Guide to light-sheet microscopy for adventurous biologists

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Ten years of development in light-sheet microscopy have led to spectacular demonstrations of its capabilities. The technology is ready to assist biologists in tackling scientific problems, but are biologists ready for it? Here we discuss the interdisciplinary challenges light-sheet microscopy presents for biologists and highlight available resources.

Light-sheet microscopy has the unique ability to image biological systems for extended periods of time and in three dimensions without damaging the specimens. This ability is in large part due to the uncoupling of the illumination and detection axes of the microscope, so that only the part of the specimen that is imaged gets illuminated. Additionally, the detection of fluorescent signals with a wide-field detection device such as a charge-coupled device (CCD) camera allows stunning imaging speeds. Together these properties open up windows into biological processes at unprecedented spatiotemporal scales, but as usual, there is no free lunch. The impressive capabilities of light-sheet microscopes come at a price: they demand a level of interdisciplinarity previously unseen in the biological sciences.

This became particularly apparent during the first European Molecular Biology Organization (EMBO) practical course in light-sheet microscopy, in August 2014, at the Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG) in Dresden. Two intense weeks of imaging diverse samples revealed the formidable expertise required to perform a successful light-sheet microscopy experiment. Although the course was focused on biological applications, it had to cover such diverse disciplines as physics, engineering, chemistry, computer science and information technology. As it turns out, biologists' desire to study biological systems using light-sheet microscopy is stronger than their reluctance to venture outside their comfort zone. Such interdisciplinary forays are made easier by a number of commercial and academic resources assembled by the light-sheet microscopy community over the past 10 years. In what follows we discuss the accessible light-sheet microscopy hardware, the changing sample-preparation techniques, the challenges of running light-sheet microscopes in a multiuser environment, image processing and strategies for dealing with the truly massive volumes of data. The growing light-sheet research community will help biologists jump-start the next decade of discovery.

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The light-sheet microscopy hardware

Biologists wishing to use light-sheet microscopy essentially have three options for gaining access to this technology: purchasing a commercial system, collaborating with a "light sheet" lab and building a microscope of their own.

There are currently three commercial setups on the market, which cover distinct flavors of light-sheet microscopy. The Lightsheet Z.1 from Carl Zeiss Microscopy (**Fig. 1a**) is a classical selective-plane illumination microscope (SPIM) offering illumination of the sample from two sides with a broad range of excitation wavelengths. Two cameras (CCD and scientific complementary metal-oxide semiconductor (sCMOS)) and a precision sample-positioning system allow simultaneous dual-color, multiview imaging of living samples for extended periods of time. It is a horizontal setup, so the sample is presented vertically from above and moved through the light sheet using a stage that also includes rotation for multiview imaging. The microscope is based on the setup introduced originally by Huisken and Stelzer[1] and also reduces stripes by pivoting the light sheet[2]. The primary application of this microscope is long-term time-lapse multiview fluorescence imaging of developmental processes, but it is versatile enough to allow for other applications, such as the imaging of fixed and cleared specimens. The imaging of tissue samples up to centimeters in size cleared in aqueous or organic solvents is the domain of another commercial setup, the Ultramicroscope from LaVision BioTec (**Fig. 1b**). This microscope combines a very large field of view and uniform light-sheet thickness with a broad range of excitation wavelengths from a supercontinuum laser. The Ultramicroscope is the instrument of choice for high-resolution multicolor imaging of large, cleared, fluorescently labeled tissue samples, particularly for neurobiology applications. It is based on the Ultramicroscope design published by Hans Ulrich Dodt[3]. In this upright setup, the sample is immersed in a clearing medium and positioned on a dedicated platform, which is moved vertically through a horizontal light sheet. No rotation, and thus no multiview imaging, is possible, but this is not always needed for cleared specimens. Finally, in contrast to the established light-sheet

geometries (**Fig. 1c**), Applied Scientific Instrumentation offers components for a single- or dual-sided light-sheet arrangement (iSPIM or diSPIM, respectively) on an inverted microscope with a so-called inclined or tilted configuration (**Fig. 1d**). The system also does not feature sample rotation, but instead it allows rapid scanning of large areas of samples mounted conventionally on coverslips. It is based on the instrument published by Hari Shroff[4] and is particularly well suited for imaging and high-throughput screening of tissue culture cells and other samples conventionally prepared on glass. While these systems cover a very broad range of applications, they cannot easily be modified to cover additional samples (for example, novel model organisms and exotic species) or additional imaging and photo-manipulation techniques. But light-sheet technology can be 'built around the sample'—that is, the necessary optics can be tailored to the sample and its mounting and culturing requirements—potentially leading to a plethora of home-built systems optimized for particular applications (**Fig. 2a**).

Commercial systems are relatively recent; most published applications of the technology therefore come either from the physics labs that developed the microscopes or from their collaborations with biologists. This is most spectacularly demonstrated in a recent publication by Eric Betzig, where he introduces lattice-light-sheet microscopy taking advantage of nondiffracting Bessel beams and structured illumination to reduce out-of-focus excitation[5]. However, it is not always practical or possible for interested biologists to visit the lab of an optical technology developer; therefore Betzig now offers access to the lattice light sheet through the newly established Advanced Imaging Center at Janelia Farm. This situation is reminiscent of the 'big physics' approach, whereby scientists travel to an instrument rather than the other way around. Alternatively, biologists can build the lattice-light-sheet microscope according to available blueprints, but this adventure requires a bona fide physicist, as the instrument is complex. In developmental biology, where multiview imaging is mandatory for samples ranging in size from hundreds of microns up to a few millimeters, four-lens light-sheet microscopes (**Fig. 2b**)[6–8] are now state of the art.

Last but not least, over the past 10 years, during light-sheet technology's infancy, many biology labs built basic light-sheet microscopes themselves. Recently, two platforms, OpenSPIM[9] and OpenSPINMicroscopy[10], formalized the do-it-yourself (DIY) process and introduced very detailed instructions for the assembly and operation of basic SPIMs (**Fig. 2a**). These platforms should help to build a community of researchers that can modify the design and promote further technological development on a collaborative basis. Roughly 50 OpenSPIM setups are being built around the world; however, thus far very little of this activity is reflected in the public OpenSPIM wiki. Unlike with open-source software, the sharing of ideas and solutions appears not to have taken root in the optical-technology-development research community.

The DIY option, which might sound the least attractive, turns out to be surprisingly viable. We built four OpenSPIM microscopes during the EMBO course with researchers who had never touched any optics before. Although these microscopes are not able to compete with more advanced setups, the fun and educational aspects of putting an imaging device together from scratch are not to be underestimated. The open-access platforms make the technology very 'honest' and easily reproducible, in contrast to many other, more complex microscopy technologies.

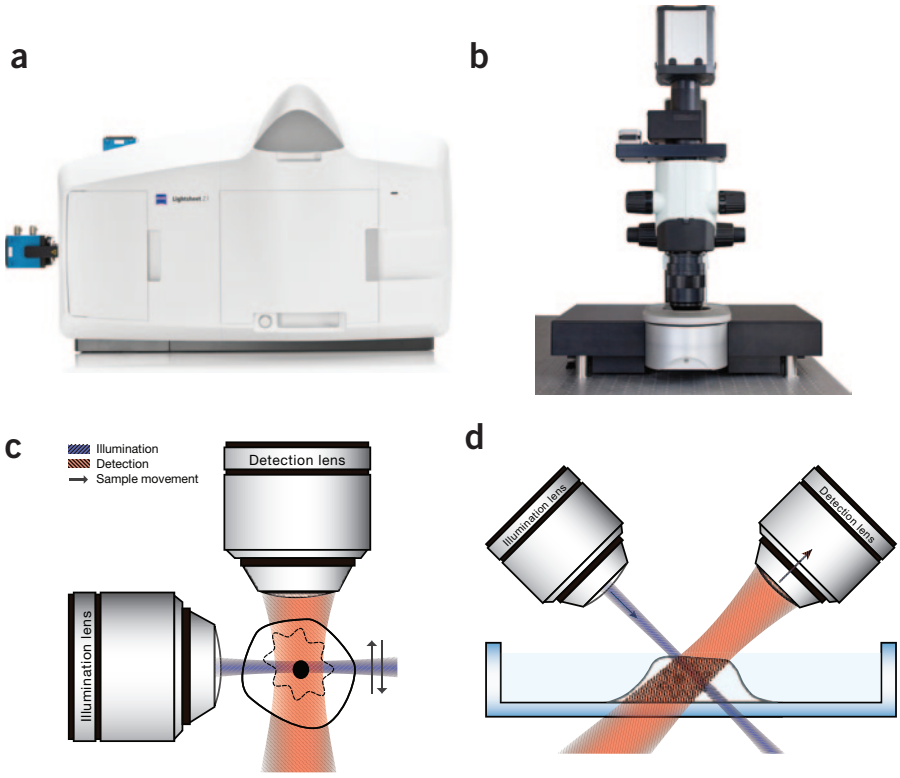

Figure 1 | Current light-sheet microscopes. (**a**,**b**) Available commercial systems are the Lightsheet Z.1 (**a**) and the Ultramicroscope (**b**). (**c**,**d**) Optical architecture of a typical horizontal orthogonal arrangement in SPIM (**c**) contrasted with the tilted architecture (for example, iSPIM) (**d**).

Forget the coverslip

In bioimaging the central element of the experiment is—or should be—the sample. Biological samples come in many sizes, and their optical properties vary drastically. For decades it was the microscopes that dictated how an ideal sample should look: thin, flat and transparent. Light-sheet microscopy offers the possibility of designing microscopes for specific, often three-dimensional (3D), samples, but this can mean doing away with the coverslip and, with it, many well-established protocols for sample mounting. Sample preparation for light-sheet microscopy is in fact so new that it is itself a subject of research, and new mounting techniques are regularly published in scholarly articles (for example, fluorinated ethylene propylene tubes[11] and 3D printed chambers[12]). The five SPIM-specific mounting techniques—embedded, clipped, enclosed, flat and flow-through[13] (**Fig. 2c**)—are described in detail in the Zeiss Lightsheet Z.1 user manual.

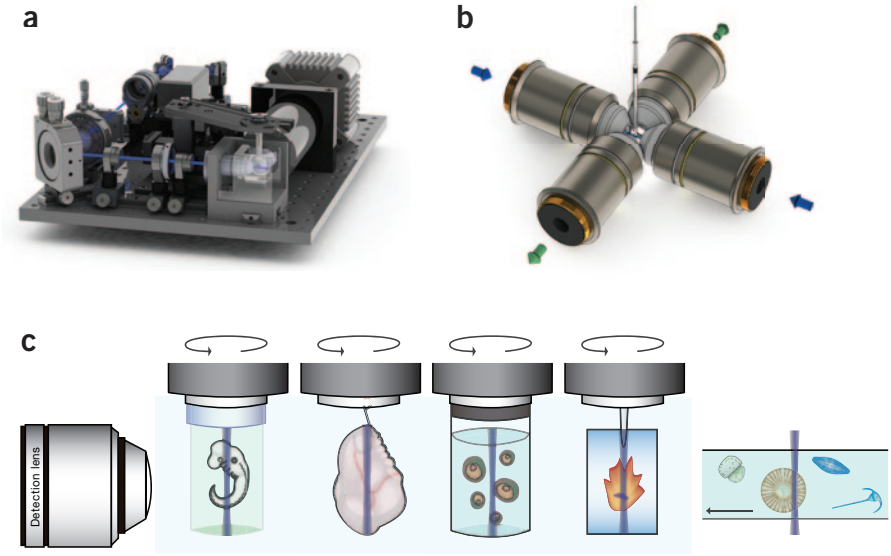

Figure 2 | (**a**) 3D rendering of the OpenSPIM design. (**b**) Schematic of four-lens light-sheet microscope for dual-sided illumination and detection. Image reproduced from Weber, M. & Huisken, J. Omnidirectional microscopy. *Nat. Methods* **9**, 656–657 (2012), Macmillan Publishers Limited. (**c**) Overview of sample-mounting methods typically used on horizontal multiview SPIM setups: from left to right, embedded, clipped, enclosed, flat and flow-through.

Stability is key in light-sheet microscopy, as the sample can be imaged for days. The evident but sometimes overlooked issues are the optical layout of the microscope and the way the sample is presented and scanned. Horizontal setups (such as SPIM) require stable mounting that can be viewed from every angle and are affected by gravity and lateral and rotational movement. Vertical or tilted systems (such as ultramicroscopes or lattice light-sheet microscopes) can rely on a slide or platform but might not be convenient for large developing samples or multiview imaging. A glass-fish-glass sandwich is simply not on the menu anymore.

Over the past 10 years all major model organisms, as well as more exotic samples such as planktonic algaes[13], living corals[14], infected mosquitoes and aphids, have been imaged with light-sheet microscopes. Thanks to the low phototoxicity, one can typically image a sample for hours or days; the primary requirement for sample mounting is thus the proper environment for the sample's survival. This necessitates thinking about optimal 'physiological' conditions before, during and after the imaging. Making sure that the imaged sample survives and produces viable offspring becomes an integral part of light-sheet experiments. In this approach the sample typically suffers not so much from the light exposure (as occurs in many other fluorescence microscopes) but rather from inadequate mounting. Overall, sample preparation is the aspect of the technology for which biologists are best prepared: they know their samples, and once they get to know the microscope they are best positioned to devise a solution that keeps the sample happy.

SPIMaging in a multiuser environment

Light-sheet microscopy is gradually entering imaging facilities across the globe. Importantly, getting this technology into a core facility is not a matter of a simple upgrade; it calls for a complete change in how imaging experiments are performed. Data from the Light Microscopy Facility of the MPI-CBG show that a commercial light-sheet system (Lightsheet Z.1) was used more than all other imaging systems installed in the facility, with total use reaching 5,000 h of instrument time per year. This was achieved, however, by only a few users who imaged developmental processes in *Drosophila* and zebrafish; other users imaging fixed samples did not have enough access, which necessitated the purchase of a second system.

As was also apparent during the EMBO practical course, anyone running an imaging facility has many decisions to make in order to accommodate multiple light-sheet microscopes. What sample should be imaged at what microscope includes considerations such as how to mount the sample, how long the imaging will take, how much data will be generated, where the data will be transferred to, how long the data transfer will take, and what strategy should be used for processing and analyzing the data. Careful planning involving biologists, imaging specialists, computer scientists and information technology specialists is necessary in order for an experiment to be performed successfully.

Making Pixar movies with SPIM data

Data acquisition is only the first, relatively simple step in generating biological insight through microscopy. Light-sheet microscopes typically produce enormous amounts of data (typically in the range of terabytes) streamed from digital cameras to computer hard discs for hours or even days. Since the volume of data far exceeds the available computer memory, it is challenging to even look at the data. Moreover, particularly in the case of multiview acquisitions, the images need to be processed before they can be productively viewed and analyzed.

Since light-sheet microscopes are still relatively new, commercial solutions for data processing are only beginning to materialize. For the most common processing tasks, applicable to most light-sheet microscopes, open-source bioimage analysis platforms have emerged as viable solutions. In the early days it could take weeks before acquired data could be examined. Nowadays, plug-ins developed for the Fiji platform[15,16] allow for the transformation of a massive time-lapse, multiview data set into a dynamic 3D-rendered movie in real time. The process can be further sped up by parallelization on a cluster computer, and the open-source model is particularly enabling in this context. No matter how well documented, however, the plug-ins are the products of research in applied computer science, and to use them biologists need to get their hands dirty with the tools of the computer science trade.

Since every entity developing light-sheet microscopes uses different formats to store the image data, it is often difficult to export data sets outside of the lab or company that created them. One challenge for the community is to adopt a standard data format that can accommodate all the flavors of light-sheet imaging. Moreover, it is not sufficient to adopt existing standards such as Tagged Image File Format (TIFF). The data containers storing light-sheet imag-

ery must allow access to arbitrary slices through the data across many dimensions for efficient visualization and processing. One attempt to develop such a container based on the HDF5 "filesystem in a file" is the BigDataViewer (BDV). This Fiji plug-in allows seamless navigation of large image data sets as if they were loaded in memory. Similarly, the Collaborative Annotation Toolkit for Massive Amounts of Image Data (CATMAID)[17,18] has been modified to provide similar functionality in a web browser, which additionally enables collaborative crowdsourced annotation.

Methods for the analysis of light-sheet data are as diverse as the applications for which the technology can be used, spanning all scales of biological systems from molecules to organisms. Special attention has been given to tools for extracting developmental lineage trees from recordings of embryonic development. Recently published automated segmentation and tracking solutions do a very good job at this task[18], but they still require manual curation using tools such as BDV or CATMAID. In addition, applications go well beyond the canonical developmental biology problems. High-resolution images of fixed and cleared brains or ultra-high-speed recordings of a beating fish heart or of neuronal activity in living animal brains require specifically tailored software solutions. The bioimage informatics community provides extensible and scalable platforms, but it is ultimately the role of biologists to mix and match these tools in order to solve the challenging image analysis problems[19]. Thus basic programming knowledge, at least on the level of scripting languages and familiarity with command-line Linux, becomes essential for light-sheet devotees.

SPIM redefines Big Data

What sets light-sheet microscopy apart from most, if not all, other imaging modalities is the volume of raw data produced in even a routine experiment. The output of light-sheet setups is orders of magnitude greater than that of confocal microscopes, and when state-of-the-art setups are used to their full potential, the data volumes can dwarf even those produced by physicists at CERN (the European Organization for Nuclear Research) (**Fig. 3a**). But even for routine applications, the data volume is clearly a challenge.

This challenge starts at the microscope's computer, which has a finite storage capacity (meaning that the data have to be moved off it). There are bottlenecks at every step, from the speed of the hard drive on the microscope's computer to the bandwidth of the network connection, the speed of the network switch, the load on the network from other users and the writing speed of the destination computer (**Fig. 3b**). These issues must be handled by professionals experienced in centralized network architecture. We learned during the EMBO course that simplistic solutions, such as giving every student a 4 TB hard drive to take the data home on, simply do not work.

Light-sheet microscopy is like a demanding state-of-the-art computer game. Biologists most likely cannot run it on their existing computers; they can make it work with a minimal configuration, but to really enjoy the experience they need high-end hardware (**Fig. 3c**). The technology is still moving forward at a rapid pace, so it is necessary to upgrade the computer quite regularly in order to stay in the game.

An unimaginative solution to the data-volume problem is to transfer the 'cold' data (the data nobody is working on at the moment) to a tape back-up, at the cost of losing immediate access. In this case 'warm' data coming off the microscopes should stay on a big central-network storage unit, and 'hot' data that are being actively processed should live on a cluster or powerful processing computer. Such hierarchical arrangement of data clearly requires complex system administration and should be implemented at the university or institute level.

Another strategy is to avoid acquiring new data until the data at hand have been processed. Raw data could even be deleted after processing, since it may be cheaper to repeat the experiment than to store the massive amount of raw data. However, most biologists are likely to be reluctant to follow this strategy; who would want to delete spectacular recordings and blindly trust processing pipelines that are far from perfect? There may also be legal considerations: in Germany, for example, raw data must be kept for 10 years by law. It is time to think about what exactly "raw data" means in the context of light-sheet microscopy.

The most promising approach for taming the data deluge is to employ clever compression strategies. One can reduce the dimensionality of the data on the fly—for example, by transforming 3D stacks to 2D cartographic projections[8]—although such solutions typically cannot be easily gener-

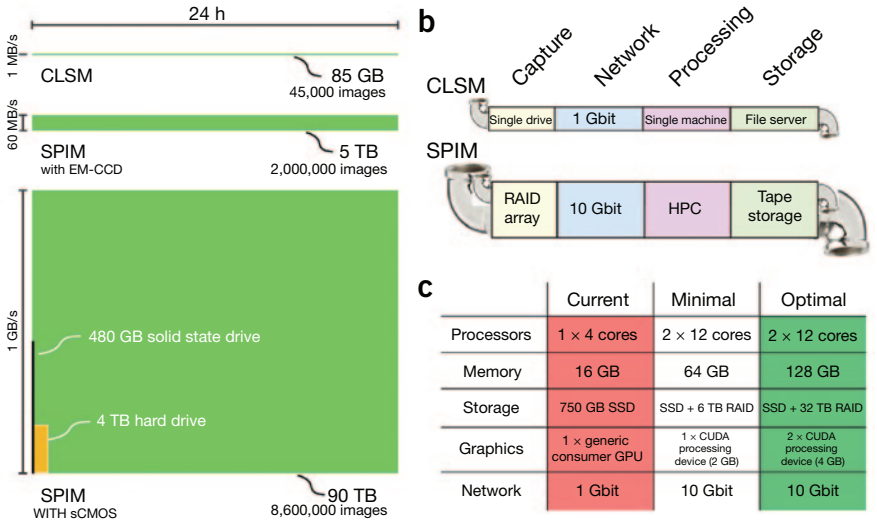

Figure 3 | The Big Data Challenge. (**a**) Comparison of the amount of data typically generated over 24 h by the indicated microscope and different camera combinations (confocal laser scanning microscope (CLSM), SPIM using EM-CCD camera and SPIM using sCMOS camera). Image adapted from Supplementary Figure 1 in Schmid, B. *et al.* High-speed panoramic light-sheet microscopy reveals global endodermal cell dynamics. *Nat. Commun.* **4**, Article no. 2207 (2013), Macmillan Publishers Limited. (**b**) Comparison of the hardware components of data pipelines dealing with CLSM and SPIM data volumes. HPC, high-performance computing; RAID, redundant array of independent disks. (**c**) Comparison of system requirements for 'playing' with light-sheet microscopy data. Analogously to computer games, light-sheet data require at least the minimal configuration, but the optimal configuration is recommended for full performance. SSD, solid-state drive; GPU, graphical processing unit. CUDA is a commonly used parallel computing platform and programming model for graphics cards.

alized. Existing compression algorithms, although able to reduce raw data volumes impressively, come with a cost in regard to interactive access to the data. An ideal scheme would be fast and reduce the data to a fraction of its original volume using the temporal dimension. Although such compression schemes are under intense research in computer science labs, biologists have to get seriously geeky to be able to handle light-sheet microscopy data sets. Information technology (IT) is barely on the curriculum of most biology departments, and yet in the absence of professional IT support, which is often lacking, the burden will fall on biologists. Fortunately, IT companies, such as Acquifer, see a business opportunity in helping biologists shuffle their piles of light-sheet imagery.

There is a strong light-sheet microscopy community

There is no doubt that biologists will need support from technology developers to exploit light-sheet technology to its full potential. Fortunately, the light-sheet research community is well organized and interactive. The first workshop on light-sheet fluorescence microscopy brought the community together in Dresden in 2009. This workshop has been organized yearly ever since; initially it was an invitation-only event, but in 2014 it became an open conference.

Light-sheet microscopes have been introduced in the context of various general imaging practical courses organized by EMBO, Cold Spring Harbor Laboratory or Woods Hole; talks from last year's EMBO course have been recorded and provide a nucleus of material for teaching the light-sheet paradigm. In addition to the growing number of publications on light sheets, several open-access online resources have been established to collect useful information about light-sheet sample preparation, instrument building and image processing. We encourage researchers who develop or use light-sheet microscopy to find time to contribute to the expansion of these online repositories by sharing their experiences and expertise.

It is all worth the trouble

What has the light-sheet microscope brought us in 10 years? We have seen hearts beating in real time, the full development of various embryos from eggs to viable and fertile adults, bacteria colonizing sterile guts, growing microtubule asters, plant roots growing for weeks, neuronal activity in live fish brains and deep views inside tissues on scales that had seemed inaccessible to microscopy. Light-sheet microscopy makes it possible to image any organism in a near-physiological context without damage and loss of signal. These microscopes can open windows into living cells to reveal the spectacle of biological processes with high resolution in space and time or can travel the planet to reveal the stunning biodiversity of plant and animal life (http://oceans.taraexpeditions.org/). Maybe we could even image thoughts as they are realized in brains. We do not have flying cars, tractor beams, tricoders or transporters a la *Star Trek*, but we do have light sheets!

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The authors declare no competing financial interests.

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