An open-hardware sample mounting solution for inverted light-sheet microscopes with large detection objective lenses

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Summary

Implementations of light-sheet microscopes are often incompatible with standard methods of sample mounting. Light-sheet microscopy uses orthogonal illumination and detection to create a thin sheet of light which does not illuminate the sample outside of the depth of field of the detection axis. Typically, this configuration involves a pair of orthogonal objectives which constrains the positioning and length of cover slips in the range of the detection objective. Here, we present an open-hardware sample mounting system for light-sheet microscopes using large detection objectives, built using 3D printed components and demonstrate the chamber’s efficacy on live biological samples in a custom light-sheet microscope.

Introduction

During the past decade, light-sheet technology has emerged as an alternative to the well-established confocal microscope. A significant drawback of light-sheet fluorescence microscopy (LSFM) is that sample mounting can be cumbersome. There have been several attempts at imaging on flat glass in order for light-sheet microscopes to be compatible with favoured sample mounting techniques. The simplest method involves using a pair of objectives with an aperture angle of less than 90° and mounting those above a sample holder. Figure 1(A) shows how the di-SPIM (Kumar et al., 2016) uses a pair of 40 × 0.8 numerical aperture (NA) objectives to allow a glass slip to be inserted, consequently sacrificing NA when compared to larger detection objective lenses. The NA is then reclaimed through image fusion, albeit after the image data for two volumes are acquired from two cameras, thereby halving temporal resolution. The lattice light-sheet (Chen et al., 2014), for instance, chooses a special excitation objective pair such that the sum of its solid angles of its NAs does not interfere with the glass raised from below, as seen in Figure 2. To do so, a custom excitation objective is mounted along with Nikon 25 × 1.1 NA objective at angles to the sample mounting section that allow a sample chamber to be inserted, as shown in Figure 1(B).

As depicted in Figure 3, other techniques for light-sheet imaging require specialist sample mounting and preparation to be employed. These methods present a barrier that can discourage new users of light-sheet microscopy: this paper presents protocols and open-source designs, using 3D printing technology, to help dispel this barrier. 3D printing allows the home user to print structurally integral and disposable shapes with the ability to reconfigure these shapes to their needs.

Here, we present open-source CAD files (Russell, 2020) for a design that we have optimized for a sample chamber that is compatible with an inverted selective-plane imaging microscope (iSPIM). Our design allows for the imaging of cell culture and embryos in their native conditions for long-term microscopy.

Design and construction

The sample chamber presented here is designed around a pair of objective lenses, a 25 × 1.1 NA long working distance (LWD) objective and a 10 × 0.3 NA long working distance water-dipping Nikon objective (Russell et al., 2018). The detection objective (the same as the lattice light-sheet detection objective) is the largest Nikon NA long working distance water-dipping objective lens,¹ whereas the excitation lens is the largest Nikon long working distance water-dipping lens that will fit next to the imaging lens. Both choices are standard parts and do not require custom engineering. In addition, both lenses are used for physiological imaging, and are widely employed in the field of light-sheet microscopy (Chmielewski et al., 2015; Chen et al., 2014). Figure 1(C) shows how this

¹ Olympus provide a similar lens with 1.0 NA and 2.0 mm working distance.
Fig. 1. Sets of objective lens pairs for large NA light-sheet microscopy. (A) Matched objective lenses with an accessible sample plane (diSPIM). (B) Asymmetric objective lenses with an accessible sample plane (lattice lightsheet). (C) Asymmetric objective lenses with an inaccessible sample plane (objective lenses used here).

Fig. 2. The combined solid angles of two objectives must not exceed 180° in the plane of orthogonality of the objectives.

At the core of the design is a right-angle shelf on which a cover-glass bearing the specimen is mounted. This gives access to a 2 × 20 mm area of accessible cover-glass at one of its edges. Beneath the shelf is a recess which allows for sample positioning from below using a Raspberry Pi-Cam (see Fig. 4 for a standard image), as well as mitigating any reflection or scattering from the laser illumination source. Figure 5(B) presents an illustration of the recess. The sample is held down firmly by a 3D printed brace with embedded magnets (3 mm neodymium). The brace is coupled to magnets inserted below the sample and mounted in a printed tray, which inserts into a slot. The slot also allows for a heating module, making it suitable for cell culture, embryos, microbes and more. The heat from the heating element is transmitted through the plastic and so does not interfere with the sample, the objective lenses or the sample medium. The temperatures used are not hot enough to damage the printed chamber material (acrylonitrile butadiene styrene begins to deform at 140°), but are sufficient to reach physiological temperatures, a thermocoupler and temperature controller may be used to control the temperature as required.

Perspex windows are (optionally) laser-cut and inserted into the side of the chamber (for viewing by eye) and below the sample (for additional illumination and camera viewing). Cyanoacrylate (Super-Glue) is used to secure the windows to the sample chamber, which ensures a liquid-tight seal. The adhesive is applied whilst the surface’s protective film is attached to the Perspex and the film is removed only when the cyanoacrylate is fully set, as cyanoacrylate vapour settles on surfaces and causes opacity. This allows both objectives to be accurately positioned, with respect to the mounted sample, by eye. Fine correction can then be achieved using

2 A Perspex-free design has also been included in the design package that is more difficult to work with by eye and with small samples.
Fig. 3. Strategies for mounting cell culture: (A) shows a cylinder of a gel used to scaffold a 3D cell culture; (B) shows cells cultured on a cuvette with the illumination arriving through the orthogonal flat window; (C) shows a reflective cuvette being used to redirect a concomitant illumination and detection into orthogonal, similar reflection strategies exist such as mounting small mirrors to a single objective or positioning small mirrors carefully near the sample; (D) shows cells cultured on a small pedestal and imaged using an iSPIM configuration, difficulties arise in keeping such pedestals sterile during incubation and then attaching to the system whilst submerged.

Fig. 4. Representative image from the RaspberryPi Cam of a wild-type zebrafish embryo. Specifications: FOV: 650 µm – sensor resolution: 2592 × 1944 pixels – pixel size: 1.4 × 1.4 µm – size: 25 × 24 × 9 mm – focal length: 3.60 mm.
Sample preparation

In Figure 6, zebrafish were manually dechorionated, with tweezers, on a bed of solid agarose immersed in embryo medium, using a separate stereo-microscope for dissection. The dechorionated zebrafish were then transferred into molten agarose and gently drawn into a length of fluorinated ethylene propylene (FEP) tubing attached to a pipette tip. Organisms are mounted in agarose within FEP tubing to help match refractive indices between immersion medium and the agarose; 0.8% agarose VII was used, as higher percentage agarose may restrict the growth of a developing embryo as well as cause more scattering and refractive index mismatch. Thus, agarose VII produces the best imaging conditions of the agarose variants (Flood et al., 2016). Once the embryo was embedded in its agarose tubing it was transported (made safe and convenient in the FEP) for imaging. It was then adhered to a 25-mm cover slip using colourless nail varnish at each end of the tubing as in Figure 7(B), whilst avoiding the active imaging area along the FEP tubing.

For cell culture imaging, SH-SY5Y neuronal cells were cultured on cover-glass that had been functionalized with poly-l-lysene (which is positively charged and glass adherent) to encourage cell adhesion, as shown in Figure 7(A). In Figure 8, cells were fixed using formaldehyde and imaged in phosphate-buffered saline. For live cell studies, a medium that does not contain phenol red (fluorescent) and that does not require atmospheric control should be used (e.g. HEPES).

Sample mounting and positioning

Samples mounted on cover slips are held in the chamber using a magnetically positioned bar to grip the coverslip.
Fig. 6. Raw, optical, two-colour slice image of a transgenic zebrafish 24 hours post fertilization. The left-hand image shows a cell-membrane-local beta-actin: mcherryCAAX probe; the right image shows fluorescent histones within the nucleus using a h2b:GFP probe.

Fig. 7. Strategies for mounting using the edge of a cover slip as proposed in this work. In (A), cells are cultured on cover-glass for which protocols for sterile cell mounting are widespread. In (B), organisms are mounted in FEP tubing containing agarose and are adhered to cover-glass using epoxy.

Fig. 8. A three-colour composite 3D image, as rendered in Imaris, of HSV-1 viral proteins in a pair of SH-SY5Y cells imaged in fixed cells using our custom light-sheet microscope. The labelled proteins are VP2:mTurquoise (blue), VP16:cYFP (yellow) and VP:1/2td-Tomato (red) VP26 is a capsid protein and marks where the viral capsid is in the nucleus. VP1/2 is a capsid protein that is recruited by the capsid and transcribed by the cell. VP16 is a tegument protein that is recruited to the viral tegument and also transcribed by the cell.

Pre-warmed immersion medium is added slowly and filled to 3 mm below the top of the chamber. The entire chamber is then driven below the objective pair by eye and carefully raised. Lateral positioning is best achieved by matching eye-level with the sample and adjusting the stage as required. Axial positioning is best coarsely adjusted by driving the sample slightly away from the laser illumination so as to not harm the sample. The scattered spot on the immersion–medium–glass interface should be minimized by eye. Finally, the sample chamber should be moved laterally, again, until a fluorescence signal on the camera can be detected. The secondary Raspberry Pi-cam below may also be used as an additional positioning camera. This is more useful for embryos, as the coarse adjustment method can be challenging due to early embryos being mostly transparent, even when using guiding marks on the cover-glass. When mounting very large samples or samples that cover large areas, multiple images may need to be stitched together to create a mosaic. For the objective pair as used here, the sample chamber allows for 2 mm by 20 mm lateral and 1 mm axial movement. For systems with piezo objective scanners, the mosaicking of volumes may drastically reduce the overall time acquisition of a mosaicked volume by requiring fewer overall steps. Both approaches are available for the design presented here, provided that care is taken to limit the range of movement that the automated stage affords the sample chamber.

Conclusions

We have presented an open-hardware solution to address the challenge of mounting a breadth of biological samples in large NA inverted light-sheet systems. Our sample mounting chamber compares favourably to current solutions in the literature for light-sheet microscopy with respect to features, flexibility, ease-of-use, cost and allowance for high NA imaging. Solutions, such as the single objective SPIM (soSPIM; Galland et al., 2015), that require custom-manufactured, single-use sample holders, become prohibitively expensive: our design is affordable (~£2 per unit) and reusable for most biological applications. Our design is open and adaptable for the wider community to build the most suitable chamber using the ideas we have presented. The principles behind the design can be used directly, applied and extended to provide a robust starting point for which users can easily modify for their needs. Moreover, the design is adaptable to any objective lens pair without any additional limitation on NA, as opposed to the open-top SPIM (McGorty et al., 2015) which requires an intrusive water prism. Finally, the design requires minimal or no remounting of a sample, as culture and preparation of a sample can happen directly on the substrate that is eventually imaged, in comparison to the systems that require a skillful mounting of the potentially fragile sample on a pedestal (e.g. the lattice light-sheet; Chen et al., 2014).
Using a monolithic piece of 3D printed material means the unit is robust (as it has no moving parts), disposable, sterile and mass producible. The unique design also allows for a large volume of travel, allowing for volumetric mosaicking and medium-throughput imaging. The device includes many useful features such as a sample mounting camera below, a Perspex window for precision positioning, multiple safety features and sample heating. Future models of this design could also include atmospheric control for long time-lapse imaging of cell cultures. Such a sample chamber may be applicable for the imaging of many other biological samples in the size range of the samples presented in this work.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material