Tuning donut profile for spatial resolution in stimulated emission depletion microscopy

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In stimulated emission depletion (STED)-based or up-conversion depletion-based super-resolution optical microscopy, the donut-shaped depletion beam profile is of critical importance to its resolution. In this study, we investigate the transformation of the donut-shaped depletion beam focused by a high numerical aperture (NA) microscope objective, and model STED point spread function (PSF) as a function of donut beam profile. We show experimentally that the intensity profile of the dark kernel of the donut can be approximated as a parabolic function, whose slope is determined by the donut beam size before the objective back aperture, or the effective NA. Based on this, we derive the mathematical expression for continuous wave (CW) STED PSF as a function of focal plane donut and excitation beam profiles, as well as dye properties. We find that the effective NA and the residual intensity at the center are critical factors for STED imaging quality and the resolution. The effective NA is critical for STED resolution in that it not only determines the donut shape but also the area the depletion laser power is dispersed. An improperly expanded depletion beam will have negligible improvement in resolution. The polarization of the depletion beam also plays an important role as it affects the residual intensity in the center of the donut. Finally, we construct a CW STED microscope operating at 488 nm excitation and 592 nm depletion with a resolution of 70 nm. Our study provides detailed insight to the property of donut beam, and parameters that are important for the optimal performance of STED microscopes. This paper will provide a useful guide for the construction and future development of STED microscopes. © 2013 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4799665>]

I. INTRODUCTION

Since the first proposal in 1994¹ and subsequent experimental demonstrations in very recent years,² stimulated emission depletion (STED) microscopy has proven to be a powerful optical imaging technique that offers sub-diffraction limited resolution.^{3–5} Compared to other high-resolution microscopies such as electron microscopy, STED microscopy offers the ability of observing samples *in situ* and in real time. This provides a brand new opportunity for studying highly dynamic events where such requirements are of critical importance. For example, STED fluorescence correlation spectroscopy (FCS) has been applied to study molecular adsorption/diffusion on cell membranes;⁶ the self-assembling of nanoparticles at controlled solvent evaporation condition has been studied with STED imaging;⁷ the nanoscale morphology of block copolymers including swelling induced mesopores and other convoluted structures was imaged at a scale that usually requires electron or scanning probe microscopic resolution,⁸ etc.

Depending on the laser sources, STED microscopes can be operated in pulsed mode² or in continuous wave (CW) mode.^{9–11} Multi-photon excitation (MPE) can also be incorporated.^{12–14} More recently, STED is combined with total internal reflection fluorescence (TIRF) microscopy to improve the axial resolution.¹⁵ The reported resolution of STED microscopy is in the range of 20–150 nm in most of

the cases,^{2,9,10,13,16,17} with a best case of 5.8 nm for a special luminescent color center in diamond crystals.¹⁸ So far, STED microscopy is still in the development phase and several groups are actively involved in improving its various aspects.^{19–23}

It is well known in optical imaging community that STED microscopy resolution is determined by a few important factors including dye properties, the depletion beam power, the donut profile, etc. Most of the current STED microscopes adopt a confocal design: the images are acquired by raster scanning using a point detector. A donut-shaped depletion laser beam having a wavelength within the fluorescence emission spectrum of the probe molecule is superimposed on the tightly focused excitation beam, bringing the excited probe molecules at the outer rim of the excitation spot back to the ground state through the stimulated emission process. The fluorescent emission spot size is effectively reduced, leading to sub-diffraction limited resolution in the focal plane. The donut-shaped beam is also used in up-conversion depletion type of super-resolution optical microscopy. In this approach, the wavelength of donut-shaped laser beam is chosen to be sufficiently far from the fluorescence emission band so that the probe molecules at the outer rim of the excitation spot are turned off mainly through the stimulated absorption process followed by non-radiative relaxation.^{11,24} Up-conversion depletion microscopy provides resolution similar to that of STED microscopy.

Apparently, the donut beam profile is of critical importance to STED and up-conversion depletion microscopy. The

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most common method to generate a donut-shaped beam is to pass a Gaussian beam through a circular π -phase plate (π PP)^{2,25} or a 0- 2π vortex phase plate (VPP).²⁶ The π PP consists of two concentric regions. The size of these regions is designed in a way that the two beam components transmitted through possess a relative phase of π and interfere destructively at the focal plane. The intensity profile of a π PP-generated donut in the focal plane after passing through a low NA lens has a ρ^4 -dependence, where ρ is the distance to the donut beam center. However, recent theoretical study shows that the focal plane intensity profile becomes tighter and surprisingly approaches a ρ^2 relationship when the beam is focused by a high NA objective, which is more suitable for super-resolution optical microscopy.²⁷

As a comparison, the VPP is designed in a way that electric field of the transmitted beam around the optical axis changes continuously from 0 to 2π . The electric field cancels out completely on the axis, yielding a donut-shaped beam. VPPs are convenient to use but difficult to manufacture in the early days. With the development of photolithography, they are becoming more popular in generating donut-like beams. When donut beam formed this way is focused by a low NA lens, the beam profile can be characterized as a first-order Laguerre-Gaussian (LG) beam, with a parabolic center.²⁸ However, the transformation of a donut beam by a high NA objective can be complicated and subject to other factors such as the polarization of the incident beam.^{29,30} Donut images and/or their intensity profiles focused by a high NA objective have been published in the literature.^{9,11,20,30} Discrepancies can be identified in terms of residual intensity in the center and their intensity profile: some resemble a parabola¹¹ while others more like a ρ^4 function.²⁰ To the best of our knowledge, there is no conclusion what the donut beam profile that works for STED microscopy should be like. We deem how donut beam transforms after passing through a high NA objective is an important question as it establishes the basis for further discussion of STED point spread function (PSF) and resolution.

In addition, it is well recognized that complete dark in the donut center is important for STED imaging. Under ideal situation when the donut center has a zero-intensity, the resolution scales with the inversed square root of the donut beam intensity. However, it can be envisioned that practically, the zero-intensity at the donut center cannot be achieved for many reasons: mismatch between phase plate and laser wavelengths, imperfectly aligned laser beam, laser beam defects, optical aberrations, improperly polarized laser beam, etc. It is unclear to what extent the resolution is affected by the presence of a residual intensity in the donut center.

In this study, we constructed a 488 nm excitation/592 nm depletion CW STED microscope. The donut beam profile and transformation after being focused by a high NA objective (NA 1.4) were investigated. Considering CW illumination and depletion, we derived the mathematical expression for STED PSF as a function of focal plane donut and excitation beam profiles, as well as dye properties. We systematically investigated the effect of these parameters on the STED PSF. Such information is important in guiding researchers in constructing and further developing STED microscopes. Finally, we

demonstrated that our STED microscope has a resolution of 70 nm, which is approaching the limit under practical conditions. The resolving power of our STED microscope was demonstrated by imaging closely spaced 45 nm and 200 nm fluorescent nanoparticles.

II. EXPERIMENTAL SETUP

A. Materials

Poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,10,3)-thiadiazole)] (PFBT; MW, 157 000 Da; polydispersity, 3.0) was purchased from American Dye Source, Inc. (Quebec, Canada). Polystyrene-grafted ethylene oxide functionalized with carboxyl groups (PS-PEG-COOH; MW 21 700 Da of PS moiety; 1200 Da of PEG-COOH; polydispersity, 1.25) were purchased from Polymer Source, Inc. (Quebec, Canada). Green fluorescent, carboxylated polystyrene nanoparticles (45 nm and 200 nm) doped with FITC were purchased from Invitrogen. Their actual sizes are 40 ± 5 and 190 ± 5 nm, respectively, measured on a JEOL JSM-6400F field emission scanning electron microscope. Gold nanorods with a size of 25×51 nm were purchased from Nanopartz (Nanopartz, Inc., CO).

The preparation and photophysical properties of the 18-nm, fluorescent nanoparticles (Pdots) have been described previously.³¹ Briefly, a tetrahydrofuran (THF) solution containing 50 $\mu\text{g/ml}$ of PFBT and 16 $\mu\text{g/ml}$ of PS-PEG-COOH was prepared. A 5-ml aliquot of the mixture was quickly injected into 10 ml of water under vigorous sonication. THF was removed by blowing nitrogen gas into the solution at 90 °C. The THF-free Pdot solution was sonicated for 1–2 min and filtrated through a 0.2- μm cellulose membrane filter. The size of the as-prepared Pdots was measured to be around 18 nm with a dynamic light scattering instrument (Malvern Zetasizer Nano ZS, Worcestershire, United Kingdom).

B. STED microscope setup

A schematic of our homebuilt CW-STED microscope is shown in Fig. 1. The excitation was provided by a 488 nm laser line from an air-cooled Ar ion laser (35-LAP-431-240, CVI/Melles Griot). The excitation beam was circularly polarized by a quarter-wave plate (QWP) (CVI/Melles Griot, ACWP-400-700-06-4) and collimated to overfill the back aperture of a microscope objective (Nikon, Plan Apo, 100 $\times$ /1.40–0.7, Oil). The depletion laser line was provided by a fiber laser (592 nm, 1.0 W, MPB communication, VFL-P-1000-592-OEM1). The depletion beam was collimated to a proper size and passed through a 0- 2π vortex phase plate (RPC photonics, VPP1a) to generate a donut-profiled beam. The polarization of the depletion beam was then cleaned with a Glan-type polarizer (Thorlabs) and circularly polarized by another QWP. The excitation and depletion beams were guided to the microscope objective back aperture by the combination of a 505 nm long-pass and a 570 nm short-pass dichroic mirrors. The fluorescence signal was collected and filtered by a 594 nm notch filter (Semrock, NF03-594-E) and a 535 ± 25 nm band-pass filter. The collected signals

FIG. 1. Schematic of the construction of the CW STED microscope. The excitation was provided by an Ar ion laser (488 nm) and the depletion was from a 592 nm fiber laser. BPF: bandpass filter; VPP: vortex phase plate; L: lenses; P: polarizer; QWP: quarterwave plate; DCSP: dichroic mirror (short pass); NF: notch filter; DCLP: dichroic mirror (long pass); PH: pinhole; APD: avalanche photodiode detector.

were imaged into a piece of multimode fiber optics (Thorlabs) and detected by an avalanche photodiode (Perkin Elmer, SPCM-AQRH-15-FC). The diameter of the fiber optics was $50\ \mu\text{m}$ (~ 0.8 AU). A programmable counting board was used for photon counting. Sample scanning in XYZ directions was achieved by a piezo-stage (PI Nano, Physik Instrumente, P-545) mounted on a manual XY translational stage. The precision and scanning range of the piezo-stage was $1\ \text{nm}$ and $200\ \mu\text{m}$, respectively, in all three directions. The integration time was $1.0\ \text{ms}$ unless specified. The acquired data were converted to images using a home-written NIH ImageJ program. MATLAB and NIH ImageJ were used to analyze the collected images.

C. Measurements of donut profiles and STED imaging

For donut profile measurement, VPP was inserted in to the optical path of the expanded laser beam. The laser beam was then cleaned with a Glan-type polarizer and then circularly polarized by a QWP. The donut of different sizes (in focal plane) was obtained by changing the laser beam diameter at the objective back aperture using a home-built Keplerian type telescope. For the 488 nm laser line, the donut profile was obtained by measuring the fluorescence of $45\ \text{nm}$ fluorescent nanoparticles or the scattering/luminescence of $25 \times 51\ \text{nm}$ gold nanorods. The fluorescence signal or the scattering/luminescence was collected between 510 and $560\ \text{nm}$. For the $592\ \text{nm}$ laser line, the donut profile was obtained by measuring the anti-Stokes Raman scattering signal from $18\ \text{nm}$ conjugated polymer nanoparticles immobilized on the coverslip ($22 \times 22\ \text{mm}$, No. 1.5, Corning, NY). The signal was also collected between 510 and $560\ \text{nm}$.

For STED image collecting, $45\ \text{nm}$ and $200\ \text{nm}$ yellow-green fluorescent particles were diluted to proper concentrations with $18.2\text{-M}\Omega$ Milli-Q water and immobilized/pre-assembled on cover glass surface in the presence of relevant chemical reagents. The excitation and depletion laser powers

at the objective back aperture were $\sim 15\ \mu\text{W}$ and $\sim 800\ \text{mW}$, respectively, unless specified.

III. RESULTS AND DISCUSSION

A. Focusing the donut beam: Donut size and intensity profile in the center

The resolution of STED microscopy is determined by the intensity slope in the center of the donut, i.e., how fast the depletion beam intensity increases from virtually zero in the center to a sufficiently high intensity that effectively depletes the excited fluorophores within a range of a few nanometers. Intuitively, the steepness of the intensity slope in the donut center is determined by how tight the donut beam is focused.

To find out how to obtain the steepest intensity slope in the donut center, we tested different filling factors of the objective back aperture by expanding the donut beam to different sizes. We found that the transformation of the donut size, defined as the distance from the maximum to the maximum across the center of the donut, follows an inversely linear relationship with respect to the incident beam size, similar to the transformation of a Gaussian beam.³² Figure 2 shows two donut images and their normalized line profiles across the center when the donut beam size was adjusted to $\sim 6.0\ \text{mm}$ and $\sim 3.0\ \text{mm}$, respectively, using the $488\ \text{nm}$ excitation of fixed $45\ \text{nm}$ green fluorescent particles. Correspondingly, the donut size was $\sim 350\ \text{nm}$ and $\sim 700\ \text{nm}$, respectively. The inversely linear relationship can be clearly identified.

Importantly, the center profile of the donut can be satisfactorily fitted with a parabolic function: $h = ar^2 + c$, where h is the intensity (with a unit of power/area); r is the distance from the center of the donut; a and c are the quadratic coefficient and the constant of the parabolic equation, respectively. Especially, as the size of the donut increases by a factor of 2, the quadratic coefficient a decreases from $7.5 \times 10^{-5}\ \text{nm}^{-2}$ to $1.8 \times 10^{-5}\ \text{nm}^{-2}$ for the normalized donut profile, a factor of ~ 4 . The donut beam transformation and intensity profile indicate that the electric field amplitude increases linearly as a function of the distance from the center of the donut, and the intensity slope scales with the inverse of the donut size squared.

Clearly, the large donut is not favorable for STED microscopy although the donut is equally dark in the center. Thus, in order to achieve the highest resolution in STED microscopy with minimal depletion laser power, we need to focus the donut as tight as possible by slightly overfilling the back aperture of the objective.

B. Transformation of the donut beam

The transformation of the donut beam after a high NA objective and the parabolic intensity profile in the center are consistent with Khonina's theoretical prediction, which assumed a coherent plane wave passing through a 2π vortex phase plate and being focused by a lens.³³ The electric field amplitude of the donut is described by

$$E(\rho, \varphi) = \frac{k}{f} e^{i\varphi} \int_0^R J_1\left(\frac{k}{f} r \rho\right) r dr, \quad (1)$$

FIG. 2. Donut profile as a function of the depletion beam size. The depletion beam was first passed through the vortex phase plate to form a donut-profiled beam. The donut beam was then collimated to have a beam diameter of (a) ~ 6 mm and (b) ~ 3 mm.

where ϕ is the phase; k is the wavenumber $2\pi/\lambda$; f is the focal length of the lens; R is the effective radius of the vortex filter aperture or the radius of the focusing lens aperture, whichever is smaller; J_n is the n th order Bessel function of the first type; r is the distance from the center of the beam in the object plane. The integration $\int_0^t J_1(t')t'dt'$ can be approximated using Struve function:

$$\int_0^t J_1(t')t'dt' = \frac{\pi}{2}t[J_1(t)H_0(t) - J_0(t)H_1(t)], \quad (2)$$

where $H_n(t)$ is the Struve function:

$$H_0(t) = \frac{2}{\pi} \left(t - \frac{t^3}{8} + \frac{t^5}{225} - \dots \right), \quad (3)$$

$$H_1(t) = \frac{2}{\pi} \left(\frac{t^2}{3} - \frac{t^4}{45} + \frac{t^6}{1575} - \dots \right). \quad (4)$$

Thus, the donut intensity profile can be written as

$$\begin{aligned} h(\rho) &= \left(\frac{\pi k R^2}{2 f} \right)^2 \frac{1}{x^2} [J_1(x)H_0(x) - J_0(x)H_1(x)]^2 \\ &= C \frac{1}{x^2} [J_1(x)H_0(x) - J_0(x)H_1(x)]^2, \end{aligned} \quad (5)$$

where

$$C = \left(\frac{\pi k R^2}{2 f} \right)^2 \quad (6)$$

and

$$x = \frac{k R \rho}{f} = 2\pi \frac{NA}{\lambda} \rho. \quad (7)$$

NA is the effective numerical aperture and is approximately R/f .

Numerical calculation shows that under the plane wave approximation, the donut profile described by Eq. (5) will have the maximum intensity at the position $x = 2.45$, where

the maximum donut intensity h_0 can be calculated as

$$h_0 = 0.02585C. \quad (8)$$

At this position, the half of the donut size ρ_0 can be derived from $x = 2.45$

$$\rho_0 = 0.39 \frac{\lambda f}{R} = 0.39 \frac{\lambda}{NA}. \quad (9)$$

Equation (9) shows the smallest donut we can obtain using a high NA objective is $2\rho_0$, or $\sim 0.78 \lambda/NA$. This is consistent with our experimental observations. Figure S1 in the supplementary material shows an example of the tightest donut image obtained using the 592 nm laser beam illumination on 18 nm conjugated polymer nanoparticles.³⁴ The 18 nm conjugated polymer nanoparticles gave excellent anti-Stokes Raman signals at ~ 540 nm when illuminated by the 592 nm depletion laser beam. The donut beam was formed by passing the laser beam through the vortex filter specific for 592 nm and then expanded to overfill the objective back aperture. The objective NA was 1.4 and the obtained donut image has a size of ~ 340 nm, which is consistent with the Eq. (9) prediction.

Especially, Eq. (5) predicts a parabolic intensity profile in a region close to the center of the donut, which is also consistent with the experimentally obtained donut profiles. When ρ is small, the Bessel function can be approximated using the small argument limit that t approaching zero³⁵

$$J_0(t) \approx 1 - \frac{t^2}{4}, \quad (10)$$

$$J_1(t) \approx \frac{t}{2}. \quad (11)$$

Under this approximation, the donut intensity profile close to the center can be written as

$$h(x) = C \left(\frac{2}{\pi} \right)^2 \frac{1}{36} x^2 = ax^2. \quad (12)$$

Putting Eq. (8) into Eq. (12), we can obtain the quadratic coefficient a ,

$$a = 0.4355h_0. \quad (13)$$

Thus, the intensity of the donut near the center can be simplified as a parabolic function of x or ρ :

$$h = 0.4355h_0x^2 = (0.4355h_0)(kNA)^2\rho^2. \quad (14)$$

Again, Eq. (14) is consistent with the experimental result, which shows the normalized 592 nm donut has a slope of $9.0 \times 10^{-5} \text{ nm}^{-2}$ when the donut beam is focused to the tightest.

Our result shows that the donut beam intensity profile in the center is parabolic after being focused by a high NA objective. Such information is important for further discussions about STED PSF and resolution.

C. Theoretical resolution

In the literature, the STED beam has been approximated as a standing wave,^{36,37} thus the intensity profile in the focal plane is³⁶

$$h(\rho) \propto \sin^2\left(\frac{1}{2}kNA\rho\right). \quad (15)$$

The resolution of STED microscopes δ , defined as the FWHM of the STED PSF, was estimated by Westphal and Hell,³⁶

$$\delta = \frac{\sqrt{8}}{\pi\sqrt{1+h_0/h_{Sat}}} \frac{\lambda}{2n\sin\alpha} \approx 0.45 \frac{\lambda}{NA} \frac{1}{\sqrt{h_0/h_{Sat}}}, \quad (16)$$

where the saturation intensity h_{Sat} is the power of the STED beam (power/area) that depletes the fluorescence intensity to half; $n\sin\alpha$ is approximately the numerical aperture NA. The h_{Sat} is an intrinsic parameter of the dye. Equation (16) shows a square root relationship of the STED resolution with respect to the maximum power of the donut beam h_0 , which is confirmed by experimental results.³⁶

The same group in a later paper obtained a similar equation by assuming that the electric field amplitude in the center of the donut to be linearly proportional to the radial position³⁸

$$\delta \approx \frac{1}{b\sqrt{h_0/h_{Sat}}}, \quad (17)$$

where b is the slope of the electrical field amplitude as a function of the distance from the center of the donut. Equation (17) shows a similar square root relation of the resolution and the STED beam power. However, the field amplitude slope b is not easy to measure practically, and the estimation of the resolution is not straightforward according to Eq. (17).

Here, we further derived the expression of the resolution using Eq. (14) under the small argument approximation of the Bessel function since the STED resolution is only related to the center portion of the donut intensity profile (several 10 s of nanometers). According to the definition, the FWHM resolution describes that at a distance r from the center of the donut, the STED beam intensity increases to h_{Sat} that it depletes the fluorescence intensity by half. When the excitation beam intensity is weak, the resolution is independent of the

excitation laser power (the weak excitation approximation is discussed in the supplementary material).³⁴ From Eq. (14), the resolution is

$$\delta = 2\rho = \frac{2\sqrt{h_{Sat}}}{\sqrt{0.4355h_0}} \frac{\lambda}{2\pi NA} = 0.48 \frac{\lambda}{NA} \frac{1}{\sqrt{h_0/h_{Sat}}}. \quad (18)$$

The result is similar to Eq. (16), with a slight difference in the coefficients. This shows that both $\sin^2$ function and parabolic function are good approximations for the donut intensity profile in the center.

D. Donut size and STED resolution

The donut profile transformation effectively bridges STED resolution with the effective NA in the image collection. Superficially, Eq. (18) shows that the resolution is proportional to the inverse of NA and we know that the best resolution can only be obtained when the STED beam is focused to the tightest.

Moreover, it is important to note that the effective NA has a much more profound effect on STED resolution as indicated in Eq. (18)! This is because the maximum donut intensity h_0 is also subjective to the effective NA. Assume the total power of the STED beam is I_{Total} (with a unit of power), the maximum donut intensity h_0 (with a unit of power/area) can be approximated as $h_0 \propto \sqrt{I_{Total}/\rho_0^2} \propto NA\sqrt{I_{Total}}$ from Eq. (9). Thus, the STED resolution is

$$\delta \propto \frac{\lambda}{NA^2} \frac{1}{\sqrt{I_{Total}}} \quad (19)$$

when the total depletion laser power is fixed while the donut size is variable.

Equation (19) shows that the effective NA in STED microscopy, or as a result the size of the donut in the focal plane is *not* trivial. For example, if the STED beam is not *fully* expanded but only filled half of the objective, the effective NA decreases by a factor of 2, and the observed resolution will deteriorate by a factor of 4! This will almost fail any attempt to improve the spatial resolution in STED microscopy even the donut center is tuned completely dark.

E. Residual intensity in the center of the donut

Above discussions are all based on ideal situations that the donut has a zero-intensity in the center as predicted by Eq. (5). However, in the experiments, we noticed that there is always a residual intensity in the center of the donut. The residual intensity may come from various factors including the sample size used in the donut profile measurement, wavelength mismatch between the vortex phase plate and laser line, imperfect alignment of the laser beam, laser beam quality, etc. It is noteworthy that the polarization of the laser beam also plays an important role on the donut formation. For example, Torok and Munro²⁹ showed in simulation that circularly polarized light gives a symmetrical depletion volume and a perfect zero at the center of the donut using a NA 0.9 microscope objective. Using a NA 1.2 objective, Bokor *et al.*³⁹ showed that among the left-handed circularly, linearly, and right-handed circularly polarized laser beams, the right-handed circularly polarized light gives the ideal donut

FIG. 3. Donut profile as a function of the depletion beam polarization. (a) Right-handed circular as defined from the point of view of the receiver; (b) linear; and (c) left-handed circular. The vortex phase plate generates a right-handed spiral phase delay viewed from the receiver.

for STED microscopy for a vortex phase plate that has the same orientation of the phase delays. As a comparison, left-handed circularly polarized light gives the poorest donut profile whose center is $\sim 80\%$ filled. Linearly polarized light has an elongated center portion that is $\sim 50\%$ filled. Similar simulation was also shown by Hao *et al.*⁴⁰ and experimentally collected data were shown to support these simulations.^{37,39}

Here, we show high quality, experimentally collected donut images as a function of the depletion beam polarization using a NA 1.4 objective. Consistent results were observed for the right-handed circularly laser beam (as defined from the point of view of the receiver), which has a center intensity $\sim 5\%$ of the maximum value, and linearly polarized beam ($\sim 50\%$ filled in the center) (Fig. 3). However, contrary to the reported values, the left-handed polarized light gave a center-to-maximum ratio of $\sim 30\%$, much lower than the earlier reports of $\sim 80\%$.^{39,40} For the elliptically polarized lights between left-hand/linearly and linear/right-hand polarized light, the center-to-maximum ratio are both $\sim 20\%$. A donut with center portion 80% filled was never observed unless the whole laser system is misaligned.

What causes the discrepancy is still under investigation. It should be noted that our experimental setup was different from those used in the simulations.^{39,40} In the simulations, the phase plate was assumed to be placed at the back focal plane of the microscope objective. In our experimental setup, the laser beam was first collimated to a proper size and then passed through the vortex phase plate to form the donut-profiled beam. The donut beam was cleaned by a linear polarizer and polarized by a QWP before being sent into the microscope. By rotating the QWP, we can obtain left-handed, right-handed, elliptically, and linearly polarized light.

F. Practical donut profiles and STED resolution

The residual intensity at the center of the donut makes the prediction of STED resolution much more complicated. To test how the residual intensity is affecting the STED image brightness and resolution, we performed a series of numerical simulations with an imperfect donut that the center

is filled with different fractions of the maximum intensity. The methods of simulation are discussed in detail in the supplementary material.³⁴ Continuous wave STED microscopy was assumed. In the simulation, a $15\text{-}\mu\text{W}$, 488 nm laser focused to the diffraction limit was used as excitation and a 592 nm laser up to 1.0 W was used as depletion. The donut profile was obtained experimentally and smoothed and further treated by adding or subtracting a diffraction limited, Gaussian-distributed intensity profile to vary the filling level in the center (Fig. 4(a)). The size of the donut was 340 nm , which is the smallest donut achievable for the 592 nm laser. The dye was assumed to be FITC and the spectroscopic parameters were found in the literature (see Table 1 in the supplementary material).³⁴

Figure 4(b) shows the simulated STED PSF at different depletion laser power. The PSFs show typical Lorentzian profiles and the FWHM decreases as the depletion laser power increases. A square root relationship between the FWHM resolution and depletion laser power can be obtained.

When the donut profile is not ideal, the first thing we will notice is that the STED image intensity drops quickly as the center intensity increases. Figure 4(c) shows the simulated brightness of the STED image as compared to the confocal image when the donut center is filled to different levels. The total depletion laser power was assumed to be 1.0 W . With a donut center filled to 5% of the maximum intensity, which is similar to our experimentally collected donut, the STED image drops to $\sim 50\%$ of the confocal image. This is in line with our experimental results that our STED image is $\sim$ half of the confocal image in the absence of the depletion beam. As the donut center is filled to 50% , the STED image would drop to 10% . This drop in intensity makes collecting high quality images virtually impossible.

More importantly, the resolution of STED microscopy is drastically affected by a partly filled donut in the center. Figure 4(d) shows how STED microscopy resolution changes with laser power for the donut filled to different levels in the center. Under ideal conditions, the limit of the resolution is $\sim 40\text{ nm}$ for FITC dyes with a depletion laser power up to 1.0 W . Especially, when the donut center is filled to 5% , the

FIG. 4. The effect of the center profile of the donut on STED images. (a) Assumed donut profile with the center portion filled to different levels of the maximum intensity; (b) simulated STED resolution at different filled levels; (c) simulated maximum STED image intensity at corresponding filled levels; and (d) resolution-depletion power relationship at different filled levels. The 340 nm donut was used in the simulation. The excitation and depletion laser powers were assumed to be $15 \mu\text{W}$ and 1.0 W , respectively.

predicted resolution is $\sim 60 \text{ nm}$, which is consistent with our experimental experience that will be discussed in Sec. III H. As the center portion is more filled, the STED resolution deteriorates quickly. For a donut center filled to 50% level, the resolution limit is $\sim 160 \text{ nm}$, barely any noticeable improvement even for very high depletion laser power.

G. Other factors affecting STED resolution and implications

Using the same simulation program, we explored other factors that may affect the STED microscopy resolution. Figure 5(a) shows that the dye fluorescence lifetime is also important to the resolution. The improvement of resolution drops quickly as the dye fluorescence lifetime decreases to below 4 ns .

In Fig. 5(b), we explored how excitation beam width is affecting the STED resolution. The result shows that the res-

FIG. 5. Other factors affecting STED resolution. Simulated spatial resolution by varying (a) dye fluorescence lifetime; and (b) excitation beam width. The 340 nm donut was used in the simulation. The excitation and depletion laser powers were assumed to be $15 \mu\text{W}$ and 1 W , respectively.

olution is essentially limited by the donut size rather than the excitation beam size. This is consistent with our observation that defocusing the excitation beam slightly is not changing the STED microscopy resolution significantly. However, when the excitation laser beam becomes larger than the donut beam size, the STED PSF line profile shows tails at the outer regions. This will result in higher background for sub-diffraction limited images.

H. Experimental resolution of a continuous wave STED microscope

Finally, with above understanding, we constructed a sample scanning, CW-STED microscope using 488 nm excitation and 592 nm depletion. Figures 6(a) and 6(b) show the confocal and STED images of the same area of 45 nm fluorescent polystyrene nanoparticles adsorbed on glass surface, respectively. 150 mM NaCl solution was added to assist the adsorption of particles on glass surface. STED microscopy resolved a lot of features that were impossible to resolve using conventional epi- or confocal fluorescence microscopy. Figure 6(b) shows that smeared images in Fig. 6(a) are clearly seen as individual particles. The line profiles of one randomly selected particle for both confocal and STED are shown in Fig. 6(c). Since STED microscopy gives a Lorentzian shaped PSF, Lorentzian fitting was applied to the intensity profiles of all individual nanoparticles in Fig. 6(b). The average width was $80 \pm 10 \text{ nm}$ (for an example, see Fig. 6(c)) for all selected individual particles in Fig. 6(b). Considering the convolution of the laser beam with the 45 nm nanoparticles, the effective FWHM width of the STED PSF, or the spatial resolution of

FIG. 6. Confocal and STED point spread function. (a) and (b) Confocal and STED image of 45 nm nanoparticles immobilized on glass surface, respectively; (c) intensity profiles across the images and their Lorentzian fitting of a particle in (a) and (b).

our CW-STED microscope, is ~ 70 nm, which is significantly improved over conventional confocal microscopy. It is important to note that this resolution is approaching the limit (~ 60 nm) with a practical donut profile (5% filled in the center) and a practical laser source (on the order of magnitude of 1.0 W).

FIG. 7. Confocal and STED images of 200 nm nanoparticles immobilized on the water/glass interface in the presence of a blanket of 1-fluoroheptane. (a) Confocal image; (b) original STED image.

To further demonstrate the resolving power, we imaged the self-assembly of 200 nm fluorescent particles on the buffer/glass interface in the presence of a blanket of 1-fluoroheptane on top (Fig. 7). Interestingly, we noted that the nanoparticles in aqueous solution forms a non-contacting, chain-like structure when being sandwiched between the 1-fluoroheptane layer and the glass interface. Such self-assembling behavior is currently being investigated. Note that in the confocal image, nanoparticles that are contacting or in close proximity to other particles are completely unresolvable but can be resolved *individually* in the STED image. STED microscopy is especially useful for *in situ* observation because such information is not available using other microscopic techniques.

IV. CONCLUSIONS

To summarize, we systematically studied the transformation of donut-shaped depletion laser beam after passing through a high NA microscope objective in STED microscopy. We found experimentally that the donut beam size transformation at the focal plane follows an inversely linear relationship with respect to the incident beam size. The intensity profile of the dark kernel of the donut can be approximated as a parabolic function with respect to the distance to the donut center. The transformation of the donut beam and its intensity profile should establish the basis for any further discussion about STED point spread function and resolution.

Especially, the effective NA is critical for STED microscopy resolution in that it not only determines the donut profile but also the area the depletion laser power is dispersed. A decrease of the effective NA by half will increase the STED resolution by a factor of ~ 4 , high enough to fail any efforts to improve the resolution by the application of the depletion laser beam. This shows that the effective NA in STED microscopy, or correspondingly the size of the donut in the focal plane is *not* trivial.

Polarization of the depletion beam plays an important role in the residual intensity in the center of the donut, which is also critical for STED resolution. A non-ideal donut, i.e., a donut has significant residual intensity in the center, will have (1) low STED image intensity and (2) poor resolution. Compared to the depletion beam profile, the excitation beam profile has little effect on STED resolution.

With a continuous wave STED microscope operating at 488 nm excitation, we report a resolution of 70 nm, approaching its practical limit with a powerful depletion laser up to 1.0 W. The resolving power of the microscope is demonstrated by imaging self-assembled 45 nm and 200 nm fluorescent nanoparticles at liquid/solid interfaces. We believe that this paper will provide a useful guide for the construction of STED microscopes.

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