Welcome to the Zeiss LSM 700 Upright tutorial.

Before using the LSM 700 upright,

- You will need to put down your name on the reservation system=
  http://svintranet.epfl.ch/index.php?option=com_view&task=view&id=52
Microscope Confocal LSM700 Invert

- Reservation System.
- System Start up: Hardware. (page 3-6)
- How to use the TFT Monitor. (page 7-8)
- How to use the Microscope. (page 9-11)
- System Start up: Software. (page 12)
- ZEN Software Presentation. (page 13-14)
- ZEN Software Acquisition Control. (page 15-25)
- ZEN Software Microscope Control / Acquiring an image step by step (page 26-27)
- ZEN Software build your Configuration / Acquiring an image step by step (page 28-32)
- ZEN Image Optimization. (page 33-43)
- ZEN Z-Section Acquisition (page 44-52)
- System Shut Down (page 53)
System Start Up = 4 Steps

1) Turn on the electric cord switch
System Start Up = 4 Steps

- 2) Turn on the **HXP 120 lamp**.
System Start Up = 4 Steps

3) Turn on the **Power Supply**.
System Start Up = 4 Steps

4) Turn on the computer.
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

- 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 Microscope

IMPORTANT

• The Upright Microscope is not a full motorized version.

• If you want to change the objective magnification, you should rotate the turret yourself to the desired objective.

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

• The Lsm700 upright contain 2 Watter immersion objectives. (20x ant 63X) This 2 objectives are using/ deeping directly into the medium + petris dish.
How to use the Microscope

IMPORTANT

• If you want to **check your sample through the ocular**, you should change the port yourself.
• Push the 2 slider port into the microscope

• If you want to **scan**, get the slider port off the top of the microscope. Keep the second slider port on the previous position.
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.
• 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.
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 (nb of the pixel).

You can change the Speed of the scan (dependant 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.
ZEN Software Acquisition Control

- 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.

• 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).

• 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.
Acquiring an image step by step

- 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.
ZEN Software build your Configuration

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

XY-plane resel = 157 nm  Axial resolution = 580nm

With Nyquist criterion the ideal voxel is 78.5 nm in XY and 290nm in Z
Resolution

- 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.

<table>
<thead>
<tr>
<th>LSM 700-710 Voxel Size Table</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ideal voxel size for confocal microscope (in nm) @ 488nm</td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>lsm700-710</td>
</tr>
<tr>
<td>10x 0.3 air</td>
</tr>
<tr>
<td>20x 0.8 air</td>
</tr>
<tr>
<td>20x 0.5 water</td>
</tr>
<tr>
<td>63x 0.5 water</td>
</tr>
<tr>
<td>40x 1.3 oil</td>
</tr>
<tr>
<td>63x 1.4 oil</td>
</tr>
</tbody>
</table>

**SVI Formula**

\[
XY = \frac{\lambda \text{ex}}{(2 \pi \text{sin} \alpha)} \quad Z = \frac{\lambda \text{ex}}{4 \pi (1-\cos \alpha)}
\]

**Resol* /2**

\[
XY = \frac{(0.44 \lambda \text{ex} \text{NA})}{2} \quad Z = \frac{(1.5 \pi \lambda \text{NA})}{2}
\]

Nyquist Calculator from SVI = [http://support.svi.nl/wiki/NyquistCalculator](http://support.svi.nl/wiki/NyquistCalculator)

Resol* = the resolution can be defined as the radius of the first dark fringe in the the diffraction pattern, or half the diameter of the Airy disc.

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.
• The signal to noise ratio can be substantially improved by:

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

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

3. Decrease the *Pmt Value* (800-600..or less)
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 pixels.
- If the image is not bright enough, it appears blue = zero. Now you should set the Digital offset until you see only few blue pixels.
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 offert 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.

Click *continuous* to scan your sample.
ZEN Z-Section Acquisition

- Then focus on the upper specimen area
- Click on the *Set First* button to set the upper position of the Z stack
- 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.
ZEN Z-Section Acquisition

- 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 = 6 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.
- Step 5 = Turn off the electrical cord switch.
- Step 6 = Turn off the HXP 120 power supply.