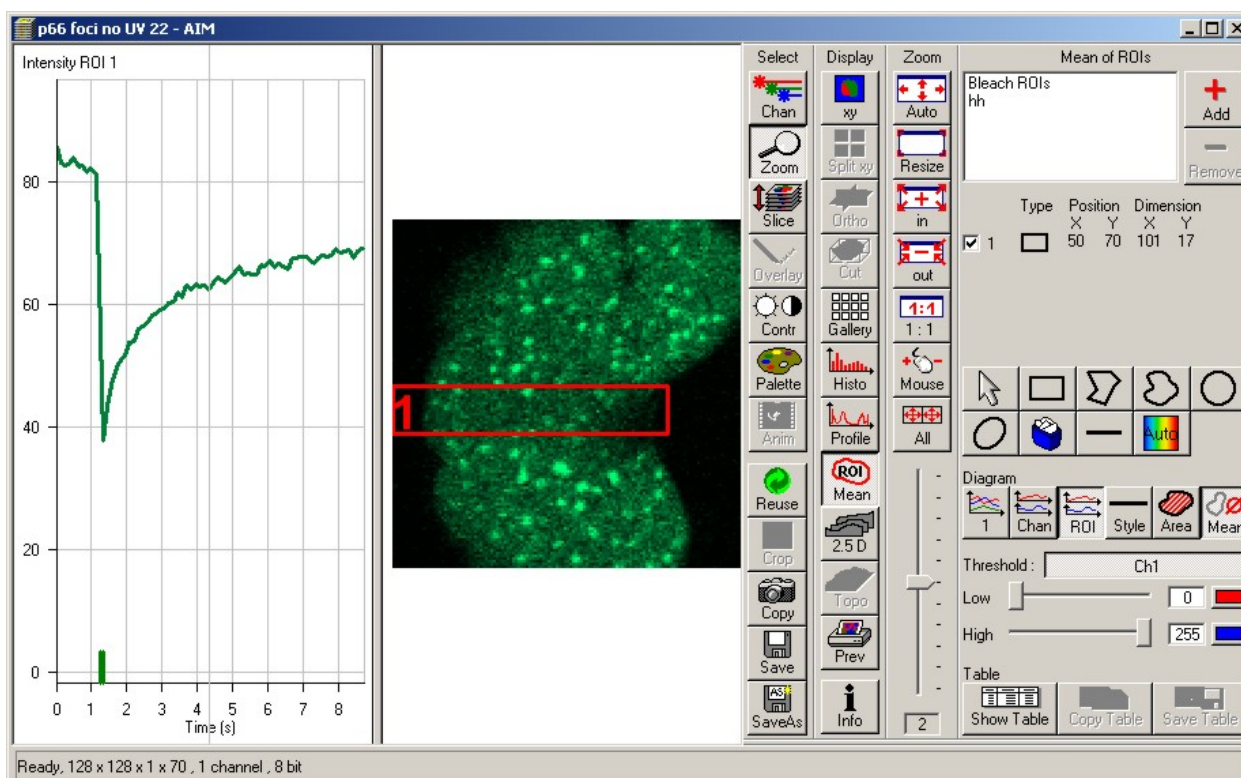
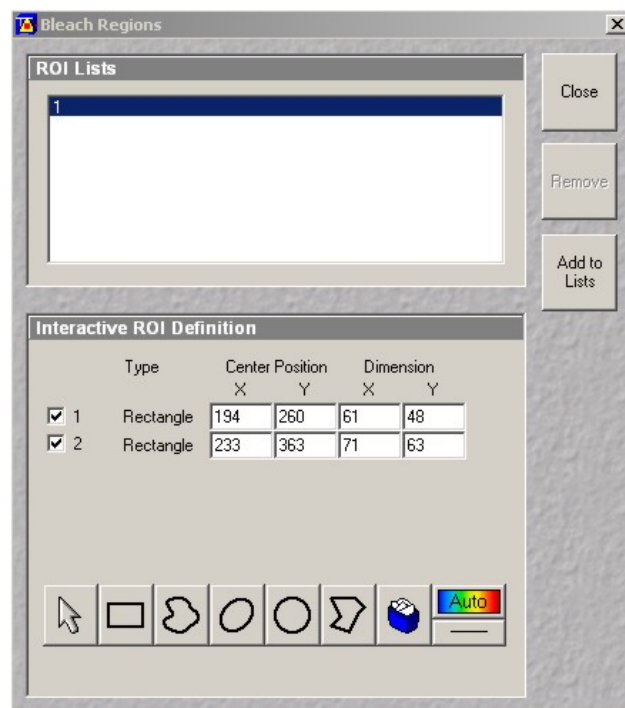


beam combiners are not replaceable in the field, but other filters could be installed by replacing the filter cartridge with new factory-mounted beam combiners.

4.2) Photobleaching

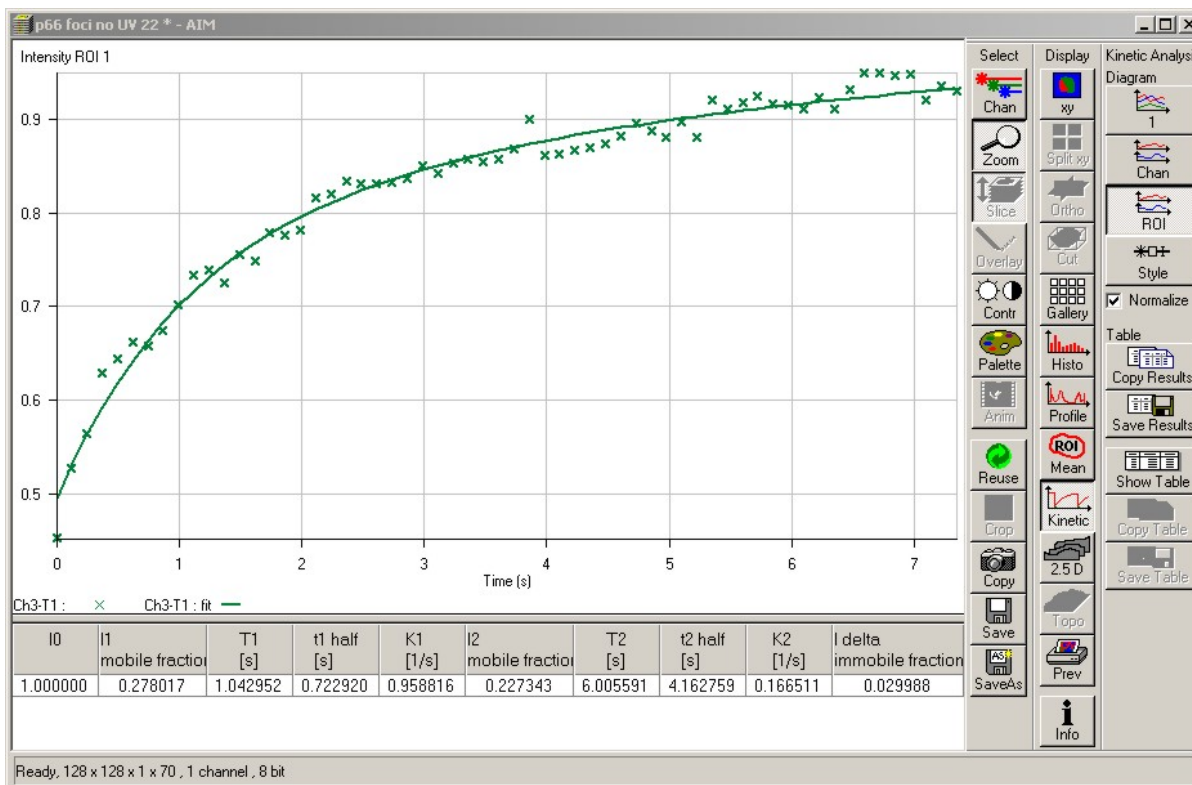
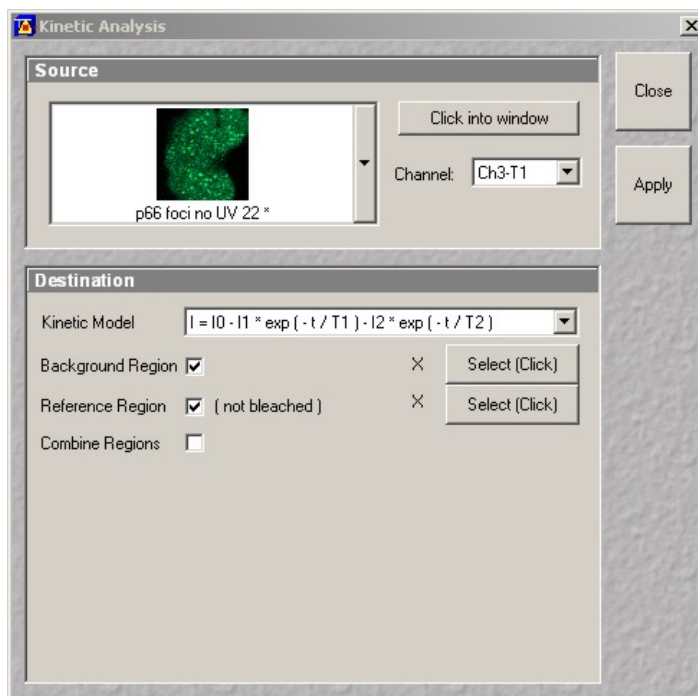
Once a satisfying image is obtained, click on the Live DuoScan tab in the configuration control window and insert the T80/R20 beam combiner. Click on the Edit Bleach button on the Acquire main menu. On the Bleach Control window, click on Bleach After Number of Scans and enter the number of imaging scans before bleaching. Select the bleaching laser line and intensity at the bottom. For FRAP, more efficient bleaching is obtained by using all available laser lines at maximum intensity. For photoactivation, use the 405 nm line and the R(405+532) beam combiner. Enter the number of bleaching scans (Iterations) under Bleach Parameters then click on Define Region to open the Bleach Region window. Remove all existing regions –if any- then click on one of the region tool at the bottom and select the bleached region on an image. Make sure the image is displayed on the monitor with a zoom of 1 or the bleached region will not be positioned properly (software bug). Click on Add to Lists to save the region. Open the Time Serie window and set up a time serie (see section 5.1), then click on StartB.

Advanced options available in the Bleach Control window include: FLIP (Fluorescence Loss In Photobleaching) with defined bleaching intensity drop, vertical (Z) bleaching, adjustable bleaching scan speed, and the option to bleach with different lasers when more than one region is selected.



A common approach to test the suitability of a specimen for photoactivation is to record a time series with the imaging laser line at low settings and the converting line at 100% transmission. For PA-GFP (for example), there should be an increase in intensity at 488 nm while the protein is slowly converted by the 405nm line. Optimizing photobleaching experiments can be complex, contact the M.I.C. staff for help.

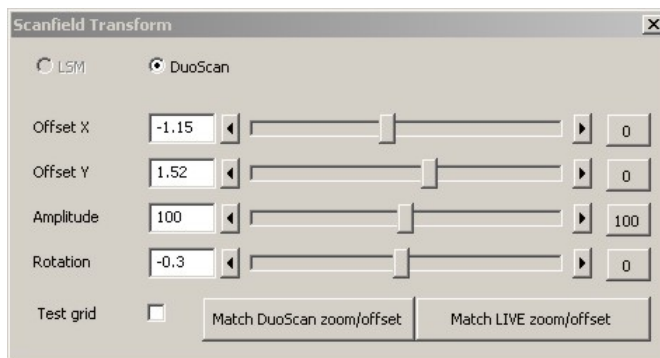
Due to the peculiar nature of FRAP on a confocal microscope and the complex underlying diffusion processes (for example diffusion in the Golgi), it is difficult to quantitate the recovery with an absolute diffusion coefficient. With the exception of low magnification / low NA objectives, flat specimens and membrane-bound markers, the post-bleach marker concentration gradient is so complex that it can hardly be analyzed by a model. However, the Zeiss software allows for simple analysis of kinetics. In the main menu, click on Process then on Kinetics. On the Kinetic Analysis window, select the image and channel to analyze, then the kinetic model (single or double exponential). Provisions for background correction and non-specific bleaching (due to imaging) are available by defining specific ROIs. Click on apply to see the results, then on Save Results to save the coefficients as a text file.



4.3 Image Match

When performing a photobleaching experiment, the bleached area is defined on an image acquired with the Live scanhead, but the actual bleaching is performed by the DuoScan exciter via the beam combiner. The

two region coincide only when the Live and DuoScan scanners are properly aligned. Before any bleaching experiment, it is strongly recommended to check for alignment using a fixed specimen. Note the alignment depends on the specific beam combiner used, T80/R20 or R(405+532). After recording a short photobleaching experiment, click on ROI in the image window toolbar and select Bleach ROI. The outline of the ROI appears on the image. Click on Slices, move the time cursor immediately after the bleaching step and increase the zoom. If adjustments are necessary, click on Macro in the main menu bar, then on Image Match in the macro toolbar. The scanfield transform window appears. Depending on the observed shift, adjust the X and Y offset and perform a new bleaching experiment to check for alignment. Repeat until alignment is acceptable. In case of severe misalignment, it may be necessary to adjust the amplitude (zoom factor) and rotation values. Note that zooming on the Live scanhead affects the registration.



5) Advanced functions

5.1) Time series

To acquire a series of images at specific time intervals, adjust first the gain, offset 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. Start Serie is the event that will trigger acquisition, enter Manual. Stop Serie is the event that will terminate acquisition, select Manual and enter the number of images to collect. To estimate the total duration of the experiment, refer to the scan time shown below the scan speed slider in the Scan Control window. 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, then click on Data to show the time on each image. Once recording is complete, click on Overlay and draw one or more regions in the image to plot their intensity versus time. Click on Show Table and Save Table to display and save the results as a text file, respectively.

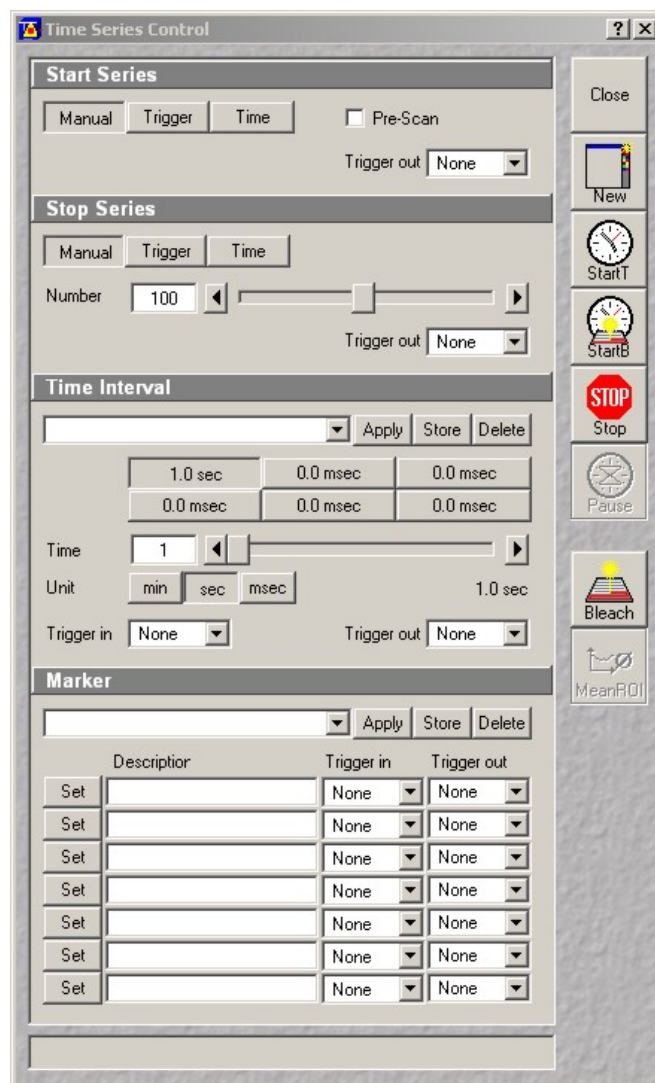
When acquiring a volume, scan times are greatly reduced by using the piezo focus drive (Hyperfine sectioning) rather than the microscope focus drive. Bidirectional scanning in Y (imaging sweep) marginally improve the speed. Bidirectional scanning in Z (both up and down) also produce small improvements. Both bi-directional mode introduce some hysteresis in the images that is difficult to compensate, especially in Z.

5.2) ROI scanning

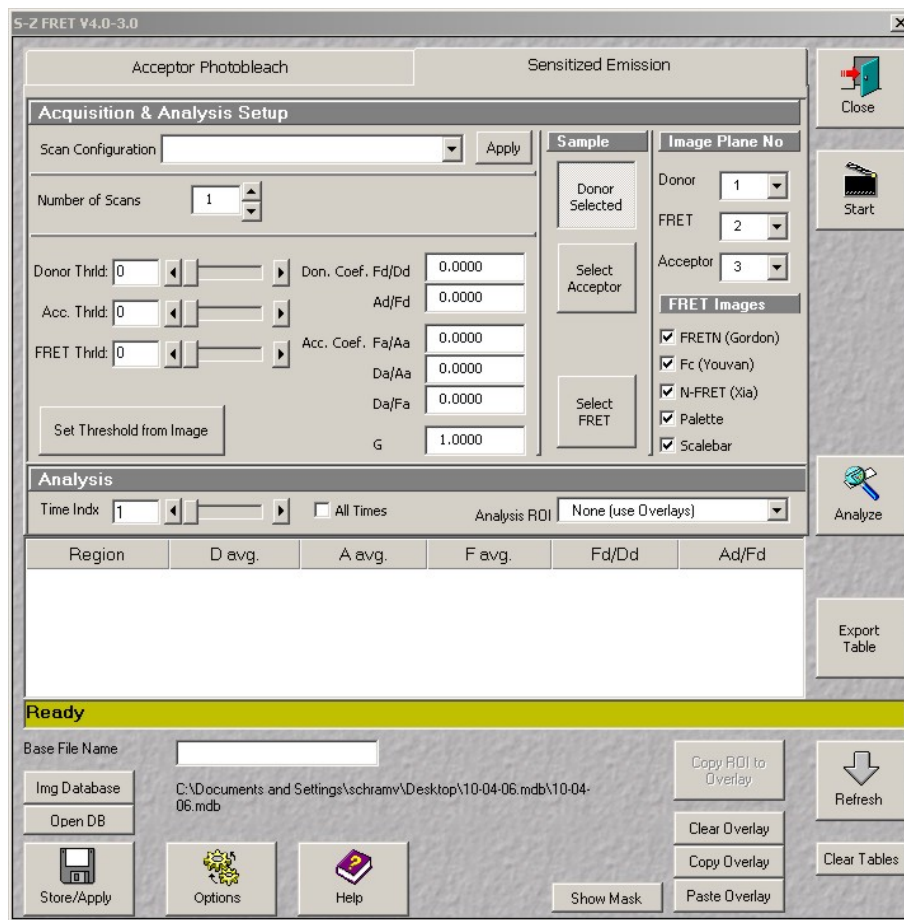
Due to the principle of the Live scanner, only horizontal rectangular regions may be selected. As mentioned earlier, the Live scanhead has no zooming optic on the excitation path. Zooming is exclusively accomplished on the emission side by inserting a smaller confocal slit. Since the laser power per surface area remains constant, zooming on the Live greatly reduces signal. Zooming can be done in a number of ways, either by using the zoom slider at the bottom of the Scan Control window or the crop tool in the Image window toolbar. There is also the option to use the Edit ROI tool in the Acquire submenu on the main toolbar, select a region then click on Use ROI at the top of the Scan Control window.

5.3) FRET

Fluorescence (Foester) Resonance Energy Transfer (FRET) is a non-radiative transfer of energy between a donor and an acceptor pair than can be used as a measure of proximity between the two molecules. FRET pairs have extensive overlap between the emission spectra of the donor and the excitation spectra of the acceptor. Both molecules must be in close spatial proximity, between 2 to 20 nm. FRET should not be used without a thorough understanding of its underlying principles and pitfalls. If you are not familiar with FRET, we recommend you acquire a basic knowledge of this application before planning experiments.



FRET can be measured either as an increase in donor emission when the acceptor is removed (acceptor photobleaching) or as an increase in the acceptor emission when exciting the donor (sensitized emission). Sensitized emission is more sensitive than acceptor photobleaching but also more prone to false positive results. Zeiss has developed a macro to quantitate FRET measurements. 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 under the same conditions and on the same day 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 supported by the macro. Note that beyond the most common artifacts (bleed-through, cross-excitation and background fluorescence), further corrections may be required for greater accuracy.

FRET between Cyan and Yellow Fluorescent Proteins and Green and Red fluorescent proteins is fully supported on the Live DuoScan using the 405 / 488 nm lasers and 488 / 561 nm laser, respectively (the instrument lacks a 514 nm line to selectively excite YFP). In the case of Chameleon indicators where the stoichiometry between each partner is fixed at 1:1, a simple, semi-quantitative experiment can be done by selectively exciting the donor and looking at both the donor and acceptor emission.

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 4.13 of the LSM documentation available from our web site. The free version of the software, LSM Image Browser, can be used to visualize, annotate and export images but lacks all measurement functionalities. A free software package, ImageJ, offers excellent image analysis 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 high-end software packages running on dedicated workstations: Universal Imaging's Metamorph for image analysis, Improvisation's Volocity and the full 3D rendering package of the Zeiss LSM software for 3D reconstruction, real-time visualization and deconvolution, Contact us for details.

6.1) Image annotations

To add text annotations, scale bars, 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 edited or erased later. To display the time or Z-position (in a time serie or a Z-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 modify all channels at once by choosing All on the bottom

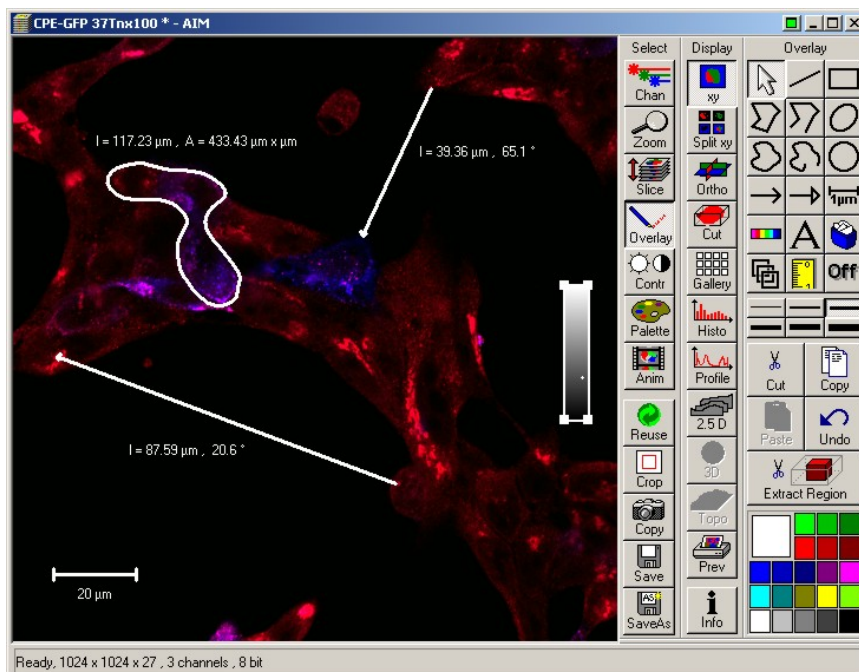
toolbar. To change the palette used to display the image, click on Palette and select a palette from the list. Range Indicator is commonly used to check for under- and over-exposed pixels during acquisition. Each channel may be turned on or off independently by clicking Chan in the image window toolbar 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 they do not alter the original image unless it is saved again. Such post-acquisition image processing has to be fully documented.

Several options are available to visualize images in a stack, regardless of its nature (time or Z 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

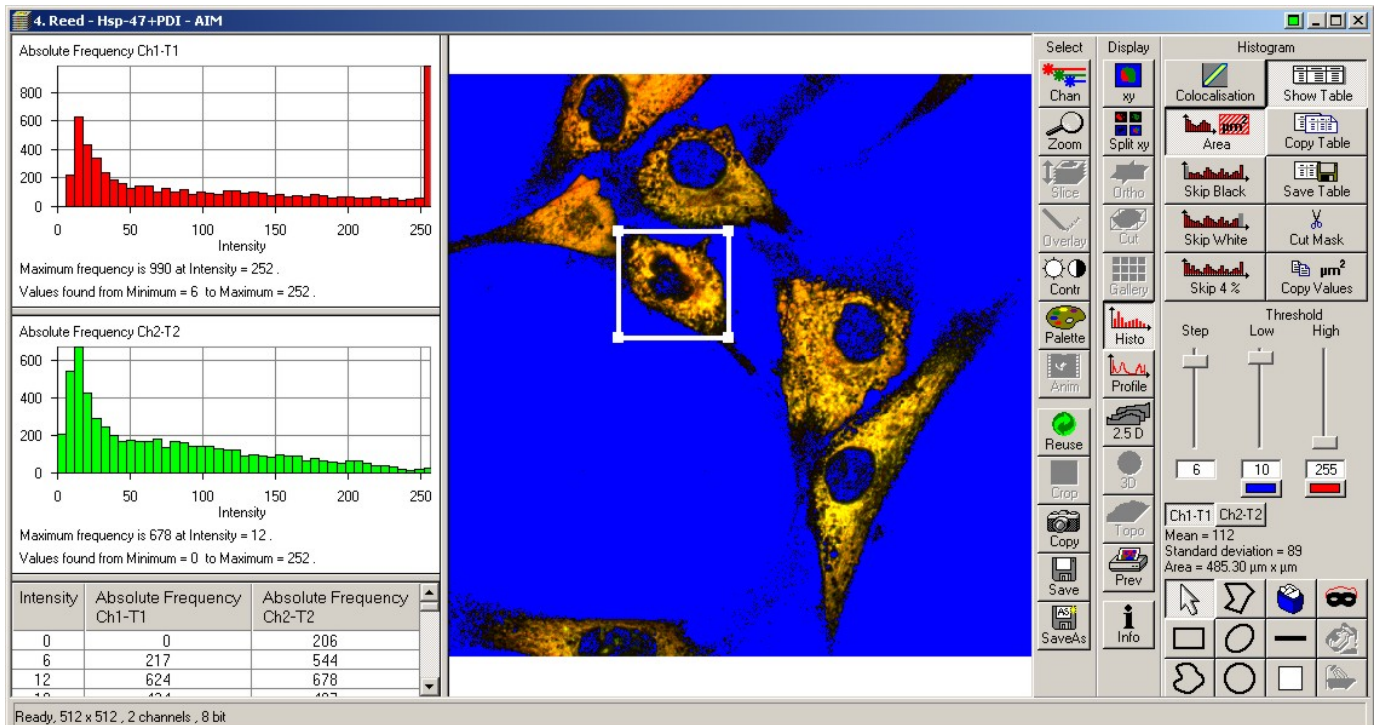
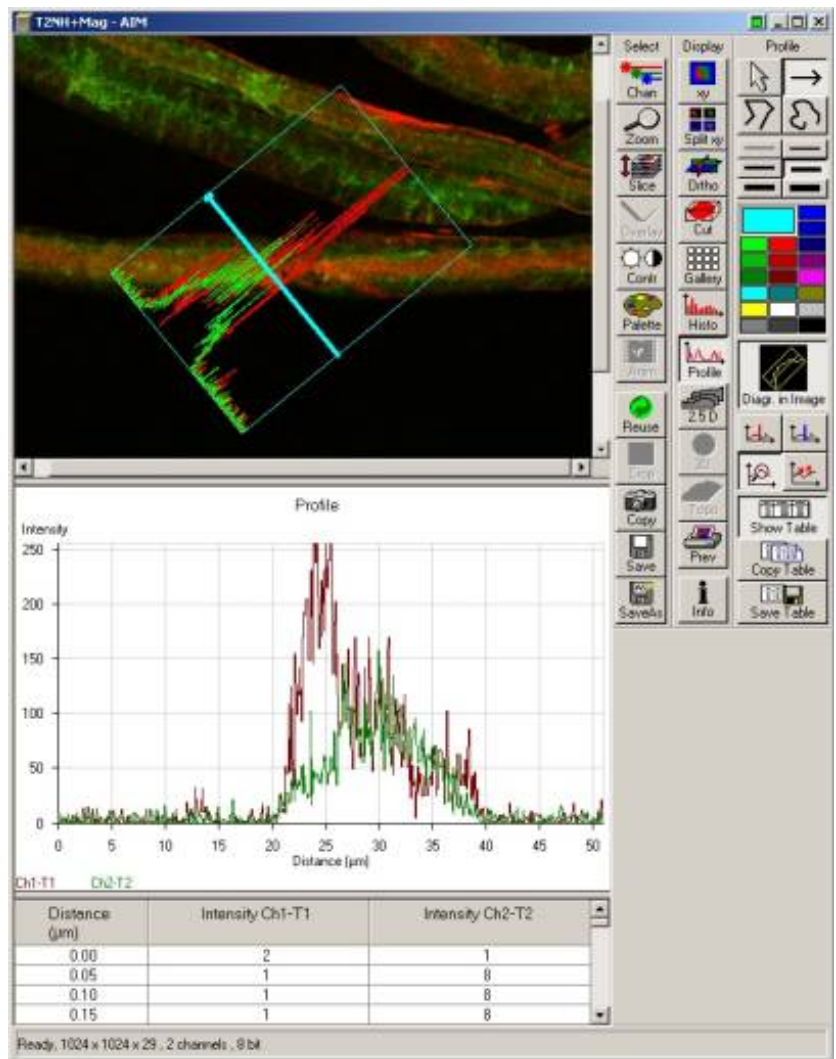
To display intensity across a line, click on Profile in the image window toolbar. Select a line thickness (1 to 6 pixels) from the menu to the right and drop it on the image to show the intensity profile plot. If more than 1 channel is present, a profile is shown for each channel. Click on Show Table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

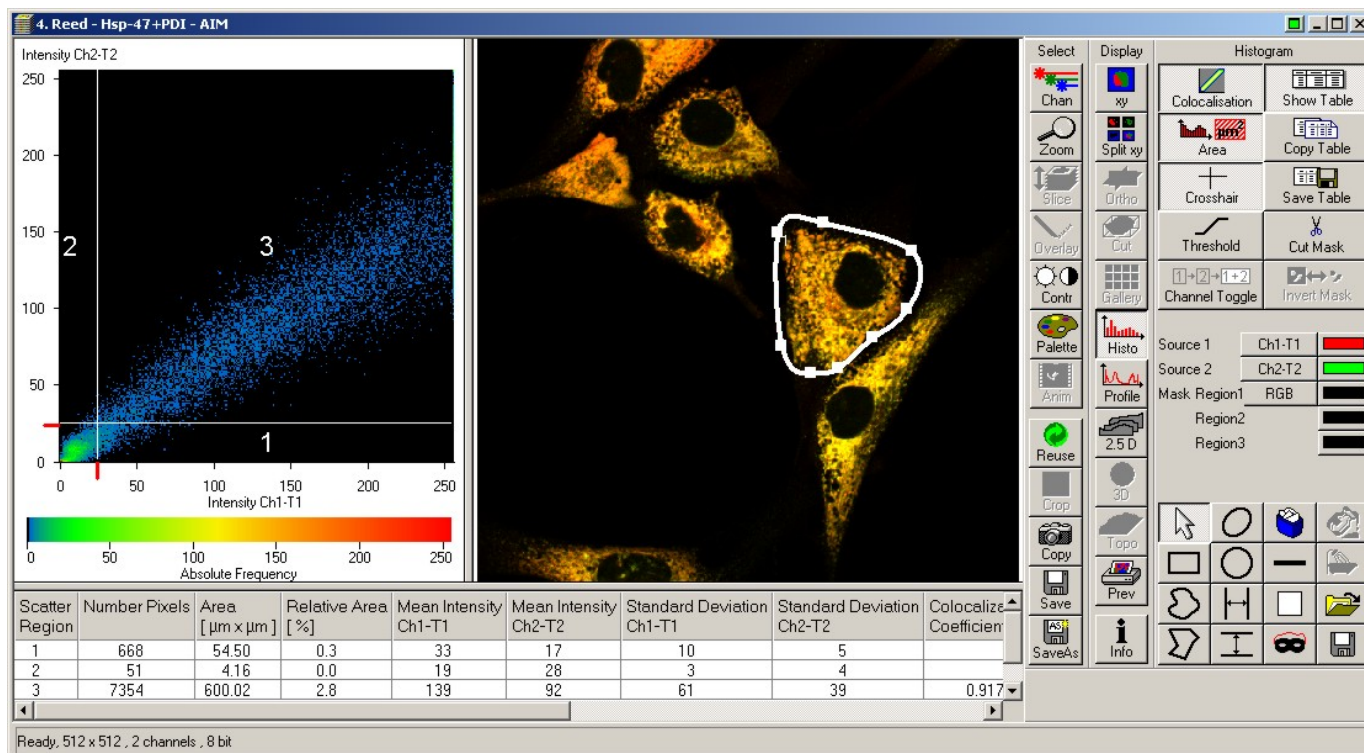


shown above the tool palette (lower right). For an image serie, the results may be viewed and saved by clicking on Show Table and Save Data. Use the high and low threshold sliders to threshold the image for the calculations.

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 for each pixel pair (i.e. identically located pixel on each channel). A perfect correlation (using twice the same image for ex.) shows on the scattergram as a perfect diagonal line. Realistically, the correlation between 2 channels shows as an elongated cluster of points with a slope varying as the overall 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 (top right) 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 several ways to quantitate colocalization. We recommend using 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

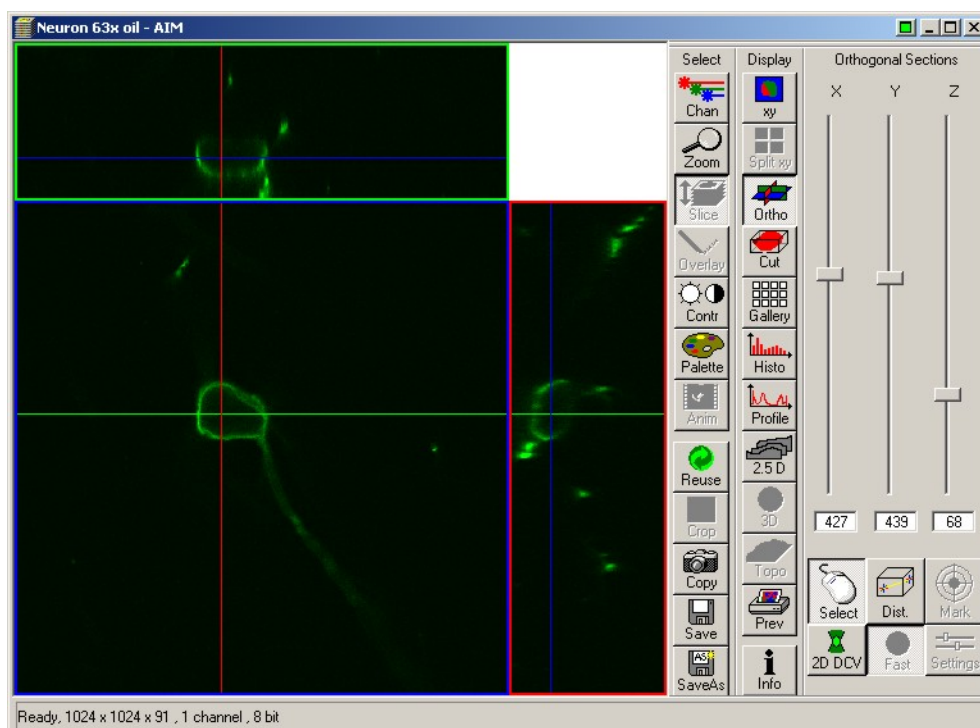


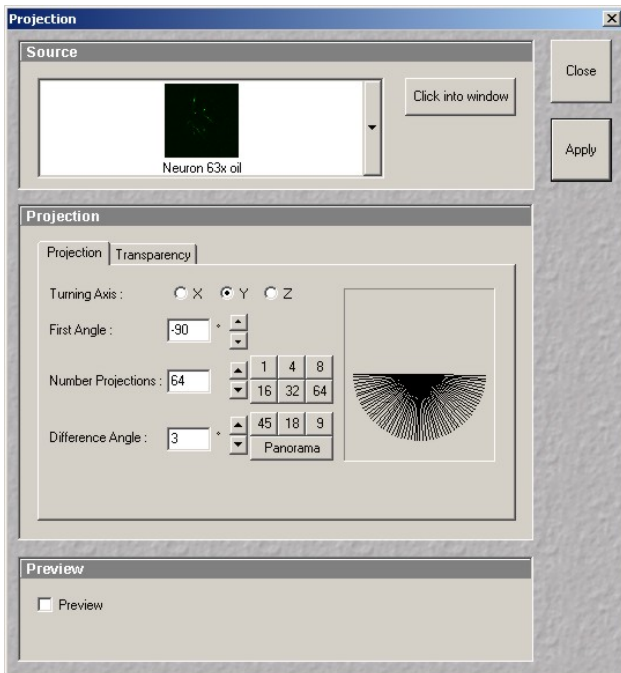
only be compared between specimen imaged and analyzed with the same parameters and threshold values.

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 panes, respectively. When the crosshair is moved, the top and right images are updated. Similarly, the top and right images have crosshairs controlling the other two panes. 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 displays a flat image as a pseudo 3-D object, with the intensity in the vertical dimension.

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 may be saved independently from the source image.

The Core also owns the full 3D rendering package from Zeiss, which allows for real-time volume rendering, rotation and zoom. This software is available on the two Dell workstations located in 5A10.

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. Only specialized software packages such as Metamorph, ImageJ and Volocity can import lsm files.

To convert individual images to a more generic tiff file format, select Export from the File main menu toolbar. Two options are available: a monochrome image or a color one. The default is color. Images recorded on the microscope are grayscale, not color. 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. A convenient alternative is to select Content Of Image Window, which exports images with the zoom factor at which the image is shown on the monitor. This option must be used when channel colors are other than red-green-blue (yellow, purple, brown...) or when annotations are desired.

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

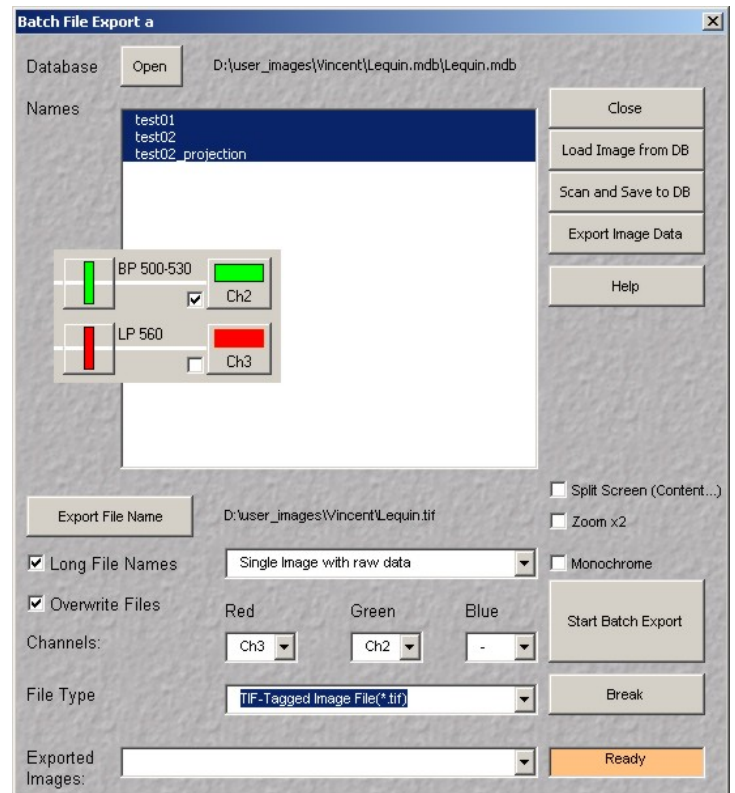
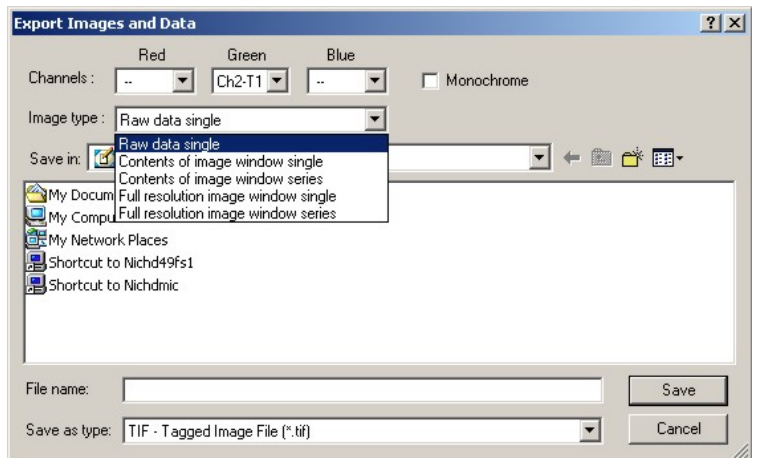


image with a channel (refer to the Configuration Control window to determine which channel is what: load your image and click on Reuse to restore the original configuration). Click on Export File Name and select a destination and a generic file name. Always use a different folder to avoid overwriting 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. The macro does not work when identical image filenames are used twice in the database, or when the same detector is used multiple times in frame switch configurations.

6.5 Transferring images

All 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) 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. Most computers on campus log onto the NIH domain. In that case, use your full name (John Doe with uppercases and a space) to log onto the MIC server.

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

We strongly recommend that users do not rely solely on the Core servers to safeguard their data. CD and DVD burning are available free of charge.

7) Turn off procedure

7.1) After saving the acquired images, open the Lasers control panel and turn all lasers off .

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 nosepiece to its lower position using the coarse focus knob on the microscope

7.4) Quit the LSM software and shut down the computer.

7.5) Shut down the system using both switches on the remote

7.6) Turn the Xcite lamp off

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

8) Troubleshooting

Error messages at startup

The LSM software cannot communicate with a component. Contact the M.I.C. staff for help.

No image in widefield fluorescence

Is the Xcite lamp on? Is there light coming from the objective? Did you click on the Vis icon in software?

No image on scanning

Is there light coming from the objective? Did you click on the LSM button before scanning? If not, a bug in software changes focus. Try reducing the scan speed. Check the gain and laser transmission settings. Is the condenser arm fully upright? A safety switch at the base of the arm shuts the lasers if the arm is tilted back. Due to a software bug, the lasers go on standby quickly even when the microscope is in use. Contact staff for a remedy.

Sample bleaching too fast

You may have left the scan speed at a needlessly high setting and increased the laser to get an image. Evaluate if the speed is truly needed and lower the laser accordingly.

Transmission imaging

Aug 07: as explained in section 3.5, DIC imaging on the Live DuoScan is severely limited. We do not recommend using it.

FRAP / Photoactivation issues

Is the adequate beam combiner in place? The T80/R20 is a good choice in most cases. Make sure the lasers are not on standby. If so, contact staff. Bleached region at the wrong location? Select the bleached area on an image displayed on the monitor with a zoom of 100% (software bug). Fine adjustments with the Image Match macro may be required. Due to instrument constraints, the 635 nm line is not available for bleaching

Calendar reservation

1) Got to our website (<http://mic.nichd.nih.gov>) and click on Reservations, 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.

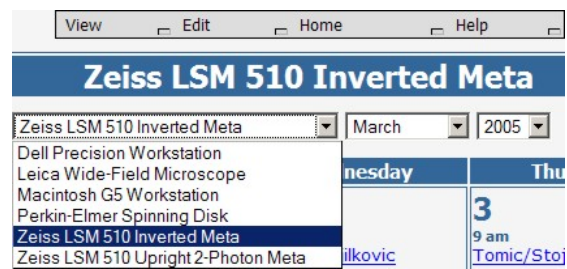
4) Click on a date to open the reservation page. Fill all the fields as shown:

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

The screenshot shows a web form with several tabs: General, Text, Recurrence, Resources, and Exceptions. The 'General' 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), 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 link 'Return to Calendar' is at the bottom left.

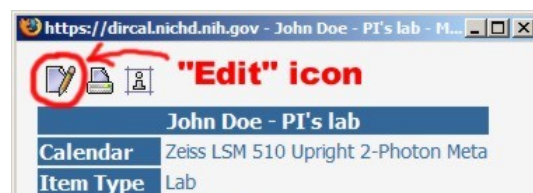


use 24-hour time (18:00 instead of 6:00 pm).

This screenshot shows the login page for the 'Connect Daily Version: 2.5.8 Web Calendar Program'. It includes a URL bar with <https://dircal.nichd.nih.gov/calendar/login.html>, the NICHD logo, and a login form with fields for 'User ID' (doej) and 'Password' (masked). There is a 'Remember Password' checkbox and a 'Login' button. A link 'Click Here' is provided for forgotten passwords.

Do not use anything other 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., Sep 2007. National Institute of Child Health & Human Development, NIH.

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