LUXENDO InVi-SPIM

USER MANUAL

Contents

Knowledge of this manual is mandatory for the operation of the instrument. It is thus crucial to make yourself familiar with the contents of this manual. Please pay special attention to the hints concerning the safe operation of the instrument.

The specifications are subject to change; the hard copy of the manual, however, does not follow any update services.

1.   Hardware Overview

The InVi SPIM was original designed with the ideal geometry to image small, delicate samples as e.g. single cells, spheroids and organoids with high magnification and resolution. Luxendo InVi SPIM Light-Sheet technology is the gentlest way to observe living samples over everything from fast short periods to longer time periods lasting several days. Luxendo Light-Sheet Microscopy systems enable high-resolution including simultaneous dual channel imaging of delicate samples in their native, stable controlled 3D environment.

This inverted geometry allows the InVi SPIM to image long-term 3D incubated samples at high speeds, while minimizing the effects of photobleaching and phototoxicity.

The InVi SPIM contains a set of internal X, Y, and Z stages that hold living samples in a distinct sample holder with incredibly high mechanical stability, allowing for maximized acquisition speed and high precision of data acquisition.

The InVi SPIM's light-sheet is created by rapidly sweeping a Gaussian beam through the focal plane. The light-sheet forming optics in the illumination pathway allow the users to optimize the beam's length and diameter for an individual sample. Homogeneous illumination of the field of view can be achieved by adjusting the light-sheet thickness and the magnification of the detection optics. About half the field of view can be illuminated with a 2µm thick light-sheet at 31.3x. The laser light used to create the beam is supplied by the InVi SPIM's expandable, alignment-free light source and allows for a range of up to six lasers (405, 445, 488, 515, 532, 561, 594, 642, and 685).

Live sample detection optics include one 25x NA 1.1 water immersion objective lens. The two detection paths are each equipped with ten position high-speed filter wheels, allowing users to collect multiple spectral channels sequentially or dual channel acquisition simultaneously using the motorized dichroic switch carrying three positions. Filter selection includes both long pass filter to maximize photon efficiently and band pass filter that minimize bleed through during multi-channel experiments. The detection paths also include a two-position magnification changer that can adjust the overall magnification of the 25x NA 1.1 objectives from 31.3x to 62.5x with a click of the mouse. The included sCMOS Orca Flash 4.0 V3 cameras from Hamamatsu are well-suited for experiments that require high detection efficiency, quantification, and speed.

The Inverted View Single Plane Illumination Microscope (**InVi SPIM**), including its original accessories and compatible accessories from other manufacturers, may only be used for microscopy methods, the intended use.

Light-sheet fluorescence microscopes (LSFM) are designed for fast and gentle acquisition of multi-dimensional, high-resolution images of living or non-living specimens. Therefore, the temperature-controlled sample chamber is filled with medium adapted to the objective lenses used and the sample carrier is filled with sample medium. The sample is inserted into the small volume of the sample carrier inside the sample chamber. The sample carrier is connected to a holder that can be moved in three dimensions. The sample is illuminated by a light-sheet generated by laser light. The light-sheet is positioned in the focal plane of a detection objective lens, whose optical axis is arranged perpendicularly to the plane of the light sheet. Hence, only light from the focal plane is registered by the system and used for image formation.

⚠️ The manufacturer assumes no liability for any malfunction or damage caused by any non-intended use of the **InVi SPIM** system as a whole or any of its components or resulting from any repair or other maintenance services carried or tried out by any non-authorized personnel. Any such activity will void the warranty for the whole system including parts not directly affected. This includes any modification of the system computer (PC), for example equipment with new hardware or software components by the user. Every manipulation voids the warranty of the laser safety.

Furthermore, to guarantee your own safety you are strongly advised to read the manuals, in particular the safety instructions, of any modules and additional components of the **InVi** SPIM system as not all features might be listed here explicitly.

☢️ The lasers built into the **InVi SPIM** microscope are classified as laser safety class 3B. The safety interlocks ensure that the user is not exposed to dangerous laser radiation. The **InVi SPIM** itself is a laser safety class 1 product.

The operator of the system must comply with all applicable accident prevention regulations enforced by law.

The Luxendo **InVi SPIM** system complies with the following standards:

EN 61000-6-3:2007 + A1:2011

IEC 61000-6-3:2006 + A1:2010

EN61000-6-2:2005

IEC 61000-6-2:2005

DIN EN 60825-1:2008

DIN EN 50581: 2013

InVi SPIM (Inverted Light-Sheet Microscope)

SCMOS Camera
- 2048 x 2048 pixels
- 100 fps
- 83% QE
- Low noise

Two High-speed Filterwheel
- Up to 10 positions for each detection arm

Incubation chamber

Environmental Control
- Temperature control from 10°C to 40°C
- Atmosphere: CO2, N2, O2 and humidity

Magnification Changer

Lasers
- Up to six wavelengths from 405 nm to 785 nm
- Optional NIR lasers from 785 nm to 1310 nm

PC, Monitor and Joystick
- 256 GB RAM
- Dual socket Xeon processor
- High-end graphics card
- High performance RAID (32 TB)
- LuxControl

LuxDATA
- LuxStorage
- LuxProcessing

Photomanipulation Module
- Diffraction limited spot generation
- Galvometer-based precision control
- Flexible patterns and hand drawing
- Pulse (780 nm to 1060 nm) and/or continuous lasers (405 nm to 685 nm)

Sample holder classic

The sample holder classic consists of two parts, the sample carrier (Figure 1 / 2) and a mounting adapter (Figure 13 / 1) connecting the sample carrier with the translation stages.
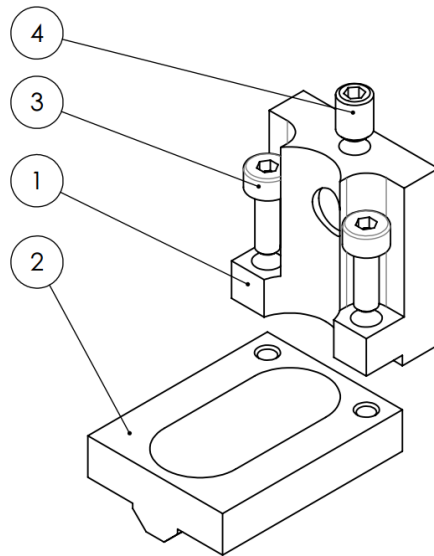

Figure 1: Exploded assembly drawing of sample holder

In order to clean/change the sample carrier, open the lid of the sample chamber. To remove the sample holder, loosen the setscrew (Figure 3 / 4) in the mounting adapter and pull the holder off the metal rod. The mounting adapter can be detached from the sample carrier by removing the two screws (Figure 3 / 3).

To reattach the sample holder onto the microscope, first connect the mounting adapter to the sample carrier, and then put the assembled sample holder on the metal rod. The setscrew orients the sample holder, ensuring a proper angle between the objectives and the sample holder.

TruLive3D Dishes

 The TruLive3D Dishes are ready-to-use sample holders for the Luxendo InVi SPIM, InVi SPIM Lattice Pro and the TruLive3D Imager light-sheet microscopes. The TruLive3D Dishes have been sterilized and are shipped in sealed sleeves. To retain sterile conditions, open the packaging sleeve only under a cell culture hood.

Similar to the classic sample holder, the FEP foil lining the holder serves the dual function of a "curved coverglass" and a physical barrier between the immersion medium and sample medium.
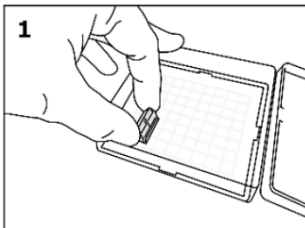
The new ready-to-use TruLive3D Dishes are fully compatible with the InVi SPIM. The possibility to enable the parallel imaging of samples grown under different media conditions.

Sample preparation:
1. Remove the dish from packaging and place it in the specialized culture stand.
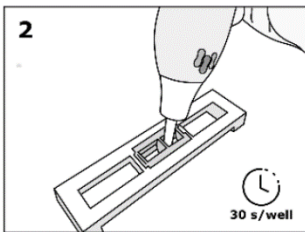
2. Carefully functionalize the TruLive3D Dish with cold plasma (PiezoBrush) for 30 seconds per well. For optimal ion transfer to the FEP foil, a metal working area of a culture hood below the dish is essential. It functions as a counter electrode.
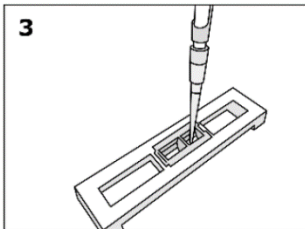
3. Subsequently, apply your sample to the dish.

Glass slide holders

For live cell samples, customized glass slide sample holders can be used for the convenience of sample preparation and cell adhesion. Cells should be seeded on the 3mm x20 mm glass slide with normal cell preparation protocols. During imaging, glass slide surface with cells seeded should be facing downwards, such that both illumination and detection light path do not need to pass through glass slide, which has a large Refractive Index difference with water.

The cross section view of the objective lenses and glass slide sample holders is shown below:
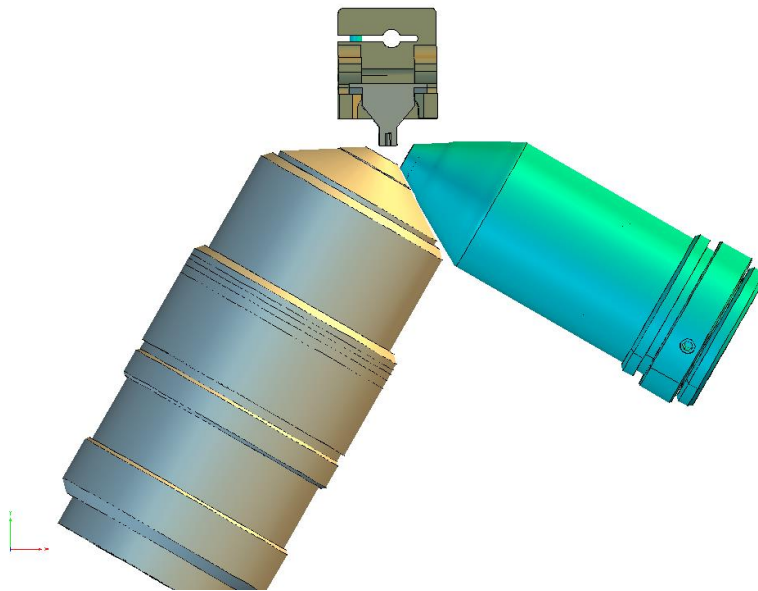
1.1. Additional Options

1.1.1. InVi SPIM Photomanipulation Module, Future Upgrade Available

The InVi SPIM photomanipulation beam is coupled to the 25x NA 1.1 detection objective lens, to create a diffraction-limited illumination spot at the focal plane of the detection objective. A 2D scanner allows the user to freely position the illumination spot in all spatial dimensions of the sample while it is being imaged; this feature is fully integrated in the acquisition software. Complex illumination regions (point, square, straight and freeform line) can be configured as part of the experimental workflow or "on the fly" while a 3D imaging experiment is performed. A pulsed laser can be use for ablation experiments while visible lasers can be used for other photomanipulation experiments like FRAP, photo-conversion and uncaging.

The InVi SPIM has the only commercially available light-sheet system that uses a detection objective to deliver the photomanipulation beam, allowing the user to draw photomanipulation regions directly on images collected by the detection objective. In contrast, other light-sheet systems utilize extra, long working distance, low NA objectives opposite the light-sheet objectives to deliver the photomanipulation light. This configuration makes it impossible photomanipulate and image the same regions in scattering samples.

1.1.2. Lux DATA Image Processing Unit, Future Upgrade Available

Cutting-edge biological imaging like light-sheet, super-resolution, or multiphoton microscopy, produces a vast amount of data; however, storage, transfer, and processing of the generated data remains a challenge. With the InVi SPIM, data can be transferred to the Lux DATA processing system by two 10 Gbit/s LAN connections. There, the data is saved to the 150 TB RAID storage unit and processed on a high-performance computation unit with two 14-core Intel Xeon CPUs, three GeForce GTX1080 GPUs, and 256 GB RAM.

Lux DATA is a comprehensive data processing and storage solution, designed to effectively handle the vast amount of data generated by light-sheet fluorescence imaging. It boosts productivity by relieving the need to constantly duplicate or transfer data sets in processing and analysis workflows. Lux DATA enables fast data transfer, even during acquisition, and large-capacity storage; as well as remote access and high-performance processing of multi-dimensional image data and leveraging multi-core and multi-GPU-based

computation. The Lux DATA approach seamlessly complements image acquisition with Bruker Light-Sheet microscopes – by enabling all subsequent data processing steps and saving time and money.

2. System Startup

2.1. Power on the system

- Turn **ON** the system by flipping the switch on the power supply box. Make sure the emergency button is in its release position (rotate to release). After approx. 1 minute the stages will initialize.
- Turn **ON** the camera.
- Start the **LuxControl** software and make sure the **LuxDatahub** is running and has connected with the camera. If the LuxDatahub is missing the camera that is turned **ON**, restart the camera and the LuxDatahub.
- The system requires about 1 hour to reach temperature equilibrium with absolute stable optical properties.
- Stack and Channel properties are stored in the embedded controller of the microscope. Power outage or shut-down will therefore result in loss of Stack, Channel and Event settings.
- The optical alignment of the microscope correlates with system temperature and may change due to temperature shifts. To ensure optical stability, the microscope needs a constant room temperature and requires about 1 hour to reach its working temperature and have thermally stabilized.
- Coarse alignment (if required see below) can be conducted 15 minutes after turning on the microscope, while the fine alignment should be conducted shortly before acquisition and in the required imaging solution and can be adapted later in the sample itself. Changes in refractive index, e.g. due to evaporation influence the alignment properties and will induce optical shifts. It is recommended to use the cuvette lid to avoid evaporation, especially if toxic reagents are used.

3.   Lux Control Overview

3.1   Abbreviations

- LSA: light-sheet alignment
- ROI / FOV: region of interest / field of view
- CS: cleared sample
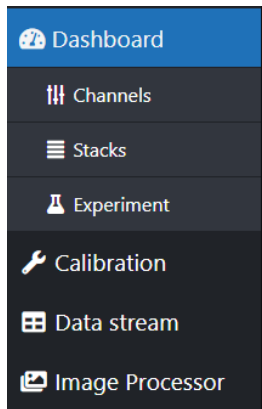- N.V.:   not visible
- RI: refractive index

3.2 Software

Navigation Tabs

- **"Dashboard":** All control panels needed for an experimental setup
- **"Channels":** Selected control panels that contribute to the channel setup
- **"Stacks":** Selected control panels that contribute to the stack setup
- **"Experiment":** Selected control panels that contribute to the experiment setup
- **"Calibration":** All control panels needed for alignment and calibration of the microscope
- **"Data streams":** Opening the window showing the different camera streams
- **Image Processing**: Selected control panels that allow setting up and starting the Image processing pipeline for image registration, fusion, resizing and storage

Control buttons
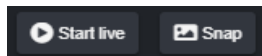
- **"Start live":** Starting live mode of all cameras
- **"Snap":** Acquires a single image of all cameras
- **"Run":** Starts the image acquisition, defined by the "Channels", "Stack" and "Experiment" setup
- **"Stop":** Halts and finishes the running acquisition

Illumination

Laser

- Laser "ON" or "OFF" setting.
- Laser intensity setting between 0 and 100% with "0%" being the lowest and "100%" the highest possible laser power.
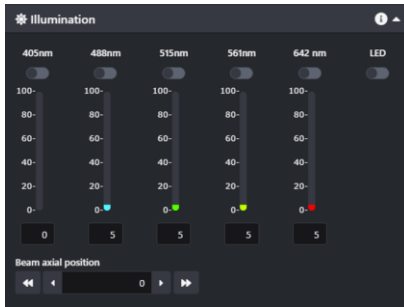
Beam axial position
- This setting changes position of the center of the beam in lateral direction, which is reflected as moving the beam left and right on camera FOV.

Detection
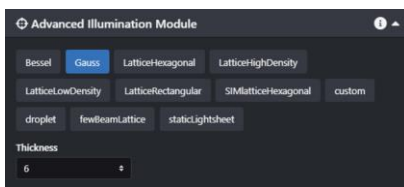
Selection to change light modalities for the respective channel. Set the beam modality and patterns according to sample size, signal strength and FOV.

- **"Bessel":** A non-diffractive beam pattern that has uniformed beam profile over larger field of view;
- **"Gausssian":** Typical beam profile used on light-sheet microscope. It is a diffractive beam so the depth of focus is relatively small as compred to Bessel, smaller beam width results in more concentrated energy but smaller depth of focus;
- **"Lattice":** Carefully crafted coherent superpositions of Bessel beams to provide a compromise between light-sheet thickness and sidelobe energy density;
- **"Lattice hexgonal":** The hexagonal lattice has a narrow propagation invariant beam waist surrounded by a series of sidelobes that grow in strength with increasing propagation length, which provides superior axial resolution;
- **"Lattice rectangonal":** Consists of a thicker central beam lobe that grows in size with

increasing propagation length but has small side lobes, achieves a more favorable balance between optical sectioning and axial resolution;

- **"Static lightsheet":** A static planar sheet, which provides a stable and static light dose on the sample, is usful for applications that require quantitive analysis of photons;

Detection
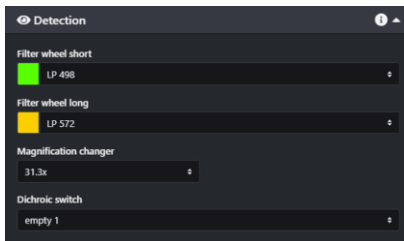
Selection to change filters for the respective channel:

- **Filter wheel:** Setting the filter for the actual channel. "Empty" positions will allow all wavelengths to be transmitted to the camera.
- **Magnification Changer:** Setting individual tube lens for differnet levels of magnification.
- **Dichroic Switch:** Setting the dichroic switch to seperate channels between Cam_Short and Cam_long.

Camera
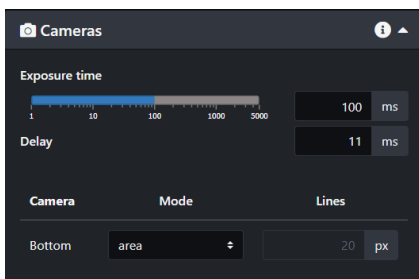
Setting of camera acquisition properties.

- **Exposure time:** Possible exposure time range from 1 – 5000 ms.
- **Delay:** Camera delay is set to 11 ms by default. This delay is the time that is required to readout data from the full camera chip (2048 px x 2048 px → 11 ms). A cropped FOV in the Y-axis allows to reduce the delay (e.g. 2048 px x 1024 px → 8 ms delay possible).

Stages
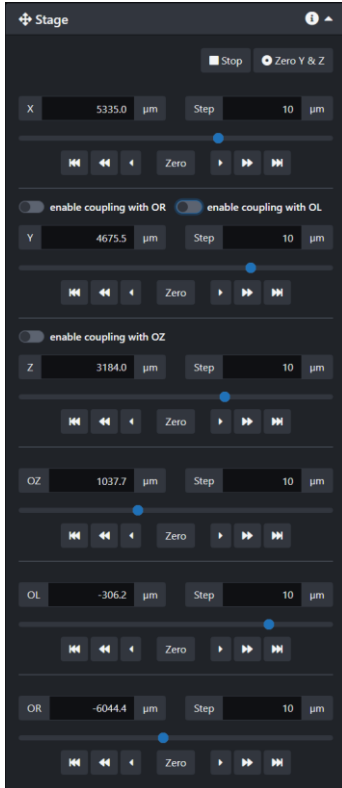
Buttons:

- **"Stop":** halts all stage movements
- **"Zero Y & Z":** Moves Y and Z stage to zero position
- **Store icon:** Sets current position to zero
- **"Zero":** Move to zero position
- **"|<< / >>|":** Move to minimum / maximum
- **" << / >> ":** 10x increment
- **" < / > ":** Single increment
- **"Enable coupling with":** Turns ON synchronous movement of coupled stages (coupling is done via the calibration tab). Coupled stages are not displayed.

Input:

- **Stage Position (µm):** Input for specific stage position
- **Step (µm):** Sets increment (default 10 µm)

Stages:

- **X:** horizontal (back to front)
- **Y:** horizontal (left to right, along the illumination axis)
- **Z:** vertical (along detection axis)
- **CR:** Correction Ring

Channel

All "Channel" relevant settings are combined in a preset labeled *channel_#*. Channel presets can be selected from the drop-down list

Settings include:

- **FOV (bottom)**: cropped detection
- **Laser**: active laser wavelength and intensity
- **Scanner**: amplitude and offset (view corresponding setting)
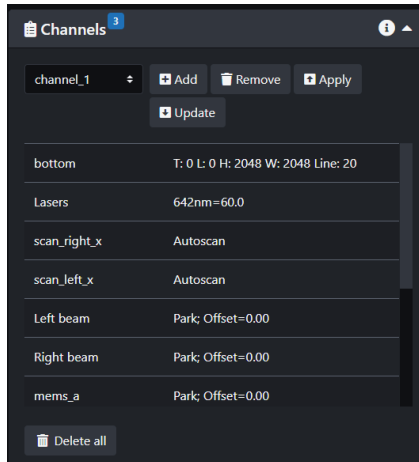
- **Camera timing (n.v.)**: exposure time, delay and chip mode (area vs. line mode)
- **Filter Wheels (n.v.)**: selected filters
- **External Trigger(n.v.)**: Customizable trigger
- **Beam** (n.v.): beam expander setting
- **LSA** (n.v.): light-sheet alignment setting

Buttons:
- **"Add"**: stores current settings to preset
- **"Remove"**: deletes current preset
- **"Apply"**: loads preset settings to the UI panels and sets the components on the microscope accordingly
- **"Update"**: overwrites current preset with modified/current settings from the UI panels
- **"Delete all"**: deletes all presets
- **"Save channels"**: Stores current preset to be available after a power cycle
- **"Load channels"**: Loads previously stored channel preset
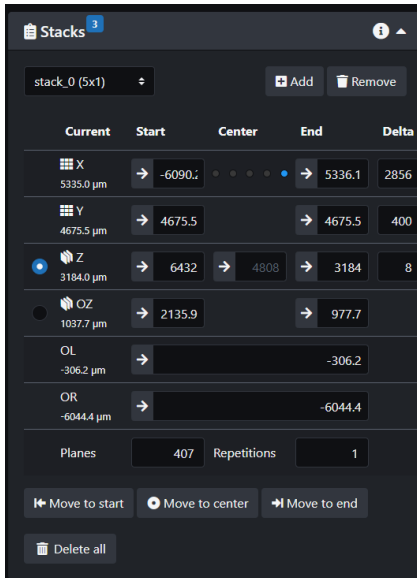
Stack

All **Stack** relevant stage settings are combined in a *stack_#*. Stacks can be modified within the panel and selected from the drop-down list.

Buttons:

- **Arrow button:** Inputs current stage position
- **"Add"**: stores current settings to a new stack preset
- **"Remove"**: removes current stack preset
- **"Move to start"**: moves stage to first slice of the stack
- **"Move to Center"**: moves stage to mid slice of the stack
- **"Move to End"**: moves stage to last slice of the stack
- **"Delete all"**: removes all configured stack presets
- **"Save stacks"**: stores current stack presets to be available after a power cycle
- **"Load stacks"**: loads previously stored stack presets

Stack settings:

Current position can either be used by pressing the arrow button or a number can be typed into the input box.

- **Start:** First slice of stack
- **End:** Last slice of stack
- **Center:** Mid slice of stack
- **Planes:** Total number of planes distributed equally between first and last slice. For stack defined by center slice an uneven number of planes is required

- **Repetition:** Number of repetitions of the stack. To increase speed, the stack is acquired bidirectional with every uneven repetition acquiring from first to last and every even repetition acquiring from last to first slice.
- **Delta:** Required value for stack and tiling properties. In a stack delta defines the distance between each Z-slice. In a tiled acquisition (X and Y) the delta defines the maximum allowed distance between subsequent tiles in X and Y (should be chosen to be slightly smaller than the actual FOV). The delta is calculated from the start and end position and distributes the tiles equidistant in X and Y.

Event
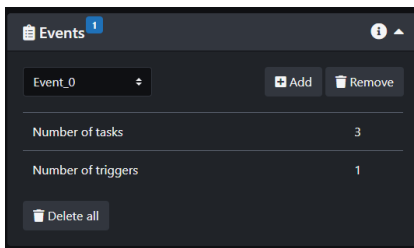
An *Event_#* arranges the acquisition and defines which Task (combination of *Stacks* and *Channels*) is acquired in which timely manner (*Trigger*).

Events are acquired in sequence as defined by their delay.

Buttons:
- **"Add"**: opens a new Event
- **"Remove"**: deletes current Event
- **"Delete all"**: deletes all configured Events
- **"Save events"**: stores current Events to be available after a power cycle
- **"Load events"**: loads previously stored Events

Tasks
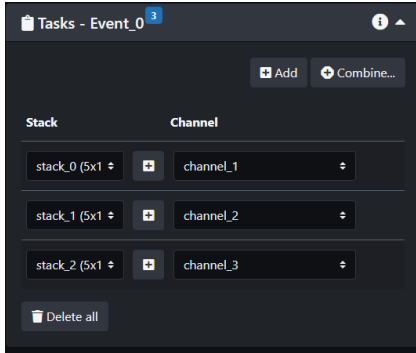
A *Task_#* defines the acquisition sequence of stacks and their optical configuration.

Buttons:

- **"Add"**: adds a new Task
- **"Combine"**: allows to choose suitable combinations of different Stacks, Channels and their optical Configuration with a defined hierarchy.
- **"Delete all"**: deletes all configured Tasks
- **Minus Button:** deletes single Task combination

Trigger
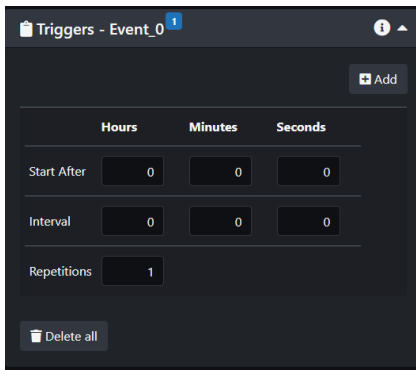
A *Trigger_#* defines the timing of the Task.

Buttons:

- **"Add"**: adds a new Trigger
- **"Delete all"**: deletes all configured Triggers
- **Minus Button:** deletes a single Trigger
- **Start After:** sets the delay of the Event in Hours, Minutes and Seconds
- **Interval:** sets the interval of the Event in Hours, Minutes and Seconds. If the interval is set shorter than the duration of the Event, the next Event acquisition will start directly after the previous
- **Repetition**: sets the number of repetitions of the Event

Scanner calibration (Calibration Tab)
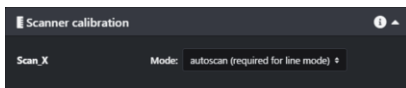
Calibration of the scanner properties, how the beam is scanned across the field of view

- Mode:
  - **Park:** Parks the Gaussian beam at the zero position
  - **Scan:** Scans the Gaussian beam across the FOV with the set amplitude / scan range.
  - **Automatic scan:** Sets the zero position and amplitude detected during "Line calibration". Set for "Line" acquisition mode (obligatory).
  - **sim:** Define number of phases (3, 5 or 7) and period dimension (*please refer to Beam Profile doucment for the measured period of differnt beam patterns*) for Structctured Illumination.
- **Offset:** Manual displacement of the beam's zero position
- **Store icon:** Sets current beam position to new zero position
- **Amplitude:** Sets the scan range of the beam

Line calibration (Calibration Tab)
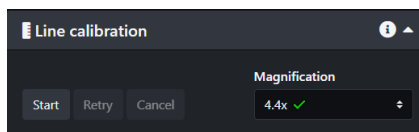
Line calibration synchronizes the "slit detection" readout of a defined number of horizontal pixel lines on the detector with the scanning movement of the Gaussian beam across the FOV. For a proper detection of the Gaussian beam, the "parked" beam needs to be clearly visible.

Buttons:
- **"Start" / "Next":** Starts the automated calibration or moves the beam to the next of three detection positions.

- **"Retry":** For correct alignment the blue line needs to be positioned precisely on top of the Gaussian beam to indicate a correct detection. In case the blue line does not coincide with the position of the beam, press "Retry" to acquire a new frame and initialize the beam detection on that new frame.
- **"Cancel":** Aborts the process of line calibration.

Tips:

- If the blue line does not detect the Gaussian beam accurately, increase laser power or exposure time and try different filters.
- Changing the scanner offset requires a new line calibration.
- Low or inhomogeneous signal in line mode but a correct detection in area mode hints toward a faulty line calibration.
- The calibration has to be done for each magnification individually

A successful calibration will be indicated in the panel by a green checkmark next to the magnification in the dropdown menu that has been calibrated.

Lightsheet alignment
(Calibration Tab)
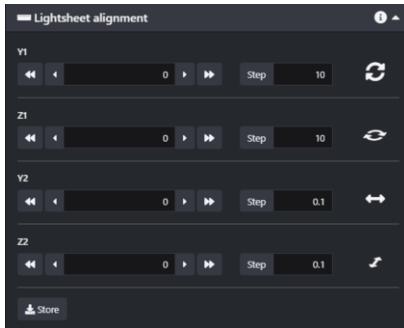
Light-sheet alignment in Y and Z

Buttons:
- **"Store:"** Stores current settings as default for next microscope start up
- **"Y1":** Rotating the light-sheet direction on the focal plane, can be adjusted by observing the camera live view when beam is parked;
- **"Z1":** Rotating the light-sheet direction perpendicular to the focal-plane, tricky to tell directly from camera live view, can be adjusted by moving the beam left and right on FOV and observe if there is any focus change;
- **"Y2":** Shifting the beam waist on the focal plane, can be adjust by observing the beam center position using camera live view when beam is parked;
- **"Z2":** Shifting the beam waist perpendicular to focal plane, can be adjusted by observing the beam focus using camera live view;

Objectives
(Calibration Tab)

Upon objective exchange, the objective properties have to be selected for accurate metadata configuration.

- **"Right/left illumination objective":** Dropdown menu for illumination objective configuration selection
- **"Detection objective":** Dropdown menu for detection objective configuration selection

- **"Store:"** Stores current selection as default for next acquisition and microscope start up

Stage coupling
(Calibration Tab)

Stage coupling

Ref stage	Linked stage	
select ref stage ⇕	select linked s ⇕	➕ Add

Coupling Y - OR

Y = 4486.1	OR = -5906
Y = 3415	OR = -5261
Y = 1224	OR = -3822

➕ Add stage positions

Coupling Y - OL

Y = 3050	OL = -1351
Y = -114	OL = -3337
Y = -4455	OL = -6125

➕ Add stage positions

Coupling Z - OZ

Z = 5929.2	OZ = 1942.3
Z = 6394.8	OZ = 2132.3
Z = 4950.7	OZ = 1612.3

➕ Add stage positions

Required stage coupling pairs (master – slave):

- **Z – Y:** horizontal moving of sample holders

Stage coupling allows the synchronous movement of two stages in a master-slave fashion with a linear correlation between the two stages. Two or more stage positions are required. With three or more reference stage positions a linear interpolation is calculated from all position pairs.

Buttons:

- **Ref stage**: Select master stage
- **Linked stage**: Select slave stage
- "**Add**"**:** Adds a new coupling pair with current stage positions of the selected two stages.
- **"Add stage position":** Adds new (current) stages position pair to existing coupling list.
- **Trash icon** (next to the coupling pair – n.v.)**:** Removes value pair from coupling list

The master stage of a coupled stage pair shows the "**enable coupling with…**" switch in the "**Stage**" panel as soon as coupling (two or more coupling pairs exist for the according master-slave pair) is performed. Enabling coupling, removes the coupled (slave) stage from the Stage panel. Moving the master stage now also moves the coupled stage synchronously. Coupled stages can be made visible again via
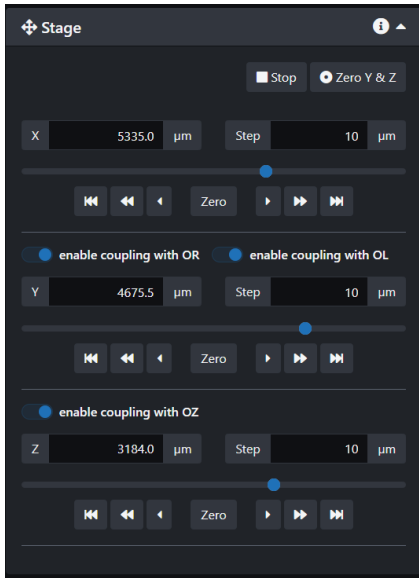
disabling the stage coupling or in the "**Calibration**" tab.

Camera Properties (Data Stream)
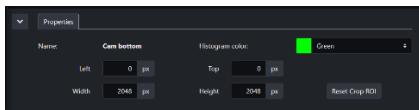
Possibility to input ROI cropping parameters, by defining the **Top**, **Left** pixels as well as **Height** and **Width** of the ROI.

"**Reset Crop ROI**" resets cropping to full 2048 px by 2048 px.

Data Stream
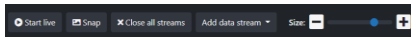
In the Data Stream window, the camera is shown as a sub-window. The ROI can be adjusted by drag-&-drop of the blue squares in the corners of the live image. For the selected stream the LUT can be adjusted manually by drag-&-drop of minimal and maximal grey value slider in the histogram panel.

Buttons:
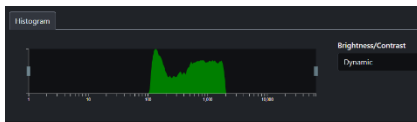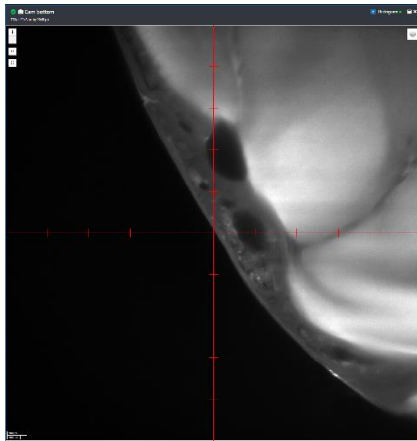
- **"Close all streams"**: Closes all stream windows
- **"Add data stream":** Opens new stream selected from dropdown menu
- "**Size" slider**: Changes window size of all streams
- **"Crosshair" / "Ticks" (n.v.):** Overlays Crosshair / Ticks on stream
- **"+ / -"** : Zooms in and out of stream
- **Brightness/Contrast**: Switches between automatic and manual LUT settings

4. Microscope alignment

4.1 Alignment

- The alignment is required to compensate for temperature shifts in the room and for small optical and chromatic shifts in the optical components of the microscope to achieve maximal acquisition quality.
- Coarse alignment should be conducted upon objective exchange or long down time of the system, prior to mounting the sample. Water with a very low concentration of fluorescent dye (text marker / fluorescein) or cell culture medium provides good fluorescent properties to find and coarse-align the Gaussian beam.
- Fine alignment is required prior to each experiment and has to be conducted in the imaging medium (e.g. refractive index matching solution - RIMS) as small changes in the refractive index can cause large changes in image quality.
- Ultra alignment (color channel shift / focusing) can be conducted in the sample but with delicate and photosensitive samples the alignment procedure should be conducted outside the ROI and with carefully selected illumination parameters or by only using the "Snap" functionality.

4.2 Coarse alignment

AIM: Visible Gaussian beam

- Use water with small amounts of fluorescent dye added or culture medium. Plain water also works but requires higher laser power for the alignment.
- Move both y and z axis to "Zero" position.
- Close sample chamber cover and trigger the laser safety button (on the side of the sample chamber).
- Switch to "Calibration" Tab
- In the "Scanner Calibration" panel: set Scanner to "Park"
- Turn ON 488 laser (or any other low wavelength laser available on the system) at 30-100% output power depending on media fluorescent content.

- Select corresponding filter and dichroic mirror (Filter short: LP498, Filter long: LP 498, Dichroic: empty).
- Set exposure time to 50 - 200 ms.
- Set beam to Gaussian and diameter to 1.
  - ➔ **The Gaussian beam should be now visible**
- If the beam is not directly visible check the following steps:
  - o Recheck settings from above:
    - Are both objective lenses fully immersed?
    - Sample chamber cover closed?
    - Interlock triggered? Press Interlock release button (listen to faint clicking noise)
    - Scanner set to "park"?
    - Laser switched on?
    - Appropriate filter chosen?
    - In "Live Mode"?
    - Is the LUT setting wrong? Set the LUT to "Dynamic" and full zoom out in the FOV using "minus" button.
  - o Moving Alignment Z by in 0.1 steps in both + and – directions to find the beam if needed.

Tips:

- If you use water: adding small amounts of fluorescent dye or using culture medium instead helps increasing the fluorescent signal. Plain water also works but requires higher laser power for alignment.
- Cell Culture Medium, PBS or organic clearing solutions have usually a higher autofluorescence / fluorescent particle and therefore require less laser power, shorter exposure times.
- Setting the LUT range of the camera histogram manually (instead of using "dynamic") can avoid the image fluctuations seen in low fluorescent alignment solutions.

4.3   Fine alignment

AIM: Focus beams and move focused beam waist to the desired lateral position of the FOV

- Switch to the "**Calibration**" Tab.
- In the Scanner calibration panel, set scanner to "park".

- Position the beam waist into the focal plane
  - Set beam pattern to "Gaussian" and diameter to "1".
  - Focus the beam with Alignment Z2+/- (in steps of 0.01).
  - Move beam waist to center of the FOV with Alignment Y2+/- (in steps of 0.01).
  - Adjust horizontal beam tilt with Alignment Y1 +/- (in steps of 0.01)
  - Adjust orthogonal beam tilt with alignment Z1 +/- (in steps of 10). The orthogonal beam tilt might not be directly visible from the beam appearance. To test for orthogonal tilt, focus the beam in the center position, and move the beam waist left and right using Alignment Y axis, if the beam is orthogonal tilted, it will get out of focus towards edge of camera FOV.
  - Press the "Store" button to save the LSA Z position for the next startup of the microscope

Tip:

To have an extra criterion of being in focus with the beam during LSA in Z (besides the criterion of having the smallest beam waist), look at the histogram: the brightest value moves up and down in intensity when moving the beam in Z. The beam is in focus when the histogram shows the brightest broad peak at the highest achievable value.

4.4   Ultra alignment

For ultra-alignment, please refer to the "Service & Application Manual AIM". Normally it has been completed during installation and regular maintenance.

4.5   General topics about alignment

- In general, the alignment of the microscope will be marginal, with only minor corrections to the startup settings, if the external conditions have not changed.
- Key reasons, that can result in major misalignment and the need of a coarse alignment of the system are:
  - Exchange of objectives
  - Substantial temperature differences
  - Difference of refractive index
  - Non corrected chromatic aberrations (405 nm laser)

General pitfalls:

If the sample is mounted, the stages moved to the imaging positions, and the first settings have been adjusted to the specific sample, but still no sample can be detected in the "Live" mode or the image quality is extremely bad, check / try the following:

- Not enough water/medium in the imaging chamber to fully cover the objective lenses and touch the bottom of the sample holders.
  ➔ reduce the Z-stage position

- Z-stage is raised too high, resulting in the sample being above the illumination optical axis
  ➔ reduce the Z-stage position

- Wrong illumination/detection settings
  ➔ Adjust laser, laser power, filters, exposure time

- No illumination upon pressing "Start Live" / closed interlock
  ➔ Press interlock release button

5. Sample preparation

A good sample preparation and mounting is key to a successful imaging session. Proper sample orientation allows to minimize the distance light has to travel inside the sample. Additionally, for multi-colour, multi position, multi time point imaging, a proper mounting ensures stable imaging properties.

The following tips should be considered for sample mounting:

- Is my sample properly attached to the imaging plate?

FEP surfaces may not provide sufficient adhesion for cells. Hydrophilization treatment can increase adhesive properties of the sample chamber. Plasma treatment the FEP sample holders before seeding cells will help cell adhesion. Glass slide can also be used for cell samples.

- What is the optimal orientation for my sample?

Proper orientation of sample can improve in-depth imaging, Z-stack alignment, and minimize aberrations. In general, reducing the distance the light has to travel between the region of interest and the detection objective as well as an orthogonal angle between detection optical path and sample surface improves image quality. Therefore, it is better to make sure the sample is close to the bottom of the sample chamber with the regions of interests facing towards detection objective.

- What makes navigation within the sample chamber easier?

Use LED light to quickly browse through the sample chamber and look for samples. Please make sure LP (not BP) filters are selected, camera mode should be selected as Area mode with an exposure time of 5-20 ms to avoid over-exposure.

- The heated incubation plate can be pre-warmed:

As soon as the temperature controller is turned ON and the heated incubation plate is connected to the microscope it will be heated to 37°C even if it is not mounted to the microscope stage. This will enable a faster temperature stabilization and optimal culture conditions.

- Optimal imaging plate mounting for long time lapse acquisition:

Sample shifts in time lapse acquisition can be a result of inadequate imaging plate mounting or instable mounting solutions (agarose / hydrogels). To ensure the optimal mounting of the imaging plate to the heated incubation plate, make sure the springs fastening the imaging plate are attached properly and the imaging plate is in full contact with the glass by gently pressing the imaging plate down, before mounting the set onto the stage.

- Autofluorescence for quicker sample navigation:

Agarose, Hydrogels and tissues always display a low to medium amount of autofluorescence. By using the 488 nm laser at low intensity and detection without any filters, this autofluorescence can be detected and facilitates sample navigation, with a minimized photo induced effect on the sample, due to the extremely low laser power required.

6.   Image acquisition

Tips to improve image acquisition quality and help image processing/analysis are:

- Adjust beam diameter to sample size:

A thinner waist will increase signal intensity and reduce signal-to-noise ratio on a narrow ROI, while for homogenous illumination throughout the FOV, a thicker beam diameter should be selected

- Focus experimental setup and reduce data size:

As the data size increases significantly with every additional time point, additional tile, additional Z-stack slice number, and additional channel, a key focus should be to avoid an immoderate amount of data, as this will facilitate faster data processing and analysis. Especially during the establishment phase, a reduced time resolution and minimal Z-stacks will improve the overall experiment development process.

- Homogenous refractive index:

Changes in the refractive index will introduce optical aberrations in the detection path. Materials like low melting Agarose, Matrigel, salt concentrations in imaging media or degraded clearing solutions can have small but noticeable differences in refractive indexes. Equilibration between different chemical components such as Agarose and the surrounding imaging solution or old and fresh clearing solution will improve the image quality over time (1-2 hours), but all components should be applied, to provide a homogenous refractive index. If RI mismatches, such as introduced by Matrigel, cannot be compensated the interface between Matrigel and the surrounding culture medium should be orthogonal to the detection axis to reduce spherical, aberrations.

7. Data Review

7.1. Luxendo InVi SPIM File Naming Conventions

7.1.1. Folder Hierarchy

After an acquisition is initiated, a new experiment folder is created and named with the date and time the experiment was initiated; for example, an acquisition on November 15, 2019 at 01:50:54 am becomes 2019-11-15_015054.
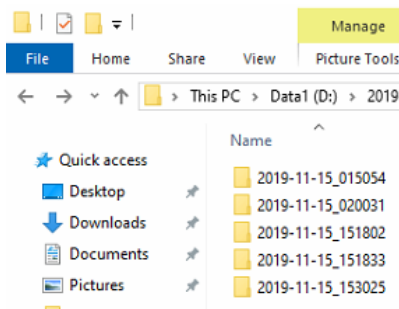
Within the main experiment folder, sub folders are created for every stack (ie. stage position) and channel combination. Each folder will be named, stack_(n)_channel_(n).
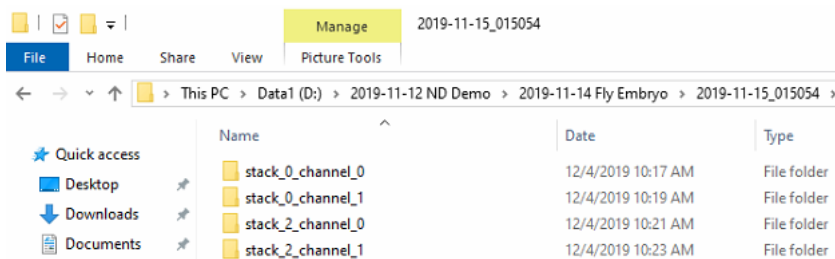

The sub-folders created for tiled montage acquisitions will have the same stack number and include the X and Y coordinates of each tile position. These sub-folders contain files related to the images and meta files generated by the short and long cameras.
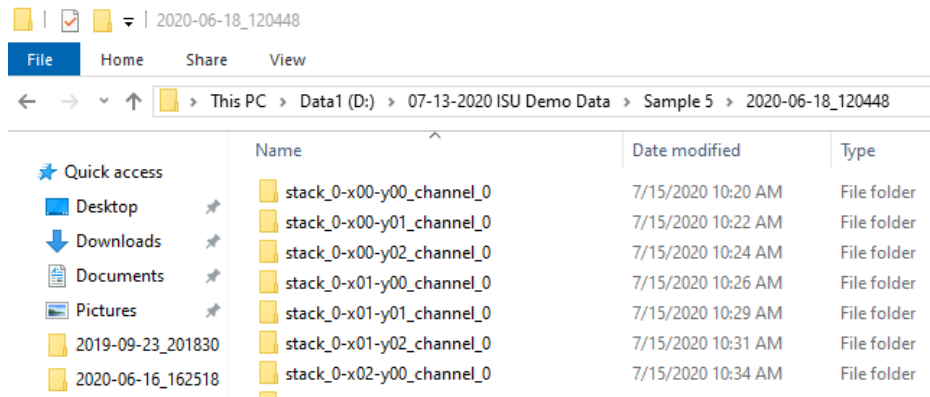
7.1.2. Image Data

- Image data for each stack and channel combination is stored in the Cam_Long_00000.h5 and Cam_Short_00000.h5 files
    - H5 files are an open source format that can be opened in FIJI
- Time series will have multiple .h5 files and be named according to time point (ie. Cam_Long_(*****).h5 where (****) is the timepoint
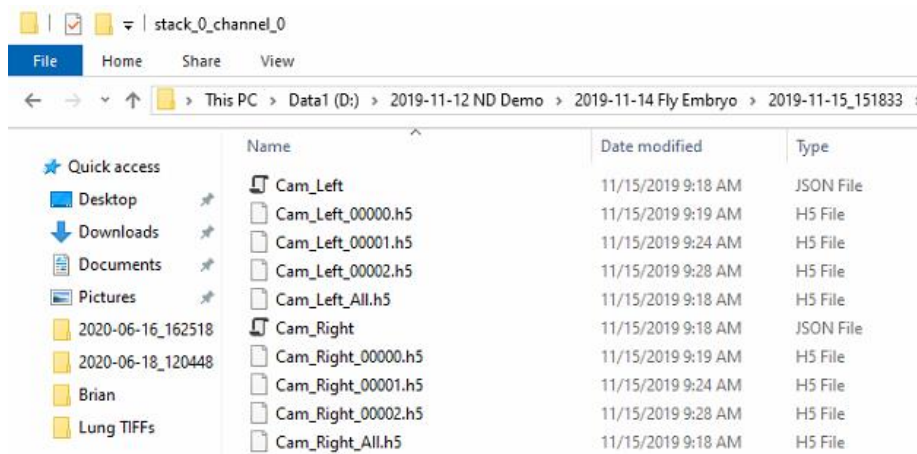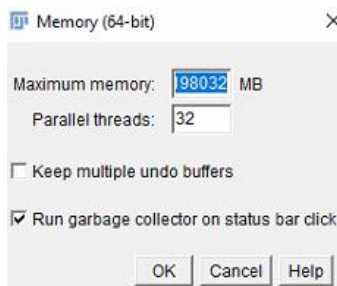

7.1.3. Meta Data

- The Cam_Long.JSON and Cam_Short.JSON files contain the metadata for each acquisition and can be opened in Notepad to view the microscope setting each for the experiment

Cam_Left - Notepad

File Edit Format View Help

{"MetaData":
{"MicroscopeInfo":{"scopetype":"MuVi-SPIM","serialnumber":"","softwareversion":"1.11.0","timestamp":"2020-06-18 12:04:48"},"objectives":[{"roles":
["detection"],"position":"right","pairedTubeLens":0,"properties":{"manufacturer":"Olympus","model":"XLUMPLFLN
20XW","magnification":20,"na":1,"wd_mm":2,"immersion":"water","focalLength":9}},{"roles":
["detection"],"position":"left","pairedTubeLens":0,"properties":{"manufacturer":"Olympus","model":"XLUMPLFLN
20XW","magnification":20,"na":1,"wd_mm":2,"immersion":"water","focalLength":9}},{"roles":
["illumination"],"position":"front","pairedTubeLens":200,"properties":{"manufacturer":"Nikon","model":"CFI Plan Fluor 10x
W","magnification":10,"na":0.2999999999999889,"wd_mm":3.5,"immersion":"water","focalLength":20}},{"roles":
["illumination"],"position":"back","pairedTubeLens":200,"properties":{"manufacturer":"Nikon","model":"CFI Plan Fluor 10x
W","magnification":10,"na":0.2999999999999889,"wd_mm":3.5,"immersion":"water","focalLength":20}}],
"filterwheels":[{"Name":"fw_left","Filter":"BP497-554"},{"Name":"fw_right","Filter":"BP497-554"}],
"fpga":{"Laser":[{"Wavelength nm":405,"Intensity":0,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":10},{"Wavelength
nm":488,"Intensity":15,"ON?":true,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":20},{"Wavelength
nm":515,"Intensity":0,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":30},{"Wavelength
nm":561,"Intensity":8.5,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":40},{"Wavelength
nm":594,"Intensity":0,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":50},{"Wavelength
nm":642,"Intensity":0,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":60},{"Wavelength
nm":100,"Intensity":0,"ON?":false,"Manufacturer":"Coherent","Model":"Obis LX","MaxPower mW":70}],"Timings":{"Delay after ms":11,"Exposure ms":50,"Delay
before ms":0,"Ablation ms":0},"Scanners":[{"Name":"Scan_Back","Amplitude":2.4772109862671660707,"Offset":-0.071910112359550693029,"# of Scans":1},
{"Name":"Scan_Front","Amplitude":2.4772109862671660707,"Offset":-0.071910112359550693029,"# of Scans":1}]},
"magchangers":[{"Name":"mc_left","Filter":"150"},{"Name":"mc_right","Filter":"150"}],
"lightsheetalignment":[{"Name":"Y1","Filter":""},{"Name":"Z1","Filter":""},{"Name":"Y2","Filter":""},{"Name":"Z2","Filter":""}],
"beamexpander":[{"Name":"beamexp","Filter":"3.0µm"}],
"Stack":{"elements":[{"name":"x","target":"x","start":2769,"end":2769,"instack":false,"canTile":true},{"name":"y","target":"y","start":-571,"end":-
571,"instack":false,"canTile":true},{"name":"z","target":"z","start":-667,"end":-9,"instack":true,"canTile":false},
{"name":"r","target":"r","start":29,"end":29,"instack":false,"canTile":false}],"n":659,"reps":1,"stageSet":"live"},"Triggers":[{"start delay in
secs":0,"type":"StartIntervalRepeats","interval in secs":0,"repeats":1,"tag":"Trigger_000","loop index":0,"text":""}]},"CameraData":
{"Manufacturer":"Hamamatsu","Model":"C13440-20C","SerialNumber":"302114","Name":"left","SensorSize":{"width":2048,"height":2048},"PixelSize":6.5,"ROI":
{"width":2048,"height":2048,"left":0,"top":0},"SensorMode":12,"ConfocalSettings":
{"SlitSize":35,"LineExposure":0.75406476817894552,"LineInterval":0.02154470766255587,"Direction":1,"TriggerDelay":1.914845739893144,"ScanAmp":2.47721098
62671661,"ScanOff":-0.071910112359550693,"Exposure ms":50,"Turn":0.10000000000000001},"k0 (pixels)":1022,"k1 (pixels/scanner units)":-
445,"Calibrated?":true}}

7.2. Viewing InVi SPIM Data in FIJI

7.2.1. Downloading and Configuring FIJI

1. Download the latest 64-bit version of FIJI from https://fiji.sc/
2. Open FIJI > select Edit > Options > Memory and Threads
3. Set the Maximum Memory to match to the amount of RAM installed on the computer
   a. This setting will limit the size of the data sets that can be directly loaded in FIJI
   b. Maximum Memory can exceed the amount of RAM if Virtual Memory is enabled in Windows

Memory (64-bit)

Maximum memory: 98032 MB
Parallel threads: 32

☐ Keep multiple undo buffers

☑ Run garbage collector on status bar click

OK   Cancel   Help

4. Select Help > Update > Manage Update Sites > check boxes for EMBL-CBA and HDF5 > Close > Apply Changes
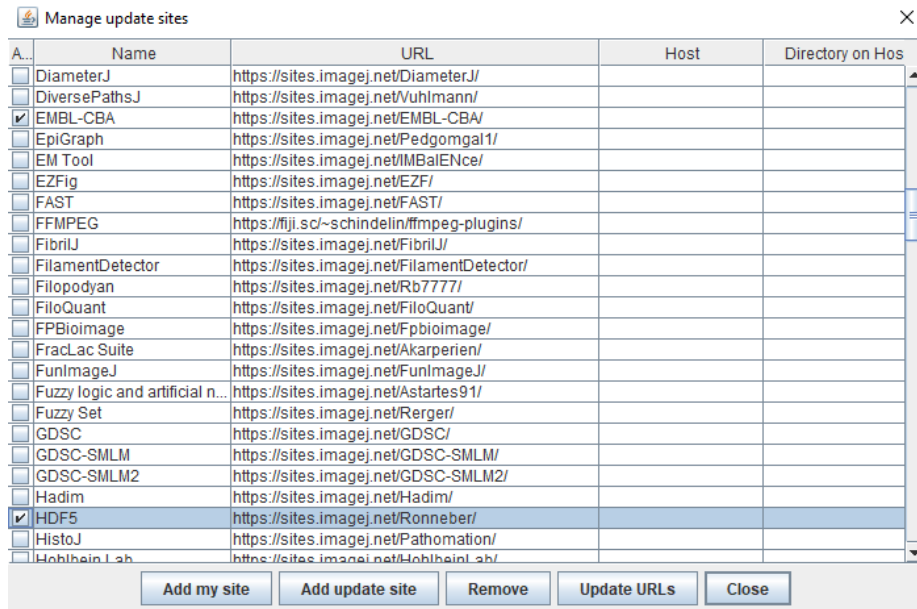
## 7.2.2. Opening Single Cam_(Long or Short)_00000.h5 Files

5. For single H5 files, Select File > Import > HDF5 > select Cam_Long_00000.h5 or Cam_Short_0000.h5
6. Repeat this process to open additional data from other stacks, channels or camera angles
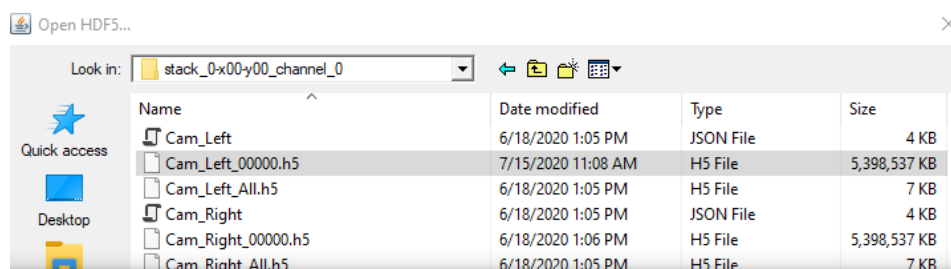

- Note: file sizes will be limited to the memory allocation configured in the FIJI Settings menu. Any files opened with this method will be stored in the computer's RAM.

## 7.2.3. Streaming Time Series with the Big Data Processor

7. Stream large data sets, such as time series with the Big Data Processor in FIJI to bypass the memory limit of the computer and take advantage of the H5 file structure
    a. Select Plugins > BigDataTools > BigDataProcessor > select folder of interest
   b. Steaming data with the BigDataProcessor does not load the entire file to RAM
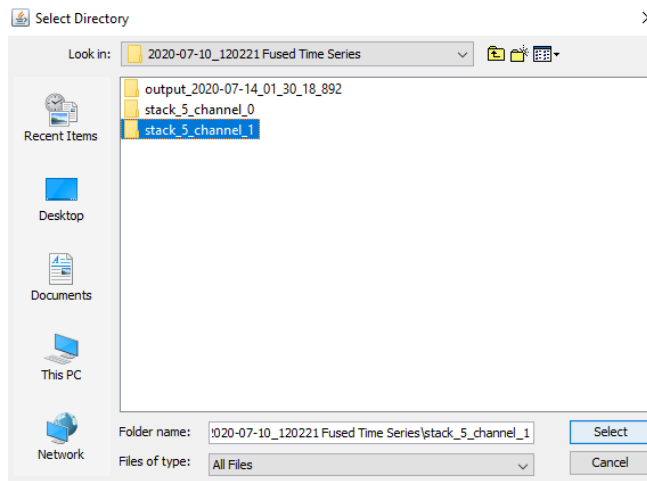
    c. Input camera specific information into the Only load files matching: field, ie. Cam_Long_0.*h5
        i. The file name information will be found in the data folder
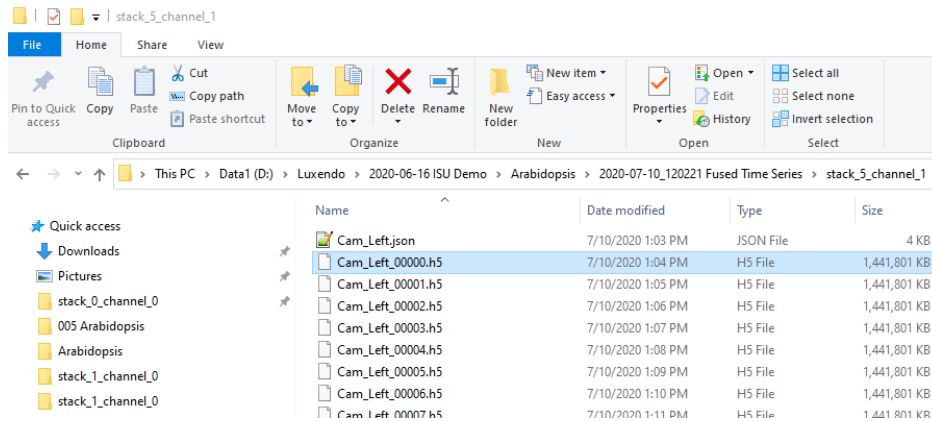
ii. Remove any numerical characters at the end of the file name and replace with "0.*h5"
   1. For example, Cam_Long_00000.h5 would be inputted in the Only load files matching: field as Cam_Long_0.*h5
d. Select Hdf5 data set name: Data from the drop-down menu
e. Click Open Virtual
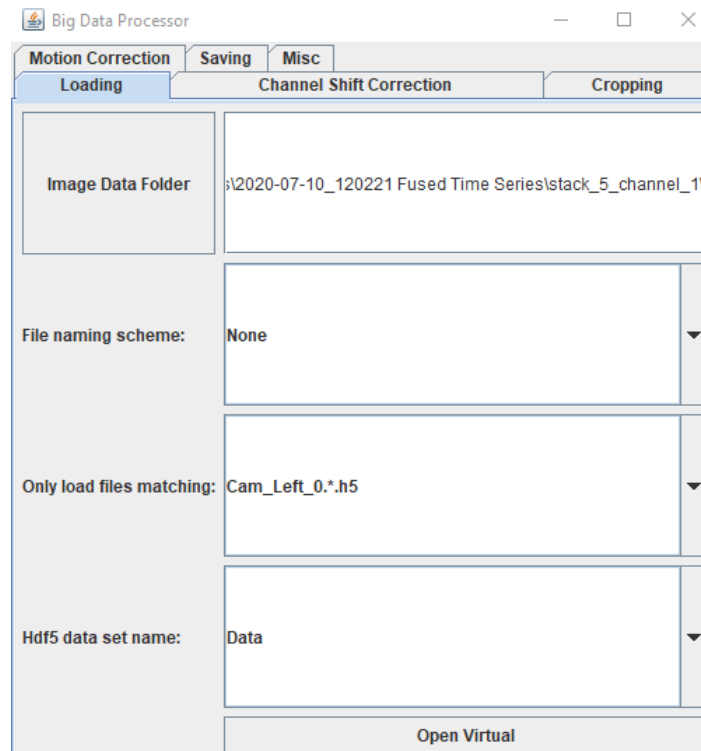
a. To open other camera angles, repeat process starting at Step 3a by inputting Cam_Right_0.*h5 into the Only load files matching: field of the Big Data Processor window
b. To open other channels or stage positions, repeat process and select appropriate folder in Image Data Folder window of Step 3a
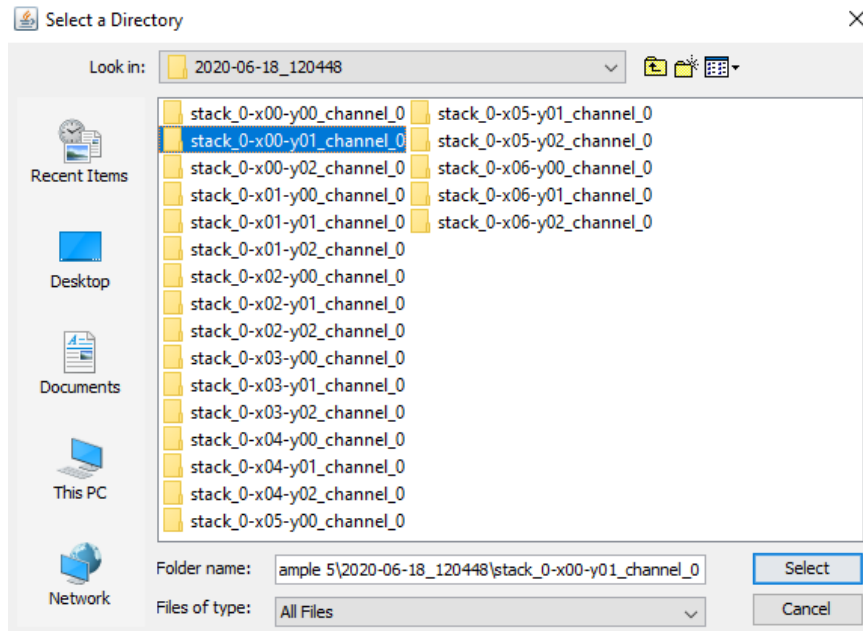
7.2.4. Opening Data Collected in Repetition Mode

8. For data acquired in Repetition mode, open data in FIJI using File > Import > HDF5 file > select Cam file of interest > Open > highlight data set path "Data" > click Load
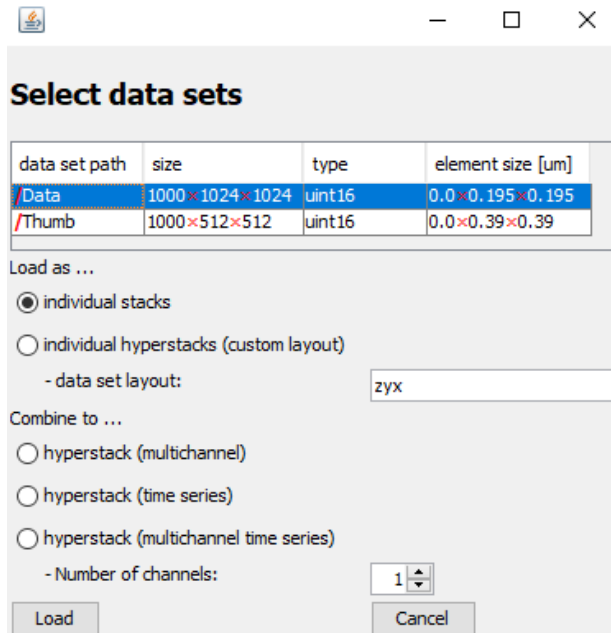
a. If two channels were acquired, repeat this process to open the second Cam view

    i. For two color samples imaged using dual channel simultaneous capture, one data set may need to be flipped (depends on the model InVi SPIM used for acquisition)

        1. Select Image > Transform > Flip horizontally

    ii. The two channels can be overlaid by selecting, Image > Color > Merge Channels
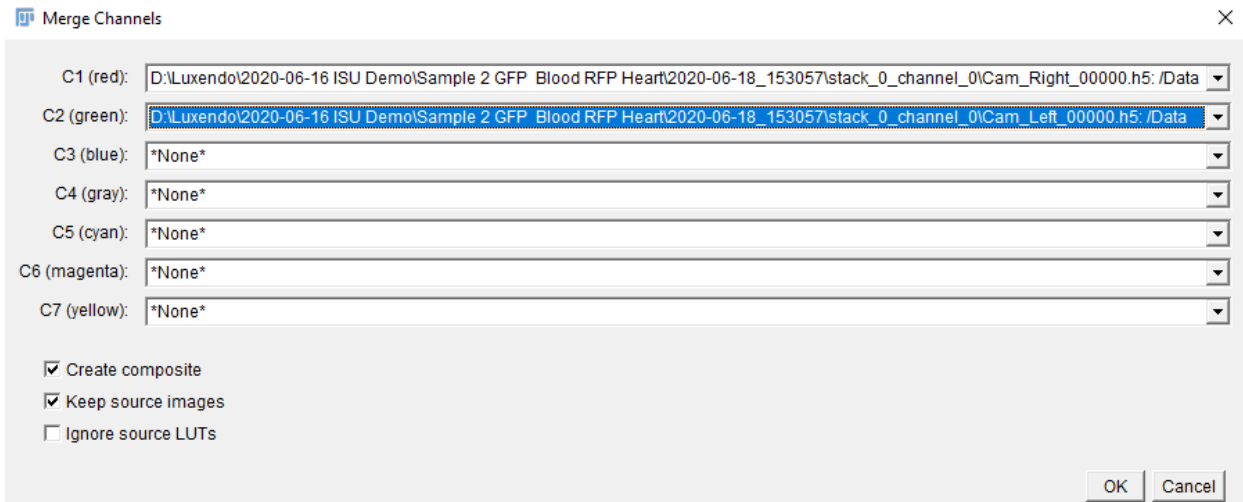
a. During conventional capture time points are saved as distinct files, this takes a lot of computer overhead and makes the acquisition slower. When using Repetition mode the files are saved as one file - this format is typically reserved for Z. To correct this, select Image > Properties > switch values automatically generated in Slices (z) to Frames (t)

   a. In the below example, Slices (z): 1000 becomes Slices (z): 1, and Frames (t): 1 becomes Frames (t): 1000

b. The Frame interval can be determined from the JSON file by adding the "Delay after ms" and "Exposure ms" values

7.2.5. Opening Data Processed with the Luxendo Image Processing Pipeline

9. For raw data fused or stitched using the Luxendo Image Processing Pipeline, an "Output" subfolder will be present in the experiment's main folder. Depending on how the fusion was performed, this folder may contain compressed versions of the raw data and the fused data.
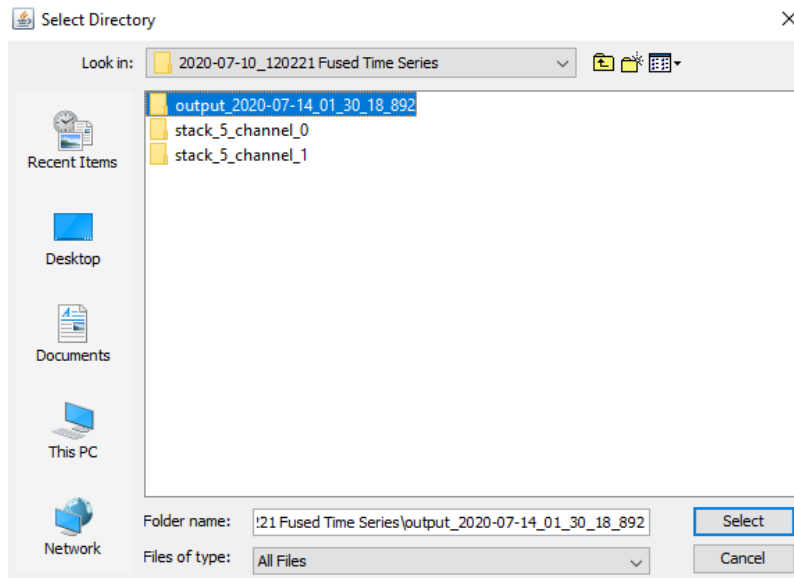
a. These data can be opened within FIJI using File > Import > HDF5 (Step 2) or with the BigDataProcessor plugin

   i. To use the BigDataProcessor plugin, follow the directions outlined in Step 3 with the following data-type specific changes:

      1. The Open load files matching: field must contain the H5 file name without the numerical characters + 0*.h5

         a. For example "out_stack0-x00-y00_chan0_camFused_tp00000.h5" becomes "out_stack0-x00-y00_chan0_camFused_tp).*h5"

      2. Select Data111 from the Hdf5 data set name: drop-down menu
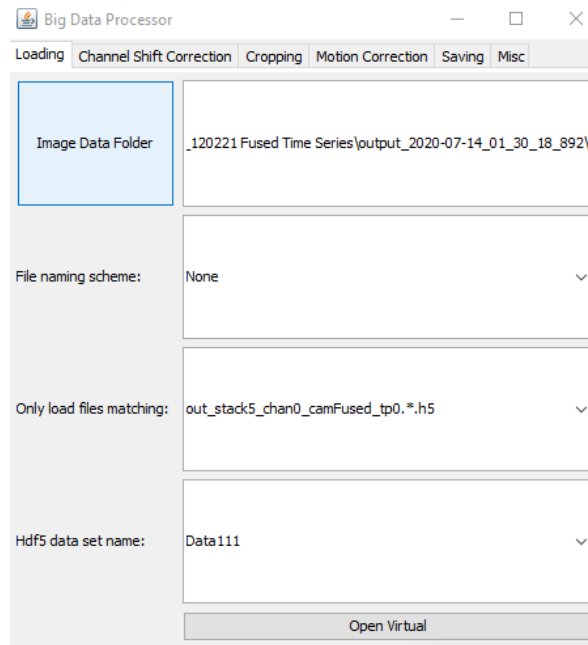
10. To use Big Data Viewer plugin, select Plugins > BigDataViewer > Open XML/HDF5 > select "_master_bdv" XML file of interest > Open
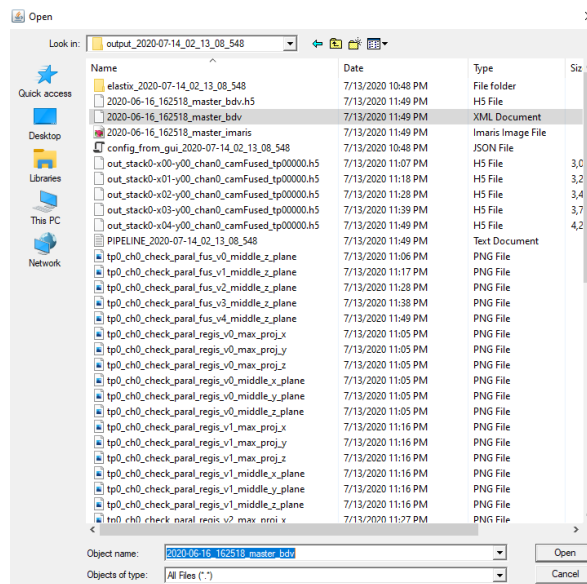
7.3. Opening InVi SPIM Imaging Data in Arivis Vision4D and Imaris

Once the imaging data has been appropriately fused/stitched in the Luxendo Image Processing Pipeline, the channels and time points will open in a single window in Arivis Vision 4D or Imaris (depending on the features of the software package being used).

- The "_master_bdv" XML file can also be dragged and dropped into Arivis Vision4D for rendering and analysis

- The "_master_imaris" Imaris Image file can be double clicked to open in Imaris or Imaris Viewer
    1. The free Imaris Viewer for Mac and PC can be download from: https://imaris.oxinst.com/imaris-viewer

7.4. Luxendo Image Processor

Image processor is integrated part of our Lux Control instrument software interface.

Images given to the Image Processor are in the Luxendo-Image format (.lux.h5) and contain important metadata, including transforms that bring the image data from the "pixel space" of the cameras to the "sample space", the 3d-coordinate system in which the sample is represented.

The Image Processor performs the following steps:

1.	(Optional) Registration (content-based): align the different objective views, tiles, and rotated views against the selected "reference" view, to get finer "correction" transforms to sample space (in addition to the transforms from the image metadata, e.g. from stage positions). The resulting transforms are saved to .transf.json files in regis... subfolders in the output folder.

2.	Fusion: the different views are transformed to sample space and fused. This produces a separate output .lux.h5 file for each time point and channel.

3.	Generate main files that link to the output-data files for all time points, channels, etc (e.g. a .ims main file to open the output images in Imaris, and .xml, .h5 files for Fiji BigDataViewer).

The settings for each step can be found in the corresponding sections in the GUI. For specific information on the individual settings, please also see the tool tips in the GUI (they are shown when pointing at the label of the respective setting).

When Fusion is deactivated, no output images will be produced. This is useful if only the transforms from the content-based registrations are needed.

7.4.1. Tasks

The Image Processor allows to set up multiple separate processing "tasks", with different settings and/or different input images. Then many tasks can be enqueued and run one after the other, without need of supervision or interaction from the user, and the results of all completed tasks can be evaluated at a later time. Each task is run as a separate process on the computer. A task's output is saved to a separate output folder (named with task name, time stamp, and user-provided task description) within the processed folder, inside the "experiment" folder that contains the used raw input images from the acquisition.

A task can have different statuses:

• Not started: a new task.

• Queuing: the task is enqueued and waiting to be run, when the tasks that are ahead of it in the queue are completed or have been stopped by the user.

• Running: the task is currently running, and output is being written to its output folder.

• Stopped: the taks was manually stopped by the user. For instance when the user realized that the settings needed to be changed or another task behind it in the queue was more important and should be run first.

• Failed: the task has failed with an error. This error is shown in a red error notification. A Failed task can be started again, e.g. after adjusting its settings, or to try it again with the same settings.

• Completed: the task is completed. It can not be run again.

All present tasks are shown in the task-list panel. When clicking on a task in the list, its settings (configuration) are shown in the configuration panel, and can be changed (unless the task is currently Running or is already Completed). For each task, all its settings are saved in a config_task.json file in its output folder, when the task starts to run.

- Newly added by clicking the Add button. A new task will show up in the task list with a new name (number), for which the user can then provide a description to easily identify the task later, and adjust the settings of the task.

- Copied by clicking the Copy button. All settings of the task will be copied to a new task created in the list. This is particularly useful when e.g. another task should be run that has mostly the same settings but only a few specific settings need to be changed.

- Exported (saved) as a .json file that contains all the settings of the task, by clicking the Export button.

- Imported, by clicking the Import button and selecting the .json file of a task that is e.g. in the output folder of a previously run task, or that was exported before.

- Saved as new default settings: by clicking the Save as default button, the settings of the currently selected task will be saved as new defaults, so that whenever a new task is added afterwards, it will at first have these settings. This function can be very useful, when the user often needs the same settings, but these settings are different from the pre-configured defaults.

- Deleted: the task will be only deleted from the task list, but its output folder will not be deleted.

- Run: the task will be added at the end of the queue of tasks (enqueued) and will be waiting until it is its turn to run.

- Stopped: the task can be manually stopped by the user, e.g. to change the settings, or to let other more important tasks that are behind it in the queue run first.

7.4.2. Usage Level

With the Usage Level slider, the power/difficulty level can be chosen, and the corresponding controls for the settings of a task will be accessible in the user interface. The settings that are not shown at a chosen level are set to their default values. The levels are:

- Basic (0): only the most commonly used controls are accessible.

- Intermediate (1): settings are accessible that provide more fine-grained control, e.g., if a sample requires specific registration settings.

- Advanced (2): these are settings which advanced users can use to fine-tune the processing results.

- Additional (3+): if additional features are installed with the Image Processor, these can be accessed with level 3 or higher. If no additional features are installed, the Usage Level slider will only go up to 2.

Should a setting that is mentioned in this guide not be visible in the user interface, please try if it shows up in a higher Usage Level.

7.4.3. Typical Workflow

A typical workflow has usually two phases:

1.   Run multiple fast low-resolution "test" processing tasks, to test different settings quickly and find the settings that lead to the desired result. We recommend setting the parameter Output > Down/Up-sample Factor to 2 or larger (e.g. factor 4), to speed up the processing and keep the output small. This factor has a very large effect on run time and output size, since it is applied in all three dimensions of the output images.

2.   Once good settings have been found in one test task, copy the task to a new task and run this task with full-resolution output (factor 1), to produce the final result.

In Phase 1, often the following tasks with low-resolution output are useful (Tip: copy the first task and only change specific settings):

1.   A task without content-based registration (Registration tab switched off), to see if the alignment of views / tiles from only the transforms in the input-image metadata is roughly correct. Tip: To also get the original views in sample space as output, in addition to the fusion result, choose the option Output > Add Views Separately. To be able to view the original, aligned data separately can often be helpful.

2.	A task with the desired content-based registrations, e.g., Objective Registration, Tiles Registration, to see the effect of the content registration.

3.	When satisfied with the content alignment, copy this task to a few tasks in which different settings for the Fusion are tried out, e.g., different choices for Mode: WeightedSum or HighestWeight. For these tasks, you can use Registration > Reuse Registrations from Task (folder) to select the previous task with the good alignment, to reuse its registrations. This can shorten the processing time significantly.

In Phase 2:

•	When good Registration and Fusion settings are found in a task in Phase 1, copy this task and set the output resolution to full resolution (if desired), i.e., Output > Down/Up-sample Factor to 1. Also here, to shorten processing time, you can use Registration > Reuse Registrations from Task (folder) to select a previous task which obtained satisfying content alignment. (If the selected previous task had a lower output resolution, this does not affect the accuracy of its registrations -- its registrations can be reused by a task with higher output resolution.) Then run this final task. Due to the high resolution, this task will take significantly longer and create much larger output-image files. Tips:

o	Note that choosing full resolution (especially isotropic) can lead to very large output-image data, and may not always be necessary, depending on further use of the data.

o	Add Views Separately usually only makes sense for low-resolution "test" tasks (Phase 1). It should be switched off in high-resolution tasks, since there it leads to much longer processing times and much larger output data (each view added will have the same size as the fusion output).

7.4.4. Registration (content-based)

The Registration phase of the Image Processor tries to improve the rough to-sample-space transforms from the input-image metadata by aligning (registering) the content of different views / tiles. Only content from the same channel will be registered, because the content in different channels can of course be quite different and does not necessarily match.

The content-based registration is not guaranteed to succeed. Depending on the sample / content, it can also fail. It can, for instance, be thrown off by "noise" or other content not of interest to the user, such as edges (borders) of images, or small features that are only present in one of the views to be registered. Sometimes, it may seem surprising that a seemingly easy alignment task does not give a satisfactory result. Please keep in mind, however, that the alignment algorithm does not necessarily know what content is of interest to the user and what content is irrelevant. The fewer different features / content there are in the views to be registered, the more likely it is that the registration will be successful. We try to make our registration algorithms more and more robust. So it may be that some registration task that did not work, will work in future versions of the software.

Tip: If the alignment after content registration looks wrong, then run the task again without content registration and check that the views are roughly correctly aligned based on the transforms from the input-image metadata.

MuVi-SPIM Registration

Objective Registration:

- For the MuVi, when input images from different objectives/cameras are present, the option Registration > Objective Registration > Register Different Cams needs to be switched on.

Rotation Registration: The content-based Rotation Registration is only performed on rotated views from the "reference" objective / camera (selected in the Input tab). And the resulting transform is then also applied to the images from the other objective / camera.

- It is recommended to calibrate the stage-rotation axis before acquisition, to facilitate the rotation registration of the MuVi images.

- If the different rotated views have significant content/features outside their intersection volume, then the option Rotation Registration > Views Are Pre-Aligned should be selected, to use only the intersection volume of the views for registration instead of registering in the full volume. Because the content outside the intersection volume can throw off the content-based registration. However, this option requires that the stage-rotation axis had been calibrated (at least roughly) before acquisition.

Tips:

- If the result of Objective Registration (i.e. multiple objective views present) and Rotation Registration and Fusion looks misaligned, then, in a separate task, first only do registration and fusion of the opposing objective views (no rotated views), and check that the result is aligned. If not, then the problem already is in the registration of the opposing objective views.

- If the rotation registration fails, try

i.   Select a different "reference" objective / camera in the Input tab.

ii.   Calibrate the stage-rotation axis before the acquisition (check that fusion without content registration aligns the rotated views roughly), and then select Views Are Pre-Aligned.

iii.   Switch Do Fine Alignment off.

iv.   Subtract a bit more or less background with the Subtract Constant Background Value parameter.

QuVi-SPIM Registration

- For registration of two different objective views for the same stack, use Objective Registration.

- For registration of two different stacks with different objectives, use Rotation Registration.

7.4.5. Fusion

In the Fusion phase, all selected input views, i.e., objective views, tiles, and rotated views, are fused at once. This happens in a chunk-wise manner, to allow

- fusion of very large images, without running out of RAM

- (optionally) calculating the image quality in each chunk for each view and in the fusion weighting each view's chunk based on its image quality

- efficient parallel processing of chunks

The fusion Mode: WeightedSum and HighestWeight enable weighting/selection of different views based on their local image qualities with the granularity of the chunk size -- typically 64 pixels (for details, please see the descriptions in the tool tips). Such weighting can be very useful, because it allows "stitching" fusion. This provides, for instance, a way to suppress doublings from inaccurate alignments in the output image, which can occur in many situations.

Tip: To ensure that the fused output images can be viewed in Fiji BigDataViewer, the option Fusion > Half Intensity should be switched on. This will divide the intensity of the output images by two.

MuVi-SPIM Fusion

- For the MuVi, when input images from different objectives/cameras are present, the option Fusion > Fuse Different Cams needs to be switched on.

QuVi-SPIM Fusion

- For the QuVi, the option Fusion > Load Only Full Chunks should be switched on, to avoid empty spots in the fused output images, at the boundary between views (though some content at the edges of the input images may be lost when this option is used).

7.4.6. Stitching

When doing stitching of multiple tiles (fields of view), the following points are important:

- For all tiles, the "reference" objective selected in the Input tab needs to be present in the list of input images.

- Content-based alignment of the tiles can be switched on and configured with Registration > Tiles Registration. If this is switched on, all overlapping tile pairs are registered based on the content in their overlap regions. Afterwards, the resulting tile-pair registrations are used in a global-optimization procedure, to

find the best overall arrangement of the tiles. If any of the registered tile pairs contribute an unusually high "cost" during this global optimization, a warning will be shown, since this likely means that the corresponding tile-pair registration is wrong or inaccurate and thus disagrees with the other tile-pair registrations. In this case, it is recommended to copy the task and run it again, but with Tiles Registration > Remove Outlier Pairs > Automatic with Cost Threshold with threshold 10 enabled, to automatically remove these "outlier" tile-pair registrations in the global optimization. (Note that often there are anyway more tile-pair registrations than needed for a good overall arrangement of tiles, so removing some inaccurate ones will usually lead to a better result.) For this new task, the tile-pair registrations from the previous task can be reused, with Registration > Reuse Registrations from Task, to avoid doing all the tile-pair registrations again, to save time.

- For images acquired with relatively small magnification (e.g. < 10x), depending on the use case, the alignment of the tiles may already be accurate enough *without* content-based registration (Registration > Tiles Registration switched off), i.e., using only the stage-position-based alignment from the input-image metadata. If this is not sufficient, the Registration > Tiles Registration can be switched on, to add content-based alignment.

- For stitching of tiles, under Output > Bounding Box, the option Union should be selected (because Intersection will yield only the overlap of the tiles).

- If there are strong intensity borders visible between the stitched tiles, you can try to achieve smoother transitions between tiles by stitching again with Fusion > Mode set to Maximum.

Typical workflow for stitching

Run different "test" tasks with low-resolution output (e.g. Output > Down/Up-sample Factor set to 4 or larger), and finally one task with high-resolution output:

Task 1 (low-resolution output):

o   Registration off, to see arrangement of tiles based on only stage positions (no content alignment).

o   Fusion > Half Intensity on, to be able to view result in Fiji BigDataViewer.

o   Output > Add Views Separately can be used to enable seeing tiles separately in the viewer later on, for easier evaluation of alignment and overlap of tiles. However, this will add a dataset of the size of the full stitched image, for each tile to the output, i.e., multiply the output size by the number of tiles. Thus, this is only recommended for either smaller numbers of tiles, or strongly downsampled output (large Output > Down/Up-sample Factor).

o   Open result in Fiji BigDataViewer, and see how much the sample moved between acquisition of different tiles, and whether the chosen overlap size between the tiles was large enough.

o   If the overlap of tiles is too small compared to the movement of the sample (i.e. corresponding features are not in the overlap region), then the sample should be re-imaged with

        better mounting

        less stage acceleration

        more overlap

o   If the overlap was sufficiently large, proceed with Task 2.

Task 2 (low-resolution output):

o   Registration on.

o   Are there warnings about tile-pair registrations with high cost?

        If so, proceed with Task 3, otherwise go directly to final Task 4.

Task 3 (low-resolution output):

o   To shorten processing time, you can select Registration > Reuse Registrations from Task > Task 2.

o   Switch on Tiles Registration > Remove Outlier Pairs > Automatic with Cost Threshold with threshold 10.

o   If in the result the alignment of the tiles is satisfying, proceed with final Task 4.

Task 4 (high-resolution output):

o   To shorten processing time, you can select Registration > Reuse Registrations from Task > Task 2.

o   Set the Output > Down/Up-sample Factor to the desired final resolution.

o   For stitching, we recommend to set Fusion > Mode to Maximum, for a smoother transition between tiles.

7.4.7. Output

The option Output > Down/Up-sample Factors > Depth can be switched off to produce isotropically sampled output images. For the QuVi, the output should normally be sampled isotropically. For the MuVi, isotropic output makes usually only sense when rotated views are fused (angular fusion). In the case of only one stage-rotation angle, for one objective or two opposing objectives, it is recommended to use the original z (depth) sampling, i.e., the same number of planes as the input images: Depth (z) sampling factor of 1.

7.4.8. Advanced topics

To only run a conversion of the input images separately to the formats readable by Imaris or Fiji BigDataViewer, do the following:

1.   In the Input tab of a new task, select only a single view to be converted.

2.   Switch Registration off completely, and switch Fusion on.

3.   Select output resolution etc in Output tab.

4.   Start the task.

5.   Copy the task for each view to be converted, and in each task only select the respective single view in the Input tab.

6.	Run all these tasks.

One separate task needs to be run for each view to be converted. The resulting Imaris and Fiji-BigDataViewer files will be in the output folders of the different tasks.

(If there are multiple different views selected in the Input tab in one task, then these will be fused.)

Run with terminal window

To run the Image-Processor Server with a terminal window, to see additional status, progress, or error messages from the processing tasks, do the following:

1.	Open the Windows Task Manager and go to the "Details" tab.

2.	Sort the processes according to "Name", and end the task "ips.exe".

3.	Open the File Explorer and go to the C:\Luxendo\Image Processor\<latest version> folder.

4.	In the address bar, enter cmd to start a command terminal at this location.

5.	In the terminal, type ips.exe to start the Image-Processor server.

Now the Image-Processor Server is running with a terminal window, and relevant content (such as error messages) can be read and copied from the window.

7.4.9. Troubleshooting / FAQ / known issues

•	If the output-image files are unexpectedly large, please consider / keep in mind that:

o	the output sampling may accidentally have been set to isotropic (Output > Down/Up-sample Factor > Depth is not checked)

o	the output-image size is rounded to integer multiples of the chunk size (usually 64 pixels) in each direction

o   the output image usually has multiple resolution layers

•   When a task that does Registration or Deconvolution is started while another application (e.g. TeamViewer) blocks the GPU, this task may hang (i.e., be blocked in the GPU-based step) until the application blocking it is closed.

•   Currently, it is not supported to run Image-Processor tasks and acquisition on the same PC simultaneously, because these processes may compete over the access to the RAID / hard drive and this way slow each other down. Also sample tracking should currently not be run simultaneously with Image-Processor tasks, because they may compete over the GPU as well as the RAID / hard drive.

•   Heavy processing of images in other applications (e.g. Imaris or Fiji) can also negatively interfer with running Image-Processor tasks, when they compete over RAID / hard drive or GPU resources.

•   Input images being open in other software, e.g., Fiji, while also being used as input image in a running Image-Processor task, can lead to access problems on the side of the Image Processor, and the processing task can fail. Please close the input images in other applications before starting the task using them as input.

•   For each task, the Image Processor estimates the disk space required to store the task's output data. If there is not enough free space available on the target drive for the output data (e.g. the local RAID), it will warn about this and say how much free space is needed. However, third-party software producing data on the same target drive, or copy processes to the same target drive, while Image-Processor tasks are already running, can still cause the tasks to run out of disk space and fail. We thus recommend to stop or pause such external processes that generate data on the same target drive, while Image-Processor tasks are running. This will also lead to better performance of the Image-Processor tasks, since input/output from/to a drive is often a speed bottleneck for the tasks, and so external processes reading from / writing to the same drive, may slow them down significantly.

8.   Cleaning, Maintenance and Service

The manufacturer cannot be held liable for damages caused by faulty operation, negligence or non-authorized procedures, particularly for the removal or exchange of components or use of improper equipment from other manufacturers. Any such action voids the warranty and laser safety is not guaranteed anymore.

8.1. Maintenance by manufacturer

To ensure a smooth operation of the Luxendo **InVi SPIM** over a long period, we recommend concluding a service contract with Luxendo GmbH.

Damaged devices or parts may only be repaired or replaced by the Luxendo service team. Modifications of system components have to be performed by the manufacturer or authorized and trained personnel.

We ask the customer to keep a safety distance during service and maintenance and to wear laser protection glasses if necessary. If the Luxendo personnel should give any further safety instructions, these are to be obliged accordingly and immediately.

During service, the room is classified as a laser safety class 3B area.

8.2. Exchange of fuses and electric connections

Only use original parts when changing fuses or replacing power cords.

In case of a malfunction of the power supply the customer may change the built-in fuses. The two fuses are located in the fuse holders right above the kettle plug as shown in Figure 2.

Figure 2: Position of fuses

The built-in fuses are type T 5 A/H, 250V, ø 5 mm x 20 mm cartridge fuses.

To change the fuse, proceed as follows:

- Unscrew protection cap (Figure 2, blue arrows)
- Pull out protection cap and replace the old fuse with a new one
- Reinsert and firmly screw in protection cap

The customer may not alter the wiring of the system. In case the power cord detached, reconnect it to the AC network as described above.

8.3. Maintenance by the customer

The maintenance by the customer is limited to cleaning the housing, cleaning and disinfection of the sample chamber, the illumination and detection optics in it, and the *sample* carrier.

a) To clean the housing proceed as follows:

- Shut down the microscope and unplug the power cord.
- Make sure liquids do not enter the system.
- Wipe the housing using a clean cloth wetted with water and a bit of detergent. Dry off using a lint-free cloth.

For sterilization wipe the surfaces with a cloth wetted with 70% ethanol or 70% isopropanol.

b) To clean the *sample* holder, proceed as follows:
- Remove and disassemble the *sample* holder as described above.
- Remove the FEP foil and residual glue.
- For sterilization wipe the sample carrier and all parts of the sample holder (screws, mounting adapter) with 70% ethanol, then rinse them with distilled water.

The sample carrier and mounting adapter may also be autoclaved.

c) To clean the sample chamber, proceed as follows:
- Shut down the microscope and unplug the power cord.
- Make sure liquids do not enter the system.
- Remove the *sample* holder as described above.
- Remove remaining water from the water bath
- Wipe the chamber using a lint-free cloth wetted with distilled water or 70% ethanol.
- Pay special attention to avoid scratching the objective lenses during the cleaning process.

d) To clean the objectives, proceed as follows:
- Remove the *sample* holder as described above.
- Clean the front lenses as commonly recommended using optics detergents and lens cleaning tissue.
- Do not wipe the front lens of the objective with dry lens cleaning tissue to prevent scratching.

9.   Dimensions

Component	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
InVi SPIM main module	72	43	41	75
Power supply	30	30	14	12

10.   Environment

The system must be installed in a closed room.

Operation	T = 22°C ± 5°C without interruption (24 h per day independent of system operation)
Storage	T = 15°C … 40°C
Temperature gradient	± 0.5°C/h
Relative humidity	< 40% at 30°C
Warm-up time	1-2 h
Maximum elevation	2000 m

11.   Power supply configuration

The Luxendo **InVi SPIM** uses an external power supply module. This module is connected to the AC network using the provided country-specific power supply cord. The PC and the camera(s) for detection are connected to the AC network using separate power supplies and cords.

Voltage Frequency	90 V AC … 240 V AC 50 … 60 Hz
Power consumption **InVi SPIM**	Max. 200 W

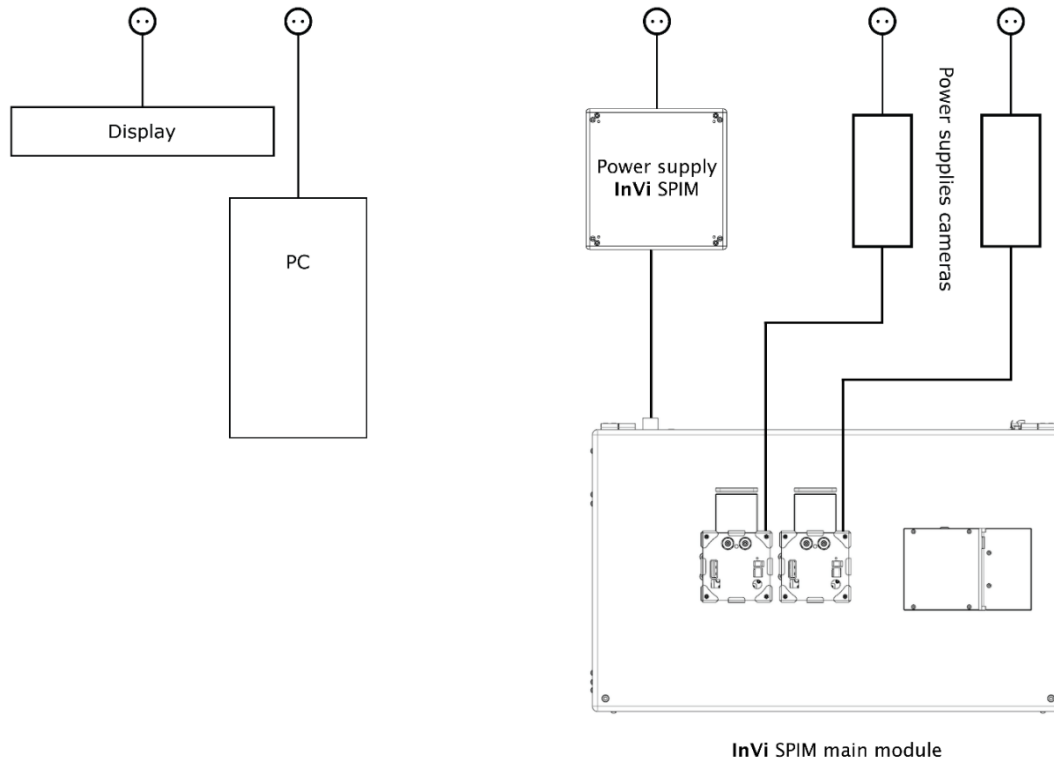| Power consumption per camera	Max. 70 W
Power consumption PC	Max. 600 W

Figure 3: *Wiring scheme of **InVi SPIM** system*

Power on the microscope by using the switch on the plugged power supply module. Initialization period is approx. two minutes; the system is then ready for usage and can be controlled via PC.

To completely disconnect the system from the AC network, turn the power switch on the power supply to "OFF" and pull the plug out of the power socket. Access to this power socket must never be obstructed.

In case of an emergency, press the kill switch installed on the power supply's front panel. This powers down the microscope immediately.

To reset the kill switch, turn it counter-clockwise.

12. Setting up the Luxendo InVi SPIM

Installation and initial startup of the **InVi SPIM** has to be carried out by authorized Luxendo service personnel. Before working with the system for the first time, a training by a Luxendo representative is inevitably required.

Do not set up the **InVi SPIM** close to a heat source, i.e. radiators or direct sunlight. An influence by air conditioning units is also possible and should be avoided.

Do not set up the system in an explosion-hazardous area.

Always use the provided power supply cord to connect the system to a properly installed safety socket. The protection by the protective ground contact must not be influenced by using an extension cord.

The system contains components carrying hazardous voltage. It may only be opened by authorized Luxendo service personnel. The power cord has to be disconnected prior to opening the system.

Maintenance and repairs, modification, exchange or removal of parts, the transport of the **InVi SPIM** module or any other encroachment on the system not described in this manual have to be executed by Luxendo or by personnel explicitly authorized by Luxendo.

This specifically applies to the microscope system, the built-in laser module, the lasers and cameras, the PC, power supplies, cable connections and other components of the system.

The system contains a safety interlock that shuts off the lasers as soon as the sample chamber's lid is opened. A notification will be displayed in the software user interface. In order to make the lasers operational again, close the sample chamber's lid and reset the lasers using the reset button next to the lid.

After installation or modification, the **InVi SPIM** system has to be inspected thoroughly by Luxendo technical staff to ensure the system functions properly - particularly the mechanisms for protection from dangerous laser radiation.

Optional components such as the temperature control unit should not be put on the same table as the **InVi SPIM** system itself to avoid vibrations.

13. Warning labels

Please pay special attention to the warning labels on the **InVi SPIM** system. Check if all labels listed below are clearly visible on your system and contact Luxendo if any of those are missing. You will receive a free substitute.

Meaning of labels:

Attention:
Possible exposure to hazardous laser radiation when working with the system.

Attention:
Malfunctions and dangers that can occur during operation and can possibly lead to damages of the system or injury of the user.

Attention:
Risk of electric shock. Possible exposure to hazardous voltage.

14. General information on handling laser components and illumination elements

The **InVi SPIM** is a laser safety class 1 product.
The lasers built into the system are encapsulated class 3B lasers. Exposure to hazardous laser radiation is prohibited by full housing and safety interlocks on all customer-accessible parts.

Attention: By using control elements or performing adjustments or procedures not described in this manual, you risk exposure to hazardous laser radiation.

When using the Luxendo **InVi SPIM** in compliance with the given instructions the user will not be exposed to hazardous laser radiation. Nevertheless, note the following warnings:

- Under no circumstances are you permitted to remove any cover panels nor any parts that are not explicitly described in this manual.
- Do not manipulate any safety interlock under any circumstances. Luxendo assumes no liability for damages caused by manipulations of the security mechanisms.

Proper precautionary measures are mandatory, if health-damaging gases, dust and vapors, secondary radiation or explosive substances are produced due to laser radiation.