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STED Super-resolution Microscopy in *Drosophila* Tissue and in Mammalian Cells

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Abstract

Far-field super-resolution microscopy is a rapidly emerging method that is opening up opportunities for biological imaging beyond the optical diffraction limit. We have implemented a Stimulated Emission Depletion (STED) microscope to image single dye, cell, and tissue samples with 50–80 nm resolution. First, we compare the STED performance imaging single molecules of several common dyes and report a novel STED dye. Then we apply STED to image planar cell polarity protein complexes in intact fixed *Drosophila* tissue for the first time. Finally, we present a preliminary study of the centrosomal protein Cep164 in mammalian cells. Our images suggest that Cep164 is arranged in a nine-fold symmetric pattern around the centriole, consistent with findings suggested by cryoelectron tomography. Our work demonstrates that STED microscopy can be used for superresolution imaging in intact tissue and provides ultrastructural information in biological samples as an alternative to immuno-electron microscopy.

Keywords

STED; super-resolution microscopy; single-molecule; *Drosophila*; centrosome

1. INTRODUCTION

Almost all biological fluorescence microscopy in the visible is restricted to the diffraction limit of resolution of ~200 nm, which is too large to resolve many processes in the cell below the organelle level¹. In 2008, Nature Methods awarded the Method of the Year to super-resolution microscopy, a set of imaging approaches that allow the “visualization of cellular structures smaller than can be visualized with conventional microscopy” which is poised to “revolutionize the current understanding of the workings of the cell²”. These super-resolution techniques commonly rely on the ability to modulate the fluorescence of the dye, allowing adjacent emitters more closely spaced than the width of the conventional microscope point-spread function (PSF) to be distinguished. Two major approaches have been identified^{1,3}: (F)PALM/STORM type imaging^{4–6}, which we collectively call Single-

Molecule Active-Control Microscopy (SMACM), a sequential method requiring active control of the fluorescence of dye labels to maintain the emitting concentration at single-molecule levels followed by PSF localization and image reconstruction^{7–8}, and structured illumination⁹ or nonlinear methods including Stimulated Emission Depletion (STED) microscopy¹⁰, which optically engineer the PSF to be smaller than the diffraction limit by utilizing spatial control of the emission by nonlinear processes.

Due to its ability to non-invasively detect specific sub-cellular objects with optical sectioning to reject out-of-focus background, STED has been successfully employed for immunofluorescence cell imaging¹¹, live cell imaging with sectioning of the cell interior¹², and video-rate imaging of cell dynamics¹³. In addition, for STED microscopy, the resolution enhancement arises intrinsically from a sub-diffraction sized PSF. STED images are not reconstructed from images of photoactivated or photoswitched single dye labels; therefore the STED method excludes image artifacts such as under-sampling of dye labels which may be present in SMACM-type methods. In this paper, we have implemented a home-built Stimulated Emission Depletion (STED) microscope to image single dye, cell, and tissue samples with resolution down to ~50 nm. We characterized the STED performance of single molecules of several common dyes and a novel dye (Section 3), imaged planar cell polarity protein complexes at the intercellular junctions of intact *Drosophila* wing as a proof-of-principle (Section 4), and initiated studies of the centriolar protein Cep164 in mammalian cells (Section 5). Our work demonstrates that STED microscopy can be used for ultrastructural imaging in biological cell and tissue samples.

2. STED METHODOLOGY

In a STED microscope, a diffraction-limited excitation focus pumping the S_0 - S_1 transition is overlaid with a red-shifted donut-shaped STED focus designed to deplete the singlet excited S_1 level via stimulated emission at the periphery of the excitation spot¹⁴. The stimulated emission light is not used for imaging, rather the desired signal comes from those emitters who were allowed to emit normally at the dark center of the STED donut. The excitation wavelength is chosen to be near the dye's absorption maximum for most efficient excitation, the STED wavelength is tuned to the red tail of the emission spectrum and carefully filtered out from the detection path, and the fluorescence is collected in between¹⁴. The choice of STED wavelength λ_{STED} is crucial and usually empirically determined on a dye-to-dye basis. Tuning the intense STED beam too blue will result in direct absorption from S_0 to S_1 via weakly absorbing intermediate states corresponding to the far red tail of the absorption spectrum¹⁵. As the resulting signal is true fluorescence from S_1 , this background signal due to the STED beam alone cannot be rejected by filtering. Tuning the STED beam too red results in low depletion efficiency and hence poor resolution since the stimulated depletion cross section is proportional to the dye emission spectrum for small changes in wavelength¹⁶. As the choice of λ_{STED} is a compromise between minimizing undesired absorption and maximizing resolution, a criterion for good STED dye performance is large fluorescence Stokes shift.

Because the residual population of the S_1 fluorescent state, N_1 , is given by $N_1 \propto \exp(-I_{\text{STED}}/I_S)$ where I_{STED} is the STED maximum intensity at the focus and the

saturation intensity I_S is a dye constant defined as the STED intensity at which the fluorescence has dropped to $(1/e)$ of the initial value, the STED resolution r is determined by how well one can saturate the depletion transition¹⁷:

$$\Delta r = \frac{\lambda}{2n \sin \alpha \sqrt{1 + I_{STED}/I_S}}$$

where λ is the wavelength and $n \sin \alpha$ is the numerical aperture for imaging index n . In theory, the limiting factor to resolution is the intensity I_{STED} applied to the dye. However, as STED is a scanning technique, to generate an image, each dye molecule is typically exposed to $\sim 10^6$ excitation-STED pulses or $\sim 10^{18}$ photons of the STED beam with peak I_{STED} of GW cm^{-2} . Hence another requirement of a good STED dye is photostability against the successive cycling by the intense excitation-STED beams. A common bleaching pathway is absorption of the STED beam from S_1 to S_n states and/or possible triplet state generation. Hofkens and coworkers showed that for two similar rylene dyes with similar spectra, their performance as STED dyes crucially depended on their robustness against STED pumping in the S_n manifold¹⁸. A more pervasive photobleaching pathway is via triplet and dark states as these states tend to be long-lived ($\sim$ microsecond lifetimes). Bleaching via these states can be prevented by waiting for relaxation to S_0 before re-excitation as in Triplet-Relaxation STED¹⁹ or by quickly transferring the population to S_0 via reduced and oxidized intermediates with a Reducing and Oxidizing System²⁰.

3. STED FAR RED DYE CHARACTERIZATION

As mentioned in Section 2, in addition to tuning wavelengths and intensities for optimal STED performance, the proper choice of the dye itself is crucial. Using a home-built STED setup (Fig. 1), we chose to characterize far red dyes excitable by $\lambda_{\text{exc}} = 635 \text{ nm}$ since these dyes have been most commonly used in successful STED microscopes and their use avoids cellular autofluorescence. The excitation pulse of $\sim 90 \text{ ps}$ in duration is produced by a pulsed diode laser (PicoQuant), and the subsequent Ti:Sa 200 fs pulse is broadened to $\sim 100 \text{ ps}$ by dispersive glass rods and 100m of polarization-preserving fiber to produce the STED pulse. The pump laser is triggered by the Ti:Sa oscillator.

To compare dye performance for STED, we prepared a series of immobilized single-molecule samples of the following dyes: ATTO 647N, ATTO 655 (both from ATTO-TEC GmbH), and Alexa 647 (Invitrogen Corp.) mounted in Mowiol 4-88 (Polysciences Inc.) and dinaphthoquaterylene diimide DNQDI (a kind gift from Klaus Müllen, Max Planck Institute for Polymer Research) mounted in poly(methyl methacrylate) (PMMA). A small representative region of each sample was repeatedly imaged with the constant experimental parameters $\lambda_{\text{exc}} = 635 \text{ nm}$, $\lambda_{\text{STED}} = 775 \text{ nm}$, fixed pulse lengths, and fixed pixel dwell times. For each sample, we characterized imaging metrics such as the resolution, signal-to-background, and number of scans before bleaching, where resolution is defined as the full-width-at-half-maximum of the image spot in the STED image, background is the averaged image offset, and bleaching is defined as photodestruction of the single molecule to the background level at a given excitation intensity (Table 1).

For the dyes and parameters given, we observe that STED performance strongly depends on the choice of the dye, which is a different trend from a previous comparison of dyes excitable by $\lambda_{\text{exc}} = 570 \text{ nm}$ ²¹. For the given experimental parameters, DNQDI displayed the best resolution of $46 \text{ nm} \pm 16 \text{ nm}$ (Fig. 2) while the bleaching of Alexa 647 was so severe not even one STED image was possible. A simple explanation might be provided by the optical spectra of DNQDI: at the choice of $\lambda_{\text{STED}} = 780 \text{ nm}$, there is a significant increased probability of stimulated emission due to the presence of a local maximum in the emission spectrum (Fig. 2). The presence of vibrational progressions in spectra is typical of such large aromatic dyes due to the rigidity in molecular structure. In addition, DNQDI has a large extinction coefficient of $\epsilon = 143\,000 \text{ M}^{-1} \text{ cm}^{-1}$ and fluorescence quantum yield of 40%, making it a strong absorber and bright dye²². In fact, large aromatic dyes such as xanthene, perylenediimide, and terylenediimide dyes have been successfully used for STED due to their large extinction coefficient, brightness, and photostability^{17–18}.

Further investigations such as those by Hofkens and coworkers¹⁸ would be needed to explain severe bleaching of Alexa 647 under the STED conditions used here. In addition, the ATTO dyes might have exhibited better resolution since our choice of $\lambda_{\text{STED}} = 775 \text{ nm}$ is at the red end of their optimal STED range of $750\text{--}780 \text{ nm}$ ^{13,23} and the laser power available in these experiments was limited. Since we are pumping with pulse trains at the full 80 MHz rate of the Ti:Sa, we anticipate by implementing Trex STED, we would experience decreased photobleaching as well. As shown by the Sauer and coworker studies²⁰, we anticipate the STED performance to depend strongly on the molecular environment and investigations of this effect are currently in progress.

4. PLANAR CELL POLARITY IN *DROSOPHILA* WINGS

During development, epithelial cells in some tissues develop a polarity orthogonal to their apical-basal axis, called planar cell polarity (PCP), an area of intense study in the biological community. Although the cell signaling pathways underlying PCP are still incompletely understood, the asymmetric localization of PCP proteins in the cell membrane is hypothesized to play a significant role²⁴. In collaboration with Dr. Maja Matis of the laboratory of Prof. Jeff Axelrod, we are imaging PCP protein localization patterns at the intercellular junction in *Drosophila* wings.

We have imaged a key PCP protein Flamingo (Fmi) immunostained with an Atto647N dye-labeled antibody (Fig. 3). STED microscopy resolves distinct Fmi protein complexes whereas in the confocal microscopy image, the same substructure is not revealed. Near the intercellular junction (hypothesized spacing of $250\text{--}400 \text{ nm}$ ²⁵), we observe distinct complexes of Flamingo immunostained with Atto647N-labeled antibody. Spots of $\sim 90 \text{ nm}$ FWHM are observed in the cytoplasm, which would be consistent with protein-embedded trafficking vesicles taking into account the STED PSF of $\sim 80 \text{ nm}$ in these experiments (hypothesized vesicle sizes of $<100 \text{ nm}$, data not shown).

These images were obtained near the surface of the wing. Note that STED's optical sectioning ability rejects background from this relatively thick sample, allowing Flamingo protein complexes to be detected at high signal-to-background ratio. Thus we demonstrate

that STED is a useful method to probe sub-cellular organelles below the diffraction limit in whole tissue.

5. ULTRASTRUCTURE OF CENTROSOMAL PROTEINS IN MAMMALIAN CELLS

The centrosome in animal cells is comprised of a pair of barrel-shaped centrioles made of triplets of microtubules and surrounding pericentriolar material. The triplets of microtubules of the centriole are arranged with nine-fold symmetry around the central lumen; this symmetry is a highly conserved feature of centrioles. The centriole duplication cycle results in each centriole pair consisting of an older mother centriole and a newer daughter centriole. The mother centriole is distinguished morphologically from the daughter centriole by the presence of distal and subdistal appendages near the end of the structure, which also have a nine-fold symmetric arrangement around the nine triplets of microtubules²⁶. Based on immuno-electron microscopy, the Cep164 protein was found to be a component of the distal appendages²⁷.

In collaboration with Yin Loon Lee in the laboratory of Prof. Tim Stearns, the images obtained suggest that Cep164 is arranged in a nine-fold symmetric pattern around the mother centriole with average measured cluster diameter of ~130 nm (Fig. 4). Considering the STED resolution of ~60 nm in these experiments, we measure an average peak-to-peak centriole diameter of ~400 nm, which is consistent with the facts that the centriole diameter is ~160 nm and distal appendages are approximately 100 nm long with a 20 nm diameter²⁸. Thus given our STED resolution, the observed ultrastructure of Cep164 is consistent with its localization to distal appendages observed in immuno-electron microscopy images.

As mentioned, STED images are not reconstructed from photoactivated dye labels; therefore STED excludes image artifacts such as under-sampling of dye labels which may be present in SMACM-type methods. Under-sampling artifacts may lead to false measurements especially in the case of cluster analysis of Cep164 structures. At the same time, a disadvantage is that our current resolution may not be as high as some other methods. Obviously, the proper choice of super-resolution method must be made for the system of interest as each method has its strengths and weaknesses. Our result demonstrates that super-resolution microscopy can be used to obtain ultrastructural information about molecular components of the centrosome, as an alternative to the complexity of immuno-electron microscopy and the potential interfering artifacts that can arise from metallic enhancement procedures.

6. CONCLUSION

We have implemented a STED microscope to study single-molecule, cell, and tissue samples with resolution down to 50 nm, taking advantage of STED's optical sectioning and representative dye sampling capabilities to generate superresolution images with high labeling concentration. In our hands, we find that STED performance strongly depends on the choice of the dye, with DNQDI giving the best resolution. More studies are in progress to investigate and optimize other parameters such as the STED wavelength and molecular

environment. We have directly demonstrated that STED is a useful super-resolution technique to probe sub-cellular organelles in intact tissue. Lastly, we have obtained ultrastructural information about the molecular components of the centrosome, demonstrating that STED can be used as an alternative to immuno-electron microscopy.

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Figure 1.

Experimental STED setup. APD, avalanche photo diode; cam, camera; DC, dichroic mirror; DGR, dispersive glass rods; HWP, half-wave plate; PH, pinhole; PPF, polarization-preserving fiber; Pol, polarizer; QWP, quarter-wave plate; SF, spectral filter; PD, pulsed diode laser; Ti:Sa, Ti:sapphire modelocked oscillator.

Figure 2.

(left) structure and spectra of DNQDI in toluene (figure taken from reference 22). (middle) Confocal (upper) and STED images (lower) of single DNQDI in PMMA. Exc: 635 nm @ $P_{\text{focus}} = 2\text{uW}$, $I_{\text{focus}} = 4\text{kWcm}^{-2}$ avg = 0.5MWcm^{-2} peak, STED: 775 nm @ $P_{\text{focus}} = 35\text{mW}$, $I_{\text{focus}} \sim 50\text{MWcm}^{-2}$ avg = 6GWcm^{-2} peak. (right) Representative Gaussian fits of the intensity line profiles of single molecules of DNQDI showing STED enhancement of resolution compared to confocal. STED FWHM is $46\text{ nm} \pm 16\text{ nm}$ while the confocal FWHM is $274\text{ nm} \pm 37\text{ nm}$.

Figure 3.
(left) confocal and (right) STED images of Atto647N-IgG immunostained Flamingo in *Drosophila* wing with 60 nm Gaussian deconvolution to filter noise. $P_{\text{excitation}} = 15 \mu\text{W}$, $\lambda_{\text{STED}} = 765 \text{ nm}$, $P_{\text{STED}} = 45 \text{ mW}$, $50 \mu\text{s}/32 \text{ nm pixel}$. Note the intrinsic resolution enhancement in the STED image.

Figure 4. (left) confocal and (right) STED images of Atto647N-IgG immunostained Cep164 in fixed IMCD3 mouse cells. $P_{\text{excitation}} = 10 \mu\text{W}$, $\lambda_{\text{STED}} = 759 \text{ nm}$, $P_{\text{STED}} = 80 \text{ mW}$, $0.5 \text{ ms}/60 \text{ nm pixel}$ (25 nm pixel size for STED).

Table 1

Comparison of STED imaging performance for single molecules of far red dyes immobilized in polymers. For typical intensities used, see Fig. 2 caption.

Dye	Resolution	Scans before bleach: STED (Confocal)	Signal: background
DNQDI	~50 nm	1 (2)	6:1
ATTO 655	~70 nm	1 (1)	7:1
ATTO 647N	~80 nm	2 (2)	8:1
Alexa 647	X severe bleaching prevents STED image		