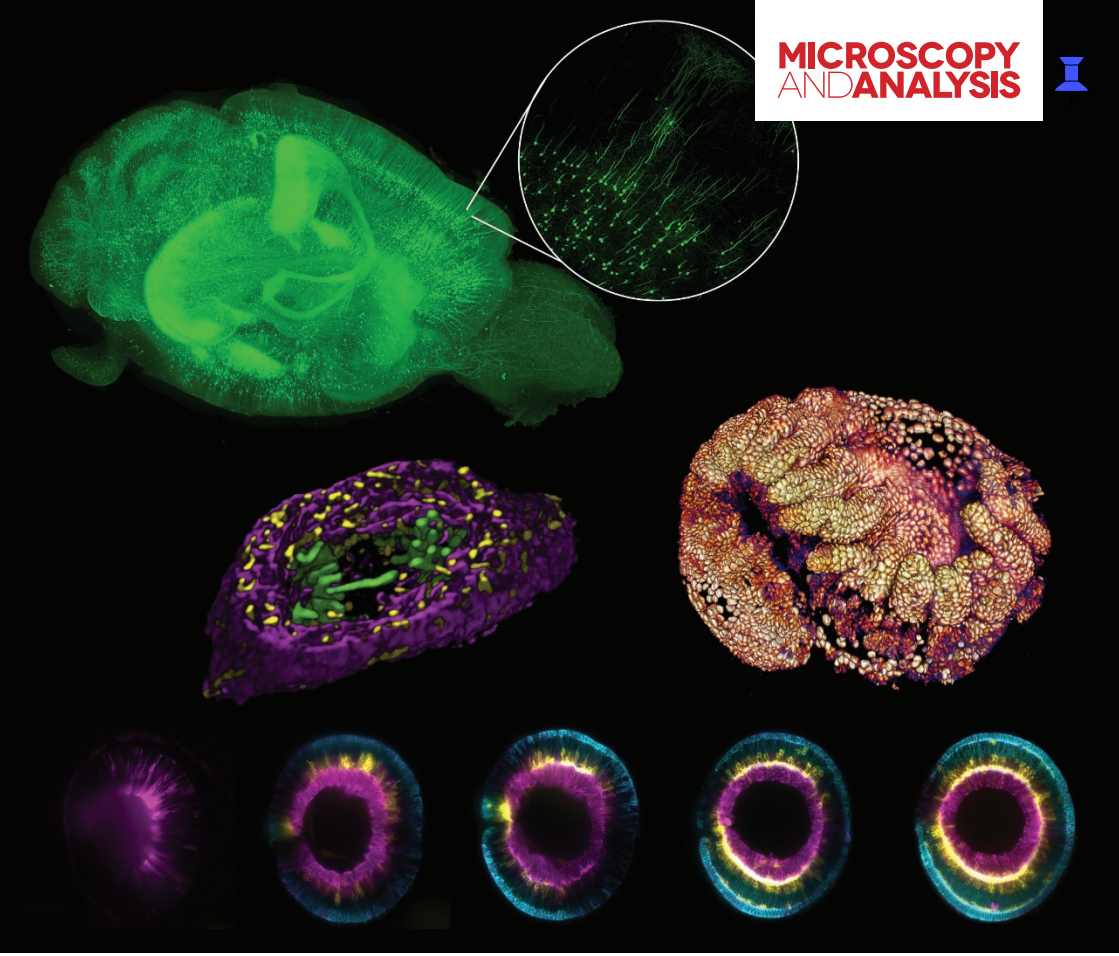
MICROSCOPY AND ANALYSIS



Light-Sheet Fluorescence Microscopy

Essential Knowledge Briefings

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Front cover image.
Top: Thy1-GFP-labeled, cleared mouse brain (CLARITY). Acquired on ZEISS Lightsheet Z.1, processed in arivis Vision4D. Imaged with a 5x objective, using 6x7 tiles from two sides. Insert: Digital zoom from the cortex region, showing that single neurons can be identified and analyzed. Image by Douglas S Richardson; reproduced with permission from ZEISS.
Middle-left: 3D rendering of a HeLa cell in mitosis. Snap from a 300 time points image series. Chromosomes are labeled green (mCherry-H2B), mitochondria yellow (mitotracker–deep red), and endoplasmic reticulum magenta (mEmerald-calnexin). Organelle structures are clearly resolved. Acquired using a lattice light-sheet microscope by Wesley Legant and Eric Betzig. Image from Chen *et al. Science* 2014;346:1257998. Reprinted with permission from AAAS.
Middle-right: 3D rendered volume data set of a six-day old embryo of the marine crustacean *Parhyale hawaiensis*. One time point from a seven-day time lapse. Acquired on ZEISS Lightsheet Z.1, data processed and fused in Fiji. Image by Tassos Pavlopoulos.
Bottom: The development of a zebrafish retina captured on a light-sheet microscope every 12 hours from 1.5 days to 3.5 days after birth. Labels: retinal ganglion cells with Ath5:RFP (magenta), amacrine and horizontal cells with Ptf1a:YFP (yellow) and photoreceptors and bipolar cells with Crx:CFP (cyan). Image by the Norden lab, Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden (licensed under the Creative Commons Attribution – Share Alike 4.0 International licence https://creativecommons.org/licenses/by-sa/4.0/deed.en).

Microscopy EKB Series Editor: Dr Julian Heath
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INTRODUCTION

Fluorescence microscopy is an extremely powerful tool for biological imaging from the subcellular to the organism level. The outstanding image contrast achieved by specifically labeling the molecules, organelles or structures of interest makes it the most widely used contrast method in biological imaging. It was only in the 1990s, with the development of genetic tools to program cells to self-label specific proteins with a fluorescence tag, such as the green fluorescent protein (GFP),[1] that fluorescence live imaging took a huge step forward. Introducing genetically encoded fluorescent proteins[2] does not require chemical or mechanical treatment of the specimen, and thus enables imaging of specimens in a 3D context much closer to their native state.

Based on this paradigm shift in fluorescence labeling, optical sectioning techniques, such as confocal laser scanning microscopy (CLSM), were increasingly and very successfully employed to map 3D samples at high resolution in a close-to-natural context.[3,4] Those techniques, however, typically use slow voxel-by-voxel image acquisition to achieve optical sectioning, and require high illumination intensities for fluorescence excitation. Both properties make them poorly suited for fast and long-term repetitive imaging. In live imaging the limiting factors are phototoxicity and photobleaching: shining the amount of laser light required by these techniques onto the specimens can kill them and fluorophores are used up. Thus, to ensure sample viability, the dose of excitation light has to be kept as low as possible and signal detection has to be as efficient as possible. Improvements were introduced to 3D optical sectioning, for instance with parallelized confocal raster-scanning (line scanning confocal microscopes, spinning disc

confocal microscopes) and more sensitive detectors and camera sensors. However, high light exposure to the sample remained a major limitation for live imaging.

One of the fundamental restrictions of established microscope systems is the geometry of the epi-illumination optics, *ie* the same lens is used for illumination and detection. Fluorescence is thus excited in the entire sample even when the signal is collected only from the focal plane (with the notable exception of two-photon microscopy). Therefore, the sample is exposed to much more light than necessary, especially when imaging an extended volume with a significant number of optical sections. This prevents long-term live imaging.

With light-sheet fluorescence microscopy (LSFM) – also known as selective plane illumination microscopy (SPIM) – a conceptually new method was introduced to fluorescence live imaging in 2004. This development by Ernst Stelzer and his group at the European Molecular Biology Laboratory (EMBL) in Heidelberg, published in Huisken *et al* 2004,[5] overcomes the above-mentioned limitations. LSFM employs a combination of efficient illumination for optical sectioning and detection parallelization to make long-term 4D (x, y, z and t) microscopic imaging with minimal phototoxicity and rapid acquisition possible. LSFM allows the user to virtually eliminate photo-damaging effects to the sample. Additionally, the optical concepts of LSFM make it easy to integrate the microscope with dedicated incubation chambers. This ensures that the specimen can be kept in stable physiological conditions during long-term experiments.

The principle of LSFM is to selectively illuminate only those parts of the specimen that are in focus of the imaging optics. To

achieve this, fluorescence is excited with a thin sheet of light from the side that is co-aligned with the focal plane of the detection objective (Figure 1). A camera acquires the image of this optical section through widefield optics. A volume is acquired by moving the specimen through the light sheet and taking a series of images that can be processed to a 3D data set. The image of a single optical section can be acquired in one shot with millisecond exposure. This is quick enough to resolve the dynamics of some of the fastest biological processes, such as heartbeat or vascular flow of a zebrafish. Entire volume data sets, for instance a map of all cells in a *Drosophila melanogaster* embryo or a 3D map of neuronal activity in organisms such as *Caenorhabditis elegans* or zebrafish can be acquired in seconds.

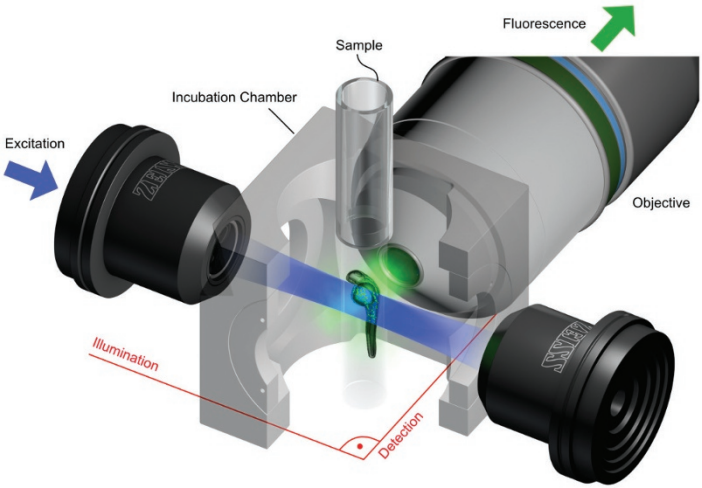

Figure 1. In LSFM, fluorescence excitation (blue arrow) and detection (green arrow) are split into two distinct optical paths. The illumination axis is orthogonal to the detection axis. The LSFM concept is compatible with imaging in dedicated incubation chambers that keep the specimen in a close-to-natural environment over long periods of time. Reproduced with permission from ZEISS

Because of its obvious advantage for imaging the development of entire embryos like *Drosophila*, zebrafish or various marine species, the popularity of LSFM first gained momentum in developmental biology. Here, it allows the study of cell migration and embryo morphogenesis over extensive time courses (up to days and weeks). However, since its introduction in the life sciences more than 15 years ago, an enormous variety of LSFM implementations have been developed. New sample mounting techniques have been established, encouraging more and more researchers to use LSFM in a wide range of biological disciplines from cell biology and neurosciences to plant biology. For its potential to have a profound impact on life sciences, LSFM was chosen as Method of the Year 2014 by *Nature Methods*. The quickly growing popularity of LSFM in laboratories all over the world goes hand in hand with the availability of easy-to-use commercial LSFM systems, as well as a growing pool of hardware and software solutions to handle, store and process the unprecedented amount of data.

This Essential Knowledge Briefing provides a general introduction to the field of LSFM, explaining the technique and its most important adaptations. Examples of LSFM applications are provided, and the briefing also discusses practical issues as well as potential advances in the near future.

HISTORY AND BACKGROUND

Unlike in conventional epifluorescence microscopy, in LSFM the illumination is decoupled from the detection light path. In the most simple case, two objective lenses are used: one to focus the light sheet on the specimen and one to collect the fluorescence. The fluorescence excited by the light sheet is collected in the direction orthogonal to the sheet: in essence, the light sheet illuminates the specimen from the side, while the fluorescence is collected from the front (Figure 1).

This arrangement automatically generates the image of an optical section that can be directly detected by a camera. To acquire a 3D data set, the specimen is simply moved through the light sheet step by step and a series of images is captured on the camera in a synchronized manner. As an extra practical benefit of this setup, each objective lens can be optimized for its specific role, *ie* generation of thin light sheets or high-resolution fluorescence collection.

The light sheet is either generated statically by using a cylindrical lens or a combination of a cylindrical lens and a high-quality objective, or alternatively a virtual light sheet is generated by fast scanning of a laser beam in the focal plane of the detection objective, often referred to as digitally scanned light-sheet microscopy (DSLM).

LSFM has several important advantages over other forms of fluorescence microscopy, such as confocal microscopy (Figure 2). Most importantly, as a direct result of the illumination concept, only those fluorophores in the sample that are close to the focal plane of the detection lens are excited. All other fluorophores do not contribute any out-of-focus light, which would blur the image, and they are not photobleached or inducing any phototoxic effects.

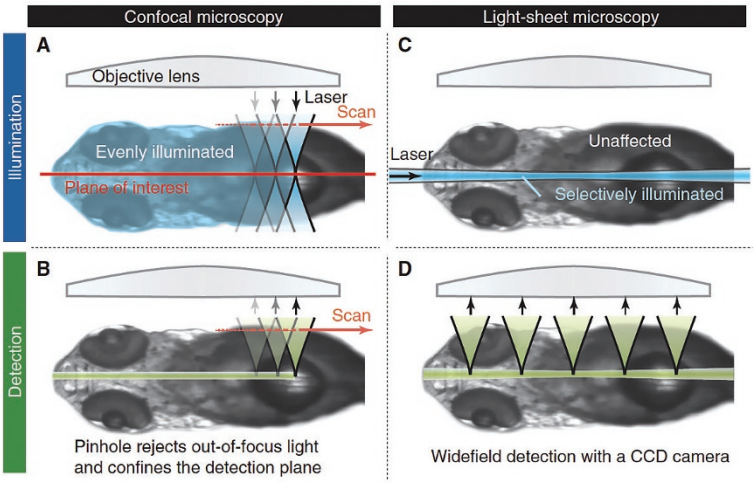

Figure 2. Advantages of light-sheet microscopy compared with confocal microscopy. Light-sheet microscopy features faster acquisition and less photobleaching than confocal microscopy. To illustrate the difference between laser scanning confocal microscopy (LSCM; A,B) and light-sheet microscopy (C,D), the processes of illumination (A,C) and detection (B,D) are split. (A,B) In LSCM, a tightly focused laser beam is scanned across the sample (A), thereby exposing the sample to high-intensity light not only in the plane of interest, but also above and below. (B) A pinhole rejects much of the excited fluorescence and confines the image to the plane of interest. (C,D) In light-sheet microscopy, a light sheet from the side (C), which overlaps with the plane of interest, illuminates the sample in a thin slice. Photobleaching is thereby considerably reduced. (D) All the fluorescence is collected and imaged onto a charged coupled device (CCD) camera. Such widefield detection is fast and benefits from modern CCD technology. Reproduced with permission from Huisken, Stainier, *Development* 2009;136:1963–75 (doi:10.1242/dev.022426)

The light dose the specimen is exposed to during acquisition of a 3D image stack is massively reduced, depending on the number of required sections, enabling long-term observation of living specimens with almost no detrimental effect to the specimen.

A second advantage of LSFM comes from illuminating the entire optical section at once, allowing the use of fast cameras. This accelerates image acquisition and at the same time exposes the specimen to much reduced laser intensities to collect sufficient signal per volume element. As a result, volume time-lapse data with unprecedented temporal resolution can be acquired over

hours and days, making LSFM the ideal tool for observing living organisms or 3D cell cultures.

In summary, both limiting factors from conventional fluorescence live imaging – photodamage and low acquisition speed – are dramatically improved by LSFM.

The concept of decoupling illumination and detection optics of a microscope and illuminating the sample at right angles with a sheet of light has already been employed by H Siedentopf and R Zsigmondy in 1903.[6] They used this approach to visualize small colloidal particles in solution. Over the following 90 years, several scientists developed similar light-sheet-based microscopy techniques, but these were used primarily to study the surface of specimens and did not become widely used.

Only with the availability of fluorescence microscopy, suitable lasers and cameras, as well as the necessary computer technology, could the great potential of light sheets in 3D bioimaging be demonstrated. The first system to utilize a light sheet to stimulate fluorescence in a biological specimen was developed in 1993 by a team from the University of Washington, Seattle, led by Francis Spelman.[7] It was used to study the anatomy of the guinea pig cochlea, which they first made transparent using a special clearing solution.

Ten years later, in 2004, a team led by Ernst Stelzer at the EMBL in Heidelberg, showed the advantages of light sheets for imaging living organisms and marked a milestone in the renaissance of the technique. Stelzer and his team called their design selective plane illumination microscopy (SPIM) and used it to study living fish and *Drosophila* embryos expressing GFP in muscle tissue and in cell nuclei, respectively. In the

following years, LSFM quickly caught the attention of the wider biological community.

In classic SPIM, the light sheet was applied by focusing a laser on a cylindrical lens placed at right angles to the fluorescence detector, with the sample positioned at the intersection between the two. In the horizontal setup, the sample was suspended from above, allowing it to be moved with precision in all three directions, x, y and z, and to be rotated around its vertical axis. Thus, the embryo could easily be moved through the light sheet step by step to acquire a z-stack of images that could then be combined into a single 3D data set.

With this first LSFM implementation for live imaging of entire embryos, Stelzer and his team also demonstrated the power of combining multiple z-stacks acquired from different angles by rotating the specimen. This imaging mode is now widely established and referred to as multiview imaging. The advantages of multiview imaging are essentially two-fold.

First, the resolution of a data set can be improved by combining angular views – if the sample is transparent enough to image the same volume with a comparable image quality from multiple sides. This is possible since the structures resolved with the typically lower axial resolution from a given angle can be imaged with the higher lateral resolution of the imaging system from a 90° perspective. In a post-acquisition data processing step, this information is combined to a better resolved output data set. Generally, two views are insufficient to provide truly isotropic resolution, but the acquisition of additional views reduces the temporal resolution, exposes the sample to more light and also generates an increasing amount of redundant data. Thus, increased

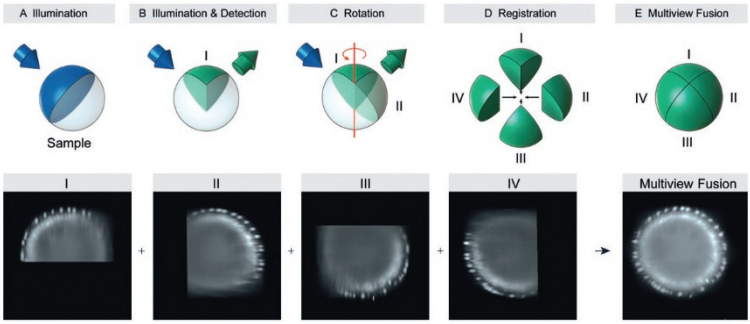

Figure 3. Multiview fusion: in a scattering sample the illumination light will only penetrate part of the sample, *eg* the half facing the light. Similarly the fluorescence may only be detected on the half facing the detection optics. Consequently only the quadrant facing illumination and detection will be well imaged. The sample is rotated to make all quadrants accessible. The individually recorded data sets are then registered and fused to yield an image of the entire sample (multiview reconstruction). Reproduced with permission from Photonik International

image resolution, as always, comes at the cost of temporal resolution and sample viability, a dependency that has to be balanced for each individual experiment.

The more important advantage of multiview imaging is that large and opaque samples can be imaged in their entirety by sequentially recording image stacks from different viewing angles and computationally fusing the complementary image information to produce a single high-resolution data set covering the full sample volume (Figure 3).

Multiview imaging thereby directly addresses the general effect of light scattering in biological tissue that limits how deeply a specimen can be imaged with any light microscope. It is important to note that although multiview imaging is not limited to LSFM geometries, it is the speed of LSFM that allows the user to take full advantage of this imaging mode. Still, acquisition of such sequences of z-stacks obviously takes longer than imaging just

one angle. To speed up multiview image acquisition, advanced implementations of LSFM have been established that show two illumination and, in some cases, two detection light paths for better illumination and simultaneous imaging of multiple angles. Ultimately, four views of the specimen can be imaged during one sweep of the sample through the focal plane (see Figure 3 and the four-lens geometry in Figure 4).

Multiview imaging also already partially compensates for problems present in nearly all microscopic imaging techniques: regions situated behind strongly absorbing or scattering objects (*eg* melanophores, chloroplasts, pigment grains, *etc*) are affected by the scatter or shadows cast by these structures. Thus, an image is perceived as having stripes from the illumination beam. This effect is apparent in many images in the LSFM literature.[5,7-11] Since LSFM is particularly used for imaging larger 3D objects, measures to reduce or compensate for stripes and blur are important. Fortunately, the concept of decoupled illumination and detection light paths facilitates this: in 'multidirectional SPIM' (mSPIM),[12] Jan Huisken summarized two fundamental ways to evenly illuminate the sample in the imaged region. First, pivoting the light sheet within the focal plane of the detection optics illuminates the sample from a range of angles during the camera exposure time. Second, two opposing objective lenses illuminate the sample from the side. mSPIM concepts have been shown to improve image quality dramatically in many living, scattering organisms and have been adopted by a range of LSFM implementations.

Further established ways to improve image quality in an optically dense specimen are the above-mentioned mode of light-sheet generation by scanning a laser beam to form a virtual light sheet

(DSLM), and based on this, the more recent introduction of a confocal slit scanning detection mode for increased contrast in strongly scattering tissue.

Confocal slit detection significantly improves the image contrast by spatially filtering the blurring from scattered light and potential remaining out-of-focus fluorescent signal.

In summary, LSFM provides a multitude of possibilities to generate excellent image quality from thick and optically dense samples. Most of these have their basis in the flexible optical concept of decoupled optics for illumination and detection.

Now that live imaging is possible over very long time periods, the maintenance of the viability of the specimen during the whole imaging process becomes much more important. This is the main reason that the various LSFM system designs are virtually built around the incubation chamber and mounting solution optimized for the sample, taking advantage of the extra degrees of freedom from choosing suitable long working distance objective lenses (see also the Case Studies and Figure 5).

IN PRACTICE

The LSFM setup by Stelzer and Huisken from 2004 has since formed the blueprint for many derived technical variations.

Some implementations cover technical advances to further improve the performance of LSFM in terms of acquisition speed and image quality, while others optimize specific features of LSFM for certain experimental requirements. Typical topics are the generation of thinner light sheets for better axial resolutions, the fastest possible volume acquisition strategies for best time resolution, adapted incubation chambers and sample holders to optimally support a specific sample type in long-term imaging, implementing imaging modes such as fluorescence lifetime imaging (FLIM), fluorescence correlation spectroscopy (FCS), Förster resonant energy transfer (FRET), super-resolution modes such as photoactivated localization microscopy (PALM) or structured illumination microscopy (SIM), special objective lenses and more. The principle geometries and common types of LSFM that most of these newer variants build upon are shown in Figure 4.

Publications on new applications or technical demonstrations appear frequently, showing that LSFM has matured to an established optical sectioning microscopy technique widely spread in live imaging applications and in imaging optically cleared samples.

The following is a list of applications that LSFM has been successfully adapted to.

Fast *in toto* volume imaging in developmental biology
This focuses on imaging fluorescently labeled embryos or organisms as a whole. The goal is the reconstruction of cell lineages, cell tracking, mapping of gene-expression pattern in space and time. Typical

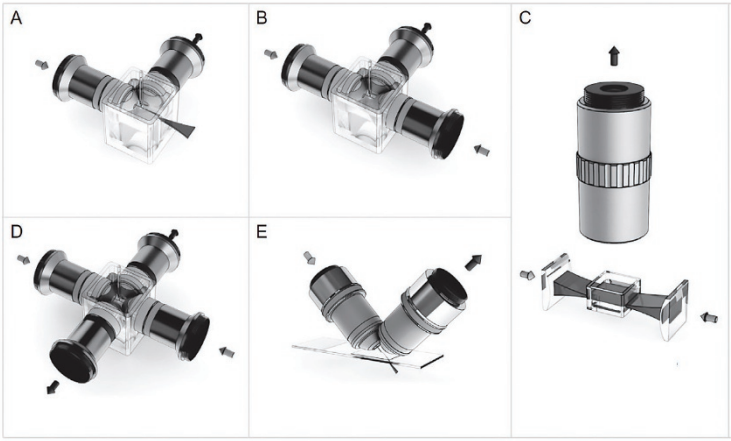

Figure 4. Implementations of light-sheet microscopy and benefits of multi-lens setups. Light-sheet microscopy is built around the sample and thus comes in numerous implementations. (A) A basic SPIM setup with one objective for illumination and one for detection. The sample is oriented vertically in the medium-filled chamber. (B) In this three-lens configuration, a second illumination objective is added for dual-side illumination. (C) An ultramicroscope with two illumination arms in an upright configuration with a low-magnification objective. The imaging chamber is typically isolated from the optical components to hold large, fixed samples in clearing agents. (D) A configuration with a second detection objective can be used to acquire images from two sides simultaneously for faster volume imaging. (E) A special objective configuration (iSPIM, diSPIM, Bessel and Lattice Light Sheet) for using cover-slip-mounted samples with light-sheet microscopy. Reprinted from Weber *et al. Methods Cell Biol* 2014;123:193–215, Copyright 2014, with permission from Elsevier

samples are *Drosophila*, *Tribolium* (flour beetle), zebrafish, *Parhyale* (amphipod crustacean), *Platineris* (annelid worm), sea urchin embryos, *etc.* For fast multiview imaging the sample is typically mounted vertically in a medium-filled chamber. Implementations are called MuVi-SPIM, SiMView, Panoramic LSFM, mSPIM and other acronyms. See also references 5, 12–17.

Functional neuroimaging in *Drosophila* and zebrafish

This enables visualization of brain activity in 3D at the single-cell level. It has been shown with LSFM in *Drosophila* embryos and zebrafish larvae and involves volumetric imaging of transgenic

calcium indicator dyes at maximum temporal resolution: whole-brain volumes are recorded in intervals of about one second, typically with single-view imaging. See also references 18–21.

Imaging of plants

This 3D long-term live imaging is used to study morphogenesis and genetic patterns in the development of *Arabidopsis*, soy beans and other plants. The sample is mounted vertically in a medium-filled chamber and imaged from the side. Adapted sample holders and incubation chambers are essential to keep roots under water and leaves in the air. Dedicated light control simulates day–night cycles while having the light off when imaging. See also references 22–25.

Ultrafast and high-resolution cellular imaging with lattice light-sheet microscopy

This enables the observation of dynamic processes with high acquisition rates at subcellular resolution, mostly for cell biological applications and small specimens. See Case Study 1 and references 26–32.

Imaging optically cleared samples

This involves imaging large, fixed, optically cleared, fluorescently labeled tissue samples. It was recently extended by Expansion Microscopy, a sample preparation method that allows the resolving of very small structures by enlarging them using a polymer system.[33] This use has become a key enabler for brain mapping and connectomics projects in modern neurosciences. The acquisition speed of LSFM makes imaging centimeter-sized, optically cleared samples possible. At the core of the adaptation for the required

refractive indices are special chambers and objective lenses. Fast imaging with high resolution is done in adapted horizontal SPIM-like geometries as in Panels A, B and D in Figure 4 (see also Case Study 2); more overview-like imaging with easy access for extremely large specimens can be done in an upright setup (Panel C in Figure 4). See also references 7, 9, 34–38.

Imaging of small organisms, spheroids and organoids

This is the 3D imaging of, for example, *C. elegans* neural development or spheroids and organoids growing in glass-bottom dishes or similar sample mounts. The focus is on fast imaging of small live samples with conventional sample handling, and not so much on incubation. Systems are called iSPIM, diSPIM, as per Panel E in Figure 4. An advantage is that it can be realized with add-ons to inverted microscopes. See also references 39–41.

Imaging of 3D cell culture, spheroids and organoids in a 3D environment

This refers to 3D time-lapse imaging of cell culture in 3D matrices, toxicology and oncology studies, stem cell differentiation in 3D, spheroids in pharmaceutical research, and the development of tumor spheroids. Live imaging often requires sterile culturing conditions in special incubation chambers. See also references 42 and 43.

Single molecule tracking in tissue, FCS and super-resolution imaging

Super-resolution LSFM has been reported in adherent cells, FCS in single cells in glass-bottom dishes and in zebrafish tissue, single molecule RNA tracking in insect tissue. Mostly these applications

involve dedicated setups in upright geometries (Panel E in Figure 4). See also references 44–47.

Imaging early mouse embryo development

In a special incubation stage, this enables observations from first cell divisions to multiple days. This has been a recent breakthrough in imaging these extremely photosensitive samples and was developed on an inverted setup, dubbed InVi-SPIM. See also reference 48.

Ultrafast imaging of smaller volumes with HILO/SCAPE

This describes the very fast and gentle imaging of small volumes with special single objective LSFM setups. They are called oblique plane microscopy, highly inclined and laminated optical sheet (HILO), swept confocally-aligned planar excitation (SCAPE) microscopy. These particular setups are used for coverslip-based imaging or in situations where the area to be imaged can only be accessed from one direction. The light sheet comes through the detection lens and the volumes can be scanned without mechanically moving optics or sample. See also references 49–51.

Deep and fast live imaging with two-photon LSFM

This serves for LSFM imaging in highly scattering embryos and tissue with a two-photon excitation scanned light sheet. See also references 52–54.

In practice, if you consider using LSFM for your scientific projects, first assess if your types of samples have been imaged before with LSFM and verify which type of optics geometry, sample mounting and incubation is required. Considering the

relatively active do-it-yourself (DIY) community around light-sheet microscopy, you might find yourself confronted with the question do you want to go through the effort of building your own microscope or would you rather choose to buy one of the commercially available turn-key systems?

A way to get started is to approach and participate in one of the open community platforms, such as OpenSPIM (www.openspim.org), which formalized the DIY process and introduced detailed instructions for the assembly and operation of basic SPIMs. However, the microscopes built after these open-source blueprints are usually not able to compete with more advanced setups or established reliable and high-performance commercial systems in terms of performance (speed, image quality) and opto-mechanical stability. But they might be an option to assess, as a first step, if and how LSFM can support your research. Most importantly, the platforms will quickly get you in contact with a large community of developers and users who can help you to decide whether to make or buy, and what is needed for your imaging requirements.[55]

With the introduction of the first commercial LSFM systems to the market, there is a range of easy-to-use turn-key systems available that covers a large part of LSFM applications. These commercial systems give biologists straightforward access to the technology and allow LSFM to address biological questions that could not previously be studied.

The following four Case Studies showcase recent publications as examples of research with LSFM.

CASE STUDY 1. Lattice light-sheet microscopy

Since all life is dynamic, it is clear that a better understanding of how molecules assemble to create life requires high-resolution imaging in space and time simultaneously. Imaging at higher spatial resolution and at higher frame rates exposes the specimen to more potentially damaging radiation, but this has been addressed by Eric Betzig and his team at Janelia Research Campus (Howard Hughes Medical Institute, HHMI). They employed LSFM with a scanned light sheet created from a 'non-diffracting' Bessel beam. When swept across the imaging focal plane, the beam creates a virtual light sheet of sub-micrometer thickness well suited to resolve sub-cellular structures and at the same time benefiting from the speed and non-invasiveness of LSFM for 4D live-cell imaging. Conventional light sheets from Gaussian beams do not allow comparably thin sections over reasonable large fields of view (Figure 1-1, Panels A and B).

Using an array of non-interacting Bessel beams to create the light sheet, Betzig could show even less photodamage in the sample and even faster acquisition rates compared to light sheets from a single scanned beam. The name lattice light-sheet microscopy (LLSM) is derived from the structured array of beams forming the light sheet (Figure 1-1, Panels C and D). The square lattice in (C) optimizes the confinement of the excitation to the central plane, and the hexagonal lattice in (D) optimizes the axial resolution as defined by the overall point spread function (PSF) of the microscope. The columns in (A) to (D) show the intensity pattern at the rear pupil plane of the excitation objective; the cross-sectional intensity of the pattern in the xz plane at the focus of the excitation objective (scale bar, 1.0mm); the cross-sectional intensity of the light sheet created by dithering the focal pattern along the x axis (scale bar, 1.0mm); and the xz cross section of the overall PSF of the microscope (scale bar, 200nm).

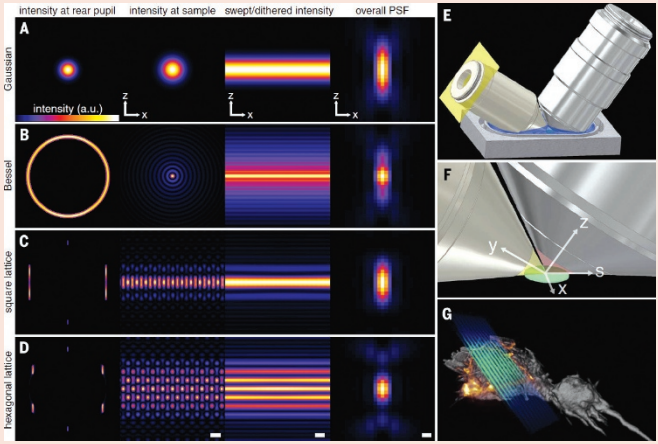

Figure 1-1. Methods of light-sheet microscopy. (A) The traditional approach: a Gaussian beam is swept across a plane to create the light sheet. a.u., arbitrary units. (B) A Bessel beam of comparable length produces a swept sheet with a much narrower core but flanked by sidebands arising from concentric side lobes of the beam. (C and D) Bound optical lattices create periodic patterns, greatly reducing the peak intensity and the phototoxicity in live-cell imaging. (E) Model showing the orthogonal excitation (left) and detection (right) objectives dipped in a media-filled bath. (F) Close up, showing the excitation (yellow) and detection (red) light cones, which meet at a common focus within a specimen on a cover glass within the media. The s-axis defines the scanning direction for 3D data acquisition. (G) Representation of a lattice light sheet (blue-green) intersecting a cell (gray) to produce fluorescence (orange) in a single plane. The cell is swept through the light sheet to generate a 3D image. From Chen *et al. Science* 2014;346:1257998. Reprinted with permission from AAAS

A key advantage of LLSM is its typical acquisition rate of hundreds of frames per second. Together with the low phototoxicity levels, this speed makes LLSM the ultimate tool for live-cell fluorescence imaging. Due to the high parallelization in illumination and the efficient objective lenses, it allows imaging of cells expressing endogenous levels of a labeled target protein and thus specimens to be studied closer to their native physiological state. It thereby becomes a key enabler of bringing imaging of genome-edited cells that have been modified with the help of the CRISPR-Cas9 system into mainstream application.

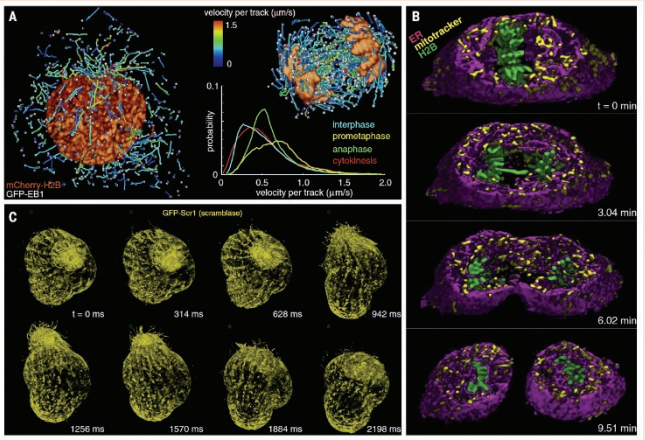

Figure 1-2. Intracellular dynamics in three dimensions. (A) Cells in prophase (left) and anaphase (right), showing histones and 3D tracks of growing microtubule ends, color-coded by velocity. (B) The 3D spatial relationship of histones (green), mitochondria (yellow), and endoplasmic reticulum (magenta) at four time points during mitosis in a slab extracted from a larger 4D, three-color data set of HeLa cells imaged for 300 time points. (C) Volume renderings at eight consecutive time points of a single specimen of the protozoan *Tetrahymena thermophila* taken from a 4D data set. From Chen *et al. Science* 2014;346:1257998. Reprinted with permission from AAAS.

Betzig and his collaborators applied this tool to numerous biological samples of various sizes with breathtaking results. They showed applications from imaging diffusion of single transcription factor molecules in stem cell spheroids over imaging the dynamic instability of mitotic microtubules and neutrophil motility in a 3D matrix to embryogenesis in C. elegans *and even* Drosophila *(Figure 1-2). Note that each image in Panel A represents a distillation of a few time points from a 4D two-color data set typically covering hundreds of time points per cell. The graph shows the distribution of growth rates at different stages of mitosis, averaged across 9 to 12 cells. Panel C shows a subset from time lapse spanning 1250 time points. Imaging at 3ms per frame in a single plane reveals the motions of individual cilia.*[26]

CASE STUDY 2. Clearing

In recent years, chemical methods for rendering light scattering tissue transparent have advanced enormously. A large variety of methods has emerged, such as Scale, SeeDB, clearsee, Ce3D, CUBIC, 3DISCO/iDISCO/uDISCO or CLARITY, most of them with a focus on a particular application or tissue. Optical clearing makes strongly scattering and opaque tissue, such as entire mouse brains, accessible for light microscopy.

With a cleared tissue sample, researchers can, in principle, choose from an array of imaging techniques to map tissue structure. 'LSFM is a natural pairing with cleared tissue' says Raju Tomer from Columbia University, New York, USA. 'Its acquisition speed of large 3D volumes makes LSFM the perfect tool to image larger numbers of intact cleared tissue at cellular resolution, a task that would be impractically slow with conventional 3D fluorescence imaging techniques.'

When he was in Karl Deisseroth's group at Stanford University, Tomer developed CLARITY-optimized light-sheet microscopy (COLM, Figure 2-1). The development of COLM involved three major adaptations of LSFM: the sample mounting to accommodate the large and soft tissue in the appropriate sample chamber with immersion medium and objective lenses adapted to the clearing medium. Second, the synchronized readout of the sCMOS camera sensor with the scanned laser beam that generates the light sheet. Today this readout mode is supported by most sCMOS cameras as 'light-sheet mode'. And last, an image-feedback driven dynamic adjustment of the light-sheet position relative to the plane of focus, to correct for position-dependent aberrations induced by the inhomogeneous optical properties of the tissue, representing one of the first examples of automatically adaptive LSFM.[35]

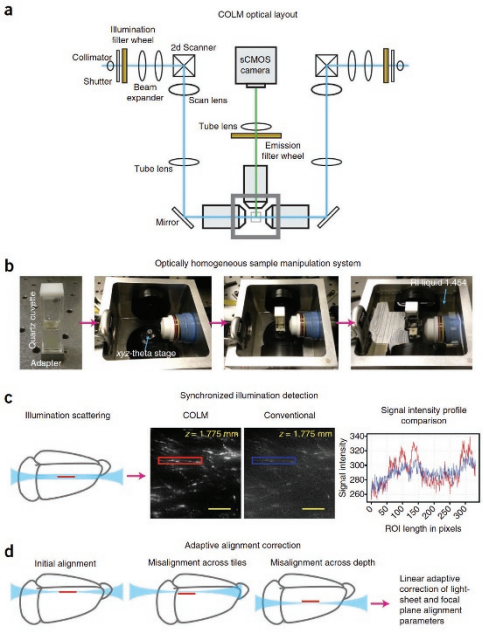

Figure 2-1. (a) Optical layout of the CLARITY-optimized light-sheet microscope. Two light sheets are created from opposite sides; shown are galvanometer scanners, scan lens, tube lens and illumination objectives. The emitted fluorescence is imaged with a detection objective, tube lens and sCMOS camera. The innovations required for COLM are discussed in b–d. (b) Optically homogeneous sample mounting framework for large intact samples. Clarified samples are mounted in a quartz cuvette filled with clearing solution such as FocusClear. The surrounding sample chamber is filled with a cheaper and non-clearing liquid with the same refraction properties. This results in an optically homogeneous sample mounting system with minimal refractive index transitions. (c) Synchronized illumination and detection is achieved by synchronizing the scanning beam with the unidirectional readout of a sCMOS camera chip, resulting in a virtual-slit effect that allows substantially improved imaging quality, as illustrated by the images shown acquired from the same plane with COLM and with conventional light-sheet microscopy. The graph on the right compares the signal intensity profile of a field acquired with COLM (red) and conventional light-sheet microscopy (blue). (d) Large clarified samples can have marked refractive index inhomogeneity, requiring correction of misalignment of illumination with the focal plane of the detection objective, achieved in this case with a linear adaptive calibration procedure. Scale bars, 100µm. Reprinted by permission from Macmillan Publishers Ltd: Tomer, *et al. Nature Protocols,* 2014;9:1682–92, copyright 2014

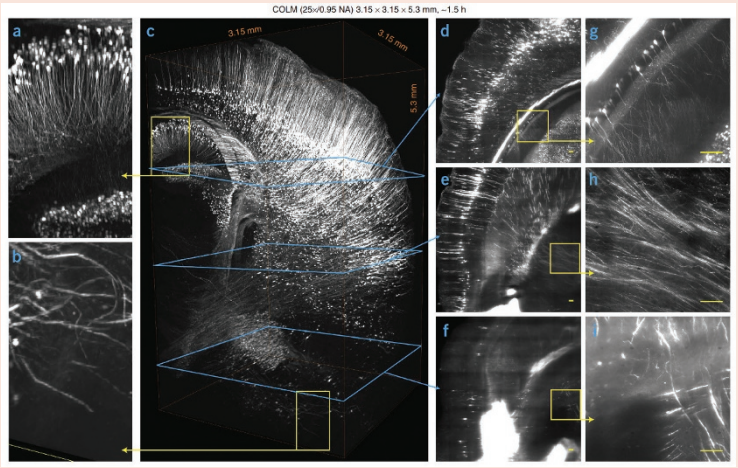

Figure 2-2. Fast high-resolution imaging of clarified brain using COLM. 3.15mm × 3.15mm = 5.3mm volume acquired from an intact clarified Thy1-eYFP mouse brain using COLM with 25× magnification. The complete image data set was acquired in ~1.5h. (a,b) Magnified views from Panel c region defined by yellow boxes. (d–i) Maximum-intensity projections over a 50µm-thick volume, as shown by the progression of cyan and yellow boxes and arrows. Scale bars, 100µm. Reprinted by permission from Macmillan Publishers Ltd: Tomer, *et al. Nature Protocols,* 2014;9:1682–92, copyright 2014

A large number of studies have either directly utilized the COLM system or used these principles for high-quality imaging of large samples. For example, in 2016, a team of collaborating laboratories used COLM and other techniques to map the organization of neuronal subtypes in the hypothalamus, the brain region with the highest diversity of neurons.[56]

Although originally developed for CLARITY cleared samples, the COLM principle is compatible with other tissue-clearing methods, and has already been successfully applied to the study of a range of tissues such as adult mouse, adult zebrafish and even adult human brain tissue, and it will facilitate the analysis of many other large biological specimens. Tomer has continued to develop and recently published improved LSFM-based methods for cleared tissue imaging in his own group at Columbia University.[57]

CASE STUDY 3. Phototoxicity

In the lab of Caren Norden at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany, scientists aim to better understand the development of organs by studying the formation of the vertebrate retina from cells to tissue. To get insights into fundamental questions of how developmental programs are coordinated during retinal formation, the Norden group uses advanced quantitative imaging and image analysis tools.

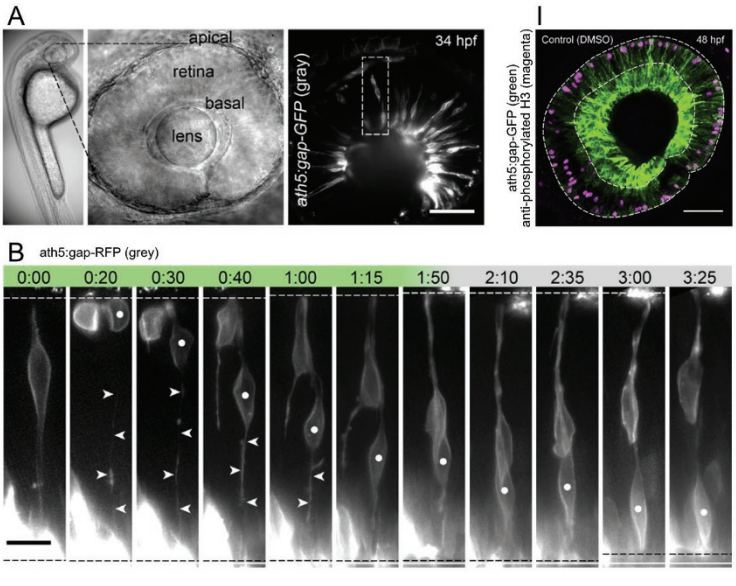

Figure 3-1. RGC translocation kinetics. (A) Developing eye of a 34-hpf embryo. *ath5:gap-GFP* transgene labels RGCs. The dashed box shows the typical area displayed in subsequent montages. Bar, 50µm. (B) Typical example of RGC translocation in LSFM. Arrowheads, basal process. Bar, 10µm. (I) RGC layer formation control. Mitotic cells are monitored by pH3 staining (magenta). Dashed lines mark the retinal outline and RGC layer. Bar, 50µm. A and B acquired with Lightsheet Z.1 from ZEISS. Republished with permission of Rockefeller University Press. Adapted from Icha, *et al. The Journal of Cell Biology*, 2016;215:259–75

As live imaging is a key tool for the study of dynamic cell migration processes, Jaroslav Icha, a postdoc in Norden's lab, used LSFM to observe retinal ganglion cell (RGC) translocation across the embryonic zebrafish retina.[58,59]

'We found that when using conventional confocal 3D fluorescence imaging techniques like, for example, spinning disk confocal microscopy, we altered the migration dynamics of retinal ganglion cells over development. Thus, we switched to light-sheet microscopy as we could show that it interferes less with the specimen,' Icha says. 'The availability of robust turn-key LSFM systems was an important enabler for our goal to characterize the underlying RGC translocation modes essential for retinal lamination and successful retina development.' Icha's work has been published in The Journal of Cell Biology.[58]

CASE STUDY 4. Multiview

The lab of Anastasios Pavlopoulos at HHMI Janelia Research Campus studies the molecular and cellular basis of tissue and organ morphogenesis during animal development and evolution. Combining functional genetic and genomic approaches with multiview LSFM and sophisticated image analysis tools, his lab follows developmental processes in vivo.

Pavlopoulos has been working to establish the crustacean Parhyale hawaiensis *as a powerful animal model to study normal development and regeneration (Figure 4-1A).* Parhyale *exhibits a remarkable diversity of limbs along their anterior-posterior axis, offering exceptional material to understand the mechanisms controlling limb morphogenesis and diversification.*

Recently, a multidisciplinary research collaboration around the Pavlopoulos lab developed a multiview light-sheet imaging and tracking workflow for studying Parhyale *limb morphogenesis.*[60]

'The availability of multiview LSFM was a major booster for the long-term observation of Parhyale *embryogenesis,' Pavlopoulos recalls. 'We were finally able to image all cells of growing limbs in intact developing embryos from early specification until late differentiation stages. We could image* Parhyale *embryos for several days or even a week under conditions and light dosages that did not compromise normal development of the specimen and did not photobleach the fluorescent markers.'*

In a typical experiment, a three-day old transgenic Parhyale *embryo with fluorescently labeled nuclei imaged on ZEISS Lightsheet Z.1 microscope (Figure 4-1B) under close-to-natural conditions in a temperature-controlled chamber filled with sea water. In each time point, sample rotation allowed to image the specimen from multiple angles (Figure 4-1C, top row). Open-source software was first used to align the input raw*

views relative to each other, and then to combine the registered views into a single output 3D image with nearly isotropic resolution (Figure 4-1C, bottom row). This process was repeated for about 1000 time points that were acquired every 7.5 minutes from day 3 up to day 8 of Parhyale *embryogenesis (Figure 4-1D).*

'Without today's robustness of the LSFM imaging and image analysis methodologies, the resolution of our analysis would have been impossible to achieve. We are finally in a stage where we can study the expression and function of genes in the context of single-cell resolution fate maps!'

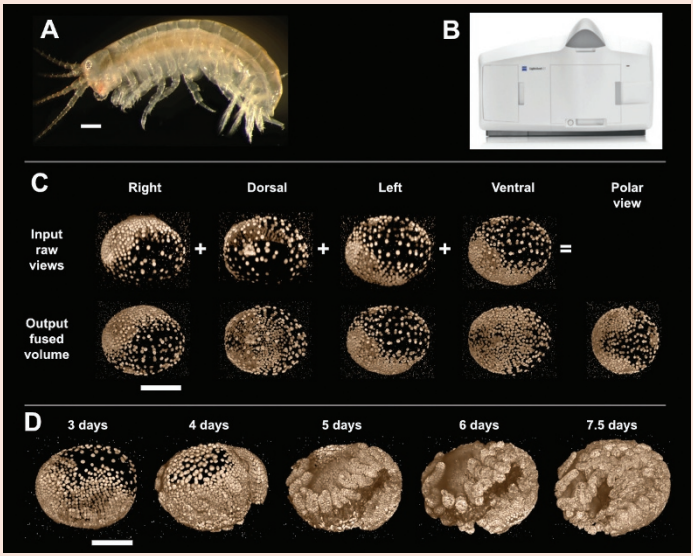

Figure 4-1. Reconstruction of *Parhyale* embryogenesis with multiview LSFM. (A) Morphology of the marine crustacean *Parhyale hawaiensis*. (B) ZEISS Lightsheet Z.1 LSFM used for imaging *Parhyale* embryogenesis. (C) A 2.5-day old transgenic *Parhyale* embryo with fluorescently labeled nuclei imaged from multiple views by rotating it around the anterior-posterior body axis (top row). Input views were registered and fused computationally into a single output volume. Each panel shows a 3D rendering of the raw (top) or fused (bottom) volumes with anterior to the left. (D) Representative time points from a five-day long multiview LSFM time-lapse recording showing different stages of *Parhyale* embryogenesis (in days after egg lay). Scale bars are 1000µm in Panel A and 200µm in Panels C and D. Reproduced with permission from ZEISS

PROBLEMS AND SOLUTIONS

In this section we have a brief look at two of the most important topics to consider when using LSFM for your experiments, namely the particularities of sample preparation and the requirements for handling and processing image data.

Choosing a microscopy method to address a particular scientific question always involves finding the optimal way to prepare the sample. The most obvious case is electron microscopy. In light microscopy, however, over more than 100 years, standardization trends, together with constraints of microscopy design, have led to a predominance of the coverslip-based sample preparation.

With 3D imaging picking up in the 1990s, mounting strategies for larger samples, *eg* tissue, organs, organisms and 3D cell cultures, were needed and the coverslip became unnecessary.

As discussed earlier, LSFM is dedicated to 3D live imaging and to imaging larger samples; therefore adoption of more suitable sample mounts is an inherent part of the technology. Although some implementations are still compatible with traditional coverslip-based sample mounting (see 'In Practice'), most of the LSFM applications literally place the sample at the centre of the microscopy process. The separate illumination and detection light paths facilitate the building of LSFM systems around the sample, using sample-specific incubation chambers and mounting strategies. The samples are maintained in conditions close to their natural enviroment, keeping them alive and healthy for the length of the study. For example, in Pavlopoulos' experiments (Case Study 4) embryos are imaged in a sea-water-filled chamber connected to a computer-controlled liquid handling system with in-line heater to supply temperature-controlled fresh medium. This setup also allows

induction of temperature changes or addition of drugs to see how living samples react to such stimuli in real time.

So what is the problem with this? Developing specific mounting techniques for each type of sample can be difficult and time consuming. This extra effort might make some researchers reluctant to use LSFM. However, there is a large pool of mounting techniques available that are described in detail, making it likely that you will be able to utilize or adapt an existing design for your samples, rather than developing new tools from scratch (Figure 5). Numerous reviews and tutorials provide quick access to helpful information to ensure successful LSFM experiments.[23,42,61–64]

Another challenge with LSFM is actually a product of its success, namely its ability to generate images both rapidly and over long time periods. Understandably, this generates a lot of data that needs to be stored and processed. In LSFM, a single imaging system today can produce almost one gigabyte of data per camera in one second. That is 100 images per second at the full sensor resolution of a modern scientific CMOS camera as it is widely used in LSFM. But even at moderate frame rates acquired over hours or days, LSFM can generate terabytes of data, which is orders of magnitude bigger than what other imaging modalities such as confocal microscopes, produce. Appropriate storage and processing solutions are required, as well as software tools and budget planning, since funding applications often require coverage of this topic.

Ideally, the image acquisition process streams the data directly to a storage location where it is safe and can be accessed at high bandwidth from high performance processing computers without interfering with the data storage from the next experiment run. It does not always take big and expensive solutions – but does require good planning since once the data is acquired, corrections are difficult.

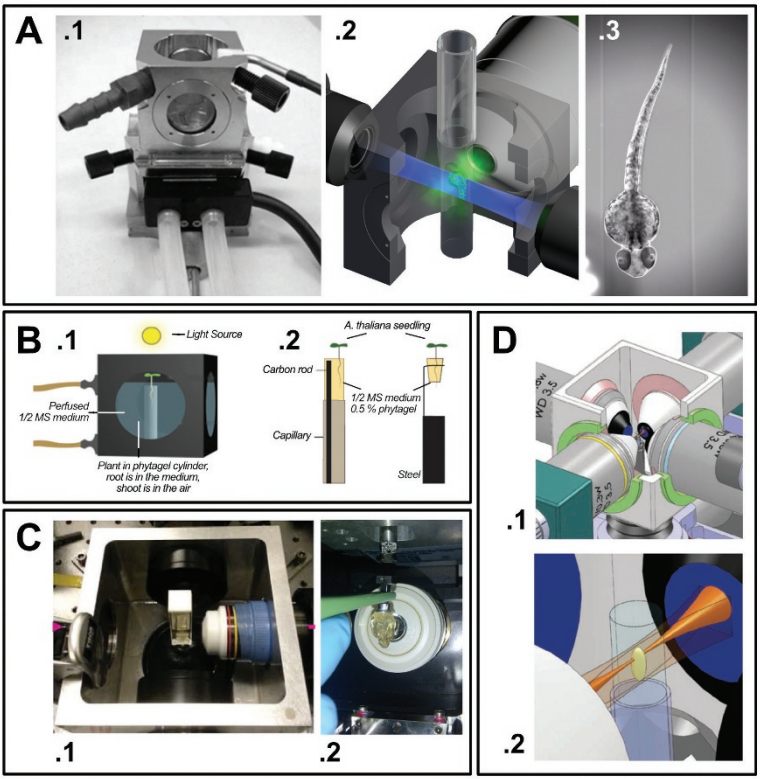

Figure 5. LSFM is built around the experiment, and the observation lens looks into the specimen chamber that serves as an incubator. The sample can be embedded in a suitable transparent 3D carrier that mimics the respective physiological conditions, such as a cylinder of a 3D matrix made of agarose or Matrigel. Other options are to use transparent fluorinated ethylene propylene (FEP) tubes, beakers made of glass, transparent polymer or membrane. The sample can also be simply attached to a stick connected to the 4D stage. Panel A shows a fully temperature-controlled sample chamber of ZEISS Lightsheet Z.1 (.1), a scheme of how a vertically hanging sample in a transparent polymer is mounted (.2), and a snapshot of a zebrafish in this configuration, mounted in an agarose cylinder or an FEP tube. Panel B shows an adapted sample mount for plant imaging in a two-lens LSFM. The chamber is equipped with daylight-cycle simulation (.1) and the holder is designed to keep the leaves in the air and the roots in a transparent polymer (.2). Panel C shows adaptations for imaging optically cleared mouse brain in a glass cuvette in (.1) and hanging after glued to a mounting rod (.2). Panel D shows sketches of a dedicated mounting of a *Drosophila* embryo in a four-lens LSFM chamber (.1 and .2). Panel A, copyright ZEISS. Panel B, from Maizel *et al. Plant J* 2011;68:377–85, reproduced with permission from Wiley. Panel C, image 1, reproduced with permission from Macmillan Publishers Ltd: Tomer, *et al. Nature Protocols* 2014;9:1682–97, copyright 2014. Panel C, image 2, copyright ZEISS. Panel D, images reproduced with permission from Macmillan Publishers Ltd: Krzic *et al. Nature Methods* 2012;9:730–2, copyright 2012

Can image storage and processing be supported by a computing department or on dedicated units as close to the microscope as possible? Is institute network infrastructure sufficient for handling LSFM data? Is a dedicated microscopy network needed? All such questions should be discussed with IT professionals at the university or institute level. Consultancy from professionals experienced in centralized network and storage architecture, as well as microscopy, is strongly recommended. Expertise and help can be found within the large Open Source LSFM community (OpenSPIM and Fiji), the microscope manufacturers, as well as independent consultants. Furthermore, IT companies are emerging that specialize in large image data solutions.

Strategies on how to handle data are equally important to appropriate infrastructure. First, reasonable measures to keep the data volume low should be taken. Deleting raw data after processing might come to mind as an approach to free the clogged data pipelines. It may be cheaper to repeat the experiment than store large amounts of raw data. But who wants to delete the great images that are the result of hard work from long days in the lab? The legal side needs consideration too: in some countries, raw data must be kept for 10 years by law. Also, some funding bodies require the long-term preservation of the raw data that back up published results. But what is 'raw data' in the context of LSFM? One ideal approach would be to use compression. But modern compression algorithms that can reduce raw data volumes significantly and at the same time ensure fast access are still under development and available to a few specialists only.

What is a typical processing workflow once the data are acquired and stored in the right place? A first step is often the alignment and fusion of multiview image data acquired from

different angles. Especially when studying developing embryos, the next step is often to identify individual cells and extract their migration paths from the image. This requires segmentation of objects from the voxel data, calculating tracks and measuring intensities, sizes, distances and velocities of objects to extract quantitative information about the imaged structures or dynamic processes. Numerous algorithms, both commercial and open-source, are available for these tasks, and some researchers have resorted to developing their own versions. But a critical criterion for the usefulness of such software remains how efficiently and easily it can be employed to the large time-lapse data from LSFM. The network of European bioimage analysts (NEUBIAS, www.neubias.org) is well connected to the light-sheet microscopy community and is a valuable resource for finding helpful software solutions.

An example of a promising strategy to streamline the amount of data produced by LSFM by online processing and discarding data from volume elements that have redundant or irrelevant information has been presented by the Huisken lab:[15] taking into account the essentially spherical shape of the early zebrafish embryo, segments of the image in which the embryo doesn't appear are skipped before data are stored. This reduces the data collection rate by a factor of about 100 and accelerates the analysis of the now condensed information. Cell segmentation and tracking are carried out in real time, rather than taking days after the experiment.

In summary, there is no single solution to address the challenges of the large image data volumes that can be easily acquired with LSFM. But there are many available solutions waiting to be employed for data analysis and information extraction from large LSFM data sets.

WHAT'S NEXT?

Over the coming years, we will certainly see a number of improvements in camera detection technology; better stages, new objective lenses and other optical elements will be able to produce thinner, more uniform light sheets. These technical improvements will make LSFM even more efficient and a more widely adopted microscopy technique.

Among the most important advances will be improvements of data storage, processing, analysis and visualization concepts for large 3D image data sets. Today's commonly available standard solutions for handling terabytes of data are still a limitation to large-scale uptake of LSFM. However, due to the growing awareness of this topic with funding bodies, manufacturers and vendors of LSFM solutions, an increasing number of scientists and developers are working on solutions for large image data handling and analysis. Funding programs for respective R&D are being established and even entire companies are founded to work on these bottlenecks. These activities indicate that significant improvements can be expected soon.

A second field of upcoming advances is the automation of LSFM imaging on multiplexed sample carriers. While LSFM is based on the parallelization of illumination and detection on a single sample, concepts for parallel imaging of multiple samples or the increase of sample throughput are still few. LSFM greatly reduces photodamage and therefore enables high-speed live imaging with no interval between time points. A parallelization of sample compromises this gain in temporal resolution, but the high acquisition rates in small volumes that can be achieved with LSFM systems make this an acceptable trade-off. Systems that are

compatible with conventional or modified multi-well plates will soon allow high content screening applications at the throughput of today's confocal or widefield screening systems – but much gentler to living samples.

A second option to increase sample throughput on multiview compatible LSFM setups could be the combination of flow-based, microfluidic technologies with LSFM to speed up systematic studies of sample-to-sample variability, large-scale phenotyping and drug screening in living embryos.

However, given that LSFM already pushes the data-handling capacity of even well-equipped laboratories, the success of such developments will strongly depend on the improvements in IT discussed above.

Last but not least, establishing easier and more flexible sample mounting techniques will continue and, with the above, will further advance LSFM to one of the most important 3D imaging methods in the Life Sciences.

FURTHER INFORMATION

History

Siedentopf H, Zsigmondy R. Uber sichtbarmachung und größenbestimmung ultramikroskopischer teilchen, mit besonderer anwendung auf goldrubingläser. *Ann Phys* 1902;315:1–39. (http://dx.doi.org/10.1002/andp.19023150102)

Huisken J, *et al.* Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 2004;305:1007–9. (http://dx.doi.org/10.1126/science.1100035)

Review

Power M, Huisken J. A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nat Methods* 2017;14:360–73. (http://dx.doi.org/10.1038/nmeth.4224)

Weber M, *et al.* Light sheet microscopy. *Methods Cell Biol* 2014; 123:193–215. (http://dx.doi.org/10.1016/B978-0-12-420138-5.00011-2)

Method of the year 2014

Stelzer EHK, *et al.* Method of the year 2014. *Nat Methods* 2014; 12:1. (http://dx.doi.org/10.1038/nmeth.3251)

Adaptive light-sheet microscopy

Royer LA, *et al.* Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nat Biotechnol* 2016;34: 1267–78. (http://dx.doi.org/10.1038/nbt.3708)

Lattice light-sheet original paper

Chen BC, *et al.* Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* 2014;346: 1257998–98. (http://dx.doi.org/10.1126/science.1257998)

Clearing review

Richardson DS, Lichtman JW. Clarifying tissue clearing. *Cell* 2015;162:246–57. (http:dx.doi.org/10.1016/j.cell.2015.06.067)

REFERENCES

1. Chalfie M, *et al. Science* 1994;263:802–5. (http://dx.doi.org/10.1126/science.8303295)
2. Tsien RY, *et al. FEBS Lett* 2004;579:927–32. (http://dx.doi.org/10.1016/j.febslet.2004.11.025)
3. Agard DA, *et al. Methods Cell Biol* 1989;30:353–77. (http://dx.doi.org/10.1016/s0091-679x(08)60986-3)
4. Conchello JA, Lichtman JW. *Nat Methods* 2005;2:920–31. (http://dx.doi.org/10.1038/nmeth815)
5. Huisken J, *et al. Science* 2004;305:1007–9. (http://dx.doi.org/10.1126/science.1100035)
6. Siedentopf H, Zsigmondy R. *Annalen Der Physik* 1903;4: 1–39. (http://dx.doi.org/10.1002/andp.19023150102)
7. Voie AH, *et al. J Microsc* 1993;170:229–36. (http://dx.doi.org/10.1111/j.1365-2818.1993.tb03346.x)
8. Buytaert JAN, Dirckx JJ. *J Biomed Opt* 2007;12:014039. (http://dx.doi.org/10.1117/1.2671712)
9. Dodt H-U, *et al. Nat Methods* 2007;4:331–6. (http://dx.doi.org/10.1038/nmeth1036)
10. Scherz PJ, *et al. Development* 2008;135:1179–87. (http://dx.doi.org/10.1242/dev.010694)
11. Keller PJ, *et al. Science* 2008;322;1065–9. (http://dx.doi.org/10.1126/science.1162493)
12. Huisken J, Stainier DYR. *Opt Lett* 2007;32:2608–10. (http://dx.doi.org/10.1364/OL.32.002608)
13. Krzic U, *et al. Nat Methods* 2012;9:730–3. (http://dx.doi.org/10.1038/nmeth.2064)
14. Tomer R, *et al. Nat Methods* 2012;9:755–63. (http://dx.doi.org/10.1038/nmeth.2062)
15. Schmid B, *et al. Nat Commun* 2013;4:3207. (http://dx.doi.org/10.1038/ncomms3207)
16. Amat F, *et al. Nat Methods* 2014;11:951–8. (http://dx.doi.org/10.1038/nmeth.3036)
17. Stegmaier J, *et al. Dev Cell* 2016;36:225–40. (http://dx.doi.org/10.1016/j.devcel.2015. 12.028)
18. Ahrens MB, *et al. Nat Methods* 2013;10:413–20. (http://dx.doi.org/10.1038/nmeth.2434)
19. Keller PJ, Ahrens MB. *Neuron* 2015;85:462–83. (http://dx.doi.org/10.1016/j.neuron.2014.12.039)

20. Vladimirov N, *et al. Nat Methods* 2014;11:883–4. (http://dx.doi.org/10.1038/nmeth.3040)
21. Chhetri RK, *et al. Nat Methods* 2015;12:1171–8. (http://dx.doi.org/10.1038/nmeth.3632)
22. Maizel A, *et al. Plant J* 2011;68:377–85. (http://dx.doi.org/10.1111/j.1365-313X.2011.04692.x)
23. von Wangenheim D, *et al. Methods Mol Biol* 2014;1062:539–50. (http://dx.doi.org/10.1007/978-1-62703-580-4_28)
24. Capua Y, Eshed Y. *PNAS* 2017;114:3246–51. (http://dx.doi.org/10.1073/pnas.1617146114)
25. de Luis Balaguer MA, *et al. Dev Biol* 2016;419:19–25. (http://dx.doi.org/10.1016/j.ydbio.2016.05.029)
26. Chen B-C, *et al. Science* 2014;346:1257998–98. (http://dx.doi.org/10.1126/science.1257998)
27. Fahrbach FO, *et al. Opt Express* 2013;21:21010–26. (http://dx.doi.org/10.1364/OE.21.021010)
28. Fahrbach FO, *et al. Nat Photonics* 2010;4: 780–5. (http://dx.doi.org/10.1038/nphoton.2010.204)
29. Gao L, *et al. Cell* 2012;151:1370–85. (http://dx.doi.org/10.1016/j.cell.2012.10.008)
30. Gao, L *et al. Nat Protoc* 2014;9:1083–101. (http://dx.doi.org/10.1038/nprot.2014.087)
31. Olarte OE, *et al. Biomed Opt Express* 2012;3:1492–505. (http://dx.doi.org/10.1364/BOE.3.001492)
32. Planchon TA, *et al. Nat Methods* 2011;8:417–23. (http://dx.doi.org/10.1038/nmeth.1586)
33. Chen F, Tillberg PW, Boyden ES. Expansion microscopy. *Science* 2015;347:543–8. (http://dx.doi.org/10.1126/science.1260088)
34. Chung K, *et al. Nature* 2013;497:332–7. (http://dx.doi.org/10.1038/nature12107)
35. Tomer R, *et al. Nat Protoc* 2014;9:1682–97. (http://dx.doi.org/10.1038/nprot.2014.123)
36. Susaki EA, *et al. Cell* 2014;157:726–39. (http://dx.doi.org/10.1016/j.cell.2014.03.042)
37. Richardson DS, Lichtman JW. *Cell* 2015;162:246–57. (http://dx.doi.org/10.1016/j.cell.2015.06.067)

38. Hama H, *et al. Nat Neurosci* 2015;18:1518–29. (http://dx.doi.org/10.1038/nn.4107)
39. Wu Y, *et al. PNAS* 2011;108:17708–13. (http://dx.doi.org/10.1073/pnas.1108494108)
40. Kumar A, *et al. Nat Protoc* 2014;9:2555–73. (http://dx.doi.org/10.1038/nprot.2014.172)
41. Christensen RP, *et al. eLIFE* 2015;4:e10070. (http://dx.doi.org/10.7554/eLife.10070)
42. Pampaloni F, *et al. Nat Rev Mol Cell Biol* 2007;8:839–45. (http://dx.doi.org/10.1038/nrm2236)
43. Hagiwara M, *et al. Sci Rep* 2015;5:8054–7. (http://dx.doi.org/10.1038/srep08054)
44. Ritter JG, *et al. PLoS ONE* 2010;5:e11639. (http://dx.doi.org/10.1371/journal.pone.0011639)
45. Capoulade J, *et al. Nat Biotechnol* 2011;29:835–9. (http://dx.doi.org/10.1038/nbt.1928)
46. Ng XW, *et al. Biophys J* 2016;111:418–29. (http://dx.doi.org/10.1016/j.bpj.2016.06.021)
47. Hoyer P, *et al. PNAS* 2016;113:3442–6. (http://dx.doi.org/10.1073/pnas.1522292113)
48. Strnad P, *et al. Nat Methods* 2016;13:139–42. (http://dx.doi.org/10.1038/nmeth.3690)
49. Dunsby C. *Opt Express* 2008;16:20306–16. (http://dx.doi.org/10.1364/OE.16.020306)
50. Tokunaga M, *et al. Nat Methods* 2008;5:159–61. (http://dx.doi.org/10.1038/nmeth1171)
51. Bouchard MB, *et al. Nat Photonics* 2015;9:113–9. (http://dx.doi.org/10.1038/nphoton.2014.323)
52. Truong TV, *et al. Nat Methods* 2011;8:757–60. (http://dx.doi.org/10.1038/nmeth.1652)
53. Mahou P, *et al. Nat Methods* 2014;11:600–1. (http://dx.doi.org/10.1038/nmeth.2963)
54. Trivedi V, *et al. Biomed Opt Express* 2015;6:2056–66. (http://dx.doi.org/10.1364/BOE.6.002056)
55. Girstmair J, *et al. BMC Dev Biol* 2016;16:22. (http://dx.doi.org/10.1186/s12861-016-0122-0)
56. Romanov RA, *et al. Nat Neurosci* 2017;20:176–88. (http://dx.doi.org/10.1038/nn.4462)
57. Migliori B, *et al. bioRxiv* 2017;March 22. (http://dx.doi.org/10.1101/119289)
58. Icha J, *et al. J Cell Biol* 2016;215:259–75. (http://dx.doi.org/10.1083/jcb.201604095)

59. Icha J, *et al. Bioessays* 2017;39:1700003. (http://dx.doi.org/10.1002/bies.201700003)
60. Wolff C, *et al. bioRxiv* 2017;February 28. (http://dx.doi.org/10.1101/112623)
61. Carl Zeiss guide for LSFM sample mounting (https://applications.zeiss.com/C125792900358A3F/0/E6504BFCBC3C2D39C1257BD500417CA6/$FILE/EN_41_011_058_LightsheetZ1_Sample-Preparation.pdf).
62. Schmied C, Tomancak P. *Methods Mol Biol* 2016;1478:189–202. (http://dx.doi.org/10.1007/978-1-4939-6371-3_10)
63. Kaufmann A, *et al. Development* 2012;139:3242–7. (http://dx.doi.org/10.1242/dev.082586)
64. Jakob PH, *et al. Cytotechnology* 2016;68:1813–25. (http://dx.doi.org/10.1007/s10616-015-9935-0)

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