PhotonIMAGER™ OPTIMA User Guide

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1 Introduction

The PhotonIMAGER™ OPTIMA is a low light level imaging equipment dedicated to bioluminescent and fluorescent imaging in vivo and in vitro. As opposed to other available instruments, the PhotonIMAGER™ OPTIMA uses a photon counting technology adapted from technologies developed by Biospace Lab for other in vitro and in vivo imaging applications, and based on intensified CCDs. This approach enables the real time display of the bioluminescent and fluorescent signals, the recording of the kinetics information, as well as improved performance in low light applications.

The PhotonIMAGER™ OPTIMA was designed with a view to make whole body in vivo acquisitions with animals easy and powerful. The system includes gas anesthesia input and heating plate for optimal physiological conditions of the animals. With its moving stage, different fields of view are available, from 21 cm x 16 cm (5 mice capacity) to 8 cm x 6 cm (optimal resolution). The system has been light proofed down to a level of less than 30 photon incident on the camera per second.

This manual aims at providing the user a quick guide to the use of the machine as well as a detailed description of its numerous features and capabilities.

2 Security and Electrical specifications

2.1 Dimensions

Width:	83 cm (without cable)
	90 cm (with cable)
Depth:	57 cm (standard version)
	87.1 cm (tunnel option)
Height:	140 cm (standard version)
	200 cm (with rolling cabinet)
	For X-Ray option, plus 13cm to the total height
Weight:	185 Kg (without options)
	220 Kg (all in one version)

2.2 Location

The system should be installed on a flat surface (less than 5° inclination). It is advised to leave some room on either side of the Photon Imager for maintenance. The acquisition computer can be installed up to 1.5 meter away from the system if needed (length of the interconnection cables).

2.3 Environmental conditions

The system should be installed in a room with the following conditions:

Temperature range: 15°C to 22°C
Humidity: < 70%
Room volume >8m^3

2.4 Installation

The installation can only be performed by Biospace Lab staff or by one of Biospace Lab trained distributors.

2.5 Remote service link

The remote service link can be performed with an Internet connection.

2.6 Electrical considerations

The maximum electrical power consumption for the Photon Imager is 1.5kW, and provision has to be made for the acquisition computer and its screen. It is recommended to supply both the instrument and its acquisition PC through an uninterruptible power supply (UPS).

2.7 Voltage and Fuses

The Photon Imager can be used in the full range (100V – 240V). Never try to use it in the wrong range of voltage.
Frequency can be either 50 or 60 Hz.

2.7.1 Main power

1 fuse (20mm, delayed) is located on the left side panel, in socket assembly. The value depends on the used voltage:

- 220V 10A if used in 250V
- 100V 10A if used in 125V

2.8 Cleaning

The internal chamber of the Photon Imager can be cleaned with ethanol. To clean the outside shell, a dry cloth must be used.

Biospace Lab cannot be held responsible in case of bad use of the instrument.

3 System

3.1 Hardware and software descriptions and requirements

The PhotonIMAGER OPTIMA is made of the main instrument and the acquisition PC on which the acquisition and analysis software programs are installed.

3.1.1 Control PC

The computer configuration is as follows:

- PC running under Windows 7 64bits ® or later, with Pentium processor, hard drive and DVD-RW reader
- 8 Go RAM
- 2x1 TB hard disk in RAID configuration
- 16 MB Video card

3.1.2 PhotonIMAGER™ software package

The acquisition computer of the PhotonIMAGER™ OPTIMA includes the following two software applications:

- Photo Acquisiton, the acquisition software.
- M3 Vision, the analysis software for processing, quantification and export of the acquisitions.

M3 Vision is a dongle-protected software. Additional licenses can be ordered to allow acquisition analysis on additional computers.

3.1.3 PhotonIMAGER™ OPTIMA hardware

Below is a schematic description of the PhotonIMAGER™ OPTIMA instrument.

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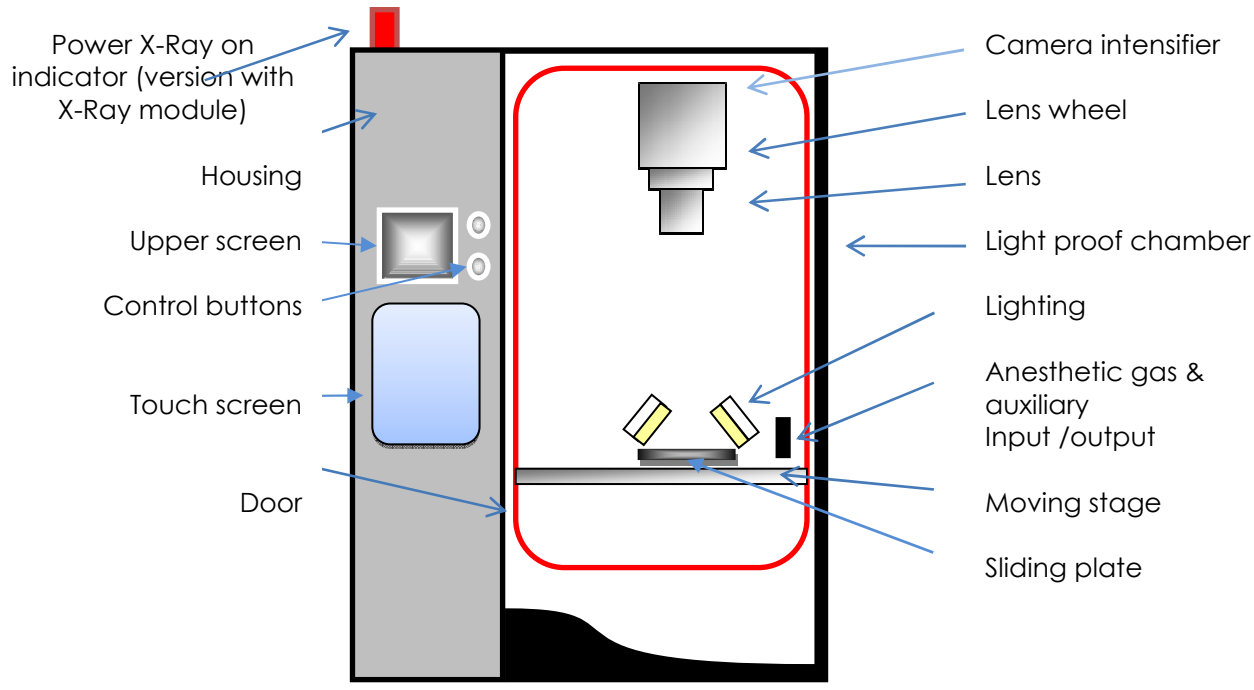


The detection system of the equipment is made of:

- a f/1.2 numerical aperture lens for optimum collection of light
- a third generation (Gallium Arsenide PhotoCathode) dual multi-channel plate intensifier tube coupled to a CCD chip (iCCD)

The system comprises also a water-cooling system, which regulates the temperature of the cryostat that cools down the intensifier tube of the iCCD.

Each photon emitted by the animal or the sample and collected by the lens is amplified by the dual channel plate intensifier by a factor of approximately 10^5. The resulting optical spots are then analyzed by a CCD chip and their centre of gravity precisely computed to ensure optimal resolution.
Resulting separating powers at the animal level are:

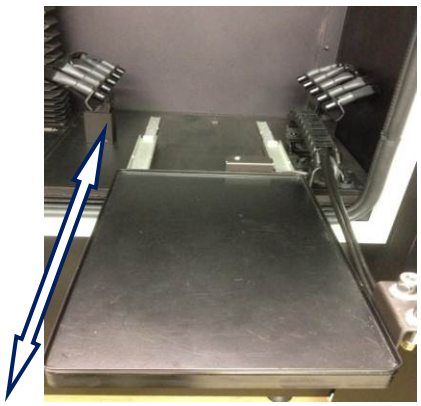
- 50 µm for the small field of view (stage in its highest position)
- 130 µm for the large field of view (stage in its lowest position)

The camera intensifier can be damaged by intense light fluxes. Avoid exposure of camera to intense light, such as flashlights or torch lamp **even when the equipment is not powered**.

- Prior to transportation, it is recommended to dismount any lens from the lens wheel and transport it separately. Contact support@biospacelab.com for instructions.

3.2 Animal and experiment monitoring accessories

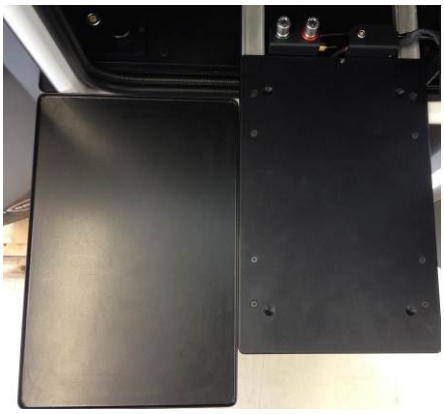
Animals must be placed on the provided sliding stage (dimensions 22 cm x 35 cm).
The stage can be slipped inside and outside of the PhotonIMAGER™ OPTIMA.



A set of black walls is available for optical isolation of animals. This may help avoiding false measurements due to lighting of an animal by another, and should be used when several animals are imaged simultaneously.

3.2.1 Heating Plate

In order to improve animal physiology, the stage is equipped with a heating plate. The upper part of the stage can be removed in order to be cleaned up. The bottom part contains the heating system and the thermal probe.

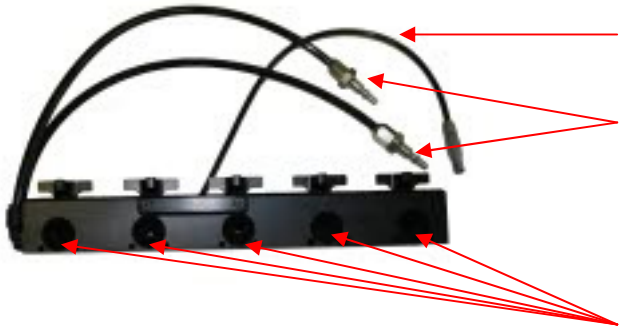


Upper and bottom part
Of the moving stage

Upper part Bottom part

3.2.2 Anesthesia Bar

The anesthesia bar includes five nose-cones for gas, to allow for a maximum of 5 animals imaged simultaneously. Fittings can easily be changed and adapted to other dimensions. This anesthesia bar includes also three light sources, emitting red, green, and blue lights, which can be used as standards. A set of "T Tap" allows gas to be distributed to each animal, whatever their number and size. A gas exhaust is provided and can be connected to an external exhaust.



Cable for diode connection

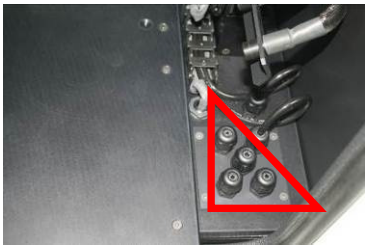
Gas input and output cables

Nose cones for gas inflow and exhaust

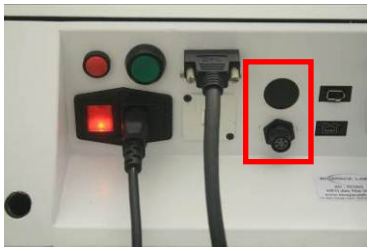
Anesthesia bar

3.2.3 Auxiliary inputs

Six auxiliary inputs are provided for animal monitoring needs (two on the left panel and 4 inside the machine on the right side of the moving stage). The outside inputs can be used to connect additional modules to the machine (e.g. MacroLens) and the internal once can serve for a catheter for instance or for cardiac monitoring.



4 auxiliary inputs inside the chamber



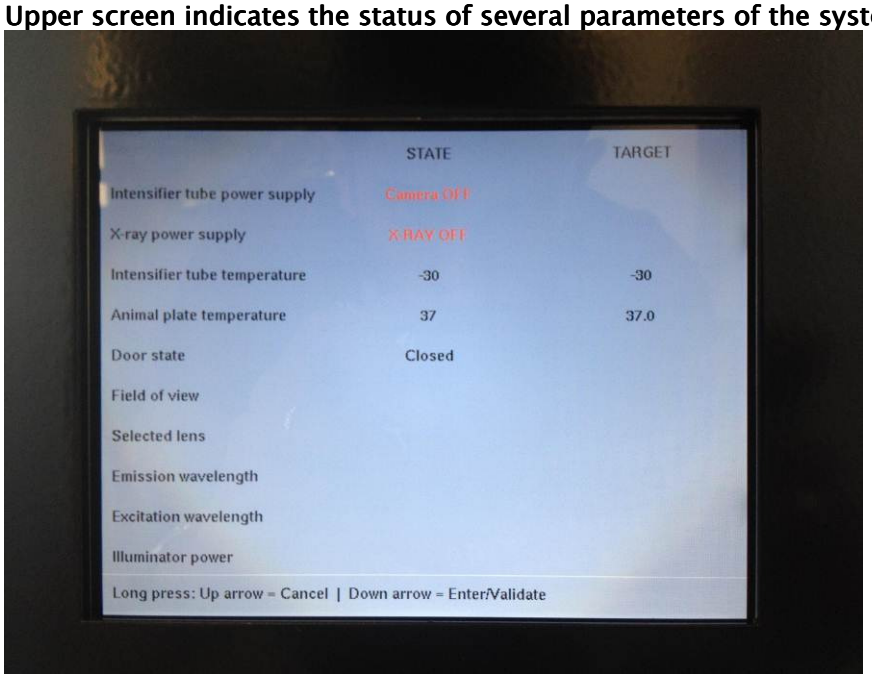
2 auxiliary inputs on the left panel

4 Getting started

4.1 PhotonIMAGER™ OPTIMA front and side panels

4.1.1 Front panel + two settings buttons

Upper screen indicates the status of several parameters of the system:



From top to bottom:

- Camera ON/OFF
- X-Ray ON/OFF (Option with X-Ray module)
- Cryostat Temperature: Red: do not start any acquisition
  Orange: you can use video and fluorescent mode
  Green: System is ready for any kind of acquisition

  You may be asked to adjust the temperature by Biospacelab staff for maintenance purpose by using the two buttons on the left side of the upper screen.
  Do not change the default settings without Biospacelab staff specific request.
- Animal plate temperature
  You can adjust the temperature to your needs from room temperature up to 45°C by using the two buttons on the right side of the upper screen.
- Door Open / Closed
- Field of view
  Regarding the height of the stage and the selected lens (option with modules), this gives you information about the dimension of the field of view. For standard option field of view changes between16 cm x 21 cm (full field of view) and 6 cm x 8 cm (small field of view)

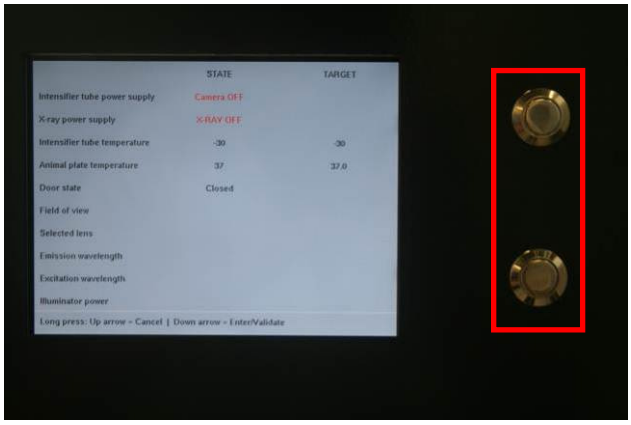
- Selected lens:
  Regarding the option installed on your system (4-View, MacroLens, X-Ray) you will have to change the lens wheel position. This indicates you which lens or tube (X-Ray) is selected.

- Emission wavelength
  Displays the value of chosen emission wavelength.

- Excitation wavelength
  Displays the value of chosen excitation wavelength

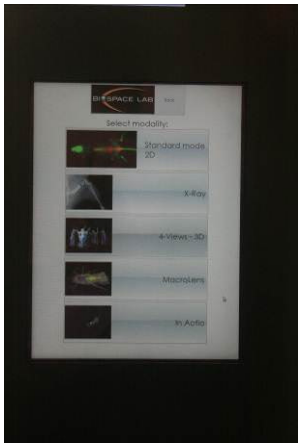
- Illuminator power
  Displays the value

Control buttons on the right side of the upper screen



Lower button :
-Long press : entering the menu; validate
-Short press: moving down in the menu, decrease the value
Upper button:
-Long press: exiting the menu; canceling the modification
-Short press: moving up in the menu, increasing the value

Lower touch screen:

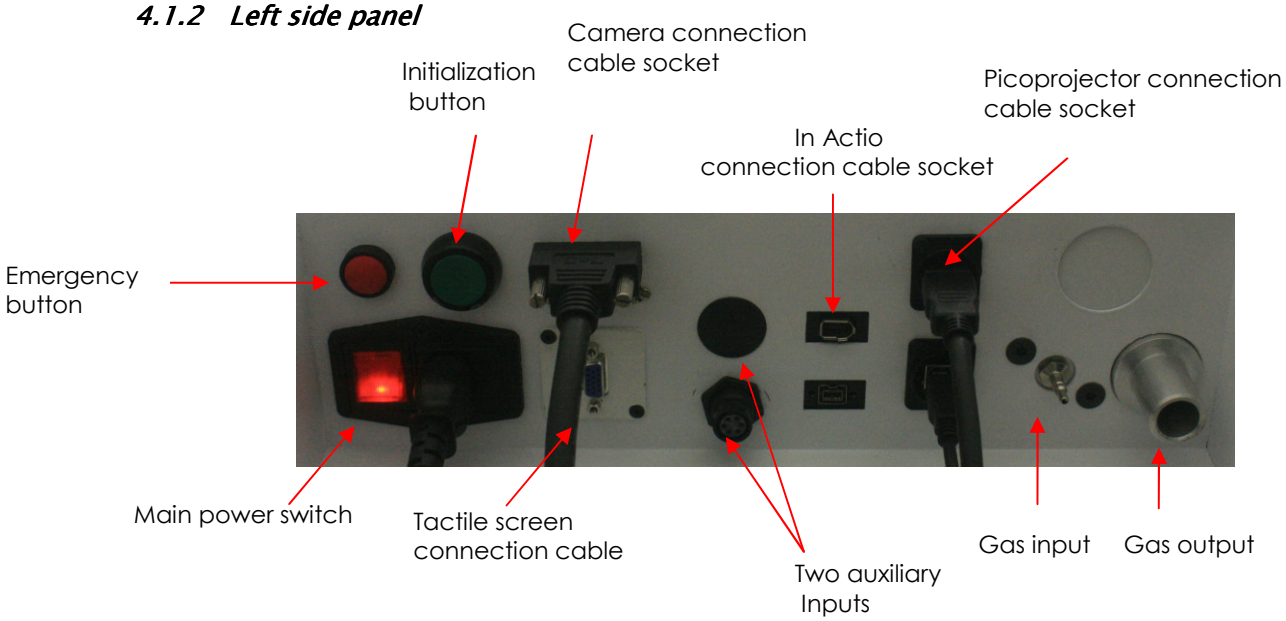


Provides an easy access to the main Acquisition Software functionalities allowing setting the parameters of your acquisition.
With the use of tactil screen you can:

- choose the module (if your machine is equipped with any)
- choose the acquisition mode
- set the parameters of the acquisition

The display of the tactile screen is identical to that of the software which makes it easy to navigate.

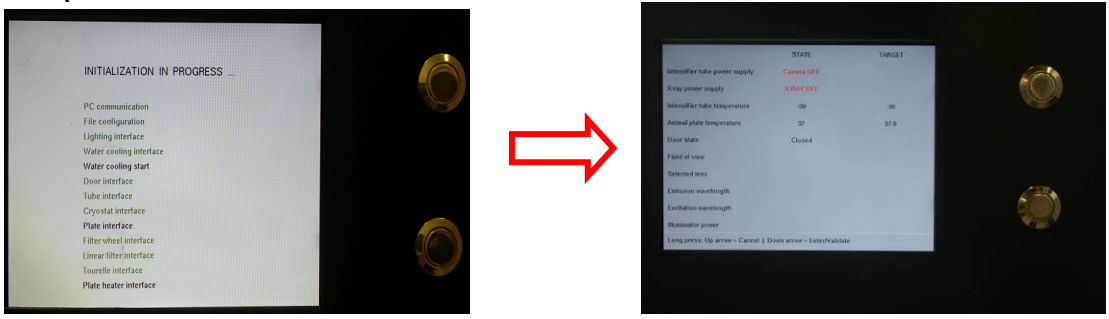
4.1.2 Left side panel



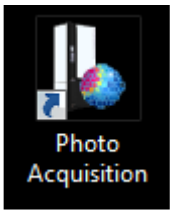
4.2 Switching on the instrument

This section describes how to switch on the instrument after it has been installed by the vendor.
The PhotonIMAGER™ OPTIMA consists of the main instrument and the computer. There are several ways to switch on the Photon Imager regarding the way it has been switched off, but in any case, there is an automatic procedure driven by the internal computer. Regarding the way the system has been switched off, it can take from 1 to 3 minutes to start. You can then follow the initialization procedure on the upper front screen. Once initialized, it takes from 10 to 15minutes to reach the cooled temperature.

You can switch on the computer at any time but **you must not launch the Acquisition software (Photo Acquisition) before the initialization of the PhotonIMAGER™ OPTIMA has been completed.**



To launch Photo Acquisition simply click on the Photo Acquisition icon on the task bar



4.2.1 After complete switch off and/or power cord removing:

Turn on the main instrument by switching on the lower switch on the back of the left side panel of the instrument

4.2.1 After Hibernation

Press once on the green button to launch the reinitialization.

4.3 Standby and switch off

Depending on the time before the next working session, you may switch off the instrument or simply put it in a standby mode.

4.3.1 Hibernation

When acquisitions are finished you should hibernate your system. To do so, press once on the green button  . On the front upper screen you will see the cryostat temperature progressively reaching room temperature and the system will then automatically switch to hibernate mode.
During Hibernation, the power consummation is reduced.
To carry on your experiments again press once on the green button to launch the reinitialization.

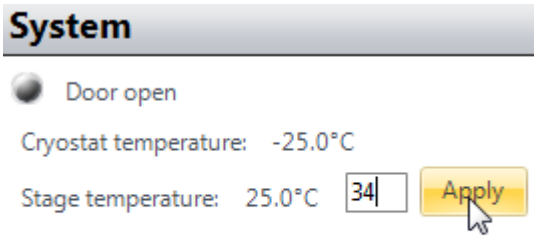
4.3.2 Complete switch off

Once in hibernation mode, the system can be completely switched off with the main switch.
When you switch on again the instrument, follow the procedure described at the beginning of this chapter

4.4 Preparation of the system before the acquisition

4.4.1 Stage temperature

Adjust the temperature to your liking and wait for the target to be reached (see 4.1.1 for temperature adjusting procedure with front panel button). You can also adjust the temperature by using the software



On the right bottom side of the Acquisition software there is a System window which gives you information about the door and temperature state.

Type the desired temperature value in the **Stage temperature** window and press **Apply** to confirm. Depending on the chosen value it may take up to few minutes to reach this temperature.

4.4.2 Anesthesia gas connections

If anesthesia gas is not used, no connection and the anesthesia bar are necessary, except if the calibration lights are needed. The positioning of animals is then free; however it is recommended to put them in the center of the plate when possible. This allows placing the stage in the highest position to get the best resolution.
If animals are anesthetized with gas, the anesthetizing gas supply should be connected to the gas input connector on the gas input and output panel. There is also a larger connector, the gas output, to evacuate the anesthetizing gas from the chamber.

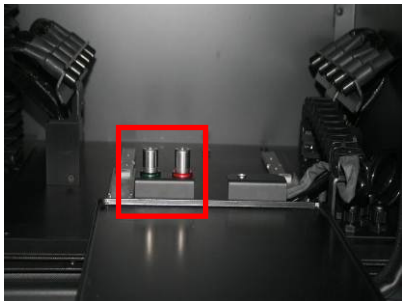
4.4.3 Anesthesia bar

The anesthesia bar brings the gas directly to mice when it is required for anesthesia. The gas is also evacuated by the same nose cones.
Some anesthesia bars do also have diodes that can be used as light standards for bioluminescent acquisitions if desired.

Gas outflow

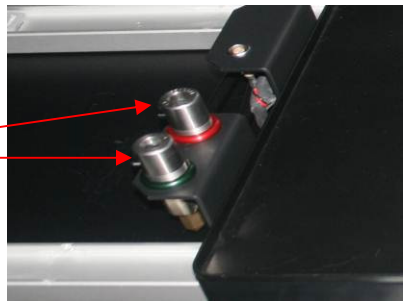
In order for the gas to flow in the anesthesia bar, the gas cable of the bar should be connected inside the Photon Imager chamber. This connection is made at the back of the

stage, on the left. The connection is obtained with a simple pressure; to disconnect the cable, simply push on the button on the side



gas connectors inside the chamber

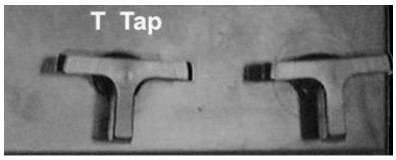
side buttons on gas connectors to release the gas cable



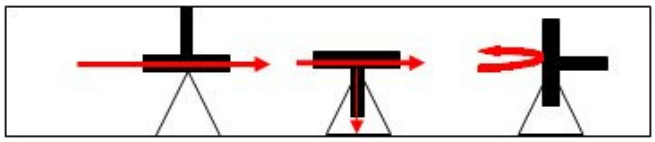
The anesthesia bar includes 5 "T taps", each corresponding to a mouse position. The T taps of the anesthesia bar should be rotated so that the selected cones are provided with gas as desired. Each T tap can allow to:

- Open or close the corresponding nose cone for the gas
- Let the gas flow to the next T tap or block it.

To know where the gas will flow, look at the branches of the T taps as the gas will follow them. This design allows the choice of any number, from 1 to 5, and any position for the nose cones to be provided with gas.



Two T taps of the anesthesia bar



Distribution of the gas depending on the position of the T-taps

Standard lights

The anesthesia bar is equipped with three calibrating lights (Blue-Green-Red) that can be used to compare measurement from one acquisition to another. In order to use this calibrating LED, you have to connect the small connector attached with the bar to the corresponding connector in the cable carrier. To disconnect them, press the lateral button on the socket as shown on the picture above.

4.5 Animal positioning

After the system has been prepared (stage temperature, gas connections, anesthesia bar, acquisition software launched), it is ready for the acquisition, and the animals or the sample can be installed on the stage. The stage can be drawn outside of the machine so that the animal can be installed more easily. The two sliders that guide it have an extension of more than thirty centimeters.
If only one animal is imaged, place it in the middle of the stage in order to use the highest position of the stage and get the best sensitivity.
How to perform acquisitions is described in the following chapter on the Photo Acquisition software.

5 The Photo Acquisition software

5.1 A software dedicated to acquisitions with the PhotonIMAGER™ OPTIMA

This software controls data acquisition on the PhotonIMAGER™ OPTIMA in the different modes (video, bioluminescent, fluorescent, and picture mode). It allows the user to get two types of images:

- Signal image

The signal image can be obtained through three different modes, namely bioluminescence, fluorescence photon counting, and fluorescence integration.

- Photographic image

The photographic image provides a black and white image of the animal(s).

The two images, signal and photographic, are automatically overlaid with the analysis software, M3 Vision, when they have the same filename (only the extensions differ: .bvr for bioluminescence or fluorescence images; .trp for black and white photographic images).

The software is designed and set to be very user friendly and to give optimal results with basics settings. It also offers the opportunity to change every setting according to your needs.

5.2 Launching Photo Acquisition

Photo Acquisition runs under Windows 7 64Bits or later, and is installed on the acquisition computer.

Before launching Photo Acquisition, it is necessary to make sure that the door is closed and the system properly initialized.

As the software is launched, the system puts the stage in its default configuration, with the stage down.
The Photo Acquisition software can be launched from the shortcut that is on the desktop, or from the START menu in the lower left corner of the Windows desktop screen, with the following path:

- Start > Biospacelab > Photo Acquisition > Photo Acquisition

5.3 The User Interface

The interface window obtained at start up is shown below.

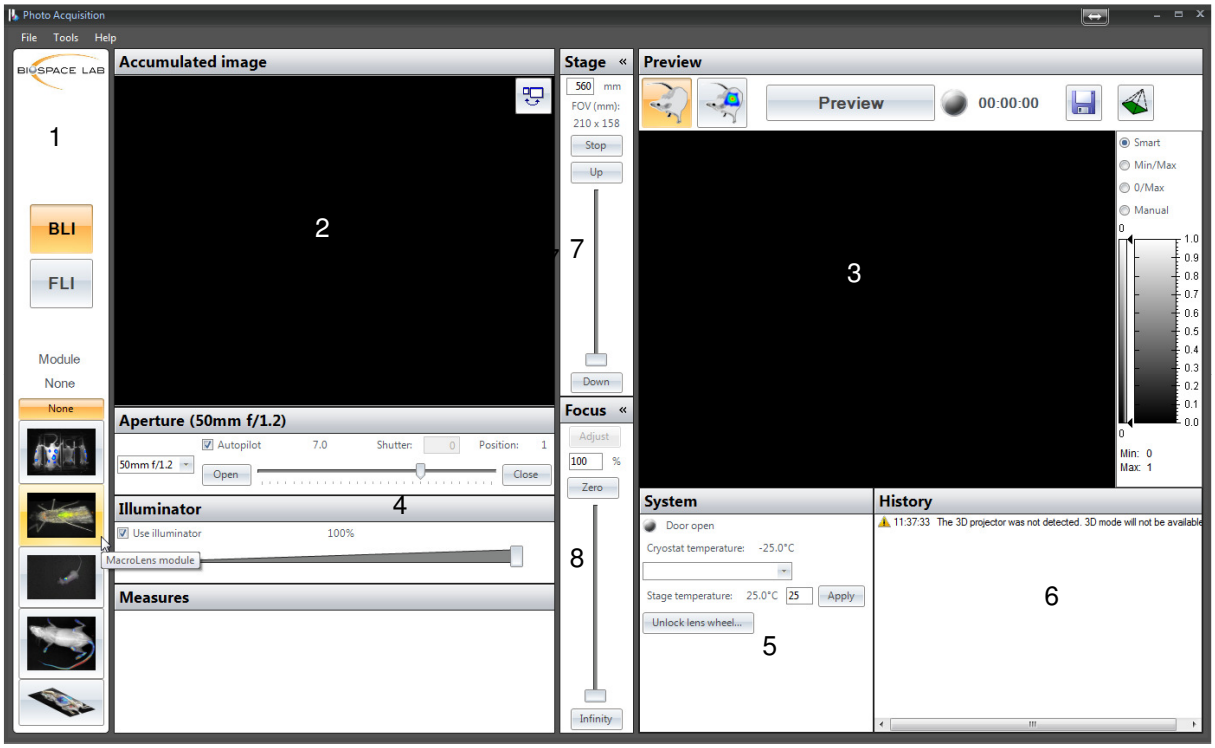


Photo Acquisition software interface

1: Menu
2: Accumulation window
3: Detection window
4: Optical parameters
5: system state
6: History window
7: Stage height control
8: Focus control

The different elements of this interface are described below and in the following chapters.

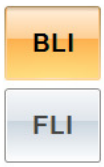
5.4 Photo Acquisition toolbar

It gives access to the different acquisition modes as well as to some major features of Photo Acquisition.

5.4.1 Acquisition modes

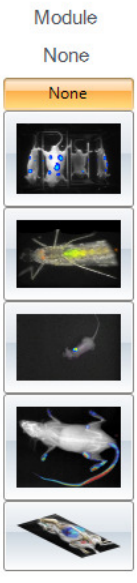
The toolbar makes it possible to choose the acquisition mode. The different modes are described in the section 6.1 Description of the acquisition modes.

Photo Acquisition toolbar:



Bioluminescence mode

Fluorescence mode



Added module:

4-View module, 3D Pack

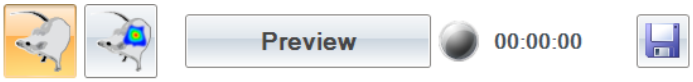
MacroLens module,

In actio module,

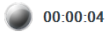
X-Ray module

Stereo CT

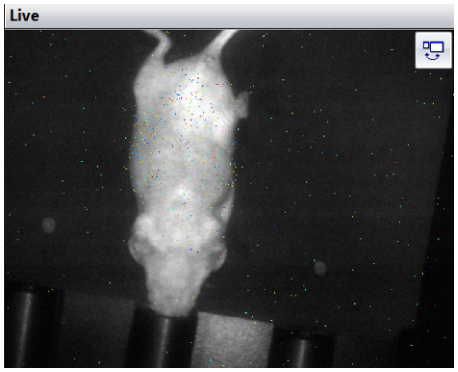
5.4.2 Acquisition display options



Launch and save an acquisition

The Tab 'Preview' allows to launch an acquisition in Preview mode  or Bioluminescence / Fluorescence mode  . The time of acquisition is also indicated 

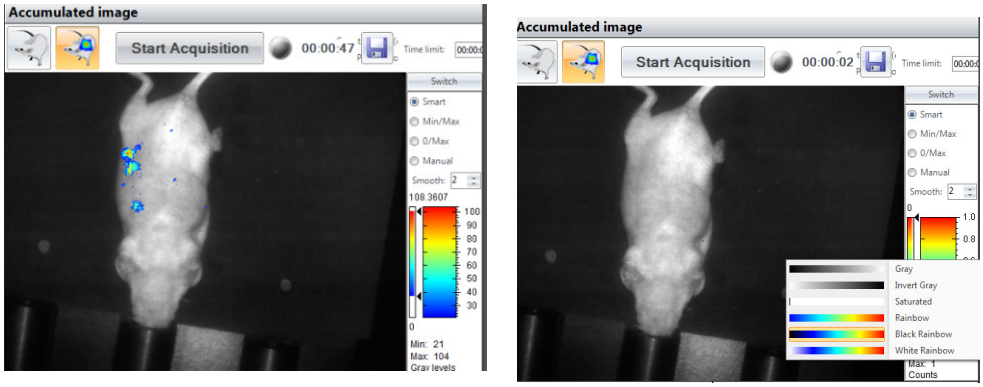
The button  allows saving a photographic black and white image after launching an acquisition in Preview mode. It is also useful, to bring back the saving data window after BLI/FLI acquisition. If you cancelled your acquisition by mistake instead of saving it, you have an opportunity to get it back by pressing on this button.



The detection display window

In this window the photons detected by the system are displayed on an almost real-time basis and are displayed as color dots on a black background with a 1 µm pixel resolution. This window can be toggled with the accumulation window with the use of this button  .
You can change and adjust the look up table to your liking with a right click on this window.

5.5 The accumulation window

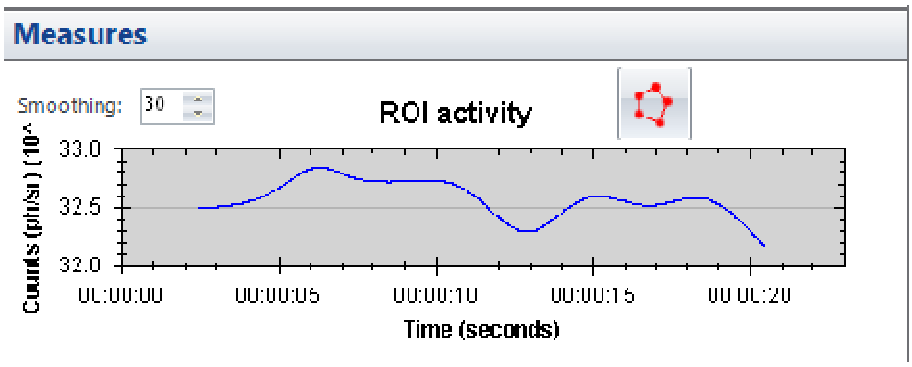


The Accumulation window

This window displays the image that is produced by the accumulation of all the photons detected. This image is refreshed every 5 seconds. The upper border of this window contains the following information:

- the time since the acquisition started
- the number of photons detected (counts)

On the right the intensity scale is displayed, for the correspondence between colors and signal intensities. The lower, respectively upper, end of the scale represents the low, respectively high, intensities. In this example, low signals are coded with dark blue and high signals with dark red. The palette (choice of colors) and the intensity scale can both be changed during an acquisition for a more comfortable display if need be, with a right click on the window.



Kinetic curve

With this window, the user can display, in real time, the kinetic of the signal. Default signal taking into account on the graphic will be full field signal if no ROI (ref next described feature) has been drawn and signal within the ROI if there is one.

ROI creation

It is possible to display the kinetics of the signal of one or several regions of interest in real time during acquisition. To create such ROI(s) press down the ROI creation button. The ROI is then created by selecting several points and double clicking to close the region. Repeat this operation for each new ROI you wish to create. Each ROI will then have different color (Picture)
You can move and modify the shape of your ROI with a single click and drag on each point. To remove ROI press on the button below.

ROI removal

Smoothing can be adjusted by clicking on the arrow or by entering a value manually.

6 Acquisition Settings

The PhotonIMAGER™ OPTIMA provides the user with the possibility to use different acquisition modes. Such modes are described in this section.

6.1 Description of the acquisition modes

Different acquisition modes can be used with the PhotonIMAGER™ OPTIMA. An acquisition session usually involves the use of several of these modes, as explained in the following section.

Preview mode

This mode provides a white light video of the animal(s) or the sample. It can be used to check the position of the animal, the position of the stage and the focus. This is also the mode to use to change the height of the stage and the focus.

Bioluminescence mode

This is the mode dedicated to bioluminescent acquisitions. Bioluminescence is the production of light by living organisms due to a biochemical reaction involving an enzyme and a substrate. With this mode, users get an image of the so-produced photons, with the information of the localization of the photons and the resulting intensities, and the temporal information of each photon (signal kinetics).

Fluorescence mode

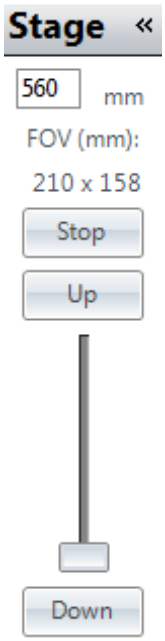
This is the mode dedicated to fluorescence imaging. Fluorescence refers to light production by specific molecules, after they have been excited with a specific wavelength illumination. It allows recording the signal by integrating it over several frames.

6.2 Changing the field of view: the stage height

The PhotonIMAGER was designed to provide optimal settings for imaging one to five mice simultaneously. The field of view can therefore be adapted to the number and size of animals or samples, to get the best trade-off between a large field of view, and maximal resolution.
Large fields of view, 21 x 16 cm², are obtained with the stage in its lower position. If the animals or samples to image do not fill the entire field of view, the stage can be lifted to improve resolution. In its highest position, the field of view is 8 x 6 cm².
To change the field of view, activate the preview mode. The stage height control, is situated in the middle up of the software interface. The position can therefore be moved, either by moving the cursor, or by pressing one of the 'Up' and 'Down' buttons. When moving the cursor, the following parameters, corresponding to the targeted stage position, are updated and displayed on top of the control window:

- Height: distance between the stage and the objective, in mm.
- FOV: size of the field of view, in mm².

The stage moves only when the cursor has been released. It gets to its new position in a few seconds. The button 'Stop' makes it possible to stop the stage while it is moving.



Stage height control

Note: changing the position of the stage does not affect the quantification values. Data are corrected to correspond to an acquisition with the stage in its lowest position. This is also true for the kinetic curve that can be displayed in real time
during the acquisition.

Warning!

Before moving the stage, make sure that nothing can hit the lens or any module inside the darkroom. **BiospaceLab cannot be held responsible in case of bad use of the instrument.**

6.3 Changing the focus

The focus can be modified with the focus control, in the middle down of the software interface. The focus is adapted automatically, depending on the position of the stage; yet this control gives the possibility to the user to change the value of the focus if the sharpness of the image is not satisfactory with the predefined setting. This can be necessary when imaging animals or samples with special thicknesses, or when using specific holders.

To check the focus, choose the preview mode and start the acquisition. Open the aperture. If the quality of the video image obtained is not satisfactory due to a bad sharpness, the focus may be changed.

To change the focus, move the cursor of the focus control. The value of the focus is given in percentage between 0 (upper position of the cursor, for a very close object) and 100% (lower position of the cursor, for a very far object). It is possible to check the result of the focus change on the image in real time with the video acquisition.

Note: the depth of field is greater when the aperture is closed than when it is opened. For this reason, the focus has little effect with a closed aperture, and the object will always look sharp. A correct focus value can thus only be found with an aperture opened.

Focus control

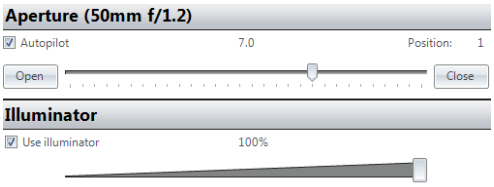
6.4 Optical parameters

The controls allow the user to modify the parameters listed in the table below. Some parameters are optional for certain acquisition modes; for example, there is no need to use a specific excitation wavelength and an emission filter in the preview mode, but this can be used for specific needs.

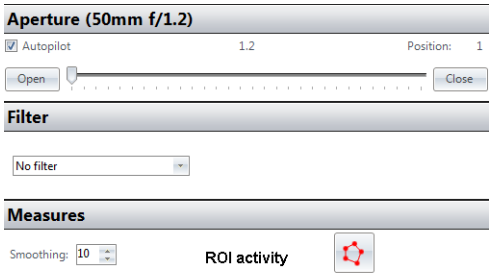
Parameters of the optical parameters window	Video	Bioluminescence	Fluorescence
Illumination power (illuminator)	X		X
Aperture	X	X	X
Auto pilot aperture		X	X
Focus	X	X	X
Image delay persistence		X	X
Emission filter	O	O	X
Excitation wavelength	O		X

X: parameter to define for the given acquisition mode
O: optional feature for the given acquisition mode

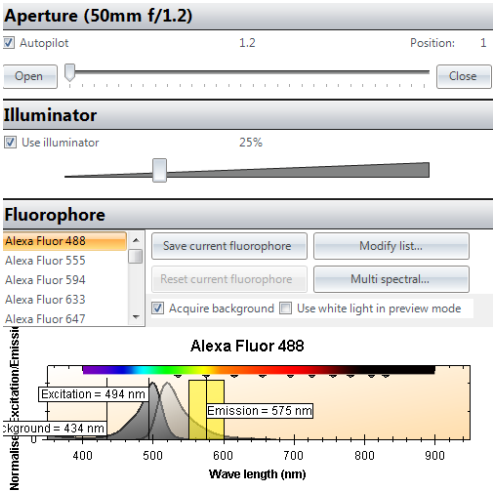
Below is represented the optical parameters window depending on the active mode.



Preview mode



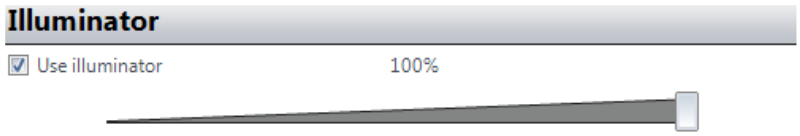
Bioluminescence mode



Fluorescence mode

6.4.1 Illumination

The illumination can be set between 0 and 100% with the control bar called 'illuminator' for the video and fluorescence mode.



NOTE : Strong illumination settings can damage the detector.Illuminator power must be increased progressively.

6.4.2 Emission filters

A set of ten emission filters is available. The standard filter set is composed band-pass filters with the following cutoff wavelengths: 510-560 nm, 550-600 nm, 590-640 nm, 630-680 nm, 670-720 nm, 700-750 nm, 730-780 nm, 760-810 nm, 790-840 nm, and 830 nm (high-pass filter). Such filters, necessary for fluorescence acquisitions, can also be used in the other modes.
In preview mode, it can be used for a very accurate adjustment of the focus. Because focus adjustment depend on the wavelength, it can be wise to adjust previously the focus with the same wavelength that will be used for a fluorescence acquisition
Filters are chosen depending on the fluorophore selected for fluorescent acquisitions.

6.4.3 Excitation wavelength

The excitation wavelength needs to be defined by choosing a fluorophore for the fluorescent modes (cf. the section on fluorescent acquisitions). In fluorescent mode excitation light is used by default to get the image of the field of view in Video.

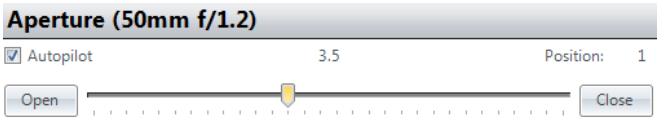
6.5 Aperture

6.5.1 Setting the aperture

The aperture is a parameter to define for any type of acquisition. It must be set between f/1.2 (aperture opened) and f/15.3 (aperture closed) for the standard acquisition, f/2.8 – f/16 for MacroLens add-in module, and f/1.4 – f/22.6 for X-ray. The control for the aperture is in the optical parameters.

Opening the apertures gives a better sensitivity; closing the aperture results in a greater depth of field and reduces the risk of saturation. The appropriate aperture is therefore a trade-off between sensitivity and non-saturation: the aperture should be as opened as possible without saturation.

The quantification values are corrected to correspond to an aperture fully opened. The unique real time capability of the Photon Imager makes therefore possible to change the aperture during the acquisition, depending on the strength of the signal: as the value of the aperture is saved for each frame, the values are corrected for the aperture with which they were obtained. The results are recalculated as if being taken with an aperture f/1.4. The kinetic curve displayed during the acquisition takes also into account such a correction.
This offers a real-time control over this parameter for the acquisition to avoid saturation but with the maximal sensitivity.



Optical parameters window of the bioluminescent mode. The aperture is defined in this window, with the corresponding cursor or with the 'Open' and 'Close' buttons.

6.5.2 Aperture auto-pilot

The aperture autopilot is a unique feature of the Photon Imager. It sets automatically the aperture to the best value corresponding to the signal intensity
This optimizes the aperture to avoid saturation but provide the best sensitivity. The way of functioning of the auto aperture depends on the mode of acquisition:

- In Bioluminescence mode, it sets automatically and dynamically the aperture to the best value corresponding to the signal intensity during the entire course of the acquisition parameters window.
- In fluorescence mode, it sets automatically the aperture and shutter to the best values corresponding to the signal intensity before the acquisition is started and keeps these settings during the entire course of the acquisition

The auto pilot is an option to activate by checking the 'Auto pilot aperture' box.
We recommend using at all times the auto pilot option to avoid saturation while ensuring optimum sensitivity

6.5.3 Stop conditions

With the single image option, the acquisition is performed in a single shot. The acquisition can be stopped by two ways:

- By clicking on the 'Stop acquisition' button (replace 'Start acquisition' after the beginning of the acquisition). This requires no stop conditions to be defined.
- By defining stop conditions. The stop conditions can be defined as a time limit (ex.: stop the acquisition after 3 minutes).
-

Even when stop conditions have been defined, the acquisition can be stopped with the 'Stop Acquisition' button at any time.

Note: with the fluorescence mode for single image acquisition, it is necessary to define the acquisition time. No stop conditions based on counts can be used.

6.6 Acquisition session

This section describes the different steps of a typical acquisition for bioluminescence and fluorescence imaging in vivo. Acquisitions in each of the modes are described in the next sections.

6.6.1 Standard acquisition session in bioluminescence

1 System preparation

The system should be switched on and Photo Acquisition launched as instructed in the previous sections (cf 4.2 Switching on the instrument and 5.2 Launching Photo Acquisition). Check the following points have been correctly installed / set:

- Anesthesia gas connections (inputs and anesthesia bar)
- Stage temperature

2 Substrate injection

Inject luciferin (or the appropriate substrate for the bioluminescence reaction) to your animal(s).

3 Installation of the animals

Draw the stage and install the animals. If you use gas anesthesia, make sure the animals are correctly with their nose in the appropriate nose cones of the anesthesia bar, with the T taps positioned to make the gas flow to such cones. Get the stage back in and close the door of the system.

4 Bioluminescence signal acquisition

Select the bioluminescence mode with the

button of the toolbar.

First, select the preview preview mode with the  button. Set the aperture and the illumination power.
Click on 'Preview': the white light video is displayed in real time in the main window. You may then:

- Change the stage height (cf. 6.2 Changing the field of view: the stage height)
- Change the focus if the automatic focus is not satisfactory (cf. 6.3 Changing the focus)
- Adapt the illumination power if necessary

This step gives the possibility to check that the animals are correctly positioned and that the optical parameters are correctly defined to provide a good image.

When you are satisfied with your video image, click on the signal tab:  . Check the aperture value or select the auto pilot aperture option (recommended). Define the stop conditions if needed.
Click on the 'Start acquisition' button.

The acquisition starts and the signal is displayed in real time in the detection and accumulation windows.

To stop the acquisition if you have not defined stop conditions, click on the 'Stop acquisition' button that has replaced the 'Start acquisition' button after the beginning of the acquisition.

5 Saving

When the acquisition stops, the saving dialog box is displayed. Choose a folder and a name for the group of files of the acquisition. It is recommended to create a folder for the group of files by selecting this feature with the first check box of the dialog box, and to record a picture image with the last check box.

Click on OK to validate. Wait before the system has finished acquiring the picture before opening the door of the Photon Imager. The files are saved in the selected folder and can now be analyzed with the analysis software M3 Vision.

The 'Saving process' dialog window is described in the section 9 Saving an acquisition.

6.6.2 Standard acquisition session in fluorescence

1 System preparation

The system should be switched on and Photo Acquisition launched as instructed in the previous sections (cf.4.2 Switching on the instrument and cf. 5.2 Launching Photo Acquisition). Check the following points have been correctly installed / set:

- Anesthesia gas connections (inputs and anesthesia bar)
- Stage temperature

2 Installation of the animals

Draw the stage and install the animals. If you use gas anesthesia, make sure the animals are correctly with their nose in the appropriate nose cones of the anesthesia bar, with the T taps positioned to make the gas flow to such cones. Get the stage back in and close the door of the system.

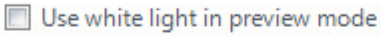
3 Fluorescence signal acquisition

Select the fluorescence mode with the 

button of the toolbar.

First, select the preview mode with the  button of the toolbar. Set the aperture and the illumination power.
Click on 'Preview': the video is displayed in real time in the main window. You may then:

- Change the stage height (cf. 6.2 Changing the field of view: the stage height)
- Change the focus if the automatic focus is not satisfactory (cf. 6.3 Changing the focus)
- Adapt the illumination power if necessary

This step gives the possibility to check that the animals are correctly positioned and that the optical parameters are correctly defined to provide a good image.
Note that in this mode by default video acquisition is done with the light of excitation wavelength to obtain better homogeneity of the acquisition. If you wish to obtain white light video, tick the option 

in the left window of the fluorescent mode.

When you are satisfied with your video image, click on the Signal tab:  . Define or check the optical parameters (illumination and aperture/shutter). Select a fluorophore and click on 

to start an acquisition.

If you want to use a new fluorophore, contact Biospace Lab and give the following information: excitation wavelength, emission filter, and the optional background excitation wavelength.

The acquisition starts and the signal is displayed in real time in the detection and accumulation windows.

To stop the acquisition if you have not defined stop conditions, click on the 'Stop acquisition' button that has replaced the 'Start acquisition' button after the beginning of the acquisition.

6 Saving

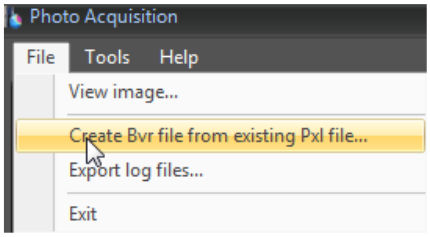
The saving dialog box is displayed at the beginning of the acquisition process. Choose a folder and a name for the group of files of the acquisition. It is recommended to create a folder for the group of files by selecting this feature with the first check box of the dialog box, and to record a picture image with the last check box.

Click on OK to validate. Wait before the system has finished acquiring the background image and the picture before opening the door of the Photon Imager. The files are saved in the selected folder and can now be analyzed with the analysis software M3 Vision.

The 'Saving process' dialog window is described in the section 9 Saving an acquisition.

6.7 File formats

The Photon Imager™ Optima generates different type of data files: signal files and photographic files.
The information relative to the signal (bioluminescence or fluorescence) is saved in two files with the extensions .bvr and .cri. The files contain the signal image and the temporal information. Signal files (.bvr and .cri) have a special format: they are list mode files. Each photon detected by the system is recorded in a new line in these files, with the information about its location and timing. As a consequence, the size of the files is directly dependent on the intensity of the signal and acquisition duration. Long acquisitions may result in large files.
Note: fluorescence integration acquisitions are saved in bvr files, but with a different format than list mode files. They do not include the kinetics information.
In order to avoid hard disk saturation, Photo Acquisition spawns also a compressed data file which extension is **.pxl**. If hard drive is an issue, when the pxl file is available, it is possible to delete the bvr and cri files, as they can be regenerated later using the **create Bvr file from existing Pxl file** option of the Photo Acquisition software.



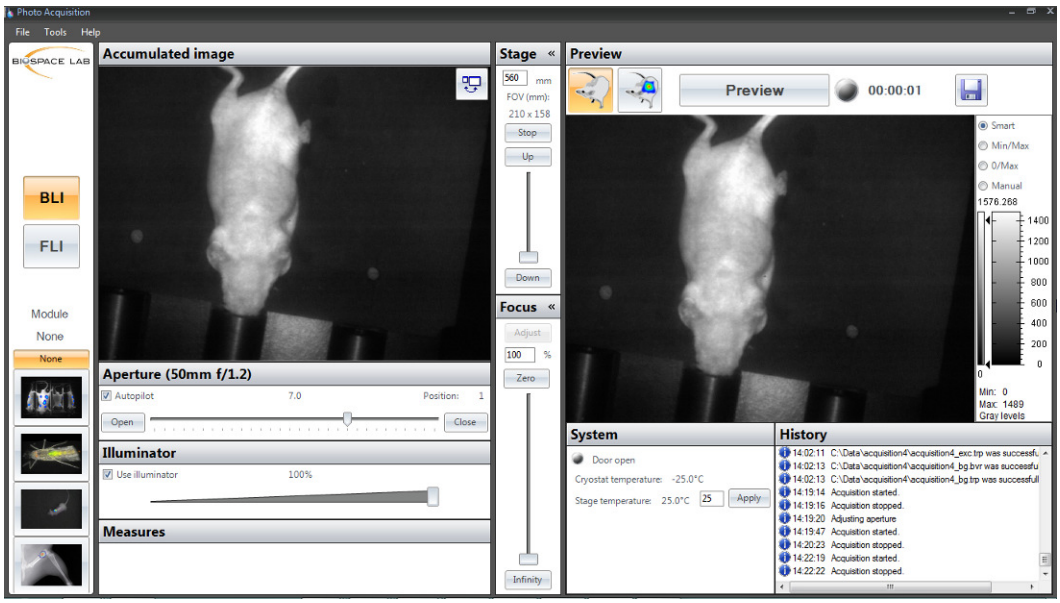
Photographic images are saved in files with the extension .trp.

For the analysis software to overlay automatically the signal image and the photographic image, the files should have the same name and differ for their extensions only. This is the case if the standard procedure for the acquisition session, described above, is followed.

7 Acquisitions in the different modes

7.1 The Video acquisition

Preview mode is activated with the  button. The white light image of the animal is displayed.

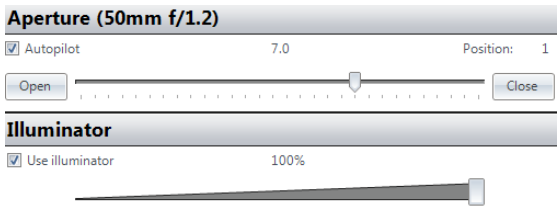


The preview mode

Preview mode parameters:

The following parameters can be changed or defined before or during the acquisition:

- Stage height (cf. 6.2 Changing the field of view: the stage height)
- Focus (cf. 6.3Changing the focus)
- Aperture (cf. 6.5 Aperture)
- Illumination power

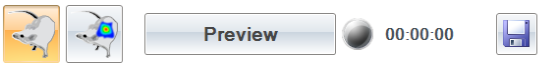


Optical parameters window of the preview mode

The preview mode offers also the possibility to use a specific wavelength for the illumination and an emission filter.

7.1.1 Video acquisition and saving

To start video, press the 'Preview' button in the Preview window.
To stop video, press the 'Stop' button which replaced the 'Preview button.

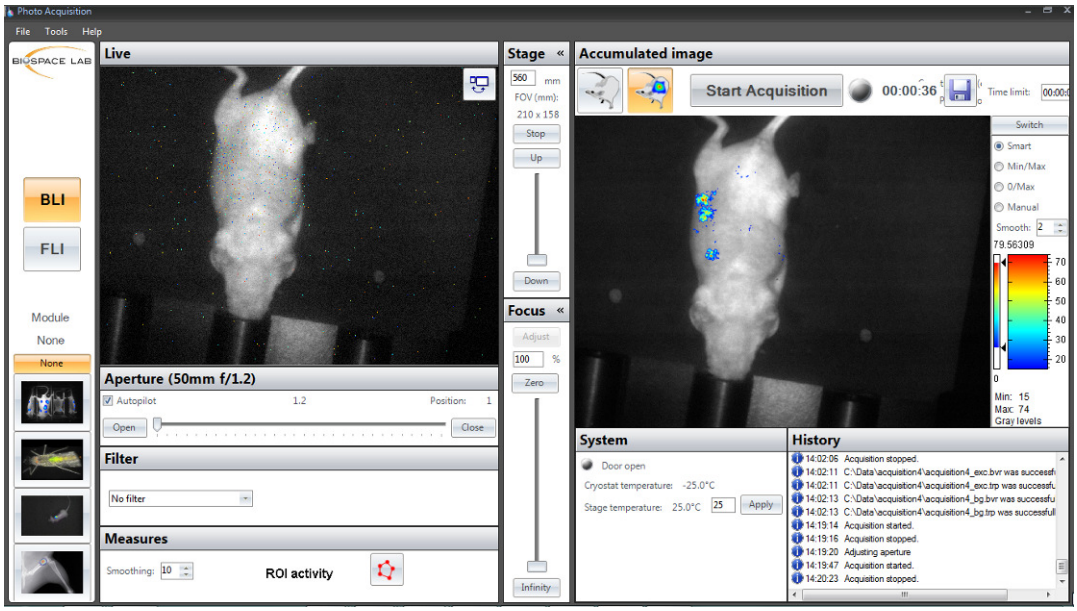


This mode can be used in order to position the animals or the sample before starting a bioluminescence or fluorescence acquisition. No data is recorded from the preview mode, unless the user specifically saves a photographic image (.trp file) with the  button.

7.2 The Bioluminescence Mode

The bioluminescence mode is activated with the 

button from the toolbar. It records and displays in real time the photons detected.



The bioluminescence mode

7.2.1 Bioluminescence mode parameters

The following parameters must be defined before the beginning of the acquisition, which is done in the Preview mode:

- Stage height (cf. 6.2 Changing the field of view: the stage height)
- Focus (cf. 6.3Changing the focus)
- Aperture (cf. 6.5 Aperture)
- Illumination power

The aperture can be changed before and during the acquisition. It is recommended to use the aperture autopilot to get the best aperture depending on the signal intensity. The aperture and the display persistence can be changed with the Optical parameters window.

7.2.2 Bioluminescence acquisition and saving

To start acquisition, just press the 'Start acquisition' button.
To stop acquisition, press the 'Stop acquisition' button which replaced the 'Start acquisition' button.

The 'Saving process' dialog window is described in the section 9 Saving an acquisition.

7.3 The Fluorescence Mode

Fluorescence is an optional feature of the Photon Imager. In this mode, the animal or the sample is excited with light at the 'excitation wavelength'. This results in the emission, from excited fluorescent molecules, of photons at a wavelength longer than the excitation wavelength (Stoke shift).

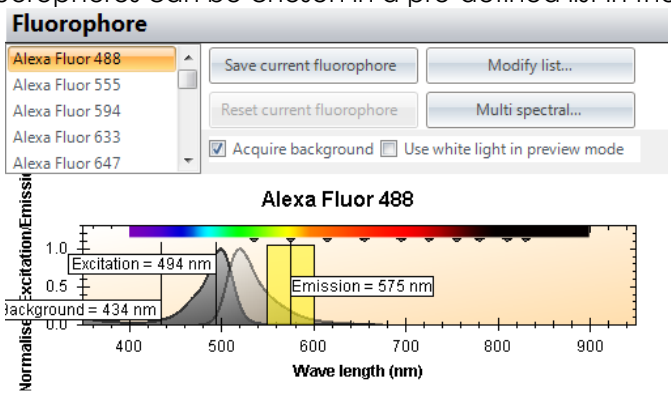
The PhotonIMAGER™ OPTIMA is equipped with a linear variable interferential filter that allows choosing any excitation wavelength between 450 nm and 1000 nm, with an accuracy of 5 nm. It also includes a set of ten band-pass filters that cover the range from 530 nm to 830 nm. The Optical Parameters window allows the control of these parameters.

The fluorescence mode is activated with the 

button from the toolbar. It records and displays in real time the photons detected.

7.3.1 Choice of a fluorophore: excitation, emission and background wavelengths

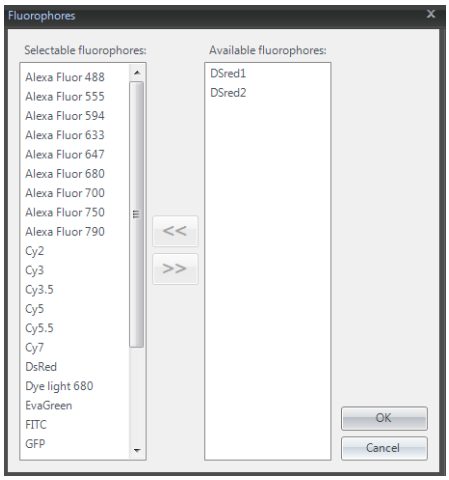
The fluorophores can be chosen in a pre-defined list in the optical parameters window.



The optical parameters window in fluorescence integration mode

To select a fluorophore, click on the corresponding line. It selects the appropriate excitation and background wavelengths and emission filter which are displayed on the graph below. You can also modify the wavelengths by moving the Emission, Excitation and Background bars while preserving the appropriate gaps between them (the choice of the appropriate wavelengths described below in this chapter). If you wish to preserve the changes press on the Save current fluorophore button. You can at any time come back to the original fluorophore settings by pressing on Reset current fluorophore. This button will be activated once you change the default settings of the fluorophore. Background image is acquired by default but if you don't want this option simply untick the Acquire background check box.

Modify list option. Information about each fluorophore is stored as Excel file in the software settings. If your fluorophore is not present on the pre-defined fluorophore list, you can add it by pressing on the button Modify list... and the window with two lists will pop out



You can now choose the desired fluorophore from the Available fluorophore list on the right and press on the arrow to place it on the Selectable fluorophore list (on the left). The software will automatically upload the appropriate Excel file containing information about this fluorophore. It will then appear in the pre-defined fluorophore list. The same way you can remove the fluorophores you don't use from the Selectable to Available fluorophore list. This function serves to keep in the pre-defined list only fluorophores that you are working with, in

order to make a selection of fluorophore easier and faster but if you wish you can keep all available fluorophores on that list.
If you want to use a new fluorophore that is not on the list contact Biospace Lab and give the following information: excitation wavelength, emission filter, and the optional background excitation wavelength.

For a given fluorophore, the following parameters need to be defined:
- Excitation wavelength
- Background
- Emission

The excitation and emission wavelengths selection:
The high pass filter is automatically selected as the filter with the cut off wavelength the closest to the emission wavelength.
Note: the excitation wavelength and the emission filter should not be too close, in order to avoid reflection problems. It is recommended to have a shift larger than 80 nm between those two wavelengths. Studying the absorption and emission spectra is recommended in order to find the best couple (excitation – emission) that gives the best efficiency with the 80 - nm shift rule.

The background wavelength is an optional parameter that can be defined in order to help remove autofluorescence signals. With living animals, there is usually a natural fluorescence of certain tissues, including hair and skin. This phenomenon, commonly referred to as 'autofluorescence', interferes with the relevant signal of fluorescence.
A simple model can be used in many cases to manage this issue. This model is based on the assumption that an excitation with a wavelength minimum 50 nm shorter than the excitation wavelength of the fluorophore results in an image of autofluorescence only. Furthermore, this autofluorescence image is supposed to be similar, in its intensity distribution, to the autofluorescence in the standard acquisition. Therefore, the analysis software, M3 Vision, can easily subtract the two signals in order to isolate the relevant fluorescence signal.
The background wavelength can therefore be set to minimum 50 nm below the excitation wavelength.

7.3.2 Fluorescence acquisition

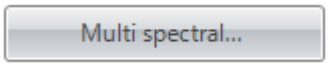
In addition to the choice of a fluorophores, the following parameters must be defined before the beginning of the acquisition (cf. 6 Acquisition Settings)

- Stage height
- Focus
- Stop conditions
- Aperture/shutter
- Illumination

The acquisition is started with the 'Start acquisition' button, and stops when the time limit is reached or with the 'Stop acquisition' button. The 'Saving process' dialog window is described in the section 9 Saving an acquisition.

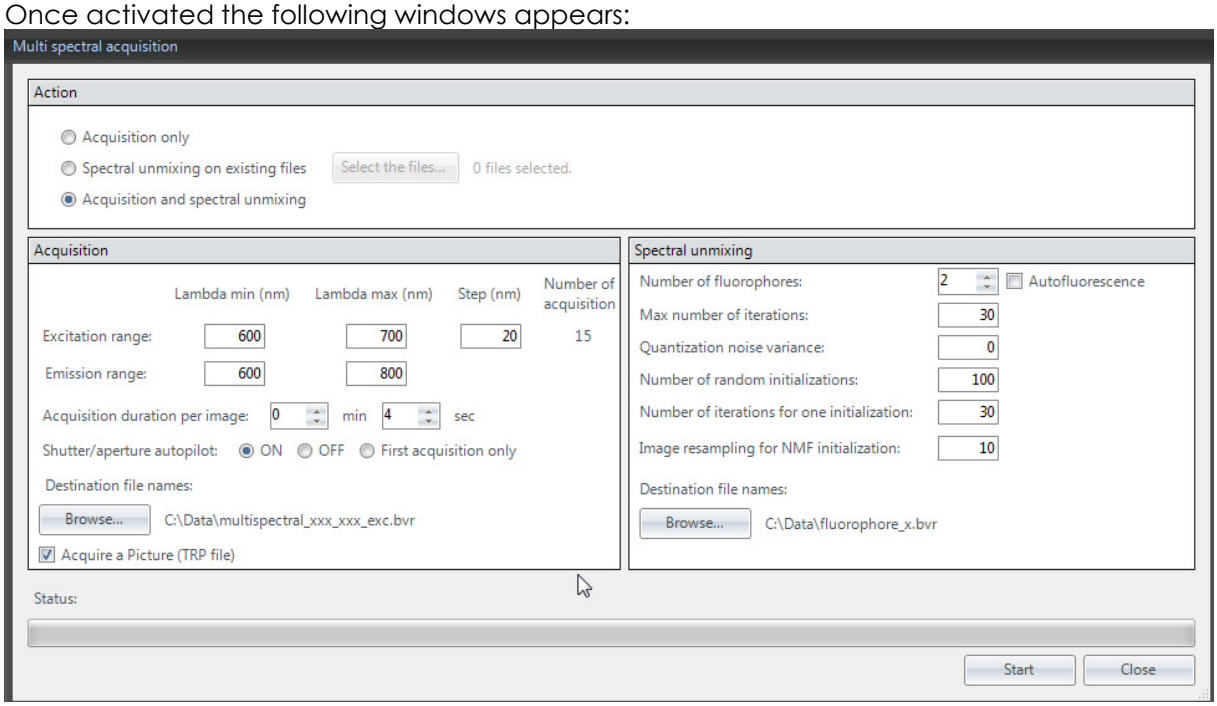
7.3.3 Multi spectral option

If two or more fluorophores are to be imaged in one animal, this option can be chosen in order to obtain a separate image for each fluorophore in one process, without a need of running few acquisitions and defining conditions for each fluorophore separately .
During a multispectral acquisition the software proceeds the number of scans through predefined range of wavelengths in order to choose the best excitation/emission pair for each fluorophore. As a result it gives one final image for each fluorophore and an image of the background if required.

Mutispectral option is activated with 

button which appears in the fluorescence options window once FLI mode activated.

Once activated the following windows appears:



In the right Spectral unmixing window define the number of fluorophores you are going to image.
According to the fluorophores to be imaged, the excitation and emission wavelengths range needs to be predefined in Acquisition window on the left. All imaged fluorophores need to

have their emission and excitation spectra in the chosen range. Number of scans and total duration of the process will depend on the excitation and emission range, the number of steps and the acquisition duration per image which can also be chosen in the same window (default settings are recommended). Shutter and aperture can be adjusted for each fluorophore separately when Shutter/aperture autopilot is on, or if option First acquisition only is ticked, the same shutter/aperture settings will be applied for all the acquisitions. Once all conditions of the acquisition are defined press on Start to begin with spectral unmixing.

In Action window of multispectral acquisition Acquisition and spectral unmixing is a default option but you can also choose to acquire the images without applying spectral unmixing algorithm by choosing an option Acquisition only and proceed with spectral unmixing at any time later by choosing Spectral unmixing on existing files.

8 Real time controls during an acquisition

This section describes the different controls that are available during bioluminescence and fluorescence counting acquisitions.

8.1 Aperture

The aperture can be modified during the acquisition, without interfering with the correction process (reminder: all quantification figures are corrected for the value of the aperture. The reference configuration is the aperture fully opened). The changes can be done manually, or with the help of the aperture autopilot, that can be selected with the check box in the optical parameters window.
The aperture can be set manually for the entire acquisition if desired. Beware that a wide opening with an intense light source may result in damages to the camera.

For more information on the aperture, see the section 6.5 Aperture.

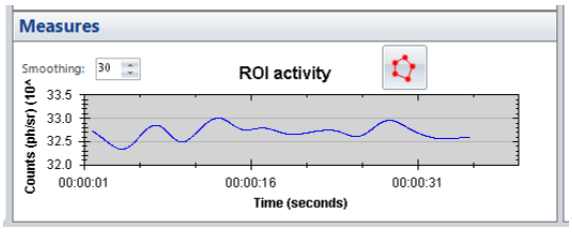
8.2 Acquisition starting point

The detection display may change just after the beginning of the acquisition if the autopilot aperture is selected. This corresponds to the time necessary for the system to find a stable position for the aperture, depending on the signal strength; the acquisition starts only after this position has been found.

8.3 Real time display of the signal kinetics

8.3.1 Displaying the kinetics

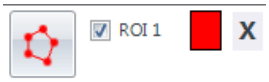
The kinetics of the signal detected can be displayed in real time during the acquisition.



Real time kinetic curve

8.3.2 Kinetics of a region of interest

If desired, the curve can be specific to the signal detected in a given region of interest. A click on the ROI button  allows to define a region of interest (a click for each point of the polygone, and a double click for the last point). The curve is then updated to correspond to the kinetics of the signal of the region of interest. It is also possible to have the kinetics of several regions of interest during the acquisition.

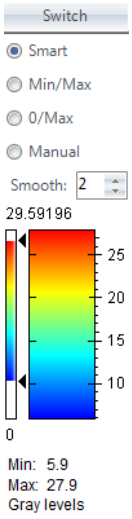
To delete the ROI(s), click on the cross 

button. When no regions of interest are created, the kinetics is displayed for the signal over the entire field of view.

8.4 The scale

8.4.1 Relation between scale and display

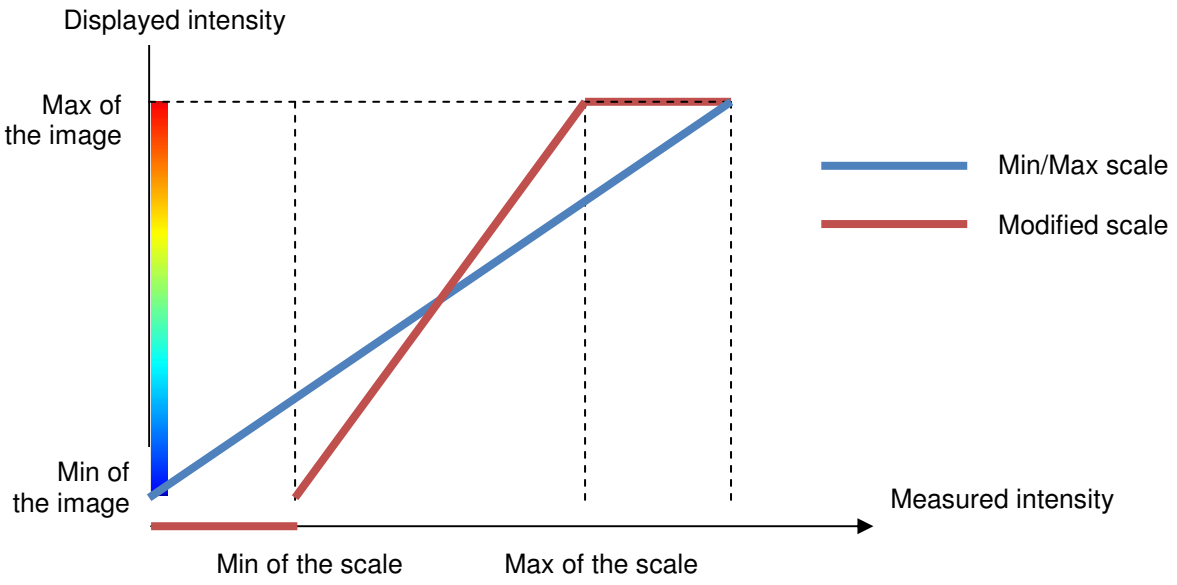
During an acquisition, it is also possible to adapt the dynamic range and the contrast of the signal displayed to focus on specific signal intensities; this can help a better visualization of low signal sources for instance.
The 'change scale' tool is displayed on the right of the accumulated window.



The 'Change Scale' window

The minimum value represents the number of counts in the lowest pixel (minimum value in the image). The maximum value represents the number of counts in the hottest pixel (maximum value in the image).
The scale makes it possible to change the relationship between the measured intensity and the displayed intensity.

Two parameters, the maximum and the minimum value of the scale, define the scale. The max and the min of the scale have to be between the max and the min of the image. The gray or color scale used in the display is thus defined by the value of the hottest pixel (11 in the example above) and the lowest pixel (O in the example) as explained with the curves below.



Effect of the scale on the displayed intensities

The blue curve corresponds to a scale where the max and the min of the scales are the max and the min of the image. In such a case, there is a linear relationship between the measured and the displayed intensity. The red curve corresponds to a scale with different values for the max and min of the scale and of the image. All the pixels with an intensity below the min of the scale are displayed as background. All the pixels with an intensity above the max of the scale are displayed with the color of the highest signal value (red with the color scale of this example). This setting focuses on the dynamic range between the min and the max of the scale; if most of the pixels are in this range, this improves the contrast.

Changing the min and the max of the scale can be done either with the cursors, or with the text boxes on the right hand side. If the min is set to 2 and the max to 6, the contrast of the image is modified: the background becomes less intense (all pixels below 2 are considered as background) and the active parts of the image become brighter (all pixels above 6 are displayed with the color of the highest value, with the color scale of this example). This can help the visualization of the weak signals. It has no effect on the recorded data and quantification values.

8.4.2 Smart scales

As the max of the image is constantly increasing, if the max remains constant, all pixels may be displayed with the same color as their intensity gets higher than the initial value of the max. To avoid this problem, autoscales can use algorithms to adapt automatically the max and the min of the scale in order to provide a good contrast, even with the accumulation of the signal detected.

The smart autoscale: the max is set to a robust maximum (less sensitive to variations of isolated high intensity pixels) and the minimum to an estimation of the noise level.

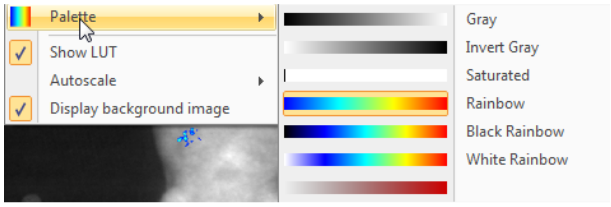
Note that when the min and max parameters of the scale are modified, the autoscale is inactivated, and the scale is in a manual mode. To re-activate the autoscale, select the desired autoscale (Min/Max or smart autoscale) in the 'Change scale' dialog window.

8.4.3 Smoothing

The signal can be displayed with a smoothing effect. This is performed by the use of a Gaussian filter on the signal image. The size of the filter, in pixels, can be modified with the cursor of the smoothing control.

8.5 The palette

During an acquisition it is also possible to change the palette. The palette is the choice of a set of colors to code for the different intensities for the signal. To change this setting, right click in the 'detection display', and select 'Palette' in the list. The different possibilities for the 'palette' are then shown.



The choice of palettes

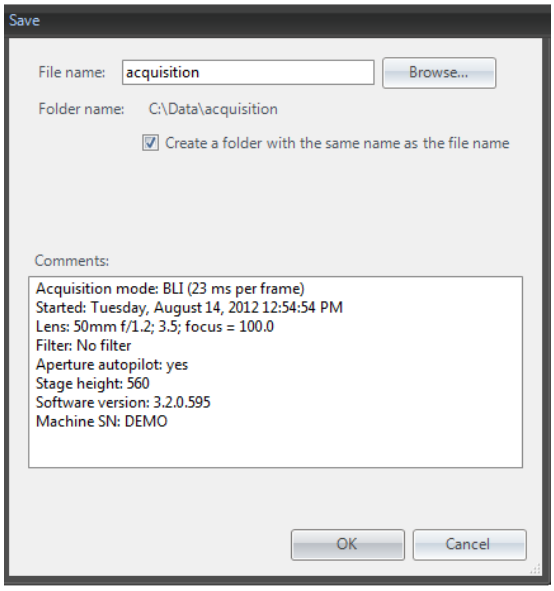
Select the desired palette from the list.

8.6 The overlay

The overlay option allows the user to display or not the photographic image simultaneously as the signal. To change this setting, right click on the 'display background image'. This activates or inactivates the overlay mode.

9 Saving an acquisition

At the end of an acquisition in bioluminescence or at the beginning of an acquisition in fluorescence mode, the 'Saving process' window helps define the properties and options of the acquisition to save.



The 'Saving process' dialog window

The following parameters and options can be defined with this dialog box:

- File name: this defines the name of the files to be saved. The different files will be saved with the same name, and a different extension (bvr for the signal file, cri for the temporal information, trp for the picture image, etc.) The button 'Browse' can be used to select a new directory folder.

- Create a folder with the same name as the file name: this option is highly recommended, as an acquisition always generates several files, and since the files should all be in the same folder for the analysis software to make correctly the link between the different files and their information.

Furthermore, it is possible to add comments, in addition to the image properties that are automatically added to the comment field.

It is not possible to save the kinetic curve that may have been created during the acquisition; however, this curve is easily created with M3 Vision.

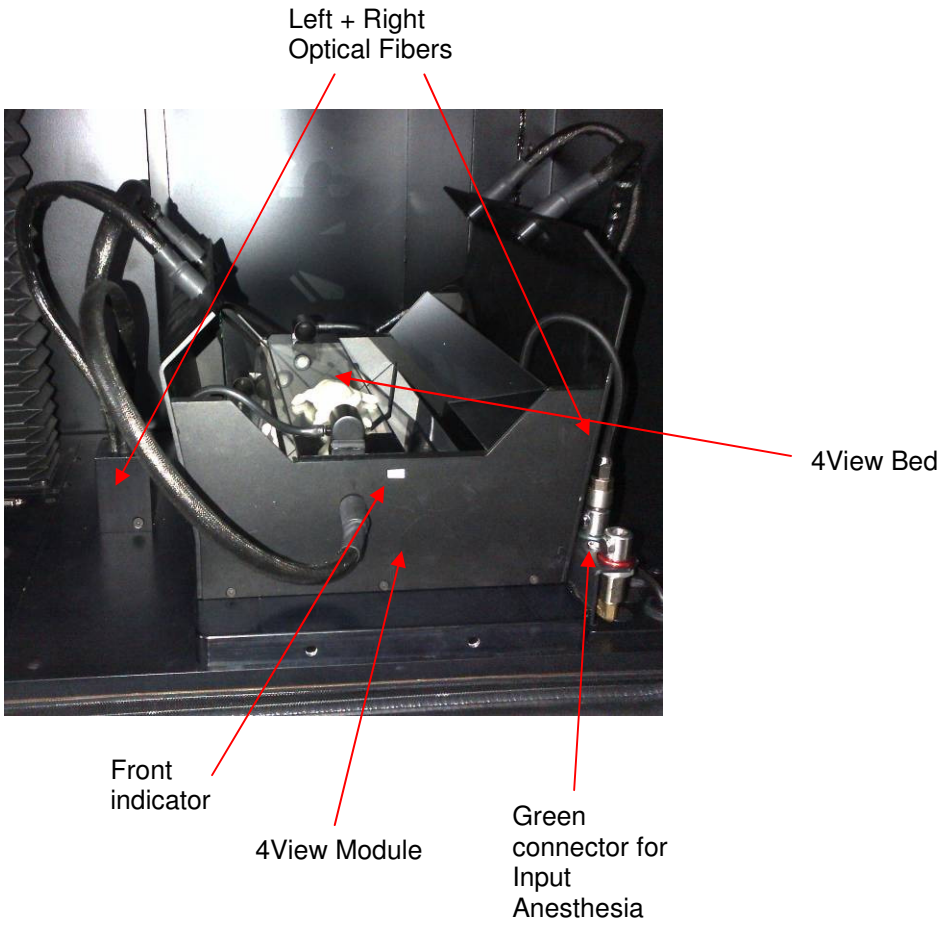
Important: Be aware that acquisitions in fluorescence include a step where the background image is acquired, provided the Acquire background option is ticked (set by default).

10 Modules

10.1 4View Module and 3D/4D reconstruction

10.1.1 4View module description:

The 4 View module is an optional add-on module for the Photon Imager. It makes it possible to acquire simultaneously 4 views of up to 2 animals: dorsal, ventral, and both lateral views. The module can be used both for bioluminescence and for fluorescence.
The 4View module consists of a set of mirrors and optical fibers that direct the light for optimal illumination and excitation for the 4 different views, and an animal holder that makes it possible to image the ventral side.
The 3D feature allows surface reconstruction of your animals and accurate localisation and discrimination of the emitting sources inside.
A specific device allows keeping two animals under anesthesia.
Acquisitions are performed with a specific mode of the Photo Acquisition, and can be analysed with the analysis software, M3Vision.



10.1.2 4View Acquisition panel overview

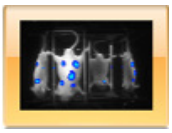
4View Installation and Activation

Before the installation:

-The moving stage of the Photon Imager should be in its lowest position
-Remove any parts that are present on the sliding plate or any anesthesia device
-Remove any optical fibers

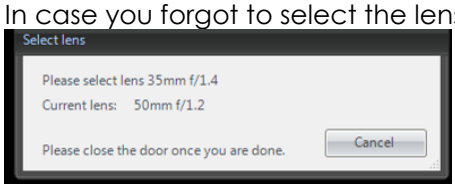
Insertion and positioning of the module:

-Slip the sliding plate of the Photon Imager outside. Put the module on the heating plate in the corresponding positioning plot front side directed on the door.
-Slip back the sliding plate inside the Photon and connect both optical fibers
-Put the animal holder in the dedicated slot and connect the anesthesia tubing on the green connector. Make sure that the tubing is not going through the field of view
-Select the 35mm Lens
-Close the door



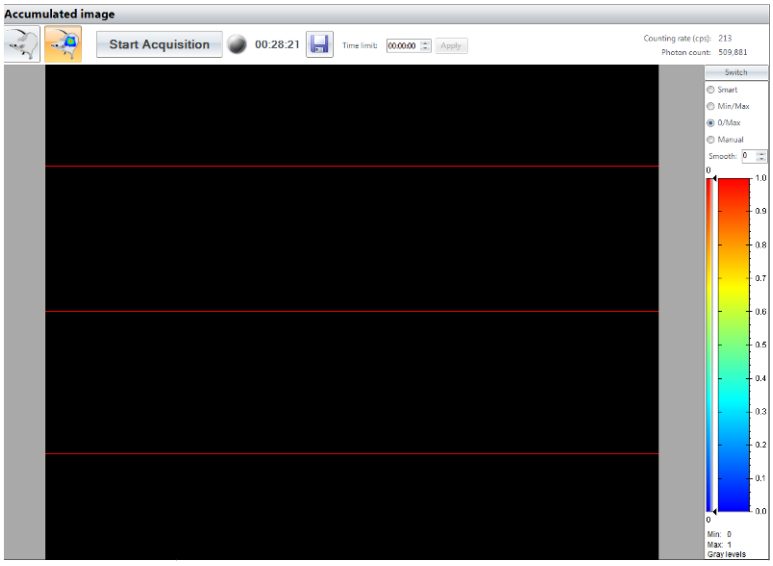
4View mode Button

In case you forgot to select the lens, the following message will appear.



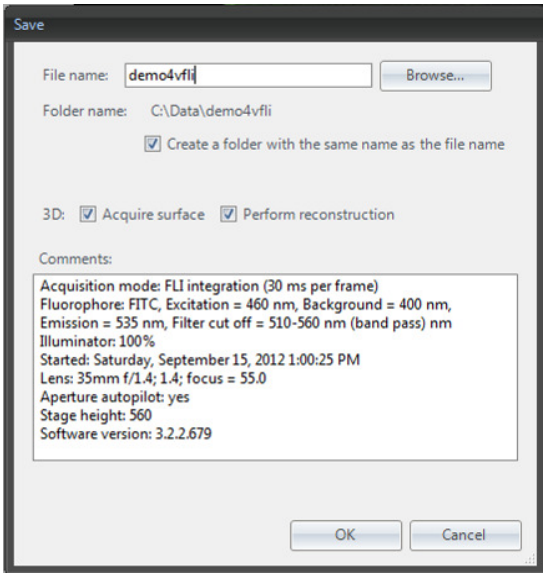
In the Photon Acquisition software, the activation of the 4View mode is enabled by the 4View mode button located in the "Module" panel on the left side of the main window. Before clicking on it, make sure that:

The picture above shows the 4View acquisition window corresponding to the 4View mode



The field of view is divided in 4 parts corresponding to each view. From top to bottom: Left, Top, Bottom and Right

10.1.3 4View Acquisition and saving



Once 4View mode selected, the acquisition works exactly the same way than for standard mode.

On the saving wizard, you have the opportunity to acquire the 3D surface and perform the reconstruction. (Ticked by default)

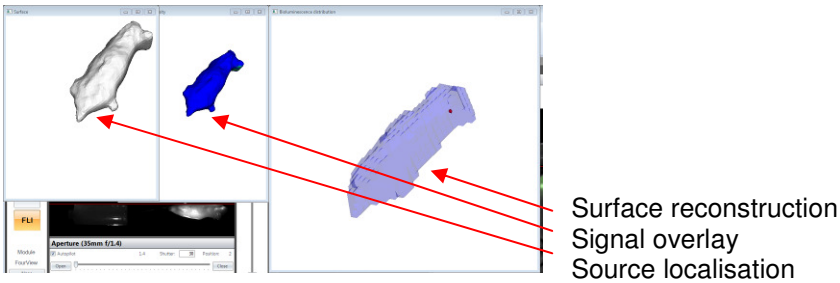
If you just want to save the bvr, untick Acquire surface. In this case M3Vision will consider the acquisition as a standard .bvr file

If you want to save time, you can untick "Perfom reconstruction" and postpone it

Note: Fluorescence reconstruction can only be performed if background image was acquired.

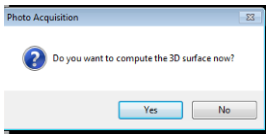
The process of reconstruction is divided in 3 parts:

- Surface reconstruction
- Overlay of the signal acquisition
- Localization of the source



For each step, a dedicated window will open and show the 3D model.

Perform the surface acquisition only:

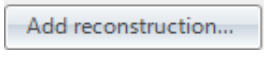


In preview mode you can acquire surface only by clicking on the dedicated button. 

A save wizard will automatically open to perform the acquisition. Enter the name you want and validate. A second wizard gives you the choice to perform the reconstruction immediately or to postpone it.

10.1.4 Perform the 3D/4D reconstruction or schedule it for later:

In preview mode select reconstruct: The following wizard will appear. You can add as many acquisitions to reconstruct as needed by clicking on add.



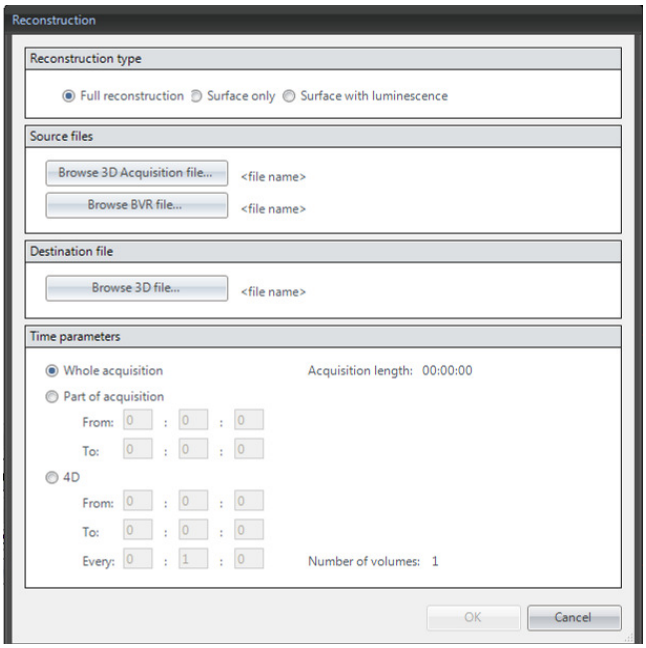
The following wizard will appear:

Select if you want to reconstruct only the surface or both the surface and the signal.
Select the corresponding 3D acquisition file (.3Dacq) and the related bvr file.
Enter the name of the destination file.

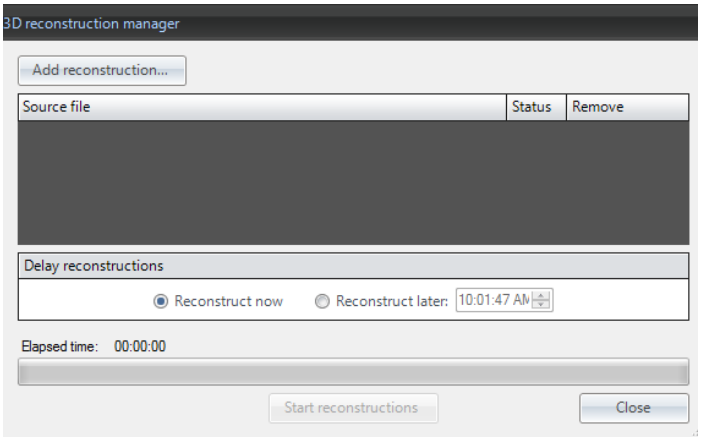
At this step 3 possibilities are available:

1) Perform the whole acquisition
2) Perform a partial acquisition
3) Perform a sequence of acquisitions so that you can look at the evolution of the signal regarding the time

Validate to finalize.



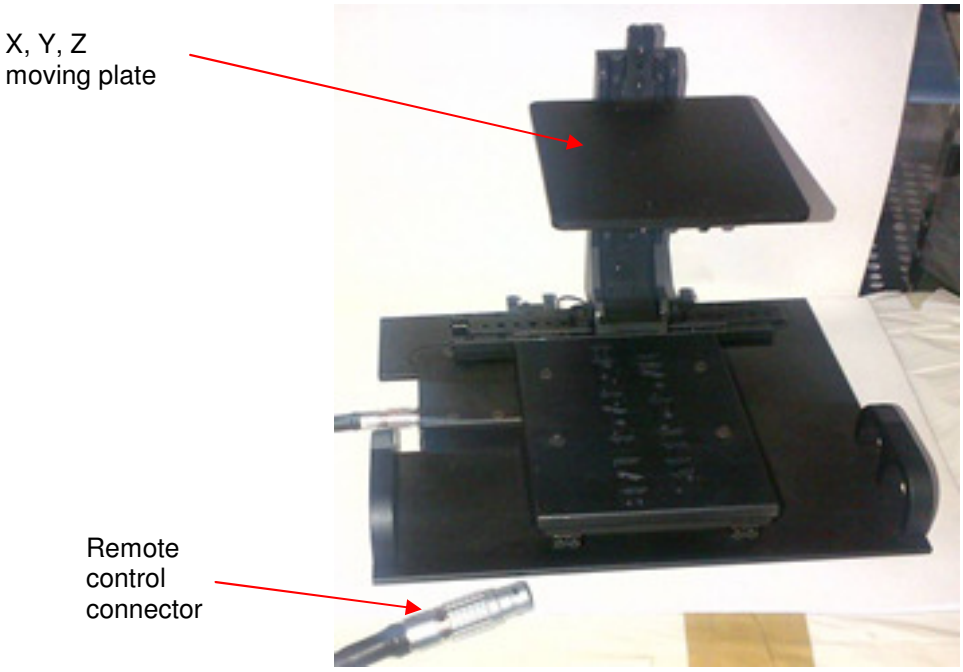
As the reconstruction may take time and CPU resources, you can postpone the process and plan a task schedule by selecting reconstruct later and entering the time of execution.
Then press on start reconstruction.



10.2 MacroLens Module

10.2.1 MacroLens module description:

The MacroLens is an optional add-on module for the Photon Imager. It was designed by Biospace Lab to open a new door to the world of bioluminescence microscopy and to build the missing link between macroscopic and microscopic acquisitions for fluorescence. This module makes it possible to get a spatial resolution as high as 2.5 µm in bioluminescence or fluorescence for in vivo or ex vivo imaging.
The module is made of an X, Y, Z remote plate, two specific optical fibers with an optical zoom on the lens wheel with a magnification from 1x to 5x a dedicated anesthesia line for one animal and a specific acquisition mode in PhotoAcquisition.
The three dimensions moving remote stage enables accurate positioning and focusing.
Acquisitions are performed with a special mode of the acquisition software PhotoAcquisition, and can be analyzed with the analysis software, M3 Vision.



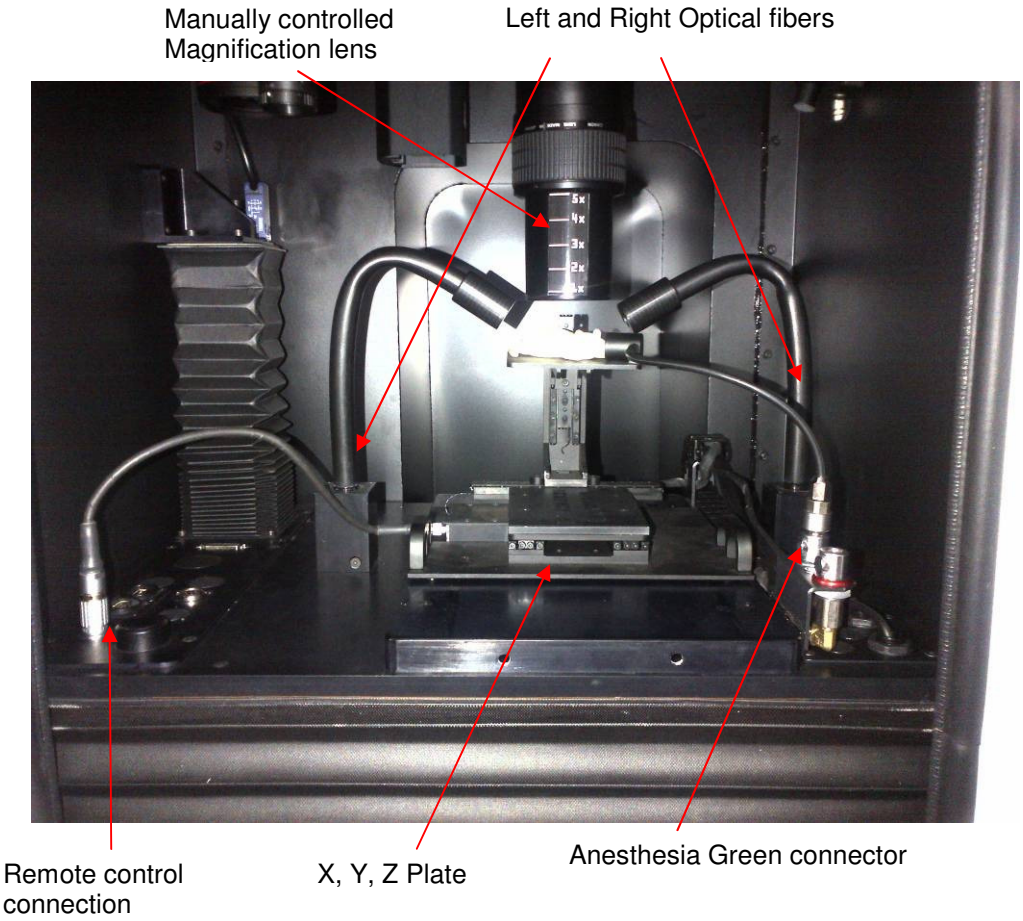
10.2.2 MacroLens Acquisition panel overview

MacroLens Installation and Activation

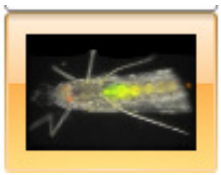
Before the installation:
-the moving stage of the Photon Imager should be in its lowest position.
-Remove any parts that are present on the sliding plate or any anesthesia device.
-Remove any optical fibers.

Insertion and positioning of the module:
-Slip the sliding plate of the Photon Imager outside. Put the module on the heating plate in the corresponding positioning plot front side directed on the door.
Warning: Always handle the module by the dedicated holding mount. Never hold the module by the plate or any other part.
Never try to move the plate manually.

-Slip back the sliding plate inside the Photon and connect both optical fibers
-Put the animal holder in the dedicated slot and connect the anesthesia tubing on the green connector
-Select the 65mm Lens and choose the appropriate magnification
-Connect the remote control connector on the left side of the elevator
-Close the door
-Switch on the remote controller



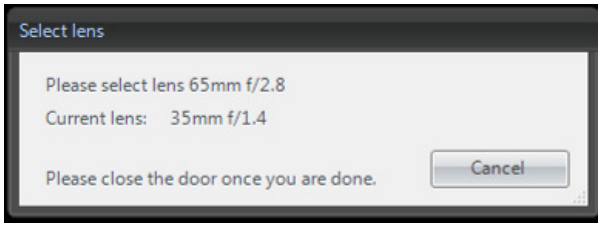
In the Photon Acquisition software, the activation of the MacroLens mode is enabled by the MacroLens mode button located in the "Module" panel on the left bottom of the main window. Before clicking on it, make sure that:



MacroLens Activation button

The stage will automatically move to the adequate altitude to provide an optimal focused image.

In case you forgot to select the lens, the following message will appear.



10.2.3 MacroLens Acquisition and saving

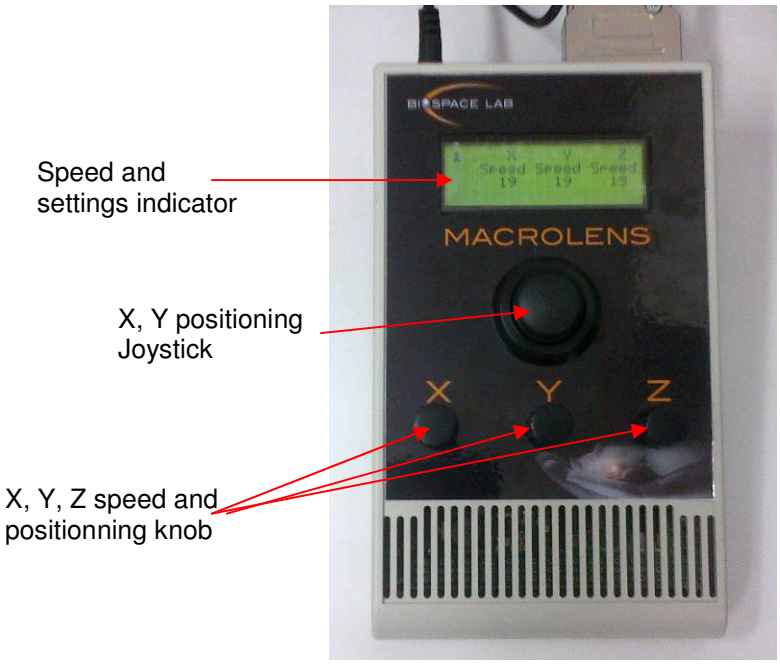
Once MacroLens mode selected, the acquisition and saving proceeds the same way than for standard mode in Bioluminescence and Fluorescence. The only difference remains in the positioning of your sample and the focusing method.

As only the aperture is computer controlled, the focusing and the positioning have to be adjusted with the remote controller.

Functioning of the remote controller:

The joystick is used to set the position of you sample in the filed of view along the X and Y axes.

The three knobs are used for adjustment of each corresponding axis and the speed change.
To change the speed press on the knob, then turn clockwise to increase and counter clockwise to decrease it. Speed can be set between 1 and 19.
Press again on the knob to validate.



To change the magnification, place the plate at the lowest position.
If you want to **increase** the magnification:
-Stop the preview acquisition
-Select the magnification on the folding, wait for the stage to mode and then open the door to change the lens magnification configuration.

If you want to **reduce** the magnification:
-Stop the preview acquisition
-Open the door to change the lens magnification
-Close the door and wait for the stage to reach its position

Note: Each time you select a new magnification, you have to validate the proper detection on PhotoAcquisiton. For magnification x1 and x2, you must precise which magnification has been selected.

10.3 X-Ray module

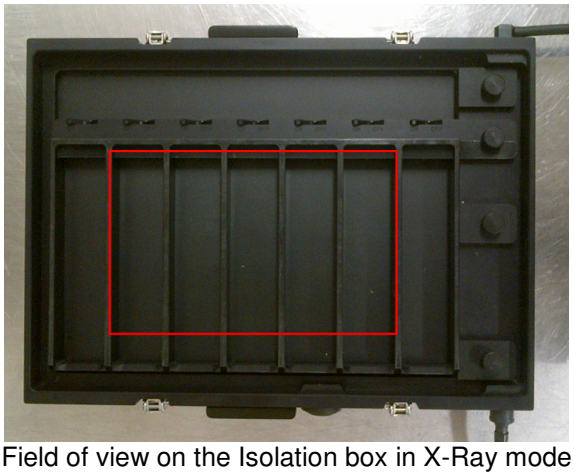
10.3.1 Overview

It is possible to retrieve anatomical information of the bone structure of the subject With the X-Ray module. Bioluminescence and fluorescence acquisitions can be overlaid to the 2D X-Ray images for precise co-localization of the signal with the subject's skeleton.

10.3.2 Hardware

The Hardware of the X-Ray module is composed of an X-Ray sources located on the automated Objective Turret and a Flat Panel X-Ray detector integrated in the lower part of the dark chamber and protected with a carbon fiber window.
For safety purposes, there is also a warning light on the top left corner of the front panel indicating whether the X-Ray source is on or off and a key lock on the bottom left corner of the front panel to allow or prevent activation of the X-Ray source.

A heated Carbon Fiber sample plate can be place on top of the detector window for precise control of the temperature of the subjects. The size of the flat panel is substantially smaller than the maximal of view of the camera. For this reason acquisitions in X-Ray mode are limited to 5 small subjects.
X-Ray acquisitions can be carried out either with a heated carbon fiber sample plate or with a heated high Throughput (up to 14 animals) Isolation box, or directly on the Carbon fiber window (highest X-Ray resolution). In Any case, a maximum of five subjects can be imaged in one acquisition.



Field of view on the Isolation box in X-Ray mode

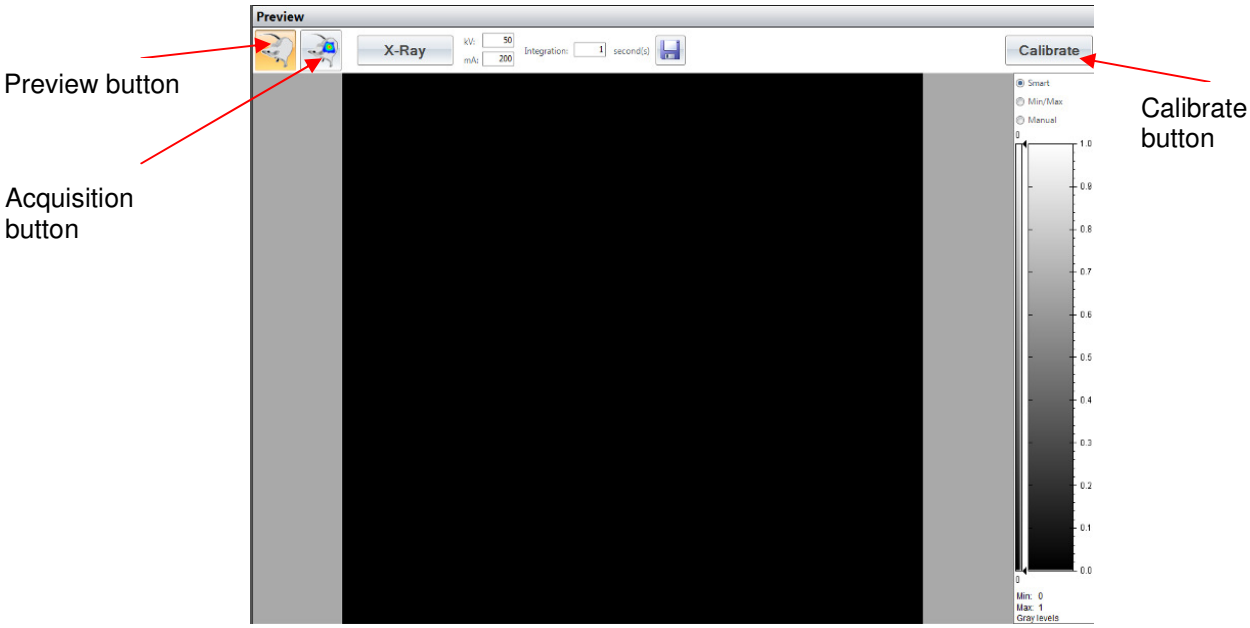
10.3.3 X-Ray Acquisition panel overview

The X-Ray mode can be accessed from PhotoAcquisition by clicking on the X-Ray Icon

Acquisitions can be carried out either in BLI of FLI by clicking on the corresponding Icon.
The X-Ray mode will only be accessible if the front Panel X-Ray Key Lock is switched on.

The Preview button allows to carry out an acquisition in X-Ray for precise positioning of the Subject's Skeleton
The Acquire button starts the acquisition in BLI or FLI
The Calibrate Button creates the offset image of the sample support plate.

The picture below shows the XRay acquisition window corresponding to the XRay mode.



X-Ray bar

On the top of the "Preview" panel is the XRay bar. The function of the different buttons and parameters are detailed below.

Radiographic mode

BLI / FLI mode for XRay fusion

Start radiographic acquisition

X-rays configuration
The x-rays energies are adjustable thanks to the high voltage of the x-ray tube (range 20kV-50kV)
The x-rays intensity is adjustable thanks to the filament current of the x-ray

tube (range 0 – 200µA)

Adjustment of the integration time of the detector (> 500 ms)

Save the radiographic acquisition

Calibration of the detector
It is advised to complete the calibration every two weeks to improve the efficiency of non-homogeneity correction. If the calibration is too old, the button turns red

10.3.4 X-ray Acquisition Workflow

1. Select the imaging mode (FLI or BLI)

Note : if using the FLI imaging mode select the fluorophore to be used at this stage in the fluorophore list Acquire background should be ticked and use white light in preview mode should be unticked.

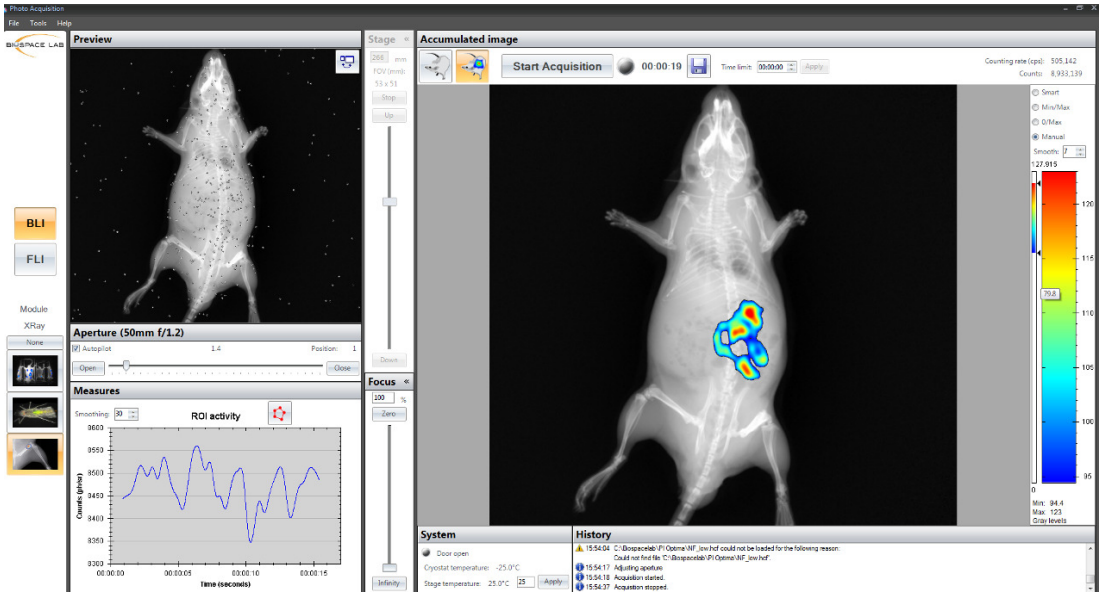
2. Select the **X ray** module
3. Place the **empty** sample holder and Anesthesia bar (sample plate or Isolation box) in the dark chamber.

Note: if using no tray, remove the nose from the field of view as it may move between calibration and acquisition and result in the wrong offset calibration picture

4. Press the **calibration** button to calibrate the offset of the image without the subjects.
5. Place the subjects to be imaged.

Notes: When using the isolation box, you should ensure that the connector for the heating element is connected connected (connecting the heating is not necessary for the calibration).

6. Select **Preview** to acquire the X-ray image.
7. Select **Start Acquisition** to acquire the luminescence signal (cf picture below)



8. The acquisition will stop automatically in FLY mode in BLI mode a stop condition can be entered or press **stop acquisition** to stop and save the acquisition at any moment.
9. Give a name to the acquisition to be saved. (By default it will be saved in C:\Data).

Note :

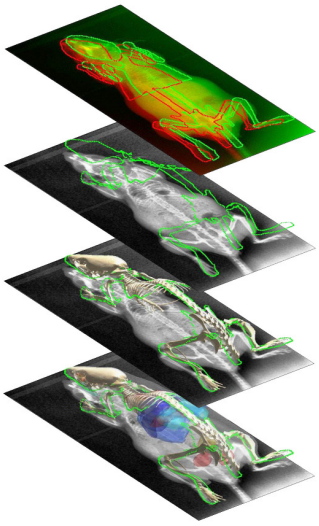
- Avoid symbols and long names when saving acquisitions
- Several files are created for each acquisition click on "Create a folder with same name as the file name" to save all the files of each acquisition one separate folder.
- Text can be added in the comment window and will be searchable in the image database of the analysis software.

1- Once the acquisition stops, save your data. The XRay acquisition and the BLI or FLI acquisition will be saved on the same time.
2- Open the bvr file on M3Vision so that the fusion appears

10.4 Stereo-CT Module

10.4.1 Overview

The Stereo-CT module allows 3D Volumetric analysis of BLI and FLI signals of up to 2 mice per acquisition and estimation of the organ localization of this signal thanks to the High resolution Digimouse CT Atlas.
The process of a stereo-CT acquisition can be divided in 4 stages:



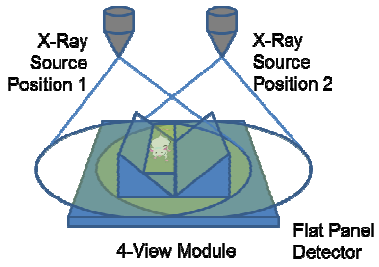
Atlas Aligment with stereoscopic X-Ray

Aligment of the Volumetric data with the mouse atlas

1. The 3D Luminescence acquisition is acquired with the 3 View module.
2. A surface reconstruction is acquired by scanning a picoprojector light over the subjects.

3. A precise estimation of the 3D bone structure of the subject is extrapolated from two stereoscopic X-Ray images of the subjects.

4. The information of the mouse atlas is aligned to the 3D bone structure which is then matched to the surface and 3D volumetric signal data.

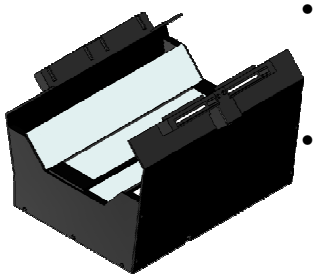


Acquisition of the stereoscopic R-Ray data

10.4.2 Hardware

The hardware components of the Stereo-CT module consist of :

- The 4View Module: a box consisting of mirrors allowing the simultaneous visualization of the ventral, dorsal and lateral views of up to two subjects. There is also a transparent bed fitted with two nose cones to maintain the subjects under gaseous anesthesia. The mirrors and bed are transparent to X-Rays.



4-View module

- A Pico projector, situated in the top right corner of the dark chamber which projects a lines of dots across the subjects, which is recorded for the camera to generate the surface reconstruction.
- An X-Ray source fitted on and a motorized drive allowing lateral movement of the source between two positions separated by approx. 20 cm for sequential acquisition of the two stereoscopic X-Ray images. The X-Ray source and drive are located on the automated Objective Turret As for the X –Ray module.
- As for the X-Ray module, A Flat Panel X-Ray detector integrated in the lower part of the
- For safety purposes, All PhotonIMAGER systems fitted with X-Ray sources have a warning light on the top left corner of the front panel indicating whether the X-Ray source is on or off and a key lock on the bottom left corner of the front panel to allow or prevent activation of the X-Ray source.

10.4.3 Stereo-CT Acquisition Panel

The Stereo-CT Acquisition Panel can be accessed by clicking on the Stereo-CT module icon

Acquisitions can be carried out either in Bioluminescence or Fluorescence mode by clicking on the corresponding icons.

The Stereo-CT Acquisition Panel is composed of four Main buttons related to the four stages of the acquisition process.

- **Preview –** To assess the positioning of the animal and acquire the black & white 4-View image overlay.
- **Signal –** To acquire the 4-View FLI or BLI signal.
- **Surface Reconstruction -** To carry out the surface reconstruction for 3 D Volumetric analysis.
- **X-Ray –** To acquire the stereoscopic R-Ray images for 3D skeleton alignment.

A green tick mark will appear on the corresponding button to indicate when a stage has been carried out and saved. All four buttons should be ticked before the Stereo-CT acquisition is complete. When all four buttons are ticked, the save window will open automatically.

There are also four Accessory buttons:

Calibrate – To carry calibrate alignment between the 4-View module, X-Ray source and flat panel.

- **Start -** To start the preview, Luminescence acquisition, Surface reconstruction or Stereoscopic X-Ray acquisition.
- **Stop -** To stop the preview, Luminescence acquisition, Surface reconstruction or Stereoscopic X-Ray acquisition.
- **Save -** To intermediately save any of the 4 stages.

10.4.4 Stereo-CT Acquisition Workflow.

1. Make sure the X ray key is in the system and turned ON.
2. Remove the 2D illumination pods. Place the 4-View module in the dark chamber and connect the optic fibers to the front left, front right and back right illumination connectors of the dark chamber. When using gaseous anesthesia connect the gas connectors of the 4 View bed.
3. Select the imaging mode (FLI or BLI)

Note : When in FLI imaging mode, select the fluorophore to be used in the fluorophore list at this stage. ***Acquire background*** should be ticked and ***use whitelight in preview mode*** should be unticked.

4. Select the Stereo-CT module
5. Press the ***calibrate*** button to calibrate the alignment of the 4 View module and the X-Ray flat panel and offset of the Animal bed image in position Without the animal
6. Place the animal on the imaging bed of the 4View module
7. Select **Preview**
8. Press the **Start** button to start the preview - make sure the subjects is well positioned with their legs as spread as possible but visible on all four sides.

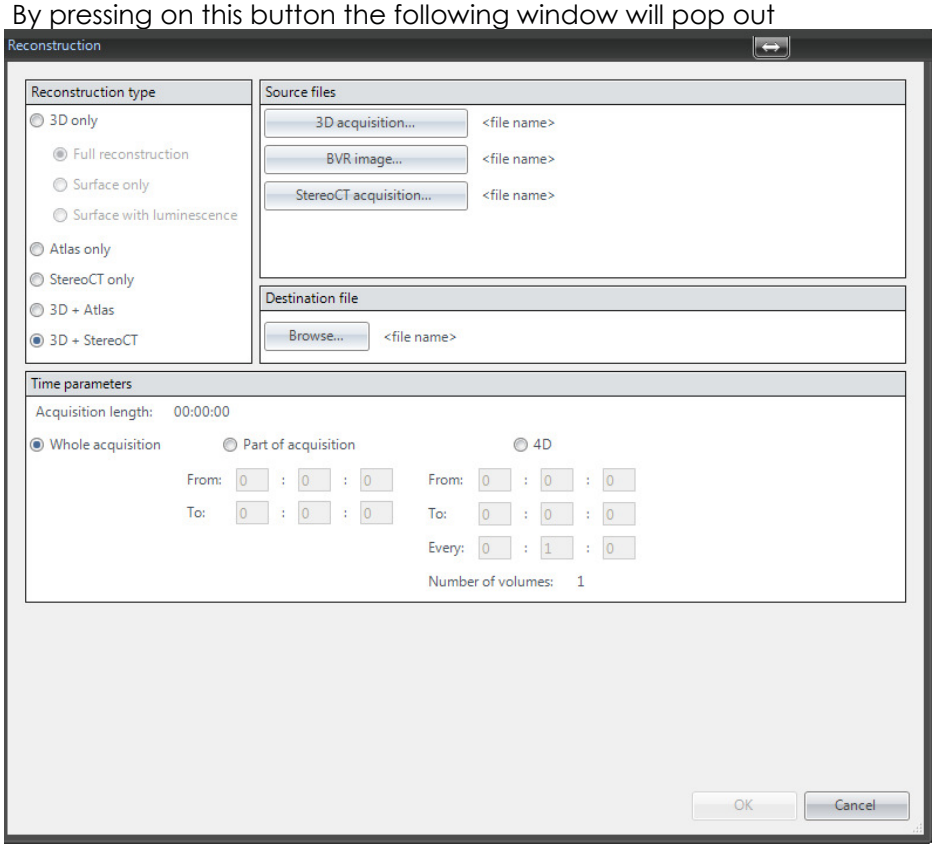
9. Press the **Stop** button to stop the preview (the last 50 ms of the preview will be automatically saved as the B&W overlay image. A green tick mark will appear on the **Preview** button, validating the preview stage.
10. Select **Signal**
11. Press the **Start** button to start the Luminescence acquisition
12. The acquisition will stop automatically in FLY mode in BLI mode a stop condition can be entered or press stop acquisition to stop and save the acquisition at any moment. A green tick mark will appear on the **Signal** button, validating the signal stage.
13. Select **Surface reconstruction**
14. Press the **Start** button to start the surface reconstruction at the end of the surface reconstruction the data will automatically be saved and a green tick mark will appear on the **Surface reconstruction** button, validating the surface reconstruction stage.
15. Select **X-Ray**
16. Press the **Start** button to start the Stereo X-ray acquisition the data will be saved automatically and a green tick mark will appear on the **X-Ray** button, validating the X-Ray stage.
17. When all four acquisitions have been carried and all four buttons are ticked out, the automated save window will open. Give a name to the acquisition to be saved. By default it will be saved in C:\Data . You can browse for an alternative folder.

Notes :

- Avoid symbols and long names when saving acquisitions.
- Several files are created for each acquisition. Click on Create a folder with same name as the file name to save all the files of each acquisition one separate folder.
- Text can be added in the comment window and will be searchable in the image database of the analysis software

18. Select start reconstruction now to start the reconstruction 

By pressing on this button the following window will pop out



One can choose either full 3D+ Stereo CT reconstruction (ticked by default) or choose to reconstruct one of the available reconstruction types.

Note: StereoCT reconstruction can take up to 20 min. when working with several animals reconstructions can be carried out after the acquisitions from the PhotoAcquisition reconstruction manager in stereo-CT mode.

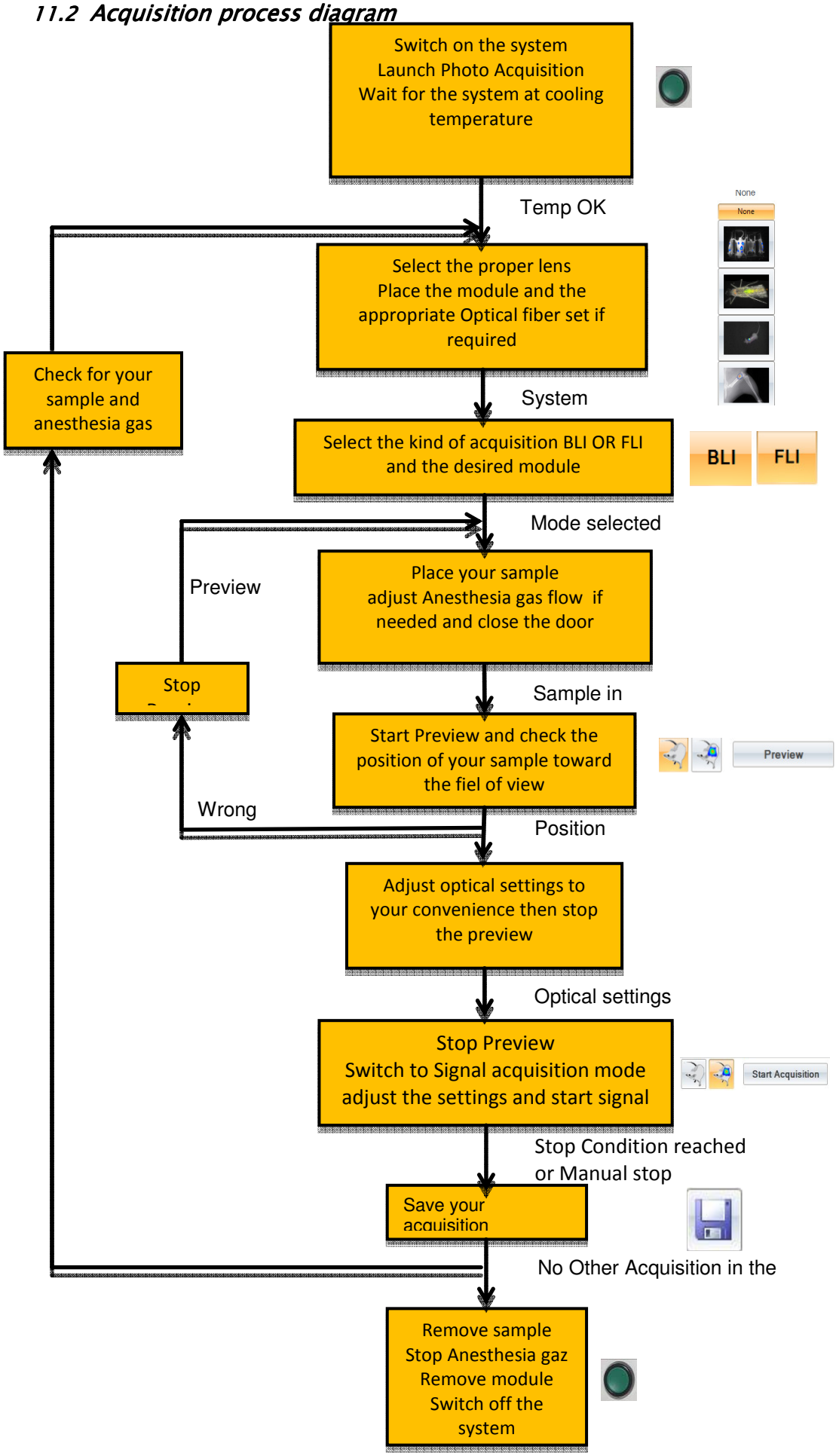
11 Quick reference tables and additional information

11.1 Lens selection

Regarding the acquisition Mode and the module you've selected, you will have to select the appropriate Lens on the Lens Wheel. Refer to the quick reference table for proper selection.

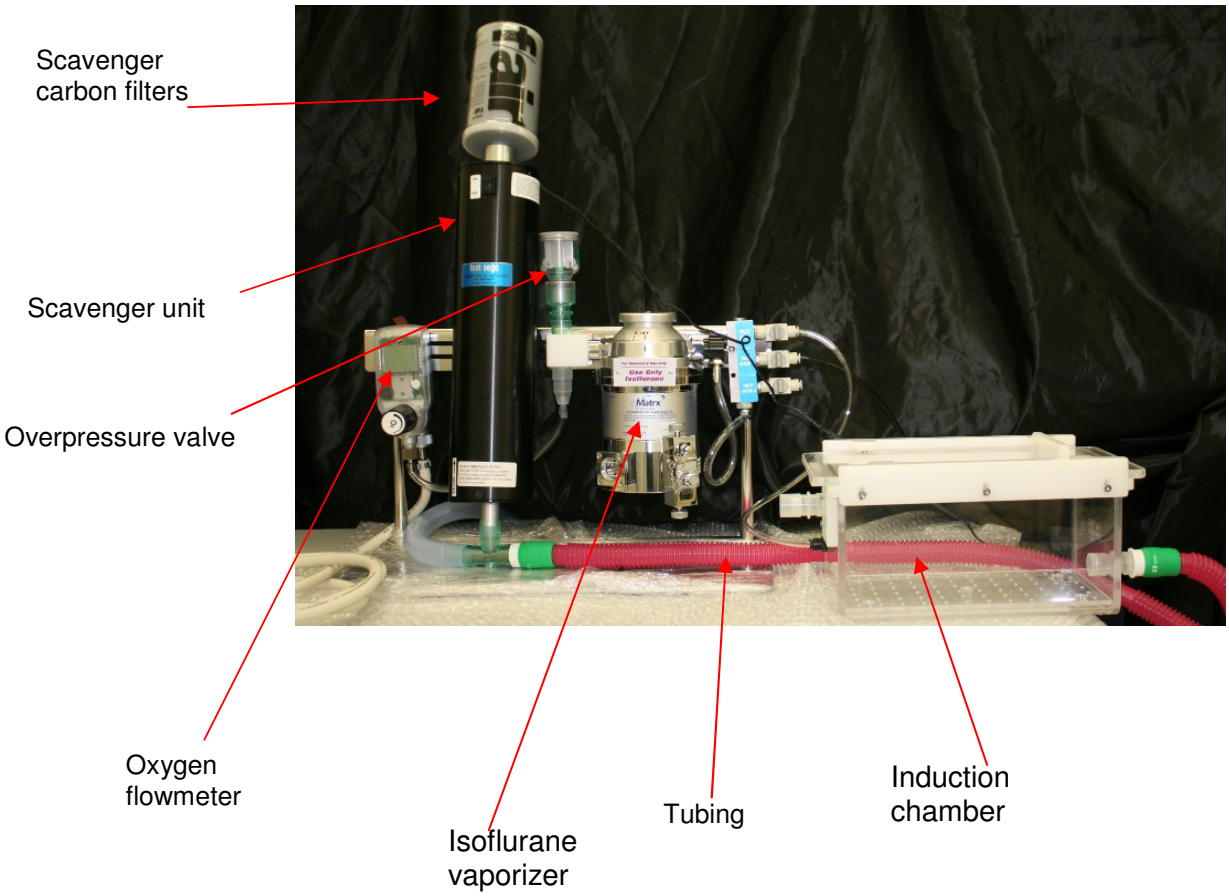
	Main field of view	InActio	X-Ray	4View 3D/4D	Large field of view / Tunnel	Macrolens
50mm f1,2	✓	✓	✓			
35mm f1,4				✓	✓	
MPE65 f2,8						✓
X-Ray Tube			✓			

11.2 Acquisition process diagram



11.3 Anesthesia system

On the clients request Biospace Lab provides also complete anaesthesia system.



12 Customer Support

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