Confocal microscopy

Zeiss LSM 510 and Zeiss LSM 510 META

Visualisation of biological structures in 3D
The Confocal Principle

Signals from above and below the plane of focus fall outside the pinhole and are blocked.

Only signals from plane of focus pass the pinhole and are detected - producing a “optical section.”
Zeiss LSM 510 META - Guided Tour

Upright Zeiss LSM 510 confocal microscope

- LSM 510 scan head
- Axioplan 2 imaging
Inverted Zeiss LSM 510 confocal microscope

Axiovert 100M

LSM 510 scan head in base port position
Upright Zeiss LSM 510 META confocal microscope

- Axioplan 2 imaging
- LSM 510 META scan head
Inverted Zeiss LSM 510 META confocal microscope
Contents

• Starting the Zeiss LSM 510 microscope, software and laser
  Selecting an objective and focusing the microscope

• Selecting an objective and focusing the microscope

• Configuring the laser scanning and detection for confocal image
  acquisition

• Acquiring a Z- and Time - Series

• Data storage

Descriptions also include the LSM 510 META
Zeiss LSM 510 META - Guided Tour

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Zeiss LSM 510 META - Guided Tour

Start the Zeiss LSM 510 Confocal Microscope

1) First switch on the mercury lamp

2) Turn on the remote control switch

3) Wait for the computer to boot up and Login by simultaneously pressing the Ctrl, Alt and Delete keys
1) Double click the LSM 510 icon
2) Select “Scan New Images”
3) Select “Start Expert Mode”
Creating a database for acquired images

1) In the main menu *File* select *New* database

2) Select drive C or D: from pull down menu

3) Create a new directory if needed
1) Select Acquire

2) Select Laser

3) Switch required laser/s to Standby

4) When status is Ready, click On

5) Set Output [%] so that the tube current is between 5.5 and 6.5 A
Change between direct observation and laser scanning

**Upright Microscopes: Axioplan 2 imaging and Axioskop 2 FS**

- For direct observation of transmitted light and fluorescence:
  - Set slider to “VIS” (push it in)

- For laser scanning image acquisition:
  - Set slider to “LSM” (pull slider out)
Change between direct observation and laser scanning

Inverted Microscope: Axiovert 200 M

Toggle between Vis and LSM button in main menu, automatic switching between direct observation and laser scanning (no slider)
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Selecting an objective and focusing the microscope

1) Select Micro (Main menu: Acquire)  
(For Axioskop 2 FS these settings have to be adjusted manually)

2) Microscope settings can be stored and up to 8 buttons assigned for fast retrieval and adjustment

3) Objective lens can be selected from a pull down menu by clicking onto the Objective button
Focusing the microscope in fluorescence mode

Click onto *Reflected Light* to open the shutter of the HBO lamp.

Fluorescence is observed by selecting the appropriate filter set in the pull down menu of *Reflector*.
Focusing the microscope in transmitted mode

Click onto *Transmitted Light* and move the slider to set the intensity of the HAL illumination.

Use no reflector cube in the reflector turret, chose *None*. 
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### Choosing the configuration

<table>
<thead>
<tr>
<th>SINGLE TRACK</th>
<th>MULTI TRACK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use for <strong>single</strong>, double and triple labelling</td>
<td>Use for double or triple labelling</td>
</tr>
<tr>
<td>Simultaneous scanning only</td>
<td>Sequential scanning, line by line or frame by frame</td>
</tr>
</tbody>
</table>

#### ADVANTAGES
- Faster image acquisition
- When one track is active, only one detector and one laser is switched on. This dramatically reduces crosstalk.

#### DISADVANTAGES
- Cross talk between channels
- Slower image acquisition
Configuration of the filters and storage of the track configurations

1) Select Config in the Acquire menu

2) Select Single Track

4a) Select the appropriate filters and activate the Channels

4b) The Config button opens the pull down menu to load/store Track configurations

4c) Transmitted light image can also be generated. Transmission channel is usually set to white colour.

3) Click Excitation to select the laser and attenuation
Applying a stored configuration and checking the settings

5) Choose a configuration in the **Track Configuration** menu. Select **Apply**

6) To check for correct settings, click the **Spectra** button.

The **Spectra** button opens a window to display the activated laser lines for excitation (colored vertical lines) and channels (colored horizontal bars).

If you select **Store** by mistake, the software will ask you if you want to overwrite the configuration. **ANSWER NO**!

Each new login loads a predefined set of correct configurations.
Multi Track Configuration

1) Select *Multi Track* for sequential scanning

2) Select *Config*

3) Select a stored track from the pull down menu, click on *Apply*

This button stores only the highlighted single track or applies a single track.
Cy5-Cy3-FITC Multi Track

Three laser lines and channels activated sequentially

- **Excitation**
  - 633 nm, using the META detector in Channel mode
  - 543 nm
  - 488 nm

- **Detection**
1) Select *Scan*

2) Select *Mode*

3) Select the *Frame Size* as predefined number of pixels or enter your own values (e.g. 300 x 600). Use *Optimal* for calculation of appropriate number of pixels depending on N.A. and \( \lambda \).

**The number of pixels influences the image resolution!**
Setting the parameters for scanning

**Note:** When using a Axioskop 2 FS, indicate the Objective that is in use in the Scan Control window. This ensures correct calculation of pinhole, z-stack optimization etc.
Adjusting the scan speed

Adjust the scan speed - a higher speed with averaging results in the best signal to noise ratio. Scan speed 8 usually produces good results. Use 6 or 7 for superior images.
Choosing the Dynamic Range (8/12 Bit per pixel)

Select the dynamic range - 8 bit will give 256 gray levels, 12 Bit will give 4096 levels. Photoshop 5 will import 12 and 16 Bit images.

Publication quality images should be acquired using 12 Bit.

12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.
Channel Settings - Adjusting the Pinhole

Set pinhole size to 1 Airy unit for best compromise between depth discrimination and efficiency.

Pinhole adjustment changes the “Optical slice”.

*When collecting multi channel images, adjust the pinholes so that each channel has the same “Optical Slice”.*

This is important for colocalization studies.
**Image Acquisition**

1) *Find* opens new image window and automatically pre-adjusts detector sensitivity

2) Select *Fast XY* for continuous fast scanning - useful for finding and changing the focus

3) *Single* records a single image

4) *Stop* blanks the laser beam and stops the scanning mirrors

5) Select *Cont.* for continuous scanning with selected scan speed
Minimal Pixel Size determined by Nyquist Sampling

<table>
<thead>
<tr>
<th>Magnification</th>
<th>NA</th>
<th>Pixel size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.15</td>
<td>1.03 µm</td>
</tr>
<tr>
<td>10</td>
<td>0.3</td>
<td>0.51 µm</td>
</tr>
<tr>
<td>20</td>
<td>0.5</td>
<td>0.31 µm</td>
</tr>
<tr>
<td>40</td>
<td>1.3 (oil)</td>
<td>0.12 µm</td>
</tr>
<tr>
<td>63</td>
<td>1.4 (oil)</td>
<td>0.11 µm</td>
</tr>
<tr>
<td>100</td>
<td>1.4 (oil)</td>
<td>0.11 µm</td>
</tr>
</tbody>
</table>

Values are for scan zoom = 1.0

Adjusting the field size ("XY") to 56 µm with the 63× lens, would produce a pixel size of 0.1 µm

Brightness of image = Magnification²/NA²

Field size can be adjusted by changing the objective magnification, or by optical zooming. Changing from 63× to 100× will reduce the field size, but will also reduce the amount of light available.
Optical Zooming

The level of zoom can be changed either by using the Zoom, Rotation & Offset control in Mode menu of the Scan Control, or by selecting Crop in the image menu.

The image can also be rotated by selecting and dragging the bars.
Selecting gain and offset – Choosing a lookup table

1) Select Palette

2) Select Range Indicator

Red = Saturation (maximum)
Blue = Zero (minimum)
Scan Control – Setting Gain and Offset

*Detector gain* determines the sensitivity of the detector by setting the maximum limit. *Amplifier Offset* determines the minimum intensity limit. *Amplifier Gain* determines signal amplification.

Saturation at the maximum → reduce *Detector Gain*

Saturation at the minimum → increase *Amplifier Offset*

Gain set correctly

Offset set correctly

*Amplifier Gain* increases the whole signal, and the *Amplifier Offset* will need to be decreased.
Saturation of Signal Intensity with Laser Power

- Fluorophore saturates at 6% laser transmission
- Photobleaching is linear

Laser transmission should not be set higher than the saturation level.
Adjusting the Laser Intensity

1) Set Pinhole to 1 Airy unit
2) Set Detector Gain high
3) When the image is saturated, reduce AOTF transmission in the Excitation panel to reduce the intensity of the laser light at the specimen.

Image with saturated pixels:
Adjusting Gain and Offset

1) Increase the Amplifier Offset until all blue pixels disappear, and then make it slightly positive.

2) Reduce the Detector Gain until the red pixels only just disappear.

Both Detector Gain and Amplifier Offset saturated

Gain and offset not correct

Gain and offset correct

No blue
No red
Adjusting the Laser, Gain and Offset using a Multi Track Configuration

Each channel is selected independently by clicking on the colour button indicating the channel i.e. Ch2-T1 (Channel 2, Track 1). The laser power and all other parameters are optimised as described in the previous slides for each selected channel.

For accurate colocalisation, adjust each Pinhole so that each channel has the same Optical Slice.

To adjust laser, gain or offset for a single track in a multi-track configuration it is possible to temporarily deactivate the other tracks in the Configuration control.
Setting up Gain and Offset - Multi Track

1) Select Split XY in the Image window

2) In Palette, select Range indicator

3) Select each channel separately under Channels in the Scan control window and adjust the Laser intensity, Detector Gain, and Amplifier Offset as described previously.
Line Averaging

Averaging improves the image by increasing the signal to noise ratio.

Averaging can be achieved line by line, or frame by frame.

1) Select Line or Frame under Mode in Scan Average within the Mode panel of the Scan Control window.

2) Select Number for averaging. The more the better for the signal to noise ratio (max 16) in this case, each line will be scanned 4 times. But: Averaging increases the exposure time of the sample!!
Frame Averaging

1) Select *Frame*

Frame averaging helps reduce photobleaching, but does not give quite such a smooth image. There is also a longer delay between each track when using “Multi Track”.

2) Select the *Number* for averaging - The more the better for signal to noise ratio (max 16). Continuous averaging is possible in this mode.

Continuous averaging has a *Finish* button which allows the scan currently in progress to be completed before stopping.
Collecting an Averaged Image

1) Under Scan Average select the Number for the average.

In the Channels panel of the Scan Control window select Single. An averaged image will be collected.

Range indicator set to No Palette
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**Scanning a Z-Series using Mark First/Last**

1) Select Z Stack

2) Start scanning using Fast XY or XY cont

3) Keep your eye on the image and move the focus to the beginning of the Z-Series, then select Mark First

4) Move the focus back in the opposite direction to the end of the Z-Series, then select Mark Last

5) X:Y:Z = 1:1:1 sets the Z-interval so that the voxel has identical dimensions in X, Y, and Z.

6) Start will initiate the acquisition of the Z-Stack. The acquisition can be stopped at any time.

NOTE
Focusing can be achieved manually (preferred), or using Stage in the LSM menu if there is a motorized scanning table.
Using Auto Z Brightness Correction

Auto Z provides an automatic gradual adjustment of Detector Gain, Amplifier Offset, Amplifier Gain, and Laser intensity setting between the first and last optical slice of a Z Stack.

1) After defining the Z position of the first and last optical slice activate Auto Z.

2) Move to the First Slice and adjust the parameter for the image acquisition in the Channels panel for each used channel as described in the previous slides. Then click on Set A to store the values.

3) Repeat the procedure after moving to the Last Slice. Click on Set B to store the parameters for the last slice.

**Note:** Positions A and B do not have to be the first and last slice of a stack and can also be defined simply by focussing to the appropriate positions, adjusting the parameters and pressing Set A or Set B.

4) The parameters for image acquisition will be gradually and linearly adjusted between the first and last slice of the Z Stack. Thus signal intensity and image quality is comparable throughout the Z Stack.
Confocal Z Sectioning
Number of Sections for correct sampling

Optical thickness \( d \) depends on:
- Wavelength \( \lambda \)
- Objective lens, \( N.A. \)
- Refractive index \( n \)
- Pinhole diameter \( P \)

\[
\text{d} \sim \frac{\lambda}{(N.A)^2} \sim 0.5 \mu m \text{ @ 63x1.4}
\]

The optical slice thickness is displayed in the Scan Control

For Z-sectioning it is optimal to have:
no missing information @ minimal number of sections

"Nyquist-“ or Sampling- Theorem
### Z Stack – Number of Slices and Increment

1) **Select Z slice** - the window *Optical Slice* will appear

2) **Select Optimal interval** the computer will calculate the optimum number of sections
   - For more or less sections - adjust *Num Slices*

3) **Select Start** to acquire the complete stack
Z Series using Z Sectioning

1) Select Z Stack
2) Select Z Sectioning
3) Select Line Sel
4) Select the large arrow button and position the XZ cut line

XZ cutline will be displayed as diagram within the XY image
Z Sectioning – Setting Range

1) Decide whether to Keep Interval (number of slices will change) or Keep Slice (Interval between slices will be adjusted)

2) Select Range and position bars to decide where the Z-Series begins and ends

3) Select Start for image acquisition

Pressing Range produces an XZ image of selected Z-range, plus 50% above and below the selected stack.

Pull red lines to set limits for Z-Series
Drag green line to change focus position
Viewing a Z-Series

In the image window:
1) Select xy
2) Select Slice
3) Use scroll bar to view individual sections
Viewing a Z - Series using *Gallery*

1) Select *Gallery*

2) Select *Data* for scale

Use *Subset* to extract sections
Viewing a Z- Series using Orthogonal Sections

1) Select *Ortho*

2) Select mouse (Select)

3) Using the mouse, position the cut lines.

To save orthogonal sections, select *Export* and save as contents of image window.
Selecting and Saving a Region of Interest (ROI)

1) Select Overlay and define shape of ROI
2) Extract region creates a Z-Stack from the ROI
3) Save data
Using a ROI for faster image acquisition and data saving

1) Select *EditROI* from the LSM menu bar
2) Select *Fit Frame Size to bounding Rectangle*
3) Choose shape of ROI
4) Position and size the ROI in the image with the mouse
5) Start Scan

To remove ROI and overlay select blue bin or deactivate ROI. Closing the window only removes overlay, ROI is still active. Deactivate *Use ROI* in the LSM menu.
Multiple Regions of Interest

1) Un-select *Fit Frame Size to bounding Rectangle*, Choose shapes of ROIs

4) Position and size the ROIs with mouse

5) Start Scan

To remove ROIs and overlay select blue bin or deactivate ROIs. Closing the window only removes overlay, ROIs are still active. Deactivate *Use ROI* in the LSM menu.
Time Series

- Set up scanning parameters for image acquisition as described in previous slides
- Select *TimeSeries* from the LSM menu
- Enter the *Number* of cycles
- For a Time Delay between image acquisition select *min, sec* or *ms* and set time with the slider
- Select *Start T* to start image acquisition
- Instead of using *Manual* you can select *Time* to start and stop the series at a certain system time!
Viewing a Time Series of a Z Stack

- **Z Sections for any time**
- **Time points for any Z Section**
- **Both Z sections and time series**
Time Series – Physiology Experiments

1) If required, use multiple regions of interest

2) Set up Time Series as before

3) Instead of using StartT select MeanROI to start scanning

View and save data by selecting Mean in the image window
Imaging a large area using *Tile Scan*

This function is only available with a motorized stage.

1) Select *Stage* on LSM menu

2) Enter the *Tile Numbers*

3) Select *Start*

The maximum size is 4096 x 4096 pixels.
Any position can then be marked and a single image acquired by selecting *Move to* and then single.
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Saving Data - Using Database

1) Select Save or Save as on image window or LSM menu bar

2) Enter file name and notes if required

3) Select OK
Zeiss LSM 510 META - Guided Tour

Saving Data – Using Export

1) Select File from LSM menu
2) Select Export
3) Select Image type
4) Select Single image with raw data (No overlay or Look up table etc. is saved), Series with raw data, or Contents of the image window (Saves the image as shown on the screen)
5) Select Save as type

Tif - Tagged image File" is OK for 8 bit - use “Tiff -16 bit” for 12 bit acquired images (Most other software will not recognize 12 bit)
Shut Down Procedure

1. Go to: Acquire in the LSM menu - Laser – and deactivate HeNe Lasers by clicking Off to switch off Lasers

   ![Laser Control screen](image)

   **Note:** To turn off the Argon laser, first click on Standby, then reduce output power to 25%. Select Off.

2. Go to File - Exit to leave LSM 510 program
3. Shut down the computer
4. Wait until fan of Argon laser has switched off.
5. Turn off the remote control box
6. Switch off the mercury vapour lamp.
Please note:
This guided tour is intended merely as a quick introduction into the Zeiss LSM 510 software and does not cover all aspects of the system.

Please consult the manual for detailed instructions!
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