Time to Upgrade: A New OpenSPIM Guide to Build and Operate Advanced OpenSPIM Configurations

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OpenSPIM is an Open Access platform for Selective Plane Illumination Microscopy (SPIM) and allows hundreds of laboratories around the world to generate and process light-sheet data in a cost-effective way due to open-source hardware and software. While setting up a basic OpenSPIM configuration can be achieved expeditiously, correctly assembling and operating more complex OpenSPIM configurations can be challenging for routine standard OpenSPIM users. Detailed instructions on how to equip an OpenSPIM with two illumination sides and two detection axes (X-OpenSPIM) are provided, and a solution is also provided on how the temperature can be controlled in the sample chamber. Additionally, it is demonstrated how to operate it by implementing an ArduinoUNO microcontroller and introducing µOpenSPIM, a new software plugin for OpenSPIM, to facilitate image acquisition. The new software works on any OpenSPIM configuration comes with drift correction functionality, on-the-fly image processing, and gives users more options in the way time-lapse movies are initially set up and saved. Step-by-step guides are also provided within the Supporting Information and on the website on how to align the lasers, configure the hardware, and acquire images using µOpenSPIM. With this, current OpenSPIM users are empowered in various ways, and newcomers striving to use more advanced OpenSPIM systems are helped.

1. Introduction

Multiview light-sheet imaging has become an important, often mandatory, technique to study developmental dynamics of living biological samples.[1] Due to its meticulous instructions and various solutions regarding assembly, operation, and image processing, the open-source platform OpenSPIM (https://openspim.org/) makes it possible to custom-build affordable and robust light-sheet microscopes capable of acquiring high resolution multiview optical sections of fluorescent samples.[2,3]

However, setting up a light-sheet microscope and acquiring multiview datasets remains a complex task.[4] In particular for newcomers, every step, from purchasing parts over assembly to operation and finally processing light-sheet data, can pose unexpected challenges, such as incompatible hardware pieces, driver issues, software crashes, etc., which quickly accumulate when modifications from the most basic OpenSPIM design are made.[5]

Nevertheless, the original OpenSPIM design is well-known for its extensibility and options for customization, a key feature as many laboratories seek to adapt it to their unique imaging applications. When the OpenSPIM platform was launched back in 2013, selective plane illumination microscopy (SPIM) was already a well-established imaging technique and led to several types of four-lens light-sheet microscopes equipped with more than one camera.[6–8] Therefore, it was anticipated that the original design, which comprises only one illumination lens and only one detection lens, also known as the L-configuration, would be extended into more complex configurations featuring geometries with three or four lenses.[2]

A major difference of such geometries is that illumination and acquisition can happen from different views simultaneously. This is advantageous over setups where the sample is rotated and subsequently imaged from multiple angles, because it reduces phototoxicity or bleaching of fluorophores and reduces acquisition time by the factor of additional cameras used.

An OpenSPIM assembled with a four-lens geometry is referred to as the X-OpenSPIM configuration (see Figure 1) and is potentially, for many users of the OpenSPIM community, the obvious next step. An X-OpenSPIM demands aligning two illumination axes, two opposing cameras as well as coaligning the focal plane of both detection objectives. Additionally, the imaging software must be configured accordingly to synchronize all devices with each other. This can be improved by using an Arduino board, a single piece of affordable hardware (https://www.arduino.cc/), which can control several hardware...
devices used in light-sheet systems such as laser shutters, stepper motors, filter wheels, and galvanometric mirrors[9] and makes it much easier to control, e.g., multiple lasers.

Besides phototoxicity, temperature is a significant factor that influences all biological processes including developmental speed and viability of living samples. For these reasons, we show how the chamber temperature can be controlled by installing a Peltier element below the acrylic chamber.

In summary, building X-OpenSPIMs creates additional complexity and may come with the danger that such OpenSPIM systems may become abandoned due to challenges in operation and maintenance that are already present in the early test stages (hardware- or software-wise), a time where the ground still needs to be prepared to generate satisfying acquisition results or any acquisition results at all.

It is the aim of this publication to guide OpenSPIM users to build more advanced OpenSPIM configurations such as the X-OpenSPIM and to facilitate image acquisition, in both, basic and advanced OpenSPIM systems, and to help operating these systems by providing a completely overhauled Micro-Manager (μManager)-based[10] OpenSPIM plugin, namely, μOpenSPIM.

2. Implementation

2.1. Overview for Assembling an X-OpenSPIM

The assembled X-OpenSPIM is based on the original L-OpenSPIM design but has an improved acquisition speed, a higher magnification, simultaneous multiview acquisition, and more evenly distributed sample illumination. The temperature within the sample chamber can be controlled. It features two laser lines (VersaLase), two illuminations, and two detection axes, for which new objective lenses (Nikon, 10× objectives for illumination and 40× objectives for detection), and appropriate tube lenses and adapters must be acquired. The latter also requires minor modifications of the infinity space tube. Two cameras (Andor) are used and aligned to each other by an newly installed corner mirror mount holding an elliptical mirror into one of the detection axes as demonstrated, e.g., with the educational selective plane illumination microscope (eduSPIM).[11] For coaligning the focal plane of both detection objectives, we propose a simple design for their manual adjustments along the z-axis.

Finally, one of the two cameras is used to trigger the available laser lines as well as the second camera by interposing the ArduinoUNO controlled circuit.

2.2. Overview of the New OpenSPIM Plugin (μOpenSPIM)

OpenSPIM systems are typically run by μManager, a ready-to-use open-source image acquisition software that is compatible with a wide range of hardware devices and therefore gives OpenSPIM users a fast entry point. With μManager’s “multidimensional acquisition tool” any OpenSPIM is functional. However, upon the initial release of OpenSPIM, a specific plugin was developed to provide a place, which would make it easier for OpenSPIM users, to set up complex time-lapse recordings with multiple angles (multiview imaging) and featured a function, which compensates for samples drifting out of the field of view.[2]

The plugin itself relies on μManager version 1.4, comes with its own specific graphical user interface (GUI) (see https://openspim.org/images/Stagecontrols.png) and is tailored for the original OpenSPIM hardware with a single detection axis and one camera. Therefore, it supports, e.g., only a single laser line (OBIS 488 nm LS 100 mW, Coherent, Inc., Santa Clara, CA, USA) and because of such limitations, the plugin has become obsolete for users with more complex OpenSPIM systems. Additionally, μManager has gone through significant changes and improvements and is currently available for download as μManager 2.0.0 version.

Here we provide a detailed description how an X-OpenSPIM and other OpenSPIM configurations can be operated using μOpenSPIM, which runs on the newly available μManager 2.0.0.
2.2.1. Summary of New Plugin Features

- A complete overhaul of the GUI has been made including simple graphic visualizations and an improved control over Picard’s 4D-stage.
- A user-friendly way of setting up multiview time lapse recordings with several positions and the option to acquire periodic and sporadic intervals with optional breaks during time-lapse recordings.
- A quick save function for user specified acquisitions settings to save time in case an imaging session is interrupted, or a similar session will take place at another time.
- Different saving formats are available including single plane tiff files, whole stacks and the n5 format. The latter is optimized for browsing through big data.
- ArduinoUNO support provides more efficient control of several connected hardware devices, which can improve acquisition speed and hardware synchronization.
- The possibility for on-the-fly GPU-accelerated image processing using the CLIJ library\(^{[12]}\) has been implemented and is demonstrated by an on-the-fly fusion option of two simultaneously acquired views.
- A revised drift-correction function with new options that can help with keeping a drifting sample within the field of view during long term image acquisition.

2.3. Upgrading the Original OpenSPIM Design into an X-OpenSPIM Configuration

As mentioned above going from the original L-configuration\(^{[2]}\) to an X-OpenSPIM requires doubling all optical elements for the illumination and detection axis and the purchase and manufacturing of additional parts that are described below and summarized in Tables S1–S3 of the Supporting Information. All additional parts needed for the upgrade and blueprints of all self-made parts can be accessed under the following link: https://openspim.org/table_of_parts_xopenspim.

A complete overview of the fully assembled X-OpenSPIM is shown in Figure 2.

2.3.1. New Illumination/Detection Objectives and Tube Lenses

Two illumination objectives and two detection objectives are needed. They should be suitable for the four-lens geometry of the acquisition chamber by providing long enough working distances. This gives more freedom regarding sample positioning. It is worth noting that new detection objectives require new holder rings and appropriate tube lenses; e.g., using new Nikon objectives (CFI Apochromat NIR 40× W), as demonstrated with this X-OpenSPIM, requires a new tube lens with \( f = 200 \) mm, as well as new tube lens adapters (SM2A20, Thorlabs) and an additional self-made linker part to connect the tube lens adapters (SM2A20, Thorlabs) with the previously used Olympus U-TV1X-2 Camera Adapters. Details of such objective holder rings and linker parts can be found and downloaded on the website (https://openspim.org/Table_of_parts_X-OpenSPIM).

Attention should be paid to the installation of both tube lenses, meaning that the same distance between tube lens and camera chip must be ensured on both detection axes respectively to avoid potential magnification deviations. For verification, we recommend measuring the distance between beads that have been acquired with both cameras after the alignment.

Figure 2. Rendering of the X-OpenSPIM (top view). The light path of the laser beam is shown in cyan.
of the X-OpenSPIM has been completed (see Figure S1, Supporting Information).

2.3.2. Acquisition Chamber Modifications to Fit New Objectives

To fit four water dipping objectives, a new acrylic sample chamber and a new metal chamber holder must be purchased or self-made by a mechanical workshop. Drafts of the described X-OpenSPIM chamber (see Figure 3A) created with Inventor can be used as an inspiration and have been uploaded to the original OpenSPIM website where they can be accessed under the following link https://openspim.org/table_of_parts_xopenspim. Notably, slight shape differences between the illumination and detection objectives, specifically at the approaching angle at the end of the objectives, led to a rectangular shaped and larger acrylic sample chamber with a bottom size of 56 × 40 mm, as can be seen in our drafts on the website. The sample chamber holder is also larger to adjust for the new objectives with a bottom size of 123 × 123 mm. The acquisition chamber therefore requires more space on the optical breadboard as it is the case for the original I-OpenSPIM sample chamber.

We would like to point out that similar X-OpenSPIM chambers can be purchased from Pieter Fourie (http://www.pfde.co.uk/) for Olympus and Nikon objectives.

2.3.3. Modifications of the Objective Holder Ring and Chamber Holder for Coaligning Both Detection Objectives

To coalign the focal plane of both detection objectives to the light-sheet, at least one of the two objective holder rings must be equipped with a small handle (Figure 3A). Both handles can be grabbed to gently slide the objective forward or backward along the detection axis. This modification is necessary to coalign the focal plane of both detection objectives.

The sample chamber holder modifications include corresponding rails and cut-outs to facilitate in- and outward gliding of the objectives along the detection axis. The inner part of the infinity tubes, which connect to the sample chamber holder, also need to undergo minor modifications (cutouts), to provide enough space while coaligning the detection objectives.

We recommend making both detection objectives adjustable.

2.3.4. Upgrading the Picard Industries USB-4D-STAGE

New objectives can lead to slightly larger acquisition chamber dimensions and may require replacing the original sample arm holder (Picard Industries) with a new custom-made one. In our case the self-made acquisition chamber required a 2 cm longer sample arm holder, which we additionally equipped with a novel syringe holder. The holder can be glued above the pulley and allows to quickly immobilize the syringe using a single screw wrapped in Teflon tape to increase the friction in the thread (Figure 3B).

Finally, the standard O-ring belt drive of the rotational stage axis arm should be replaced with a new tooth-belt drive to achieve a more precise sample rotation. This can be purchased from Picard Industries (http://picardindustries.com/).

2.3.5. Replacing Telescopic Lenses, Mirrors and Adding a Beam Splitter

We suggest replacing the lenses of the first telescopic system by two lenses of 19 and 75 mm focal lengths, respectively, resulting in a slightly thinner light-sheet. The original OpenSPIM design by contrast comprises two lenses of 25 and 50 mm focal length. Another minor change concerns the orientation of the lenses. The first telescopic lens (with 19 mm of focal length) should face the arriving beam, while the second telescopic lens (with 75 mm of focal length) faces the direction of the beam as shown in Figure 3C.

We replaced all Ø1" Mirrors by Ø1/2" Broadband Dielectric Mirrors (BB05-E02, Thorlabs) mounted on a Kinematic Mount (KM05/M, Thorlabs), respectively. However, this is not an essential modification and there is no reason why other mirrors, including Ø1" Mirrors should not be used. What we do recommend is using a Ø1" Mirror (BB1-E02) mounted on a Gimbal Mirror Mount (GM100/M, Thorlabs) instead of the original corner mirror (see Figure 3D), because it gives better control for the final beam aligning step on the sample. Additionally, we placed one of the reflecting mirrors into a pedestal post holder (PH20E/M, Thorlabs), which was clamped to the breadboard by a clamping fork (CF125, Thorlabs) and mounted on an optical post (TR20V/M, Thorlabs). This, in our opinion, gives higher flexibility to adjust the reflecting mirrors while rough aligning the beam along the rails. To split the laser beam for each illumination axis a beam splitter must be installed (e.g., BS004, Thorlabs) and therefore mounted into a cube adapter (BS127CAM), which in turn must be placed into a cage cube (CCM1-4ER/M, Thorlabs).

Another small modification worth mentioning is abrading the emission filter holder top by about 1 mm to better fit the filter holder into the infinity space slits.

2.3.6. Corner Mirror Installation for One of the Two Detection Axes

To correctly align both fields of view from the two Andor sCMOS cameras, a dielectric elliptical mirror (BBE2-E02, Thorlabs) has to be installed into one detection axis. Using a 2" corner mirror mount (KCR2EC/M, Thorlabs) allows to flip one half of the detection axes, comprising the tube lens and camera adapters by 90°. The weight of the camera can be sustained, e.g., by using cut off ends of optical rails (RLA300/M, Thorlabs) as shown in Figure 3E. Now the field of view of both cameras can be matched mechanically by using the fine adjustments of the corner mirror mount knobs (Figure 3E, white arrows).

2.3.7. Additional Acquisition Chamber Modifications to Obtain Temperature-Control

For some OpenSPIM users, it can be crucial to control the temperature within the acquisition chamber, e.g., when it
Figure 3. New upgrades and modifications to assemble the X-OpenSPIM. A) Depicted is the fully assembled X-OpenSPIM acquisition chamber with two adjustable 40× Nikon detection objectives. B) The modified 4D-USB stage from Picard Industries with a replaced self-made sample arm holder, a new syringe holder, and the installed upgrade kit from Picard, which uses a more precise tooth-belt drive. C) Rendering of the two telescopic lenses with 19 and 75 mm focal length with arrows depicting the direction of the laser beam. D) Gimbal Mirror Mount with two adjustable knobs. Within its central mirror the horizontal light-sheet has been indicated in green. The cylindrical lens creating the light-sheet is visible on its left. E) The new corner mirror is shown, that is now present in one of the two detection axes to mechanically align the two camera views by using the two mirror mount knobs. F) Cross-section of the assembled cooling chamber showing the Peltier element resting between the heat sink and the acrylic chamber. G) Top view of the assembled acquisition chamber with cooling function mounted into the metal chamber holder of a fully assembled X-OpenSPIM. The indicated plastic handle, Teflon spacers, and nylon screws are needed thermally to insulate the objectives from the heatsink.
comes to in vivo long-term recordings or imaging of temperature-sensitive organisms. Adding a temperature control to the sample chamber can be achieved by placing a Peltier element (TEC12706, 40 × 40 × 3.8 mm, 65 W cooling power) under the sample chamber. We kept the acrylic chamber, typically used in OpenSPIM systems, for its biocompatibility, despite its low temperature conductivity compared to metal. Additionally, acrylic can easily be machined in most workshops and OpenSPIM user might be able to reuse an already existing acrylic chamber for implementing temperature control. To compensate for the low thermal conductivity, the acrylic chamber thickness at the bottom has to be reduced to 1 mm thickness. And to better distribute the heat flux and to improve temperature homogeneity, we recommend adding thermal mass around the acrylic chamber by installing four cooling walls made out of aluminum.

The Peltier element is placed inside a compartment on top of a customized heat sink and contacts an overlying 2 mm thick aluminum plate (cooling plate), which in turn contacts the acrylic sample chamber and the four cooling walls (Figure 3F). The heat sink has a circular deepening to fit an additional O-ring that seals the Peltier and prevents it from getting wet by water leakage or condensation. The Peltier element has to be inserted evenly by using four metallic screws mounted on Belleville and fiber washers to ensure thermal insulation. The heat sink is attached to the chamber holder, carrying the four objectives, using four M6 screws. Details of the assembly are shown in Figure S2 of the Supporting Information.

The four water dipping objectives contact the liquid inside the chamber and represent a major source of heat dissipation. Therefore, they need to be thermally insulated from the heat sink with 1 mm Teflon rings and nylon screws before mounting them into the sample chamber holder. The sample chamber holder is sitting on rails that are screwed to the optical breadboard, which acts as a large heat sink.

Temperature control is implemented with a temperature sensor (NTC probe, K-type, etc.) placed inside the chamber, with direct liquid contact and connected to a PID controller (TLK33, Ascon Tecnologic). The PID controller is powered with a switching power supply (for instance, Mean Well RD-125A, or TRACOPOWER TXL 100-0512DI) with sufficient output power and voltage to drive the Peltier unit. The Peltier controller allows for both cooling and heating.

Thermometer stickers can be added to the cooling walls and the sample chamber holder for direct visual indication of the temperature and efficiency of the cooling and heat dissipation.

To ensure proper heat transfer, the interfaces between the individual parts are filled with a thin layer of thermal paste (Silicon thermal grease S606C, RS PRO). All places that could expose the Peltier unit to liquids should be greased with silicone grease, namely, the O-ring, the screws attached to the sample chamber and the holes for the Peltier wires.

Drafts and detailed assembly figures for the described cooling chamber have been created with Inventor and uploaded to the original OpenSPIM website, where they can be accessed under the following link: https://openspim.org/table_of_parts_xopenspim.

2.4. Configuring Multiple Cameras (Andor sCMOS) in µManager

The described X-OpenSPIM features two Andor sCMOS Neo-5.5 cameras of which the exposure digital output of the designated ("master") camera is wired to the second ("slave") camera as shown in Figure 4. In case Andor sCMOS cameras are used, the Andor SDK3 has to be downloaded from Andor and installed into the working µManager directory (see also the µManager website: https://micro-manager.org/wiki/Andor_SDK3).

If one is not yet familiar with µManager’s Hardware Configuration Wizard, its Device Property Browser and how to create Configuration “Groups” and “Presets”, we recommend reading through µManager’s Configuration Guide: https://micro-manager.org/wiki/Micro_Manager_Configuration_Guide.

It is also recommended to read through µManager’s Multi Camera configuration guide, which covers the same steps in greater detail on the website: https://micro-manager.org/wiki/Utilities#Multi-Camera.

We depicted all necessary steps on how to configure the two sister cameras in µManager (see Guide S1, Supporting Information). Furthermore, the guide can be followed and accessed on our website (https://openspim.org/micro-openspim_micro-manager-configuration), where additional video guides are available.

2.5. Setting Up the ArduinoUNO Microcontroller

Arduino is an open-source project that provides a low-cost solution to control all kinds of devices through its microcontroller, a programmable digital input/output board.

The described X-OpenSPIM takes advantage of this microcontroller to deal with the synchronization of both cameras and to control the two laser lines. The initial TTL trigger-pulse is provided by the “master” camera and received by the Arduino board, which has six digital output pins (Pin-8 to Pin-13), of which two, namely Pin-13 and Pin-12, are used to trigger the two laser lines (488 and 561 lasers of the VersaLase System) as depicted in Figure 4.

To work with µManager, the Arduino board must be initially programmed, meaning that an appropriate “firmware” has to be uploaded. We chose the ArduinoUNO board, because it benefits from its known functionality with µManager. Detailed installation guidelines can be accessed at the µManager website: https://micro-manager.org/wiki/Arduino

To facilitate setting up an Arduino board in µManager, we created a detailed step-by-step guide (see Guide S2, Supporting Information), which is also available on the OpenSPIM website (https://openspim.org/micro-openspim_micro-manager-configuration).

2.6. Laser Alignment of the X-OpenSPIM System

It can take time to learn how to optimally align an OpenSPIM system and there are different ways and always room to improve. With two illumination sides and two detection objectives this task becomes particularly challenging. Therefore, we provide a simple, reproducible way. Our alignment guide describes how to initially align the beam along the rails, how to visualize and
tune the beam within the field of view of the cameras and finally aligning the created light-sheet on the sample. The guide is accessible in Guide S3 of the Supporting Information. We recommend also accessing the original description of calibrating the light-sheet in an OpenSPIM (https://openspim.org/Light-sheet_Calibration) where alignment is shown in a slightly different way.

2.7. Getting Started with \( \mu \text{OpenSPIM} \)

\( \mu \text{OpenSPIM} \) is a \( \mu \text{Manager} \) Version 2.0 gamma plugin, written in Java, that allows image acquisition for all OpenSPIM configurations. It comes with its own GUI and several features that we present here for the first time.

2.7.1. Requirements and Installation for \( \mu \text{OpenSPIM} \)

All hardware components of an OpenSPIM system (Laser, Camera, Stage, etc.) have to be preconfigured with \( \mu \text{Manager} \) using Version 2.0 gamma on a computer running Windows 10. \( \mu \text{Manager} \)’s hardware configuration wizard has to be used to create a working configuration (.cfg) file containing all hardware devices. This configuration file will be read by \( \mu \text{Manager} \) upon the startup of \( \mu \text{OpenSPIM} \).

In case one is not yet familiar with \( \mu \text{Manager} \)’s Hardware Configuration Wizard, we advise reading through \( \mu \text{Manager} \)’s Configuration Guide first (https://micro-manager.org/wiki/Micro-Manager_Configuration_Guide) or watching the available screencast (https://micro-manager.org/Screencasts). We also provide a video showing how a working configuration file was created from scratch for the described X-OpenSPIM using \( \mu \text{Manager} \)’s Hardware Configuration Wizard. The video can be accessed on our website: https://micro-manager.org/wiki/Micro-Manager_Configuration_Guide.

To install \( \mu \text{OpenSPIM} \), please visit https://openspim.org/micro-openspim for installation files and guides.

2.7.2. The GUI of \( \mu \text{OpenSPIM} \)

\( \mu \text{OpenSPIM} \) consists of several configurable tabs (see arrow in Figure 5), which are explained in more detail below, in particular when it comes to the acquisition panel. They comprise a) Acquisition, b) ArduinoUNO, c) Console, d) Editor e), Lasers, and d) Stage.

a) Acquisition: In the “Acquisition” panel (Figure 5A–I) users can acquire single images, stacks and set up long-term image acquisition sessions. We recommend visiting our website for more detailed descriptions: https://openspim.org/micro-openspim.

Defining Imaging Stacks: To add a 3D stack to the positions list (Figure 5A), a new stack must be defined using the blue “Z-start” and “Z-end” buttons and by selecting the desired Z-step size (Figure 5B). The Z-step size value is predefined according to the positional stage resolution specified in the...
hardware configuration, e.g., the minimum step size of a Picard
4D-Stage is 1.524 μm.

After the stack has been defined, it can be added to the “Posi-
tions/Angles” window by pressing the “Add Z-stack” button.
This also takes the current rotational position into account. In
this way, views or positions can be added to the list. Within the
“Position” panel, positions can also be added without a defined
z-stack and wrong position settings can be edited or whole posi-
tions (XYZR) either deleted or dragged into different orders
using the arrow buttons.

Setting Up Time Points (Using Periodic or Sporadic Intervals):
Time points are added within the “time points (TP)” panel
(Figure 5C). Users can set up the desired number of time points
with either periodic or sporadic intervals. It is also possible to
add breaks to skip, e.g., unwanted time points during acquisi-
tion. This can be useful when the precise timing of develop-
mental events of interest is known or to reduce vast amounts of
SPIM data after key events have already taken place.

Adding Channels: The channels area (Figure 5D) is used to set
up multiple laser lines or other devices wired to the ArduinoUNO
board. They can be selected simply by ticking the available check
boxes, which correspond to the digital output pins of the Arduino
board (also shown in Figure 5D). In case the OpenSPIM is not
equipped with an Arduino, laser channels can be added under
the “Software Controlled” tab by clicking the “Add a channel”
button (depicted in Figure S3, Supporting Information).

Saving Images and User Settings: Images can be saved in three dif-
ferent formats 1) as single plane tiff files, 2) as ometiff image stacks,
and 3) as a N5 format (see https://github.com/saalfeldlab/n5)
(Figure 5F). The advantage of the latter file format is, that it can
be directly opened with Fiji’s BigDataViewer.[13] It is also pos-
sible and recommended for time-lapse recordings in particular
to enable the “save Maximum Intensity Projections of each TP/
stack” option (Figure 5F). Maximum intensity projections will
then be saved while image acquisition takes place (on-the-fly).

Users also have the possibility to save current or load previ-
ously specified acquisition settings by using the “Save/Load
Settings panel” (Figure 5G).

Acquisition Features and Acquisition Preview: Other acquisition
options, that can be enabled, are drift-corrections, specifying a
region of interest and changing the desired camera binning
values (Figure 5H).

Figure 5. The acquisition panel allows users to A) add positions/angles, B) define the z-stack, C) add time-points, D) select created channels and change their exposure times, E) take a look at a summary of the imaging ongoing session based on its current settings, F) define the saving folder and saving options such as save maximum intensity projections of acquired stacks on-the-fly and G) Save all current user settings and load them at a later imaging session. H) Acquisition options allow to enable drift-correction function, change the binning values of the camera(s), specify a region of interest and to enable the “On-the-fly processing” option. I) “Preview of imaging session” panel at the bottom provides a schematic visualization of the current time-lapse session and indicates overall acquisition progress during imaging. Other panels can be added to the acquisition panel such as J) the “Stage” controls for Picard’s USB 4D-Stage and K) the Console panel.
We added a “Preview of imaging session” panel at the bottom (Figure 5J), which provides a schematic visualization of the current time-lapse session and indicates overall acquisition progress during imaging.

**Customization of μOpenSPIM:** All panels within μOpenSPIM can be customized and saved as shown in more detail on the OpenSPIM website (https://openspim.org/micro-openspim-GUI). As depicted in Figure 5, it is also possible to add other panels such as the “Stage” controls for Picard’s USB 4D-Stage (Figure 5J) or the Console (Figure 5K) to, e.g., the “Acquisition” panel.

b) ArduinoUNO: Within the “ArduinoUNO” tab any triggered device can be named inside the row of its corresponding output pin. Once specified, a device name will show up within the “Channels/Pins” area of the “Acquisition” panel when the “Arduino Controlled” option is selected.

In case of our X-OpenSPIM, the Arduino pins 12 and 13 are wired to the digital beam modulation inputs of a 488 and 561 nm lasers as depicted in Figure 4 and renamed in the software accordingly (see Figure S3, Supporting Information). The digital outputs across pins 8 to 13 are set via the Arduino-Switch-State number, which μOpenSPIM sets automatically by taking the selected devices in the “Channels/Pins” area into account.

c) Console: The console window can be useful to see what is going on in the background of the plugin and to spot potential error messages.

d) Editor: Similar to μManager’s “ScriptPanel,” which provides the possibility to create and load scripts written and run in BeanShell, there are two editors accessible in μOpenSPIM: Java and BeanShell. The μManager website explains how a BeanShell script can be edited and run (see https://micro-manager.org/Script_Panel_GUI) and provides a number of example scripts, which can change, e.g., the acquisition behavior of μManager.

We added, next to the BeanShell editor, also the Java editor, where example script can be loaded, tested, and modified.

**On-the-Fly GPU-Accelerated Image Fusion Using the Java Editor:** There are two types of methods supported μOpenSPIM’s Java editor: The “main()” method is invoked once by clicking the “Run” button and processes an open image. The “process()” method is called every time an image is received during acquisition. Hereby, the “Run” button compiles the user script, invokes the “main()” method once and attaches the “process()” method to the acquisition event listener. Users can easily check the compile errors if there are errors to be corrected as well as print the system information within the Console window with relevant methods called in the “main()” method. The provided “ClijxMaxFusionDoG” example script enables the fusion of two acquired stacks, generated by each of the two cameras. For this the “on-the-fly image processing” box must be enabled in the Acquisition panel tab “On-the-fly” (Figure 4H). According to the example script, fusion takes place via the “maximumImages” operation ([https://clij.github.io/clij2-docs/reference_maximumImages](https://clij.github.io/clij2-docs/reference_maximumImages)) and is then followed by the “differenceOfGaussian3D” function (see [https://clij.github.io/clij2-docs/reference_differenceOfGaussian3D](https://clij.github.io/clij2-docs/reference_differenceOfGaussian3D)) and finally by a maximum intensity projection of the image stack along Z ([https://clij.github.io/clij2-docs/reference_maximumZProjection](https://clij.github.io/clij2-docs/reference_maximumZProjection)). Notably all these operations take place on the graphics processing unit (GPU) using the GPU-accelerated image processing library (CLII2) and without the need of initially saving the two stacks to the hard drive. The script can be edited and the operations replaced by hundreds of other image processing functions provided and explained on the CLIJ platform (see [https://clij.github.io/](https://clij.github.io/)). In this way, on-the-fly GPU-accelerated image processing becomes an easily accessible tool within μOpenSPIM.

Be aware that, depending on the size of acquired images and the indented operations on the GPU, a good graphic card may be required with memory capacities of 4GB or higher.

e) Laser (VersaLase): The laser power can be changed using various laser lines, e.g., Coherent OBIS Laser, Vorton Stradus VersaLase and Cobolt Lasers (Hübner Photonics). However, there is a possibility that for some lasers this control window may not work properly. In such a case we recommend using μManager’s GUI to set and change the laser power.

Note that other settings beside laser power (e.g., exposure time) can also be made using μManager’s GUI. We recommend not to change the same settings in both interfaces but rather stick to one interface.

**Stage Control:** In case the Picard stage is connected, all stepper motors (X, Y, Z, R) can be controlled immediately after μOpenSPIM’s startup. An example of the R stage control is shown in Figure 5J.

In case the sample holder rotation deviates from a single full revolution when moving the R stage from 0° to 360°, we added a calibration window to find the correct step size value for the R-stage. The window can be accessed by clicking “Calibrate” button. The correct value, once found, should replace the incorrect value of the Picard Twister step size, which can be accessed using μManager’s “Hardware Configuration Wizard and by selecting the Picard Twister.”

3. Demonstration of the Described X-OpenSPIM System

We tested our four-lens X-OpenSPIM on embryos from the flour beetle *Tribolium castaneum*. Because our OpenSPIM is equipped with 40× detection objectives, these embryos are too large to fit into a single field of view. To acquire the entirety of the embryo we set up two positions, position 1 (top) and position 2 (bottom), and imaged 3D-volumes with a Z-step size of 3 μm using both Andor cameras simultaneously.

**Figure 6** and Movie S1 (Supporting Information) show the development of the beetle as maximum intensity projections. Both views (Camera 1 and Camera 2) were captured simultaneously every 6 min for more than 30 h. The dataset was not processed in any way except for background subtraction and bleach correction using Fiji and demonstrated that the X-OpenSPIM is capable of producing 4d-volumes of high quality over long acquisition periods. It becomes also clear how much information is gained from the second view, which is simultaneously captured without the need of rotating the sample.

We would like to point out that the axial (z) resolution in the described X-OpenSPIM could be greatly improved by
generating more advanced light-sheets (e.g., nondiffracting light-sheets such as Bessel beam\cite{14,15} or a light-sheet that is axially swept through the sample (ASLM\cite{16}). This could be a desirable enhancement for many applications.

4. Sample Preparations

_Tribolium castaneum_ embryos used for live-imaging came from the EFA-nGFP transgenic line,\cite{17} where a nuclear-localized GFP reporter is ubiquitously expressed in the animals.

Live embryos were briefly checked for fluorescent signal, then incubated in 1% low melting agarose and immediately sucked into glass capillaries. The capillary was mounted into the X-OpenSPIM chamber using a 1 mL BD Plastipak syringe (REF 300013) and imaged at room temperature.

For the alignment guide, 1% of low melting agarose containing beads (1:4000 XC Estapor Fluorescent Beads) was used for visualizing the laser beam and the alignment of the light-sheet.

5. Conclusion

OpenSPIM is a widely used open-hardware platform to build light-sheet microscopes. But to upgrade to more advanced OpenSPIM systems, less well described hardware and software modifications become necessary. To simplify and accelerate this process we provided detailed instructions of how to get from a basic OpenSPIM L-configuration to a more advanced multilaser controlled and hardware synchronized X-configuration system, which is capable of acquiring data from two views simultaneously.

We describe in detail necessary hardware modifications, e.g., to coalign the focal plane of the two detection objectives, to coalign both cameras and other minor modifications, provide the appropriate software configuration changes that have to be made within μManager, present a new plugin, which we developed for OpenSPIM users with the aim to operate basic as well as more advanced systems such as the one described here and created detailed guides that depict step-by-step how to align an X-OpenSPIM and how to configure μManager and implement...
new hardware, which is recommended for more advanced OpenSPIM systems.

The user-friendly GUI of μOpenSPIM makes it easy to set up complex multiview time lapse recordings and supports an ArduinoUNO microcontroller, which is a worthwhile enhancement for OpenSPIM systems in general.

OpenSPIM users and the OpenSPIM community will benefit from several features our new plugin provides. We encourage them to build more advanced OpenSPIM configurations in case their biological applications will require or benefit from it. All our modifications are open access and explained in detail on the OpenSPIM website and we welcome contributions by the community.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
J.G. and P.T. designed the project and wrote the manuscript. J.G. built and tested the X-OpenSPIM, acquired and processed imaging data, troubleshoot the software, and prepared the figures for the manuscript. H.M. created, programmed, and tested μOpenSPIM. C.B. modified the acquisition chamber and sample arm holder of Picard's 4D-USB stage, installed the cooling chamber, and provided renderings of the X-OpenSPIM and individual parts and improved the manuscript. R.H. modified μManager to make it suitable for GPU-accelerated image processing (CLIJ), helped to implement on-the-fly fusion via CLIJ in μOpenSPIM, and improved the manuscript.

Data Availability Statement
MicroManager-gamma nightly builds are available at https://micro-manager.org. μOpenSPIM is available at https://openspim.org/micro-openspim. The software plugin "μOpenSPIM" is openly available in "Github" at https://doi.org/10.11101/0000-0007-F040-1.

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μOpenSPIM, CLIJ, light-sheet microscopy, micro-Manager, open sources, OpenSPIM, plugins, softwares

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