

**Zeiss LSM 510 META NLO User's Manual.**  
**Scientific Imaging- Fred Hutchinson Cancer Research Center.**

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Scientific Imaging

This manual is still under revision, and therefore may contain errors. Please consult with Scientific imaging staff if in doubt.

***Before you start:***

*Get proper training from Scientific imaging staff before you use the microscope.*

*To use the microscope after normal hours (after 5:00 PM and during week ends), you need to know the start-up, shut-down, and basic operation and maintenance procedures.*

*During normal hours, leave system ON. If you will be using the microscope for 4 hours or longer, you may power OFF the mercury lamp or any lasers that you do not need.*

*To extend laser life, put the Argon laser on Stand-by when not needed, or when done.*

*When working after normal hours, SHUT DOWN the system, UNLESS you KNOW that someone else will need it shortly afterwards. This generally means that someone else is SIGNED-UP, AND you have verified with them that they will be using the microscope. If in doubt, shut down the system.*

*ALWAYS LOCK DOORS after hours.*

# **Zeiss LSM 510 META NLO User's Manual, Part I.**

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## **I. Introduction.**

The FHCRC LSM 510 META NLO is configured around the Zeiss Axiovert 200M, a fully motorized inverted microscope. Control of the confocal system is done through a Pentium IV Windows 2000 platform and Zeiss LSM510 software. META stands for the META detector, a 32-PMT detector array capable of imaging the entire emission spectrum. NLO stands for Non Linear Optics (i.e. multi-photon).

### **Microscope:**

The Zeiss Axiovert 200M is a fully motorized inverted microscope. The microscope is equipped for transmitted light (including DIC), epifluorescence in the FITC and DAPI/FITC/TRITC channels (for quick sample finding and focussing), and confocal and two-photon microscopy. All microscope actions

(change of objectives and filters, etc) can be done either at the microscope (manually and/or by push-button), or can be controlled through the LSM software. Operating the microscope through the software interface is easier and is recommended for new users.

### **Objectives:**

A variety of objectives are available, including conventional air and oil immersion objectives, water immersion objectives optimized for confocal imaging, and long-working distance IR objectives for two-photon imaging. A chart listing all available objectives is available near the microscope. The microscope nosepiece carries six objectives. Other objectives can be installed if needed. Users are strongly encouraged to discuss their special imaging needs with the Scientific Imaging staff. Exchanging objectives requires re-configuration of the microscope; always consult with staff before swapping objectives!

### **Lasers:**

The Zeiss LSM 510 META NLO has three conventional lasers in the visible range, as well as a Coherent Chameleon Ti:Sapphire near-IR tunable pulsed laser for two-photon microscopy:

30 mW Argon (458nm, 477nm, 488nm, 512nm; note that the 458 and 477 are weaker lines; the 488 and 514 nm lines give about 10 mW output)

1 mW HeNe (543 nm)

5 mW HeNe (633nm)

1.4 W Ti:Sa Chameleon (tunable 720-930nm)

The visible lasers allow excitation of most common fluorophores from CFP to Cy5. The tunable IR laser allows excitation of a variety of fluorophores, including UV-excited dyes such as DAPI.

## **Detectors:**

The LSM 510 META NLO has two conventional detectors (PMTs), plus a spectral detector (META detector), for confocal imaging. The META detector can be used as a user-defined bandpass filter, as a multi-channel detector (up to 8 channels), or as a spectral detector (lambda scans). The META can be used alone, or in combination with other PMTs.

Two non-descanned detectors are available for two-photon imaging (two-photon imaging can also be performed using any of the confocal PMTs). Non-descanned detectors are inherently more sensitive, since photons are directly imaged without having to travel inside the scanner.

## **Special features:**

The LSM 510 META NLO is capable of imaging most fluorescent dyes, from UV-excited dyes such as DAPI (with two-photon excitation) to red excited dyes such as Cy5.

The LSM 510 can image samples in up to eight channels, can image the entire emission spectrum (spectral scans), can separate similar but distinct dyes with linear unmixing. Time-lapse imaging and Photobleaching are straightforward. The LSM 510 can collect images up to 2048x2048 pixels. A tile function in the stage control panel allows to collect images up to 4096x4096 pixels, by automatically scanning the stage. The Zeiss 510 also comes with a Multi-Time Series Macro, that allows to collect complex time-lapse sequences (for instance tiled stacks, or multi-point visiting).

A full Zeiss software suite is available off-line on our Dell Xeon imaging workstation. Software includes various 3D rendering modes, colocalisation, and 3D measurement functions.

## **Quick guide to image acquisition:**

The basic steps to acquire an image are the following:

- Turning ON the System and Software if needed (during normal hours, system will normally be ON).
- Turning ON all peripherals as needed (basically, the mercury arc lamp for viewing fluorescence through the eyepiece, and the Chameleon laser if needed for two-photon microscopy)
- Turning ON all required lasers through software
- Setting your sample (choosing objective, finding sample and focusing); this is done in VIS mode at the microscope (i.e. while viewing your sample through the eyepiece).
- Setting the microscope for confocal imaging (scanning); this is done by selecting LSM mode in software main menu
- Selecting (or creating) a "track" for your specific dyes. Two modes are available:

Single Track is designed for viewing one dye, or a limited number of compatible dyes; imaging in single track mode is done with all needed laser lines and all needed detectors ON.

Multi-Track is designed for running up to four distinct tracks sequentially. See note below.

- Optimizing your image (adjusting laser power and detector gain and offset)
- Setting the image parameters (image size, zoom factor, scan speed, averaging, z-sectioning)

- Collecting your image
- \* Saving your image to your personal database; Exporting files to your fred account or CD.
- Cleaning up microscope area and objectives. Shutting down the system if you are working after hours.

You can further view your images, and perform various image analysis tasks off-line, using Zeiss confocal software or other software such as ImageJ, Volocity, or Imaris, on our Dell workstation.

Note about tracks:

For example, you could have a single track configuration for fitc and Texas red, where the 488 and 543 lasers are ON, and you detect fitc on detector 2 and Texas red on detector 3. Other dye combinations are possible. For instance, fitc on channel 2, Texas red on channel 3, and Cy5 on Channel1 (or channel S, i.e. on the META detector). This is the fastest way of imaging multiple channels. The number/type of channels you can image in single track mode is limited by hardware configuration (i.e. available mirrors and filters in the scanner), and by cross-talk between channels (for instance, to view fitc and rhodamine simultaneously, you need to detect both dyes on a narrow band in order to get clean signals, therefore losing some of the signal intensity).

Multi track mode allows more flexibility, since you can combine up to four tracks. It also allows optimization of the detection of each dye; It is also slower, since tracks are done sequentially. Note however that it is often possible to collect a 4-channel image (or higher) with two tracks, with minimal cross talk, by doing for example DAPI and Texas red in one track, and fitc and Cy5 on a second track.

Please check with Scientific imaging staff for tips on how to design the most optimal configuration for your specific samples

Detailed procedures are described below.

## **II. Powering ON the System.**

During normal working hours, the system will be ON. Use the procedure below after-hours, week-ends, or if the system is OFF.

1. Turn ON mercury arc lamp if needed.

The simplest rule for Hg lamp is to leave it OFF unless you need it, but to leave it ON at the end of your session if it is still ON, so that the next users do not have to wait if they need it. If you are going to do a long time-lapse and do not need the Hg lamp, you may turn it OFF. This will save a few hours of lamp life.

The lamp has a dial that allows to regulate the amount of light reaching your sample. Use the lowest amount of light requires to protect your sample from photobleaching.

2. Turn ON system with the remote control switch on the monitor desk (powers microscope and Visible lasers). Allow enough time for system to initialize before starting software (about 30 seconds).

If you start the software too early, connection to hardware will fail, and you will have to quit and restart.

- 2b. If needed, turn ON the Chameleon (2 Photon laser) by turning the key switch on power supply from STANDBY to ON.

3. Generally, the PC will be ON. If needed, power ON computer; allow time for Windows 2000 to launch.

4. If needed, log in by pressing CTRL+ALT+DEL; Username: LSM\_USERS; Password: fhrc.
5. Start LSM 510 software by double-clicking icon.
6. Click SCAN NEW IMAGES (otherwise software will work as a viewer only)
7. Click START EXPERT MODE. (if launching fails, e.g. because not enough time was allowed for system to initialize, quit software and restart. If you encounter problems, you may need to log off and log back on again with CTRL+ALT+DEL)

Turning ON the lasers:

8. Select ACQUIRE>LASER

(will open Laser control panel)

9. Select the lasers you need:

Click STANDBY for Argon (458, 488, 512); and allow to warm up. When "ready", click "ON" and set current to ~6 Amp. (While you wait for the Argon laser to warm up, it is a good time to set up your database; see below)

Click ON for HeNe 543 and/or 633 as needed

Click ON Ti:Sa if needed. (Will not turn ON laser, but just connect the software to the laser).

10. Close Laser Control window.

**Setting up your database:**

Zeiss software stores files in a database format. A database is basically a folder or directory that contains all the images you collect. In addition to the image files, the folder also contains a "database" file. Zeiss software can read information from the database file, and can display all the images in a database format. Image files in a database can be browsed, copied and pasted between databases, and various information about image acquisition is displayed. If you copy an image from one database to another through Zeiss LSM software, both databases will be updated. If you move image files between databases through windows or Mac, files will be copied and pasted, but database will not be updated. You can always view individual files (i.e. not inside a database) using the "Import" function in Zeiss software. ImageJ and other imaging software such as Volocity can also read individual files.

Temporary user's databases are created on Drive D:/. We recommend you create a new database for each session. You could reuse an older database, and keep saving new images to it. However, we do not recommend this, as we would run out of space rather quickly if everybody stored their files on the PC. You can however store a reference database on drive( E:). In this manner, you could recall all the settings used for collecting a given image at a later date. Please keep your reference database below 1 GB.

Two methods to save your images are described at the end of this tutorial. Below is a quick start method.

11. In the Main Menu, select "FILE".

12. Click "New". In the "Create New Database" window, select drive D:/

13. Under "File Name" enter a name for your database (for example your fred username and date "jdoe\_052304"). Click "Create". A new (empty) database will be displayed. Close it.

14. Go to OPTIONS>SETTINGS in the Main menu. In the new window "Settings for user LSM\_USERS", click the AUTOSAVE tab.

15. Choose "USE LSM IMAGE DATABASE AND AUTO INCREMENT IMAGE NAME".

16. Under DATABASE, select your newly created database.

17. BASE IMAGE NAME: enter desired file prefix (e.g. somefile\_). Set "Counter" to 1. Click OK.

Every time you collect an image, you can save it by just clicking "Save". The software will use your file name, and increment the file name by a value of one each time you save. Each file will be saved inside the specified database.

### **III. Viewing specimen at the microscope (VIS mode).**

#### **Selecting the Visible light path configuration**

1. Click ACQUIRE on main toolbar.

2. Click VIS on the main menu bar.

3. Click on the MICRO button to open microscope control panel.

4. Select objective on the microscope control panel ( or by using the objective up and down buttons at the microscope). If using an immersion objective, apply a small amount (~10 microliters) of the appropriate immersion fluid to your sample (double check objective chart) and place sample on stage with coverslip facing down.

Note:

Please gently push back the condenser stand before placing/removing samples on the stage. This will reduce the risk of scratching the front condenser lens. Hold the condenser tower by its metal rod handle on top, not by grabbing the condenser, lamp housing, or PMT.

## **Viewing your specimen in transmitted light**

1. Click VIS and MICRO on main menu bar to open microscope control panel if not already open.
2. Click the "VIS" button. This will automatically set the microscope for transmitted light viewing.

( reflector turret will be set to NONE

TRANSMITTED LIGHT will be turned ON automatically. If not, or if you want to turn it OFF, use the HAL lamp button on the left front side of the microscope)

3. Select an Objective if needed. Consult chart and/or objective for immersion fluid compatibility. Normally, oil objectives will have a red hair band near top, multi-immersion objectives an orange/yellow one, and water objectives may or may not have a blue band.
4. Find specimen. Use Joystick for fast x/y translation of the stage. For slow translation, press "X" or "Y" button, and rotate wheel.
5. Adjust light intensity with front switch if necessary.

## **Viewing your specimen in DIC (Nomarski).**

1. Click VIS and MICRO on main menu bar to open microscope control panel if not already open.
2. Click the "DICII" or "DICIII" button. This will automatically set the microscope for DIC viewing. Most objectives will work with DICIII. Use DICII for objectives  $\leq 10\times$  or  $NA \leq 0.7$ .

( reflector turret will be set to "Analyzer D"  
Condenser DIC prism II or III will be selected; you can view/change settings  
by clicking the "condenser" button in the micro control panel)

Note: Objectives up to NA 0.7 generally use DIC II, Higher NA's use DIC  
III, Consult objective chart on wall. You can always try out DIC II and/or  
DIC III and see which one you like best.

A more detailed description of DIC, including getting a DIC image, are  
described in Part II of this tutorial.

### **Viewing your specimen in Fluorescence mode.**

**WARNING:** be familiar with and follow the operations below. Always select  
the light path and filters BEFORE looking through (as opposed to WHILE  
looking through) the eyepiece. Because of the microscope configuration, it is  
possible to expose your eyes to bright mercury arc lamp light if you do not  
follow proper procedures.

1. Click VIS and MICRO on main menu bar to open microscope control  
panel if not already open.
2. Select the appropriate filter set ("reflector") by clicking the "FITC" or  
"DAPI\_FITC\_TRITC" button:

Filter set #09: FITC/GFP

Filter set #25: DAPI/FITC/TRITC

We also have DAPI and TRITC filters that can be installed if needed.

Always minimize exposure of your sample to light. Use arc lamp dial, and/or  
microscope epifluorescence aperture diaphragm, to attenuate light intensity.

If you do not see anything, press Hg lamp button on the right side of  
microscope or make sure Hg lamp is selected and ON in MICRO control

panel. Make sure Hg lamp is ON. Make sure the manual shutter in front of the Hg lamp fiber optic port is open.

4. Close the microscope control panel. View your sample.

5. Click LSM on main menu bar to start scanning mode.

Note: Most microscope operations can be controlled both with the LSM510 software, AND at the microscope. The buttons on the Axiovert 200M are well labeled, and generally self-explanatory. Pressing most buttons will display the specified action or setting on the microscope's LCD screen. Consult with Scientific Imaging staff if you are unsure about the microscope's operation. Generally, controlling the microscope via software is a bit easier for new users.

Note that if objectives were switched, and the switch was not recorded in the software, the actual objective may not match the objective displayed in the LCD screen. Know your objectives and use common sense.

#### **IV. Acquiring a confocal image (LSM mode).**

Before starting, make sure you know how to save images. If necessary, read the procedure for saving images at the end of this tutorial.

Note: the confocal can be used in SINGLE TRACK (one or several channels acquired SIMULTANEOUSLY), or MULTI TRACK, which consists of one or several sets of single tracks, acquired SEQUENTIALLY.

For simplicity and maximum versatility, you may want to do all your imaging in MULTI TRACK mode. Single track mode will be explained first.

#### **Selecting filter configuration in single track mode.**

0. Select ACQUIRE and LSM in main menu bar to switch to scanning mode.

1. Select CONFIG on main menu bar

2. Select CHANNEL MODE. This mode will set the META detector to act as a standard channel.

3. Select SINGLE TRACK in CONFIG window. Also make sure that CHANNEL MODE is selected.

(A "track" can be a single channel or set of channels that are acquired simultaneously with a specific hardware configuration (e.g. filters and dichroic mirror sets).

4. Click CONFIG button on right side of CONFIG window. This will display a menu window.

5. Click down arrow to view track list menu. Select an appropriate configuration if available. If your favorite configuration is not listed, make your own (see below), or ask Scientific Imaging staff to make one for you.

6. Click APPLY. This will configure the scanhead filters and mirrors for your dyes.

Notes:

Once you have a configuration that works for you, save it with a personal name, and use it in the future. You can modify your own configurations.

**PLEASE do not modify configurations that do not belong to you!**

it is preferable to store only a limited number of configurations in the Lsm\_users account. Users may, however, store sample images in a reference database. This images can be used to recall specific configurations and settings for re-use. Consult with Scientific Imaging staff for preferred practices.

## Setting the scanning parameters.

**Note: DO NOT change hardware settings while scanning (i.e. mirrors, image size, zoom, etc...). ALWAYS STOP scanning before you modify those settings. You may however change laser power and PMT settings while scanning (e.g. to optimize image acquisition).**

1. Select SCAN in main menu bar to open scan control panel.  
(make sure LSM and ACQUIRE are selected on main menu bar)
2. Select CHANNELS.

Under CHANNEL SETTINGS, adjust settings for each of your channels (display color, pinhole size, etc...):

### 2.1 Pinholes:

Typically, set pinhole size to 1-1.5 Airy units. This gives the best compromise between image brightness and resolution. A larger pinhole will collect more light, but will also generate thicker optical sections.

If you are imaging multiple fluorophores, you may set individual pinholes to achieve optical sections of comparable thickness. This is especially important if you are planning to do co-localization studies. Longer wavelengths will require smaller pinholes. The procedure for matching pinholes for multiple channels will be explained under "collecting z-stacks".

### 2.2 Laser attenuation:

Under EXCITATION OF TRACK, set TRANSMISSION % for the laser lines you need. Use these values as a first approximation:

458nm: 12%

488nm: 5%

514nm: 12%  
543nm: 50%  
633nm: 15:

Ti:Sa (2 Photon) : ~3% **WARNING: DO NOT** exceed 5% for the Ti:Sa laser without consulting with staff!

For a normal sample, these laser settings should be sufficient to get a good image.

With experience, you will learn what the appropriate values are for your particular samples.

3. Select **MODE** and **FRAME**. Make sure "z-scan" is not selected.

3.1 Under **OBJECTIVE LENS & IMAGE SIZE**, select field size in pixels. For setting up scanning parameters, use 512x512 (or smaller). For imaging, click **OPTIMAL** to see what the software recommendations are. Find the best compromise between resolution, image size and scanning speed.

Image size determines the number of pixels sampled, **WHILE KEEPING THE SAME FIELD OF VIEW**. Increasing the number of pixels increases the size ("resolution") of the **IMAGE**, but does not necessarily increase the true resolution (which is determined by the objective and wavelength). For any combination of objective and zoom factor, there is **ONE** optimal image size. If you click "**OPTIMAL**", the software will display the optimal image size for a given objective and zoom factor. Note that if you zoom, the optimal image size will decrease by the same factor).

3.2 Under **SPEED**, select scan speed (laser dwell time). Reasonable values are in the 1.6-6 microsecond range.

Choosing a lower speed increases the dwell time (duration of laser excitation for each pixel); i.e. you get better images with less noise (but also potentially more bleaching and longer imaging times). To reduce noise, it is recommended you increase the laser dwell time to about 6 microseconds (if

necessary); if you need further noise reduction, use averaging. To protect your sample, use the lowest pixel dwell time and averaging that gives a satisfactory image.

3.3 Under PIXEL DEPTH, SCAN DIRECTION & SCAN AVERAGE, chose appropriate settings.

Generally, 8-bit (256 levels of grey per channel) is fine. Increase to 12 bit (4096 levels) for publication quality images if you find that image quality is improved. Using 12-bit also increases file size (and does not necessarily add much information to your image if you have poor signal to noise).

Unidirectional scan may be more accurate, but also slower. If you chose bidirectional scan, click "CALIBRATE" to align the tracks. This will align the scan lines (works if your sample has good contrast; if sample is poor, use unidirectional scan, or adjust manually).

Averaging increases the duration of image collection, but also improves the image (better signal-to-noise ratio). For weak samples, use sum instead of mean. For moving samples, use a line averaging. Otherwise, frame averaging may cause less bleaching.

Notes:

All the parameters above affect the quality (resolution) of your images, but also the bleaching rate of your sample, and file size. The best settings generally are those where you sample at about 2-3 times the resolution of the objective (i.e. pixel size equal to 1/2 or 1/3 Airy).

4. Select FIND to let software determine the gain and offset settings for the detectors (PMTs). This will open an image window, and scanning will start. The active window is overwritten every time you do a scan, unless you save it, or open a new window by clicking "NEW".

a) Start scanning mode by using FASTXY or CONT (Under SCAN CONTROL).

"Continuous" will do live scanning with your set parameters. "Fast" will use factory-set parameters.

If you use "Fast Scan", software may change some of the scanning parameters, so you may be better off using "Continuous" rather than fast. Also, adjustments may be more accurate in this mode, as you will be collecting your image at your actual image settings; the drawback is that scanning may be slower; hence the necessity to adjust your image using a small image size (512x512) and no averaging). Once your image is optimized, you can change the size and the averaging without affecting the image brightness.

b) Move stage with the remote joystick/wheel. You can also move stage through software by clicking the arrows under STAGE POSITION. Focus your sample.

(to optimize your image, see below)

c) Click STOP when done.

6. Click SINGLE to collect a single image at the current settings.

7. Image display options:

a) SPLITXY displays a composite image with each channel shown separately, as well as a merged image.

b) XY shows a single large image with all channels.

c) CHAN allows you to turn individual channels ON/OFF, and select display color.

## **Optimizing detector settings.**

Whether in single track or in multi track, this is best done one channel at a time. (i.e. only one laser line active, and one detector). This will increase speed, and reduce bleaching of sample. Therefore, deselect laser lines if you are in single track with multiple channels, and/or deselect individual tracks in multi track mode.

1. Open a new image window (NEW) and click FIND.
2. In the Image Window, select PALETTE.

Under COLOR PALETTE LIST, select RANGE INDICATOR. This LUT will show out-of-range pixels: zero values in blue, 255 values in red.

3. Start "Continuous" scan and adjust DETECTOR GAIN until few or no red pixels remain. Adjust AMPLITUDE OFFSET until most blue pixels are just gone.

Note: if the image is weak, you may use a higher AMPLIFIER gain. This will boost the image intensity (but will also increase noise). If you can not adjust the image with the PMT settings, you may need to increase/decrease laser power.

4. Select NO PALETTE to return to normal channel display.
5. Run a SINGLE scan.
6. Repeat for all channels/tracks.

## **Zooming.**

The scanner zoom allows users to "zoom in" on a region of interest and collect a magnified image. This is done with the "crop" function in the image

window, or by adjusting the zoom settings in the Scan control window (Mode panel). Zooming does affect image resolution, and also sample bleaching. There is usually ONE optimal zoom setting for a given objective and image size, as described above. For some objectives, you may need to zoom to obtain an optimal image (i.e. to get all the resolution the objective can provide).

The CROP function is used to zoom in on a region of interest.

0. Collect an image.

1. Select CROP in the image window. A square frame with red and blue lines will appear. The blue line defines the top. The red lines define the other three sides of your new image.

2. You can adjust the shape/size of cropping frame with the left mouse button (within certain limits imposed by the hardware). To move the entire frame, drag inside the frame. To change size, drag the corners. To rotate frame, drag on the frame axis. To adjust height or width, drag edges. For each of these actions, cursor will take a specific shape.

The new image size and zoom settings will be updated in the SCAN CONTROL window (MODE panel).

3. Click SINGLE to scan the defined region.

Note: the crop function adjusts the zoom setting and scanning mirrors to collect a specific field of view. If you resize and/or rotate the imaging area, your image size parameters may no longer be optimal. Resizing and rotating image area may also change some of the scanning parameters such as scan speed. Therefore, you may need to slightly readjust your image.

## **V. Acquiring a Z-series Stack.**

Note: Make sure that you have adjusted the pinhole for optimal Z-sectioning (1-1.5 Airy units) before setting parameters for z-scanning. The procedure for matching pinholes will be given at the end of this section.

### **Method 1.**

(in this method, you define the bottom and top of your sample by focusing while collecting a live image).

1. Click Z-STACK to open the Z-stack collection control panel.
- 1b. Click "Z-settings" (this button is active only if you selected Z-STACK).
2. Click MARK FIRST/LAST.
3. Click "XY cont" (or FAST XY) to start continuous scanning. Use fine focus knob to find one edge of your sample.
4. Click MARK FIRST to define the beginning of your stack.
5. Focus in the opposite direction until you find the other edge. Click MARK LAST.
6. Click STOP to stop scanning.
7. Click ZSLICE to open the OPTICAL SLICE control panel.
8. Click OPTIMAL INTERVAL and close window. This will set your focus steps to the optimal value. You can always enter a different value if needed.
9. Click START to perform Z-scan.

Note: While doing a live scan, you can focus with the fine focus knob. Alternatively, you can open the STAGE CONTROL panel, and click the UP and DOWN arrows to focus (you can define the step size).

## **Method 2.**

(in this method, you perform a cross section (xz scan) through your sample to find the top and bottom).

Find range.

1. Click Z-STACK to open the Z-stack collection control panel.
2. Click on LINE SEL. This collect an xy image in a new window.
3. Click on the arrow tool. Drag the center line over the image to define the line you want to scan. Chose an area with good signal, if possible in the thickest part of your specimen.
4. Click Z-SECTIONING tab.
5. Set INTERVAL to 1 micron and # SLICES to 1.5 times the approximate thickness of your sample in microns (or start with 20-50 microns if you have no idea). Otherwise, it will collect using the previous z settings.( For thick samples and faster settings, use a larger interval (e.g. 5 microns) and reset interval to an optimal value before acquisition) .
6. Click RANGE to begin xz-scan.
7. Choose desired interval. Select KEEP INTERVAL.
8. Move red lines to desired start (top) and end (bottom) positions . If your sample is too thick (beyond range), increase the number of slices and redo an xz scan (RANGE). Use C button to center the stack.
9. Use the FIRST, MID, and LAST buttons to view the specified sections. Readjust if necessary.
10. Click START to collect Z-stack.

Note: It is often most convenient to use the MARK FIRST/MARK LAST to find the overall settings. Later, you can use the Z SECTIONING method to fine tune the stack for different parts of the sample.

### **Matching optical sections of multiple channels.**

A pinhole of 1 Airy unit provides the optimal compromise between collecting enough light from your section, and removing out-of-focus light. The pinhole diameter also affects the thickness of the optical section you are collecting. Longer wavelengths offer less resolution. Therefore, for a pinhole of 1 Airy unit, a FITC section will be thinner than a Rhodamine or Cy5 section. For weak samples, you may use up to 1.5 Airy units. This will collect about 10-15% more light, without noticeably affecting resolution. At larger pinholes, your confocal sectioning will start degrading (i.e. you will start getting thicker optical sections).

To match the thickness of optical sections for multiple dyes (e.g. for colocalization studies). you need to adjust the pinholes accordingly:

1. in the SCAN CONTROL window, select Z-STACK and Z-SETTINGS.
2. Click on Z-SLICE; this will display a window that gives a graphic representation of the optical sections for all channels selected.
3. In SCAN CONTROL window, select channels. Adjust pinholes and see the effect on the optical sections.
4. Match the thickness of the sections manually by adjusting the different pinholes. Start at  $\sim 1$  Airy unit for all channels, and increase pinhole size for shorter wavelengths (you could also decrease it for longer wavelengths, but you will lose a lot of light and is therefore not recommended).

## **VI. Acquiring a Time Series.**

Before collecting a Time Series, you need to set all parameters for collecting either a single xy section, or a z stack. The TIME SERIES macro will apply to your current scanning settings.

1. Select ACQUIRE>TIME SERIES
2. Under START SERIES, select manual (or use TIME to set a pre-determined start time)
2. Under STOP SERIES, enter number of time points
3. Under TIME INTERVAL, select unit and number of units for the intervals between your time points. Click APPLY.
4. Click START.

Note: A DELAY is the interval between the end of the first scan (e.g. stack) and the beginning of the new scan. An INTERVAL is the interval between the start of the first scan and the start of the next scan. Under OPTIONS>TIME SERIES, you may chose INTERVAL instead of DELAY.

## **VII. Photobleaching a region (FRAP).**

A photobleaching experiment is a standard time series, with an additional bleaching step either at the beginning of the series (Method 1) , or at some arbitrary position between the pre-bleach and post-bleach frames (Method 2).

### **Method 1.**

1. Select ACQUIRE>TIME SERIES. Define all parameters as indicated in

## Section VI.

2. Select "EDIT BLEACH" in main menu
3. Click DEFINE REGION. This will open a BLEACH REGION control panel.
4. Under INTERACTIVE ROI DEFINITION, choose desired shape, and draw shape in your sample image.
5. Under BLEACH PARAMETERS, choose # ITERATIONS (number of bleach scans), for instance 50.
6. Under EXCITATION OF BLEACH (Bleach Control window), adjust laser transmission for the bleach step. (experiment to find parameters that provide good bleaching within the ROI, with minimal effect outside ROI). Typically, you will use 100% for the 543 and/or 633. The Argon laser is stronger, so you may want to try somewhere around 30-50%, as a start.
7. To bleach your region, click BLEACH in the BLEACH CONTROL window. You can see the laser scanning (no image will be collected while bleaching).
8. Run your time series.

### **Method 2.**

Add Step 5b to the procedure above:

- 5b. Under BLEACH PARAMETERS, click BLEACH AFTER NUMBER OF SCANS. Fill in the number of control (pre-bleach scans).

This number will also be counted as part of the total number of time points in the time series settings.

in Step 7, click STARTB in the TIME SERIES CONTROL window. This will start your time series, and will include your bleach routine after the specified number of scans.

## **VIII. Saving your images.**

Images are saved on the [D:] drive. At the beginning of a session, create a database in drive D, and then save each new image into that database. At the end of each session, users should transfer their files (or TIFF/QT movies) to their fred/ial account. Databases older than six months will be deleted by Scientific Imaging staff.

In addition to the working database, users may create and keep a database on drive E:\LSM\_USERS. After a confocal session, it is possible to transfer specific files (or portions of files) from the working database to the reference database. This database is for the purpose of storing reference sample files, in order for users to reapply the same imaging settings to future confocal sessions. The size of these individual databases is limited to 1GB per user. Scientific Imaging may decide to reduce this size in the future if the E:\ drive becomes cluttered, or if additional storage space becomes necessary.

To copy files between databases, use Zeiss LSM software. If you just open the folders and move files around in Windows 2000, the Zeiss software will not update the information. Your files will be OK, but the database will not.

Method 1. Save each image individually.

For the first image (if you have not already created a database):

1. Collect one image.
2. In the image window, click "Save As"

3. In the window that appears ("Save Image and Parameters As"), click "New MDB"
4. In the "Create new database" window, navigate to drive D:\
5. In the field "File name", enter your a name for your database (e.g. your fred username); Click "Create"
6. In the window "Save Image and Parameters As", select your newly-created database (it should be selected already).
7. In the field "Name", enter the new file name (we recommend you use the format ddmmyyuu\_01, such as 021904jd\_01, etc..., but you can use anything you want).
8. (optional): you may enter additional info for your reference in the fields "Description" and "Notes".
9. Click "OK".

For the next images (or if you already have a database):

1. Collect your image
2. Click "Save as"
3. Select your database in the pull-down menu
4. enter a file name in the field "Name".
5. Click OK.

Method 2. Autosave.

1. Create a new database if you do not have one already. Close it. If you

already have one, go to 2 (close it if it is open).

2. Go to **OPTIONS>SETTINGS** in the Main menu. Select the **AUTOSAVE** tab.

3. Choose **"USE LSM IMAGE DATABASE AND AUTO INCREMENT IMAGE NAME"**.

4. Under **DATABASE**, select your personal folder (**USERNAME**)

5. **BASE IMAGE NAME**: enter desired file prefix (e.g. **mmddyuu\_**). Click **OK**.

6. Each file you collect from now on can be saved by just clicking **"Save"**. The Software will automatically increment the index digits.

Note: You could record the autosave settings at the start, without having to collect an image first and saving it. However, this makes it easier for you to find the right drive for your database, since it will already be selected when you go to the **"Autosave"** settings.

If you use the **"Autosave"** mode, click **"Save as"** if you want to add comments to your files.

## **IX. Exporting your images.**

1. Click **FILE** on the main menu. Click **OPEN**.

2. Navigate to your database (**AIM\_DATA** (**D:**)/username/userdatabase.dbm).

3. Open your database. Select the file you want to export. Open the file.

4. Click EXPORT.
5. Select: Full Resolution Image Window Single for a single section, or Full Resolution Image Window Series for a series (z-stack, time-lapse,...).
6. Select or create a folder for your files.
7. Select the file type you want to generate (e.g. TIFF, QuickTime movie, etc...).
8. Click Save.
9. Repeat for all your files.
10. Transfer your files, original and/or exported, to your ial folder in your fred account, using the "Fred Users" link on the Desktop.

Note:

Zeiss has a batch export utility that allows to automatically save all, or a subset of files from a database. Check with S.I. staff for instructions on how to run the macro.

## **X. Shutting down the system.**

Generally, during normal hours (9:00 AM- 5:00 PM), leave the system ON. After normal hours (evenings and week ends), leave the system ON ONLY if you KNOW that someone else is going to use the microscope (someone is signed-up AND you have verified that they are coming). If you are in doubt, shut down the system.

During normal hours:

**When your session is over:**

1. Close all open windows;
2. Select ACQUIRE; Open the laser control panel; select the Argon laser and set to "Standby" (unless there is someone already waiting for the microscope).
3. EXIT LSM software. A Dialog box will appear warning you that the system should not be shut down while lasers are ON. Click OK. (This is optional; you may leave LSM software running).
4. Wipe clean all the immersion objectives you used with lens tissue (DO NOT use the same tissues for dry, water and oil objectives!). Clean up the microscope and monitor area.
5. Log your use time and billing information.

**After Hours (Shut Down Procedure):**

1. Open the LASER CONTROL control panel in the main menu.
2. In the software, turn OFF all lasers.
3. Close all open windows. EXIT LSM 510 program.
4. If you used the Chameleon, switch the key from ON to STANDBY on the Power Supply under microscope table.
5. Wipe clean all used immersion objectives with lens tissue. Clean up the microscope and monitor area.

Note: you should clean immersion objectives as soon as you don't need them any longer (for example, if you are switching to a different objective). Any Oil left for too long on an objective will eventually end up in the wrong places and will be costly to clean up.

6. Log your use time and billing information.

7. WAIT for Argon laser to go OFF by itself (fan will go OFF, and purple light will go OFF); this will take less than five minutes after you turn laser OFF with software.

8. Once the Argon laser fan has stopped switch OFF the remote control. (If you really are in a hurry, leave system ON, rather than shutting it down before laser has cooled down).

9. Turn OFF the Mercury arc lamp, if used.

**Warning: The remote control shuts down the entire system, including the laser power supply. Never turn OFF while Argon laser is still running!**

Revised 06/22/04

## **Zeiss LSM 510 META NLO User's Manual. Part II.**

**Specialized topics. This tutorial assumes you are already familiar with the basic acquisition techniques described in part I.**

### **Contents of Part II:**

- I. Scanning in Multi-Track Mode**
- II. Collecting z-stacks in Multi-Track mode**
- III. Collecting a DIC image**
- IV. Using the META detector**
  - 1. Channel Mode**
  - 2. Lambda Mode**
  - 3. Linear Unmixing**
  - 4. Online Fingerprinting**
- V. Collecting Two-Photon Images**

### **I. Scanning in Multi-Track mode.**

The Zeiss 510 Single-track mode allows the collection of images in one single channel, or the simultaneous acquisition of several channels with a single hardware setting. This means a single primary beamsplitter for all channels, and simultaneous excitation with all laser lines used.

In some cases, such as FITC/Cy5, this will give good results. In other cases, however, there may be substantial cross-talk (bleed-through) between channels. In addition, multiple-channel beamsplitters may not be optimal for certain dyes, or you may have laser line reflections in some channels (e.g. the 453 laser line may show up in the FITC channel). Finally, multiple channel beamsplitters may not be available for specific combinations of dyes, making it impossible to image those two channels simultaneously.

The Multi-Track mode allows one to collect different channels sequentially,

and therefore optimally and without (or with minimal) cross-talk. This mode of image collection is more efficient and accurate, but slower, than the Single-Track mode. If only laser lines and PMT settings are switched between channels, channels can be collected on a line by line basis (line mode), or in fast frame mode. This will be relatively fast. If hardware is also switched (such as beamsplitters), then the channels can be switched only on a (slow) frame by frame mode. This will be substantially slower.

The best approach is to create, optimize, and save your favorite single tracks in single track mode (each track can be one or more channels). Then, in multi-track mode, you can load up to four single tracks (for example DAPI/FITC/Rhodamine/Cy5), save that configuration with a new name (e.g. "myname\_4-channel"). When you use this new multi-track configuration, you can turn individual tracks ON and OFF. For instance, you can use your DAPI-FITC-Rhodamine-Cy5 configuration to collect only FITC and Rhodamine.

Alternatively, you can load up to four blank tracks in multi-track mode, name them, configure each of those manually, and save the multi-track configuration.

Below, we describe how to set up multi-track configurations. However, we strongly recommend that users do not create their own configurations without consulting with Scientific Imaging Staff, until they are thoroughly familiar with the microscope. This will prevent cluttering the configuration menu, and possibly the overwriting of better configurations. If specific configurations are needed, we will help users create and optimize them.

You can load a suitable multi track setting, rename it with a personal name, and save it. You can then reuse, and/or modify, those settings without destroying the original settings.

**Note: None of the configurations on the Zeiss 510 are write protected (i.e. they can be overwritten, and therefore, lost). therefore, USE CAUTION before you re-save a configuration, to avoid overwriting a stored configuration.**

Another option is to save sample images in your individual reference database on drive E:/. Reference images can be loaded, and the specific configuration used for the collection of those images can be loaded and reused. This is a convenient and safe method for storing your configurations. This method has the benefit that all settings used for a particular experiment can be recalled, making it really easy to reproduce an experiment, without the risk of overwriting a perfectly good configuration.

**Again, we strongly recommend you consult with Scientific imaging staff to create an optimal configuration for your samples.**

Loading a Multi-Track configuration.

1. Set up the microscope for scanning. Make sure Channel Mode is selected, not Lambda mode. This will ensure that the META detector is used as a regular channel. In the CONFIGURATION CONTROL window, select MULTI-TRACK. The previous Multi-Track settings will be loaded.
2. On the right side of the Configuration window, click the "Config" button. This will display a menu window.
3. From the menu, select the desired configuration (e.g. "DAPI\_FITC\_Rhodamine\_Cy5"). Click "Apply".
4. The selected configuration will be loaded. Individual tracks (with either one or multiple channels) will be listed in the Configuration Control window, under "List of Tracks". The "Line" button will be depressed if the tracks can be switched on a line by line basis. The "Frame" button will be depressed if the tracks are switched only on a frame by frame basis. Fast Frame mode can be used as an alternative to Line mode.

**Caution: If you have a set of tracks in normal (frame) mode, and you click on "Line" or "Fast Frame", tracks will be overwritten and may become useless. Consult with staff if you are unsure.**

5. You can activate or deactivate individual tracks by checking their individual check boxes on the left. The track currently selected is highlighted (you can select other tracks by clicking on any specific line). The hardware configuration displayed in the lower part of the Configuration Control window applies to the currently selected track.

6. To run a scan, open the Scan Control window ("Scan" button on main menu bar). The currently selected track will be displayed under "Channels" in the Scan Control window. To display a different track, select it in the Configuration window, or click its button in the Scan Control window. Only active (checked) tracks will be listed in the Scan Control window.

7. Adjust imaging parameters as for single tracks. **It is HIGHLY recommended to adjust the settings one track at a time (i.e. uncheck all other tracks). This will reduce the rate of bleaching of your sample, will be much faster, and will reduce stress on the confocal hardware. If you use the range function (xz scan) to set your z-stacks, you MUST use only one track, unless your tracks are in Line or Fast Frame mode.**

8. After all channels have been optimized, activate (check) all the required tracks and run your scan.

Creating a multi-track from stored single tracks.

1. Set up the microscope for scanning. Make sure Channel Mode is also selected, not Lambda mode. This will ensure that the META detector is used as a regular channel. In the CONFIGURATION CONTROL window, select MULTI-TRACK. The previous Multi-Track settings will be loaded.

2. The loaded settings may include one to four tracks, that will appear as lines in the menu. If you need more tracks, you can create them by clicking "add new track". You can also delete unwanted tracks by selecting them and clicking "Remove".

3. Select one individual track by clicking on it (the entire line is highlighted; if you check the box on the left, this activates a track for scanning, but does not necessarily select that track for editing). Load the desired single track configuration by clicking "load from single track". Select one track from the menu (e.g. Cy2) and click "Apply".

4. Create and/or select a new track in the Multi-Track window. Repeat the procedure described in 3. Repeat for up to four tracks.

5. Check the tracks you want to activate.

5. Open the SCAN CONTROL window.

6. Enter your image parameters under MODE.

7. In the SCAN CONTROL window, click CHANNELS. All the tracks selected (check box) in the Multi-Track window will be displayed here. The track selected (highlighted) in the Multi-Track window will also be the one selected here.

8. Adjust the image acquisition parameters (gain, offset, pinhole) if needed. The Active track will be displayed in the SCAN CONTROL window.

**It is HIGHLY recommended to adjust the settings one track at a time (i.e. uncheck all other tracks). This will reduce the rate of bleaching of your sample, will be much faster, and will reduce stress on the confocal hardware. If you use the range function (xz scan) to set your z-stacks, you MUST use only one track, unless your tracks are in Line or Fast Frame mode.**

9. When you have adjusted all the tracks, check (activate) all the tracks you want in the CONFIG window.

10. Start scan.

Note: you can check all the tracks and run "Find" to let the software find the

optimal settings for each track. Note that "Find" does a good job at adjusting the gain, but not so good with the offset.

## **II. Collecting z-stacks in Multi-Track mode.**

The procedure is the same as above, except that you also need to set your z-stack parameters (see "Acquiring a Z-series stack" in Part I of this tutorial).

**Note: If using the "Range" function (xz scan) when adjusting your z-stack parameters, use a single track. Otherwise, the microscope will try to switch hardware settings every line, making it very slow, and putting unnecessary stress on the hardware. If your tracks are set in Line or fast Frame mode, then it is OK.**

## **III. Collecting a DIC image.**

(this section subject to revision)

To collect a DIC image, you can use any of the lasers you use for fluorescence. It is best to use a single laser line though. Our pre-set DIC configurations use the 543, which seems to be more stable than the 488, but you can try it with any laser line. To save time, the best is to combine a DIC image with one of your other channels. Basically, all you need to do is to check the transmitted light detector (Channel D) and adjust it as any other channel.

Setting up your sample in VIS mode:

1. Select VIS in main menu, and set your sample. In the MICRO control panel, select "DICII" or DIC III". This will engage the Analyzer module and condenser DIC prism.

Note: Select DIC II or DIC III to match the DIC Prism on the objective (DIC II up to 20x, DIC III for 20x or higher). You may also select the condenser DIC prism by pressing one of the buttons on the right side of the condenser until you see the desired setting on the LCD screen, or in the MICRO control panel, by clicking "condensor" and selecting the desired prism in the pull-down menu.

2. Make sure the Polarizer above the condenser is engaged in the light path and set to the left.

3. View your sample in DIC and adjust focus.

Collecting a DIC image:

4. Switch to LSM in main menu.

5. In the configuration panel, select SI\_DIC(543). This will activate the 543 laser line, select one of the two DIC prisms in the condenser, and activate ChannelD (transmitted light detector).

6. Click Find, and/or adjust PMT gain and offset manually for Channel D.

7. Run your scan.

Note: You may try to experiment by collecting a DIC image with the polarizer set to the right, and/or with or without the condenser DIC prism.

Notes:

Typically, you may want to add DIC to a fluorescence image. If you already have a configuration for fluorescence, you can add a DIC (transmitted light) channel by choosing one of your fluorescent channel tracks (such as FITC),

and just checking the ChannelD detector. adjust channel D gain and offset as for any other channel (except that typically the gain settings will be substantially lower, since you are imaging the transmitted laser light directly).

#### **IV. Using the META detector.**

The META detector can be used in three modes: CHANNEL MODE, LAMDA MODE, and ONLINE FINGERPRINTING.

In Channel Mode, the META is used just as another regular detector, except that it can be used to collect up to 8 channels simultaneously. (This sounds exciting, but in practice you probably won't collect more than 1-3 channels on the META). In Lambda Mode, the META collects spectral scans; i.e. it images the emitted light at different wavelengths. This can be used to generate an emission spectrum to select the optimal imaging window for a particular dye. Spectra can also be used for linear unmixing (separation) of dyes. In Lambda Mode, the META detector is used by itself, and can not be combined with other detectors. In online fingerprinting, the META is used to separate specific channels (for which you have spectra) in real time.

##### **4.1. META in CHANNEL MODE.**

This example will describe how to set a single track for Rhodamine and Cy5, with Cy5 on the META detector.

1. In the Configuration Control Window, select "CHANNEL MODE". This mode can be used in single track, or multi-track mode. Here, select "Single-Track".

2. Activate the 543 and 633 laser lines. Set laser power to 50% and 25% respectively (as a start). When using the META, you may need a slightly higher laser intensity.

3. Select the HFT 488/543/633 primary beamsplitter to reflect the two laser lines toward the sample.

4. Select the NFT 635 VIS secondary beamsplitter just above the HFT. This will send all light above 635nm to the META. Everything below is reflected to PMT 2 and PMT3.

5. Select NFT 545 beamsplitter and BP 565-615 in front of PMT3 (Ch3). This will be the Rhodamine channel.

6. Activate (check) one box on the META. (ChS).

7. Click ChS, You will see the spectrum of light, and the two laser lines. Also, you will see that one channel is active.

8. Move the sliders of the active channels to include the part of the spectrum to the right of the 633 line (but not including the line).

9. Select a color for the channel (e.g by clicking on ChS1). Close the sub-window.

10. In Scan Control window, select the image parameters. Click "Find". You should now get a two-channel image.

11. Click the "Config" button. Enter a name for your track (e.g. Rhodamine/Cy5\_META) and save it ("Store").

Note: You can collect two (or more) channels on the META. However, since there is only a single setting (gain/offset) for this detector, the only way you can balance two unequal channels is by changing laser power.

As an exercise, change the configuration above to collect both Rhodamine and Cy5 on the META.

## 4.2. META in LAMBDA MODE.

1. In the Configuration Control Window, click "LAMBDA MODE". Select the laser line and matching beamsplitter (e.g. 488 line and HFT 488). Use more laser power than you would normally (e.g. set the 488 at ~10%, and the 453 and 633 at 100% and 50%, respectively).

2. In the scan control window (Mode) , set the image parameters. Start with a small image size (e.g. 512x512) and no averaging.

3. In the scan control window (Channels), set pinhole to 1 Airy Unit or slightly higher to gain some light (e.g. 1.5).

4. Click "Find". With the META, often "FIND" may not work. If that is the case, increase the PMT gain and/or laser power, or adjust gain manually while doing a fast scan (FastXY).

5. Adjust image size, and/or averaging, and collect your final image. You may find that using "Sum" may be better than using "Mean", as this mode adds the different scans instead of averaging them, resulting in brighter images with less laser power and/or PMT gain. You may also benefit from using a slow scan speed (e.g. ~ 6 microseconds pixel dwell time) to collect more photons on the tiny META detectors.

6. A gallery of images will be displayed, representing the emission at different wavelengths. You can display the wavelength by clicking "Data" in Gallery mode.

In the "lamda-coded" view (button in the image window), the software will try to guess if there are different types of pixels (channels), and encode them in different colors matching the emission maxima.

In "lamda-max" mode, the brightest channel will be displayed.

7. In "MeanROI" mode, you can select a tool (e.g. a cross-hair), and obtain the emission profile for a given region of interest (ROI). Click on a region tool again to add more regions. You can save individual spectra by clicking "Save Spectra to DB", and selecting individual ROIs.

### 4.3. Linear Unmixing.

#### Method 1: direct unmixing.

1. Follow the procedure above and get the spectra for your various dyes by selecting appropriate ROIs. Ideally, each ROI should correspond to a pure dye. ( dyes should be well-separated spatially). You can take several reads until you get spectra you are satisfied with.
2. Discard all the bad spectra and keep only one for each putative dye by clicking on the ROIs and pressing the "Del" key.
3. Click "Linear Unmixing" button in the image window.

#### Method 2: unmixing from stored spectra.

Often, you will be unmixing the same combination of dyes (or using the unmixing feature to remove autofluorescence). In this case, you may want to store the relevant spectra. If the different dyes are well separated spatially, you may use a single sample to get all spectra. If there is some overlap, the spectra will not be pure, and unmixing will not be optimal. In this case, it is best to have separate samples for the different dyes (or have an unstained sample for autofluorescence).

1. Follow the procedure in 4.2 to measure and save spectra to the database. Use the same META settings for all the channels you want to unmix from a given sample. Also, save a sample image to your image database so that you can reuse the META settings in the future.
2. Image your new sample in lambda mode, using the same settings you used to record the spectra (for example, open your reference image and click "reuse").

3. In the Main Menu bar, select PROCESS>UNMIX. This will open a new window titled "Linear Unmixing".
4. In the "Linear Unmixing" window, select the image you want to unmix.
5. Select the number of channels in your image, and load the reference spectra by clicking "Dye...". Select color for that channel. Repeat for all channels.
6. Check the boxes: "ignore overexposed pixels" and "ignore underexposed pixels". Click Apply.

Note: you can also load spectra from a reference image ("Load from image")

#### 4.4 Online fingerprinting.

Online Fingerprinting is the third mode for the META channel, and can be selected in the configuration control panel.

In this mode, a lamda stack is collected as described in 4.2. However, instead of generating a lamda stack, reference spectra are used to generate a multi-channel image "on the fly". Use this mode once you have tested your stored spectra to unmix images as described in 4.2 and 4.3.

1. In Configuration Control, select ONLINE FINGERPRINTING.
2. Set the configuration (laser line, beamsplitter, start and end points, step size). Use the same settings (e.g. same bandwidth) you used to record the spectra.
3. Set the number of channels you want to unmix by clicking individual channel boxes.
4. Select the spectra for each channel from the spectra database. Select the

color for displaying each channel.

5. In Scan control window, set your acquisition parameters and start scan.

## **V. Collecting Two-Photon Images.**

**Caution: Two photon imaging is tricky, and potentially dangerous (for your sample, and for the optics). Before you use the two photon mode, consult with staff! We will provide some useful tips.**

Collection of two photon images requires excitation with the tunable Near Infra-Red Chameleon Laser. Images can be collected on any of the three regular PMTs in the scan head (PMT2, PMT3 and META). This is called the normal, or descanned mode. In this mode, the Chameleon is used just as another laser line, and can be used in single track or multi-track mode in combination with the visible lasers.

In the second mode, called Non-Descanned, light emitted under two photon excitation is imaged by a set of two special detectors, called NDD (non-descanned) detectors. These are located in the back of the microscope. Non-descanned mode should be more sensitive. However, at this moment, you are limited to DAPI/FITC/Rhodamine and Cy5 channels. In addition, you can not combine non-descanned detection with visible laser confocal detection (i.e. you can not mix visible confocal and two-photon channels). Since dyes have different two photon excitation optima, and since dye intensities may greatly vary, it is generally not recommended imaging two channels simultaneously.

### 5.1 Two-Photon imaging: standard mode.

As an example, we illustrate how to collect a DAPI image in 2 photon mode.

1. Turn the key switch of the Chameleon power supply from "Standby" to ON. Laser will take a few minutes to warm up.

2. In the main menu bar, select LASER, to open the laser control panel.
3. Select "Chameleon". Click "ON".(Contrary to the other lasers that are switched ON and OFF through the software, this will only tell the software that the Chameleon is ON, and will establish contact.)
4. The current status of the laser, power, and wavelength will be indicated. For DAPI, the Chameleon is set at 780nm. To change wavelength, click "Modify" and enter the new value. Click "Store". The Chameleon will try to lock at the new wavelength (this may take 1-2 minutes). Note: It is recommended to change the wavelength settings in steps of no more than 30-40 nm at a time. Trying longer steps may result in a loss of mode-locking and may require a new calibration.
5. When Chameleon is ready, close Laser control window.
6. In the Configuration Control window, select SINGLE TRACK and CHANNEL MODE.
7. Click the "Config" button. Select "DAPI\_2P" Click "Apply"
8. Verify that the Chameleon is ON (under "Excitation in Configuration Control Panel, or under "Channels" in Scan Control Window) and power is set to no more than about 3%. Verify that an IR primary beamsplitter is selected (e.g. HFP KP 650 or HFP KP 700/488; the latter type is designed to image VIS and 2P simultaneously).
9. Adjust scan parameters as for any other channel. Just be more careful, as 2P can result in faster photobleaching, and/or sample damage. **Note that the pinhole for the detection of 2P excited dyes should be completely open for maximum sensitivity.** With 2P excitation, the pinhole has no effect on the thickness of the optical sections.

Notes:

This mode of 2P excitation can be used as another channel; i.e. it can be used

in single track or multi track mode, and can be combined with a Visible laser line to image 2P and VIS channels simultaneously. It can also be used with the META, either in Channel mode or in lambda mode.

You can combine channels in 2P and normal confocal mode in a single track. For instance, the 700/488 beamsplitter allows you to use 2P excitation above 700nm (e.g. for DAPI), and the 488 VIS laser for FITC. The 700/543 allows you to do DAPI and Rhodamine.

## 5.2 Two-Photon imaging: non-descanned mode.

This mode uses two special detectors in the back of the microscope that collect light directly from the objective, therefore bypassing the scanner (Non Descanned Detectors or NDDs).

**IMPORTANT: Before starting, close the manual shutter for the Hg lamp (metal slider in front of Hg lamp housing). Omission of this step will result in a flooding and saturation of the NDD detectors, making them unusable for the next 10-24 hours, and may damage them.**

1-5 as above.

6. Select "Non Descanned" in the Configuration Control window.

7. Click "Config" button. Select a 2P configuration. Alternatively, manually select the 2P laser line, set the laser power, HFP primary beamsplitter (e.g. HFP KP 700/488 or HFP KP 650) and NDD LP680 reflector.

8. Verify that you have an appropriate secondary beamsplitter, and that the appropriate detector and emission filter is (are) selected.

9. Proceed with setting the scan parameters in the Scan Control panel.

Note: for best results, it is advised to turn OFF or DOWN all lighting in the microscope room. In 2P mode, all light that reaches the objective (including

ceiling lights and emission from the computer monitors) will be recorded as noise in the final image. (note that this is true to some extent in all microscopy applications, but is more important in 2P because of the low intensity of the signals, and because there is no pinhole to block out of focus noise).

Also note that for best efficiency, it is advised to remove the objective DIC sliders. If you do so, PLEASE store them in a safe place (DIC containers located near scope... Please ask) and protect from dust!

Updated: 06/22/2004.

## **Zeiss LSM 510 META NLO User's Manual. Part III.**

### **Advanced Techniques for multiple location imaging.**

#### **Contents:**

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- II. Calibrating stage.**
- III. Marking Multiple locations.**
- IV. Collecting Tiled Stacks.**
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- VI. Collecting stacks at multiple locations.**
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#### **I. Collecting a tile image.**

1. Set up your sample and imaging parameters. Optimize image.
2. In the main menu, open the Stage control panel: ACQUIRE>STAGE
3. At the bottom, under Tile Scan, enter the tile dimensions (n(x), n(y)).  
Maximum tile size is 4096x4096 pixels. Therefore, you can collect at most a 4x4 tile at 1024x1024 pixels, but 8x8 tile at 512x512. The lower the resolution, the larger the field of view you can collect.
4. Save your tile image.

#### **Notes:**

It is best to collect a small tile first at good resolution (e.g. 2x2 at 1024x1024 at zoom 1-2) to test stage alignment. If there is an offset in the tiles, proceed with stage calibration as described below.

This method only allows the collection in a single plane (not z stacks). For tiled z stacks, see section IV.

## **II. Calibrating stage.**

You need a sample that has good contrast.

1. Place sample on stage. Choose one channel that shows good image definition and brightness (overall good contrast).
2. Select objective and zoom factor. Optimize image.
3. In main menu, select ACQUIRE>STAGE, and set for tile collection under "Tile Scan", e.g. 2x2 tiles.

Collect an image and check alignment in x and y. If Alignment is good, the stage is calibrated: proceed with your experiment. Otherwise:

4. In the main menu, select MACROS>Rotation
5. In the calibration menu window, click "Calibrate". Microscope will start automatic scans and attempt to calibrate stage. When calibration is done, a correction factor will be displayed.
6. Collect a new tile to test the new calibration. Repeat if you are not happy with result. You may also try to change the numbers manually until satisfied. Note that alignment may not be perfect.

Note: Calibration will be best if you select "speed 1" in the Stage Position

panel (Stage Control window).

### **III. Marking Multiple locations.**

The Zeiss 510 allows you to mark locations in your sample and store them. Once stored, you can navigate to any of those locations for further imaging.

The recording of stage positions is done within the Stage control window. You can mark positions in two different manners: manually, or using a tile scan.

#### **a. Manual marking:**

1. You can select any position, for instance the center of your sample, and mark that as the "center": Find the position you want to be the center by visual inspection (e.g. you can use the 2.5x objective to do a rapid survey of the sample.

2. In the stage window, under "Stage Position", click "Zero", and click "Mark Pos."

3. Find a new location by visual inspection through the eyepiece, or by moving the stage in fast scan mode. Find a new interesting location. Click "Mark Pos."

4. Repeat for all locations.

5. The list of locations will be recorded in the stage control window, in the

"Marks" pull-down menu.

6. You can now select a location in the pull-down menu, and use "Move To" to move the stage to that position.

Note:

The Stage control window also remembers the z position. Therefore, you can mark different locations in x,y,z. I noticed, however, that if you mark one position in x,y,z, the system will not record a new position that has the same x,y coordinates, but a different z location. Therefore, if you want to record two z positions at the same location, you will need to slightly move the stage in x or y.

b. Tile marking.

1. Collect a tile.

2. In the Tile Scan panel, click "Mark"

3. Click on your tile scan image to mark different locations.

### **Collection of complex images.**

The Multi-Time Macro allows the collection of single z sections, or z stacks, in a tiled manner, or on a grid pattern, or at multiple locations. The various procedures are described below.

### **IV. Collecting Tiled Stacks.**

1. Select a Multi-track configuration (not single track, as single track do not remember certain settings).
2. Set your sample and optimize image collection. Adjust all parameters.
3. If you want z stacks, set the z stack parameters.
4. Save all the parameters as a new configuration: Click "Config", enter a new name (e.g. aa1), and "Store".
5. Calibrate stage as described in section II.
6. Start Multi-Time Macro (Main Menu>MACROS>MultiTime"R")
7. Click "Refresh" button.
8. Load Multi-Track configuration from pull-down menu in Multi-Time window (Config, Time-Interval and Bleach), (e.g. aa1)
9. For a single tile, make sure "number of scans" is set to "0", and z-stack is checked.
10. Under "List of Blocks", set number of repetitions to "0"
11. Make sure autofocus is NOT checked, and bleach is NOT checked.
12. Click "Single location" button on top of Multi-Time window.
13. Click "Edit Locations"
14. Select "Tile"
15. Enter the tile size (e.g. 2x2)
16. Select Overlap (e.g. 20%). The overlap is required for automatic stitching

of images. If Stitching fails, use higher overlap.

17. Click "Create Tile"

18. Click "Options" (in Multi\_Time window); Check "Keep Final Image Open" and "Save Final Image"

19. Click "Image Database", and select the database where your final image will be stored.

20. Click "Start Time"

21. Wait until images are collected and assembled. The final image will be saved in the database you selected. Intermediate images will be saved in a temporary directory in drive C.

## **V. Collecting a grid of stacks.**

The "grid" function collects single sections or stacks in a grid pattern, with current position used as the origin.

To collect a grid, follow the same procedure as for a tile, except for:

14. Select "Grid"

15. Enter the spacing of the grid in x and y.

16. Click "Create Grid"

Proceed as for a tile with steps 18-21.

## **VI. Collecting stacks at multiple locations.**

0. Mark multiple locations as described in section III.
1. Select a Multi-track configuration (not single track, as single track do not remember certain settings).
2. Set your sample and optimize image collection. Adjust all parameters.
3. If you want z stacks, set the z stack parameters.
4. Save all the parameters as a new configuration: Click "Config", enter a new name (e.g. aa1), and "Store".
5. Calibrate stage as described in section II.
6. Start Multi-Time Macro (Main Menu>MACROS>MultiTime"R")
7. Click "Refresh" button.
8. Load Multi-Track configuration from pull-down menu in Multi-Time window (Config, Time-Interval and Bleach), (e.g. aa1)
9. Make sure "number of scans" is set to "0", and z-stack is checked in the "Config, Time-Interval and Bleach" panel.
10. Under "List of Blocks", set number of repetitions to "0"
11. Make sure autofocus is NOT checked, and bleach is NOT checked.
12. Click "Multiple Location" button on top of Multi-Time window.

13. Verify that the list of locations in the Multi-Time series window menu matches the list in the stage control panel. If not, click Refresh.

14. Click "Options" (in Multi\_Time window); Check "Keep Final Image Open" and "Save Final Image"

15. Click "Image Database", and select the database where your final image will be stored.

16. Click "Start Time".

17. Macro will start collecting z sections or stacks at the different locations. Each location is displayed as a different time point.

## **VII. Collecting a Time series at multiple locations.**

Proceed as described in section VI.

In addition:

15.1. Under List of Blocks, enter the number of Block repetitions (e.g. 3, for three time-points)

15.2. Under Block 1 parameters, enter the wait interval.

Proceed with 16 as in section VI.

## **VIII. More complex imaging patterns.**

Ask Scientific Imaging Staff.