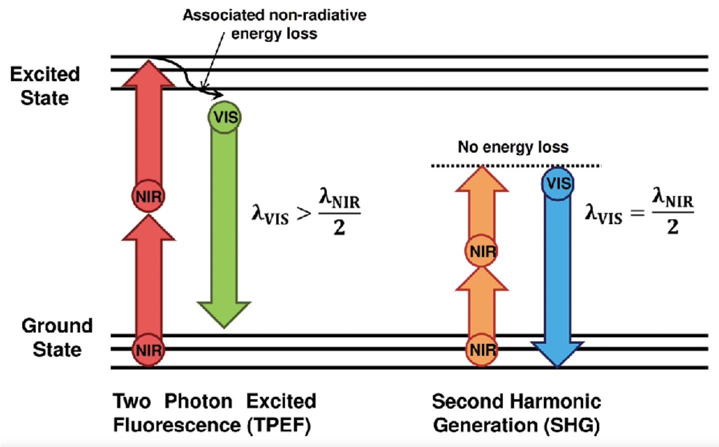
SHG with Multi-Photon laser

SECOND HARMONIC GENERATION IMAGING WITH MULTI-PHOTON LASER

This manual is used to create Second harmonic generation images on one of the microscopes equipped with a Multi-photon laser. Within the OIC there are 2 microscopes that u can use namely the **SP5Intravital_880 and the SP5-778**. This manual is a continuation on the basic SP5 Quick start manual. Before starting your SHG imaging contact OIC for an additional introduction. For SHG question contact us via: Gert-Jan Kremers; tel. 43578; g.kremers@erasmusmc.nl .

Second harmonic generation (SHG) background

SHG is a nonlinear optical process in which highly polarizable, noncentrosymmetric materials emit photons with half the wavelength of incident light. This means that these materials can be visualized without staining with a primary/secondary antibody. One of these highly polarizable, noncentrosymmetric materials is collagen.
To visualize these materials you need a high voltage Multi Photon laser. The MP Laser is a pulsed laser, this means that the laser gives high peaks of 3000mw instead of the usual 3mw that the normal laser create. The high power and pulse is needed to make sure the 2 photons are combined in the same spot. When the two photons meet, and only then, they will excite the material and visual light will be emitted. SHG is based on the fact that the tissue “scrambles” the laser with 880nm wavelength to 440nm. In SHG the light is so precisely scattered that it is always half the wavelength that you started with. This is because there is no loss of energy during the decay.
In theory you can use every wavelength that you want. The tissues structure will half the wavelength (1000nm > 500nm) but because the MP laser has the most power at 880nm, we choose this wavelength. This high power is needed because the signal of the tissue is very low, so you need to have a high power to visualize the tissue.



Adjust the MP laser offset slider before you begin imaging

Before use of the MP laser you always need to calibrate the offset of the laser. If you want to use the same offset between all your samples/experiment you need to have an Orange/yellow fluorescent Chroma slide (ask OIC). Otherwise, you can just follow the steps to calibrate the MP laser power.

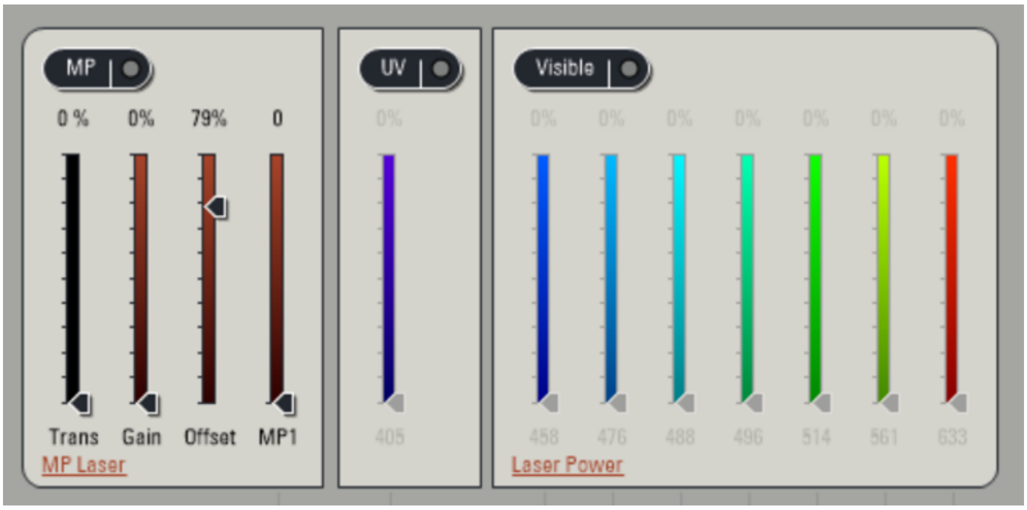
MP laser power calibration

A calibration needs to be done because the offset, set by the software, is most of the time not correct.

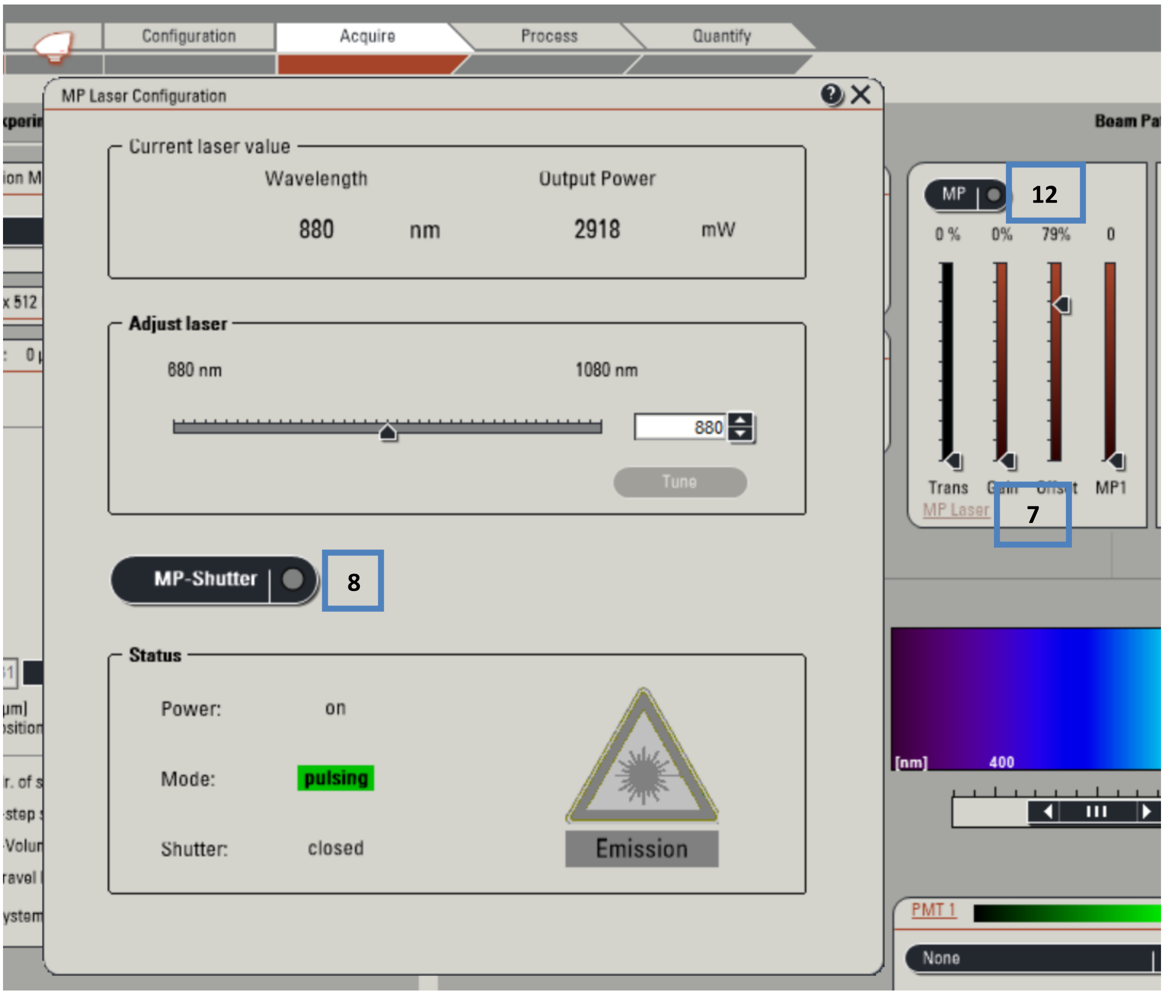
1. First check if you have the correct objectives. Otherwise, change these before you start the system.
2. Start the MP laser, by turning the key, before turning on the confocal.
    a. Check if the power goes up (final +/- 3000mw)

       SP5-intravital_880: located in the small extra room at the end of the room.
       SP5-778: located underneath the microscope

3. Start the system by pressing the green buttons from left to right and turn the key.
4. Start the LAS AF software using the configuration “Configuration_MP-Laser_on without OPO”.
5. In LAS AF go to the Configuration tab and activate the additional lasers.
6. In the Acquire tab you will see, next to the Visible laser lines, an additional MP laser control panel with Trans, Gain, Offset and MP1.



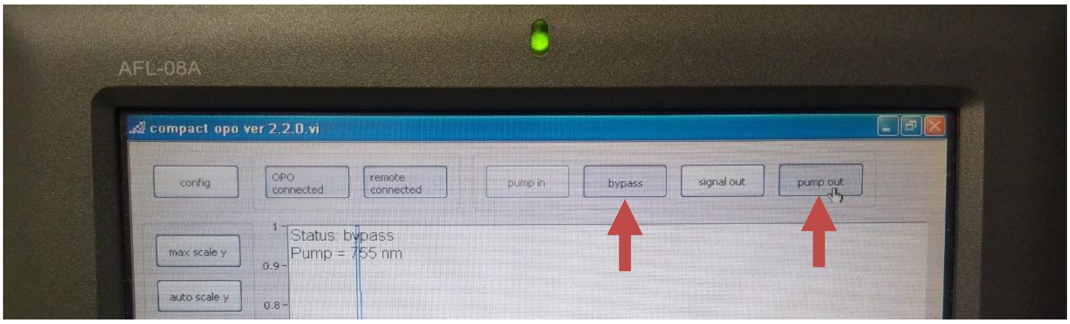
7. Open the MP laser wavelength configuration menu
8. In the MP Laser Configuration menu that pops-up, click the MP shutter button and hold for 2 seconds (dot turns red).



9. Tune the wavelength of the laser (if the laser is already set to the correct wavelength, change it by 10 nm and then tune to the correct wavelength again).
    a. Type the correct wavelength and press enter
    b. Click "Tune"

10. Set the MP laser slider to: Trans 10%, Gain 30% and XXXnm 0 (MP1 is changed to the wavelength value). The Offset slider is automatically set upon tuning the laser wavelength, but, as said before, this value is most of the time not correct.
11. Activate the transmission detector (PMT trans) and adjust the detector gain to 300V. Leave the PMT for the visible lasers activated!
12. Open the MP shutter (dot turns red)

13. Make sure the "bypass" and "pump out" buttons, on the touchscreen are activated.



14. Start live imaging with all lasers off. Increase the gain of the transmission detector until you pick up signal from the room lights. Turn off the room lights and increase the transmission detector gain until you observe a signal again (This is the light from the MP laser). Adjust the MP laser offset slider in order to minimize the signal on the transmission detector (i.e. the laser is really off).
15. When you set the XXXnm slider from 0 to 1 you should see an increase in signal (Transmission images will be completely white).
16. You can now start imaging with the MP laser. Increase the Trans slider to increase the laser power.

SET UP SHG IMAGE

1. Put your samples under the microscope and focus by eye with the bright-field light.
2. In the software, setup the system to create a transmission image with the visible lasers.
3. Press 'live' and look at your Transmission image to focus.
4. If correct press 'stop' and turn to the MP laser by closing the visual laser shutter and open the MP laser shutter.
5. Select the correct NDD PMT's for you experiment and set the NDD's Gain to 1250V. When selecting the NDD's you should see a pop-up "Switching to internal Detectors".
6. Put the black microscope cover over the microscope to block all the light from the room and equipment surrounding the microscope (located on the shelf in the microscope room)
7. Make sure the bypass and pump out on the touchscreen are activated.
8. Set the XXXnm slider to 1 and press 'live'.
9. The laser power (MP Trans) is still 10%. By turning up the power, you can gradually increase the power until 100% (if needed). Stop when the Second Harmonic structures are visible.

   If your image is to weak you can increase the MP gain. You want to increase the MP gain and try to lower the NDD's gain so that the signal becomes less grainy.

   Averaging, Gain and Format can be changed according to you sample and the details you want to see. The more averaging and higher Format the longer it takes to create the picture. So decide what works best for you. MP Gain % depends on the intensity of your signal.

10. When your settings are correct you press ‘start’ or ‘capture image’.
11. Save your images in the OIC transport drive.

Notes:

MP laser and normal lasers cannot be used together. If you want to combine the SGH image with the transmission image or other fluorophores you have to create two separate pictures and combine them in Image J.

The NDDs have no pinhole and capture all the light from the spot where the 2 photons interact. NDD = Non Descanned Detection. NDD’s have different filter sets to collect the specific light. NDD1: 430-450nm, NDD2: 465-500nm, NDD3: 520-550nm, NDD4: 565-605nm.

Materials that have SHG:

- Collagen I and IV: 880nm laser and NDD1 (SGH emission : 440)
- Elastin in blood vessels: 880nm Laser with NDD2 or NDD3

FIRST TIME CALIBRATION OF MP LASER POWER WITH ORANGE/ YELLOW FLUORESCENT CHROMA SLIDE

This calibration method is to assure you that you use the same MP laser power for experiments done on different days. The calibration is done for the first time AFTER you have performed your experiment for the first time. Subsequently you begin with the calibration each time you repeat the experiment.

Required: Orange/yellow fluorescent Chroma slide (ask OIC)

1. Place the orange/yellow Chroma slide on the microscope. Use one of the visible lasers (Ex 488nm Em 500-550 nm, or Ex 561nm Em 570-625 nm) to locate the focus plane with the highest fluorescence signal (typically at a depth of ~10um into the fluorescent slide).
2. Switch to the MP laser and use the same trans, gain and offset settings as during your biological experiment.
3. Adjust the following hardware settings:
   Objective: same as for experiment
   Format: 512x512 pixels
   Scanspeed: 400Hz
   BiX: active
   Zoomfactor: 1x
   Pinhole: 1AU
   PMT2 active, start at 850V

4. Start “Live” and adjust the detector gain for the active channel to get a bright image. Make sure to avoid saturated pixels (average intensity around 100 is good). Measure the mean intensity of the image (use the analysis tab to do this). Note the average intensity as well as the PMT detector gain in your lab journal.
5. This is your reference image. Save it so you can always get the settings back.
6. Calibration of MP laser power before repeating your experiment
7. To verify you have the same MP laser power during your next experiment, first image the calibration slide with exactly the same settings as during your first calibration. If the average intensity is different, you can adjust the MP trans slider to get the same fluorescence intensity and thereby the same MP laser power.

CONTACT INFORMATION

For direct support at the microscope call 31105.

For SHG question contact us via: Gert-Jan Kremers tel. 43578 g.kremers@erasmusmc.nl