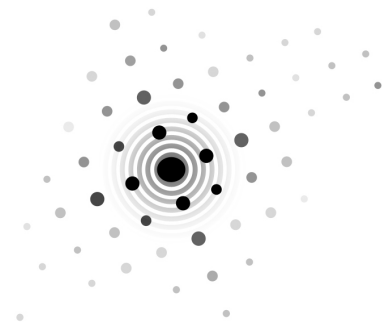


# Bruker Luxendo InVi SPIM Lattice Pro CBIS user manual



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## Prefatory note

Welcome to the Bruker Luxendo InVi SPIM Lattice Pro light sheet microscope at CBIS and SingaScope. The latest version of this user guide can be downloaded at <https://www.dbs.nus.edu.sg/2022/07/13/cbis-lm-core-bruker-luxendo-invi-spim-lattice-pro-light-sheet-microscope/>.

This document contains not only the microscopy workflow but also instructions to adapt the hardware and software for special purposes beyond the instrument's advertised functionalities. Please email me with queries, corrections and prospective research projects.

I specially thank SANE ASIA engineers Zheng Chao and Goh Wei Jia for their meticulous work on the instrument and guidance to the facility managers. I am also grateful to Daniel Aik, Anurag Chaudhury, Björn Eismann, Kathy Fan, Goh Wah Ing, Goh Yun Jing, Rutuparna Kulkarni, John Lim, Liou Yih-Cherng, Christine Low, Radek Macháň, Ashwin Nelanuthala, Pathoor Nithin, Mario Adan Mendieta Serrano, Shang Wanqi, Aravind Sivakumar, Avinaash Subramaniam, Cathleen Teh, Tong Yan, Malte Wachsmuth, Thorsten Wohland, Zhong Jun, Zhu Chennianci and Zhu Shiwen for their assistance and discussions relating to the operation of the microscope and for providing samples for testing.

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# 1 Switch on the system

## 1.1 Microscope

Rotate the two overhead plugs clockwise. The red indicator lights on upper right of plugs should turn on. Check that the switch on the microscope power supply box is in the on position and lighted. Check that the switches on both cameras are in the on position. If you're the first user of the day, you may wish to turn on the microscope early to give it some time to warm up before performing beam alignment (§3).

Turn on the computer. Wait a few minutes for the computer to boot up before turning on the monitor using the remote. The red light on the lower right side of the monitor should turn green. If you turn on the monitor too soon, it may go back to sleep. Log in to PPMS and log in to local account `lmconfocaluser`

Launch LuxBundle. If not using incubation, check that lid heating is toggled off in the software.

The system is designed to work whether the microscope or the software is turned on first. If the software freezes or doesn't detect the microscope after some time, check the red emergency shut-off knob on the power supply box. Even if power to the microscope has been cut off by the emergency knob, the power switch beside the knob will be lighted. Look at the base of the knob. You will see a yellow band if the knob hasn't been activated. If you can't see the yellow band, pull the knob to reset it. If the software still doesn't detect the microscope, go to Task Manager, end all `Lux` and `Luxendo` tasks and relaunch LuxBundle.

Lift the lid of the sample chamber and check that the front lenses of the objectives are clean. We advise you to use the torchlight and observe at a shallow angle, as some residues are almost invisible otherwise. If the objectives are dirty, clean them with lens paper and purified water followed by 100% ethanol if water is inadequate.

*Switch on the system*

## **1.2 Incubation**

### **1.2.1 Hardware setup**

If you need incubation, you may wish to check if any previous user is using incubation, and request that the incubation be left on or come earlier to turn it on yourself if necessary.

For CO<sub>2</sub>, turn on the main valve of the CO<sub>2</sub> tank. Wait a while for the pressure to stabilize. Check that the left-hand pressure gauge reads 1 bar, otherwise adjust using the black knob. Check that the under-table pump pressure is also 1 bar.

Turn on the gas mixer beside the microscope on the left (switch is on the back). Check that the water level of the humidifier column is between the two horizontal red lines. Top up with purified water if necessary.

If incubation temperature is 37 °C, turn on lid heating five minutes before activating the temperature controller, to prevent condensation on the inside of the lid of the sample chamber, which can drip onto the sample and cause the humidity sensor in the lid to mistakenly measure the humidity as 100% and stop the humidifier system from working.

For temperature control, turn on the temperature controller on the floor below the optical table. The switch is on the front upper right. Check that the water level is between the L and H markings. If it is too low, top up with tap water. Do not use purified water as the ions in tap water are required for the heater to work properly.

If necessary, sterilize sample chamber with 70% ethanol and wipe the chamber lid with tissue wetted with 70% ethanol.

Fill chamber with about 20 ml of (sterile if necessary) imaging medium pre-adjusted to the required temperature if applicable. The liquid level should be a few millimeters below the top and cover both objectives. Use only media compatible with water-dipping objectives, such as water, PBS, cell culture medium or aqueous dye solution. For live cells we recommend adding antibiotics to the medium, especially for long-term imaging.

### **1.2.2 Set parameters**

Launch the IncuControl software and click **gas control**. Set the desired values and click the top right button until it shows **Run** in green. Click **Record** to record data at your desired time intervals, and click the button again when you wish to stop recording.

Note that there is no O<sub>2</sub> control in this system and that the displayed live O<sub>2</sub> level is just the set value, not the actual level.

Alternatively, you can set the parameters directly on the gas mixer controller unit:

- Press circular button until square bracket appears around one of the parameters.
- Press NE/SW triangular buttons to select parameter to adjust.
- Press circular button to toggle square brackets to chevrons <>.
- Press triangular buttons to set desired value.
- Press circular button to toggle back to square brackets to lock set value.
- If the word **Run** appears at the bottom of the display, the controller is already running and you can leave it. If the word **Stop** is shown, press triangular buttons to select it with square brackets, press circular button to toggle square brackets to chevrons, press triangular buttons to toggle to **Run** and press circular button to lock the setting.

How to set temperature controller:

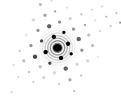
- press **RET** then **SEL** buttons, and check that the < symbol points at **SV** (set value) on the display.
- Press up/down triangular buttons to set temperature, then press **RET**. The display should show **MODE Learn**. **PV** (present value) is the current temperature.

Recommended settings for typical live-cell experiment:

- temperature 37.0 °C
- flow 10.0
- CO<sub>2</sub> 5.0%
- humidity 90.0%
- T-C (temperature of humidifying water column) 50.0 °C

*Switch on the system*

Give the system an hour to reach equilibrium.



## 2 Sample preparation

### 2.1 Holders for thick samples

#### 2.1.1 Sample dish

For thick samples e.g. zebrafish.

Pre-sterilized and ready-to-use ibidi TruLive3D single-use dishes (catalogue n<sup>o</sup> 80-0031-00-00) are available from Bruker Luxendo. If you wish to use these, please order through your lab. Place dish in its holder while preparing sample.

For most purposes, you can use one of the recycled TruLive3D dishes whose original foil has been removed.<sup>1</sup> Its capacity is about 300  $\mu\text{l}$ .

Using scissors, cut a rectangular piece of replacement FEP<sup>2</sup> foil measuring roughly 2 cm by 3 cm. Handle the foil by its edges and check that it is not creased or soiled. Put it down in a clean place.

Using a toothpick, apply a thin coat of fresh silicone sealant<sup>3</sup> to the outside of the dish. Avoid the lip. Place the dish upside down. Take the foil with both hands, pull it taut, drape it over the dish, push down against both sides to adhere and hold for a minute. Give it another hour to cure. Trim edges of foil with scissors.

Another dish option is the reusable PEEK<sup>4</sup> dish but this is more troublesome. First check that the edges of the bottom slot are not chipped off, and try threading the screws into the corner holes to make sure the threaded holes are not worn.

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<sup>1</sup> Thanks to Goh Yun Jing for the idea.

<sup>2</sup> Fluorinated ethylene propylene has an almost identical refractive index to water so it will not cause spherical aberrations. Do not heat it to high temperatures as it will release toxic fumes.

<sup>3</sup> If biocompatibility is critical, you can use medical-grade Silpuran® 4200. For zebrafish mounted in agarose, Cathleen Teh has successfully tested 5% methyl cellulose as a foil adhesive. It holds for at least 24 h yet is very easy to wash off.

<sup>4</sup> Polyether ether ketone is a very strong substance with high resistance to both organic and aqueous chemicals.

### *Sample preparation*

Apply silicone sealant around upper part of inside of dish. Put dish on holder. Drape foil over tapered end of aluminium mould and pull taut so the foil forms a neat fold along tapered ridge. Don't pull too hard on the foil as you may leave a mark on the foil along the ridge. Insert tapered end smoothly into the dish. Inspect the foil from the side and bottom to ensure that the foil has formed a straight trough with no dents or wrinkles. Secure the mould to the holder using a rubber band or clamp, and let it stand for a few minutes. If necessary, trim the edges of the foil with scissors.

Dishes are not recommended for use with lattice beam patterns (§4.4) as the FEP foil may affect the beam.

#### **2.1.2 Sample tube**

FEP tubing can be used for samples such as plant meristems. The high curvature of the tube may affect image quality since the refractive index of FEP isn't exactly the same as that of water. Obtain your own supply in the required diameter. Cut to length with scissors, fill with nutrient medium and grow sample. To mount, stick the tube through the gap at the bottom of the reusable dish and hold it in place with agarose.

#### **2.1.3 Bulky sample mount**

Wedge mount 3D-printed from PLA+ for bulkier expansion microscopy gels or other bulky samples. But can image no more than about 2 mm deep due to objective working distance. Can adjust objective correction ring to get slightly more  $z$  space for sample.

Affix sample with super glue with the region of interest on the top away from the glue. Press for flat samples, dig edge for blobby samples. When mounting in microscope, rotate to appropriate orientation to avoid sample hitting objectives.

Can drill holes to grow plants.

## **2.2 Glass slide mount**

Glass slide (1 mm × 3 mm × 20 mm)<sup>5</sup> mounted on holder, for samples such as adherent cells and objects that can be stuck upside down on the glass.

During imaging, live cells will be hanging upside down. They may have a lower survival and division rate or fall off the slide. It may help to activate the slide surface with plasma (§2.3) followed by a fibronectin coating before seeding the slide.

If mounting a piece of gel such as from an expansion microscopy preparation, apply a small dab of super glue onto the slide and gently slide the gel off a piece of Parafilm onto the glue, with the cells on the side away from the glue. Be sure to orientate the gel correctly as you cannot undo it once stuck!

If necessary, sterilize holder prior to mounting. Position the slide in its slot, making sure it sits properly. Using a PH00 screwdriver, tighten screws just enough to hold slide securely. Careful as the 3D-printed screws are fragile. Make sure the driver is fully engaged with the head before turning, and minimize vertical and lateral forces on the screw. Maintain inward pressure on the slide while tightening so the slide doesn't get dislodged by contact with the rotating screw head.

## **2.3 Plasma activation of mounting surface**

You can use cold plasma to treat the surface of the FEP foil or glass slide to clean it and/or make the sample adhere better, such as when preparing stationary fluorescent microspheres or a fibronectin coating for adherent cells.

Take the piezobrush® PZ2 out of box. Change to small nozzle by pressing small buttons at side. Connect power.

Place sample dish on metal surface to act as counter-electrode. Sweep nozzle to and fro near surface of foil or glass for a few minutes without touching it.

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<sup>5</sup> If you have suitable machinery, you can cut these yourself from a regular microscope slide.

## **2.4 Fluorescent microspheres**

You can use 100 nm TetraSpeck™ microspheres to collect experimental PSF or check for colour shifts.

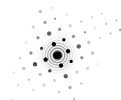
Sonicate stock suspension for 10 min. Dilute 50 times with 100% ethanol [or PBS?] and vortex. Plasma-activate (§2.3) mounting surface for stronger adhesion. Use a micropipette to transfer 2.5  $\mu$ l of working suspension to mounting surface. Spread out the microspheres using the pipette tip and air-dry.

## **2.5 Sample mounting**

If using the sample dish, push your sample to the bottom of the dish and ensure it doesn't float back up. Otherwise you may not be able to focus. Avoid positioning the sample near the ends or the central divider of the dish, as the plastic may cause stray reflections or scattering.

If you have just turned on the microscope, wait a few minutes until the stage initializes and moves through its travel range before mounting the sample holder on it.

Mate the sample dish or sample mount to its respective microscope mounting adapter and tighten screws using 1.5 mm hex key where applicable. Then slot the adapter onto the horizontal stage pin in the sample chamber, rotate the sample holder into a roughly upright position and lightly tighten with 1.5 mm hex key. Do not force the holder onto the pin, as excessive force will push the pin out of its calibrated position. The pin cannot be rotated so you have to loosen the screw to rotate your sample manually if necessary to fine-tune sample orientation. But be very careful with this as the sample holder may hit one of the objectives as the stage approaches its travel limits. If you rotate the holder anticlockwise, it may also become impossible to get the sample close enough to focus.



## 3 Microscope alignment

### 3.1 System setup

Fill chamber with about 20 ml of imaging medium if not already done so. There is no need to add fluorescent dye, although using it will yield higher-SNR beam images if needed. Even water works as it gives Raman scattering.

Opening the chamber lid will activate the safety interlock and disable the lasers, and trigger a pop-up message on screen. To enable lasers again, press button on the side of the sample chamber. If you are not using the lasers just yet, you can just close the pop-up box to access the on-screen microscope controls.

If camera feed windows are absent, click the **connect** button(s) at upper right. If that doesn't work, right click and restart Lux Datahub at lower right corner of taskbar. If that fails, try flipping the switch on the camera off and on again. When camera is acquiring images, the LED on the camera will flash alternately green and orange.

Alignment should be done after the microscope has reached thermal equilibrium.

If you plan to use previously saved settings for your experiment later, load them (§5.2) now before you start alignment, otherwise the alignment values stored in the saved settings will override the current alignment.

Select **Calibration** tab on the left. Set the magnification you will be using for the experiment, either  $31.3\times$  or  $62.5\times$ . They have the same optical resolution but the camera undersamples at  $31.3\times$ . Align separately for each magnification. After aligning at a given magnification, try not to switch to the other magnification and back, as this may cause the alignment at the first magnification to shift.

If you are calibrating after the sample has been mounted, **Zero** the  $z$  position to temporarily move the sample out of the beam path. If you have already located the sample on camera, first save the sample position (§5.1) so you can return to it later. Set the objective correction ring (§5.1) if applicable.

Set the cameras and filters (§5.2). Use the same dichroic mirror and emission filters that you will be using for the experiment, especially the dichroic, as the

beam may shift laterally with a different dichroic. You can use a longer exposure, like 500 ms for water, to get a brighter beam for now.

## 3.2 Beam alignment

Select Gauss beam, thickness:0 (see §4.1 for explanation). In the **Scanner settings** box, set mode:park. You can also align using the specific beam you will use for your experiment if the beam has a narrow and well-defined main lobe.

Turn on a laser that you will be using for the experiment and click **Start live**. A horizontal beam appears in the camera window. The light travels from left to right in the image. Increase exposure time and laser power to see the beam more clearly.

First focus the beam waist using **Focus** slider. Reduce the step size<sup>6</sup> as you zero in. Don't click the arrows too fast as there's a time lag. You can try narrowing down the histogram display range to see the focus more easily.

If moving **Focus** causes beam waist to move left/right, or if moving **Horizontal Displacement** causes waist to become defocused, **Horizontal Tilt** is not parallel to screen plane. In that event, adjust **Horizontal Tilt** until it is parallel. You can also use the Bessel beam to check **Horizontal Tilt** without moving **Focus**. The main lobe of the beam should look uniform.

Turn on the crosshairs and/or ticks by clicking the icon at top right of each camera feed.

Center **Horizontal Displacement** and make **Z Rotation** horizontal. For adjusting **Z Rotation**, it will help to temporarily use a Bessel beam, as it is easier to visually gauge the tilt of this beam.

Click **Store** if you wish to zero the values.

In the **Scanner settings** box, adjust offset to move beam up/down until centered. Click the icon button beside the text box to store the value.

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<sup>6</sup> The step size setting is related to the galvanometer voltage rather than the physical distance traveled by the beam.

Next is line calibration, which determines the correct voltage range to scan the field of view and aligns the beam with the rolling shutter. It only has to be done once for all lasers. If you are using only 405 nm for your experiment and the signal at this wavelength is too weak for calibration, calibrate with 488 nm instead. If using both cameras, the cameras will be calibrated simultaneously. Bessel is the best beam to use. In the **Scanner Calibration** box, click **Start**. After a few moments, a horizontal magenta line will appear in the camera view. If the magenta line doesn't coincide with the beam waist, click **Retry** until it does. Click **Next** and repeat. You can help it by increasing exposure time and/or changing emission filters (don't change the dichroic). You may see a noisier image, but it actually helps the magenta line find the beam, as it does so by averaging pixels across the entire frame. The noise doesn't matter as long as you can discern the beam.

If using two or more lasers, calibrate each one separately, especially if extreme accuracy is required for co-localization. You may also wish to verify with beads (§2.4). Although the detection objective is apochromatic up to NIR, some chromatic shift is present. **Horizontal Displacement** can also be out of alignment if different dichroic settings are used. Click **Add** in the **Channels** box under the **Dashboard** tab to hold the separate alignment settings in their own imaging channel (see p. 23 for more details of the **Channels** box). Do not use the zeroing buttons.

Once done, select one of the scanning modes for image acquisition. In the **scan** mode, you can reduce the **amplitude** (travel range expressed as voltage) of the galvanometer to scan a smaller height for smaller FOV and shorter scan time, or to dither the lattice patterns (§4.4). The **autoscan** mode covers the full FOV of the camera. [unidirectional (which direction up or down image frame) or bidirectional scanning up/down image frame?]

You can further fine-tune light-sheet focus (**Focus**) using FCS autocorrelation function [1]. Set the beam to scan and launch the ImFCS plugin in Fiji (see §7). Define a small ROI and set **Mode** in camera window to **Calibration**. You can try an exposure of 3 ms and **In-camera Pixel Binning** of  $4 \times 4$ . **Start**. In the **Acquisition** mode window, click the button beside *Correlation function type* until **NonCumulative** appears. In the popup, click **Plot Interval** and try 500. Adjust **Focus** in **Lux Calibration** tab to maximize ACF peak height in the live plots, which corresponds to the waist of the PSF and the point of sharpest focus.

*Microscope alignment*



## 4 Beam patterns

Beam patterns are selected in the **Advanced Illumination Module**.

The cross-sectional views of all supplied beam patterns in both parked and scanned modes, obtained using a mirror (§4.6) that reflects the beam from the illumination objective into the detection objective, can be found in [upcoming folder]. You can use these images to measure and compare pattern characteristics such as sheet thickness.

### 4.1 Gaussian beam

#### 4.1.1 Scanned Gaussian

Gaussian beam waist has higher intensity than Bessel but smaller area covered by thin part of light sheet. See Table 4.1 for measured beam waist diameters at different **Thickness** settings in the software drop-down menu.

The thinner and more intense the waist, the shorter it is. Select the beam whose waist length is no more than the area of interest on your sample, so as to minimize out-of-focus signal. Use a thicker Gaussian to get a wider usable FOV.

There is a beam of thickness 0 in the drop-down menu. Its waist is even thinner than thickness 1, but the beam is not perfectly Gaussian. This is because the rectangular SLM (§4.5) is generating a pattern for a beam whose cross-sectional diameter matches the length of the SLM's diagonal, so the parts of the cross-section outside the rectangle are missing. This beam is good for calibration (§3.2) but not recommended for normal imaging.

#### 4.1.2 Static light sheet

Non-scanning Gaussian formed by simulating a cylindrical lens with the SLM gives constant light dose good for photon quantification such as in FCS (§7).

**Table 4.1.** Waist cross-sectional diameters of available Gaussian beam **Thickness** settings.

Thickness	Beam waist* ( $\mu\text{m}$ )	FWHM† ( $\mu\text{m}$ )
1	2.132	1.255
2	2.295	1.351
3	2.346	1.381
4	2.477	1.458
5	2.635	1.551
6	2.911	1.714
7	3.015	1.775
8	3.576	2.105
9	4.150	2.443
10	4.882	2.874
11	6.718	3.955
12	9.886	5.820

\*Width of fitted Gaussian curve at  $1/e^2$  of maximal fitted intensity.

†Full width at half maximum of Gaussian-fitted intensity distribution.

Actual thickness of settings 1 and 2? Waist characteristics? Set **Magnification** to what you set on the microscope.

## 4.2 Airy beam

A curved beam that can reduce shadowing and striping in highly scattering or pigmented samples. See VETTENBURG *et al.* [2] for more details, and LATY-CHEVSKAIA *et al.* [3] if you wish to generate the SLM pattern yourself (see §4.5). You can try comparing its effectiveness with that of the Bessel beam (§4.3).

## 4.3 Bessel beam

Bessel is a self-healing beam, which gives it a more uniform profile over larger FOV and less shadow/stripe artifact. The best part of the beam for imaging is from where the light cone on the right of the camera view converges to a point, going leftwards until the brightness of the main lobe begins to fall off. In general, the best diffraction-limited resolution on this microscope is obtained

using a Bessel beam with cameras in line mode, which blocks out the side lobes, followed by deconvolution. But this may not always be the case, as factors like sample characteristics and image processing workflows can affect the final image characteristics.

If using this beam, increase `Beam axial position` until dispersed part is outside FOV or area of interest, and increase length to make beam more parallel.

[table of beam diameters and usable lengths]

### **4.3.1 Droplet**

Bessel with reduced side-lobes. Good axial resolution, better image contrast, less photodamage and deeper penetration of thick turbid samples [4, 5].

## **4.4 Lattice patterns**

Lattice light sheets [6] even out the Bessel side lobes through interference, reducing the thickness of the light sheet over a wider FOV and reducing photodamage. They can also scan the frame faster as the stationary beam covers a larger area. They have been used successfully in whole-brain nanoscale imaging [7]. But they are sensitive to variations in refractive index so samples should be relatively homogeneous. Always set the microscope magnification to  $62.5\times$  when using lattice beams. Do not use the 405 nm laser with lattice patterns as the SLM is not optimal for beam patterns with non-circular cross-sections at that wavelength.

`LatticeRectangular` and `SIMlatticeHexagonal` are the recommended patterns and are also used for SIM (§6). The rest of the lattice patterns have varying beam densities and characteristics along the different spatial dimensions, which affect resolution and other imaging properties. For example, patterns with lobes spaced more widely in the vertical direction allow a greater height of the sample to be imaged. Images of the cross-sectional beam profiles can be found in [directory path] in the workstation.

When a lattice beam pattern is selected in the `Advanced Illumination Module` box, one or more of these additional parameters appear:

- Numberofbeams
- Spacing
- Xspacing
- z-period for varying the pattern period, or vertical distance between adjacent lobes.
- height for varying the total height of the stationary beam pattern. Patterns with greater height have a larger FOV and can scan through the FOV faster as mentioned above. Patterns with smaller height concentrate more light in the middle, yielding higher SNR.
- Numberofsimimages should be the same as SIM number (§6.1).

The values in the drop-down menus of the above parameters are just a numbering system, not the actual parameter value. For **z-period**, a larger number means higher frequency and smaller period.

**Table 4.2.** Galvanometer voltages corresponding to the periods of selected lattice patterns.

$\lambda$ (nm)	Voltage (V)	
	SIMlatticeHexagonal (height 2)	LatticeRectangular (period 2, height 3)
488	0.0138	0.0177
515	0.0146	0.0146
561	0.0158	0.0203
642	0.0182	0.0233

If dithering the lattice pattern, the dither can be set using the **amplitude** (galvanometer voltage) field in **scan** mode in the **Scanner settings** box under the **Calibration** tab. Dither at slightly more than the pattern period in case of imperfections in beam calibration. For the **LatticeRectangular** and **SIMlatticeHexagonal** patterns, you can use values slightly higher than those shown in Table 4.2 for the given **z-period**.

For the rest of the lattice patterns, you have to determine the appropriate **amplitude** yourself using the cross-sectional beam profiles obtained with the stage mirror (§4.6). Get the stationary beam profile on camera and find a stack of about 10 horizontal lobes with equal vertical spacing in the centre of the field of view. Turn on the crosshairs. Go to the **Scanner settings** box in the **Calibration** tab and adjust the offset to move the pattern up and down so that the horizontal crosshair intersects the top and bottom lobes of the stack in

turn. Take the difference in offset value between the two end points and divide by the number of interlobe spaces to get the required **amplitude**.

The relationship between **amplitude**  $A$  and pattern period  $d$  in  $\mu\text{m}$  is defined by the equation

$$A = \frac{488d}{0.016365\lambda} \quad (4.1)$$

where  $\lambda$  is the laser wavelength in nm.

## 4.5 Custom SLM patterns

Fourier transform of the pattern on the front focal plane of the illumination objective, to be projected on its back focal plane. This uses the light much more efficiently than projecting on the front plane.

Upload custom pattern in landscape-format  $800 \times 600$ -pixel 8- or 16-bit greyscale (not RGB, not binary). [What file format?]

You can use commercial software, the SSPIM toolbox (<https://github.com/aakhtemostafa/SSPIM>, where a detailed manual is available) or your own scripts to generate SLM patterns.

SLM settings?

**Amplitude mask** cuts out higher frequencies and reduces the effective working area of the SLM.

**Blaze grating** simulates a diffraction grating with serrated grooves optimized for a desired diffraction order.

**Hologram Type:** choose **Gray pattern**.

See TANG *et al.* [8] for a review of fluorescence imaging with tailored or structured light.

## 4.6 Beam cross-section viewing mirror

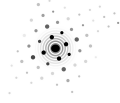
A special mirror can be mounted in the sample chamber to reflect a beam into the detection objective so you can inspect the beam's cross-section on camera. This procedure is allowed only under the supervision of core facility staff, as there is a high risk of the mirror hitting the objectives.

- Zero stage axes  $y$  and  $z$ .
- Check that the mirror is clean. Attach it to the sample mounting adapter (§2.5) and mount it in the sample chamber with purified water. Before tightening the mounting screw, use an L-shaped hex wrench pushed against the side of the adapter and against the horizontal top of the microscope body to make the side of the adapter vertical. This will orientate the mirror at the correct angle. Do not restart the system while the mirror is mounted, as the stage initialization will crash the mirror into the objectives.
- Set magnification to  $62.5\times$ .
- Move stage in the  $x$ -direction until the mirror is roughly aligned with the optical path. Then, slowly, move the other two dimensions until  $y$  is somewhere between 1400 and 1700 and  $z$  is somewhere between  $-2400$  and  $-2600$ . While doing this, keep a close eye on the mirror and ensure that it does not get too near to either objective, as the coordinates may drift over time. The mirror should now be at or very near the correct position for reflecting the beam into the detection objective.
- Set the cameras (§5.2) and filters.
- For the purpose of fine-tuning mirror position, set the beam to **Gauss** of **Thickness 0** and **mode:park**. After adjusting the mirror you can switch to other beam patterns of interest and scan the beam to see the resulting sheet.
- Turn on the laser, click **Start live** and fine-adjust  $y$  and  $z$  in both directions until you locate the reflection on camera. Reduce the stage movement to increments of  $1\ \mu\text{m}$  as you zero in on the cross-section of the narrowest part of the Gaussian beam and center it in the camera view.

For critical inspection of beam cross-sections without emission filters or dichroic mirrors, an ND filter has to be inserted in the microscope to prevent camera pixel saturation. Ask facility staff if you require this.

Clean the mirror with lens tissue wetted with isopropanol or purified water. Do not use ethanol.

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## 5 Image acquisition

### 5.1 Find and focus sample

Set folder to save in. Only D drive available as it is the partition with enough space for potentially very large datasets. Create folder with PI's name if it doesn't already exist and, under that, a subfolder with your name if it doesn't already exist. Create lower subfolders as necessary to organize your research.

Click **Start live**, or **Snap** a single image to minimize exposure. Note that a **Snapped** image cannot be saved; to save a snapshot you have to run an experiment (§5.6). [future software update may fix this]

First mount the sample (§2.5). Dismiss the laser interlock warning (§3.1).

With the chamber lid still open, move stage  $x$  to roughly center the sample to the detection objective at its working distance of about 2 mm. Move  $y$  to around 1000 and  $z$  to around  $-3500$  and search from there.

- Increasing  $x$  moves the sample towards you as you face the chamber, and the sample moves upwards in the camera view.
- Increasing  $y$  moves the sample left and up in the chamber and farther from the illumination objective, and the sample moves to the right in the camera view.
- Increasing  $z$  moves it farther from the detection objective along the objective's optical axis. Focal plane moves towards bottom of sample.

Next, close the lid, press the interlock button and turn on the transmitted bright-field LED by clicking the toggle switch at the top right of the **ILLUMINATION** box. As the LED is red, use either a long-pass emission filter or no emission filter to let the light through. Find and focus sample on the camera by moving sample using software (recommended) or 3D mouse. Change step setting to adjust movement step size.

The image scales as calculated from the optical specifications are  $0.208 \mu\text{m pixel}^{-1}$  at  $31.3\times$  and  $0.104 \mu\text{m pixel}^{-1}$  at  $62.5\times$ , by dividing the camera pixel size of  $6.5 \mu\text{m}$  by the magnification. The camera sensor has  $2048 \times 2048$  pixels, giving a field of view of  $426 \times 426 \mu\text{m}$  at  $31.3\times$  and  $213 \times 213 \mu\text{m}$  at  $62.5\times$ .

To save a stage position to return to later in the session, click **Add** in the **Stacks** box. If you change the position subsequently, click the arrow buttons beside both the start and end values of the relevant axes  $x$ ,  $y$  and  $z$  to update.

Turn off the **LED** and turn on the laser(s).<sup>7</sup> At this point you may wish to adjust **Focus** (§3.2) in the **Calibration** tab to fine-focus the beam waist or main lobe. Otherwise the signal and resolution may be suboptimal. It may help to switch to a multi-coloured **LUT**.

Also fine-tune the correction ring while observing the live view. The optimal value would be close to 0 for a sample similar to water, and higher if the sample is deep and/or has a higher refractive index than water.

Remember to click **Stop** to turn off live view when you don't need it.

## 5.2 Exposure and camera settings

If you wish to recall saved settings from a past experiment, click the **Load settings** button in the **Experiment settings** box.

Use **camera long** (nearer back of microscope) for longer wavelength, and **camera short** for shorter wavelength. Select the appropriate filters and dichroic mirror in the **Detection** box. When using both cameras, if you see a double image in **camera short** caused by second reflection of longer wavelength from rear surface of dichroic mirror, try bandpass instead of longpass filter for **camera short**. Ensure that **camera short** has an emission filter when a dichroic is in the light path, otherwise direct laser will hit and damage the sensor. If using one camera, use **camera long** with dichroic in empty position to minimise light loss.

In the **Cameras** box:

- When the **link** checkbox is checked, whatever setting you select for one camera will be automatically applied to the other. Be sure to uncheck the box if you do not want that.
- **Mode**: line mode is usually better, as a rolling shutter will follow the scanning beam and prevent the sensor from collecting background noise from

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<sup>7</sup> For live viewing only, you could have both LED and laser on at the same time to view the fluorescence signal with respect to the rest of the sample.

**Table 5.1.** Measured laser power values.

Wavelength (nm)	Power* (mW)
405	
488	
515	
561	
642	

\*Maximal power at stage with empty sample chamber.

areas of the FOV that are not being illuminated at any given instant. Use line mode to exclude the side lobes of the Bessel beams. Use area mode when using the static light sheet or for particle tracking. Turn off the camera you are not using, or else it will record noise into an extra folder in your data.

- The `lines` setting controls the vertical width of the rolling shutter. You can start with the default of 20 px. If your signal is weak, try increasing the width to maximize collection of signal from the periphery of the scanning beam.
- The minimum safe `Delay` between snaps is 11 ms. You can try shortening this if using smaller ROI, but the experiment will abort with an error popup if data cannot be written to disk fast enough. To get higher frame rates and shorter delays, trigger the camera internally (§5.7).

Adjust `Exposure time`, together with laser power (see Table 5.1 for measured values) in the `Illumination` box, for optimal SNR and minimal photodamage. In line mode, the exposure time is constrained by the minimum and maximum speed of the rolling shutter; the allowed range depends on line calibration (§3.2) and ROI height (§5.4.1).

When laser power is set to 0, a very small amount of laser still comes out. You can try this for very sensitive samples. But the laser may be unstable below 1%.

Check histogram for exposure. Camera bias offset is 100. The signal intensity should be at least 1000–10000 for satisfactory processing later. Make sure signal is not saturated, especially if you are planning to deconvolve the data (§8.4). Select `dynamic` or `range` (settable by dragging sides of darkened box) to adjust the displayed brightness.

Check for shadow and stripe artifacts. The beam illuminates left to right in the image. To reduce the artifacts, try Bessel (§4.3) or Airy (§4.2) beam.

The sensor in each camera comprises two chips and you may see a fine horizontal line of darker pixels (with a different readout amplifier) separating the upper and lower half of the image when the signal is weak.

If you are doing super-resolution microscopy or FCS (§7) or measuring low signal intensities at small spatial scale, you may wish to maximize accuracy by calibrating the camera sensor for intrinsic pixel-to-pixel variations [9].

For full technical documentation of the Hamamatsu ORCA-Flash4.0 v3, see [https://www.hamamatsu.com/content/dam/hamamatsu-photonics/sites/documents/99\\_SALES\\_LIBRARY/sys/SCAS0134E\\_C13440-20CU\\_tec.pdf](https://www.hamamatsu.com/content/dam/hamamatsu-photonics/sites/documents/99_SALES_LIBRARY/sys/SCAS0134E_C13440-20CU_tec.pdf).

In the **Channels** box under **Dashboard** tab, click **Add** to store settings. If you make changes, click **Update** to overwrite settings in current channel. The settings are lost if you turn off the microscope. Save button coming in future version of the software.

If you change the magnification, wait a few seconds for the tube lens to move into position before adding or updating a channel, and check that the correct magnification is shown in the channel data.

### **5.3 Sequential and simultaneous detection**

To set up sequential detection of two or more channels, adjust the exposure and camera settings for each laser with only that laser turned on, and add/save a separate channel for each laser.

For simultaneous detection by two cameras, which is not recommended on this system, turn on all required lasers, adjust global exposure and camera settings and add as one channel. During acquisition, the SLM will optimize the beam for the laser with the highest percentage power setting and then shortest wavelength [double check].

If you are acquiring more than two channels, you have to reuse one or both cameras. In this case try to allocate cameras and filters in a way that requires

minimal changes in filter and dichroic mirror configuration from the first two channels, in order to minimize delay during acquisition.

You can also acquire images using the bright-field LED—just allocate it to a channel as you would for a laser. This is useful for assessing cell health over the course of a time-lapse. You may want to acquire only one slice of bright-field instead of an entire  $z$  – *stack*—just move the stage to the centre of the stack and add a new stack which will automatically contain only one plane. Set the camera to area mode.

## 5.4 ROI, stacks and tiles

### 5.4.1 Regions of interest

Where possible, define an ROI to speed up acquisition and reduce file size. Also crop the ROI to the thin part of the light sheet that gives a usable image. Click on the white border of the image view and it will turn green. Drag corners to change the dimensions. You can also specify dimensions in the **Properties** tab to the right of the camera feeds. Type in the width and height (in pixels) first, then the top and left pixel coordinates. **Width** must be a power of 2 and at least 128. **Height** must be a multiple of 4. After defining the initial ROI, you can adjust it by dragging the corners of the ROI box in the camera view. Drag central square to move the ROI. The ROI must be set for each channel. Update the channel to store the ROI.

### 5.4.2 Slice scan stack

Move stage  $z$  to find your desired start point. If your sample is deep, there may be different optimal objective correction ring positions for the start and end points, so you may wish to adjust the correction ring too. To set the position, go to the **Stacks** box and click the arrow beside the Z box under the start column. Do the same for the correction ring position if applicable. Repeat the whole procedure for the end point of the stack.

Alternatively you can set a central position for the stack and specify the total number of planes. Use an odd number to have the same number of planes on either side of centre.

If you are collecting experimental PSF and/or planning to perform deconvolution, ensure that the stack covers all of the out-of-focus light.

The **Delta** field allows you to set the  $z$ -step size in  $\mu\text{m}$ . The optimal step size is beam-dependent and can be calculated at <https://svi.nl/NyquistCalculator>,<sup>8</sup> especially if you are planning to deconvolve the data (§8.4) or do SIM (§6). For this microscope it is generally around 0.1 to 0.2. But you have to weigh the benefits against potential photodamage and reduced acquisition speed.

Don't forget to set the  $x$  and  $y$  stage positions. Click the start and end arrows as for Z and check that the start and end values are identical unless you are tiling (§5.4.4).

### 5.4.3 Stage scan stack

You can use stage scanning for thin part of light sheet to cover wider FOV for thin samples that cover a large area of the microscope slide. Use a beam with a short and narrow waist.

Stage scanning setup has two parts: specifying two pairs of  $y$ - $z$  stage coordinates for the software to compute the trajectory of the coupled stage, and then setting the start and end  $z$  positions for  $z$ -stack acquisition. If you are imaging only one location in your sample, you can set the  $z$ -range of the  $z$ -stack to be identical to that of the stage coupling coordinates. If you wish to image multiple locations in your sample, define an overall stage-coupled area that covers all the imaging locations and then define separate  $z$ -stacks within.

To set up coupling, first go to the **Stage coupling** box in the **Calibration** tab and set reference stage to Z and linked stage to Y.

Locate one  $y$ - $z$  end of the overall area you wish to image by moving the stage  $y$  and  $z$ . Center the focused part of the sample on the vertical crosshair in the camera view. Add the position in the **Stage coupling** box. If you wish to set this position as the start of the  $z$ -stack, set it now (§5.4.2).

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<sup>8</sup> Enter 0.5 in the light sheet NA field and 1 in the fill factor field. If using Gaussian beam, the **Width of Gaussian light sheet** to enter in the calculator is the beam waist value in Table 4.1.

## *Image acquisition*

Also double check that the beam itself is centered by temporarily setting the emission filter to **empty**, cutting laser power to 0% and shortening exposure, to more clearly see the scanning beam waist's reflection off the glass slide while minimizing photodamage to the sample. Fine-tune the beam's centering if necessary by adjusting **Horizontal Displacement** in the **Lightsheet alignment** box.

Locate the other  $y$ - $z$  end of your desired imaging area and repeat the procedure above to set the second position in the **Stage coupling** box and fine-tune beam centering and, if applicable, set the position as the end of the  $z$ -stack.

Toggle **enable coupling with y** above  $z$  stage position control. Now, when you move  $z$ , the focused part of the image stays on the vertical crosshair. The stage is moving in a straight line between the two specified positions. Set a narrow vertical ROI (see §5.4.1) to reduce acquisition time and file size.

**Warning:** do not zero the  $z$ -axis of the stage while it is coupled to  $y$ , otherwise the coupled  $y$ -value will run to the bottom and the sample holder will hit the illumination objective!

If you are acquiring multiple locations within the stage-coupled area, define your  $z$ -stacks now (§5.4.2).

For  $z$ -stacks, set **Delta** (optical section thickness) as for slice scan (§5.4.2).

You may wish to double check that the beam is perfectly focused by fine-tuning **Focus** in the **Calibration** tab.

When done with stage scan, toggle  $y$ -coupling off to avoid accidentally hitting the objective if you zero  $z$ .

Data acquired using stage scan should be deskewed in Lux Image Processor (§8.3.1) before analysis in external software.

### **5.4.4 Tiles**

For tiling, specify the ranges of **X** and **Y** in the **Stacks** box. The **Delta** value specifies the amount of translation between adjacent tiles in  $\mu\text{m}$ . As the FOV is about  $426 \mu\text{m}$  at  $31.3\times$  and  $213 \mu\text{m}$  at  $62.5\times$ , the **Delta** values must be

smaller than these, otherwise there will be no overlap between the tiles. We recommend having a 10–15% overlap depending on the amount of sample detail in your image. See §8.3.3 for tile registration and stitching.

## 5.5 Time lapse

### 5.5.1 Normal time lapse

In the **Triggers** box, set time interval and number of repetitions. A time interval of 0 means as fast as possible. If you want it as fast as possible for a specified total time, you have to manually calculate the required number of time points by summing the exposure time and delay of each frame and multiplying by the number of  $z$ -slices to get the duration of each time point.

For long-term experiments lasting more than about a day with incubation at 37 °C, seal the edges of the chamber lid with Parafilm to further reduce evaporation of imaging medium.

### 5.5.2 High-speed video

Instead of setting the repetitions in the **Triggers** box, set the number of repetitions in the **Stacks** box and control the interval in the **Cameras** box (§5.2). The interval is the sum of **Exposure time** and **Delay**.

This is the fastest way to get 2D or 3D video using Lux software. For even higher frame rates, you can trigger the camera internally (§5.7) but it is more complicated.

When acquiring 3D video this way, adjacent time points are  $z$ -sectioned in opposite directions for speed, and the entire time series is saved as a single stack. You can separate the time points and flip the stacks into the same direction using your own script or the executable<sup>9</sup> in [file path]. Analyze the data with caution due to the inversion of time in every other stack, unless the height of your ROI is small enough for the time direction mismatch to be insignificant.

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<sup>9</sup> written by Goh Wei Jia

## 5.6 Run the experiment

In the **Events** box, first delete any existing events from finished experiments, otherwise they will be repeated. Then click **Add event**.

In the **Tasks** box, click **Add** and select the desired stacks and channels. Click **Combine...** to specify whether you want stack or channel to take precedence during acquisition.

If you are not doing time lapse, check that the number of repetitions in the **Triggers** box is set to 1.

In the **Experiment settings** box, double check the correct folder is chosen for saving, and check the required and available disk space. Click **Save settings** if you wish to save your channels and stacks for future use.

Make sure nothing is resting on the floating optical table and its frame at the same time, to minimize transmission of vibrations.

Start recording incubation data in IncuControl if applicable.

Click **Run**.

Do not touch any part of the setup during imaging, but you may use the workstation. View images in the **Data stream** tab.

Remote monitoring and control is available on special request.

When the experiment is done, turn off the laser(s) in the software.

If applicable, stop the incubation data recording and save it.

Click **View last experiment** at upper left to open the data in Lux Image Viewer (§8.2).

## 5.7 Direct camera control

You can decouple the cameras from Lux control software and internally trigger the cameras using other software to get very high frame rates with small ROI for purposes like FCS (§7). However, you cannot do *z*-stacking or time-lapse imaging.

- Exit Lux Datahub by right-clicking its icon at the bottom right of the Windows task bar.
- Flip the physical switch on **camera short** to the off position so as to make the internal trigger software connect to **camera long**, which doesn't require the dichroic to be in the optical path and can therefore gather more signal.
- Turn off the lasers if they are on.
- Click **Start live** to keep the laser shutter open, and leave it like this for the duration of internal trigger control. See §5.7.1 if you need direct laser control.
- When finished, exit the ImFCS plugin if it is running, click **Stop live** in Lux software, turn on the physical switch on **camera short** and restart Lux Datahub.

### 5.7.1 Direct laser control

You also have to decouple the laser control from Lux so the lasers can be controlled separately and kept continuously on instead of blinking according to the exposure setting in Lux.

- Launch Omicron Control Center software and select the correct laser.
- Turn the laser off using the button at lower left of the Omicron window until just before triggering the camera.
- In the **operating modes** box, set **preset** to **APC Mode - CW only** for 405, 488, 515 and 642 nm, and **CW APC Mode - no modulation active** for 561 nm. The laser controls in Lux are now disabled.
- Adjust laser power in the Omicron window.
- Perform the experiment.
- To return lasers to Lux control, first change laser power back to 100% in the Omicron window.

### *Image acquisition*

- Then set `preset` in the `operating modes` box back to `ACC Mode - Analog modulation active` for 405, 488, 515 and 642 nm, and `CW ACC mode - Digital and Analog modulation active` for 561 nm.
- Turn on the laser(s) in the Omicron window. Close the Omicron software.

#### **5.7.2 Micro-Manager**

Launch Micro-Manager. Click `Live` and adjust `Exposure`. Define ROI.

Click `Multi-D Acq.` button. In the `Multi-Dimensional Acquisition` window, check `Time Points` and set. Leave `Save Images` unchecked so the data will be written to RAM which is faster than writing to disk, but be careful of exceeding the RAM.

Click `Acquire!` and note the total run time (lower left of image window) for calculating actual frame rate, which may vary slightly across replicate runs. Save.

#### **5.7.3 Tokupic**

Tokupic is Hamamatsu's own camera software. It is less user-friendly but gives more advanced control. Launch the software and it should detect the cameras automatically. Our Tokupic license allows only one camera to be operated at a time. Check the box for the camera you want. The `camera long` is 305318 and `camera short` is 305320.

Under the `Trigger` subtab in the `Camera Properties` tab, set the trigger to `INTERNAL` and `TRIGGER DELAY = 0`. Set `Exposure Time` in the `Acquisition` subtab. In the `Mode and Timing` subtab, set `SENSOR MODE` to `AREA` and `READOUT SPEED = 2`.

Set ROI in the `Image` subtab. In the `Sub Array` box, click `Select Sub Array`. Unfortunately you can't see the ROI boundary in live view.

To acquire, click `Record` and `Stop`.

#### **5.7.4 Connecting your own camera**

This must be assisted by core facility staff unless you have obtained prior approval for independent execution.

Your camera should be swapped with **camera short**. Do not touch **camera long** as it is a calibrated reference for the microscope optics.

The camera port uses the C-mount (flange focal distance 17.526 mm). If your camera is too big to fit in the available space, an extension tube and appropriate photo eyepiece is needed.

Removal of **camera short**. Set screws. Place dust cap on sensor.

Attachment of your own camera. C-mount docking procedure.

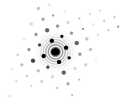
Reattachment of **camera short**.

Realignment of mirror.

### **5.8 Luxendo API**

Use Python to control microscope via MQTT client. Contact the facility managers.

Could be scripted for example to calculate transformation of growing and moving zebrafish while time-lapse experiment is running, and tell microscope to move stage to compensate and keep fish within FOV



## 6 Structured illumination microscopy

No rotation of the structured light. Gives super-resolution along  $x$  and  $z$  axes if hexagonal lattices are used. Interpret your images accordingly.

Do not use the 405 nm laser for SIM as the SLM is not optimal for lattice patterns at that wavelength.

SIM is generally susceptible to noise speck artifacts. Minimize SNR during acquisition.

### 6.1 Acquisition

Select `SIMlatticeHexagonal` beam pattern (see LI *et al.* [10] for an application of a hexagonal lattice), or `LatticeRectangular` if signal is weak.

In the `Scanner` calibration box under the `Calibration` tab, switch to SIM mode. Set `amplitude` according to Table 4.2 (p. 16). Set `SIM number` to 5 (recommended for `SIMlatticeHexagonal`) or 7. Individual SIM images [exactly how in time?] will be spaced evenly within pattern period. More phases give more accurate reconstruction but will be slower with more photodamage, as each slice has to be imaged more times. Fewer lattices faster but less accurate. In any case, do not use `SIM number` 3 as the phases are too far apart to light the sample evenly when combined. Remember to add or update `Channels` (see p. 23) to store these settings.

Only a small area near the centre of the field of view is optimal for SIM. You should set an ROI approximating one of those specified in Table 6.1.

**Table 6.1.** Recommended ROIs for SIM.

Beam pattern	Left	Top	Width	Height
<code>SIMlatticeHexagonal</code> (height 2)	860	792	256	456
<code>LatticeRectangular</code> (period 2, height 3)	780	748	384	580

Define your  $z$ -stack in slice scan mode (§5.4.2) and time lapse (§5.5) as usual. The  $z$ -step size should fulfil the Nyquist criterion (see §5.4.2) for super-resolution SIM reconstruction.

## 6.2 SIM reconstruction

You may wish to denoise the data first to minimize the generation of noise speck artifacts in the subsequent reconstruction.

You can use SIMToolbox [11] or the fairSIM plugin [12] in Fiji. If using SIMToolbox, first convert the data to `tif` using Fiji (§8.6). [Exclude out-of-focus slices at both ends of the  $z$ -stack?] Do SIM reconstruction before deconvolution (§8.4).

Launch SIMToolbox and browse to the data. Set the number of angles to `1`, since this microscope does not rotate the lattice pattern. The number of phases is the **SIM number** you used during imaging (§6). Enter the number of  $z$ -planes (total number of planes divided by **SIM number**) and the number of time points in the data. The projected pixel sizes are `0.104` for  $x$  and  $y$  and the `Delta` value you set for  $z$  (§5.4).

### 6.2.1 FFT peaks

Next, find and exclude the peaks in the FFT of the data that correspond to regular harmonics and high frequencies that originated respectively from the beam pattern and noise in the system rather than from biological structures in the sample. Click **Find peaks** and select **Spot finder (using data)**.

Enter a starting value of `1250 nm` for **Radius/pattern spacing**. Click **Refresh** to update the preview. If you see fewer magenta crosses than **SIM number**, decrease **threshold** until you get the same number of magenta crosses as **SIM number**. If you see lots of green crosses (weak peaks) and the magenta crosses are not lined up vertically, increase **threshold** until the magenta crosses are lined up vertically and the angle is indicated as `90°`. When satisfied, click **OK**.

### 6.2.2 MAP-SIM

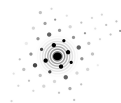
There are two reconstruction methods: automatic convergence using MAP-SIM , and the standard SR-SIM method [13].

Check **enable MAP-SIM processing** in SIMToolbox. MAP-SIM [14] uses automatic convergence and is less sensitive to parameter values than SR-SIM [13] which cannot be used here as the lattice beams are not single-harmonic patterns.

The cut-off frequency is that above which signals will be removed. It tries to reduce noise. Try 0.25 for a start. Too small a value will blur the actual structures. For **spectral merging** you can try 0.65–0.8 as suggested by the microscope manufacturer. A smaller value will produce fewer noise speck artifacts.

Under **OS-SIM processing**, a simple form of SIM that separates in-focus from out-of-focus signal, check **Average**, **Homodyne detection** and **Stripe removal**. You can optionally subtract the background. Set it at no more than the bias offset of the camera you were using, which is 100 in the case of the factory-installed Hamamatsu cameras.

Click **Preview** to see a sample slice of the reconstruction. The **Average** output should resemble a normal non-SIM image. **Save options** choose **16-bit**. Click **Run**. You can subsequently try deconvolution (§8.4) to further enhance resolution.



## 7 Fluorescence correlation spectroscopy (FCS)

### 7.1 Acquisition setup

Camera-based FCS using light sheets [15]. See SANKARAN *et al.* [16] for guidelines on obtaining accurate and precise camera-based FCS measurements. Note that the sCMOS cameras on this microscope are noisier than the EMCCD often used for FCS.

Select  $62.5\times$  magnification. Set the beam to static light sheet (see §4.1.2 for settings, beam characteristics and thickness). Park the beam to prevent brightness oscillations caused by the galvanometric mirror, although parking may increase the tendency for shadowing and striping. Set up the system for internal camera trigger and direct laser control (§5.7). You may wish to calibrate the camera sensor for intrinsic pixel-to-pixel variations [9], especially for spatial flow analysis. This microscope is periodically corrected for wavefront errors.

Acquire and analyze data using the ImFCS plugin in Fiji ([https://www.dbs.nus.edu.sg/lab/BFL/imfcs\\_image\\_j\\_plugin.html](https://www.dbs.nus.edu.sg/lab/BFL/imfcs_image_j_plugin.html)). Launch the plugin through Plugins → Imaging FCS. Dismiss the popup saying Missing PVCAM driver.

In the main plugin window click once on Live Readout. An Experimental Settings window will appear. Leave the settings as they are and click Set, as these don't affect acquisition.

Camera settings window appears. Click ROI in the window to show and hide ROI window and ROI Selector Window. Click Full in the ROI window. In camera window, set Mode to Live Video and click Start. Turn on the laser in Omicron. Position the sample area of interest in the centre of the frame, where the parked static light sheet is more uniform. You may wish to position it slightly lower or higher to avoid the central line of darker pixels, but it doesn't affect FCS analysis. Focus the sample using the stage controls in Lux. Set the exposure in the camera window and adjust laser power in Lux. Click Stop in camera window.

Set Mode to Single Capture and click Start. Turn off the laser in Omicron. The ROI Selector Window will be refreshed with an image of your sample. Now you can set the ROI by entering numbers and clicking on different parts of the preview to reposition the ROI, or using Fiji's rectangle tool to draw an ROI.

Centering the ROI vertically will give you the highest possible frame rate, as the camera reads out data from the centre upwards and downwards. Set **In-camera Pixel Binning** to your requirements for sensitivity and diffusion speed. Save the full-frame view showing the overlaid ROI boundary.

Click **Settings** in camera window. In the **Settings** window, check **GPU**. You can also adjust display settings of live plots to show during the experiment by clicking **Options**.

In the camera window, set **Mode** to **Acquisition** and specify **Total Frame**. Turn on the laser in the direct control software (§5.7.1). Click **Start** to run the experiment. When done, click **Save** and immediately turn off the laser.

At the end of the session, hand camera and laser control back to Lux software (§5.7).

## 7.2 Experimental PSF

The **ImFCS** plugin can handle PSF from both stationary and moving beads. It assumes there are no optical aberrations such as coma or astigmatism.

Stationary beads can be prepared according to §2.4. To get the  $xy$  and  $z$  values required by the plugin, first fit a Gaussian distribution to each of the three dimensions of the image of a bead. For  $xy$ , take the mean  $\sigma$  of the  $x$  and  $y$  fits and multiply it by 2. For  $z$ , calculate  $2\sigma$ . You should do this for multiple beads and average the values.

For moving beads, obtain 50000 frames of 20 nm beads at high laser power using the same exposure time as your experiment. This will get you PSF that accounts for random movements at your experimental time scale. Load the raw data into the plugin and it will use brute force to find the lateral PSF with constant diffusion coefficient.

## 8 Image processing

### 8.1 Data overview

In the folder you selected during image acquisition, there will be a subfolder for each experiment, named by date and time. In each of these experiment folders, there is a `Raw` subfolder. There is also an `xml` file that you can drag into image processing software to open the data.

In the `Raw` folder, there is a subfolder for each channel. In the subfolder are one `json` file and one `h5`<sup>10</sup> file for each time point. The `json` file contains the microscopical parameters and the `h5` file contains the image stack.

Recommended methods for common processing tasks:

- quick 2D or 3D view on microscope workstation: arivis Vision4D (§8.5)
- landmark registration: Lux Image Viewer (§8.2)
- deskew: Lux Image Processor (§8.3)
- shadow and stripe artifact reduction: deep learning [17]
- deconvolution: Huygens (§8.4)
- 3D animation: arivis Vision4D (§8.5), Fiji `3Dscript` plugin
- very large datasets: Lux Image Processor (§8.3), arivis Vision4D (§8.5), Fiji `BigDataProcessor2` plugin (§8.6)
- stitching: Lux Image Processor (§8.3), Fiji `BigStitcher` plugin (§8.6)
- export to Imaris format: Lux Image Processor (§8.3)

You can view the `json` file with a text editor. A free and cross-platform text editor you can use is Visual Studio Code. If it shows all the text in a single line, right-click inside the window and select **Format Document**. This will introduce line breaks and you can scroll through the parameters easily.

Note that for SIM data, the voxel  $z$  dimension recorded in the `json` file is the physical  $z$  section thickness divided by the **SIM number**. This enables the data to be displayed as a single stack of the correct total height in image processing software.

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<sup>10</sup> HDF5 is an open-source hierarchical file format for storing large amounts of scientific data.

## **8.2 Lux Image Viewer**

Fast viewing and landmark registration to correct chromatic shifts.

Click “i” icon at top right to view keyboard shortcuts.

Click **View last experiment** at upper right, or browse computer for earlier data.

Click **Add task in Image Processor** when done.

### **8.2.1 Landmark registration**

For correcting chromatic shifts. Use beads or make sure the different fluorophores are labelling exactly the same spot.

- Click **Add landmark**
- Select channel on right
- Find slice in which landmark shows brightest signal in selected channel
- Click on landmark
- Repeat with same landmark in another channel

Registering one landmark enables simple translation. If chromatic shift varies across area or volume, register more landmarks across entire range.

To actually do the chromatic shift correction, check **Landmarks registration** in the **Registration** subtab in Lux Image Processor (§8.3). **Usage level** must be at least 2.

## **8.3 Lux Image Processor**

The **Image Processor** tab is where you can process the raw data in various ways before exporting it for further analysis. It will also auto-generate an **ims** file for Imaris. It can be used when the microscope is turned off.

If you see a blank page when you click on the **Image Processor** tab, go to **C:\Luxendo\ImageProcessor** and double click **ips.exe** to restart it.

In the **Tasks** box on the left, click **Add**. You can also **Import** an existing task to replicate and modify its settings. You can **Copy** a task to make multiple copies to compare the effects of different settings.

In the **Task Configuration** box on the right, give your new task a sensible name to avoid confusion later. Adjust **Usage level** to show fewer or more settings in the subtabs below, depending on how much control you want. While adjusting settings, you can mouse over a parameter's name to see a pop-up explanation.

In the **Input** subtab, browse to the **raw** folder containing your data. Select the **h5** file of the desired channel and time point.

The software uses zero indexing i.e. the first time point, first *z*-slice, first tile *etc.* is numbered 0. Index accordingly when telling the software what part of your data to process.

Click the **Export** button to select the destination folder.

### 8.3.1 Deskewing

Check the **Fusion** subtab. Shear transform only. No interpolation.

### 8.3.2 Blind deconvolution

**Fusion** subtab. Enabled in **Usage level 3**. Not recommended as it uses a blind deconvolution algorithm that does not incorporate **PSF**. Use the default values as starting points, adjust if needed and compare results. Set chunk size to maximum of 128 to stay within the workstation's **RAM**.

For more control and better results use **Huygens** (§8.4).

### 8.3.3 Tile stitching

If you wish to deconvolve (§8.4), do it before stitching.

You can alternatively do stitching in arivis (§8.5), Imaris or Fiji (§8.6).

In the **Input** subtab, choose channel, time point and tile with best signal as reference.

Tick the checkbox on the **Registration** subtab if you wish to include it in the task. Parameters:

- **subtract background** to make stitching accuracy less susceptible to noise. Generally, subtract the camera bias offset of 100. This setting is used for registration only and does not affect the final output.
- **minimum content** is the minimal summed pixel intensities of a processing chunk in order for the corresponding tile to be included in the stitched image. The recommended value is 5000.
- **Do fine alignment**: you should tick it, especially if there was sample movement.
- **Objective registration**: uncheck it as it doesn't apply to our system.
- **Tile registration**: select **re-register each time point** if the sample moved or changed shape during acquisition.
- **Remove outlier pairs** will compute the so-called cost of aligning each pair of adjacent tiles. High cost means the computed alignment is too good to be true, perhaps due to periodic artifacts in the data. The **Automatic with cost threshold** parameter lets you set a cost higher than which the computed alignment will be disregarded.
- **Maximum translation** is the maximal number of pixels you allow the tiles to be shifted to find alignment. Reduce it if two neighbouring bright points are mistakenly aligned as the same object.
- **Rotation** is not applicable to this system so ignore.

Tick the checkbox on the **Fusion** subtab if you wish to include it in the task. Parameters:

- **Image quality weighting**: uncheck as it is for rotating stages and multiple detection objectives which are not applicable to our system.
- **Blending**: tick it to get consistent intensity across tiles.
- **Subtract constant background value**: set to 0 if doing deconvolution. Otherwise recommended to use 80 which is slightly below camera bias offset, to avoid accidentally removing weak signals.
- **Load only full chunks/Fuse different cams**: not applicable to our system.

- **Half intensity:** divides the 16-bit intensities by 2. Tick for compatibility with Fiji's BigDataViewer (§8.6).

### 8.3.4 Output

Switch to the **Output** subtab.

**Bounding boxes options:**

- **UnionAll:** Creates a rectangular view of the full  $xy$  data range with any empty space (such as between two sets of tiles in different locations) filled with camera offset value.
- **UnionsOverlapping:** Creates a rectangular view of the full  $xy$  data range with the any space between non-overlapping tiles assigned intensity 0.
- **Intersections:** Retains data only in overlapping areas. Used for checking alignment of tiles.
- **Separate:** This will output individual tiles with no stitching. Select it only when you are making use of Lux to generate the `ims` file for stitching in Imaris.

**Down/up-sample factors** can be used to downsample large datasets to more manageable file sizes for comparing the effects of different image processing settings. A value of 2 will downsample by a factor of 2 and so on. Set the values to 1 if you wish to keep the original pixels. If **Depth (non-isotropic)** is unchecked, the software will upsample  $z$  to the  $xy$ -resolution and create an even larger file. Do not upsample **Width**, **Height** as the algorithm doesn't interpolate.

Remaining parameters:

- **Add views separately:** not applicable to our system.
- **Copy images to BigTIFF:** not recommended.

Click arrow button to run. [individual tasks vs batch]

The processed output plus the Imaris file will be written in a **Processed** folder in the same directory as the **Raw** folder (§8.1).

## 8.4 Deconvolution

Huygens is available on the microscope workstation and some of the image processing workstations. Huygens gives better deconvolution results than Luxendo's built-in blind deconvolution algorithm (see §8.3.2), although the former still doesn't account for the variation in PSF across the FOV.

If your data was acquired using stage scanning, deskew it first (§8.3.1) so you can deconvolve in 3D. If you acquired an experimental PSF, remember to deskew it too. If possible, crop your data to reduce file size before deconvolution.

Drag the `bdv.h5` file into Huygens. You can also drag individual `h5` stacks from the `Raw` folder.

In the **Microscopic Parameters** dialogue box, set the objective NA to 1.1, objective quality as good and coverslip position to a large number such as 500 to move it far away from the sample. Set the microscope type to light sheet unless you used the Airy beam for imaging, in which case select widefield. The effective light sheet NA is 0.5 as the laser slightly underfills the illumination objective of NA 0.7, so just type 0.5 in the light sheet NA field and type 1 in the fill factor field if asked. The light sheet direction is from left. If you used a Gaussian beam, look for the beam waist value in Table 4.1 (p. 14) that corresponds to the beam thickness you used for imaging, and enter the beam waist value in the sheet width field in the Huygens microscope parameters dialogue box.

You can use **Deconvolution Express** for fast and good but less reproducible results for display purposes, **Deconvolution Wizard** for more control and reproducibility, or the **Workflow Processor** for batch processing and additional control over deconvolution algorithms and parameters.

In **Deconvolution Express**, click **Adjust acuity**. Scroll through the slices and move the preview box around as you try out the different deconvolution profiles and adjust the smoothing parameter to reduce artifacts.

In **Deconvolution Wizard**, crop out any parts of the data that contain no in-focus  $z$ -slices, otherwise those parts will appear as haze in the deconvolved image. Set both background subtraction and brick layout to auto.

Save the final deconvolution output as an `ics2` file, as `tif` will clip the 32-bit float.

## 8.5 arivis Vision4D

Arivis is available on the microscope workstation. It is good for 3D reconstruction of very large datasets up to terabyte range using relatively little RAM. Imaris may produce slightly better 3D results but uses more RAM. Arivis is also good at membrane segmentation. PDF manuals are available by clicking **Help** in the menu bar.

To import data, drag the `xml` file (§8.1) into the Arivis window. If you have deconvolved the data in Huygens (§8.4) beforehand, shift-select the deconvolved stack of `ics2` files and drag them into the Arivis window. The data will convert to `sis` format, which occupies about 25% less disk space.

It is easy to make a 3D time-lapse movie in Arivis:

- **Data** → **Edit Structure**. In the window that appears, click the menu icon at upper right of **Time Points** column and select **Set Time Interval**.
- **View** → **Toggle Storyboard**.
- In the storyboard window, assign keyframes to desired time points.
- Set viewing angles and zoom factors for keyframes.
- Click **Export movie** icon at upper left of storyboard window.
- Enable time stamp with font size 15–20.
- Save as AVI at full **Data Resolution**.

## 8.6 ImageJ (Fiji)

Install the HDF5 plugin. Navigate to your data and drag the `xml` file onto the main Fiji window. Or you can open individual `h5` files with the following script:

```
file = getArgument();
run("Scriptable load HDF5...",
"load=["+file+"] datasetnames=/Data nframes=1 nchannels=1");
```

You can also open and do basic processing of large datasets with the `BigDataProcessor2` plugin. It streams `h5` datasets without loading the entire dataset to RAM. In the plugin window, go to **Open** → **Open Predefined File Series** → **Open Luxendo HDF5 Series** and browse to the `raw` folder. In the **Process** menu, you can align channels, crop your data in space and time *etc.*

To view unstitched tiles pieced together, use `Plugins` → `BigDataViewer` → `Open XML/HDF5` to open the `xml`. To stitch, use the `BigStitcher` plugin.

## 8.7 MATLAB

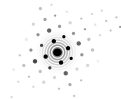
You can easily import `h5` data into `MATLAB`. See <https://www.mathworks.com/help/matlab/hdf5-files.html>. For handling large datasets with dynamically loaded filters, see [https://www.mathworks.com/help/matlab/import\\_export/read-and-write-hdf5-datasets-using-dynamically-loaded-filters.html](https://www.mathworks.com/help/matlab/import_export/read-and-write-hdf5-datasets-using-dynamically-loaded-filters.html).

## 8.8 High-performance computing

`CBIS` users can `ssh` into the facility's high-performance computing cluster (<https://www.dbs.nus.edu.sg/cbis/cbis-services/computational-resources>) and run jobs using the `pbs` scheduler. Software such as `Fiji` and `MATLAB` are available. Contact us for more information.

## 8.9 Data transfer

Portable drives are not allowed to be connected to the workstation. Please transfer via Internet to the `CBIS` server (`CBIS` users only) or other servers within two weeks of your imaging session.



## 9 Clean up and switch off

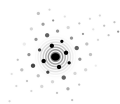
**Warning:** before zeroing the  $z$ -axis of the stage, make sure it is not coupled to  $y$ , otherwise the coupled  $y$ -value will run to the bottom and the sample holder will hit the illumination objective!

Zero all axes and remove sample. If using reusable dish, peel off FEP foil, scrape off the silicone residue with a toothpick and clean dish with 100% ethanol. Dispose of any used glass slides in the sharps bin.

Suck out the imaging medium from sample chamber with a dropper. Rinse a few times with purified water. Disinfect with 70% ethanol: rinse twice, on the second rinse leaving the ethanol inside for 3–5 minutes. Finally, rinse again with purified water. Wipe the inside of the chamber lid with tissue wetted with 70% ethanol.

Check the objectives with the torchlight. If necessary, clean them with lens paper and purified water followed by 100% ethanol if water is inadequate. Dab dry the sample chamber with KimWipes or C-fold towel. Bring down the lid but prop it open slightly to air the chamber.

Check the PPMS schedule. If you are the last user for the day, exit the software by clicking on the cross, shut down the computer and twist the two overhead sockets to off (in any order and no need to wait). Otherwise, just log out of PPMS. If applicable, turn off the temperature controller, gas controller and the main valve on the gas tank unless the next user has requested for them to remain on.



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