3D Reconstruction of High-Resolution STED Microscope Images

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ABSTRACT Tackling biological problems often involves the imaging and localization of cellular structures on the nanometer scale. Although optical super-resolution below 100 nm can be readily attained with stimulated emission depletion (STED) microscopy methods, attaining an axial resolution <100 nm with focused light generally required the use of two lenses in a 4Pi configuration or exceptionally bright photoswitchable fluorophores. Here, we describe a simple technical solution for STED microscopy of fixed samples: biological specimens are fluorescently labeled, embedded in a polymer resin, cut into thin sections, and then imaged via STED microscopy with nanoscale resolution. This approach allows a 3D image reconstruction with a resolution <80 nm in all directions using available state-of-the art STED microscopes. Microsc. Res. Tech. 00:000–000, 2008. © 2008 Wiley-Liss, Inc.

INTRODUCTION Fluorescence microscopy, one of the most common techniques in the life sciences, is currently undergoing fundamental improvements with respect to spatial resolution. Although confocal microscopy introduced optical sectioning, and thereby allowed the creation of three-dimensional (3D) images, the advent of stimulated emission depletion (STED) microscopy, has been constantly increasing (Hell, 2007).

STED microscopy typically uses a tightly focused laser beam for excitation that is overlapped with a second “STED” laser beam that features a zero-intensity point at the focal center but strong intensities at the focal periphery. If excited, fluorophores exposed to the STED beam are almost instantly transferred back to their ground state by means of stimulated emission. As a result, only molecules that are close to the zero of the STED beam are allowed to fluoresce and contribute to the fluorescence signal. Confining the signal to such a subdiffraction-sized spot results in higher resolution imaging when scanning the spot through the specimen.

The idea that underlies STED microscopy is more general and can in principle be extended to any marker that can be photoswitched between a fluorescent bright state and a nonfluorescent dark state (i.e., RESOLFT microscopy) (Hell et al., 2003). In particular, reversibly switchable fluorescent proteins and photoswitchable synthetic dyes have been used to implement various types of RESOLFT microscopes (Hell, 2007; Hofmann et al., 2005). Photoswitching is also at the heart of more recent approaches to super-resolution microscopy based on the photoactivation, localization, and subsequent deactivation of individual molecules (Betzig et al., 2006; Rust et al., 2006). Although highly effective, this strategy has its own limitations. Besides being background sensitive, photoswitching of individual molecules is inherently more sensitive to the molecular environment than is stimulated emission.

Resolution enhancement in STED microscopy can be achieved in the axial direction (e.g., by using a 4Pi setup) (Dyba and Hell, 2002), the lateral (focal plane) direction (Klar and Hell, 1999; Willig et al., 2006b), or both simultaneously (Harke et al., 2008). A common STED implementation for lateral resolution improvement uses a toroidal (doughnut-shaped) STED focal spot (Keller et al., 2007; Willig et al., 2006b) and confocal fluorescence detection to minimize background. This configuration is optimal for lateral resolution enhancement and typically provides a resolution of 20–40 nm in the focal plane, but the axial resolution remains at the confocal level (>500 nm) resulting in the fact that small and dark structures remain difficult to discern if other bright features are present within the confocal axial section. This limitation holds equally for the combination of single-molecule photoswitching microscopy with two-photon excitation (Fölling et al., 2007), which also provides lateral super-resolution with 3D optical sectioning. Increasing the axial resolution beyond its diffraction limit requires more demanding approaches, such as the use of a STED-4Pi microscope or a combination of several depletion beams (Harke et al., 2008). Additionally, 3D imaging with a resolution of a few nanometers is...
limited by the photostability of the marker dyes. Because each section of the sample is illuminated when an adjacent section is read out, the dyes are subjected to elevated doses of light.

To circumvent these difficulties, at least for fixed samples, we report on a different approach to obtain 3D STED imaging. Sectioning is achieved mechanically by cutting thin layers of the polymer-embedded sample with a microtome (Fig. 1). Although sacrificing some of the typical advantages of fluorescence microscopy, such as the rapid and convenient sample preparation, this approach eliminates problems with out-of-focus fluorescence and photobleaching. These benefits are even more important in super-resolution imaging than they are in confocal microscopy, where resin embedding and mechanical sectioning has been demonstrated (Melville et al., 1998). The method therefore offers a simple way of obtaining highly resolved 3D images of biological specimens, including thick samples, which are not optically accessible due to the limited working distance of lenses with high-numerical apertures.

MATERIALS AND METHODS

Sample Preparation

Rat hippocampal neurons were prepared and immunostained as described (Willig et al., 2006). To facilitate the registration of neighboring xy-images within a z-stack, the cells were incubated overnight with a diluted suspension of two-color fluorescent silica beads at 37°C. The beads were derived from commercially available green fluorescent silica beads (sicastar®-green/redF, 300 nm, NH₂ functionalized, 50 mg/mL, micromod GmbH, Rostock, Germany), which were additionally labeled with ATTO 633 (ATTO-TEC GmbH, Siegen, Germany). The following antibodies were used for immunostaining: anti-synaptophysin (Jahn et al., 1985), anti-SNAP-25 (71.1, Synaptic Systems, Goettingen, Germany), antisyntaxin-1 (HPC-1; (Barnstable et al., 1985), and antitubulin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

We used secondary antibodies (Dianova GmbH, Hamburg, Germany) labeled with ATTO 532 (ATTO-TEC GmbH) or ATTO 633 fluorescent dyes, followed by fixation with paraformaldehyde (PFA) and embedding in Nanoplast® resin (Polysciences, Warrington, PA). For 1 mL of hexamethylol-melamine-ether (HMME), 25 mg B52 catalyst were used. This mixture was added onto coverslips using BEEM® capsules (Plano GmbH, Wetzlar Germany). The samples were stored at room temperature for 24 h over silica gel to ensure proper infiltration of the cells with the embedding medium. Afterward, the medium was dried over silica gel for 24 h at 40°C, followed by 48 h at 60°C to effect polymerization. The polymerized blocks were detached from the coverslips by dipping them into liquid nitrogen and cut with an ultramicrotome (EM UC6, Leica Microsystems GmbH, Wetzlar, Germany) using an ultrasonic diamond knife (Diatome AG, Biel, Switzerland). The slices were directly transferred onto glass coverslips.

STED Microscopy

The setup used for the experiments was a home-built two-color STED microscope as described in detail elsewhere (Donnert et al., 2007; Meyer et al., 2008). In brief, a laser system consisting of a Ti:Sapphire master oscillator (Mira 900F, Coherent, Santa Clara, CA), a regenerative amplifier (RegA 9000, Coherent), and an optic-parametric amplifier (OPA 9400, Coherent) was used to generate STED pulses at a rate of 250 kHz and a wavelength of 603 nm for the visible STED beam. For the infrared STED beam, a second mode-locked Ti:Sapphire oscillator (Mira 900F, Coherent) with a repetition rate of 76 MHz was used at a wavelength of 750 nm. The <250 fs duration of the initial pulses was expanded to ~200 ps by means of a glass fiber. The pulse energies at the back aperture of the objective lens were up to 0.5 nJ and ~1.5 nJ for the visible and infrared channels, respectively. Fluorescence excitation was performed with two picosecond diode lasers (PicoTA-488 and LDH-P-635, PicoQuant GmbH, Berlin, Germany) synchronized to the Ti:Sapphire oscillators. To create the toroidal STED doughnut, a vortex phase plate (RPC Photonics, Rochester, NY) for the infrared channel and a spatial light modulator (Hamamatsu, Hamamatsu City, Japan) for the visible channel were used.

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Japan) for the visible channel were placed into the expanded laser beams before focusing them with a 100×/NA 1.4 oil immersion lens (Leica Microsystems GmbH, Wetzlar, Germany). Unlike the previous setup (Donnert et al., 2007) on which our experiment was based we achieved a uniform lateral resolution $< 40 \text{ nm}$ in both channels.

The images of the infrared and visible color channels were recorded consecutively, whereas the STED and confocal images were measured quasi-simultaneously on a line-by-line basis. The field of view was typically chosen as 10 $\mu\text{m} \times 10 \mu\text{m}$, which, along with a quadratic pixel size of 20 nm, and a pixel dwell time of 0.5 ms for the infrared and 6–8 ms for the visible channel, resulted in acquisition times of $\sim 60 \text{ min}$ per slice in this setup.

**Image Processing**

Registration of the two color channels was performed using the in-house data acquisition and analysis software *Imspecor* and a set of custom-written routines in MATLAB (The Mathworks, Natick, CA). We compensated a slight drift during acquisition of the slower channel by fitting a polynomial to the drift at selected positions determined from superimposing the coembedded silica beads. The alignment of consecutive slices was performed by translating and rotating the images until a satisfactory match of the coembedded silica beads was achieved. To build the 3D representations, the manual alignment tool *midas* was used, which is part of the academic *IMOD* software suite (Kremer et al., 1996). Static and animated 3D visualizations of the data stacks were produced with the commercial visualization software package *amira* (Visage Imaging, Carlsbad, CA).

For the colocalization analysis of the SNARE proteins and the vesicle marker synaptophysin, the positions of the protein clusters (syntaxin-1/SNAP-25, green channel) were marked manually by two individuals in three independent runs. All three runs provided...
consistent position data. Vesicle release sites were then identified based on the fluorescence of the labeled vesicle marker synaptophysin in the red detection channel. A map of these regions was automatically created using a MATLAB script, which performed the following image-processing steps: regions below a noise level of 50 counts/pixel were discarded, while the remaining regions were thresholded at their average intensity. (The resulting mask was stable with respect to variations of the background level.) The mask was eroded by a disk with radius $r = 80–100$ nm to exclude remaining artifacts and then dilated by a disk with $r = 260$ nm to account for the diameter of the vesicles. Clusters lying within the resulting mask were considered “inside.”

**RESULTS**

**Sample Preparation and Imaging**

The general workflow is depicted in Figure 1. To prepare the samples, we immunostained cultured rat hippocampal neurons for different proteins (Willig et al., 2006b) and then incubated them with a polymer resin. After curing the resin, the resulting polymer block was cut into 75–100-nm thick slices, which were transferred to the coverslips for imaging (see Experimental Procedures for details). During the sample preparation, we also added fluorescent silica beads (300 nm in diameter) onto the cells. The beads were also cut during sample sectioning and then identified in two to four
consecutive slices, allowing us to overlay the images taken from the different slices with high precision (Fig. 3B).

The imaging was performed with a custom-built STED microscope designed for two-color operation (see Materials and Methods section). A lateral resolution of \(<40\) nm for both color channels was typical for this setup (Meyer et al., 2008), see Figures 2B and 3A. The resolution along the optical (z) axis was determined only by the slice thickness. Figure 2B shows typical images for a number of immunostained proteins, surpassing the results obtained in conventional, uncut preparations. The high density of staining makes it difficult to observe single spots in conventional preparations, while the z-axis sectioning renders this much more feasible in the thin slices.

3D Reconstruction

Before recording high-resolution STED images of the slices, we acquired confocal overview pictures to localize corresponding regions in all slices (Fig. 4A). We then collected several series of two-color high-resolution images with up to 15 consecutive sections. Data from the two channels were registered using the images of embedded beads fluorescing in both channels. We then proceeded to reconstruct the 3D structures. During imaging, each slice is arbitrarily oriented on the coverslip, and therefore the data from different slices had to be registered using the signal from the fluorescent beads as reference marks. The alignment was performed manually. An example of such a reconstruction is shown in Figure 4.

Localization Analysis of Syntaxin-1 and SNAP-25

As shown in Figure 2B, thin cutting allows structures to be observed, which would be obscured by the out-of-focus fluorescence in a normal preparation, because they are too crowded or too close to a bright feature. This allows an improved analysis of the localization of two synaptic proteins, syntaxin-1, and SNAP-25, which play an important role in the fundamental step of neuronal communication: fusion between the synaptic vesicle and the plasma membrane (Jahn and Scheller, 2006). We immunostained preparations for these proteins and to visualize the
synaptic vesicles as well, we coimmunostained for the vesicle marker synaptophysin (Fig. 5, red).

Both syntaxin-1 and SNAP-25 showed a spotty distribution. Most of the spots were located close to groups of synaptic vesicles found within the axons. This is not surprising as both proteins are mainly found in the membrane of the axons (Jahn and Scheller, 2006). Some fluorescence was also associated with the vesicles, as both syntaxin-1 and SNAP-25 are found in the synaptic vesicles at low levels (Takamori et al., 2006), despite being plasma membrane proteins. Again, we note that this type of staining would have been difficult to observe by confocal imaging due to the small size of the synapses investigated. Even in a STED microscope with an enhanced lateral resolution, without the increased z-resolution provided by thin-sectioning, the density of the syntaxin-1/SNAP-25 staining would have obscured the individual spots (see Fig. 2).

As syntaxin-1 and SNAP-25 function exclusively in vesicle fusion, one would expect them to be concentrated in small areas of the synapse, in the release sites where synaptic vesicle fusion actually takes place. However, this is not the case, as the two proteins are distributed in spots (protein clusters) across the entire sample (Figs. 5A and 5B, green panels). To confirm this, we determined the percentage of syntaxin-1/SNAP-25 clusters in the immediate vicinity of the areas occupied by the synaptic vesicles (as identified by the synaptophysin staining). We found that only ~40% and ~50% of the clusters of syntaxin-1 and SNAP-25, respectively, are associated with the synaptic vesicles. By varying the image processing parameters, these numbers were found to be stable to within ~10%.

Overall, one is forced to conclude that syntaxin-1/SNAP-25, although associated with synaptic vesicle fusion, are not restricted to the space near synaptic vesicle accumulations. Interestingly, the wide positioning of the synaptic vesicle’s fusion partners opens, in principle, the possibility of vesicles fusing outside of their normal release sites, in regions where neurotransmitter release would not be productive and would not result in any transmission of information to the postsynaptic cell (because postsynaptic receptors are normally found only near the release sites).

**DISCUSSION**

We demonstrated that STED imaging in combination with microtome-based sectioning is capable of producing images with a resolution of well below 80 nm in 3D. We were thus able to obtain multicolor 3D reconstructions of rat-cultured neurons, which were suitable for high-resolution colocalization analysis.

Similar to electron microscopy, the slicing protocol is rather laborious but still retains many of the advantages of optical microscopy of fixed samples. Most importantly, the well-established labeling procedures from fluorescence microscopy can be used without
significant modification. A plethora of immunostaining protocols as well as genetically encoded markers such as fluorescent proteins (Willig et al., 2006a) should be available, which provide more flexibility compared to the less efficient immunolabeling in electron microscopy. Compared to all-optical 3D imaging, our approach can arguably be more easily optimized for negligible dye photobleaching and allow samples of arbitrary thickness to be investigated. Essentially, any part of an animal (or plant) body can be imaged. This is particularly important for neurobiological and other tissue samples, which are optically accessible only up to a limited depth, which is set by sample scattering and, ultimately, by the working distance of the objective lens. Besides, radical developments in laser technology such as the combination of fiber lasers, diode lasers, and photonic crystal nonlinear frequency conversion will greatly reduce the system complexity for STED.

In the future, the mechanical slicing method may further benefit from alternative sample preparation techniques such as cryosectioning, in particular, if aldehyde fixation can be omitted, and replaced with rapid freezing, as is the case in recent protocols (Donnlaar et al., 2007). In that case, faithful morphology preservation can be achieved along with optimal epitope preservation and labeling. However, it should be noted that compared to freezing techniques, resin embedding allows a greater variety of samples to be processed. Shock-freezing is limited to very thin (monolayer) samples, whereas high-pressure freezing requires samples to be housed in a 1–2-mm-sized holding chamber. In contrast, virtually any sample can be embedded in a resin after fixation as long as efficient dehydration and resin penetration is ensured. For this reason and because of the full compatibility with existing STED microscopes, including commercial ones, resin embedding, and slicing could well become a standard sample preparation in far-field fluorescence nanoscopy.

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