

# **User guide and application notes for Zeiss LSM7 Multiphoton intravital microscope**

**Biomedicum Imaging Unit (BIU) University of Helsinki**

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## 1. SYTEM LOCATION AND GENERAL RULES

### Location

Biomedicum 1 animal facility (P-floor, room AP29b)

### Contacts

This system is a part of Biomedicum Imaging Unit infrastructure. For more information please visit the BIU website <http://www.biu.helsinki.fi/>

In case you have any questions or comments regarding the system, please contact BIU personnel via e-mail [biu-support@helsinki.fi](mailto:biu-support@helsinki.fi) or by phone +358-2941-25271, +358-2941-25268.

### System reservation and use

The system is available for reservation via BIU's scheduler <http://scheduler.miu-support.org>

Please also check the BIU user policy <http://www.biu.helsinki.fi/userinfo.htm>

In order to start using the system on your own for intravital imaging several criteria should be met:

- Receive user training for the system and approval from BIU personnel.
- Make sure you have an access to the Biomedicum-1 animal facility.
- Make sure you have the right to work with live animals.\*
- Check and update, if needed, your animal experimentation license.\*

\* Researchers are fully responsible for keeping their animal licenses and permits valid for their imaging experiments. BIU is not responsible for checking researcher's animal permits and licenses but can help users to update them if requested. BIU's intravital personnel has approval for C-category animal procedures.

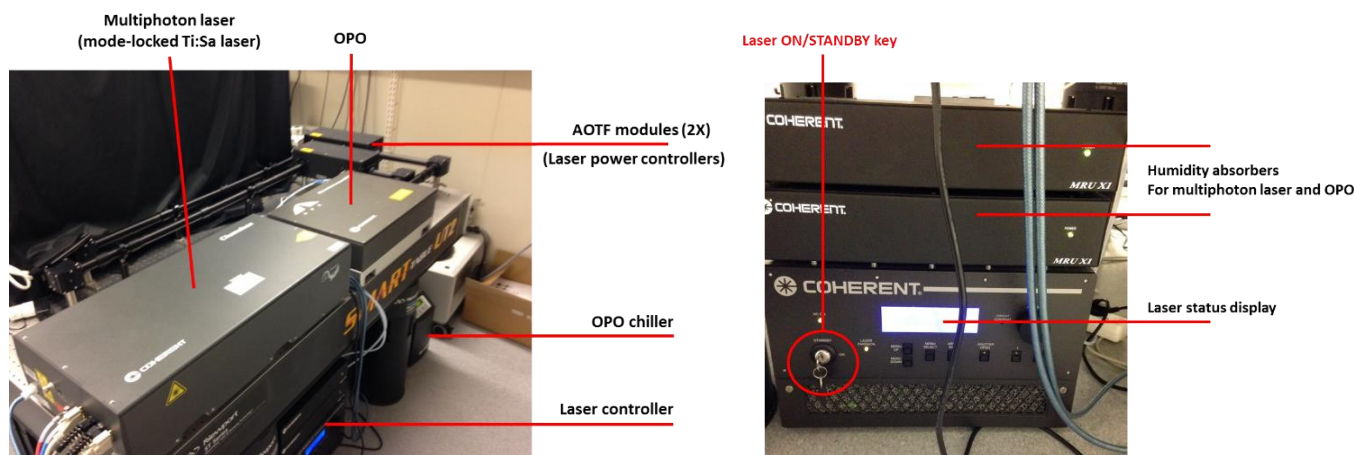
## 2. SYSTEM COMPONENTS OVERVIEW

The intravital microscope and its peripheral devices occupy a single room. The system includes a **Zeiss AxioExaminer microscope**, a Coherent **Chameleon multiphoton laser**, an APE **optical parametric oscillator** (OPO) and laser beam tracts in tubes located on an air-suspended optical table. The laser control module is located behind and under the optical table. There is an **ON/STANDBY key** for the laser on the control module's front panel (see figure below).

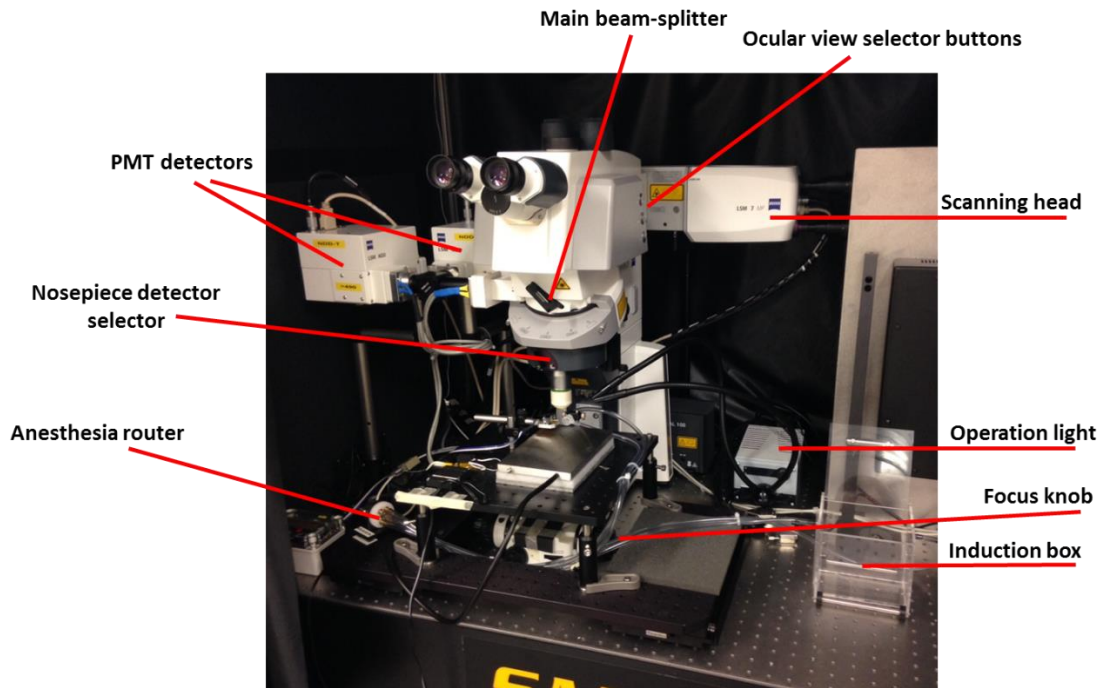
The microscope has **4 PMT Non-Descanned Detectors** (NDD) allowing simultaneous acquisition of 4 different wavelengths ranging from 400 to 750 nm. Additional **GaAsP nosepiece detector** has no optical filters and also requires manual selector switching for use (see figure below).

A customized **in vivo stage** can be moved with XY stage control wheels. There are two ways for controlling Z-position: **focus knobs on microscope stage** and **remote focusing wheel** on the left side of the operator's desk. The system is also equipped with **animal monitoring/maintenance** and **gas anesthesia units** (see figure below).

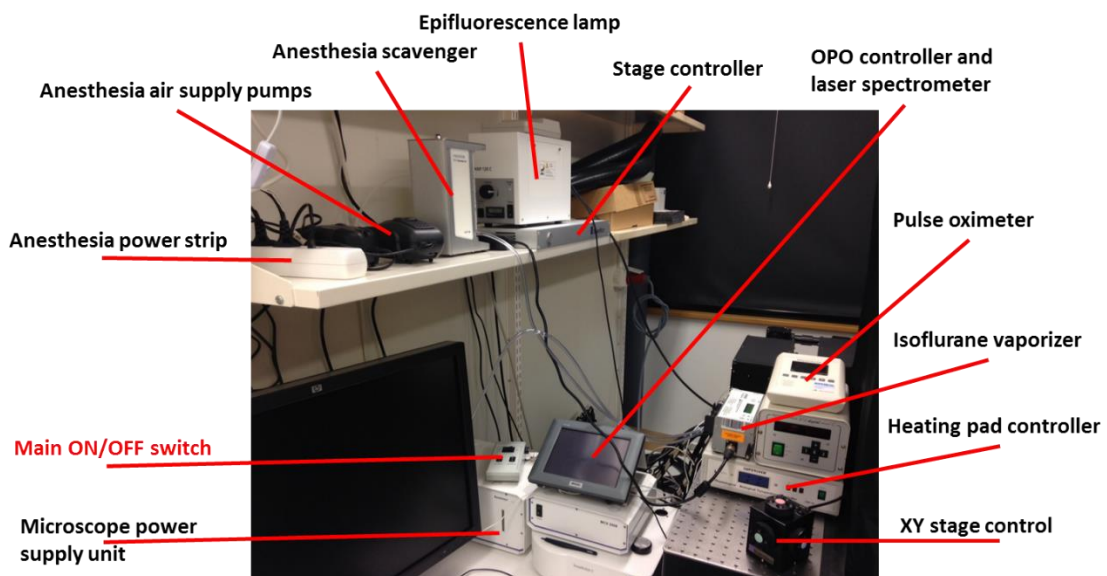
### LASER COMPONENTS



## MICROSCOPE COMPONENTS



## OPERATOR TOOLS



### 3. SAFETY RECOMMENDATIONS

- **This is a class 4 laser system!** Direct exposure to the beam can easily cause burns and is especially harmful for eyes. **Do not look at beam apertures!**
- **IR laser beam is invisible by eye!** When laser is set for wavelengths over 800 nm, the laser beam will not be visible. **Do not try to find the beam by looking under different angles!**
- **Switch off operation lights, close curtains and switch off main room light before scanning!** Non-descanned detectors are very sensitive to external light. Overflooding NDDs with light may cause permanent damage.
- **Always use the scavenger for anesthesia gases!** If the scavenger is OFF, isoflurane can accumulate inside the microscope enclosure during imaging.

## 4. STARTING THE LASER AND THE MICROSCOPE

### 1. Turn the laser emission key to ON position

**NOTE:** When the key is at upright position (STANDBY), laser status display indicates 0 mW emission and the status “standby”. When the key is in horizontal position (ON), laser status display indicates actual laser emission power (usually above 1000 mW) and the status “OK”.

**NOTE:** When started, it takes about 3-5 minutes for the laser to reach “OK” status and normal power output. *If the laser is not able to reach “OK” status in 10 minutes, please contact BIU personnel.*

2. Main switch – ON
3. Systems/PC – ON
4. Fluorescent lamp – ON
5. Scientifica stage controller – ON
6. Computer Log in as LSM User (no password required)
7. Wait for computer and Windows to boot completely.
8. Components – ON
9. Start ZEN software from desktop icon

## 5. SHUT DOWN SEQUENCE

1. Close ZEN software and switch off lasers when asked by ZEN.
2. Components – OFF
3. Scientifica stage controller – OFF
4. Fluorescent lamp – OFF
5. Shut down the computer.
6. Systems/PC – OFF
7. Main switch – OFF
8. Turn laser emission key to STANDBY position.
9. Follow shutting down sequence for anesthesia equipment (chapter 6).
10. Follow shutting down sequence for animal maintenance equipment (chapter 6).
11. Clean the objective front lens with optical tissue.
12. Make sure there is no mess, dirty instruments, used paper towels, animal cages, etc. left around.
13. Go through the essential check list below and you are done!

### ESSENTIAL CHECK LIST WHEN LEAVING ZEISS LSM7 MP

- The laser is at STANDBY.
- The main oxygen valve is tightly closed.
- Microscope main switch is OFF.
- Power strips for anesthesia and animal monitoring/maintenance equipment are OFF.
- Ear temperature controller is unplugged.

## 6. ANESTHESIA OPERATION AND ANIMAL MAINTENANCE

### 6.1. Starting anesthesia equipment

The imaging system is equipped with an **Univentor isoflurane anesthesia system** for small laboratory animals, which consists of an Univentor vaporizer, an air/oxygen source (pump and gas tank), an air sink (vacuum pump), an Univentor scavenger unit, an induction box, an animal mask, a gas selector valve and tubing. You can use either normal air or pure oxygen from the tank to mix with isoflurane. This choice is to be made based on your particular experimental design.

1. If you are going to mix isoflurane with oxygen, **check the Univentor's tubing connection to the oxygen tank.**

**NOTE:** *If you are going to use normal air to mix with isoflurane, you just need to reconnect the air supply tube to the air pump located on a shelf over the computer monitor, and make sure the pump is plugged in to the anesthesia power strip. Then proceed straight to step 3.*

2. **Completely open the main oxygen valve** on the gas tank, and **check that the pressure is between 0.5 and 1.0 kgf/cm<sup>2</sup>.** If needed, pressure can be adjusted with a pressure setting valve.

**NOTE:** *Exceeding max pressure (see figure below) can damage the anesthesia equipment.*

3. **Turn ON the anesthesia power strip** (on a shelf over the computer monitor).

4. **Turn ON the scavenger unit** (knob is on its back wall).

5. Fill a gastight 10 ml syringe with isoflurane, avoiding bubbles, and set it on the vaporizer.

**NOTE:** *The amount of isoflurane required can be estimated from a normal consumption of about 2-2.5 ml/hour.*

**NOTE:** *Do not remove the plunger from the gastight syringe, it will damage sealing and syringe will need to be replaced.*

6. Set the **air flow on the vaporizer between 300 and 350 ml/min.**

7. Check the anesthesia router position (it should be at "box" for induction).

8. Switch the **isoflurane feed ON** and **set percentage** of isoflurane in a gas mixture.

**NOTE:** *Usually 4% is used for anesthesia induction and 1.5-2.2% for animal maintenance.*

9. When the animal is asleep, **switch the gas valve** to "mask" position and place the animal on the stage with its nose to the mask.

10. Continuously **monitor anesthesia depth** with the pulse oximeter (see below) and **do not leave the animal unattended.**

## 6.2. Shutting down anesthesia equipment

1. Switch the **isoflurane feed OFF.**

**NOTE:** *Make sure the animal does not require anesthesia any more.*

2. **Disconnect the gastight syringe** from vaporizer, and pour isoflurane back to a bottle.

**NOTE:** *Do not connect the empty syringe to the system but keep it attached to the syringe holder.*

**NOTE:** *Do not remove the plunger from the gastight syringe, it will damage sealing and syringe will need to be replaced.*

3. **Allow air or oxygen to pass through the vaporizer for extra 10 minutes** to dry the system.

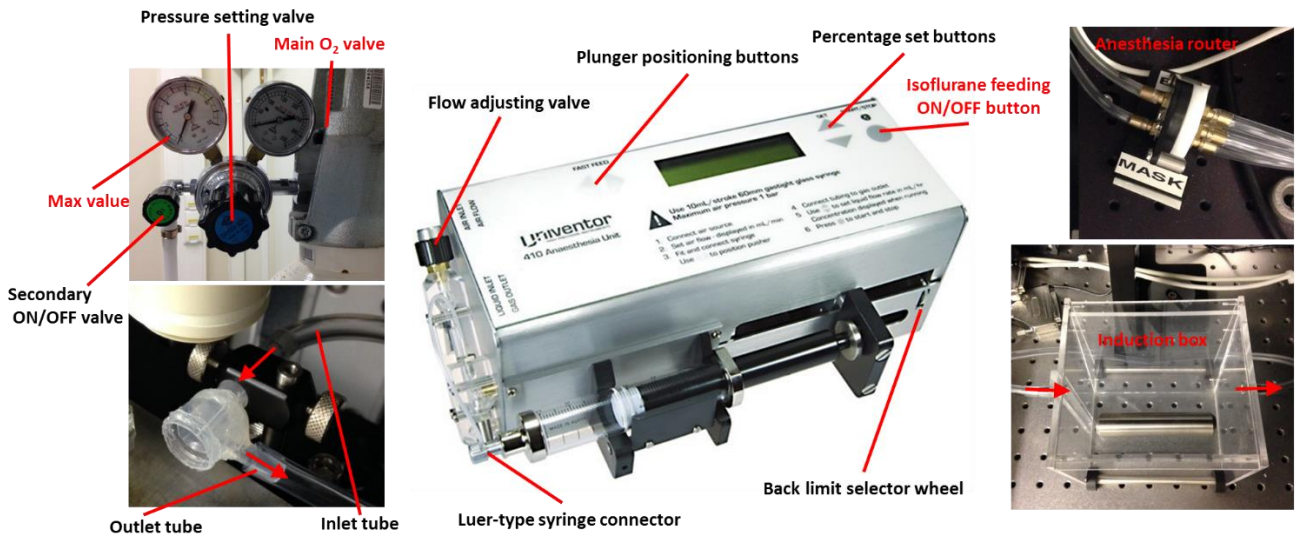
4. **Switch the scavenger unit OFF.**

5. **Switch the anesthesia power strip OFF.**

6. **Close the main valve** on the oxygen bottle if you used oxygen.

**NOTE:** *Pressure on the main monometer (right) should drop down to 0 quickly if the main valve is well closed.*

## ANESTHESIA SYSTEM



### 1.3. Starting animal maintenance and monitoring equipment

Small laboratory mammals under general anesthesia are not able to maintain their normal body temperature and easily develop hypothermia. Thus, efficient and reliable general anesthesia requires continuous animal warming and anesthesia depth monitoring. The intravital microscope is equipped with a **Supertech TMP-5b temperature control** device (controller and metal heating pad) and a **PhysioSuite monitoring system** (Pulse oximeter monitor and optical paw sensor).

1. **Turn ON the animal maintenance power strip** (floor below optical table).
2. **Turn ON the Supretech heating pad** controller and **set the temperature**.

**NOTE:** This animal heating device lacks temperature feedback from the animal, thus only passive heating is available. We recommend to use **37°C when animal is laying abdomen-down** and **38°C when it is positioned abdomen-up**. This way the animal's body temperature will be at around 36-37°C.

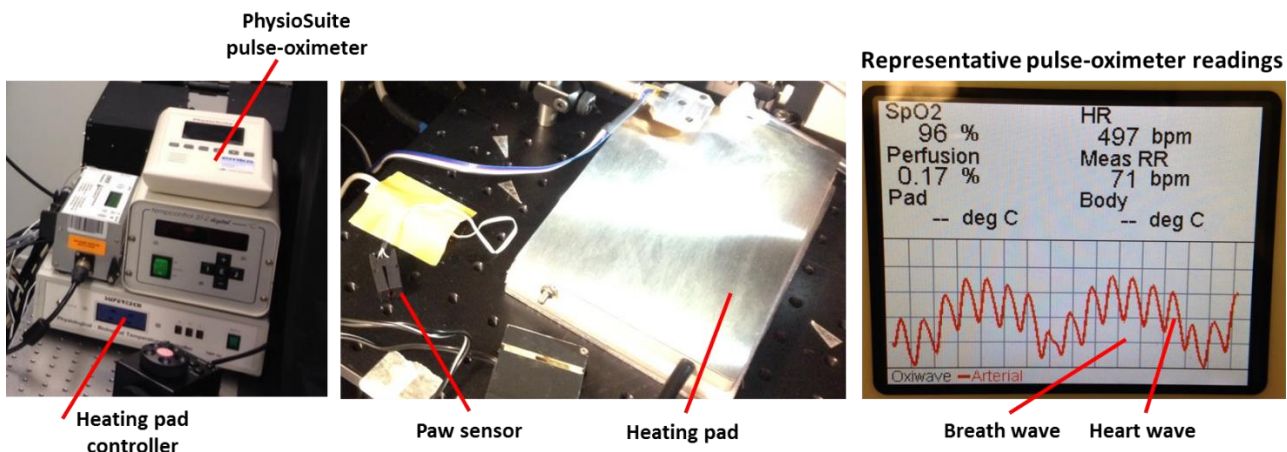
3. Wait until the pad's actual temperature will reach the set temperature, and put your pre-anesthetized animal on it.

**NOTE:** Refer to the anesthesia part of this guide for further details on anesthetizing animals.

4. **Turn ON the PhysioSuite pulse-oximeter** (knob is at the back) and **connect animal's paw sensor**.
5. Set "Arterial" on the pulse-oximeter to see real-time oxiwave readings.

**NOTE:** There should be distinguishable heartbeat-related pulsations and breathing-related pulsations. The device should be able to show numerical values of **HR** – heart rate, **RR** – respiration rate, **SpO2** – partial oxygen pressure, and perfusion coefficient. Also see a reference screenshot below for readings from a healthy anesthetized mouse.

## ANIMAL MAINTENANCE



## 7. SOFTWARE CONTROLS AND IMAGE ACQUISITION

The Zeiss LSM7 multiphoton microscope is operated through the ZEN 2011 software package. This software is very similar to newer ZEN versions installed at BIU's Axiolmagers, and LSM780 and LSM880 confocal microscopes, and thus those who have used any ZEN software before will find it very easy to navigate. Indeed, a multiphoton microscope is simpler than a confocal; it has no pinhole, just one laser, and no spectral detectors. Here is a typical start-up sequence to get the first image.

1. Using oculars in **LOCATE** mode find the region of interest and check that at least one fluorescent label is visible.

**NOTE:** *The system has 3 fluorescent filters for wide-field observation: DAPI (blue), GFP (green) and TRITC (red). These filters can be chosen from the software in the LOCATE tab. You can also use white reflected light from operation lights to see the surface of your preparation under the microscope.*

2. Switch to the **ACQUISITION** tab.

3. Switch ON the laser from the **Laser menu**

**NOTE:** *This button actually opens the laser shutter, so you should hear a click sound behind the microscope while pressing it.*

4. Activate detectors of suitable wavelength on the **Light Path** menu.

**NOTE:** *All detectors have fixed filter sets installed, they can be only mechanically exchanged by BIU personnel. Filter change procedure requires restarting the software. Please check which filters you will need to use and inform BIU personnel in order to check that the system is in correct configuration.*

**NOTE:** *A typical detection configuration is shown in the figure below. Even though the software recognizes 2 detectors for transmitted light (3 and 4) and 2 detectors for reflected light (1 and 2), they are all currently set for the reflected light pathway.*

5. In the **Channels** menu, set the **laser wavelength** (2-photon excitation) and **laser power**.

**NOTE:** *The laser has a lot of power (up to 1W at the sample plane), which is approximately 10 times higher than in a regular confocal microscope.*

6. Set a suitable **Master Gain** for every detector.

**NOTE:** *We do not recommend to use master gain higher than 750. It will increase noise more than it increases the signal.*

7. Set a suitable **Frame Size** and acquisition **Speed** (pixel dwell time).

**NOTE:** *The frame size will determine the pixel size, and thus the image resolution. This microscope has a physical XY resolution of about 0.5-0.4  $\mu\text{m}$ , and therefore setting a pixel size less than 0.2  $\mu\text{m}$  will not make a lot of sense.*

**NOTE:** *We recommend to use pixel dwell times between 1.0 and 2.0  $\mu\text{sec}$ , and improve the signal to noise ratio rather with averaging than with increased pixel dwell time (for further details please see the chapter "Imaging with two-photon excitation").*

8. Use **Live** to start fast scanning with lower pixel resolution or **Snap** for single image acquisition with your defined resolution and speed settings.

### 7.1. Thick sample Z-stack and brightness correction settings

Z-stack acquisition is used when 3D localization information is essential. Although two-photon microscopes have an excellent Z-sectioning capability and improved visibility depth, optimal Z-stack acquisition requires setting a laser power gradient in order to compensate for light scattering in thick tissue samples. The Z-stack menu is very conservative through all ZEN versions and allows the creation of smooth laser power gradients in the Correction tab, using at least 2 reference points.

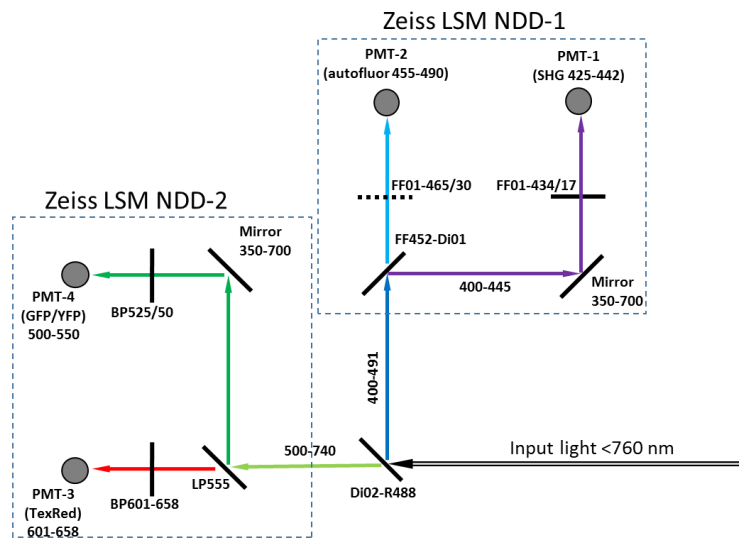
1. Activate **Z-stack menu**.
2. **Set Last** (top) and **Set First** (bottom) slices of a Z-stack.
3. **Set Interval** (z-spacing) in microns.

**NOTE:** *Axial resolution of this microscope is about 1.0  $\mu\text{m}$ , so setting Z-spacing less than 0.5  $\mu\text{m}$  will not improve an image's axial resolution.*



4. **Set the laser power** suitable for the most superficial Z-stack position and click **(L)** on the Z-stack window. The system will scan a full-resolution image in position **(L)**.
  5. If the image is good **press Add** in the Correction menu; a Z-coordinate in  $\mu\text{m}$  should appear in the position list. Acquisition settings (including the laser power value) are saved for this Z-coordinate.
  6. **Set laser power** suitable for the middle of your Z-stack and **press (C)**.
  7. If the image is good **Add** this setting to the Position list as described above.
  8. You can set as many reference points as needed and then use **Spline interpolation** to create smooth laser gradients.
- NOTE:** The correction function also allows setting different detector gains for different Z-positions, but in order to keep it simple and reliable we recommend using only the laser power as a variable.
9. Tick **Use Correction** box in order to activate the correction settings.
  10. Proceed with Z-stack acquisition by pressing **Start Experiment** button.

### TYPICAL DETECTION CONFIGURATION



The figure shows three screenshots of the microscope software interface with red annotations:

- Laser shutter control:** Points to the 'Laser' panel where the 'Chameleon' laser is set to 'On'.
- Laser AOTF ON/OFF:** Points to the 'Lasers' section in the 'Channels' panel, where the 920 nm laser is highlighted.
- Laser wavelength setting:** Points to the '920' and '1150' wavelength settings in the 'Lasers' section.
- Laser AOTF power:** Points to the '15.0' power setting for the 920 nm laser.
- Detector gain (actual sensitivity):** Points to the 'Gain (Master)' settings for NDD R1, R2, T1, and T2, all set to 700.
- Do not use digital gain and offset:** Points to the 'Digital Gain' and 'Digital Offset' settings, which are all set to 1.0 and 0, respectively.
- Z-Stack acquisition parameters:** Shows the 'Z-Stack' panel with 'Correction' and 'Auto Z Brightness Correction' checked. The 'Position ( $\mu\text{m}$ )' list shows three points: -350.63  $\mu\text{m}$ , -399.13  $\mu\text{m}$ , and -450.75  $\mu\text{m}$ . The 'Laser power' is set to 5% for the first point, 10% for the second, and 15% for the third.

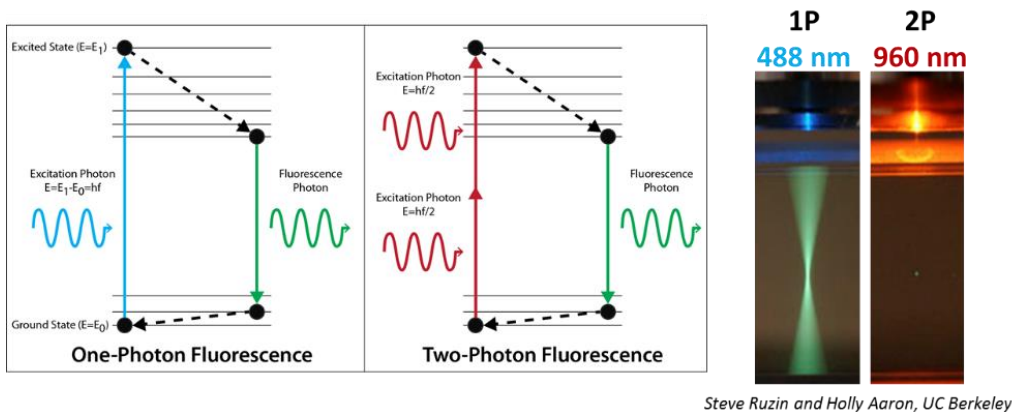
## 8. IMAGING WITH TWO-PHOTON EXCITATION

Two-photon excitation (TPE) microscopy provides resolution somewhere in between wide-field and confocal microscopy, because it actually utilizes some features from both of these widely used methods. In this chapter, we will explain some basics of TPE microscopy in order to provide an understanding of the capabilities of this powerful and elegant method, and to help you choose correct imaging settings for your experiment.

### 8.1. Two-photon excitation vs single photon excitation microscopy

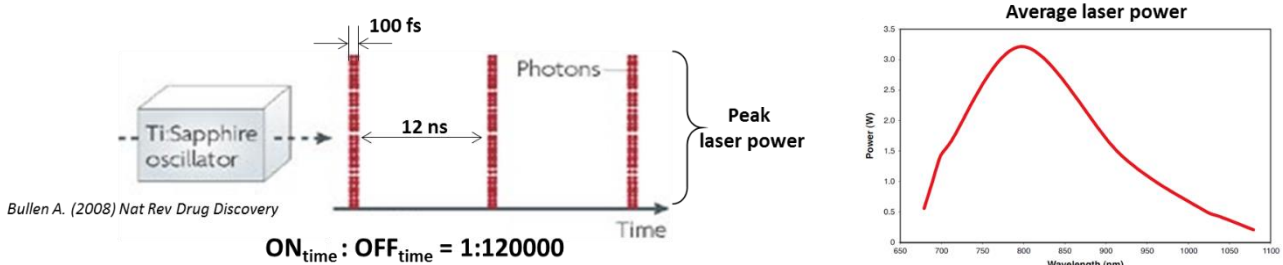
TPE is based on a principle that a fluorophore molecule can simultaneously absorb two low energy (red) photons, resulting in their energies combining to produce the same excitation effect as the absorption of a single high energy photon. When a fluorophore molecule drops back down from an excited state to a ground state, it emits a photon of fluorescence (see figure below), which happens exactly the same way for single and two-photon excitation processes. For instance, a GFP molecule emits green photons irrespective of whether it is excited with two red photons or a single blue photon.

However, since the probability for 2 photons and a fluorophore molecule meeting in a space simultaneously is much lower than for 1 photon and a fluorophore molecule, fluorescence excitation is restricted to a very small volume (typically about 1 cubic micrometer) defined by the focus of the objective lens (see figure below). This is why high N.A. objectives lenses with excellent focus characteristics are used for multiphoton imaging; this is also why TPE reduces out-of-focus fluorophore emission and bleaching when compared with confocal or wide-field microscopy.



### 8.2. Mode-locked laser and peak energy

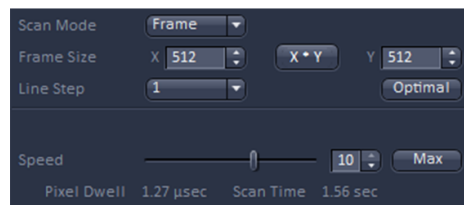
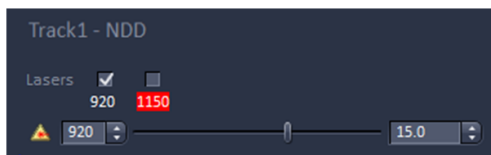
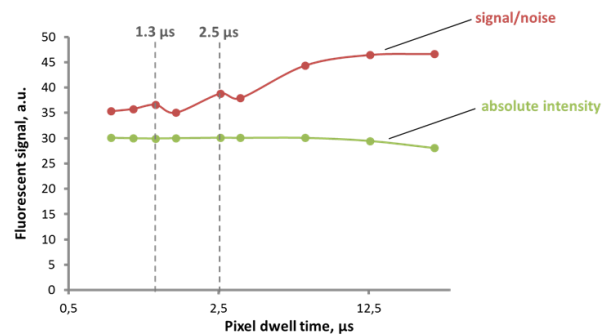
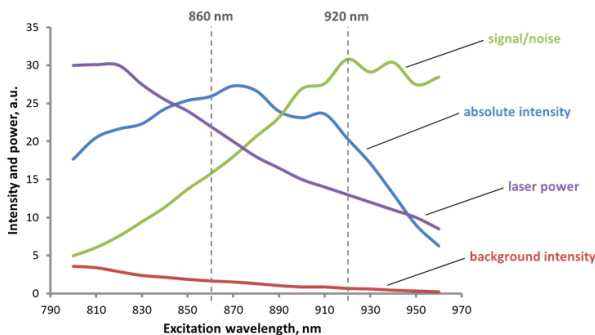
Perfect laser beam focusing *per se* is not enough to produce photon densities high enough to facilitate TPE and keep total energy load to a specimen within physiological limits. Multiphoton imaging systems utilize pulsating (**mode-locked**) Titanium-Sapphire (**Ti:Sa**) lasers, which generate very high energy but very short pulses (100-140 fs) divided by relatively long silent intervals (see figure below). These intervals are described as the laser's **repetition rate** and usually are at 80 MHz, meaning the laser produces  $8 \cdot 10^7$  pulses in a second. The **peak power**, which usually exceeds 100 kW, determines good fluorophore excitation efficiency, but a moderate **average laser power** (3 W or less, see figure below) protects biological samples from being destroyed by the enormous energy load. Another feature of Ti:Sa lasers is their ability to be **tuned for different wavelengths** in the range of 690 – 1010 nm within several seconds. The drawback, however, is that laser output power is highly dependable on the wavelength (see figure below).



### 8.3. Fluorescent TPE imaging

Fluorescent imaging with TPE is basically very similar to imaging with a confocal microscope. Since both techniques utilize pixel by pixel scanning with a laser beam, image formation and system components are very similar. However, a TPE microscope does not have a pinhole, and its **non-descanned detectors** are catching every photon that enters the microscope objective and for that reason our multiphoton system is protected from external light sources by an enclosure. There are several essential tips which are useful to keep in mind while setting up the acquisition on a multiphoton microscope:

- Ability of the Ti:Sa laser to be tuned for different wavelengths adds versatility to the system, but it is essential to choose the **correct excitation wavelength for your fluorophore(s)**. Note that TPE spectra for most fluorophores are much more complex than their single photon analogues. We recommend to test your fluorophores on different TPE wavelengths prior to starting intravital experiments. Usually, switching to a longer excitation wavelength helps to reduce autofluorescent background intensity, and increases the signal-to-noise ratio (see figure below). Most often we are using either **920 or 860 nm excitation**.
- In laser scanning systems **pixel dwell time** is the time which the laser and detector spend at every pixel location for fluorophore excitation and fluorescence collection. This parameter is very similar to (and equally important as) the exposure time in wide-field microscopy, because it determines how much the sample is exposed to a high energy laser beam, and for how long the signal is collected. Pixel dwell time is determined by scanning speed (see figure below) and should be kept between 1 and 2.5 microseconds to avoid photodamage to the sample.



### 8.4. Second harmonic generation (SHG) imaging

Besides fluorescent excitation, TPE can produce special non-linear effects which can be utilized in imaging. One of these effects is the **generation of second harmonic signal**. SHG signal appears exactly at **half of excitation wavelength**, for instance if your TPE is set to 860 nm, SHG will appear at 430 nm. SHG is an intrinsic property of some biological molecules such as **collagen, elastin** and **myosin**, and does not require any labelling. Since SHG is not fluorescence, it is not subject to bleaching. SHG is useful for visualizing collagen in connective and epithelial tissues, and it can be used for visualizing blood vessels which have elastin

in their walls, and for imaging myosin-rich sarcomeres. It worth remembering that due to its nature the direction of SHG signal radiation is the same as for excitation light radiation, meaning that only a minor part of SHG signal will be backscattered to the objective lens and detected by the imaging system- using a mirror located under a thin sample, or collecting transmitted light might improve SHG detection efficiency.

## 9. OPO USE

The Zeiss LSM7 MP microscope is equipped with an Optical Parametric Oscillator (OPO), which enables multiphoton excitation on longer wavelengths when compared to Ti:Sa mode-locked lasers. Chameleon OPO can be tuned within the wavelength range from 1050 nm to 1300 nm, which covers 2-photon excitation (TPE) spectra for red fluorescent proteins such as tdTomato (1100 nm TPE) and mCherry (1150 nm TPE), and it can also be used for 3-photon excitation and third harmonic generation imaging (THG). OPO can also be used as an independent laser source together with the Ti:Sa laser. When OPO is switched ON, only 85% of Ti:Sa beam is used for pumping the OPO. Therefore there are two beams at the OPO exit: the OPO-generated beam, and the original Ti:Sa beam attenuated by 85%. OPO power and wavelength are measured by an inbuilt spectrometer and displayed on the LCD screen of the OPO controller (see figure below). Significant laser power attenuation when the OPO is ON requires using higher power settings in the ZEN acquisition configuration.

There are certain limitations when the Ti:Sa laser and OPO are used together. The Ti:Sa pump wavelength should be between 740 and 880 nm, with the most efficient pumping at approximately 780-800 nm (see chart below). Longer pump wavelengths from the Ti:Sa laser are required to reach the longest wavelengths that the OPO can produce, so not all combinations of wavelengths are possible for simultaneous excitation (see top chart below). Similar to Ti:Sa mode-locked lasers, the OPO output power strongly depends on the wavelength (see bottom chart below).

The figure below illustrates an example of setting the Ti:Sa and OPO beams for simultaneous multiphoton excitation on two different wavelengths (800 nm and 1100 nm).

1. In the ZEN Channels menu tune the Ti:Sa laser to wavelength suitable for OPO pumping.

**NOTE:** *There is a chart below that can be used as a reference. Usually the most efficient pumping can be achieved with wavelengths between 780 and 800 nm.*

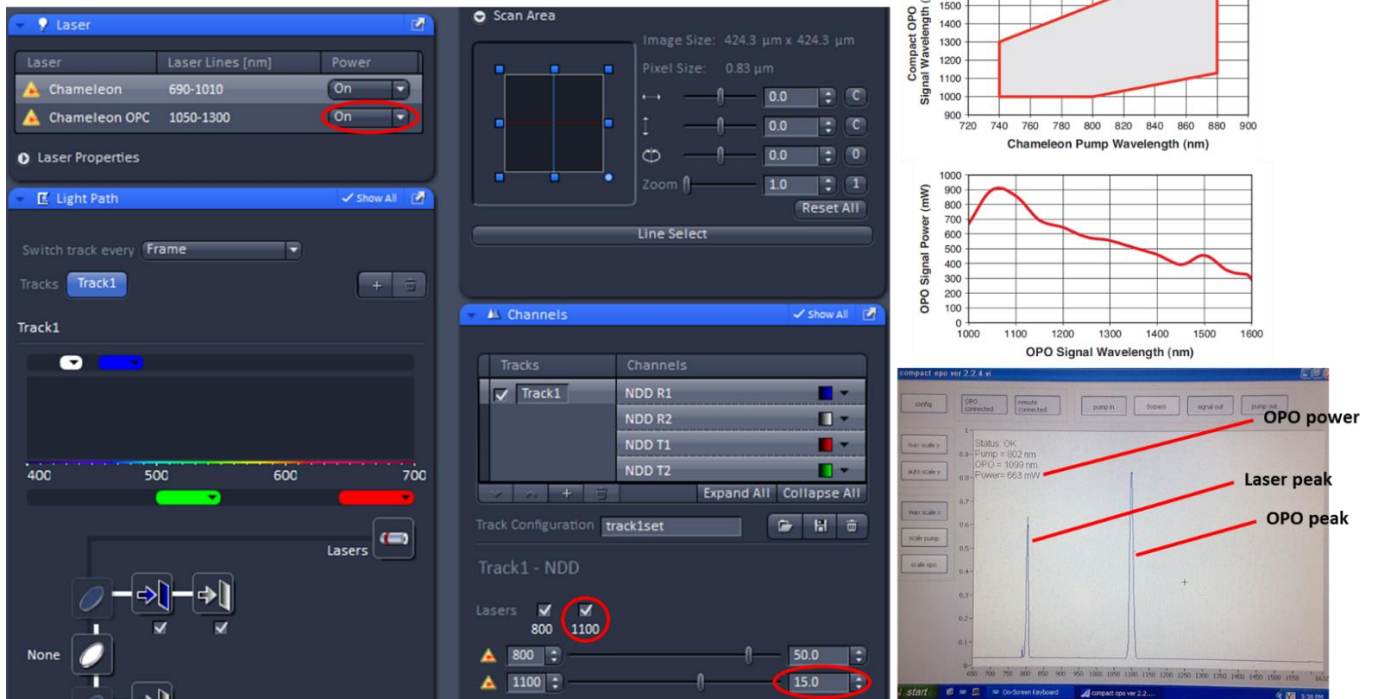
2. Activate the OPO controller screen by touching it, and check that a single laser peak is visible.
3. In the ZEN Laser menu turn the OPO ON.

**NOTE:** *The OPO controller screen should indicate Status: tuning, and you should hear servo drives operating behind the microscope.*

4. In the ZEN Channels menu tick the OPO box and set its wavelength.
5. Wait until the OPO controller indicates Status: OK and there are two clear laser peaks visible on the screen (see figure below).
6. Set the Ti:Sa and OPO powers in the ZEN Channels menu, and proceed with image acquisition.

**NOTE:** *The Ti:Sa beam power is now strongly attenuated by the OPO (reduced by 85%), so you will need to use much higher power settings in ZEN to reach the usual laser intensities at the sample plane (see example settings below).*

## OPO USE



## 10. INTRAVITAL ADAPTERS, HOLDERS AND APPLICATIONS

BIU's intravital multiphoton microscope has a unique set of custom-made animal organ adapters and holders. These holders were designed with a modular principle which simplifies their installation and reduces the number of required parts for every application. In a number of prior studies, BIU personnel together with collaborative researchers have used these adapters for the following intravital microscopy applications:

- Abdominal adipose tissue
- Liver
- Kidney
- Spleen
- Lymph node on skin flap
- Beating heart
- Mouse ear skin

In the following application examples, there are short technical recommendations for using the available intravital imaging holders.

### 10.1. Acute intravital microscopy of mouse internal organs

There are several similar holders available with different imaging window sizes available. They all were designed at BIU and tested for imaging static internal mouse organs in acute (end-point experiment) intravital preparations in collaboration with Dr. Kari Alitalo's and Dr. Karl Lemström's research groups. This holder is fixed to the organ's surface with cyanoacrylate instant adhesive Loctite 401 or 3M Vetbond.

1. Clean the holder with acetone (to dissolve leftover cyanoacrylate adhesive).
2. Glue a 12 mm coverglass inside the immersion chamber with instant adhesive avoiding glue inside imaging window.
3. Let it dry completely for several minutes.
4. Install fixation arms on the stage. Check that their position is correct and you will be able to reach the region of interest on the animal.

**NOTE:** Stage travel distance is very limited, approximately 5 cm back/forth direction and 2 cm right/left direction, so checking the holder position prior fixing the animal is very important. There are also marked holes on the stage to simplify holder positioning.

5. Prepare the animal and expose the organ of interest.

**NOTE:** Make the incision as small as possible, just enough to glue the holder reliably. Usually this requires approximately 1 cm<sup>2</sup> or less of open organ space.

6. Keep the internal organ surface moistened with physiological saline all the time.

**NOTE:** Organ drying might cause local damage of upper cellular layers which will result in the appearance of bright autofluorescent spots.

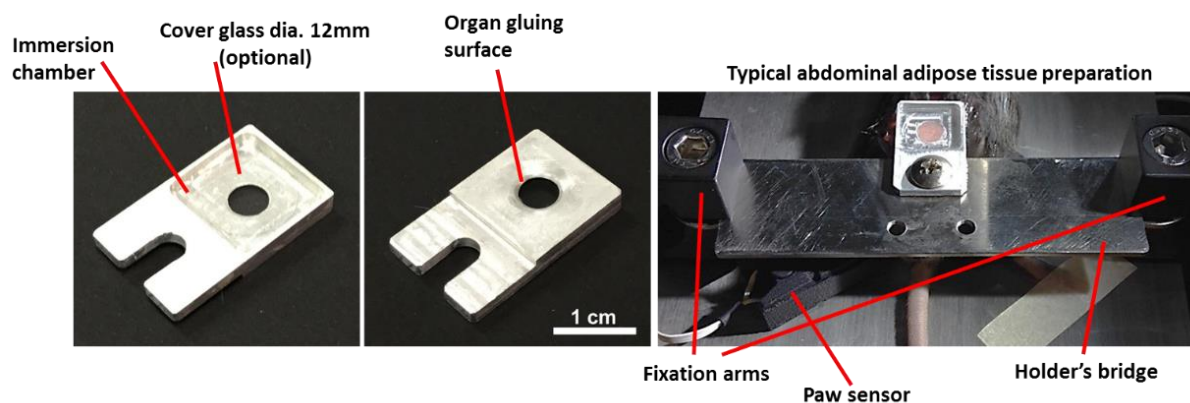
7. Apply instant adhesive on the holder's organ gluing surface.

8. Soak excess saline from the organ surface and apply a small drop of saline to ensure a tight contact between the glass and tissue.

9. Attach the holder to the organ surface.

10. Install the holder's bridge, and set the animal position so that the holder can be fixed to the bridge without tension.

11. Screw the holder to the bridge and fill the immersion chamber with physiological saline.



## 10.2. Acute imaging of mouse heart

Mouse heart is a very mobile and at the same time a very sensitive organ, and for investigation with intravital microscopy, it requires a fixation that is steady but has a contact area as small as possible. In collaboration with Dr. Esko Kankuri's research group, BIU has developed a mouse heart imaging holder (see figure below). The holder allows short-term heart fixation (about 1 hour) and imaging when glued to the myocardium with Loctite 401 or 3M Vetbond instant adhesives. This holder can be used with or without a 5 mm round coverslip. Manipulations of an open rodent heart require the animal to be intubated and supplied with additional oxygen.

1. Assemble the holder: glue the holder's arm to the holder's ring using a small drop of epoxy resin.

**NOTE:** It is recommended to use 5 minute 2-component epoxy.

**NOTE:** Holder should be pre-assembled on the day before the experiment.

2. If a coverslip is required, glue a 5 mm round coverslip in the holder using Loctite 401 instant adhesive.

**NOTE:** The coverslip can be mounted with a vacuum pick-up tool (VP10C from Thorlabs), which is available at BIU.

3. Perform anesthesia/intubation procedures and check that the animal is deeply anesthetized.

4. Perform heart exposure surgery.

**NOTE:** To fix the holder you will need at least 8x8 mm open access space, so the ribcage should be cut on both sides, and bent and fixed in upwards position with surgical clamps. All major bleeding sites should be clamped. We also recommend to supply the animal with 0.2 ml of physiological saline i.p. prior to the preparation.

5. Install the holder's bridge and check that the holder's position is correct. Move the animal if needed.

**\*\*\*For holder with a coverslip\*\*\***

- Put Loctite 401 cyanoacrylate adhesive on the holder's gluing surface and glue the holder on the myocardium surface.

**NOTE:** When the holder is glued, its screw clamp should be as horizontal as possible to avoid deforming the heart's geometry when fixed to the holder's bridge.

**NOTE:** If the holder's bridge was positioned correctly, a screw clamp should be very close to a fixation hole with M4 threading.

- Screw the holder to the bridge using a short M4 bolt.

- Use Viscotears gel as immersion medium in the holder's immersion chamber.

**NOTE:** Be careful not to damage the objective front lens. This holder's geometry was designed for being used with Zeiss 20x/NA1.0 Plan-Apochromat W objective (W.D. 1.8 mm); compatibility with other objectives should be tested separately. Deep imaging is only possible in the central 3 mm area.

**\*\*\*For holder without a coverslip\*\*\***

- Put a small drop of 3M Vetbond cyanoacrylate adhesive on the myocardium surface to create a patch 5-6 mm in diameter.

**NOTE:** This patch locally restricts myocardium movement, and is still transparent enough to allow multiphoton microscopy through it.

- Put some 3M Vetbond cyanoacrylate adhesive on the holder's gluing surface, and glue the holder to the glue patch on the myocardium surface.

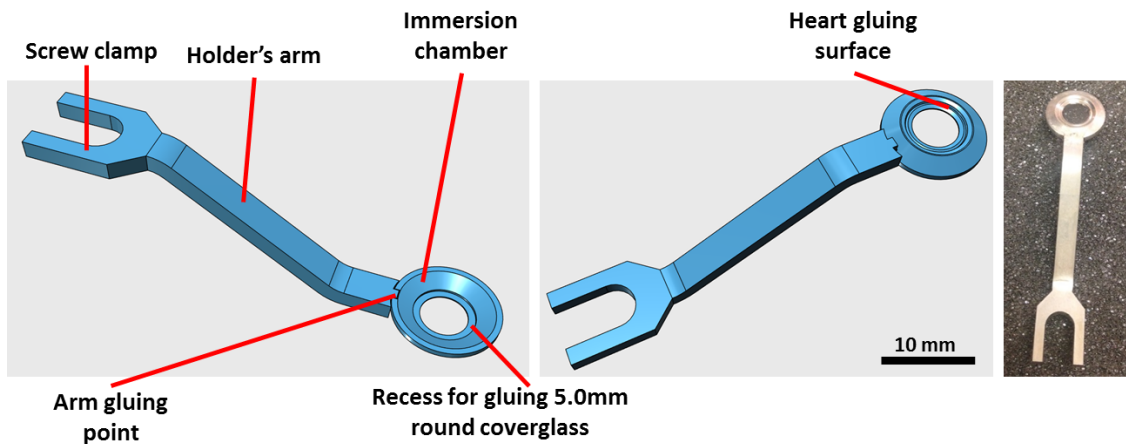
**NOTE:** When the holder is glued, its screw clamp should be as horizontal as possible to avoid deforming the heart's geometry when fixed to the holder's bridge.

**NOTE:** If the holder's bridge was positioned correctly, a screw clamp should be very close to a fixation hole with M4 threading.

- Screw the holder to the bridge using a short M4 bolt.

- Use Viscotears gel as immersion medium in the holder's immersion chamber.

**NOTE:** Be careful not to damage the objective front lens. This holder's geometry was designed for being used with Zeiss 20x/NA1.0 Plan-Apochromat W objective (W.D. 1.8 mm); compatibility with other objectives should be tested separately. Deep imaging is only possible in the central 3 mm area.



### 10.3. Acute imaging of mouse lymph node on a skin flap

The skin flap holder (see figure below) facilitates imaging structures on the inner surface of skin flaps, such as lymph nodes, inner epithelium tissues and blood vessels. This holder has passive thermal control through heat conductance from a heating pad, and a water tight immersion chamber allowing a constantly immersed preparation under a certain temperature. The holder was designed by BIU in collaboration with the University of Helsinki Hematology Research Unit.

- Install the holder on the stage (polycarbonate fixator removed), and make sure it has a good thermal contact with the heating pad.

**NOTE:** The heating pad should be set to 38 °C. Expected temperature drop on the holder's surface is approximately 2-3 °C, thus you can expect your preparation to be at approximately 35 °C.

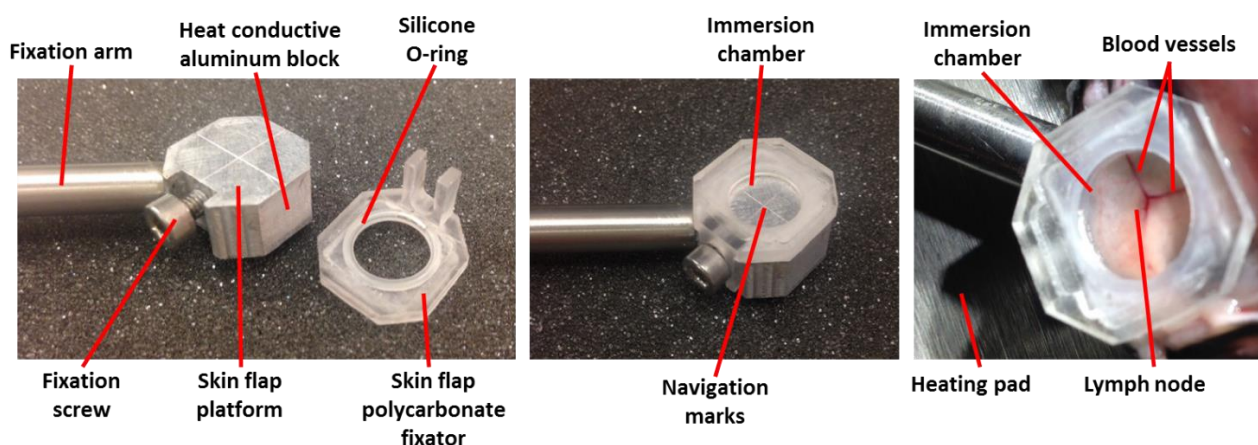
2. Anesthetize the animal and perform the skin flap dissection.
3. Fix the skin flap on the holder platform, so that the area of interest is exactly in the center of the platform.

**NOTE:** We used 3M Vetbond glue to fix the skin reliably and to avoid lateral slip.

**NOTE:** While fixing the skin flap, make sure to leave the fixator's mounting position accessible.

4. Set the polycarbonate fixator on top of the skin flap, and tighten the fixation screw.

**NOTE:** Be careful to not restrict local blood flow. The silicone O-ring is very flexible and provides good insulation without the application of too much pressure.



#### 10.4. Chronic non-invasive mouse ear imaging

Mouse ear skin is a commonly used model for studying vascular biology, immunology and blood-to-tissue exchange kinetics. The main advantage is that the mouse ear has a dense blood vessel network in a thin tissue with relatively sparse hair cover, and is therefore well accessible for non-invasive microscopic and mesoscopic imaging studies if fixed and flattened properly. On the other hand, the mouse ear serves as one of the main thermoregulatory organs and is very sensitive to temperature fluctuations, and thus proper thermostatic control is required. In collaboration with Dr. Pipsa Saharinen's research group, BIU has developed a custom ear holder and the procedure for using it. The design of this holder was inspired by a previously described preparation (Li et al., Nat Protocols 7, 221–234, 2012). The ear holder enables mouse ear immobilization, flattening and thermostabilization at a user-defined temperature. After completion of the imaging session, the ear can be released from the holder without any damage, and the animal can be recovered from anesthesia for further use in long-term chronic studies.

1. Check the connections. The holder's RS-232 port and power supply cable (7.5 V) should be plugged in the heating controller box.
2. Switch the temperature controller and holder heating ON by plugging in the power supply to the power strip below optical table.

**NOTE:** There is no ON/OFF switches on this device, it starts immediately when plugged in. Heating indicator should light continuously due to warming up, display should show actual and set temperatures.

3. Set the temperature using the white (decrease by 1.0 °C) and red (increase by 1.0 °C) buttons on the controller.

**NOTE:** We used 35.0 °C for the ear. Temperature tolerance is +/- 0.25 °C from the set value within the 33.0 – 38.0 °C range. The device heating power was optimized for the physiological temperature range of 32 – 39 °C; using this device for higher or lower temperatures is not recommended.

4. Wait until the actual temperature reaches the set temperature and the heating indicator starts blinking.

**NOTE:** The holder is now maintaining a constant set temperature.

5. Anesthetize the animal and keep it on the heating pad.



**NOTE:** The animal is positioned abdomen-down, so we recommend the heating pad temperature to be set at 37 °C.

6. Adjust the ear holder position and height using the set screws on the fixation arm.

7. Stick a strip (3x15 mm) of masking tape to the edge of the ear.

**NOTE:** Mouse ear capillaries are very fragile; do not use tweezers or forceps. Also avoid pinching it or pulling it too much.

8. Pull the tape strip and the ear through the ear-mounting gap on the holder.

9. Position the ear as shown on the figure below and gently remove tape.

10. Clean the ear using a cotton swab soaked with physiological saline.

11. Put immersion gel (Viscotears) on top of the ear and on the platform around the ear.

**NOTE:** Avoid bubbles on top of the ear.

12. Put a coverglass on top of the ear and fix the glass with magnetic clips as shown on the figure below.

**NOTE:** Avoid whiskers and hairs between the coverglass and the metal platform, they may cause drift and thus disturb the imaging.

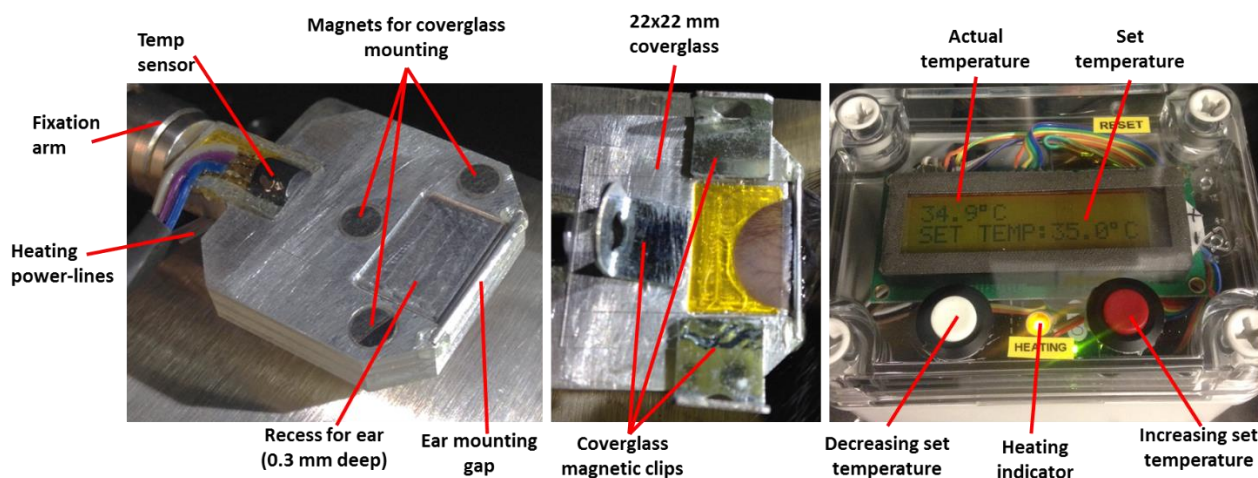
**NOTE:** We used a 22x22 mm standard square coverglass. the holder can accommodate a coverglass of width equal to or less than 22 mm.

**NOTE:** Be careful when setting the magnetic clips, the magnets are pretty powerful and dropping the clips on them can cause the coverglass to be cracked.

13. Put immersion gel (Viscotears) on top of the coverglass, position the microscope objective, and make sure you have enough immersion media.

**NOTE:** The immersion gel does not evaporate quickly, and it also does not leak too much, so usually checking immersion once in 1 hour is enough.

14. Wait for 20 minutes allowing the ear and coverglass to settle to minimize drift during imaging.



## 10.5. Fluorescent tracing of blood vessels with a tail vein injection

Fluorescent blood plasma tracing by intravenous injection is the most common animal manipulation done during intravital microscopy. Plasma labelling is necessary for sample navigation, blood flow quantification, extravasation kinetics measurements, cell tracing, drift correction and many other applications. There are a number of dyes which can be used for fluorescent plasma tracing and two-photon imaging. **FITC** and **TexasRed**-conjugated dextrans are available in different molecular weights displaying different wash-out kinetics. The cheap and widely utilized **Evans Blue** dye also can be used as a fluorescent tracer with 850-950 nm two-photon excitation and 680 nm emission peak. Compared with retro-orbital and femoral vein injection, tail vein injection is probably the most reliable way to perform intravenous administration of fluorescent tracers and experimental drugs. It is also possible to install a tail vein catheter for gradual drug delivery during intravital imaging. Tail vein injection can be tricky, and is much more likely to succeed if you have good tail vein visibility, tail immobilization and can keep the tail warm during injection. BIU's intravital microscope is equipped with a miniature **TaiLight** device (guide light for tail

vein injection) which illuminates tail veins, and immobilizes and warms up the tail for a smooth injection (see figure below).

1. Check that the animal is well anesthetized under the microscope.
2. Switch ON the TaiLight and place it on a heating pad near the animal for 1-2 minutes for more efficient warming.

3. Prepare your solution for injection and fill the syringe.

**NOTE:** We used Terumo Myjector 0.3 ml syringes with an integrated 29G needle.

**NOTE:** It is recommended not to exceed 0.15 ml (10% of total blood volume) injection volume for an adult mouse.

4. Bend the needle 45 to 90 degrees 0.5-0.7 cm from a tip using forceps as it is shown on a figure below.
5. Check that solution is able to pass through the needle and that there is no air present in the needle.
6. Put the tail in the tail fixation groove and hold it in place with your fingers.
7. Wait for approximately 30 seconds to warm up the veins.
8. Locate a lateral tail vein by slightly rotating the tail (see figure below).
9. Insert the needle to a tail vein and perform injection.

**NOTE:** When piercing a tail vein with a needle there is an obvious resistance drop. When you feel it, move the needle a bit forward inside the vein to make sure it is in. While injecting, the plunger should move smoothly almost without resistance; you should also see the solution rapidly going in along the vein. Probably the most common way to miss the vein is going too deep.

