Light-sheet fluorescence microscopy for quantitative biology

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In light sheet–based fluorescence microscopy (LSFM), optical sectioning in the excitation process minimizes fluorophore bleaching and phototoxic effects. Because biological specimens survive long-term three-dimensional imaging at high spatiotemporal resolution, LSFM has become the tool of choice in developmental biology.

Although transmitted light microscopy provides a view of the morphology of a specimen, the ability to tag specific organs, organelles or macromolecules with fluorophores allows one to localize well-defined subsets of targets in the specimen. Fluorescence microscopy images have high contrast, showing bright targets on a dark background.

In conventional wide-field and confocal epifluorescence microscopes, the same lens is used for the excitation of the fluorophore and the collection of the emitted fluorescence. The excitation light passes through the specimen and, assuming uniform fluorophore distribution, excites the same number of fluorophores in each focal plane along its optical axis. Therefore, whenever an epifluorescence microscope focuses on a plane along its optical axis, it actually excites all the fluorophores in a specimen, including those above and below the focal plane.

The optical sectioning capability of a confocal fluorescence microscope is based on discrimination against the out-of-focus fluorescence light by a pinhole in an image plane in front of the intensity detector. The excitation of a fluorophore and the detection of a fluorescence photon are independent events. Although the excitation intensity is conserved along the optical axis, the fluorescence emission intensity, which is proportional to the square of the excitation intensity, is not; it actually has a maximum in the focal plane. Only optical instruments whose fluorescence intensity detection depends on the product of two independent events have this property of optical sectioning and thus an axial resolution[1,2]. In other words, wide-field fluorescence microscopes have no axial resolution.

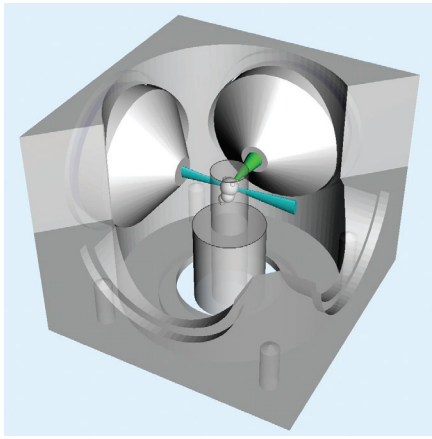

Figure 1 | Basic optical arrangement in LSFM. In contrast to an epifluorescence arrangement, LSFM uses at least two independently operated lenses. The lenses used for fluorophore excitation are arranged at a 90° angle relative to those used for detection. Only a thin planar section in the specimen centered on the focal planes of the detection lenses is illuminated. The specimen is maintained in a close-to-natural 3D state.

To make matters worse, fluorescence microscopy has several basic limitations. First, the excitation light is absorbed not only by fluorophores but also by many endogenous organic compounds, which are degraded much like fluorophores[3] and thus are unavailable for vital metabolic processes. Second, the number of fluorophores in any volume element at any given time is finite, and fluorophores can degrade upon excitation. As a consequence, the number of photons that can be retrieved from a fluorophore-labeled specimen is limited. Finally, life on Earth is adapted to the solar flux, which is less than 1.4 kW/m^2. This might not be a hard limit, but it indicates that irradiance should not exceed 1 nW/μm^2 = 100 mW/cm^2 when dynamic biological processes are observed (**Box 1**).

In toto, as long as we rely on epifluorescence microscopes, we are faced with two serious challenges. First, both fluorophores and specimens are essentially wasted during the observation process; second, all fluorophores and many endogenous organic compounds in the specimen are excited whenever we record a single plane. Obviously, the situation becomes even more challenging when we perform complex biological experiments and observe the behavior of multiple targets in three dimensions as a function of time.

For imaging living biological samples, these challenges must be addressed. LSFM[4] is perhaps the best technique we have so far with which to make a sincere and honest effort: it provides optical sectioning and a true axial resolution, reduces fluorophore bleaching and phototoxicity at almost any scale, allows one to record millions of pixels in parallel and dramatically improves the viability of the specimen (**Table 1**).

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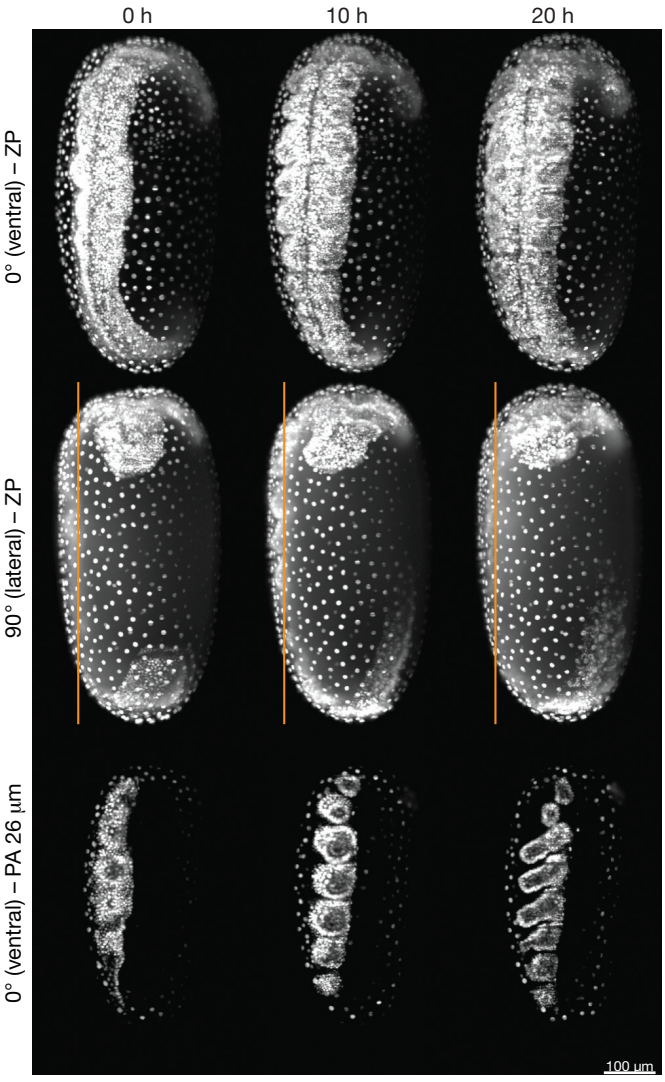

Figure 2 | LSFM imaging of a live *Tribolium castaneum* embryo expressing nuclear GFP. The ventral and lateral maximum projections as well as a ventral single plane 26 µm below the embryo surface are shown. The location of the plane is indicated by the orange line. Three time points were recorded during germband elongation. ZP, *z* maximum projections with image processing; PA, single plane with intensity adjustment. See also ref. 24. Image by Frederic Strobl.

Light sheet–based fluorescence microscopes are based on two main optical paths[5]. The detection path consists of the detection microscope objective lens, a spectral filter—which discriminates the excitation light—a tube lens and a camera. The excitation path is perpendicular to the detection path. It directs a light sheet into the side of the specimen. The light sheet and the focal plane of the detection microscope objective overlap (**Fig. 1**). Another way to look at it is that a light sheet–based fluorescence microscope is essentially the detection part of a conventional fluorescence microscope, which uses at least one further lens to direct the excitation beam into the specimen.

Because the thickness of the light sheet is smaller than the depth of field of the detection lens for low numerical apertures, and not much larger for high numerical apertures, the light sheet illuminates only the thin cuboid surrounding the focal plane of the detection path. This means only the fluorophores that are close to the focal plane of the detection lens are excited, whereas all fluorophores outside the focal plane receive no excitation light.

This has enormous consequences: fluorophores outside the thin volume close to the focal plane do not contribute out-of-focus light, which would blur the image (**Fig. 2**), and they are not photobleached. For the same reason, potentially absorbing endogenous organic molecules are not degraded and specimens are less affected by phototoxicity.

For three-dimensional (3D) imaging, which is typically of the greatest interest in the study of biological specimens, we move either the light sheet through the specimen or the specimen through the light sheet, and we record images in different planes along the optical axis of the detection lens. It turns out that light sheet–based fluorescence microscopes expose zebrafish to 300 times less energy than a conventional microscope and about 5,000 times less energy than a confocal fluorescence microscope when recording image stacks of similar quality[6].

Furthermore, a light sheet–based fluorescence microscope can massively parallelize the data acquisition process and take full advantage of recent (and probably many future) developments of modern cameras; other methods that do not introduce optical sectioning in the excitation process cannot do so. For instance, a confocal fluorescence microscope samples the specimen with a single diffraction-limited spot of light, performing each recording independently of all other recordings. Variations that do parallelize this process, e.g., spinning disk confocal fluorescence microscopes, suffer from a decreased axial resolution[7].

The generation of the excitation light sheet comes in two basic flavors. First, a single-plane (or selective-plane) illumination microscope[4,5] (SPIM) employs a cylindrical lens to generate the light sheet. A collimated laser beam is focused along the one direction parallel to the optical axis of the detection path while the other direction remains collimated. The light sheet is static, its intensity is easily controlled and it is well suited for massively parallel detectors such as very fast cameras. Alternatively, a digital scanned laser light sheet–based fluorescence microscope[6] (DSLM) generates the light sheet by moving a focused (that is, Gaussian) beam through the focal plane of the detection lens, e.g., by tilting a mirror in an appropriately conjugated plane. It relies entirely on circular symmetric optics and does not require a cylindrical lens. In contrast to a SPIM, its illumination is perfectly incoherent, and as a result it produces fewer artifacts. The beam's intensity can be modulated to generate structured illumination[8,9] or to synchronize it with a line detector. Its disadvantages are that line sampling takes more time and requires a higher excitation intensity.

Essentially all further developments of SPIMs and DSLMs affect the illumination path. Two-photon excitation[10] has

been suggested to improve the penetration depth; Bessel beams[11] and Airy beams[12] have been used for their capability to provide a longer and narrower field of view; and super-resolution contrasts such as those obtainable with photoactivated localization microscopy[13], stochastic optical reconstruction microscopy[14] and stimulated emission depletion microscopy[15] have been reported in fixed, cleared and thick specimens. A particularly interesting development by Betzig and colleagues[16] combines LSFM and the concept of coherent structured illumination microscopy[17]. It reduces the light sheet's thickness by replacing the traditional Gaussian beam with several Bessel beams. This makes LSFM suitable for the observation of thin specimens: in the study[16], one or two cell layers and the superficial volume segments of thicker specimens were observed. The resolution is comparable to that of a confocal fluorescence microscope, and the recording speed, number of frames that can be recorded and imaging duration are much improved.

Light sheets have been known to scientists for more than 100 years[18], but so have light spots. Until lasers became available in the early 1960s, neither light spots nor light sheets were diffraction limited. A confocal fluorescence microscope, which is based on the sequential illumination of the specimen by a diffraction-limited spot of light, cannot be operated without a laser, which is essential for true optical sectioning. Laser light sheet–based devices, including a macroscope[19], had been built several times, but their capability to perform at a microscopic level was not known until, starting around 2002, my group at the European Molecular Biology Laboratory (EMBL) described a diffraction-limited laser light sheet–based fluorescence microscope[4], applied it to living biological samples and evaluated its applicability for multiple-view[20,21] imaging. This developed from work in which, since the early 1990s, we systematically evaluated diffraction-limited microscopes[22,23] with two to four lenses, both in theory and in practice.

LSFM has already started and will continue to revolutionize fluorescence microscopy. It is based on an extremely simple yet ingenious optical arrangement that provides true optical sectioning over an extended field of view. Using modern camera technology, it records up to thousands of images per second. The resolution of LSFM is comparable to that of confocal fluorescence microscopy, but the signal-to-noise ratio is about two orders of magnitude better. It reduces the energy load on a specimen by 2–4 orders of magnitude so that fluorophore bleaching and phototoxic effects are dramatically reduced and biological samples including embryos[24], plants[25] and tissue sections survive the recording of millions of images. Specimens can be observed along multiple directions[20,21], and, because the technique can be adapted to any camera and camera feature, it enables the application of fluorescence correlation spectroscopy[26], fluorescence resonance energy transfer and fluorescence lifetime imaging microscopy[27] with unprecedented speed, duration and quality.

Table 1 | Advantages of LSFM over wide-field and confocal fluorescence microscopy

Property	Improvement	Quantification and/or details
Only observed plane is illuminated	Optical sectioning capability	Lateral resolution of ideal conventional fluorescence microscope
		True axial resolution
	Low fluorophore bleaching	1/300–1/5,000 energy exposure
	Low phototoxicity	Improved specimen viability
Camera records fluorescence intensity	Massively parallel detection	Several million pixels per frame
	Reduced exposure time	Micro- to milliseconds per frame
	Fast recording	10–1,000 frames per second
	High dynamic range	Several thousand gray levels
	Time gating	SPIM-FLIM/FRET
	Ultrafast	Tens of thousands of frames for SPIM-FCS/FCCS
Reduced fluorophore bleaching	Improved *z* sampling	Axial pitch similar to pixel pitch
	Longer observation periods	Days to weeks rather than hours
	Improved time sampling	More images or stacks
	Multiple-views imaging	2–18 directions per specimen
More photons per pixel	Higher signal-to-noise ratio	Improved image processing
	Excellent deconvolution	More gray levels are maintained
	Shorter imaging intervals	Several stacks per second
	Improved resolution	NA determines resolution
	Simplified 3D image processing	Segmentation more reliable
Low-excitation NA and multiple views	Thicker specimens	Deeper penetration
	Improved axial resolution	Isotropic resolution

SPIM, single/selective-plane illumination microscopy; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; FCS/FCCS, fluorescence (cross-) correlation spectroscopy; NA, numerical aperture.

LSFM has already started and will continue to revolutionize large-scale image processing[28]. Light sheet–based fluorescence microscopes allow us to record the multiple views, the many channels and the thousands of stacks that are required to follow 3D biological processes as a function of time for many hours, days and, in some cases, even weeks. Millions of images are recorded within relatively short time periods, which easily amount to several tens of terabytes. The data need to be stored, documented, retrieved and processed. The usually high image quality and the potentially high spatiotemporal resolution mean that our capability to reduce images to lists of objects and follow these objects as a function of time has become considerably more precise.

LSFM has already affected the way biological specimens are prepared and will continue to change our attitude towards specimen viability. Traditional fluorescence microscopy had enforced certain specimen preparation schemes that usually rely on hard and flat surfaces. LSFM places the specimen in the center and arranges the optics around it[29]. Specimens can be prepared in new and as-yet unimagined ways, their 3D integrity is maintained, and they can be used in experiments hitherto regarded as impossible. Large, multicellular specimens with complex structure can be observed under physiologically relevant conditions[30], and embryos can survive more than 50 hours of observation to develop into fertile adults[24]. The light sheet–based fluorescence microscope should become the instrument of choice for all quantitative aspects and thus for recording the data required for a physics-based approach to the life sciences.

Finally, we should not forget that the task of any new method is not to repeat the old experiments but to do things

BOX 1 PHOTOTOXICITY IN LIVE-SAMPLE IMAGING.

To how much light can one expose a biological specimen? There is no general answer, but all those who have worked with fluorescence and even transmission light microscopy are aware of the negative effects of illumination on the viability of a specimen. There are some general findings: (i) phototoxic effects are more obvious at lower rather than at higher wavelengths, (ii) the presence of fluorophores has a dramatic negative impact[3] and (iii) both low intensity and low energy of the illumination light are desirable. Assuming that many organic molecules will absorb the excitation light without fluorescing but will still degrade, it is probably safe to suggest that any imaging of live specimens should be performed at a level that avoids even moderate fluorophore bleaching.

A hint might be provided by the solar constant, which is around 1.4 kW/m^2 at the equator and around 1 kW/m^2 in central Europe. In microscopic terms, this is about 1 nW/μm^2 or 100 mW/cm^2. Hence, one can calculate that the maximal radiant exposure should be around 0.5 μJ/μm^2 and infer that cells and small model embryos should not be exposed to more than a few millijoules and a few hundred millijoules, respectively. Very few microscopes can operate at these low levels, i.e., in the 'single sun' regime. The light sheet–based fluorescence microscope is one of them. Confocal fluorescence and super-resolution microscopes usually operate in a 'multiple-suns' regime. This may pose a substantial problem for live-specimen imaging.

Solar constant at equator	1,366 W/m^2 = 1,366 J/s·m^2
Solar constant in central Europe	≈ 1 kW/m^2
Microscopy relevant units	≈ 1 nW/μm^2 = 100 mW/cm^2
Energy density within 600 s	≈ 0.6 μJ/μm^2
Cell diameter of 100 μm × (0.3 s or 10 min)	≈ 2.4 μJ or 4.8 mJ
Embryo diameter of 900 μm × (0.3 s or 10 min)	≈ 190 μJ or 380 mJ

differently, to seek new answers and question the ideas of the past. The fact that specimens have to be prepared differently for their observation in a light sheet–based fluorescence microscope should not be regarded as a problem but as a challenge and a chance to do it right. The huge number of images generated in a light sheet–based fluorescence microscope is not a problem but a part of the answer in our quest to understand biological processes. When we follow the cells in a developing embryo, the large number of spatiotemporal tracks resulting from the segmentation of a time series should not be seen as a source of confusion but rather as a solid basis to test our biological models against strict mathematical-physical ones. The application of light sheet–based fluorescence microscopes in the life sciences has only just started, but it has already made a tremendous difference.

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The author declares competing financial interests: details are available in the online version of the paper (doi:10.1038/nmeth.3219).

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