Even fluorescence excitation by multidirectional selective plane illumination microscopy (mSPIM)

Jan Huisken* and Didier Y. R. Stainier

Department of Biochemistry and Biophysics, University of California San Francisco, 1550 Fourth Street, San Francisco, California 94158-2324, USA

**Corresponding author: jan.huisken@ucsf.edu*

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Multidirectional selective plane illumination microscopy (mSPIM) reduces absorption and scattering artifacts and provides an evenly illuminated focal plane. mSPIM solves two common problems in light-sheet-based imaging techniques: The shadowing in the excitation path due to absorption in the specimen is eliminated by pivoting the light sheet; the spread of the light sheet by scattering in the sample is compensated by illuminating the sample consecutively from opposing directions. The resulting two images are computationally fused yielding a superior image. The effective light sheet is thinner, and the axial resolution is increased by $\sqrt{2}$ over single-directional SPIM. The multidirectional illumination proves essential in biological specimens such as millimeter-sized embryos. The performance of mSPIM is demonstrated by the imaging of live zebrafish embryos. © 2007 Optical Society of America

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Selective plane illumination microscopy (SPIM) is an invaluable tool for the high-resolution fluorescence imaging of live biological specimens [1]. The fundamental principle of SPIM is the selective illumination of a thin sheet in the focal plane of the detection objective. Images are acquired with an efficient camera along the detection axis, orthogonal to the illumination axis [Fig. 1(a)]. The benefits include optical sectioning, reduced phototoxic effects, high acquisition speed, and improved axial resolution compared with multi- and single-photon confocal microscopy [2]. Similar light-sheet-based illumination schemes have been realized for the macroscopic imaging of fixed and cleared tissue: (high-resolution) orthogonal-plane fluorescence optical sectioning [(HR) OPFOS] [3,4] and ultramicroscopy [5]. A unique feature of SPIM is the ability to rotate the sample and acquire multiple views that are then combined by image processing (multiview fusion) [6,7].

In light sheet based microscopies the sample is illuminated with a laser beam collimated in y and focused in z (Fig. 1). The height of the sheet w_y is chosen to fill the field of view (FOV, dimensions $d_x \times d_y$). The numerical aperture of the illumination NA_z is chosen to yield a light sheet with a thickness of $w_{z,0}$ in the center and $\sqrt{2}w_{z,0}$ at the edge of the FOV, hence $w_{z,0} \propto \sqrt{d_x}$. For typical biological applications $w_{z,0} \approx 1-5\ \mu\mathrm{m}$. Alternatively, the sample can be slowly scanned through the focus of the light sheet [4].

Multidirectional SPIM (mSPIM) has been developed to overcome artifacts induced by scattering and absorption in dense life tissue. All light microscopes suffer from the interaction of the excitation light with the sample. However, because of illumination from the side, some artifacts are more pronounced in light-sheet-based techniques such as SPIM, (HR) OPFOS, and ultramicroscopy. Absorption of the illumination light results in stripes and shadows along x throughout the FOV [Fig. 1(b)]. These stripes make it difficult to accurately analyze images generated by these techniques. Since the shadows constitute high-resolution information they are likely to be reproduced in multiview fusion. In mSPIM even illumination of the FOV is achieved by pivoting the light sheet in the detection focal plane [Fig. 1(c)]. The beam is scanned over an angle of 10° at a frequency of 1 kHz with a resonant mirror. Shadows are

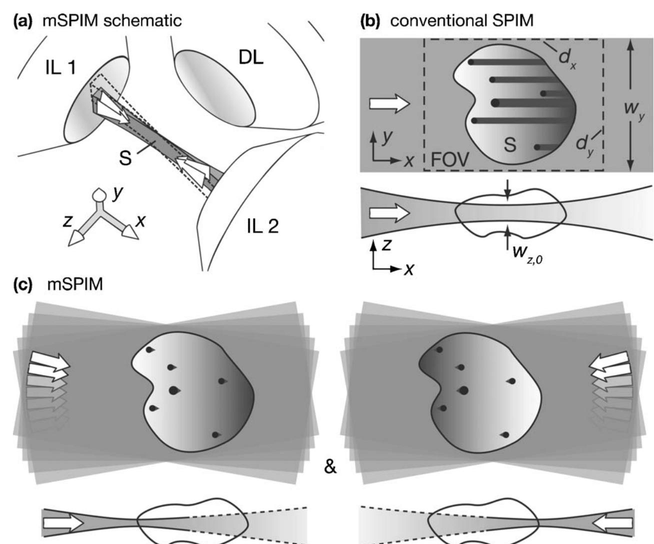

Fig. 1. (a) Schematic of mSPIM. (b) Parallel illumination of the sample in SPIM results in shadows and attenuation across the FOV. (c) In mSPIM these artifacts are severely reduced by sequentially illuminating the sample from two sides with a thinner pivoting light sheet. IL, illumination; DL, detection lens; S, sample.

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thereby significantly reduced in images taken with typical exposure times of 10–30 ms.

Two additional challenges in SPIM are the attenuation of the excitation light along the illumination axis x [Fig. 1(b)] and the spread of the light sheet in z. The illumination light entering the sample from one side can be partially absorbed by dense tissue, which results in a loss in excitation light and fluorescence on the far side of the sample. Moreover, the light may be scattered by the tissue. Scattering along y, i.e., in the plane of illumination, does not affect the light sheet quality. Scattering along z, however, will lead to a widening of the light sheet and a loss in z sectioning and resolution. Therefore images of scattering tissue show a progressive degradation in image quality from the light sheet entry site along x. The attenuation of the light sheet can be compensated in fixed and cleared tissue by the simultaneous illumination with two counterpropagating confocally aligned beams [5]. In live tissue, however, each beam is scattered, resulting in the widening of the light sheet at the distal side. Hence the overlap of two such counterpropagating beams would result in an optical section that is much thicker than that of a single sheet close to the entrance into the tissue.

In mSPIM the pivoting light sheet can be *sequentially* directed onto the sample from two opposing directions [Fig. 1(c)]. In both images the side of the sample facing the illumination is well sectioned and uniformly illuminated. These two images can then be combined by image fusion techniques [6]. In many cases this fusion can be efficiently performed by merging the well-illuminated halves from both images. Since only half of the image needs to be read out during acquisition, the camera can be operated at nearly double the frame rate, thereby partially compensating for the extra images that need to be taken. In addition each light sheet needs to fill only half the FOV, $d_x/2\times d_y$ in size. As a result, both light sheets can be made thinner by a factor of $1/\sqrt{2}\approx 0.7$, which improves optical sectioning and axial resolution.

A setup for mSPIM is shown in Fig. 2. The laser unit provides light from one or multiple lasers, which is then scanned in the vertical direction and expanded. This beam is guided into either of the illumination arms that direct light into opposing objectives. The central unit of the microscope holds three confocally aligned water dipping objective lenses, two for illumination (Leica 10×, 0.3) and one for detection (10×–40×). Hence, the quality of the light sheet is determined by the well-corrected objectives and not by cylindrical lenses as in [1,3–5]. Four stages are used to control the sample's position in x,y,z and its orientation ϕ [1]. The detection unit forms an image of the sample onto one or multiple cameras. The rays are shown in Fig. 2(b). In this telecentric setup the

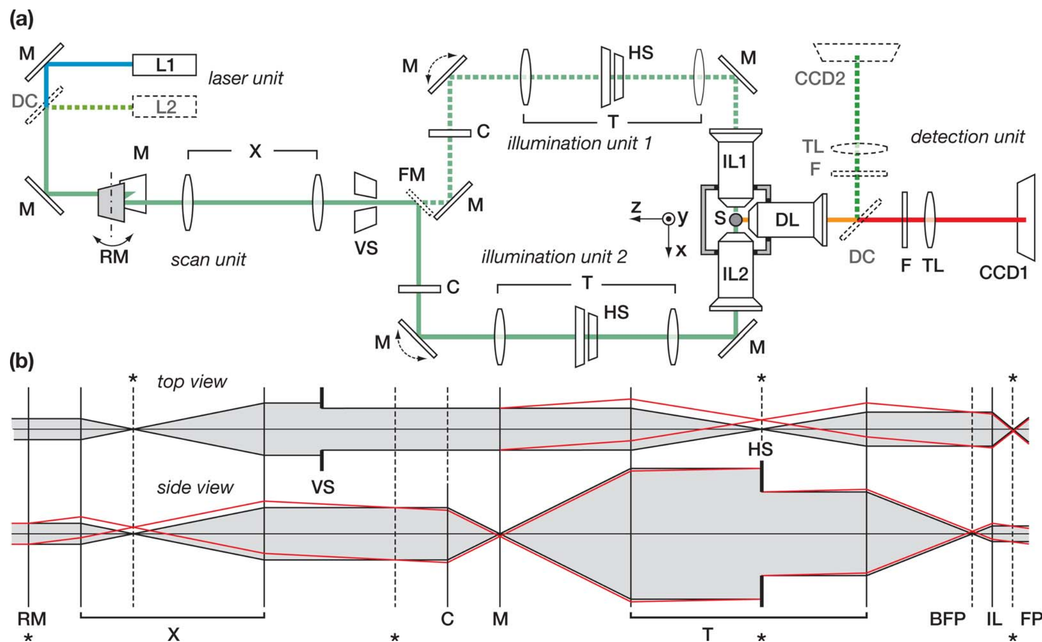

Fig. 2. (Color online) Setup for mSPIM: (a) Top view of the instrument, (b) top and side view of the beams in the scan and illumination unit (not to scale). The laser unit provides the light from one or multiple lasers (L1/2). The beam is scanned in the vertical direction by a resonant mirror (RM) and expanded by a telescope (X). A vertical slit (VS) is used to control the numerical aperture NA_z and the thickness of the light sheet. A flip mirror (FM) directs the light into illumination unit 1 or 2. In these identical units a cylindrical lens (C) focuses the light to a horizontal line that is imaged into the back focal plane (BFP) of the illumination lens (IL1/2) by a telescope (T). A horizontal slit (HS) is used to confine the excitation light along y to the FOV in the sample (S). The resulting light sheet exiting the IL is collimated in y and focused in z. It is aligned in z to match the focal plane of the detection lens (DL) by tilting one mirror (M; red rays in the top view). The scanning of the RM results in the pivoting of the light sheet (red rays in side view). The sample can be translated in x,y,z and rotated about y with micromotors (not shown). The detection unit consists of the DL and one or multiple cameras (CCD1/2) with dichroic (DC), filters (F), and tube lenses (TL). Planes conjugate to the illumination focal plane (FP) are marked with asterisks.

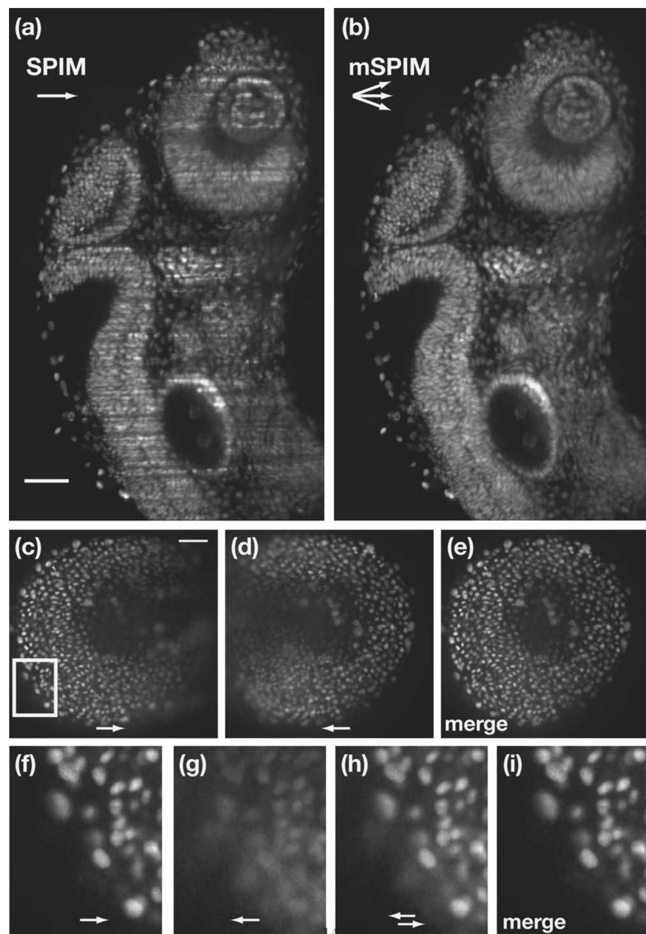

Fig. 3. Head of a live 35 h old zebrafish embryo imaged with (a) SPIM and (b) mSPIM. In mSPIM stripes are eliminated. (c)–(e) 10 h old embryo imaged with mSPIM and illuminated from (c) left and (d) right. In the merge of the two images (e) no attenuation is visible. (f)–(i) Magnified view of the area delineated in (c). Scale bars 50 μm. Sample mounted as described in [1].

scanning of the beam results in a pivoting of the light sheet about its focus. Each light sheet's dimensions are adjusted with the slit apertures, and the position is controlled with one mirror.

The zebrafish is a widely used model organism and ideal for *in vivo* analyses [8]. Imaging zebrafish expressing H2A-GFP in all nuclei [9] demonstrates the improvements of multidirectional illumination. In conventional, collimated SPIM, many stripes are apparent [Fig. 3(a)]. The stripes make it difficult to distinguish individual nuclei. In contrast, the same plane is mainly free of shadows when illuminated with the pivoting light sheet by activating the scan mirror in mSPIM [Fig. 3(b)]. Another feature of mSPIM is the ability to illuminate the sample from two sides. A 10 h old embryo is illuminated from the left [Fig. 3(c)] and the right [Fig. 3(d)]. Features on the side facing the illumination show better contrast and better sectioning. This information is preserved in the merge shown in Fig. 3(e). Figures 3(f) and 3(g) show a magnified area of the individual images. The average of these images would have been the result of simultaneous illumination from both sides as in [5] [Fig. 3(h)]. The merge of sequentially recorded mSPIM images is superior [Fig. 3(i)].

The illumination of the sample from multiple sides substantially improves image quality in mSPIM: (1) The sample is illuminated from a range of angles during the camera exposure to eliminate stripes; (2) an additional image of the same plane is taken with illumination from the opposite direction filling in missing information and residual shadows. In life specimens such as zebrafish mSPIM is superior to light-sheet-based microscopes with simultaneous, collimated illumination from one or two sides.

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