Optical Sectioning Deep Inside Live Embryos by Selective Plane Illumination Microscopy

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Large, living biological specimens present challenges to existing optical imaging techniques because of their absorptive and scattering properties. We developed selective plane illumination microscopy (SPIM) to generate multidimensional images of samples up to a few millimeters in size. The system combines two-dimensional illumination with orthogonal camera-based detection to achieve high-resolution, optically sectioned imaging throughout the sample, with minimal photodamage and at speeds capable of capturing transient biological phenomena. We used SPIM to visualize all muscles in vivo in the transgenic Medaka line Arnie, which expresses green fluorescent protein in muscle tissue. We also demonstrate that SPIM can be applied to visualize the embryogenesis of the relatively opaque Drosophila melanogaster in vivo.

Modern life science research often requires multidimensional imaging of a complete spatiotemporal pattern of gene and protein expression or tracking of tissues during the development of an intact embryo (1). In order to visualize the precise distribution of specific genes, a wide range of processes, from small-scale (subcellular) to large-scale (millimeters), needs to be followed. Ideally, such events, which can last from seconds to days, will be observed in live and fully intact embryos.

Several techniques have been developed that allow mapping of the three-dimensional (3D) structure of large samples (2). Gene expression has been monitored by in situ hybridization and block-face imaging (3). Techniques that provide noninvasive (optical) sectioning, as opposed to those that destroy the sample, are indispensable for live studies. Optical projection tomography can image fixed embryos at high resolution (4). Magnetic resonance imaging (5) and optical coherence tomography (6) feature noninvasive imaging, but do not provide specific contrasts easily.

In optical microscopy, green fluorescent protein (GFP) and its spectral variants are used for high-resolution visualization of protein localization patterns in living organisms (7). When GFP-labeled samples are viewed, optical sectioning (which is essential for its elimination of out-of-focus light) is obtainable by laser scanning microscopy (LSM), either by detection through a pinhole (confocal LSM) (8) or by exploitation of the nonlinear properties of a fluorophore (multiphoton microscopy) (9). Despite the improved resolution, LSM suffers from two major limitations: a limited penetration depth in heterogeneous samples and a marked difference between the lateral and axial resolution.

We developed selective plane illumination microscopy (SPIM), in which optical sectioning is achieved by illuminating the sample along a separate optical path orthogonal to the detection axis (Fig. 1 and fig. S1). A similar approach in confocal theta microscopy has been demonstrated to improve axial resolution (10–12). In SPIM, the excitation light is focused by a cylindrical lens to a sheet of light that illuminates only the focal plane of the detection optics, so that no out-of-focus fluorescence is generated (optical sectioning). The net effect is similar to that achieved by confocal LSM. However, in SPIM, only the plane currently observed is illuminated and therefore affected by bleaching. Therefore, the total number of fluorophore excitations required to image a 3D sample is greatly reduced compared to the number in confocal LSM (supporting online text).

GFP-labeled transgenic embryos of the teleost fish Medaka (Oryzias latipes) (13) were imaged with SPIM. In order to visu-
alize the internal structure, we imaged the transgenic line Arnie, which expresses GFP in somatic and smooth muscles as well as in the heart (14). A 4-day-old fixed Arnie embryo [stage 32 (15)] is shown in Fig. 1. SPIM was capable of resolving the internal structures of the entire organism with high resolution (better than 6 μm) and as deep as 500 μm inside the fish, a penetration depth that cannot be reached using confocal LSM (fig. S6). The axial resolution in SPIM is determined by the lateral width of the light sheet; for the configuration shown in Fig. 1, the axial extent of the point spread function (PSF) was about 6 μm, whereas without the light sheet it was more than 20 μm (supporting online text).

Any fluorescence imaging system suffers from scattering and absorption in the tissue; in large and highly scattering samples, the image quality decreases as the optical path length in the sample increases. This problem can be reduced by a multiview reconstruction, in which multiple 3D data sets of the same object are collected from different directions and combined in a postprocessing step (16–18). The high-quality information is extracted from each data set and merged into a single, superior 3D image (supporting online text). One way to do this is by parallel image acquisition, using more than one lens for the detection of fluorescence (18).

We collected SPIM data for a multiview reconstruction sequentially by generating multiple image stacks between which the sample was rotated. Sample deformations were avoided with a rotation axis parallel to gravity (Fig. 1). In contrast to tomographic reconstruction techniques [such as those in (4)], which require extensive processing of the data to yield any meaningful 3D information, rotation and subsequent data processing are optional in SPIM. They allow a further increase in image quality and axial resolution compared to a single stack, but in many cases a single, unprocessed 3D SPIM stack alone provides sufficient information.

We performed a multiview reconstruction with four stacks taken with four orientations of the same sample (figs. S2 and S3). Combination of these stacks (supporting online text) yielded a complete view of the sample, ~1.5 mm long and ~0.9 mm wide. In Fig. 2, the complete fused data set is shown and the most pronounced tissues are labeled. The decrease in image quality with penetration depth is corrected by the fusion process. It yielded an increased information content in regions that were obscured (by absorption or scattering in the sample) in some of the unprocessed single views.

The method of embedding the sample in a low-concentration agarose cylinder is nondestructive and easily applied to live embryos. We routinely image live Medaka and Drosophila embryos over periods of up to 3 days without detrimental effects on embryogenesis and development. To demonstrate the potential of SPIM technology, we investigated the Medaka heart, a structure barely accessible by conventional confocal LSM because of its ventral position in the yolk cell. We imaged transgenic Medaka Arnie embryos and show a reconstruction of the inner heart surface (Fig. 3A) derived from the data set shown in Fig. 2. This reveals the internal structure of the heart ventricle and atrium. In a slightly earlier stage, internal organs such as the heart and other mesoendodermal deriva-

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**Fig. 3. A Medaka heart imaged with SPIM (movies S3 and S4). (A) Surface rendering of the heart taken from the data shown in Fig. 2. The heart has been cut open computationally to make the internal structure visible. hv, heart ventricle; ha, heart atrium. (B) Schematic representation of a Medaka embryo at stage 26 of development (13), 2 days post-fertilization. Three optically sectioned planes are indicated. At this stage, ventral structures such as the heart are deeply buried in the yolk sphere. d, dorsal; v, ventral; a, anterior; p, posterior; y, yolk; ey, eye. (C) Optical section of an Arnie embryo showing the eye and the optic nerve labeling and the dorsal part of the heart ventricle. on, optic nerve. (D) Optical section showing the heart ventricle chamber and the dorsal wall of the heart atrium. (E) Optical section showing the atrium chamber.**
tives are deeply buried in the yolk sphere, under the body of the embryo (Fig. 3B). In Fig. 3, C to E, three optical sections at different depths illustrate GFP expression in the muscles of the living heart. Fast frame recording (10 frames per s) allows imaging of the heartbeat (movies S3 and S4); similar imaging has previously only been demonstrated at stages when the heart is exposed and by cooling the embryo to reduce the heart rate (19).

To demonstrate that SPIM can also be used to image the internal structures of relatively opaque embryos, we recorded a time series (movie S5) of the embryogenesis of the fruit fly Drosophila melanogaster (Fig. 4). GFP-moesin labeled the plasma membrane throughout the embryo (20). Even without multiview reconstruction, structures inside the embryo are clearly identifiable and traceable. Stacks (56 planes each) were taken automatically every 5 min over a period of 17 hours, without refocusing or realignment. Even after being irradiated for 11,480 images, the embryo was unaffected and completed embryogenesis normally.

In summary, we present an optical wide-field microscope capable of imaging protein expression patterns deep inside both fixed and live embryos. By selective illumination of a single plane, the excitation light is used efficiently to achieve optical sectioning and reduced photodamage in large samples, key features in the study of embryonic development. The method of sample mounting allows positioning and rotation to orient the sample for optimal imaging conditions. The optional multiview reconstruction combines independently acquired data sets into an optimal representation of the sample. The implementation of other contrasts such as scattered light will be straightforward. The system is compact, fast, optically stable, and easy to use.

SPIM is well suited for the visualization of high-resolution gene and protein expression patterns in three dimensions in the context of morphogenesis. Heart function and development can be precisely followed in vivo using SPIM in Arnie transgenic embryos. Because of its speed and its automatic operation, SPIM can serve as a tool for large-scale studies of developing organisms and the systematic and comprehensive acquisition and collection of expression data. Even screens for molecules that interfere with development and regeneration on a medium-throughput scale seem feasible. SPIM technology can be readily applied to a wide range of organisms, from whole embryos to single cells. Subcellular resolution can be obtained in live samples kept in a biologically relevant environment within the organism or in culture. Therefore, SPIM also has the potential to be of use in the promising fields of 3D cultured cells (21) and 3D cell migration (22).

References and Notes
14. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material
www.sciencemag.org/cgi/content/full/305/5686/1007/DC1
Materials and Methods
SOM Text
Figs. S1 to S6
References and Notes
Movies S1 to S5
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