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CW STED Nanoscopy with a Ti:Sapphire Oscillator

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ABSTRACT

Fluorescence microscopy has become an essential tool to study biological molecules, pathways and events in living cells, tissues and animals. Meanwhile, the conventional optical microscopy is limited by the wavelength of the light. Even the most advanced confocal microscopy or multiphoton microscopy can only yield optical resolution approaching the diffraction limit of ~200 nm. This is still larger than many subcellular structures, which are too small to be resolved in detail. These limitations have driven the development of super-resolution optical imaging methodologies over the past decade.

The stimulated emission depletion (STED) microscopy was the first and most direct approach to overcoming the diffraction limit for far-field nanoscopy. Typically, the excitation focus is overlapped by an intense doughnut-shaped spot to instantly de-excite markers from their fluorescent state to the ground state by stimulated emission. This effectively eliminates the periphery of the Point Spread Function (PSF), resulting in a narrower focal region, or super-resolution. Scanning a sharpened spot through the specimen renders images with sub-diffraction resolution. Multi-color STED imaging can present important structural and functional information for protein-protein interaction.

In this work, we presented a dual color, synchronization-free STED stimulated emission depletion (STED) microscopy with a Ti:Sapphire oscillator. The excitation wavelengths were 532nm and 635nm, respectively. With pump power of 4.6 W and sample irradiance of 310 mW, we achieved super-resolution as high as 71 nm. We also imaged 200 nm nanospheres as well as all three cytoskeletal elements (microtubules, intermediate filaments, and actin filaments), clearly demonstrating the super-resolution resolving power over conventional diffraction limited imaging. It also allowed us to discover that, Dylight 650, exhibits improved performance over ATTO647N, a fluorophore frequently used in STED. Furthermore, we applied synchronization-free STED to image fluorescently-labeled intracellular viral RNA granules, which otherwise cannot be differentiated by confocal microscopy. Thanks to the widely available Ti:Sapphire oscillators in multiphoton imaging system, this work suggests easier access to setup super-resolution microscope via the synchronization-free STED A series of biological specimens were imaged with our dual-color STED.

Keywords: Stimulated emission, super-resolution, point spread function, diffraction limit

1. INTRODUCTION

Fluorescent microscopy has become an essential tool to study biological molecules, pathways and events in living cells, tissues and animals. Meanwhile even the most advanced confocal microscopy can only yield optical resolution approaching Abbe diffraction limit of ~200 nm. This is still larger than many subcellular structures, which are too small to be resolved in detail. These limitations have driven the development of super-resolution optical imaging methodologies over the past decade [1].

Multiphoton microscopy had been placed hope on reaching super-resolution with its nonlinear excitation. It has been widely applied in both biological microscopy as well as in clinical imaging-based diagnosis, due to its pinhole-less detection, deeper penetration depth, and dramatically reduced photobleaching. The resolution of MPM is still in the regime of micron level, as the wavelength is generally doubled comparing with that of single-photon microscopy

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counterparts. On the other hand, several novel techniques on breaking the resolution barrier of diffraction limit have been reported in last decade, such as STED [2], SSIM [3], and PALM[4] /STORM [5]. Stimulated Emission Depletion (STED) is the first and optically straightforward method to effectively unlock the diffraction limit for far-field nanoscopy. Although STED is very promising and has attracted many interests in biological super-resolution imaging, the budget of affording such a system, especially the laser, is still a great barrier to its wide instrumentation and application just like MPM [6].

In stimulated emission depletion (STED) microscopy, the excitation focus is overlapped by an intense doughnut-shaped spot to instantly de-excite markers from their fluorescent state to the ground state by stimulated emission. This effectively eliminates the periphery of the Point Spread Function (PSF), resulting in a narrower focal region, or super-resolution. Scanning a sharpened spot through the specimen renders images with sub-diffraction resolution. In this paper we describe the realization of STED nanoscopy with Ti:Sapphire oscillator, a laser system typically used in MPM.

2. THEORETICAL ANALYSIS

2.1 Point spread function

The resolution of conventional microscopy is constrained by the Abbe diffraction limit:

$$d = \frac{\lambda}{2NA} \tag{1}$$

In confocal laser scanning microscopy, the PSF of the system is decided by the multiply of the excitation PSF and the detection PSF:

$$PSF_{confocal} = PSF_{ex} \cdot PSF_{\det} . \tag{2}$$

As both the excitation PSF and the detection PSF are diffraction limited, the PSF of a confocal system is 1.4 times narrower than conventional microscopy, yet still, diffraction limited. Under ideal condition (the pinhole is infinity small), the full-width half maximum (FWHM) of such a system can be described as:

$$d_{Confocal} = \frac{\lambda}{2\sqrt{2}NA} \tag{3}$$

where NA is the numerical aperture of the imaging lens. It equals to the numerical aperture of the objective in the microscope only when the back aperture is overfilled. λ is the square root of the product of the excitation and detection wavelengths. It reduces to the Abbe diffraction limit when the pinhole is widely open.

In multiphoton microscopy, the nonlinear process is restricted to a spatially and temporally confined zone. For a second-order nonlinear effect, the PSF is also the product of the excitation:

$$PSF_{TPM} = PSF_{ex}^{\ 2} . \tag{4}$$

where PSF_{TPM} resembles the PSFs of all the second-order effect such as two-photon excited fluorescence (TPEF) or second-order harmonic generation (SHG) signals. Consequently, the resolution can also be expressed with

$$d_{TPM} = \frac{\lambda_{ex}}{2\sqrt{2}NA} \tag{5}$$

As the signal is already self-modulated to a tight second-order focal area, the confocal pinhole in a single-photon microscope is usually not necessary. The FWHM can be described similar to that of Eq.(3), because the PSF is also a product of two excitation PSFs, with the nonlinear ratio of the fluorescent medium. Note that since here λ is the excitation wavelength, which is now almost two-fold that of single-photon excitation, MPM does not give better resolution than that of its single-photon counterparts.

2.2 Stimulated emission process and resolution scaling

To effectively switch-off the peripheral of the fluorescence PSF for super-resolution, a doughnut shaped PSF with wavelength locating on the tail of the fluorescent emission spectrum is overlapped on the excitation PSF. Such kind of far-field intensity distribution can be generated with a 0-2π vortex phase plate. Since the phase distribution has a constant π phase difference at symmetric position along the axis, it produces a first-order Bessel distribution.

For CW excitation and depletion, the fluorescent depletion ratio can be described by [7]

$$\eta = \frac{1}{1 + I_{mod} / I_{sat}} \tag{6}$$

The FWHM of the STED microscope can therefore be described as [8]

$$d = \frac{\lambda}{2NA\sqrt{1 + I_{mod} / I_{sat}}} . \tag{7}$$

Therefore, the resolution of STED can be increased with the increasing of I_{mod} , and the measurement of I_{sat} can be used to estimate the resolution that is achievable with the certain STED power. It should be noted that, due to the fact that the intensity profile is inhomogenous inside the focal spot (it takes a Gaussian distribution along lateral as well as axial planes), the theoretical achievable resolution versus the power applied takes the following relationship [9]:

$$d \approx \frac{\lambda}{2NA\sqrt{1 + \alpha P_{mod} / P_{sat}}} . \tag{8}$$

where α is a modulation factor, and P_{sat} is the power required (for a specific objective) to deplete the fluorescence to its half.

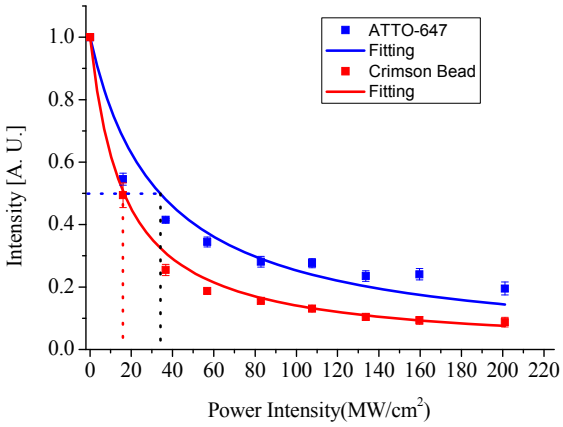

Figure 1 Saturation intensity measurement for both ATTO 647N and crimson beads, measured with Gaussian distribution.

3. METHODS AND MATERIALS

3.1 STED Instrumentation

The STED system was built based on a home-made confocal imaging system with 635 nm excitation. A Ti:Sapphire laser (Griffin-F, KMLabs, USA) pumped by a 6-Watt DPSS 532nm laser (Finesse-6W, Laser Quantum, UK) was employed as the CW STED source. The output from the laser source is ~800mW. To generate the doughnut PSF for

effective depletion, a vortex 0-2π phase plate (VPP-A, RPC Photonics) was used. We focused the beams into samples using an oil-immersion objective of NA=1.4 (100x, PlanAPO, Zeiss). To obtain a 2-D image, a piezo scanning stage (Nanomax, Thorlabs) was employed to move the specimen. A photo-counting avalanche photodiode (SPCM-AQRH-13-FC, Perkin Elmer) was used to collect the fluorescence signal. The scanning and data collection were performed with a DAQ board (USB-6259, National Instruments), and image collection was accomplished with Imspector (Max-Plank Innovation).

3.2 Microtubule Immunofluorescence

Pt2K were plated the day before fixation at 25% confluency and were fixed with 100% methanol for 10 min at -20 °C and additionally permeabilized with 100% acetone for 2 min at -20 °C. Nonspecific antibody binding was blocked with 5% bovine serum albumin (EMD) in PBS for 30 min at 37 °C. Pt2Ks were then incubated with a primary antibody against alpha tubulin (rabbit polyclonal IgG, Abcam ab18251) for 30 min at 37 °C, washed twice in PBS, and incubated with a secondary antibody (goat anti-rabbit ATTO647N IgG, ATTO-tec) for 30 min at 37 °C, washed twice in PBS, and mounted in Mowiol 4-88 (Sigma).

4. RESULTS

As the measurement of I_{sat} can reflect the resulted resolution achievable by an STED system, we have measured the saturation intensity with solutions of ATTO67N and crimson beads. They are measured to be ~17 MW/cm2 for crimson beads at 763 nm, and ~23.8 MW/cm^2 for ATTO 647N at 763 nm. The intensities are measured before the 100x objective. Since the maximum STED power of our system is 200 MW/cm^2, the estimated resolution achievable by crimson bead is ~70 nm, which is four fold that of the wide-field counterparts. For 760nm STED light, the doughnut area can be estimated to be 2.7x10^{-9}cm^2, and the Gaussian shape is only 0.8x10^{-9}cm^2, one third that of the doughnut shape [10].

With 20 nm crimson fluorescent beads (Invitrogen), we measured the resolution of our STED system. 10 beads are averaged, and the resolution is 71nm ± 9nm. The result is shown in Figure 2. The comparison showed clearly that the performance of deconvolution is much better with the STED results than the confocal results.

We have also imaged the microtubules of PtK2 cell, as shown in Figure 3. The fine structures can be distinguished from the STED imaging but not in the confocal image.

5. CONCLUSION

Ti:Sapphire laser has been widely used in multiphoton microscopy. Here, we demonstrate the application of a Ti:Sapphire oscillator with a modest intensity of 300mW, to realize the CW STED super-resolution nanoscopy. With the imaging of 20 nm nanospheres, we achieved resolution of ~70 nm optical nanoscopy. The cellular STED images revealed the fine structures of the cellular skeletons such as microtubules, which cannot be differentiated by confocal microscopy. This work suggests a general access for the synchronization-free STED super-resolution nanoscopy.

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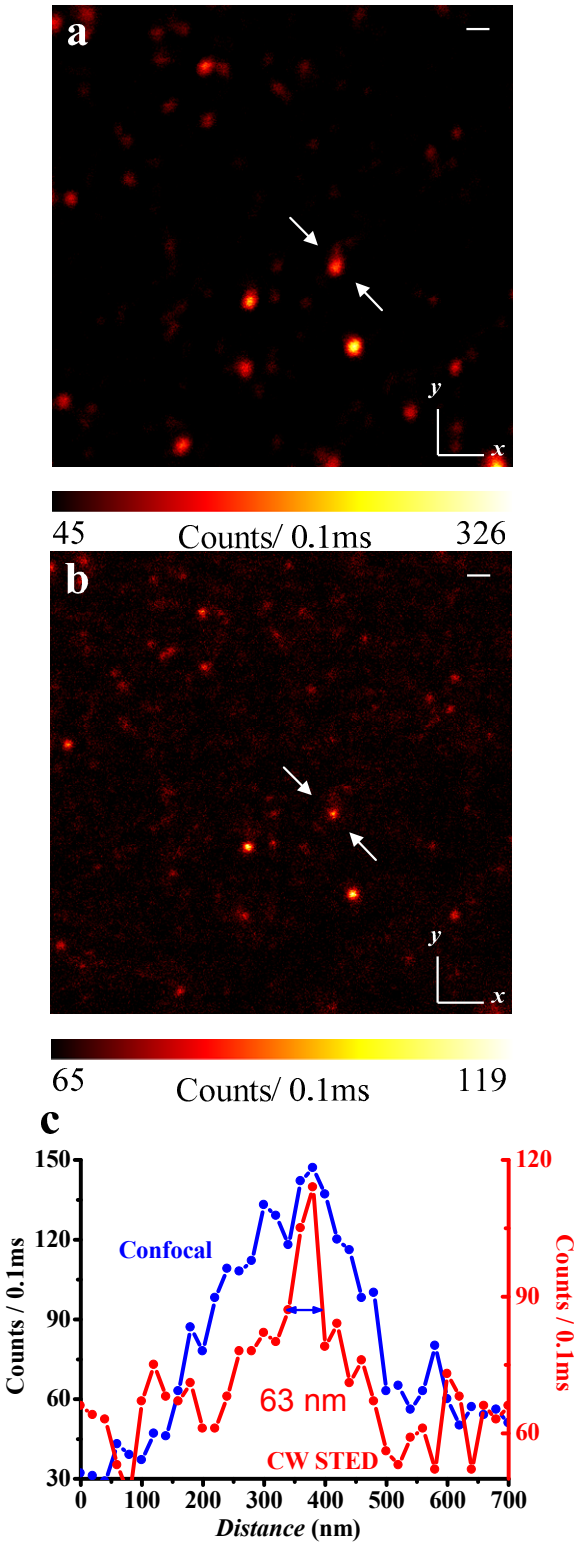

Figure 2 Resolution measured with 20nm nanospheres.

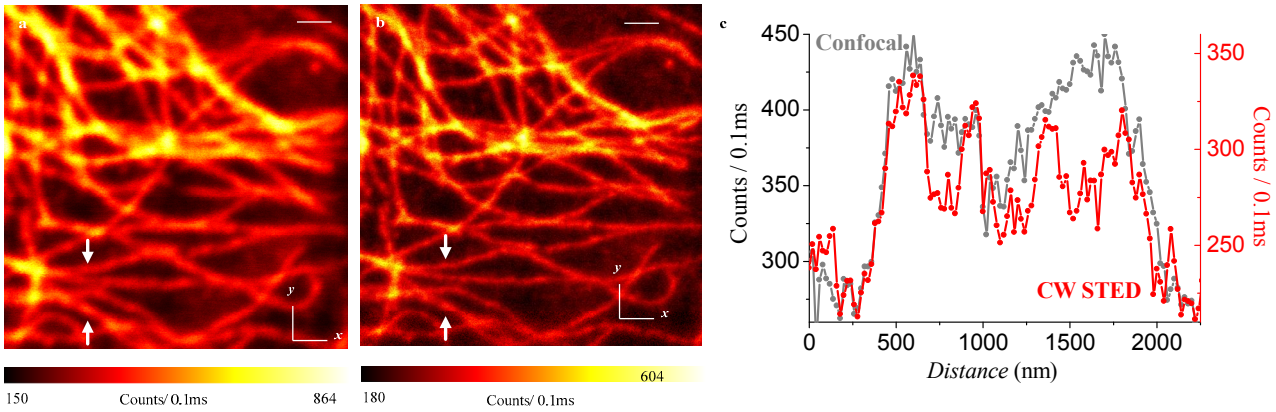

Figure 3 The cellular microtubules imaged with (a) confocal and (b) STED. The intensity profile in the inlet of (a) and (b) are plotted in (c). and the branches can be resolved in the STED results.

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