Welcome to the Zeiss LSM 710 Upright tutorial.

Before using the LSM 710 upright,

Microscope Confocal LSM710 UPRIGHT

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System Start Up = 8 Steps

• 1) Switch the *safety lock* (turn the key)

• 2) Switch the *main switch*
System Start Up = 8 Steps

- 3) Only if the ARGON laser is required, turn the key on the right, from the power supply.
System Start Up = 8 Steps

- 4) Wait 10 minutes, until you can toggle the switch, on the "laser run" position.

- Adjust the required power level with the control knob (default should be 10 to 11 o’clock) the light indicator from the "operation mode" should stay in the green range...not far to the red range.)
System Start Up = 8 Steps

- 5) Switch ON the “Systems/PC”
System Start Up = 8 Steps

- 6) Turn ON the **PC**
System Start Up = 8 Steps

- 7) Switch ON the “Components”
System Start Up = 8 Steps

• 8) Switch ON the “HXP 120” power supply (mercury lamp)
How to use the TFT Monitor

- The **TFT touch screen monitor** shows the microscope information status and can be used for controlling the microscope settings (objectives, filters, ports, etc).
How to use the TFT Monitor

• If you want to change some objective.
• Press on TFT monitor the *Control* function.
• Press desired objective under the *Objectives* function.
How to use the TFT Monitor

• For changing the port (e.g. to toggle from the side-port to the eyepiece) select the *Light path* button you want.

• **LSM = Scanning Mode**
• **TV = CCD Camera**
• **VIS = Eyepieces**
How to use the TFT Monitor

• For changing the fluorescent filter-cube chose the **Reflector** menu and select the desired fluorescent filter cube by pressing on the TFT-screen.
How to use the TFT Monitor

- Opening and closing of shutter can be found under the menu Reflector. RL shutter controls the HXP shutter.
How to use the Microscope

• The Upright Microscope AxioImager Z1 is a full motorized version.

• If you want to change the objective, filter cubes, shutter or port position, you should rotate through the TFT display or through the software.

• In case you change from a dry objective to an immersion objective, the TFT display prevents you from having to clean the objective before you turn/rotate the objective turret.

• The Lsm710 upright contain 2 Water immersion objectives. (20x and 63X) These 2 objectives are using/deeping directly into the medium + petri dish.
How to use the Microscope

• If you want to check your sample through the ocular, with the halogen lamp.
  1) Push the 2 slider port into the microscope
  2) Press the Halogen shutter
  3) Turn the intensity light knobs to the desired position

• If you want to scan, get the slider port off the top of the microscope. Keep the second slider port on the previous position.
System Start Up

• Once you have entered your login, you will see the screen fill with several icons.

• The software to control the confocal microscope is called **ZEN 2009**.

• To start it double click on it.

• Click **Start System**.
ZEN Software Presentation

- ZEN Main application Window after Startup.
- Application Bar
- Menu Bar
- Main Toolbar
- Tool Area (Acquisition)
- Tool Area (Image status)
- Image Area (Display)
- Status Area
• If you activate the checkbox *Show manual tools* new tool bars will appear which are necessary if you want to have access to all components/functions of the confocal microscope.

• Most of the tool bar contain an advanced mode, please click on *Show all* to implement the expert mode if you need it.
• Open the *Laser* Tools.
• The solid state lasers are automatically turned ON, except the *DPSS 561-10 laser*, for that one, you should connect if you need it.
Open the **Imaging Setup** menu to show the configuration of the light path.

- The detection Bands and the laser are displayed in a spectral panel.
- You can visualize the activated laser lines for excitation (vertical line) and activated detection channels (colored horizontal bars).
• Open the *Light Path* tools.
• You can build a **MULTI TRACK** set-up, for a sequential scanning acquisition.

• Each **TRACK** is a separate unit and can be configured independently from the other tracks.

• Add the new track through the *Imaging setup* and configure the new track with the *Light Path* tool.
ZEN Software Acquisition Control

- Open the **Acquisition Mode** tool.
- Here you can check which **objective** you use.
- You can change the **Scan mode** (Frame by default).
- You can change the **Frame size** (number of pixels).
- You can change the **Speed** of the scan (dependent on the zoom factor).
- You can apply **Averaging** (Methods: Frame/Line; number 2-16). Frame for fixed cell, line for living cell.
- You can change the **Bit Depth** (8 for a standard image, 12 for colocalisation, ratio or for deconvolution).
- You can change the **Zoom** factor (scanner).
• With the **Frame** scan mode you can:
  - Scanning of an XY frame (+Time)
  - Scanning of XY frame in different Z value (+Time)
  - Scanning of XY frame in defined ROIs (+Time)
  - Scanning of XY frame in different Z value, in defined ROIs (+Time)

• With the **Line** scan mode you can:
  - Scanning of a line in the XY-plane (+Time)
  - Scanning of a line with different Z value (+Time)

• With the **Spot** scan mode you can:
  - Scanning of a spot (+Time)
• Select the *Frame Size* from the default size via the drop down menu (click the X Y) button. Recommended setting to start with 512x512

• The *Optimal* button sets the image resolution to an optimal value corresponding to the optical magnification, resolution, the zoom and the emission range detected.
• Use the *Scan Speed* slider in the acquisition mode tool to adjust the scan speed.

• The signal to noise ratio of the image decreases with increasing *Scan Speed*. Speed 8-9 is a good starting value.
Averaging improves the image by increasing the signal to noise ratio.

Frame averaging is preferred for fixed samples as it reduces photo-bleaching.

Line average is preferred for moving/living specimens.
Select the dynamic range 8, 12 or 16 in the **Bit Depth**.

- 8 bit will give 256 gray value; 12 will give 4096 gray value and 16 bit will give 65536 gray level. 12 or 16 bit is recommended when doing quantitative measurement or when imaging low fluorescence intensities.
In this panel you can:

- Set the **Zoom** (through the scanner) in the range from 0.5 to the maximum of 30.
- Set the scanner rotation
- Move the offset of scanner in relation to the field of the view.
• Click on the **Channels** Menu.
• Set the **Pinhole** size to 1 AU (Airy Unit) for best compromise between depth discrimination and detection efficiency. Pinhole adjustment changes the Optical Slides thickness. When collecting multi-channel images, adjust the pinholes so that each channel has the same optical slide thickness.
• The output intensity of the lasers can be varied from 0.2% to 100%. Usually 2-15% should be enough for “normal” imaging.
• **Master Gain**: The PMT value is corresponding to the high voltage of the PMT. Typical values are 600-800 or up to a value where saturation of pixels can be detected.
• **Digital Offset**: Sets the offset of the A/D converter. It can be used to increase the dynamic range by setting the background to zero.
• **Digital Gain**: Amplification factor of the A/D converter. A value between 3 and 7 can be used to reduce the master gain value (=less noise).
Before using the scanner, check your sample under the scope using either the fluorescent or the transmission lamp.

If you use an oil immersion objective please put one drop of oil on your (cleaned) sample and not on the objective.

Don’t mix different oil brands. This might decrease the image quality considerably.

Select **Ocular**.

Select **Online**.

Select the **Objective** you want to use.
• Most microscope operations can be controlled by the Zen software.
• Under the ocular window/ menu you can control/choose/change the following parameters:
  • The objective.
  • The fluorescent filter cube.
  • The shutter(s).
• Now you are ready to find your sample with the microscope through the ocular.
Acquiring an image step by step

- Select the **Acquisition** window.

- To load pre-designated configurations, Click the button, and such as /for example (dapi43x), and Click **Enter**
If you want to create a new configuration it is very convenient to use the **Smart setup** tool.

You can use it to quickly set up the confocal beampath including the detector settings. Nevertheless check the settings as they might not be optimal for all applications.
Acquiring an image step by step

- Simply select the fluorescence dyes in your specimen from a list.
  - (for ex. DAPI)
• With this system you can easily build a configuration with several stains step by step,

• Simply select the fluorescence dyes in your specimen from a list. And choose one of the 3 images acquisition strategies.

• Then the system automatically changes all the required setting of the LSM

• Now you are ready to acquire an image. Click **Apply**
By clicking *AutoExposure* the ZEN software is trying to find reasonable PMT gains for the different channels and acquiring one image with these settings. These settings serve as a good starting point for further optimization.
In order to optimize your image you can follow the described procedures in the following.
In case of multiple channels: *Split* your image.
The sampling frequency is an important parameter which governs the resolution of the acquired image. Regarding the Nyquist theorem the smallest resolvable structure (in this case defined by the optical resolution limit) must be sampled (at least) twice in order to record all necessary information.

The maximum optical resolution resel* can be defined as the radius of the first dark fringe in the diffraction pattern, or half the diameter of the Airy disc.

\[
\text{Resel, confocal xy-plane} = \frac{0.44 \cdot \lambda}{\text{NA}}
\]

\[
\text{Resel, confocal axial} = \frac{1.5 \cdot n \cdot \lambda}{\text{NA}^2}
\]

Example \( \lambda = 500 \text{nm}, n = 1.518, \text{NA} = 1.4 \)

\( \text{XY- plane resel} = 157 \text{ nm} \)  \( \text{Axial resolution} = 580 \text{nm} \)

With Nyquist criterion the ideal voxel is \( 78.5 \text{ nm in XY and 290nm in Z} \)
The following table shows two different voxel sizes. One is the optimal voxel size following the Nyquist theorem, the other voxel size is proposed by SVI for doing deconvolution (Huygens).

For doing Deconvolution it is recommended to use the voxel size proposed by SVI if you sample allows it. But be aware of the fact that a smaller voxel size leads to more photobleaching. If bleaching is an issue, you can also use a voxel size following the Nyquist theorem.

For standard imaging a voxel size following the Nyquist theorem is totally sufficient. If you don’t have to optimize for maximum resolution even undersampling (larger voxel size) can be an option.
To optimize the digital resolution, you should:

1) Calculate your optical resolution (resel).
2) Calculate the Nyquist sampling corresponding to the optical resolution.
3) Apply the XY Nyquist sampling within the Optical Zoom factor and/or the Frame size to use as an ideal XY resolution.
4) If you are acquiring a 3 or 4D, please apply the Z Nyquist sampling within the Z interval size to use as an ideal Z resolution.
ZEN Image Optimization

Resolution

- Set the Pinhole size to 1 AU (Airy Unit) for best compromise between depth discrimination and detection efficiency.
- The Pinhole adjustment changes the Optical Slices thickness. When collecting multi-channel images, adjust the pinholes so that each channel has the same optical slice thickness.

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The signal to noise ratio can be substantially improved by:

1. Reducing the *Scan speed* to an acceptable level.

2. Increasing the *Averaging* number (2 to 16) using Mean as method.

3. Decreasing the *Pmt Value* (800-600..or less)
ZEN Image Optimization

Gray Levels

- To obtain a good image (usually) saturation must be avoided (exception small dim objects of interest and big bright objects in the same channel). Quantification within a saturated region is always leading to wrong results. Parameters which can influence saturation are:
  - PMT gain
  - A/D converter gain
  - Laser intensity used for excitation.

- Don’t push to much the Master Gain, otherwise you over-sature the image
- Don’t push to much the Digital Offset, otherwise you cut some image information.
- Let’s see next page which is the procedure to obtain a correct gray value into your image.
ZEN Image Optimization

Gray Levels

- Start to scan in continuous mode.
- Click inside the color field in the button under the channel button.
- The scanned image appears in a false-color (red and blue pixel appear).
ZEN Image Optimization

Gray Levels

• If the image is too bright, it appears red = saturation. Now you can reduce the **Master Gain** until you see only few red pixel.

• If the image is not bright enough, it appears blue = zero. Now you should set the **Digital offset** until you see only few blue pixel.
ZEN Image Optimization

Gray Levels

- If the image is too bright, it appears red = saturation. Now you can reduce the Master Gain until you see only few red pixel.
- If the image is not bright enough, it appears blue = zero. Now you should set the Digital offset until you see only few blue pixel.
Cross-talk

- The maximum of 2 channels can be defined simultaneously. But if you use 2 laser and 2 pmt in the same time, depending of which dye you use together, you may acquire some overlap in the excitation or emission peaks.

- To avoid some cross-talk you will work in the sequentiel mode (in multi-track mode) as the Smart Setup offered you.

- *Simultaneous* scanning of single or double labelling:
  - Advantage: faster image acquisition
  - Disavantage: Eventual cross-talk between channel

- *Sequentiel* scanning of double, triple or forth labelling.
  - Advantage: Only one detector and one laser are switched on at any one time. This reduces cross-talk.
  - Disadvantage: slower image acquisition.
After having a good image quality with the single scan.

Select the **Z-stack** function under the **Information On Experiment**.
• Open the **Z-stack** window.
• **Clik** *continuous* to scan your sample
• Then focus on the upper specimen area
• Click on the *Set First* button to set the upper position of the Z stack
ZEN Z-Section Acquisition

- Then focus on the lower specimen area
- Click on the *Set Last* button to set the lower position of the Z stack
Click to the *Optimal* button to set the number of slices to match a Z-interval for given stack size, objective lens, and pinhole diameter, or introduce the ideal z step size relative to the Nyquist criterion.
Click to the *Start Experiment* button to acquiring the Z section.
• You can check the z acquisition with the Gallery tool.
You can check the z acquisition with the *Orthogonal* tool.
You can check the z acquisition as a 3D volume,
System Shut Down = 11 Steps

- Step 1 = Remove your sample.
- Step 2 = Clean the immersion objective with Lens paper and ethanol 70%.
- Step 3 = Turn off the Zen software.
- If somebody would like use the microscope after you....Choose the LOGOUT option and leave the microscope like this.
- Step 4 = If nobody come after you, go to Start and shut down the Computer.
System Shut Down = 11 Steps

• Step 5 = Turn off the HXP 120 power supply
System Shut Down = 11 Steps

- Step 6) Turn the switch, on the "standby start power" position.

- Turn back the control knob
System Shut Down = 11 Steps

- Step 7) Turn the key to the left, from the power supply. (position “0”).
System Shut Down = 11 Steps

- Step 8) Switch off the "Components".
- Step 9) Switch off the "Systems/PC".
System Shut Down = 11 Steps

- Step 10) Only when the fan from the ARGON laser is off, switch off the main switch.

- Step 11) Switch the safety lock (turn the key).