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Light sheet microscopy of living or cleared specimens

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Light sheet microscopy is a versatile imaging technique with a unique combination of capabilities. It provides high imaging speed, high signal-to-noise ratio and low levels of photobleaching and phototoxic effects. These properties are crucial in a wide range of applications in the life sciences, from live imaging of fast dynamic processes in single cells to long-term observation of developmental dynamics in entire large organisms. When combined with tissue clearing methods, light sheet microscopy furthermore allows rapid imaging of large specimens with excellent coverage and high spatial resolution. Even samples up to the size of entire mammalian brains can be efficiently recorded and quantitatively analyzed.
Here, we provide an overview of the history of light sheet microscopy, review the development of tissue clearing methods, and discuss recent technical breakthroughs that have the potential to influence the future direction of the field.

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History of light sheet microscopy

The first use of light sheet microscopy dates back to the work of Siedentopf and Zsigmondy [1••]. Siedentopf was an optical physicist working at the Carl Zeiss Works and Zsigmondy a physical chemist working on colloid chemistry. They tried to visualize small colloidal particles (Ultramicronen). At that time no powerful darkfield condensers were available, and they came up with the idea to illuminate their colloidal solutions from the side. In order to illuminate a well-defined volume, they created a sheet of light by using lenses, aperture stops and microscope objectives. Lasers were not yet available and, thus, they had to use bulky carbon arc burners. Although this 'ultramicroscope' was made commercially available, it was never used widely besides in colloidal chemistry. Subsequent efforts in microscope development focused instead on fabrication of more light efficient darkfield condensers like the cardioid condenser, which allowed illumination with numerical apertures as high as 1.4. Darkfield microscopy with high numerical aperture condensers employs inclined ring-like light sheets – however, this technique will not be discussed here. Incident light darkfield microscopy was popular in the sixties in biological research before the advent of confocal microscopy and was marketed as 'Ultropak' by the microscopy company Leitz.

Real light sheet microscopy was used thereon in different fields, most prominently in material macrography. McLachlan described the use of light sheet illumination in microscopy to visualize the surface of minerals and insect eyes [2•]. A similar approach was used in light-scanning photomicrography, which was also employed to visualize the surface of insects with two-sided or three-sided illumination [3]. This arrangement was marketed as 'Dynaphot' (Irvine Optical Corporation, Burbank, CA) and was primarily designed as an imaging tool with lower magnification, complementary to the scanning electron microscope. A similar arrangement for photomicrography was described for the macroscopic imaging of screws, coins and rifle bullets [4,5]. The use of laser light sheets has also been reported in the context of fluid dynamics investigations [7]. Finally, an arrangement was developed that used only one illumination source and a clever arrangement of mirrors to surround the object with a light sheet [6]. However, all these latter approaches relied on light sheet microscopy only as a means to visualize the surface of objects.

So far, light sheet microscopy had made no relevant impact on biology, as the surface of biological objects is often not of particular interest and biological specimens larger than a few cells layers tend to be relatively opaque, preventing light from entering their inside.

The first relevant use of light sheet microscopy in biology was reported by Voie *et al.*. In their seminal paper, the authors used for the first time light sheet illumination for fluorescence excitation in a biological specimen [8••]. As the object of study they chose the guinea pig cochlea, which was made fluorescent by immersion in rhodamine. Before imaging they had to make the cochlea transparent by the use of a special clearing solution developed a century ago by Werner Spalteholz (see below). They obtained good images of the anatomy of the inner ear and extracted quantitative data, which would have been difficult to obtain with histological methods. Although all basic elements present in modern light sheet microscopes were used, the method, which was published in the

renowned Journal of Microscopy, remained largely unnoticed by the general public. There were probably several reasons. The authors had no access to powerful three-dimensional reconstruction software, although these tools were already available at that time for confocal microscopy. Therefore, they used their own custom software and rendering tools, which yielded rather unattractive wire grid visualizations. And second and most importantly, the applicability of optical clearing to more relevant biological specimens than the inner ear was not realized. Thus, light sheet microscopy had relatively little impact in the scientific community. It took an entire decade before light sheet microscopy was used again in biology, - this time for microbial studies in oceanography [9].

After the pioneering work of Voie and colleagues, it was then the ground-breaking study conducted by Ernst Stelzer's lab at EMBL, which drew the attention of developmental biologists to light sheet microscopy. The team realized that light sheet microscopy was extremely well-suited to image transparent fish embryos, which were readily available as transgenic versions with GFP expression in muscles. They combined light sheet microscopy with the concept of sequential image stack acquisition along different directions (multiple-view imaging) and were thus able to obtain good coverage of large fixed samples [10••]. However, this first implementation of multiple-view image fusion was computationally expensive and required rather stable, non-dynamic samples.

The application of light sheet microscopy to living GFP-expressing organisms attracted much attention: Now, light sheet microscopy was on the radar of biologists. But like all light microscopes, light sheet microscopy was limited by nature to rather transparent samples, such as fish embryos or, to some extent, insect embryos. However, since most multi-cellular biological specimens are relatively opaque, interrogation with the light sheet microscope requires means for making them transparent. Therefore, it became necessary to develop a method to 'clear' these opaque samples.

The first reports on clearing opaque biological samples dates back about a century, similarly to the first reports on light sheet microscopy. At that time, it was popular in medical academia to attempt rendering fixed anatomical preparations translucent. It was mainly the German anatomist Walter Spalteholtz who pursued these studies in a systematic fashion [11••]. He was working with Wilhelm His, the chair of anatomy in Leipzig, who investigated the nervous system of the heart. Spalteholtz was studying the heart's blood vessels (*rami communicantes*), which were of particular interest with respect to the development of infarction [12]. In order to obtain transparent preparations he tested numerous organic solutions to obtain optimal results. He found a mixture of benzyl alcohol and methyl salicylate to be most effective for clearing large specimens. Since developing clearing methods was 'chic' at that time, Spateholtz had a number of competitors. They eventually managed to clear large preparations in the decimeter range [13] and it is entertaining to read the very emotionally written discussions about priorities in advancing these methods.

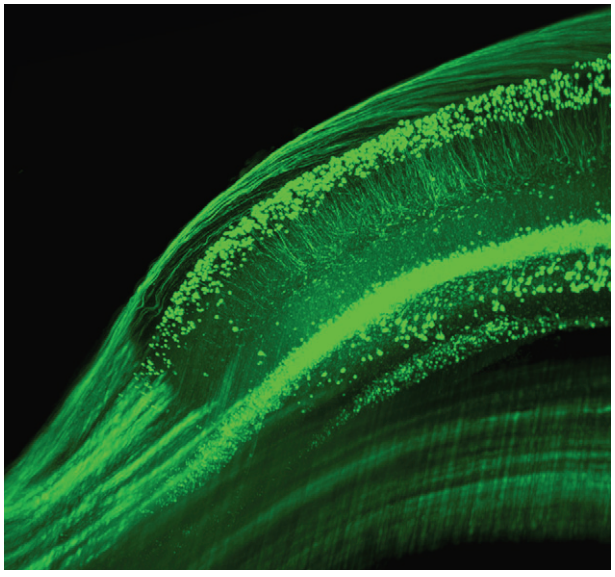
Although fashionable at that time, Spateholtz's method had no strong scientific impact: No serious attempts at quantification were made, even though a combination of clearing treatments and light sheet microscopy with carbon arc lamps would in principle have been possible 100 years ago.

Next, clearing was used by embryologists [14,15]. They developed a special clearing solution for embryo imaging consisting of a mixture of benzyl alcohol and benzyl benzoate (Murray's clear), thus replacing the methyl salicylate in Spateholtz's recipe by benzyl benzoate. Occasionally, the technique was used by surgeons and anatomists for angiographic investigations in large preparations like the human larynx [16,17].

Clearing became very important in a new imaging method, optical projection tomography, pioneered by James Sharpe [18]. Using an optical method similar to X-ray computer tomography Sharpe's team was able to image whole mouse embryos. They achieved a resolution of about 20 μm by trans-illumination imaging of the embryos, which were turned and imaged from multiple directions. Although these images were quite impressive, this first study on OPT did not yet include data on imaging fluorescent proteins, such as GFP. At that time reports appeared on the internet that GFP was destroyed by the clearing procedure. Thus, although a big step forward in imaging entire whole-mount preparations, limitations arose from the relatively low spatial resolution and challenges in using fluorescent proteins. Another group used cleared whole-mount preparations, such as mouse lung or mouse heart, using a technique called optical transmission and emission tomography [19], but the spatial resolution was even lower with this approach.

The solution to this problem was provided by Hans-Ulrich Dodt's lab through the application of light sheet microscopy to cleared whole-mount specimens, such as whole mouse brains strongly expressing GFP [20••] (Figure 1). In this report, mouse strains were used, which expressed GFP under a thy1 promoter in subsets of brain neurons. By modification of the original protocol and the first application of two-sided light sheet microscopy, the authors were able to image neuronal networks in entire mouse brains. In excised hippocampi, they imaged dendritic trees of neurons and small structures resembling dendritic spines. Taking advantage of auto-fluorescence, the typical pattern of the barrel cortex in the mouse brain and the blood vessel system in mouse embryos could be

Figure 1



3D reconstruction of a subset of GFP labeled neurons in the mouse hippocampus. The dense network of dendritic trees of the pyramidal layer is visible under a mesh of neuronal axons.

recorded. Also neuronal cell bodies could be made visible by auto-fluorescence after clearing, without the use of an additional stain [21]. In a different study, the interior of entire *Drosophila melanogaster* could be visualized in auto-fluorescence after clearing [22]. Further enhancements in image contrast became possible by applying the concept of confocality to light sheet microscopy [23]. In the meanwhile, a commercial 'ultramicroscope' version of light sheet microscopy has been made available to the scientific community (LaVision Biotec, Bielefeld, Germany).

More recently, light sheet microscopy was also applied to living uncleared brains for calcium imaging up to a depth of about 100 μm below the cortex surface [24]. Owing to strong light scattering both in the illumination and detection process, the imaging depth remained rather limited in this method termed objective coupled planar illumination (OCPI). Nonetheless, the authors achieved high imaging speeds and an image quality sufficient for simultaneous calcium imaging of a fairly large number of neurons.

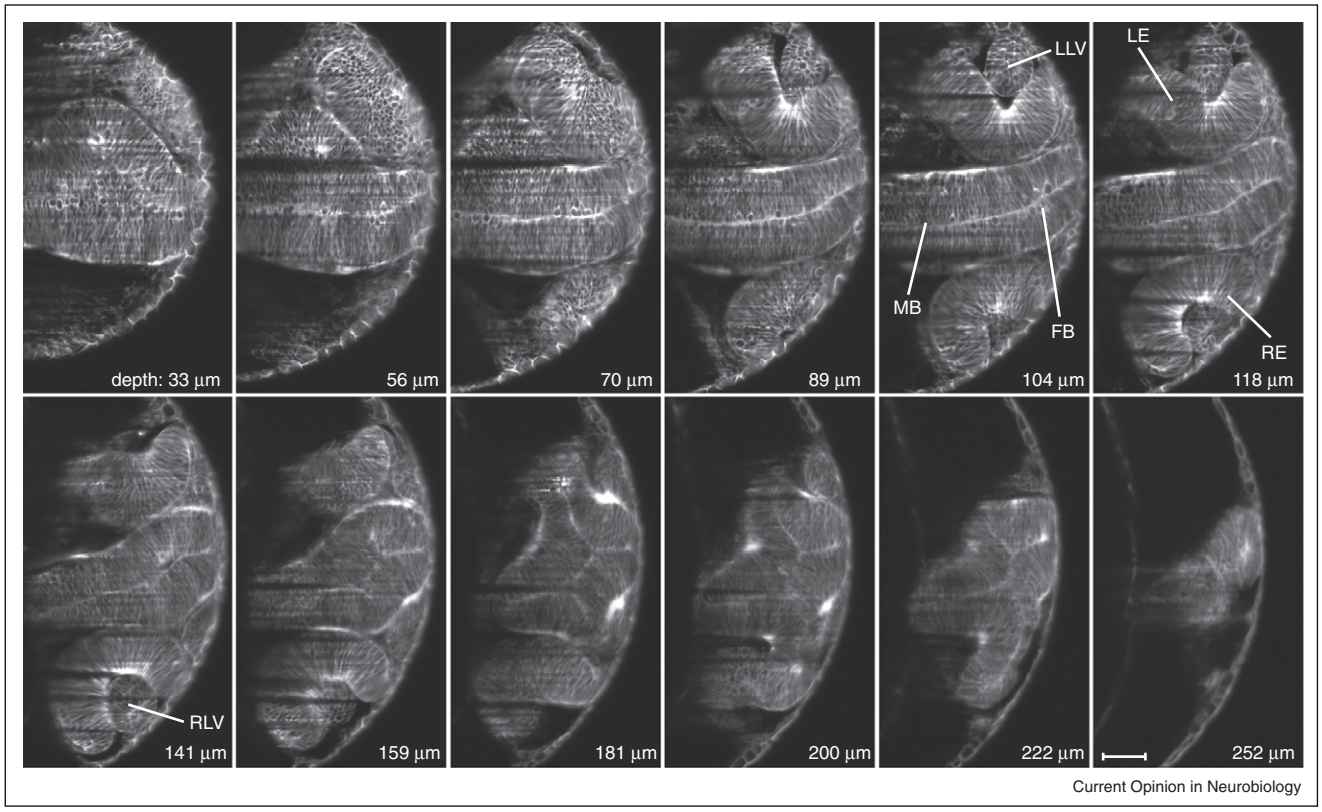
Recent advances in light sheet microscopy

The past few years have seen steady progress in the field of light sheet-based microscopy. The key advantages of light sheet microscopy – in particular high imaging speed, high signal-to-noise ratio and low levels of photobleaching and phototoxic effects – are crucial in a wide range of applications and generally highly desirable in light microscopy-based investigations. A rapidly increasing number of laboratories across the world are working on technological advances and new applications of light sheet-based microscopy [25–30]. Major improvements have been achieved and we will highlight some of the most recent novel concepts in this section.

Early implementations of fluorescence light sheet microscopy typically relied on stationary mechano-optics to create static laser light sheets for specimen illumination [8–10]. This strategy is still used in many designs and is particularly useful when implementing miniaturized instruments [31•,32] or when performing high-speed 2D imaging, for example, of the beating heart in fish embryos [33]. The recent development of scanned light sheet microscopy [34] introduced a new degree of freedom and enabled the implementation of advanced strategies to light sheet-based specimen illumination. In scanned light sheet microscopy, the specimen is illuminated from the side with a thin pencil beam that is rapidly scanned in one dimension to form a uniform laser light sheet. Using a two-axis scanner, one can furthermore quickly displace the entire light sheet and thereby perform 3D imaging without actually moving the specimen itself. Owing to the intrinsic incoherence of the illumination process in scanned light sheet microscopy, light-scattering induced artifacts are greatly reduced if compared to imaging with static light sheets [35••,36]. The scanned light sheet microscopy approach formed the basis for the implementation of high-quality structured illumination [37••], high-resolution imaging with 'self-healing' Bessel beams [35••,38••,39] and efficient two-photon (2p) excitation [38••,40••].

Combining scanned light sheet microscopy with structured illumination (DSLM-SI) allows eliminating the contribution of scattered out-of-focus light to the final image [37••]. This is particularly helpful when imaging large non-transparent specimens, in which the quality of the light sheet as well as the fluorescent signal suffer from the effects of light scattering of photons on their path through the specimen. DSLM-SI takes advantage of the fact that the light sheet is created by scanning a beam across the field-of-view in a time-dependent process. The laser light intensity is modulated sinusoidally in synchrony with the one-dimensional scanning process, thus creating an illumination pattern that consists of alternating light and dark stripes. By using a simple processing algorithm introduced by Neil *et al.* [41], the images obtained by using multiple phases of this pattern can be combined into a single contrast-enhanced image that represents only the unscattered fluorescence signal. Keller *et al.* used this approach to perform fast long-term imaging of zebrafish embryos over approximately three days of development (Figure 2), to record early *Drosophila* embryogenesis by sequential multiple-view imaging and to reconstruct a Digital Fly Embryo from the time-lapse recording of a nuclei-labeled embryo [37••] (www.digital-embryo.org).

Figure 2



DSLM-SI live imaging of zebrafish brain development. Single planes from a DSLM-SI image stack recorded at 23 h post fertilization. The planes show the head of the embryo, including forebrain, midbrain and both eyes. See Movie 3 of the Digital Embryo repository (www.digital-embryo.org/fly.html) for the complete 58-h recording. FB: forebrain, MB: midbrain, LE: left eye, RE: right eye, LLV: left lens vesicle, RLV: right lens vesicle. Scale-bar = 50 μm.

Credits: Reprinted from *Nature Methods*, vol. 7 no. 8, Keller *et al.*, ''Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy'', 637–642, Copyright (2010), with permission from Macmillan Publishers Ltd.

The recent introduction of Bessel beam-based light sheet microscopy allowed for a two-fold advance: a substantial improvement in axial resolution, achieving an effectively isotropic point-spread function [38••], and the reduction of light scattering artifacts in combination with an increased penetration depth [35••]. In contrast to the Gaussian beams that were used in light sheet microscopy so far, Bessel beams exhibit a self-reconstructing property when propagating through an inhomogeneous medium [42]. Furthermore, their longitudinal extent can be set independently of their central thickness. Planchon *et al.* developed a Bessel beam plane illumination microscope by projecting an annular illumination pattern at the rear pupil of the illumination objective and scanning the resulting Bessel beams with galvanometer scanners across the field-of-view. The authors used their scanned Bessel beams in combination with structured illumination and two-photon excitation to reduce the effective thickness of the light sheet to about 300–500 nm. This performance was demonstrated in the context of live cell imaging and revealed the dynamics of cell membranes, intracellular vesicles, organelles and mitotic chromosomes at unprecedented spatiotemporal resolution. This work opens up exciting new possibilities in high-resolution fluorescence live imaging, and could potentially also be extended to the study of large multi-cellular organisms.

Fahrbach *et al.* used a spatial light modulator, which they imaged into the back focal plane of an illumination objective, to create Bessel beams [35••]. Fast one-dimensional scanning of this beam provided Bessel beam-based light sheet illumination for imaging of biological tissues. The authors showed that this illumination strategy can be used to reduce light scattering-induced image artifacts and to significantly improve image quality and penetration depth in dense media.

In addition to devising strategies for improving spatial resolution in light sheet microscopy, the recent study by Planchon *et al.* also demonstrated that scanned light sheet microscopy allows for efficient two-photon excitation [38••]. Previously, Palero *et al.* used two-photon excitation

with static light sheets in a proof-of-principle imaging study of live *C. elegans* specimens [43]. They found that the fluorescent signal yield in their 2p-SPIM implementation was rather low, owing to the limitation in power density that can be achieved with static light sheets. To compensate for the low signal rate, the signal had to be integrated for relatively long periods of time, thus limiting the maximum imaging speed of the instrument. The studies by Planchon *et al.* [38] and Truong *et al.* [40••] illustrate that this problem can be overcome by taking advantage of scanned light sheet microscopy, which allows for high power densities due to the efficient focusing of the laser beam into a much smaller focal volume than in static light sheets [34]. This advance opens up exciting perspectives in the context of fast deep-tissue live imaging with light sheet microscopy.

Finally, there are a number of recent studies that exploit light sheet microscopy in novel combinatorial approaches. Friedrich *et al.* used the principle of stimulated emission depletion (STED) in a light sheet microscope to improve the axial resolution [44]. Wohland *et al.* combined light sheet microscopy with fluorescence correlation spectroscopy to measure diffusion properties of small particles in live zebrafish embryos [45]. Another series of technical advances, which are of particular interest to the neuroscience community, include the miniaturization of light sheet microscopy for live imaging in the mouse neocortex [31•], the introduction of adaptive optics for reduction of aberrations in light sheet microscopy [46] and the combination of high-speed light sheet-based imaging with optogenetics [47•].

Although light sheet microscopy has not yet spread much in the neurosciences, it is obvious that its key capabilities will be particularly useful in this field. The combination of high imaging speeds and low photo-bleaching rates is indispensable for live imaging studies, and recent breakthroughs in resolution enhancement and deep-tissue imaging complete the list of desirable attributes in a fluorescence light microscope. The unique strength of light sheet microscopy is the combination of all of these capabilities into a single instrument that allows for exceptionally powerful experimental investigations.

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