STED-SPIM: Stimulated Emission Depletion Improves Sheet Illumination Microscopy Resolution

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ABSTRACT We demonstrate the first, to our knowledge, integration of stimulated emission depletion (STED) with selective plane illumination microscopy (SPIM). Using this method, we were able to obtain up to 60% improvements in axial resolution with lateral resolution enhancements in control samples and zebrafish embryos. The integrated STED-SPIM method combines the advantages of SPIM with the resolution enhancement of STED, and thus provides a method for fast, high-resolution imaging with >100 μm deep penetration into biological tissue.

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The idea of illuminating a sample orthogonally to the imaging objective was first introduced by Siedentopf and Zsigmondy (1) in 1903. However, the method wasn't used extensively until almost a century later, when Voie and coauthors (2) successfully reintroduced the concept of orthogonal plane fluorescence optical sectioning to measure whole cochlea. Subsequently, investigators developed other types of selective plane illumination microscopy (SPIM) configurations to image deep into fish embryos (3) and fixed and cleared biological tissues (4,5), to remove artifacts from imaging (6), to create a light sheet of a single scanning beam and rigidly place the excitation and detection on a single system that can be maneuvered to image any biological probe (7), and to detect single molecules (8).

In the original method, a vertical light sheet is created by focusing an optically controlled laser beam with a cylindrical lens. The excitation optics are selected and positioned to achieve a light sheet with typical thickness of a few micrometers. The light sheet thickness does not vary by more than a factor of 2 over a field of view of up to many millimeters. This sheet is aligned with the focal plane of the imaging objective, which transfers this image to a CCD camera (5). The sample is rastered through the focal plane of the objective to capture the whole sample volume in a stack of images (5) (Fig. 1). Because only the object plane is exposed to the laser light, SPIM avoids the scattering and photophysical effects that can result from illuminating sample regions that are not in the focal plane (3).

Furthermore, the light sheet thickness determines the maximal effective numerical aperture of the objective, as well as the axial and lateral resolution. The theoretical maximal limit of the light sheet by Gaussian optics and the experimentally achievable light sheet for SPIM set the highest possible numerical aperture (NA) to be ~0.8. At NA $\leq$ 0.8, SPIM can provide better axial and (in some cases) lateral resolution than would be possible to achieve with a confocal microscope (9).

The resolution of an optical microscope is limited by light diffraction, which depends on the light wavelength and the objective aperture, and is also related to how well the light can be focused. Because SPIM is an optical technique in the visible wavelength range with the maximum NA, the lateral and axial resolutions are diffraction-limited to ~0.6 and 2 μm, respectively, depending on the field of view and with red (700 nm) light (9).

Over the past 15 years, researchers have developed a technique known as stimulated emission depletion (STED) to attain resolution better than the diffraction limit. STED microscopy uses a secondary, phase-modulated beam around the central excitation light source in a confocal microscope platform to switch off the fluorescence on the edge of the excitation beam and improve resolution. To date, the STED method has been successfully used to image many biological tissues with resolution as low as 20 nm, or ~10 times less than the diffraction limit (10,11). Although this technique was originally developed with temporal modulation of the STED beam for visual range excitation, new versions with nontemporal or continuous wave (CW) STED and multiphoton, infrared excitation incorporated with STED have been introduced along with other methods such as multiplexing and live-cell STED (12,13).

To incorporate a CW STED beam into SPIM, we use a modified optical pathway of the original STED method (14) by overlapping the excitation beam with a depleting laser beam, which had a beam mode of TEM_{01} (see Supporting Material) in the focus caused by the creation and insertion of a phase plate into the depletion beam before coupling with excitation. The depletion beam and its double-sheet,

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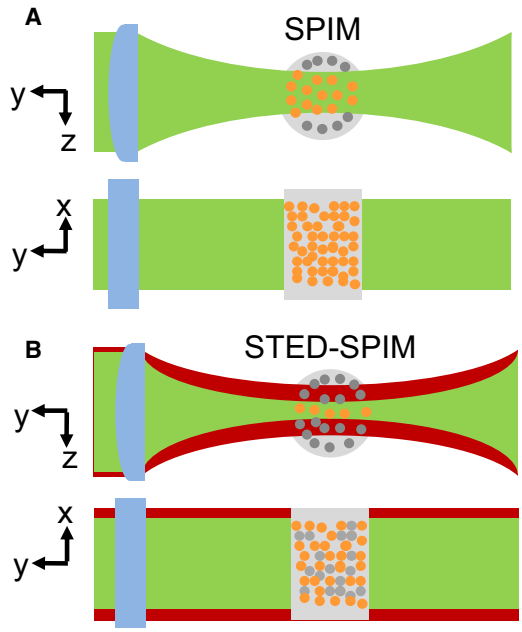

FIGURE 1 Excitation optics and sample fluorescence of SPIM (*A*, *top*) and the STED-SPIM method introduced here (*B*, *bottom*). The dots in the cartoon represent individual fluorophores, which are either fluorescent (*orange*) or nonfluorescent (*gray*). The green region represents the SPIM excitation sheet and the red regions (in *B*) represent the dual-sheet STED beam in the sample area.

single TEM_{01} profile would be just on either side of the excitation light sheet in the focus of the objective plane (Fig. 1 *A*). Our concept is that, just as with STED in epiillumination, we should be able to effectively reduce the fluorescence emission in the axial direction of the imaging plane for smaller object sectioning (Fig. 1 *B*). As a result of having a thinner beam in the axial direction, we can also foresee that the benefit of improved lateral contrast in the images could appear as a collateral lateral resolution enhancement. These improvements would be present even though the entire system has the same imaging capability as an epifluorescence system.

As a first test of our setup, we stained micrometer-sized filter particles (FF09; Hartenstein, Würzburg, Germany) with ATTO647 dye and affixed them into agarose gels. We first imaged the filter particles with our standard SPIM system with CW red laser excitation (Cube 640/40; Coherent, Santa Clara, CA) and recreated them into a 3D projection lateral image (Fig. 2 *A*). The addition of a double-sheet STED beam with laser light from a Ti:Sapphire laser (Mira-900F and Verdi-V10; Coherent) at 750 nm and 400 mW immediately revealed finer structural details of the ATTO647-stained fibers in the same region of the particle (Fig. 2 *B*). Axially, we observed an even greater improvement between the normal SPIM and the STED-SPIM acquisitions of the same filter particle region (Fig. 2, *C* and *D*). After fitting the structural profiles in the

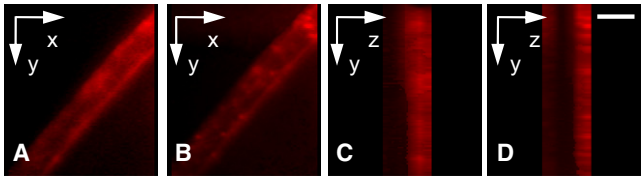

FIGURE 2 Comparison of SPIM and STED-SPIM on an ATTO647-stained control fiber sample. The lateral (*A*) and axial (*C*) details and contrast of the SPIM image are far less resolved than those achieved with STED-SPIM (*B* and *D*). A profile analysis of the same regions revealed consistent 11–40% improvements in lateral images and a 46% improvement in axial images (Fig. S2). Scale bar: 10 μm.

same specific sites to Gaussian functions (Fig. S2), we found that the lateral resolution was improved by 11–40% and the axial resolution was improved by 46%.

We then investigated the resolution improvement in biological tissue. Fig. 3 *A* shows a SPIM image of a fixed zebrafish actin stained by phalloidin ATTO647. Imaging of the cytoskeleton in developing multicellular zebrafish embryos under physiological conditions can provide informative clues about cell shape development in vivo (15). However, it is difficult to achieve deep penetration with high resolution in such embryos. Upon application of the STED beam as described above, improved structural details could be observed at penetration depths $>$ 100 μm (Fig. 3 *B*). Analysis of the structural features apparent in both recorded images revealed improvements of 30% axially (Fig. S3) and 17% laterally (Fig. S4).

Our results demonstrate the suitability and applicability of incorporating STED into the SPIM imaging technique. The 30–40% axial resolution improvement gained by including STED should enable the use of imaging objectives with a numerical aperture of $>$1 with light sheet excitation (9). Our microscope system has a calculated resolution of 0.55 μm laterally and 2.0 μm axially (see Supporting Material). From control measurements of 40 nm beads (Fig. S5), we were able to obtain resolutions of 0.61 and 0.80 μm

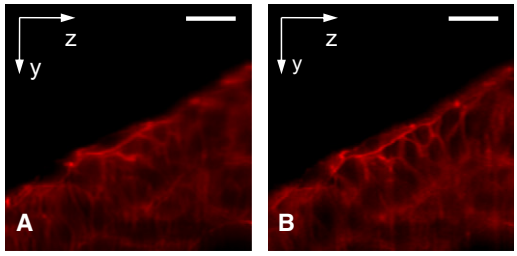

FIGURE 3 Comparison of SPIM and STED-SPIM on an actin in zebrafish embryo stained with ATTO647 phalloidin. The axial details and contrast of the SPIM image (*A*) are far less resolved than those achieved with STED-SPIM (*B*). A profile analysis of the same regions revealed a consistent 30% improvement in axial images (Fig. S3) and 17% improvement in lateral images (Fig. S4). Scale bar: 20 μm.

laterally, and 0.68 and 1.24 μm axially, with STED-SPIM and SPIM, respectively (Fig. S5). Thus, we again demonstrated improved resolution performance by point light source measurements of >40% axially (and nearly threefold greater than the diffraction limit) and confirm the collateral lateral resolution enhancement with STED, but in this case only to roughly the level of the diffraction limit. It is possible that the resolution improvement could be further extended with full depletion (the maximal depletion obtained was only 70% for this system (Fig. S6)) by the addition of scanning excitation (7,9), which would allow for higher STED intensities, and a smaller-pixel, high-sensitivity CCD camera.

In recent studies, investigators were able to achieve improved resolution for SPIM by using structured illumination techniques (16). STED incorporated with SPIM might also be beneficial because it allows for faster imaging acquisition times way beyond two frames per second due to the immediate response during the image acquisition. By changing the STED platform to a nonepiluminescence configuration, we also gain further advantages, such as much deeper sample penetration (>100 μm) with the STED effect, and less exposure and possible sample damage for out-of-focus regions. Furthermore, the high energy of the depletion laser beam is only incident on the scanned sample regions.

We find that the incorporation of STED into SPIM provides a unique method for high-resolution imaging of model organisms and tissues. This method is advantageous because it allows imaging of live organisms with high resolution and fast acquisition >100 μm deep into tissues. It also requires no histological sectioning for fixed organisms or tissues, which dramatically reduces additional artifacts. STED combined with SPIM microscopy significantly enhances the original technique's advantages and enables fast imaging of biological tissues with axial resolution beyond diffraction limits.

SUPPORTING MATERIAL

Supplemental methods and materials, data, and Figs. S1–S6 are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00303-1.

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