

CONFOCAL MICROSCOPE (Zeiss LSM 510 META v4.2)

Abridged INSTRUCTIONS

1) *STARTING THE SYSTEM*

Switch on the mercury bulb first. Ensure that no lasers are on when starting the mercury lamp as the mains emf 'pulse' can potentially damage the diode lasers and other active electronics.

Push the switch labelled **System/PC** on the remote control box to switch on the LCD monitors. Then **Switch on** the **PC** [button on PC].

Ensure that the glass slide, Labtek or multi-well plate **adapter** is fitted correctly into the **stage insert** – gently slide the adaptor in from the right into the twin locating springs at the bottom left - don't push down on the springs. Place the specimen on the stage.

2) *INITIALISING THE SYSTEM WITH THE LSM 510 SOFTWARE*

Allow computer to boot up and log on onto Windows XP.

Now **Push** the second switch labelled **Components** LASER on the remote control box for the confocal system hardware, including the Axiovert 200M microscope, to be functional.

Double click the **LSM 510 icon** on the Windows desktop. The LSM 510 switchboard window appears on the screen.

Click on the **Scan New Images** button to activate the complete hardware.

Click on the **Start Expert Mode** and the Expert Mode Main Menu will appear.

3) *TURNING ON THE LASERS*

Click on the **Acquire** button in the Main Menu to open the subordinate menu.

Click on the **Laser** button to open the Laser Control Window. Select the appropriate **Laser Unit** by clicking on the name of it:

There are four different lasers available:

<i>Blue Argon (458, 477, 488, 514 nm) for FITC/GFP green fluorescence</i>	<i>30.0 mW full power</i>
<i>Green HeNe1 (543 nm) for TRITC/Rhod red fluorescence</i>	<i>1.2 mW full power</i>
<i>Red HeNe2 (633 nm) for far red Cy5 fluorescence</i>	<i>5.0 mW full power</i>
<i>Violet Diode (405 nm) for DAPI & Hoechst DNA</i>	<i>30.0 mW full power</i>

Click on the **Standby** button to switch the argon laser to Standby. When the argon laser status is **Ready** click on the **On** button.

Argon 488-line laser only: Set **Output %** so that the **Tube Current** is **6.1 A** [it doesn't matter if the little box goes red at 6.1 amps – this is just a software warning not to exceed 6.1 Amps]. Note that the argon laser will switch back to **standby** if you come out of the LMS-510 software.

4) SETTING THE MICROSCOPE to visualise specimen

Click on the **VIS** button (in the **Acquire** subordinate toolbar) to set the microscope for direct observation.

Click on the **Micro** button to open the Microscope Control window.

Select an objective by clicking on the **Objective** button and clicking on the objective you want to use. The selected objective will automatically move into the beam path and be shown on the microscope mini LCD display.

There are six different objectives available:

<i>Plan-neofluar 10x Air</i>	<i>[Zeiss 0.3 NA no phase contrast or DIC]</i>
<i>Plan-neofluar 20x Air</i>	<i>[Zeiss 0.5 NA can work with DIC optics]</i>
<i>Plan-neofluar 20x Air Ph2</i>	<i>[Zeiss 0.5 NA with Phase Contrast optics]</i>
<i>Plan-apochromat 40x Air Ph3</i>	<i>[Zeiss 0.95 NA with Phase Contrast optics]</i>
<i>EC Plan-neofluar 40x Oil DIC</i>	<i>[Zeiss 1.3 NA oil DIC]</i>
<i>Plan-Apochromat 63x Oil DIC</i>	<i>[Zeiss 1.4 NA oil DIC]</i>

Transmitted light and Differential Interference Contrast (DIC):

Click on the **Transmitted Light** button. Click on the **On** button. Set the intensity of the halogen illuminator using the slider, and unclick to switch off. Click on **Close** to close the pop-up menu.

Alternatively simply use the **rocker switch** for illumination intensity at the front base of the microscope, where minimum intensity will switch the lamp off (i.e. 0v on the microscope mini LCD display).

Place specimen on microscope stage. The coverslip must be facing down.

Use the focusing knobs on the microscope to focus to the required object plane.

Select specimen area by moving the stage in X and Y using the XY stage controller.

Have the **Condenser** set to H for bright-field. For differential interference contrast, have the condenser ring to either DIC 2 (for 10x and 20x) or DIC 3 (for 40x and 60x). For Phase Contrast select the appropriate phase rings [20x: Ph2 or 40x: Ph3].

For best quality transmission images align the condenser for Kohler illumination [ask a member of Core staff for more details].

Reflected fluorescence light:

For viewing wide-field fluorescence down the microscope, click on the **Reflector** button and select the desired fluorescence filter by clicking on it.

Click on the **Reflected Light** button to open the shutter of the mercury lamp.

There are **three** different **fluorescence filter sets** available:

None	<i>Transmission bright-field illumination only</i>
FSet 10 wf [Green]	<i>For Alexa-488, FITC, GFP green emission fluorescence</i>
FSet 15 wf [red]	<i>For Alexa 543, Rhodamine, TRITC red emission fluorescence</i>
FSet 49 [blue]	<i>For DAPI [DNA stain] blue emission fluorescence</i>
Analyser Module D	<i>A polarising filter for visualising DIC down the microscope</i>

5) CONFIGURING THE BEAM PATH AND LASERS

Click on the **LSM** button (in the **Acquire** subordinate toolbar) to set the microscope for laser scanning.

Click on the **Config** button in the **Acquire** subordinate toolbar to open the Configuration Control window.

Select **Channel Mode**. You can choose between two different configurations: **Single Track** and **Multi Track**.

Single Track is for **Simultaneous** scanning only. Advantage: faster image acquisition. Disadvantage: possibly severe crosstalk [bleed-through] between channels. Use Single Track for single fluorochrome labelled samples.

Multi Track is for **Sequential** scanning, either line by line or frame by frame. Advantage: when one track is active, only one detector and one laser are switched on. This greatly reduces cross talk and is the preferred method of scanning. Disadvantage: slower image acquisition.

Clicking on the **Config** button (floppy disk icon) opens the **Track Configurations** window to load, store or delete **Track** configurations.

For loading a Zeiss default configuration select it in the list box and click on **Apply**.

The existing configurations are the following:

DAPI/FITC/TRITC MT

DAPI/FITC/TRITC/Cy5 MT

FITC/TRITC MT

FITC/TRITC/Cy5 MT

To add **DIC Transmission** scanning to a FITC or TRITC configuration check box for **ChD**.

6) SETTING THE PARAMETERS FOR SCANNING

Click on the **Scan** button in the **Acquire** subordinate toolbar to open the **Scan Control** window.

Select **Mode** in the **Scan Control** window.

Select **Frame** in the **Scan Control** window.

Keep default values in the **Objective Lens, Image size and Line Step Factor** panel. Make sure the objective matches the lens being used. *Recommended value for image size is 512 x 512 or 1024 x 1024 with line step of 1.*

Use the slider in the scan **Speed** panel to adjust scan speed. *Recommended scan speeds are 6 to 9.* The slower the scan speed, the better the image quality, but slower scan speeds will increase sample bleaching and scan time.

Select the **dynamic range** as 8 or 12-bits per pixel in the **Pixel Depth, Scan Direction and Scan Average** panel. 8-bits will give 256 grey levels per pixel, while 12-bits will give 4096 grey levels per pixel. Images required for pixel intensity measurements should be acquired using 12-bit data depth.

Select the **Line** or **Frame** mode for averaging. *Averaging [mean] improves the image by reducing noise, however it does increase laser dwell time, and so may lead to bleaching.*

Select the desired scan average method **Mean** or **Sum** in the **Method** selection box. Sum increases image brightness with every scan.

Select the **Number** for averaging. **Continuous** averaging is possible in the **Frame** mode. In this case a **Finish** button for ending averaging is displayed.

Select **Channels** in the **Scan Control** window.

Set the **Pinhole** size to **1 Airy Unit (AU)** for best compromise between resolution, depth discrimination, and efficiency. Pinhole adjustment changes the **Optical Slice** thickness. When collecting multi-channel images, adjust the pinhole so that each channel has the same **Optical Slice** [e.g. 0.9 μm]. Typically set the pinhole to 1 **AU** for the longest wavelength light/channel, making a note of the **Optical Slice** thickness and adjust other channels accordingly.

7) IMAGE ACQUISITION

Use one of the **Find, Fast XY, Single** or **Cont.** buttons to start the scanning procedure and acquire an image.

Select **Find** for automatically pre-adjustment of detector **Gain**.

Select **Fast** for continuous fast scanning at 512x512 at maximum scan speed (useful for finding and changing the focus). **Fast** minimises sample bleaching but has the lowest image quality.

Select **Single** for recording a single image in **high quality** mode [set scan speed and averaging].

Select **Cont.** for continuous scanning at the selected scan speed in **high quality** mode. This can be used to fine tune the image slow scan quality settings prior to using **Single**.

Select **Stop** for stopping the current scan procedure.

8) IMAGE OPTIMIZATION

Using the Range Indicator over-exposure tool:

Select **Palette** in the **Image** window of the scanned image.

In the **Color Palette List** panel click on the **Range Indicator** item.

The scanned image appears in a false-colour presentation. If the image is too bright (pure white and overexposed), the pixels appear red on the screen [Red = Saturation (maximum)]. Pure black areas of the image appear blue on the screen [Blue = zero (minimum)].

Adjusting gain and offset:

Typically set the **pinhole** to **1 Airy Unit**.

Adjust the **Detector Gain** to visualise image. High gain gives a very noisy image. Higher laser power, averaging [mean] or dwell time [scan speed] reduces image noise but may lead to fluorochrome bleaching or long scan times.

With **Range Indicator** on, typically reduce the **Detector Gain** until the red pixels disappear, or there are only a few in the entire image. Over-exposing areas of the sample will raise the background brightness.

You can adjust the **Amplifier Offset** until the blue pixels disappear, or there are only a few in the object. **Amplifier Offset** can also make dark grey regions go to pure black, which may be used to reduce background fluorescence in non-sample areas.

Adjusting the laser intensity:

If the image is saturated [over-exposed], use either the **Detector Gain**, or the slider in the **Excitation** panel to reduce the intensity of the laser light on the specimen.

9) *MOTORIZED XY STAGE*

Click the **Stage** button

FOCUS POSITION

This gives Z focus motor control for moving the focus. Generally simply using the coarse/fine focus knobs on the microscope is the preferred method of adjusting focus.

STAGE POSITION

Set the **Step** distance (16 μ m is ideal for small steps with the 63x objective). Click on the arrow pad buttons to move the stage, with each click being one precise step in that direction.

You can mark the position of the sample on the stage using the **Mark Pos** button. Use the drop down **Marks** to return to any stored stage position. Note that the stage resets to zero at the position the stage is switched on to, so you cannot return to a stored position after the microscope has been switched off [unless you always zero on a specific location within your slide when switching on and note the field XY stage co-ordinates].

TILE SCAN

To run a raster scan about your field position set the tile numbers required, e.g. 3 X and 3Y will give a raster scan of 9 fields tiled around the present field in view. The original field will be in the middle in the case of a 3x3 raster scan.

MULTI_TIME SERIES MACRO

To run the **Multiple Time Series** macro go to the main menu's **Macro**, then **Macro** button and then click **Load**. Search for and load the C:\Aim\Macros 'MultiTime4.0.lvb file'. Run the first option in the MultiTimeSeries list: A_main_multi40.A.main_multi. The Multi Time Series control panel will appear (v4.0.12).

This macro can run multi-well or multi-position time-lapses and it can raster scan entire sections using top-left & bottom-right selection. The Multi_Time series panel is initially quite challenging to use, so ask a microscopy core member of staff for help if you would like to run the macro.

10) SCANNING A Z-STACK

Select **Z Stack** in the **Scan Control** window. Select **Frame** if necessary.

The Z Settings panel appears.

Select **Mark First/Last**.

Click on the **XY cont** button.

A continuous XY-scan of the set focus position will be performed.

Use the focusing drive of the microscope to focus on the upper position of the specimen area where the Z Stack is to start. Click on the **Mark First** button.

Then focus on the lower specimen area where the recording of the Z stack is to end. Click on the **Mark Last** button to set this lower position.

The **Num Slices** and **Step Interval** is now shown (e.g. 15 slices with a step of 1 um).

Click on **Z Slice** and then **Optimal Interval** to double oversample and so maximise z resolution. On thick samples this may create too many z slices.

To review the Z Stack locations click on **First**, **Mid** or **Last** under **XY Cont**. Ensure that no slices are over-exposed and that the scan time per image isn't excessive for the number of z slices in the z-stack.

You can click on the **X:Y:Z=1:1:1** button to set the Z-interval in such a way that the voxel has identical dimensions in the X-, Y- and Z- directions (cube).

Click on the **Start** button to start the recording of the Z Stack. The time left to capture the z-stack and the percent completed bar is now shown in the bottom of the image window.

Click on the **Z Stack** button again to deselect the Z Stack and return to the Scan window.

11) DISPLAY AND ANALYSIS OF IMAGES: BASICS

(For more info please refer to the Zeiss Operating Manual and in-program help)

The **Display** toolbar in the **Image Display** window offers the following functions:

- **XY**: To display a single image in frame mode or display multi-channel images in superimposed mode.
- **Split XY**: To display the individual channels of a multichannel image as well as the superimposed image.
- **Chan**: To switch fluorescence/transmission channels off and on within the captured image and to apply a look up table [LUT] colour to the B&W image.
- **Zoom**: A digital zoom to adjust the captured image size to fit on the VDU screen.
- **Crop**: The microscope optical zoom tool for selecting and magnifying a smaller area of the captured image. It works in conjunction with the microscope confocal scanning hardware.
- **Ortho**: To display a Z stack of images in an orthogonal view and measure distances in three dimensions.
- **Slice**: Shows the z-stack/time-lapse slider for reviewing the image sequence
- **Anim**: The video player for z-slices and time-lapse. This video can be exported to *.avi or *.mov, via File, Export.
- **Copy**: To copy the image(s) displayed to say PowerPoint (at screen resolution).
- **Cut**: To display a Z stack of images at a user defined section plane.
- **Gallery**: To display a Z stack of images side by side in a tiled fashion.
- **Histo**: To display a histogram (distribution of pixel intensities) of an image; to show the histogram values in table form (**Show Table**); to copy table to clipboard or save as text file; to analyse the colocalisation between two channels (**Colocalisation**). The **Histo** button can also be used online during scanning.
- **i-info**: Displays image information, e.g laser/optic configuration, zoom and calibration.
- **Overlay**: Add a scale bar and or text/shapes to the captured image.
- **Profile**: To display the intensity distribution of an image along a straight or curved line. The line can be drawn using tools in the **Profile** toolbar. The **Profile** button can also be used online during scanning.

Note that many of the above **Display** toolbars are also available in the stand-alone **Zeiss LSM Image Browser** [available for download from our web pages]. **Image Browser** allows you to look at, edit and export your Zeiss confocal *.lsm image files.

12) SAVING AN IMAGE

Click on the **Save** or **Save As** button in the **Image** window or in the **File** subordinate toolbar of the Main Menu.

The **Save Image and Parameter As** window appears.

Open (or create a new) **MDB database**.

Open **AIM-DATA (D:) Users**.

Select Directory, enter file name, description and notes and click on the **OK** button.

The Core recommends that you leave your confocal image files on the D: drive as a secondary backup and transfer a copy to your PC at the end of your session. Note that we **do not** backup any user data, although we will not delete any user files without prior consultation – you must ensure your images are always backed up in the event of hard drive failure.

13) EXPORTING AN IMAGE

Select the image to be exported.

Click on the **Export** button in the **File** subordinate toolbar of the Main Menu. This opens the **Export Images and Data** window.

Under **Save in**, select the folder to which the image is to be exported.

Enter a name for the image under **File name**.

Select the image format in which the image is to be exported under **Image type**.

Choose a compression level for jpg.

Click on the **Save** button.

14) LIVE CELL IMAGING AND TIME-LAPSE VIDEOS

Ask the Microscopy Core staff for help on how to use the live cell time-lapse system.

14) TO SHUT-DOWN THE SYSTEM

1. **Switch off all the lasers if you are the last user.** The Argon and DAPI 405 lasers can be switched to standby if another user has booked on later that day.
2. Close all PC software windows and **exit the program.**
3. The system will prompt you to **turn off any lasers still on.**
4. **After switching the lasers off, you will need to WAIT at least FIVE MINUTES to let the lasers cool down**

You will probably hear a click and a fan turn off within the laser module when they are sufficiently cool. If you miss the click just ensure that at least five minutes have passed since the lasers were switched off.

5. **Shut down the computer** from Windows XP.
6. **Turn off the Components** LASER power button on the small control box.
7. **Turn off the System PC** power button on the small control box.
8. **Switch off the mercury bulb LAST**
[The mercury lamp should always go **ON FIRST** and **OFF LAST**].

If you have any microscope/imaging problems or queries, just ask a member of the Microscopy Core staff for help. There is additional help and advice on our Core web-pages: <http://www.well.ox.ac.uk/microscopy>.