Welcome to the Leica Sp5 2 Photons Upright tutorial.

Before using the Sp5 2 Photons

- 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
System Start up (step 1)

1) Turn the Key on position "ON" from the CHAMELEON power supply.

2) Switch on the Extension cord = (EOM, NDD, and Xcite controller.)

3) Wait 30"

4) Turn on the PC/Microscope button
5) Wait 20"

6) Turn on the Scanner Power button.
System Start up (step 2)

- Turn on the PC
- Introduce your Username
- Introduce your Password
- Introduce your login
System Start up (step 3)

• Start the LASAF software.

• If you want to use the resonant scanner (8000 HRZ) select “Active Resonant Scanner”.

• The conventional scanner is optimized for morphological studies. An 8196x8196 pixel images can be obtained in combination with large field of view.

• The Resonant Scanner work at 16000 Hz in bidirectional mode. The system acquires 25 images per sec with the 512x512 pixels.
System Start up (step 4)

• And select “OK”.

Leica Application Suite
Advanced fluorescence

Ok
If you want acquiring some Tiled image or if you want recover some xy position, select **YES**
How to turn on the Chamelon 2P laser

- Under **Hardware Configuration and Laser**
- Switch on the **Multi-Photon Laser**.
How to turn on the Chamelon 2P laser

- Select MP Laser
How to turn on the Chamelon 2P laser

• Introduce the Wavelength you want.

• Switch the **MP Shutter** (maintain the mousse 3”)
  • on the MP shutter Icon)
## Suitable dyes for MP imaging

<table>
<thead>
<tr>
<th>Category</th>
<th>Dye</th>
<th>1 Photon Ex/Em (nm)</th>
<th>2 Photon Ex (nm)</th>
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<tbody>
<tr>
<td><strong>Gene Expression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFP</td>
<td>395/509</td>
<td>780&gt;820</td>
<td></td>
</tr>
<tr>
<td>CFP</td>
<td>434/477</td>
<td>780&gt;840</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>488/507</td>
<td>860&lt;960</td>
<td></td>
</tr>
<tr>
<td>YFP</td>
<td>514/527</td>
<td>860&lt;960</td>
<td></td>
</tr>
<tr>
<td>Yellow Chameleon</td>
<td>434/477-527</td>
<td>780&gt;840</td>
<td></td>
</tr>
<tr>
<td>Ds RED</td>
<td>543/580</td>
<td>900-1064</td>
<td></td>
</tr>
<tr>
<td><strong>Mito tracers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodamin 123</td>
<td>507/529</td>
<td>780-860</td>
<td></td>
</tr>
<tr>
<td><strong>Neuronal Tracer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DID</td>
<td>633/665</td>
<td>780</td>
<td></td>
</tr>
<tr>
<td><strong>Neurotransmitter Release</strong></td>
<td>FM 1-43</td>
<td>510/626</td>
<td>830</td>
</tr>
<tr>
<td><strong>Cell Wall Stain</strong></td>
<td>Calcofluor White</td>
<td>440/500-520</td>
<td>780&gt;820</td>
</tr>
<tr>
<td><strong>Nucleic Acid Stain</strong></td>
<td>DAPI, Hoechst</td>
<td>350/470</td>
<td>780&gt;820</td>
</tr>
<tr>
<td>Feulgen</td>
<td>480/560</td>
<td>780&gt;820</td>
<td></td>
</tr>
<tr>
<td><strong>Cell Viability</strong></td>
<td>Fluorescein Di Acetate</td>
<td>495/520</td>
<td>780&gt;821</td>
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<tr>
<td>Calcium</td>
<td>Calcium Green/Texas red (770)</td>
<td>488/530</td>
<td>780</td>
</tr>
<tr>
<td>Calcium Texas red (770)</td>
<td>596/620</td>
<td>780</td>
<td></td>
</tr>
<tr>
<td>Calcium Green</td>
<td>488/530</td>
<td>780&gt;820</td>
<td></td>
</tr>
<tr>
<td><strong>Protein Conjugates</strong></td>
<td>AMCA</td>
<td>431/498</td>
<td>780</td>
</tr>
<tr>
<td>FITC</td>
<td>490/525</td>
<td>780&gt;820</td>
<td></td>
</tr>
<tr>
<td>CY2</td>
<td>489/506</td>
<td>780&gt;800</td>
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</tr>
<tr>
<td>CY3</td>
<td>550/570</td>
<td>780</td>
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<tr>
<td>CY5</td>
<td>649/670</td>
<td>780&gt;820</td>
<td></td>
</tr>
<tr>
<td>TRITC</td>
<td>541/572</td>
<td>800&gt;840</td>
<td></td>
</tr>
<tr>
<td>Texas Red</td>
<td>596/620</td>
<td>780</td>
<td></td>
</tr>
</tbody>
</table>
• **Under Hardware Configuration and Dyes,**

• **Find the Excitation and Emission spec. of your labelling into the spectral Data Base.**
2 Photon Power vs Wavelength

![Graph showing 2P laser power vs wavelength](image-url-here)
Under **Hardware Configuration** and **Crt Panel**, you can manage the control of the panel.

- **Usually the first knobs** is dedicated for the **Smart Gain**.
- **Second for Smart Offset**.
- **Third for Scan Field Rotation**.
- **Fourth for the Pinhole Aperture**.
- **Fifth for Zoom Factor**.
- **Six for the Z Position**.
Hardware Setting

- Under Hardware Configuration and Setting.

- Panning Step size = 3.

- Line average should be active.

- Data transfer mode = Direct.

- 8 bit is selecting by default. 12 or more if for a ratio, co-localisation, or high resolution image.

- Activate the z-limitation movement.
Instrument Parameter Setting

- Under **Hardware Configuration** and **IPS Mask** you can see which parameter of the Confocal that you want to recover, from the image. The image contain all parameter from the scope. You can reload the parameter if the **Apply** function still active.
The Microscope Control (1)

- To control the microscope, you should use the “Leica STP6000” Function-Keys.

- Here a message give you the microscope status concerning:
  - The Method. CS, FLUO or TL
  - The filter cube: A4 I3 N21
  - The Objective

- If IL is active = Illumination for FLUO and you can reduce or increase the % of light with the FIM function and open/close the shutter with the IL-SHUTTER.

- If TL is active = Transmission Light, and you can reduce or increase the light with the Intensity function; reduce or increase the Field of the view.
The Microscope Control “FLUO” (2)

- You can select FLUO = Fluorescence
- CS = Confocal Scanner

- For example, if you select FLUO you can use 3 different filters as:
  - A4 = Dapi
  - I3 = FITC
  - N21 = ROHD

- Select one filter position and select IL-shutter

- Now you can check your sample through the eyepiece
The Microscope Control “BF” (3)

• Press TL
• You can reduce or increase the light with the Intensity function, or you can, reduce or increase the Field of the view with the Field function.

• Now you can check your sample trough the eyepiece
The Microscope Control (4)

- Here you can select the objective you want.
- If you want to check through the eyepiece, press the Eyes piece Icon.
- If you want to scan, press the CCD Icon.
The Microscope Control (5)

• Here you read the current Z position

• You can also Set the Z position at 0 if you press this function
• Don’t use this two function =

• Touch Keys

• Language
The Microscope Control (7)

- Here you can move the XY stage in X and Y position

- Here you can move the stage in Z position
Create your experiment

- Under Acquire and Experiment

- When the software is open, an experiment name is created, you can rename it.
Choosing the objective

- **Under Acquire and Acquisition**
- Choose and select your objective in the list
- You can choose the:

  - **HCX PL APO CS 10x 0.4 IMM**
  - low magnification, low resolution and immersion,
  - as water, glycerol or oil.

  - **HCX PL APO CS 63x 1.2 WATER**
  - high magnification, good resolution,
  - deep penetration (use for living cells)

  - **HCX PL APO CS 63x 1.3 Glyc 21c**
  - high magnification, very good resolution, deep
  - penetration (use for fixed cells in mounting media)
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 software.

- Choose the correct objective and click. The objective turret automatically 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 the 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.

- 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. Always keep an eye on your sample during the focus.
To attenuate the laser power, a continuously adjustable electro-optical modulator (EOM) is used.

A fine EOM calibration gives a good power laser in relation of the offset value.

The EOM adjustment allows improving the ratio signal to noise of your image.

Because the EOM adjustment is not user-friendly, the BIOP team already saved some EOM vs Wavelengths, configurations (the most useful) into the “single setting list”. Please use it….Otherwise follow the method as it’s described on the next page.
How to setup the EOM (step 1)

- Before to setup the EOM:
  - 1) Turn on the 2 Photon laser
  - Example for GFP:
    - 2) Select the wave length (+/- 900nm)
  - 3) Select the PMT you want.
  - 4) Introduce the Spectral band pass range you want into the PMT dialogue. (505-550nm)
How to setup the EOM (step 2)

- Let's scan at 700Hz, 512x512, increase the Gain of the PMT at the max (1250 V)
How to setup the EOM (step 3)

• During the scan, Setup the FW at 12.5% (filter wheel)
How to setup the EOM (step 4)

- During the scan increase the **Offset** until the image disappear (the best Polarisation)
How to setup the EOM (step 5)

- Switch the **MP** (Modulator Polarisation) at “1”
How to setup the EOM (step 6)

- Reduce the **Gain** of the PMT (600-900 V)
- Increase the **Gain of the Laser** until the image appearing again (Laser Power Increasing)
How to setup the EOM (step 7)

• Save the configuration into the list
<table>
<thead>
<tr>
<th>Stain</th>
<th>nm</th>
<th>Filter Wheel %</th>
<th>Gain %</th>
<th>Offset %</th>
<th>mW Power</th>
<th>QE Image</th>
<th>Background</th>
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<td>86</td>
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<td>dapi</td>
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<tr>
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<td>82</td>
<td>3720</td>
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<td>no</td>
</tr>
<tr>
<td>Pacific Blue</td>
<td>800</td>
<td>12</td>
<td>1</td>
<td>82</td>
<td>3725</td>
<td>ok</td>
<td>no</td>
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<tr>
<td>Pacific Blue</td>
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<td>12</td>
<td>17</td>
<td>89</td>
<td>3495</td>
<td>ok</td>
<td>no</td>
</tr>
</tbody>
</table>
NDD = non descanned detector

• For detection, the internal spectral detector in the scan head can be used. But given the intrinsic confocality of the method, excitation is limited to the focal plane. Higher collection efficiency can be ensure by the extremely short coupling detectors. Leica TCS SP5 allows for using large photo sensor area, as found in external detectors, call non descanned detector or NDD.

• PMT NDD1 and 2 (RLD 1 and 2) = Reflected Light Detector

• PMT NDD3 and 4 (TLD 1 and 2) = Transmission Light Detector
How to use the NDD PMT

- To use the NDD PMT you should select “Additional Channels”

- Here you have 5 NDD (non-descanned detector):
  - PMT Trans = Transmission PMT
  - PMT NDD1 (RLD 1) = Reflected Light Detector position 1
  - PMT NDD2 (RLD 2) = Reflective Light Detector position 2
  - PMT NDD3 (TLD 1) = Transmission Light Detector position 1
  - PMT NDD4 (TLD 2) = Transmission Light Detector position 2
How to use the NDD PMT

- Here you can select the RLD1 and RLD2 and introduce the desired filter into the NDD chamber like:
  - Tritc (RFP) for position 1 and Fitc (GFP) for the position 2 = TRITC/FITC or
  - Fitc (GFP) for position 1 and Dapi for the position 2 = FITC/DAPI or
  - Dapi for position 1 and Tritc (RFP) for the position 2 = DAPI/TRITC
- If you select “TLD/RLD” you can coupled the TLDs and RLDs together.
- You can use the NDDs as a RLDs (reflection PMT) or TLDs (transmission PMT), to ensure full flexibility, two channels detector can be coupled on both sides (2 PMT for the RLD detection channels and 2 PMT for TLD detection channels) = four NDD channels detectors if you need.
How to use the NDD PMT

- Here as example the two NDD for TLDs are selected, and the FITC/TRITC Filter are used
- *(FITC for the TLD1 and TRITC for TLD2)*
Choose your acquisition setting.

- Select the acquisition dimension mode, XYZ by default.

X = 1 Dimension (line)
XY = 2 Dimension (plan)
XYZ = 3 Dimension (Volume)
T = Time
λ = Emission
Λ = Excitation
Choose your acquisition setting.

- Chose the **format** (nb. pixel) 512x512 by default.

  Small format = fast acquisition but bad pixel size.

  Large format = slow acquisition but contribute to a good pixel size.
Choose your acquisition setting.

- You can increase or decrease the **scanner speed**: 400 Hz by default.

Slow scanner speed = increase the photon counting but increase the time acquisition and the bleaching effect.

High scanner speed = decrease the photon counting and the acquisition time but increase the bleaching effect.
Choose your acquisition setting.

- You can change the **single direction** scan by **bidirectional** (you decrease the acquisition time by 2)
  Use only if is necessary for live sample / tracking.

- One direction = the scanner and the Pmt acquiring the line from the left to the right position (X) = good phase

- Bidirectional = the scanner and the Pmt acquiring the line with the two direction = may be produce a bad phase (you should to test) is adjustable, and you increase the acquisition time by 2.
Choose your acquisition setting.

- You can make a **zoom** into the scan field (scan zoom) as region of interest (the pixel size change).
Choose your acquisition setting.

- You can change the Pinhole aperture = 1 Airy unit by default

- **BUT KEEP ALWAYS OPEN** completely the Pinhole Photon

- In the case of two-photon excitation, the dye is excited by the simultaneous absorption of two photon. Due to the non-linearity nature of two photon absorption, the excitation is limited to the focal volume and the photobleaching outside the focal plane is reduced. Only inside the Confocal volume the photon density is sufficiently high to allow two photon absorption by the fluoro-phore.
Choose your acquisition setting.

- Use the average in **Line scan mode**:  
  2 to 4 line if it is a living cell  
  4 to 16 if it is a fix cell/tissue  
  6 to 8 is the compromise.

- You can also use the **Accu**, (accumulation) mode if the fluorescents efficiency is too low.
Choose your acquisition setting.

- Use the **Substrate** position.
Acquire an image with a good Grey level

- Press «Live» for acquiring a preliminary image in living condition.
- Focus your sample with the last button on the console.
- Select the «QLuts» Icon.
- Set the «Gain» button on the console until you see some few blue pixels = high signal.
- Set the «offset» button on the console until you see 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 (= black (0)).
- The ideal value of the GAIN is between 400 to 600.
- The maximum GAIN value is between 900 to 1000 Volt.
You can save or load your setting.
How to acquire a Z-Stack, step 1

• Go to acquisition mode.
• Select XYZ.
• Go to the Z-Stack dialogue.
How to acquire a Z-Stack, step 2

- Scan in live mode
- 1) Find the first section by moving the z motor Galvo (with the USB Control Panel) until the focus is positioned on the top of your sample.
- 2) Select Begin
How to acquire a Z-Stack, step 3

- Continue to scan in Live Mode
- 3) Draw and setup the last section by moving the z motor Galvo until the objective is positioned below to your sample (with the USB Control Pannel).
- 4) Select End
- 5) Stop to Scan
How to acquire a Z-Stack, step 4

6) Introduce the **step size** into the calculator.

Do enter.
How to acquire a Z-Stack, step 5

- 7) Select Start
The image resolution obtained with two-photon microscope (TPM) is not better than that achieved in a simple photon confocal microscope. The utilization of longer excitation wavelengths (such as red or infrared, instead of ultraviolet or blue), although an advantageous aspect of two-photon excitation, actually results in a larger resolution spot. If a biological structure cannot be resolved in the confocal microscope, it will similarly not be resolved in a two-photon excitation laser-scanning microscope.

Because the TPM wavelength is twice that of the one-photon case, owing to longer wavelength used, TPM has a wider PSF when compared with the one-photon case, we find that the lateral resolution in TPM is around 2 times worse in TPM as a confocal 1 Photon image.

So to calculate the xy and z resolution, you can use the same resolution rule as we use in the widefield case, or like we use for the confocal microscope because in this case, the wavelength of excitation into the equation is twice as high as in the case of a confocal.*

\[
\text{Resel, confocal xy-plane} = \frac{0.44 \cdot \lambda}{\text{NA}} \\
\text{Resel, confocal axial} = \frac{1.5 \cdot n \cdot \lambda}{\text{NA}^2}
\]

Example \( \lambda_{ex} = 1000\text{nm}, n = 1 \) NA = 1.33

XY- plane resel = 610 nm  Axial resolution = 2660 nm

With Nyquist criterion the ideal voxel is 305 nm in XY and 1330 nm in Z

*handbook of biological confocal microscopy claims on page 449 (chapter 28):
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 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.

### Sp5 2P Voxel Size Table

<table>
<thead>
<tr>
<th>Sp5 2P</th>
<th>SVI xy (nm)</th>
<th>SVI z (nm)</th>
<th>Resel xy (nm)</th>
<th>Resel z (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x.4 IMM</td>
<td>312</td>
<td>550</td>
<td>4060</td>
<td>6234</td>
</tr>
<tr>
<td>63x.1.2 Water</td>
<td>104</td>
<td>330</td>
<td>183</td>
<td>693</td>
</tr>
<tr>
<td>63x.1.3 Glycerol</td>
<td>96</td>
<td>341</td>
<td>169</td>
<td>674</td>
</tr>
<tr>
<td>20x.1.0 Water</td>
<td>125</td>
<td>533</td>
<td>220</td>
<td>973</td>
</tr>
</tbody>
</table>

SVI Formula: $0.44 \frac{\lambda \text{ex}}{N_a}/2$

Resel* /2: $\frac{\lambda \text{ex}/(4 \text{ k Na})}{2}$

Nyquist Calculator from SVI = http://support.svi.mtu/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 (step 1)

• Switch off the MP Shutter

• Under Hardware Configuration and Laser.

• Switch off the Multi-Photon Laser.

• Exit from the LASAF Software.
System Shut Down (step 2)

- Go to Start and shut down the computer.
**System Shut Down (step 3)**

- **1)** Turn off the *Scanner Power* button.
- **2)** Turn on the *PC/Microscope* button.
- **3)** Switch on the *Extension cord* = (EOM, NDD, and Xcite controller.)
- **4)** Turn the Key on position "**STANDBYE**" from the CHAMELEON power supply.

**Before you exit from the software**
**Please clean the objective**