Light sheet fluorescence microscopy and revolutionary 3D analyses of live specimens

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Recent technological advancements have enabled the three-dimensional (3D) analysis of live specimen via the over 100-year-old concept of Light Sheet Fluorescence Microscopy (LSFM). Compared to other microscopy methods, LSFM provides high frame rates with low phototoxicity due to a fundamentally different arrangement of specimen illumination and image detection with independent optics. As a result, LSFM-based studies are becoming the first choice in many of the life sciences, particularly when observing millimetre-sized live specimens in developmental biology and 3D cell biology.

In many biomedical research areas, analyses of dynamic processes in live specimens are increasingly gaining importance. Methodological and technological advancements have made it possible to observe and analyse processes at the molecular and cellular level in three dimensions over time in the context of entire live specimens and cell clusters. These methods provide images of structures inside the specimens, and no longer require mechanical cutting. In addition, the use of fluorescent markers like GFP (green fluorescent protein) in genetic engineering and other advancements in genetics have enabled the labeling and the observation of proteins in cell cultures and in whole organs. In this context, fluorescence microscopy techniques that create optical cross sections of three-dimensional biological specimens are key technologies [1]. The new trend is to move away from the combination of static microscopy of fixed and stained specimens and toward live imaging of the dynamics of specifically marked structures in vivo. And last but not least, recent progress in computer speeds and networking technology allows capture, storage, and further analyses of the large quantities of data that are generated by time series of 3D images. Despite these many advancements, fluorescence microscopy, using live specimens remains challenging today. Even the most modern microscopes exhibit weaknesses in several key factors, which are necessary for the complete optical imaging of an organism’s development over time. For example, the significant scattering of visible light in biological tissue limits resolution, having negative impacts on image quality as the depth of the structure being imaged in the specimen increases. Similarly negative phototoxic effects of fluorescence excitation, including the photobleaching of fluorescent dyes due to the excitation light, shorten the maximum time span for recording images before the specimen is damaged. Finally, the limited temporal resolution and sensitivity of the fluorescence detector have made it impossible to observe many interesting processes.

However, the illumination concept of LSFM has undergone a renaissance in recent years [2,3,4]. And in particular, there have been ground-breaking developments in making LSFM methods usable in 3D fluorescence microscopy of live biological objects [4]. Taken together, these steps address most of the aforementioned challenges, and as such, open up new possibilities for developmental biologists. LSFM examinations enable better understanding of processes related to organ creation, organ function, and reveal the relationships between cellular processes that lead to embryogenesis and the creation of an organism. In many LSFM examples, the zebrafish Danio rerio and the fruit fly Drosophila melanogaster serve as model organisms.

**The method of using a light sheet**

In its technical implementation, a light sheet microscope differs fundamentally from a conventional microscope stand – fluorescence excitation and image acquisi-
Microscopy

The extension of optical sections are carried out through completely separate optical paths that are arranged perpendicular to one another. The light sheet is generated by using either a cylindrical lens or a scanning laser beam. The light sheet is aligned exactly with the focal plane of the detection lens (figure 1).

Both methods of generating the light sheet have their advantages, depending on the application. While the static light sheet allows for lower laser intensities or shorter exposure times, the scanned light sheet produces a more homogeneous illumination of the image area.

The specimen is usually embedded in a transparent gel to be suspended vertically in the light sheet. A motorised mount allows the specimen to be translated as desired, as well as rotated around its vertical axis. By moving the specimen in a linear manner across the light sheet, a series of images is recorded (known as a Z-stack) that is used to reconstruct the specimen in three dimensions.

The fluorescent signal emitted by the sample is then collected by a lens aligned perpendicular to the plane of illumination using conventional wide-field technology. A fast and sensitive camera serves for detection.

Minimal photobleaching – maximum efficiency

LSFM, sometimes referred to as selective plane illumination microscopy (SPIM) uses the most efficient method for fluorescent imaging of optical sections: the light sheet specifically excites only dyes within the focal plane, and in doing so, an image of a single plane of the specimen is recorded as an optical section. Through this selective excitation, no signal is generated outside the focal plane, which minimises any contribution from out-of-focus structures. In addition, the dyes that are not involved in creating the image cannot photobleach or contribute to other phototoxic effects.

Desirable 3D image stacks are created and captured by stepping the specimen across the light sheet, while successively recording optical sections at different sample depths (a process known as Z-stacking). Typical light sheet thickness ranges from approximately 2 to 10 µm. This means that stacks of images from millimetre-sized specimens are often composed of 1000 optical sections. Especially when dealing with such large samples, established methods, such as confocal laser scanning microscopes (CLSM), have more negative effects on the sample. For example, when section number 1000 is recorded using an LSFM, it is illuminated for the first time. When using a CLSM, it has already been illuminated 999 times before being recorded. As such, the use of an LSFM reduces phototoxicity at least by a factor of 1000 in this simple example.

Because the light sheet illuminates only the area of the specimen that is actively being recorded (figure 2), the benefit of the lower light exposure scales with the relationship of the light sheet thickness to the sample thickness, or the number of optical sections required to completely record the 3D volume. This extreme advantage when working with live specimens creates the foundation for a new form of 3D fluorescence microscopy and enables biological researchers to conduct many new types of experiments [5].

A new way of preparing specimens

The geometry of an LSFM also enables a new sample preparation method (figure 3), which decisively improves the maintenance of physiological environmental conditions for the specimen. A specimen is placed inside a chamber, which is then filled with an appropriately tempered.

Figure 2: Illuminated volume of an extended specimen while recording an optical section (A). In an LSFM, only the focal plane is illuminated and imaged all at once by the camera (B); light-induced bleaching can only occur in the focal plane (C). In a confocal laser scanning microscope, each image point is recorded individually in a sequential manner, and a cone along the optical axis is illuminated for each point (D), meaning that the entire specimen is exposed to light when creating just one optical section (E).

Figure 3: Typical chamber design for live specimens embedded in 3D polymer hydrogel (A and B): in order to embed the specimen, a low-concentration transparent polymer matrix in liquid state is drawn into a hollow cylinder, together with the specimen, and once inside, passes into a gel state in cylindrical shape. For imaging the specimen with the microscope, the polymer with the embedded specimen is pushed out of the cylinder (C) or left in an FEP tube (D) and immersed into the medium in the chamber. The result: Transmission contrast image of a mounted two-day-old zebra fish embryo (E).
aqueous medium and then can be positioned as desired. In addition to the obvious three axes of movement, the specimen can be freely rotated around the vertical axis, which enables not only selection of the ideal perspective for the experiment being conducted, but also recording of multiple image stacks from different viewing angles, if so desired.

The LSFM is set up around the experiment, and the observation lens looks sideways into the specimen chamber that has been converted into an incubator. This makes it possible to gently embed the prepared sample within a cylinder of a transparent 3D matrix (such as agarose, Matrigel, or a similar material). As a result, researchers are no longer confined to the two-dimensional world of cover slips, which poorly relate to physiological conditions. This LSFM set up best supports the undisturbed development of live specimens over long periods of time in a manner that most closely matches their natural environment [11].

**Multiview image acquisition**

Suitable software can combine the complementary information in different image stacks, which have been collected from various directions when rotating the specimen cylinder. Resultant 3D images (figure 4) [12] bring together all the details from each perspective. In certain cases, doing so achieves an isotropic spatial resolution in the processed data set, overcoming the reduced resolution along the optical detection axis, which is a well-known drawback in microscopy.

In addition, illuminating the specimen from both sides also compensates for detrimental absorption and scattering effects within the tissue [13]: Instead of reaching only as far as to the middle of an extended preparation with a conventional microscope, the second half is also accessible with such multiview images recorded by an LSFM. In this way, even biological preparations of several millimetres in size can be imaged as a whole.

Now LSFM is ready for commercialisation: Lightsheet Z.1 (figure 5) has been developed by Zeiss especially for microimaging live specimens over extended periods of time. The closed housing protects the specimen and the incubation chamber from ambient influences and offers optomechanical stability required for long-term recordings of several days. Six laser wavelengths (405, 440, 488, 514, 561, and 638 nm) are available for fluorescence stimulation, and the detection optics are based on water immersion lenses. Depending on the application, either two CCD or two sCMOS cameras can be used simultaneously. Dedicated software and integrated data management support evaluation of the Multiview images.

**Applications**

The low level of phototoxicity and the extended depth of penetration make the specimen-friendly LSFM technology ideal for live images of larger biological samples, such as embryos or 3D cell cultures. Figure 6 shows six points in time during the development of a zebrafish embryo during the first day after fertilisation [11]. The ability to record its highly dynamic morphogenesis from several directions (in the image: 0° and 180°) allows researchers to continuously study specimen processes. In conventional 3D fluorescence microscopy, illumination levels are too high and the specimens are not embedded adequately for normal development over extended periods of time. Instead, the high frame rates possible by LSFM record the entire volume of the embryo in two or more views, and at one-minute intervals, making it possible to conduct completely new experiments, and thereby gain deeper insight into the processes of embryogenesis.

Similarly, LSFM is revolutionising the methods used by developmental biologists and genetic engineers, who regularly work with the model organism Drosophila melanogaster. Figure 7 shows five embryonic development points of the fruit fly, and does so in three views of the rendered volume data set of an 18-hour time series. Without the LSFM’s multiview recording ability and low level of light exposures, drosophila embryos cannot be recorded at one-minute time intervals. Other specimens that can now be examined in ground-breaking new ways using light sheet microscopy include the roundworm C. elegans; many marine organisms (Ciona, squid, many types of plankton), and cell clusters cultivated in a 3D matrix. Established fluorescence methods, such as FLIM, FCS, two-photon excitation, and...
Bessel beams can be combined with LSFM [6,7,9,10]. And both extremely small specimens, such as yeast cells, and extremely large (cm scale) specimens, which need to be fixed and optically cleared, benefit from the concept of LSFM. For the latter, researchers prefer a special variation of the LSFM, also known as an ultramicroscope. It is based on macroscopes and is becoming one of the most important tools in 3D fluorescence microscopy, not only because different methods of sample preparation and mounting are possible compared to conventional microscopes, but also because of the efficient ways of creating optical sections of spatially extended specimens.

New areas of application, driven both by technical advancements and by fundamental questions in biological research, may trigger a paradigm shift in the microscopic examination of live specimens. We expect to see improved resolution by using thinner light sheets, deeper penetration into scattering tissue, faster and more sensitive detectors, experimental variations, such as tools for image manipulation or ablation, as well as methods such as FCS or FLIM [8,9,10].

Computer speeds and networking technology are also a driving force behind exciting advancements in this field. Long time series of large volume data sets result in enormous amounts of data – up to several terabytes per experiment – which require a high-performance infrastructure for management and storage. The computer industry has already been addressing this issue for some time [15]. New software, and hardware developments during the past few years, including LSFM, continue to support methodological breakthroughs in biology and computational sciences.

Outlook

LSFM has become popular in recent years; the further development of new technical variations of LSFM illumination principles is an active field of investigation [6,7]. Now researchers can observe highly dynamic intracellular processes (at milliseconds intervals) in the overall context of the tissue and during morphogenesis in developing specimens over a period of hours and days. Light sheet illumination is becoming one of the most important tools in 3D fluorescence microscopy, not only because different methods of sample preparation and mounting are possible compared to conventional microscopes, but also because of the efficient ways of creating optical sections of spatially extended specimens.

Figure 6: LSFM microscopy of an entire zebrafish embryo (featuring nucleus staining) from two different perspectives, between 8 and 17 hours after fertilisation

Figure 7: Embryonic development and morphogenesis of the model organism Drosophila melanogaster

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Literature:


