Welcome to the Leica Sp2 Confocal Upright tutorial.

Before using the Sp2 Invert,

- You will need to put down your name on the reservation system at:
  
Leica Sp2 Confocal Upright tutorial

- Reservation System.
- Startup Device.
- Start Up System.
- Objectif chossing.
- Switch the Microscope control in scanning or visual mode.
- Sample Manipulation (fixe or live).
- Focus the sample with the eyes pieces.
- Image Acquisition Setup.
- How to make your olne configuration (no sequential).
- Open or save your olne configuration (no sequential).
- Single vs. Multi-labelling.
- Sequential scanning.
- Choosing the configuration.
- Series (Preparing the Z stack).
- Resolution Rule, Objective/Format/Zoom.
- Schutting Down the System.
System Start Up

- Turn on the **PC/Stand** button (number 0).
- Turn on the **microscope controller** (number 1).
- Turn on the **mercury lamp** (number 2).
- Turn on the **XY stage controller** (numb. 3)
- Turn on the contact for the **Argon Laser** (number 4).
- Turn on the **Scan head button** (number 5).
System Start Up

- Switch on if you needed, the key of the Argon Laser (number 6.1). Let the laser warm 2 minutes, and turn the power button at approximately 11h.

- Switch on if you needed, the key of the HeNe 543 Laser (numb. 6.2).

- Switch on if you needed, the key of the HeNe 633 nm Laser (numb. 6.3).

- Turn on the button of the Diode 405nm Laser (numb. 6.4), and switch on if you needed, the key.

- Turn on the PC.
System Start Up

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

- Double click the icon. **Leica Confocal Software**.

- The Leica software will startup and you will see the **Leica Confocal Software** window.

- You will click **Personal** and then **Start**.

- During the loading of the software, you can follow the checking and **initialisation** of the hardware.

- The stage initialise going to the extreme in XY and setting the Zero.
When the Leica software opens 2 field appear.

In the *left screen* you can find:

1. The *Beam Field* = Manage the laser, PMT and beampath.
2. Working *browser and saved* the files.
3. Picture and confocal *parameters*.

In the *right screen* you can find:

4. *Channel switcher*
5. *Scan field*
6. *Stack Scroller*
7. *ROI Parameters*
Choosing the objective

- The first thing to do, when the sample is fixed on the stage, is to choose the correct objective. You can do it directly from the microscope (on the right side of the scope) with the objective button, but it is much more convenient to do it with the software. The objective button is on the left side of the Working panel.
- You will find the list of the objectives on this microscope.
- Choose the correct objective and click. The objective turret auto-matically presents the objective to the sample. You can adjust now the focus.
- If afterwards you want to change the magnification, just choose another objective in this menu. The computer will change the objective.
- When changing objectives, be careful to take the same type of objective. Always glycerol objective together, Oil objectives together and dry objectives together. If you mix up different immersion medium, you will have a really blurry picture and you have to really well clean the objective and slide with 70% alcohol.
Switch the Microscope control in scanning or visual mode

- To visualise the cells on the microscope you have to switch the system to the «Visual» mode. You click on the «MicCtrl» and chose «Visual».
- When the focus is done and the cell chosen, switch to the «Scan» mode, with the same button, to do a confocal imaging.
- When changing objectives, be careful to take the same type of objective.

- If you do a mistake, you will have a message from the software.
- If you mix up different immersion medium, you have to really well clean the objective and slide with 70% alcool.
Sample manipulation

• **Fixed samples on slides**

  Just put a drop of glycerol (or oil) on your coverslip, clip the slide, with the coverslip downward, facing the objective you choose and focus the sample. Don’t forget to fix the slide with the iron lamellas, for more stability.

• **Be extremely careful** that the high numerical aperture objectives have really short working distance, from 360 to 100 microns. Always keep an eye on your sample during the focus.

• **Living samples on Petry dishes**

  Chose the ring corresponding to the size of your petry dish (36 or 39 mm). Put the ring containing the petry dish on the stage, fix it with the iron lamellas, chose an objective and do the focus.

• **Be extremely careful** always keep an eye on your sample during the focus.
Focus the sample with the eyes pieces.

- To focus your sample you have to illuminate the sample with the correct light.
  - The filter wheel is composed by 5 filters.
    - Cy5 (Red light) => sample in Deep Red
    - TRITC (Green light) => sample in Red
    - FITC (Blue) => sample in Green
    - CFP (UV-Blue) => sample in Cyan
    - DAPI (UV-light) => sample in Blue

- Turn the filter wheel by pressing the «filter» button.

- The buttons near the indication «Shutter» open or close the mercury light shutter.

- When the focus is done and the cell chosen, switch to the «Scan» mode, on the «MicCtrl» button.
Image Acquisition Setup.

Modes
• For fixed samples, we will work almost always in xyz mode.
• For living cells, you should certainly work in xyz yxt or yxzt mode.

Bits depth
• Clicking on the «Bit» button, you have the choice of working in 8 or 12 bits.
• Most of the work is done in 8 bit.
• 12 bit is used for quantitative work to have a bigger grey values (4095 instead of 255).

Format
• The format corresponds to the size of the image scan, the most common is 512x512.

Speed
• The scanning speed of the laser is usually set at 400 Hz to 800 Hz the most common setting.
Create your own configuration.

1) Select the laser wave length and the power (%) of the laser.

2) Choose the virtual band pass filter by clicking the collector slice, click on the grey bar and you can mechanically define exactly what is the range of your virtual band pass filter.

3) Set the colour of your choice for every labelling.

4) Select the PMT «active».

- Activate the PMT 1 preferentially for Dapi
- Activate the PMT 2 preferentially for Fitc/Rfp/alexa 488/Yfp
- Activate the PMT 3 preferentially for Rohd/Rfp/alexa 555/Texas Red
- Activate the PMT 4 preferentially for Cy5/Draq5/Alexa 633)
Open or save your own configuration.

- Press the Icone «Beam».
- Click two time for opening a configuration.
- Select «Save» for saving a new configuration.
Image Acquisition Setup.

- Press the Icon «A.GAIN» for acquiring a primary image.
- Click the «continuous» button, (All the scanning setting as: zoom/gain/offset/begin/end).
- Focus your sample with the last button on the console.
- Select the «QLuts» Icon.
- Set the «Gain» button on the console until you seen some few blue pixel = high signal.
- Set the «offset» button on the console until you seen some few green pixel = low signal.
- Increase the Gain until you have the first blue pixels on your sample (you just need to have a few blue dots (blue = saturation (255))).
- Increase the Offset, the image becomes darker and the background pixels change to green (green = black (0)).
Image Acquisition Setup.

- Before clicking on the big button “Single scan” you have to set the Averaging, «Aver» button.

- The Line Averaging «Li.A.» the laser passes multiple times in the same line.

- In normal use, we do a 4 to 8 imag averaging.

- When the Gain and Offset is setted, Apply «Single Scan». 
Single vs. Multi-labelling

- We can use multiples PMTs at the same time but be careful to use dyes that not overlap in the excitation or emission peaks, otherwise you will have «Cross-talk»

- To avoid cross-talk you will work in «Sequential mode».
Sequential scanning.

- First, you have to set the PMTs and lasers lines separately.
- Example: FITC and TRITC.
- Set the 488nm and the 561nm laser, the PMTs (2 and 3) with the correct mechanical Band pass.
- Acquiring an image and correct the blue and green pixel value.
- Save this setting as a FITC and TRITC.
- Turn the 488nm laser at 0% and turn off the PMT 2.
- Save this setting as TRITC.
- Open the FITC and TRITC setting.
- Desselect the 561 laser and the PMT 3.
- Save this setting as FITC.
Sequential scanning.

- Select «Sequential mode».
- Drag and drop the FITC and TRITC setting from the Beam path setting, in the new «sequential scan setting» window.
- Select «between line», OR select «between frame» only if you scan a sample who contain Dapi stain.
- Press «Single Scan» if «between line» is activated.
- Press «Series» if «between frame» is activated.
Choosing the configuration

**No sequential mode.**

- Use for single, double, triple... Labelling.

  **Simultaneous scanning only.**

  **ADVANTAGE**

  Fast Acquisition. crosstalk.

  **DISADVANTAGE**

  Cross talk between channels.

**Sequential mode.**

- Use for double, triple, labelling or colocalisation.

  **Sequential scanning.**

  **ADVANTAGE**

  Reduce dramatically the crosstalk.

  **DISADVANTAGE**

  Slow Image Acquisition (One channel after one channel).
Series (Preparing the Z stack)

- Click on the “Continuous” button.
- Click on the “Series” button, you will see the absolute positioning of the current position in yellow, the end of the stack in red and the beginning of the stack in green.
- Turn the “Z” button until the region on the picture you define to be the beginning of the stack and click on the “Begin” button.
- Do the same for the “End” of the stack.
- Click “Sect”. you have an infinite choice of sectioning.
- You can use the “Optimized” option, is the precise number of optical slides you need to have a good 3D reconstruction or deconvolution.
  OR.... You can use the “Voxel size table rule/Format/Zoom” as you can check on the next page.
- Then press “Series”. 
Resolution Rule

- 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 Rule

- 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>SP2-SP5</th>
<th>SVI</th>
<th>Resel</th>
</tr>
</thead>
<tbody>
<tr>
<td>xy</td>
<td>z</td>
<td>xy</td>
</tr>
<tr>
<td>5x 0.15 air</td>
<td>406</td>
<td>10783</td>
</tr>
<tr>
<td>10x 0.3 air</td>
<td>203</td>
<td>2648</td>
</tr>
<tr>
<td>20x 0.4 air</td>
<td>152</td>
<td>1461</td>
</tr>
<tr>
<td>40x 0.6 air</td>
<td>101</td>
<td>610</td>
</tr>
<tr>
<td>10x 0.4 imm</td>
<td>152</td>
<td>1981</td>
</tr>
<tr>
<td>20x 0.7 imm</td>
<td>87</td>
<td>612</td>
</tr>
<tr>
<td>10x 0.3 watter</td>
<td>203</td>
<td>3559</td>
</tr>
<tr>
<td>20x 0.5 watter</td>
<td>122</td>
<td>1250</td>
</tr>
<tr>
<td>63x 0.9 watter</td>
<td>67</td>
<td>347</td>
</tr>
<tr>
<td>63x 1.30 gly</td>
<td>46</td>
<td>151</td>
</tr>
<tr>
<td>40x 1.25 oil</td>
<td>48.8</td>
<td>185.8</td>
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<tr>
<td>63x 1.4 oil</td>
<td>43</td>
<td>131</td>
</tr>
<tr>
<td>100x 1.46 oil</td>
<td>41</td>
<td>110</td>
</tr>
</tbody>
</table>

SVI Formula:
- \[ XY = \frac{lex}{8 \cdot n \cdot \sin a} \]
- \[ Z = \frac{lex}{4 \cdot n \cdot (1 - \cos a)} \]

Resel* /2

Nyquist Calculator from SVI = http://support.svi.nl/wiki/NyquistCalculator

Resel* = 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.

System Shut down

Before you turn off the System...Please follow this rule.

1) Save your data on the \Svitsrv1\biop your group name / your name.
2) Quit the software.
3) Remove your sample.
4) Clean your objective with the specific Lens paper.
5) Verify on the intranet on the Reservation system if somebody use the scope after you.

If yes, Log off the computer, and turn down the laser 488nm power button.
If no, follow the procedure is described in the next page.
System Shut down

- Turn on the button of the Diode 405nm Laser (numb. 6.4).
- Switch off the 405nm laser key.
- Switch off the 633 nm laser key (numb. 6.3).
- Turn off the 488 laser power.
- Switch off the 488nm laser key. (numb. 6.1)
- Turn off the scanner head button. (numb. 5)
- Turn off the 488 laser button. (numb. 4)
System Shut down

- Turn off the **XY stage controller** (numb. 3).
- Turn off the **mercury lamp** (number 2).
- Turn off the **microscope controller** (number 1).
- Shut down the **PC** (go to start).
- Turn off the **stand button** (0).