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Dual-color three-dimensional STED microscopy with a single high-repetition-rate laser

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Abstract

We describe a dual-color three-dimensional stimulated emission depletion (3D-STED) microscopy employing a single laser source with a repetition rate of 80 MHz. Multiple excitation pulses synchronized with a STED pulse were generated by a photonic crystal fiber and the desired wavelengths were selected by an acousto-optic tunable filter with high spectral purity. Selective excitation at different wavelengths permits simultaneous imaging of two fluorescent markers at a nanoscale resolution in three dimensions.

Stimulated emission depletion (STED) microscopy is one of the most powerful fluorescence nanoscopy techniques, exploiting a molecular property of fluorophores, i.e. “on” and “off” states, via stimulated emission process with visible light and a conventional lens [1].

Spatially and temporally overlapping a focused excitation spot with a patterned depletion beam called the STED beam, it enables the optical imaging of macromolecular complexes and subcellular structures with a nanoscale resolution in cells [2].

Multi-color fluorescence imaging discloses proximity of biomolecules of interest and elucidates their functional relationships in cells [3, 4]. In an earlier STED implementation, two-color imaging was achieved by using spectrally well-separated fluorophores [5]. Despite its excellent performance, this method has not been widely used because it requires massive laser systems for generating two strong STED beams, one for each color. This shortcoming was alleviated by employing large Stokes shift dyes [6, 7], photochromic fluorescent proteins [8], or spectrally adjacent fluorophores [9] so that similar emission wavelengths of two fluorescent markers require only a single STED beam. Among these, the use of spectrally adjacent fluorophores has been the most successful, owing to the fact that it does not suffer from limited photostability of fluorophores and it can image different species simultaneously without crosstalk. However, previous implementations required three separate light sources, e.g. one STED and two excitation lasers that must be externally synchronized [9]. In this case, temporal jittering of each pulsed laser source can reduce its performance unless sophisticated electronics are employed. Moreover, the lack of tunability of the excitation wavelength limits its usage to a particular pair of fluorophores.

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External synchronization can be avoided if the excitation beam is obtained from a supercontinuum source generated from a mode-locked Ti:Sapphire laser which by itself can be used as the STED beam [10]. Potential advantages include: the excitation wavelength is tunable, the excitation and STED beams are automatically synchronized, and the high repetition rate of the oscillator allows faster imaging compared to the imaging systems that use a commercial supercontinuum source with a low repetition rate [11, 12]. Here, we demonstrate a compact and versatile two-color 3D-STED microscopy with a single but spectrally tunable excitation beam and a fixed STED beam, all emanating from a single laser source with 80 MHz pulse trains. Sub-diffraction resolution was achieved in fluorescence nanoparticles and immunofluorescence stained mammalian cells.

A custom microscope was constructed for two-color STED imaging [10, 13, 14]. A schematic is shown in Fig. 1. Light from Ti:Sapphire laser (MaiTai HP, Spectra Physics) with a typical wavelength in the range of 760–780 nm was divided into STED and excitation beam paths using a half-wave plate, $\lambda/2$ (AHWP05M-980, Thorlabs) and a polarizing beam splitter, PBS (PBS252, Thorlabs). The STED pulses were pre-stretched using two 15 cm long glass rods (N-SF57, Casix) and further stretched to ≈ 300 ps using a 100 m long polarization-maintaining single-mode fiber (PMJ-A3AHPC, 3S-633-4/125-3-100-1-SP, OZ optics). The power of the STED beam was adjusted using another set of $\lambda/2$ and PBS, and the STED pulse was synchronized with the excitation pulse using a manual optical delay stage (PRL-12, Newport).

In the excitation beam path, the supercontinuum light was produced by a 12 cm long photonic crystal fiber (FemtoWhite 800, NKT photonics) with an incident power of 200–300 mW, and spectrally filtered using a short-pass filter (FF01-680/SP, Semrock) to block near-IR light. Although the spectral selection can be readily achieved by a narrow band-pass filter [10] and a motorized filter wheel system, its slow on/off switching time allows only serial imaging of multiple colors. Therefore, we instead used an acousto-optic tunable filter (AOTFnc-400.650, AA Opto-Electronic) to select particular wavelengths with 1 μ s response time and 1–2 nm bandwidths. The RF frequency applied on the AOTF transducer allowed controlling of up to eight wavelength channels. The spectral purity of the selected excitation beams was further ensured by a double-pass AOTF configuration [15], yielding a low background level (< 0.5 kHz photon count rate at the detector). Excitation beams of selected wavelengths were coupled to one single-mode fiber (P5-488PM-FC-2, Thorlabs), providing us with pulse duration < 100 ps, maximum output power 30–40 μ W, and power stability of $< 2\%$.

The collimated STED beam was split using a PBS and each beam passed through a phase plate imprinted with either a 2π -helical lamp (VPP-1, RPC photonics) or a π -shifted pattern to generate a lateral or axial doughnut-shaped STED beam, respectively [16]. The recombined STED beam was reflected by a 5 mm thick dichroic beam splitter (z740sprdc, Chroma) and circularly polarized using an achromatic quarter-wave plate and a half-wave plate (RAC 4.4.15 and RAC 4.2.15, B. Halle Nachfl.). The excitation beam was reflected by a 30:70 (R:T) beamsplitter (BS019, Thorlabs). The fluorescence signal collected by an oil immersion objective (NA = 1.4 HCX PL APO 100x, Leica) was sent to two detection channels, APD1 (600–640 nm; HQ620/40m (Chroma)) and APD2 (655–690 nm;

BLP01-635R-25 (Semrock) and ET670/40m (Chroma)), after being separated by a dichroic beam splitter (FF649-Di01, Semrock). The fluorescence signal was imaged onto multimode fibers with a core diameter of 62.5 μm (M31L01, Thorlabs) and detected by avalanche photo diodes (SPCM-AQR-14-FC, Perkin Elmer). The scanning of samples was accomplished by a piezo-controlled stage (Max311D, Thorlabs) and Imspector was used as a data acquisition program.

To demonstrate two-color STED imaging capabilities, crimson and dark red beads (24 nm and 36 nm, respectively, according to the manufacturer specification (Invitrogen)) were immobilized on a poly-L-lysine coated surface and embedded in a mounting medium (Mowiol, Sigma) [17]. We measured the excitation/emission maxima to be 624/645 nm for the crimson beads and 660/675 nm for the dark red beads. Despite the strong overlap of their emission spectra [Fig 2(a)], selective excitation and linear unmixing allowed us to clearly separate the two bead signals [18, 19]. We conducted line-by-line sequential imaging of two channels with one detector (APD2) [Fig. 1 inset]: CH1 imaged with 620 nm excitation and CH2 with 650 nm excitation. For linear unmixing, fluorescence intensity contributions to each channel were obtained from single bead samples [Fig. 2(b)]. We then obtained the linear unmixed images via least-square fitting [18]. Figure 2(c) shows confocal and STED images on a mixture of crimson and dark red beads. Unlike the overlay image of two channels (left panel), the linear unmixed image clearly separated two fluorescent beads with < 5 % crosstalk (right panel). The measured spot diameters (full width at half maximum) from 15 fluorescent beads each were 48 ± 6 nm for crimson and 43 ± 5 nm for dark red with 160 mW of STED light at 775 nm (errors denote the standard errors of the mean).

Considering the size of nanoparticles, effective lateral resolutions were 41 nm and 26 nm, respectively. Additionally, two-color 3D-STED images were recorded by adding an axial doughnut-shaped STED beam to the lateral STED beam [Fig. 2(d)]. Although there was 50 nm focal shift in the axial direction between the two confocal images of different channels caused by incomplete chromatic correction of the objective lens, the single STED beam predetermined the position of channels at the same focal plane, providing completely co-aligned images with the axial resolution of 176 nm for crimson and 133 nm for dark red.

Next, a dual-labeled immunofluorescence sample was prepared using NIH3T3 cells. Microtubules and mitochondria were specifically targeted using anti- β -tubulin (mouse, Sigma) and anti-Tom20 (rabbit, Abcam), and subsequently labeled using anti-mouse-IgG-Atto594 (Rockland) and anti-rabbit-IgG-Star635P (Abberior). We chose the excitation wavelengths and detectors based on fluorescence excitation and emission spectra of Atto594 and Star635P: CH1 imaged with 585 nm excitation and APD1, and CH2 with 645 nm excitation and APD2. During each channel imaging, the other APD was turned off by modulating its gate function. There was negligible crosstalk (~ 5 %) between channels; therefore, the linear unmixing process was unnecessary for this dye pair [9]. Under very strong STED illumination, however, the fluorescence intensity of Star635P on CH1 was slightly increased, probably due to photoconversion, so in this case linear unmixing was required. Figure 3 shows dual-color STED images of microtubules and mitochondria. It super-resolved their structures and reliably separated two markers (see confocal images for comparison). With pixel dwell time of 80 μs , dual-color images of Figure 3(c) were recorded in 92 seconds, currently limited by the sample scanning system. The imaging speed can be

much improved by adapting a fast-beam scanning system [20] to fully exploit the high-repetition-rate of the laser system.

Over the past few years there have been remarkable technical advances in STED imaging, mainly focused on cost-effective and simple implementations without compromising its performance. Our new implementation reported here is a very attractive multi-color 3D-STED nanoscope; it is simple and tunable, and maintains the high resolution. In principle it is possible to cover more than two colors by spectral imaging and linear unmixing [18]. Alternatively, different fluorescence lifetimes of spectrally similar fluorophores can be used as a third channel [19]. This will be valuable in super-resolving more than two cellular components.

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Figure 1.

Schematic of dual-color 3D-STED microscope: Ti:Sa, Titanium-Sapphire laser; $\lambda/2$, achromatic half-wave plate; PBS, polarizing beam splitter; PCF, photonic crystal fiber; AOTF, acousto-optic tunable filter; PMF, polarization-maintaining fiber; PP_{xy} and PP_z : phase plates for 3D-STED; DC1, short-pass dichroic beam splitter (740 nm); BS, 30:70 (R:T) beamsplitter; $\lambda/4$, achromatic quarter-wave plate; Obj, objective lens; F1: short-pass filter (720 nm); TL, tube lens; DC2, long-pass dichroic beam splitter (650 nm); F2, band-pass filter (620/40 nm); F3, combination of long-pass (655 nm) and band-pass (670/40 nm) filter; APD, avalanche photodiode. Inset: Line-by-line acquisition mode of dual-color STED imaging.

Figure 2.

Two-color STED images of fluorescent nanoparticles. (a) Excitation (dotted line) and emission (solid line) spectra of crimson (blue) and dark red (red) beads. (b) Fluorescence intensity distributions of crimson or dark red beads obtained from photon counts at each pixel upon illumination of 620 nm (CH1) and 650 nm light (CH2). (c) 2D-confocal and STED images of a mixture of crimson and dark red beads. Left, overlay image of two channels; right, linear unmixed image. (d) 3D-confocal and STED images. All images are raw data after background subtraction and look-up tables for the false colors are provided for STED images. Scale bar, 200 nm.

Figure 3.

Two-color immunofluorescence STED images. (a–c) Atto 594 labeled tubulin (green), Star 635P labeled Tom20 (red) and their overlay image. (d–g) Magnified images of the boxed region in (a–c). The excitation and STED (760 nm) powers were 2 μ W and 130 mW, respectively. (h) 3D-STED and (i) 3D-confocal images of tubulin and Tom20. STED images were smoothed with a 1.2 pixel Gaussian filter. Scale bar: 1 μ m (a–c, h–i) and 500 nm (d–g).