Shedding light on the system: Studying embryonic development with light sheet microscopy
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Light sheet-based fluorescence microscopy (LSFM) is emerging as a powerful imaging technique for the life sciences. LSFM provides an exceptionally high imaging speed, high signal-to-noise ratio, low level of photo-bleaching and good optical penetration depth. This unique combination of capabilities makes light sheet-based microscopes highly suitable for live imaging applications. There is an outstanding potential in applying this technology to the quantitative study of embryonic development. Here, we provide an overview of the different basic implementations of LSFM, review recent technical advances in the field and highlight applications in the context of embryonic development. We conclude with a discussion of promising future directions.

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Introduction
Non-invasive three-dimensional imaging over time is indispensable for a quantitative understanding of biological processes at multiple scales, from molecular interactions to tissue morphogenesis. In particular, the in vivo study of early embryogenesis requires state-of-the-art imaging strategies that achieve high spatiotemporal resolution without compromising specimen integrity. Progress in sensor technologies, lasers and desktop computing in the past decades has led to a remarkable progress in imaging technologies and capabilities.

Specifically, the emergence of Confocal Laser-Scanning Fluorescence Microscopy (CLSM) provided means for non-invasive imaging of fixed as well as live samples for three-dimensional reconstructions and, therefore, confocal microscopes have become standard instruments in many laboratories. However, the ever-increasing demand to image for longer periods of time and at higher spatiotemporal resolution is rapidly exposing the limitations of CLSM. The point-scanning implementation of CLSM is inherently slow and causes high levels of photo-bleaching and photo-toxicity, owing to the iterative use of non-selective excitation with high-power beams. Additionally, tissue penetration depth is relatively low. These issues inspired the development of non-linear microscopy, specifically two-photon microscopy, which provided a substantial increase in the penetration depth and reduction in photo-bleaching, albeit, at the expense of spatial resolution and imaging speed.

In the past decade, Light Sheet-Based Fluorescence Microscopy (LSFM) has emerged to fill the gap resulting from the inherent limitations of CLSM and point-scanning two-photon microscopy. The key concept behind LSFM is sample illumination in a thin volume section from the side, and fluorescence detection with an independent optical subsystem at a right angle to the illumination axis. This is in contrast to confocal microscopy and conventional wide-field microscopy, which typically use the same objective lens for illumination and detection. By illuminating only the in-focus plane, LSFM provides intrinsic optical sectioning and enables simultaneous detection of the fluorescence signal from an entire plane with highly efficient detectors. Thereby, LSFM combines several crucial properties, including high acquisition speed, high signal-to-noise ratio, minimal levels of photo-bleaching and good penetration depth. Moreover, sample preparation typically involves the use of low-concentration agarose cylinders for sample embedding, which represent a less stressful environment for live biological samples than the traditionally employed glass slide/cover slip. LSFM is thus naturally well-suited for studies of early embryogenesis, since it combines high-content dynamic imaging with a more physiological imaging environment.

Here, we discuss the various implementations of LSFM, review recent technical advances in the field, and highlight novel applications in the study of early embryogenesis. Other aspects of light sheet microscopy have been reviewed elsewhere [1–6].

Implementations of light sheet-based fluorescence microscopy
LSFM development has accelerated in the past decade. Many different variants with specialized features have emerged. However, the basic concept remains similar to the design of the first light sheet microscope by
Siedentopf and Zsigmondy [7]: the sample is illuminated with a sheet of light that overlaps with the focal plane of the detection optics (Figure 1).

Almost a century later, Voie et al. developed Orthogonal-Plane Fluorescence Optical Sectioning (OPFOS) [8]. The authors used a cylindrical lens to create a light sheet and imaged the internal architecture of the cochlea by scanning the sample through the sheet, achieving lateral and axial resolutions of 10 μm and 26 μm, respectively, for a field-of-view (FOV) of 1.5 mm. The shape of the light sheet can be well approximated by Gaussian beam optics and, thus, an intrinsic tradeoff exists between the central thickness of the light sheet, which directly influences axial resolution, and the size of the FOV within which the sheet’s thickness remains sufficiently uniform. A high-resolution extension of OPFOS (HR-OPFOS) [9] was developed more recently, in which the authors used a large aperture lens to generate a thin light sheet. By moving the (fixed) sample stepwise along the illumination axis and recording the fluorescence signal from the thinnest part of the light sheet at each step, large specimens could be recorded with excellent axial resolution.

Thin Light Sheet Microscopy (TSLM) was developed to observe microbes in their natural setting with a millimeter-sized FOV [10]. Ultramicroscopy is a similar implementation optimized for imaging large fixed samples [11]. The sample is placed into a chamber containing the clearing solution with matched refractive index. Sample illumination is performed from two sides with aligned light sheets generated by cylindrical lenses. Both HR-OPFOS and Ultramicroscopy excel at imaging large fixed samples, but they are incompatible with the in vivo imaging of dynamic biological samples.

Selective Plane Illumination Microscopy (SPIM) was developed as an implementation of LSFM for non-invasive live imaging of early embryos [12]. Using fast CCD cameras and agarose cylinders for sample mounting, Huisken et al. reported long-term imaging of *Drosophila* embryonic development and high-speed imaging of the beating heart of a Medaka fish embryo. Since the one-sided illumination used in SPIM was often accompanied by shading effects resulting in stripe artifacts in the recorded images, the basic SPIM setup was further extended by multidirectional illumination (mSPIM) [13].
In addition, multi-view reconstructions and image deconvolution have been used to improve image quality [14,15]. In multi-view imaging, different views of the sample are obtained by rotating the sample. These data sets can then be combined computationally to achieve isotropic resolution in transparent samples [14] or to maximize coverage in partially opaque specimens [16,17].

Keller et al. reported the first beam-scanning implementation of light sheet-based microscopy (Digital Scanned Laser Light Sheet Fluorescence Microscopy, DSLM) [16] and used it to perform fast long-term imaging of entire developing zebrafish embryos. DSLM provides uniform intensity levels across the FOV, minimization of energy losses in the illumination process, a high-quality implementation of image contrast-enhancing structured illumination, and the capability to scan the light sheet through the sample for high-speed three-dimensional imaging. The beam-scanning approach introduced in DSLM has also been used as a basis for new LSFM techniques, such as Bessel plane illumination and efficient two-photon excitation [18**,**19**].

Other LSFM implementations include Objective Coupled Plane Illumination (OCPI) [20], Highly Inclined Laminated Optical Microscopy (HILO) [21] and Oblique Plane Microscopy (OPM) [22]. In OCPI, the light sheet generating optics is coupled with the detection objective. The authors applied their technique to the fast imaging of neuronal activity in the mouse vomeronasal organ. HILO uses the same objective for illumination and detection. A highly inclined sheet of light is created and fluorescence observation is limited to a small FOV, where the light sheet and focal plane of the detection system overlap reasonably well. The basic HILO implementation has been extended in OPM, which enables an oblique plane in the specimen to be illuminated as well as imaged with the same objective lens. This approach allows recording a large FOV using conventional glass slide-based sample preparation.

Recent advances in light sheet-based fluorescence microscopy

Many aspects of LSFM have been improved over the past few years, including the development of better illumination and detection optics, instrument miniaturization, integration of other modalities, such as Fluorescence Correlation Spectroscopy (FCS), and efforts toward resolution enhancement and implementation of adaptive optics. Two particularly noteworthy improvements were recently reported: first, the use of self-reconstructing Bessel beams to create thinner light sheets and to reduce image quality degradation by light scattering, and second, the use of two-photon excitation in scanned light sheet microscopy.

Bessel beams are created by projecting an annular pattern at the rear pupil of an illumination objective. The central peak width is determined by the thickness of the annulus and can be decoupled from the Bessel beam’s longitudinal extent. Fahrbach et al. conducted experiments with holographically shaped Bessel beams to demonstrate the self-reconstructing properties in three-dimensional scattering tissues, which reduce light scattering-induced image artifacts and increase penetration depth [18**,23].

The authors reported a prototype implementation (Microscope with Self-Reconstructing Beams; MISERB), which employs a Spatial Light Modulator (SLM) to generate scanned light sheets using Bessel beams.

Planchon et al. used an annular mask and an axicon to generate scanned light sheets from large numerical aperture Bessel beams [19**]. Bessel beams contain a substantial amount of energy in side lobes, which can be accounted for – lead to reduced axial resolution in light sheet-based microscopy in comparison to a Gaussian beam implementation. To address this problem, the authors used structured illumination and two-photon excitation, which eliminated the signal contribution of the side lobes to the recorded fluorescence images. The overall result is a marked improvement in axial resolution. Planchon et al. applied their technique to high-speed volumetric imaging of chromosomes in mitosis, three-dimensional imaging of protein pairs with isotropic resolution, and the observation of membrane dynamics in live cells. Photo-bleaching rates in scanned Bessel beam-based light sheet microscopes are significantly higher than those obtained with the scanned Gaussian beams used in DSLM. Thus, further experiments are required to determine if these principles can be extended to the live imaging of larger samples, such as entire developing embryos, over long periods of time.

Two-photon excitation has been implemented in scanning as well as non-scanning light sheet microscopy [19**,24*]. These implementations combine the intrinsic advantages of light sheet microscopy with the increased penetration depth resulting from the use of an infrared laser beam for fluorescence excitation.

Improvements in axial resolution can also be achieved by imaging the sample through the thinnest part of a light sheet created with Gaussian beams. Buytaert et al. used large aperture cylindrical lenses to generate highly focused light sheets and moved their sample step by step along the illumination axis. By stitching the resulting images, they obtained high axial resolution over a large FOV (HR-OPFOS) [9,25]. Similarly, Santi et al. reported TSLIM (Thin-Sheet Laser Imaging Microscope), where bi-directional light sheet illumination is used and the sample is moved through the thinnest part of the sheet to record a large FOV at high axial resolution [26]. Schacht et al. further improved TSLIM to provide a 70% reduction in scanning time and a 63% reduction in photo-bleaching [27]. These approaches are very useful for imaging large fixed samples at micron axial resolution. However, the
relatively slow acquisition speeds and high rates of photo-bleaching limit their applicability to live specimens.

Building upon the first implementation of scanned light sheet microscopy (DSLM) [16], Keller et al. implemented incoherent structured illumination in DSLM (DSLM-SI) to eliminate the contribution of scattered light to the fluorescence signal in partially opaque specimens [28**]. The authors demonstrated substantial improvements in signal-to-noise ratio, image contrast and lateral resolution, which allowed them to perform high-speed long-term imaging of zebrafish embryos and highly light-scattering Drosophila embryos. Similarly, Mertz et al. used scanned light sheet microscopy and a combination of uniform and incoherently modulated illumination to reject scattered signal light in their recordings of mouse brain tissue [29]. Lei et al. used a symmetric four-faceted pyramid to obtain a coherent three-dimensional structured illumination pattern. Combining this multi-layered light sheet geometry with the SI reconstruction algorithm introduced by Neil et al. [30] allowed the authors to discard out-of-focus light and improve axial resolution [31].

Some applications benefit greatly from miniaturized implementations of LSFM. Turaga et al. used an uniaxial gradient-index lens (GRIN), which replaces the cylindrical lens, to generate a light sheet in a miniaturized implementation of OCPI [32]. Similarly, Engelbrecht et al. used single-mode optical fibers, GRINs and a right-angle prism to generate a light sheet in a miniSPIM setup [33*].

In addition to two-photon excitation, several other concepts have been successfully combined with LSFM. Friedrich et al. integrated stimulated emission depletion (STED) in SPIM (STED-SPIM) and achieved a 60% enhancement in axial resolution [34]. Turaga et al. introduced adaptive optics in light sheet microscopy. The authors implemented an image-based wavefront sensor using a variant of generalized phase-diverse imaging called multi-frame blind deconvolution to calibrate the deformable mirrors in an OCPI setup [35]. Wohland et al. combined SPIM with Fluorescence Correlation Spectroscopy (SPIM-FCS) and reported its in vivo application by imaging microspheres injected into the blood stream of a zebrafish embryo [36].

Applications in embryonic development

LSFM is particularly well suited for fast and long-term imaging of developmental processes at multiple spatial and temporal scales. Its applications range from imaging molecular interactions and gene expression patterns (Figure 2) to the reconstructions of early developmental dynamics (Figure 3) and in toto imaging of large fixed specimens (Figure 4).

Taking advantage of the high imaging speed and excellent signal-to-noise ratio of LSFM, Ritter et al. observed single molecules as deep as 200 μm within salivary gland tissue of C. tentans larvae [37,38]. The authors estimated the intra-nuclear viscosity parameters by imaging tracer molecules within the cell nuclei and were able to visualize the transport of single mRNA molecules tagged by fluorescently labeled RNA binding proteins. This study shows the potential of using LSFM to investigate gene activity in developing embryos at single molecule resolution.

At the scale of entire embryos, Keller et al. used DSLM to image early development of wild-type and mutant
zebrafish for 24 h [16]. The authors performed computational reconstructions of the microscopy data sets to generate ‘digital embryos’ (www.digital-embryo.org), which comprise quantitative information on cell coordinates, migratory tracks and cell division patterns for most of the cells in the developing embryo (Figure 3). This approach provided a detailed model of germ layer formation and revealed an initial morphodynamic symmetry break in the patterns of cell division, which identifies the orientation of the dorso-ventral body axis. Taking advantage of the capabilities of incoherent structured illumination, Keller et al. extended DSLM to DSLM-SI, to image and reconstruct early embryogenesis of partially opaque Drosophila embryos [28**]. Recently, Swoger et al. used SPIM to image and quantitatively analyze posterior lateral line (pLL) organogenesis in zebrafish [39*]. The authors used a triple-label fluorescence marker strategy for recording 4D data sets of the migration of the primordium and maturation of proneuromasts. The retrospective tracking analysis enabled the identification of hair cell progenitor lineages.

Global cell tracking in zebrafish embryos with scanned light sheet microscopy. DSLM microscopy data (right half of embryo: animal view, maximum-intensity projection) and the subsequently reconstructed ‘digital embryo’ (left half of embryo) with color-encoded cell migration directions. Time points: 289 min post fertilization (mpf) (a), 368 mpf (b), 599 mpf (c), 841 mpf (d). Color code: dorsal migration (cyan), ventral migration (green), toward or away from body axis (red or yellow), toward yolk (pink). Objective: Carl Zeiss C-Apochromat 10×. Databases and high-resolution movies of the digital zebrafish embryo are available at http://www.digital-embryo.org.

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Scherz et al. used high-speed mSPIM imaging to investigate the morphological and functional development of the zebrafish heart. Their analysis revealed that the atrioventricular valve (AVV) invaginates to directly form leaflets [40]. The authors furthermore disrupted ErbB receptors, TGF-β receptors and Cyclooxygenase 2 (Cox2)-dependent signaling to dissect the role of Cox2 in regulating myocardial cell shape. In a follow-up study, Arrenberg et al. combined optogenetics and light sheet microscopy to study the emerging function of the developing heart [41**]. The authors imaged zebrafish embryos with channelrhodopsin and halorhodopsin transgenes, which provided the capability to precisely control muscle cell activity. Using mSPIM in combination with well-defined photo-stimulation patterns for the systematic illumination of cell groups, they found that the pacemaker cells converge to the sinoatrial region in the developing heart. Systematic inhibition of these cells showed that only a few dozen of these cells are sufficient to maintain a functional system.

**Future perspectives**

Although the basic concept of light sheet illumination is over a century old [7], its modern form, laser light sheet-based fluorescence microscopy, has taken shape only recently. The spectrum of applications in the life sciences in general, and in early embryogenesis in particular, is extensive and further improvements can be envisioned in a multitude of ways.

Key parameters in any light microscopy technique are temporal resolution, spatial resolution, photo-bleaching/photo-toxicity and optical penetration depth. In the context of light sheet-based microscopy, further improvements are feasible in all of these aspects. Higher imaging speeds can be achieved by using faster detectors such as cameras based on sCMOS technology (http://www.scmos.com), integration of real-time electronics for microscope control and by parallelizing sample illumination and signal detection. Similarly, a further reduction of the energy load on the specimen – and thus a reduction of photo-bleaching and photo-toxic effects – is possible by using multiple detectors to collect more signal photons. Combining photoactivatable light microscopy (PALM, STORM) with the concept of light sheet illumination might provide enhanced super-resolution imaging capabilities in three dimensions. Further improvements in imaging depth could be achieved through the development of (infra-)red fluorescent proteins with good photostability and high quantum yield.

Qualitative advances and new experimental strategies are also conceivable and complement the quantitative improvements outlined above. For example, enhanced
capabilities in specimen interaction during imaging can be realized via optical subsystems for photo-activation, laser ablation and photo-stimulation. Automation of the experimental assay will enable the design of entirely new imaging-based studies, and could be achieved by using high-throughput approaches to sample loading via microfluidics or robotics and computational approaches to online data analysis. For example, using a reference database of embryonic development with single cell resolution (‘digital embryo’), the control computer of a light sheet-based microscope could segment and structurally correlate a developing specimen online during image acquisition, thereby allowing statistical predictions of cellular or tissue-level identity for targeted laser ablation in functional studies. New approaches to in vitro tissue/organ culture may allow time-lapse imaging experiments in organisms that exhibit challenging optical properties, whereas new approaches to in vivo sample preparation may further enhance capabilities in physiological long-term imaging of entire developing animals.

Finally, it should be noted that large amounts of data result from high-speed and/or long-term in vivo light sheet-based microscopy experiments. For steady progress in the field, it will be imperative to develop advanced computational strategies to high-throughput data streaming, data management and storage, online processing and offline data analysis.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:  
* of special interest  
** of outstanding interest


The authors demonstrate self-reconstructing properties of holographically shaped Bessel beams in three dimensional scattering tissues, which lead to reduced scattering-induced image artifacts and increased optical penetration depth.


The authors used an annular mask and an axicon to generate scanned light sheets from high aperture Bessel beams. Using structured illumination and two-photon excitation, the authors achieved a marked improvement in axial resolution, which allowed them to perform fast volumetric imaging of living cells with isotropic spatial resolution.


The authors used near-infrared lasers to achieve two-photon excitation with light sheets in a SPIM set-up (2p-SPIM). They performed optical sectioning of C. elegans as a proof-of-principle application of their implementation.


The authors introduced incoherent structured illumination to scanned light sheet microscopy (DSLM-SI) to enhance the image contrast in partially opaque specimens and to eliminate image artifacts arising from light scattering. They applied DSLM-SI to live imaging of zebrafish development for three days and fast imaging of early Drosophila embryogenesis, achieving subcellular resolution. The authors performed a computational reconstruction of the Drosophila recording, creating a digital fly embryo.


Combining graded index lenses (GRIN) and single mode optical fibers, the authors developed a miniaturized version of SPIM (miniSPIM).


The authors applied SPIM to time-lapse imaging of posterior lateral line (pLL) organogenesis in zebrafish. A retrospective tracking analysis enabled the identification of the respective progenitor lineages.


The authors combined transgenic expression of channelrhodopsin/alarhodopsin and light imaging using mSPIM to precisely control and image the activity of muscle cells in the developing zebrafish heart. Using a systematic activation/inhibition approach, the authors showed that the pacemaker cells converge to the sinoatrial region, and that a few dozen of these cells are sufficient to maintain a functioning heart.