Research article

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Ultralow power demand in fluorescence nanoscopy with digitally enhanced stimulated emission depletion

<https://doi.org/10.1515/nanoph-2019-0475>

Received November 20, 2019; revised December 27, 2019; accepted January 26, 2020

Abstract: Stimulated emission depletion (STED) microscopy breaks the optical diffraction barrier and has become a powerful tool for biological study. However, its application for *in vivo* study is limited because of its high demand for depletion power. Here, we propose digitally enhanced STED (DE-STED) as a method for reducing the depletion power that is required for STED superresolution imaging. A donut image is the key in this approach, which is composed of the depleted photons by STED laser and represents the intensity difference between confocal and STED images from the same imaging position. The depletion efficiency is digitally enhanced by multiplying the intensity of the donut image with a factor greater than 1, and then the photons from the periphery of the diffraction-limited spot are fully depleted by subtracting the enhanced donut image from the original confocal image. Finally, DE-STED achieves a resolution of $\sim\lambda/8$ in biological samples with a depletion power that is an order of magnitude lower than that in traditional STED imaging. Furthermore, the proposed method helps to relax the restrictions on the fluorophore because of its low phototoxicity and photobleaching.

Keywords: fluorescence microscopy; superresolution imaging; stimulated emission depletion; digital enhancement.

1 Introduction

Optical microscopy has become a powerful tool in biology because it is minimally invasive and easy to operate and provides rich information on structures and functions of biosamples. However, the lateral resolution of a traditional optical microscope is limited to ~ 200 nm because of optical diffraction. In order to break the diffraction limit and improve the spatial resolution, several superresolution microscopy (SRM) techniques have been proposed over the last two decades, which come from the ideas of the fluorescence on-off effect and molecule localization [1–4].

Stimulated emission depletion (STED) microscopy, which is typically implemented in a laser scanning confocal microscope system, is the first theoretically proposed and experimentally realized SRM technique [1, 5–7]. Based on the idea of compressing point spread function (PSF), a donut-shaped depletion beam is overlapped with the excitation beam so that only the beam center can emit fluorescence. The depletion wavelength is typically at the tail of the emission spectrum of the fluorophore to avoid re-excitation. However, the stimulated emission cross-section of the fluorophore is becoming smaller as the distance between the depletion wavelength and the emission peak increases, thereby resulting in a huge demand for depletion power to completely deplete the excited photons in the periphery of the excitation spot. A high laser power is not desirable for *in vivo* imaging of biological samples because it can cause severe photobleaching and phototoxicity, which impedes the application of STED microscopy in biological research.

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To lower the required depletion power in STED microscopy, several methods have been proposed, such as time-gating detection, background suppression, and photon separation approaches. The time-gating detection approach can improve the fluorescence on-off contrast in continuous-wave STED (CW-STED) microscopy; however, it is difficult to improve the effective spatial resolution in the all-pulsed STED modality [8–10]. The background suppression approach improves both the resolution and the signal-to-noise ratio (SNR), but at the expense of the fluorescence signal [11–14]. The photon separation approach, which is typically combined with the fluorescence lifetime detection technique, resolves the signal into infinite dynamics components for extracting the spatial high-frequency information [15–17]. However, its performance in generating and discriminating dynamics components is limited by the noise. In addition, new materials, such as upconversion nanoparticles and quantum dots, have been synthesized with lower saturation intensity and strong photobleaching resistance and thus are suitable for STED imaging at low depletion power and long experiment duration [18–20]. However, these materials have inferior biocompatibility. It is difficult to label them to biomolecules, which hinders their biological applications.

Kuang et al. [21] proposed a method, which is called *fluorescence emission difference* (FED), to achieve higher spatial resolution by subtracting a negative confocal image from a confocal image, where these images are illuminated by a doughnut-shaped and a Gaussian excitation beams, respectively. As FED is a pure mathematical process that does not involve stimulated emission, it is simple and efficient. However, this method cannot accurately reflect the physical process of molecular transition between energy levels. Here, we present a novel method, namely, digitally enhanced STED (DE-STED), which can universally achieve nanoscale imaging with ultralow depletion power. In this approach, a donut image that is formed by depleted photons is digitally enhanced to further shrink the effective fluorescence area. We demonstrate that a donut PSF with a zero-intensity center is formed at a critical laser power for specified imaging conditions. After subtracting the enhanced donut intensity from the original confocal image under the critical laser power, the resulting image features superior image quality with theoretically unlimited resolution.

2 Operating principle of DE-STED

In DE-STED, a donut image that is composed of depleted photons is first generated by subtracting the STED image

from the confocal image, both acquired from the same imaging position. The intensity of each pixel in the donut image can be expressed as

$$I_{\text{donut}}(x, y) = I_{\text{confocal}}(x, y) - I_{\text{STED}}(x, y)$$

where I_{confocal} , I_{STED} , and I_{donut} are the normalized intensity distributions of the confocal, STED, and donut images, respectively. These images have the same pixel size and field of view (FOV).

In a scan-based optical system, an image is composed of the PSF intensity at each pixel. The confocal PSF is the product of the excitation source PSF_{excitation} and the collection pinhole PSF_{pinhole}, i.e.,

$$\text{PSF}_{\text{confocal}} = \text{PSF}_{\text{excitation}} \times \text{PSF}_{\text{pinhole}}$$

where the PSF_{excitation} is the convolution of the objective PSF for excitation wavelength and the transmission function of the excitation laser source, and the PSF_{pinhole} is the convolution of the objective PSF for fluorescence wavelength and the transmission function of the pinhole. Accordingly, the donut PSF is the product of the depletion laser source PSF_{depletion} and the collection pinhole, which can be represented as

$$\text{PSF}_{\text{donut}} = \text{PSF}_{\text{depletion}} \times \text{PSF}_{\text{pinhole}}$$

In consequence, the STED PSF is the result of subtracting the donut PSF from the confocal PSF

$$\begin{aligned} \text{PSF}_{\text{STED}} &= \text{PSF}_{\text{confocal}} - \text{PSF}_{\text{donut}} \\ &= (\text{PSF}_{\text{excitation}} - \text{PSF}_{\text{depletion}}) \times \text{PSF}_{\text{pinhole}} \end{aligned}$$

The donut PSF can be easily obtained when both the confocal PSF and the STED PSF are available

$$\text{PSF}_{\text{donut}} = \text{PSF}_{\text{confocal}} - \text{PSF}_{\text{STED}}$$

Therefore, a donut image that is generated by subtracting a STED image from the corresponding confocal image is defined by the donut PSF for each pixel. Theoretically, the intensity of each pixel in a STED image is lower than that in the corresponding confocal image due to the stimulated emission effect, inducing a donut image with nonnegative intensity.

As a universal and easy-to-use method, DE-STED is available for any CW- or pulsed-STED microscope without the modification of the optical system. The method has been implemented successively not only in a home-built STED microscope, but also in a commercial STED

microscope. In a universal pulsed STED microscope, the depletion pulse is delayed and synchronized to the excitation pulse for optimizing the depletion efficiency (Figure 1A). For traditional STED imaging, the reduction of the confocal PSF is achieved by depleting the photons in the area of the donut-shaped depletion beam via stimulated emission. With the increase of the depletion power, the central low-intensity area of the donut PSF gradually decreased, leading to a shrinking STED PSF (Figure 1B). This process can also be realized by digital enhancement approach when the donut intensity is obtained. In this way, the depletion efficiency of the stimulated emission can be enhanced by different multiples, which is equivalent to the increase of the depletion laser power. Consequently, a DE-STED image with an improved resolution and a lower background is obtained after subtracting the enhanced donut intensity from the original confocal image (Figure 1C). With increasing enhancement coefficient (K), the central low-intensity area of the donut PSF is gradually reduced, resulting in a similar area for spontaneous fluorescence:

$$\text{PSF}_{\text{DE-STED}} = \text{PSF}_{\text{confocal}} - K \times \text{PSF}_{\text{donut}}$$

Note that the DE-STED with a K value of 1 is the original STED image; therefore, the resolution improvement is realized only with an enhancement coefficient that is greater than 1.

3 Determination of the donut PSF for DE-STED

Similar to STED, DE-STED can also achieve unlimited resolution in theory. If the intensity in the center of the donut-shaped depletion beam is maintained at zero no matter what the laser power is, the peak intensity of the STED PSF after depletion is the same as that of the confocal PSF (Figure 2A, C). In consideration of the Nyquist theorem, the resolution is limited only by the size of the scanning step, namely, the pixel size of the image. In this case, the image features superresolution and low background noise. However, the actual resolution is limited to tens of nanometers in imaging biological samples. Generally, the wavefront of the depletion beam is modulated from Gaussian to donut shape by a spatial light modulator or a vortex

Figure 1: Schematic principle of DE-STED.

(A) A sketch of a universal pulsed STED microscope. (B) The simulation of STED superresolution imaging with the increase of the depletion laser power. (C) The principle of DE-STED for achieving resolution improvement in the sample of fluorescent beads with the increase of the enhancement coefficient and without increasing the depletion laser power.

Figure 2: Comparison between theoretical simulations and experimental results of a donut PSF and a STED PSF.

(A) Theoretical simulations of confocal, donut, and STED PSFs for increasing depletion power. (B) The intensity profiles of the donut PSF and (C) the STED PSF in (A). (D) Experimental results of the confocal, donut, and STED PSFs for increasing depletion power. (E) The intensity profiles of the donut PSF and (F) the STED PSF in (D).

phase plate. Because of the finite precision in forming the $0-2\pi$ phase and the aberrations that are induced by the optical system and the biological sample, the wavefront of the depletion beam is always distorted, thereby generating a donut PSF with nonzero intensity in the center. To measure the PSF, fluorescent beads of 100 nm in diameter were prepared as the sample. The images were obtained in the home-built STED microscope with pulsed excitation and depletion beams (Figure S1). The confocal and STED PSFs can be measured and determined by imaging a single fluorescent bead with the size below the diffraction limit, and the donut PSF was generated by subtracting STED PSF from the confocal PSF (Figure 2D). As the depletion power increases, the donut PSF comes into expansion, and the STED PSF shrinks (Figure 2E, F). However, the peak intensity of the STED PSF is also severely reduced due to nonzero intensity in the center of the donut PSF, which leads to limited resolution and low SNR of STED images. This is one of the reasons that STED microscopy cannot achieve infinite resolution in practice.

This phenomenon is also observed in a commercial STED microscope (Leica SP8 STED 3X, TCS SP8 STED 3X, Leica Microsystems, Wetzlar, Germany). To measure the resolution limit of the microscope, we prepared the sample using fluorescent beads of 23 nm in diameter labeled with

Atto647N dye. The excitation and depletion wavelengths were 635 and 775 nm, respectively. The STED images were obtained with increasing depletion power, and the relevant confocal images were captured without depletion laser output. Accordingly, the donut images were obtained by subtracting the STED images from the confocal images (Figure S2). After intensity normalization, we can clearly see that the resolution of the STED images improves, but at the expense of the signal intensity. The STED imaging resolution is represented by the average full width at half maximum (FWHM) of 10 typical fluorescent beads. The mean FWHM value of 246 nm for confocal image decreased to about 40 nm for STED image when the depletion power was 36 mW (Figure S3). All laser powers were measured at the front aperture of the objective. The STED images were deconvoluted with Huygens essential software (Scientific Volume Imaging) for increasing the image contrast [22].

The signal intensity of a STED image depends on the peak intensity of the STED PSF, in other words, the center intensity of the donut PSF. If the peak intensity of a donut PSF is pretty high and its center intensity is close to zero, the obtained STED images have high resolution and low background. This can be realized by digitally enhancing the donut PSF in DE-STED. The core of DE-STED method is to increase the intensity of donut PSF by K times for

shrinking the effective fluorescence area. Therefore, the value of K is critical to the performance of DE-STED, which depends on the specifics of the donut PSF. The critical donut PSF should be first confirmed, which is affected by many factors, such as the depletion wavelength and power, as well as the optical properties of the fluorophore. The intensity of donut PSF is determined by the number of depleted photons, in other words, the efficiency of stimulated emission. For a fluorescent material, it improves with the increase of depletion power and the decrease of wavelength interval between the emission maximum and the depletion laser. Therefore, the critical donut PSF can be realized at an ultralow depletion power if the wavelength interval is very small. Without the consideration of the peak intensity, the donut PSF can be classified into three types according to the intensity distribution at the center (Figure S4). In the first case, the center of the donut PSF has a zero-intensity area, which typically occurs at low depletion power. The donut PSF can be digitally enhanced by increasing the K value. However, the spatial resolution is limited to approximately the radius of the zero-intensity area. In the second case, only a point or pixel in the center of the donut PSF is zero intensity, which can be realized at a specific depletion power if the other conditions are invariable. Therefore, unlimited resolution is expected to be realized at a sufficiently large K value. In both cases, the peak intensity of DE-STED image stays the same, and the value of K could be an infinite number. In the last case, there is no zero-intensity pixel in the center, which is the most common case in STED imaging, especially under high depletion power and deep imaging. With the increase of the K value, the center radius of the donut PSF is reduced, but at the expense of the signal intensity, ultimately resulting in a limited resolution and a poor SNR. In this case, the value of K needs to be determined in terms of image quality. Therefore, it is extremely important to obtain DE-STED images by using an approximately ideal donut PSF, which can be formed at an ultralow power.

The differences between STED and DE-STED are reflected in the center intensity of donut PSF and the required depletion power when saturated stimulated emission is achieved. The digitally enhanced donut PSF in the first two cases has a high contrast between the peak intensity and the center intensity, leading to the DE-STED image with a higher resolution compared with the original low-power STED image. The fluorescent molecules both at the periphery and the center of confocal PSF can be depleted in different degrees in the last case because of the nonzero center intensity of the donut PSF, which is same as STED imaging at high laser power. In STED imaging, the donut PSF with low peak intensity will lead

unsaturated stimulated emission. However, no matter how much the peak intensity of donut PSF is, the depleted area, namely, the area of donut PSF, is constant, which depends only on the depletion wavelength for a particular optical system. In DE-STED, the compression of confocal PSF is performed by subtracting a donut PSF of enhanced intensity. The negative values will appear during the process of image subtraction when the intensity of digitally enhanced donut PSF is greater than that of the confocal PSF, which indicate the occurrence of saturated stimulated emission just as the case of high-power STED imaging. The final DE-STED image is derived from the original confocal image, and its intensity should be between the maximum and minimum value of the original; therefore, the negative values can be clipped to zero without the loss of superresolution information (the signals from the center of confocal PSF) [11].

Because the DE-STED image has lower fluorescence signal and background noise compared with the original STED image, it cannot be said that the method conduces to improve the SNR of the original STED image. However, the DE-STED image based on the critical donut PSF does have an excellent image quality with the high-power STED image as the reference. The reason is that the peak intensity of DE-STED image might stay the same no matter what the enhancement coefficient is, while it rapidly decreases with the increase of depletion power in STED image. Many objective measures have been developed for assessing the image quality, of which peak SNR (PSNR) is one of the simplest and commonly used [23, 24]. The fluorescent beads of 100 nm in diameter were imaged in confocal and STED modes, respectively. To achieve a similar resolution with the STED image under the depletion power of 12 mW, the DE-STED image was carried out under the enhancement coefficient of 7 (Figure S5). The PSNR of the DE-STED image was calculated with the STED image under the depletion power of 12 mW as the reference, and the value of 41.97 indicates excellent quality of the DE-STED image.

When imaging the sample of 23-nm fluorescent beads, the intensity of STED images rapidly reduces, leading to increased donut image intensity (Figure S2). Four donut images at depletion powers of 0.7, 1.4, 2.9, and 3.6 mW were selected for comparison. As shown in Figure 3A, most donuts have an area with zero intensity in the center. After 20-fold enhancement in the intensity, the peak intensity reaches the maximum, but the center intensity remains zero. In the donut images with depletion powers of 1.4 and 2.9 mW, the donut exhibits high contrast between the peak intensity and the center intensity (Figure 3B, C). The center intensity of the enhanced donut PSF remains very low (almost zero) even under the

Figure 3: Digital enhancement of donut images.

(A–D) Comparison between original and digitally enhanced donut images (bottom right, $K=20$) at the depletion powers of 0.7, 1.4, 2.9, and 3.6 mW, respectively. Pixel size: 6.5 nm; FOV: $12.5 \times 12.5 \mu\text{m}^2$. Scale bar, 2 μm . (E) Mean intensity profiles of 10 typical donuts in the original and digitally enhanced donut images in (A–D).

K value of 20, but the center zero-intensity area becomes much smaller. When the depletion power increases to 3.6 mW, the center intensity of the donut PSF in the enhanced donut image reaches the maximum due to the nonzero center intensity in the original donut PSF (Figure 3D). In this case, the confocal PSF is completely overlapped by the enhanced donut PSF; therefore, the DE-STED image will not contain any information from the sample. The donut PSF is expressed by the mean intensity profiles of 10 donuts in the image, and the original and digitally enhanced donut intensities are compared in Figure 3E. The critical donut PSF for DE-STED is generated at a depletion power that is in the range of 1.4–2.9 mW. We have also shown that the intensity of the STED image was gradually reduced with the scanning time at depletion wavelength of 750 nm (Figure S6). The photobleaching rate was increased with the increase of laser power; therefore, low laser power in DE-STED helped to resist photobleaching and extend the effective imaging time.

4 Superresolution imaging with DE-STED

Compared to traditional confocal and STED images, DE-STED images of 23-nm fluorescent beads demonstrate a

significant improvement in spatial resolution (Figure 4A, B). In DE-STED, we used enhancement coefficient values from 1 to 15 to observe the image changes in terms of resolution and intensity (Figure 4B). As discussed above, the donut PSF at a depletion power of 0.7 mW has a zero-intensity area in the center. With the increase of the K value, the FWHM of a single bead gradually decreases until reaching the radius of the zero-intensity area. It represents the first case of unchanged peak intensity but limited spatial resolution. The mean FWHM of 10 typical beads is ultimately limited to 55 ± 12 nm (Figure 4C). With depletion powers of 1.4 and 2.9 mW, the resolution of the DE-STED image can reach up to about 30 nm at a K value of 15 and 8, respectively. The peak intensity of DE-STED image remains constant at the depletion power of 1.4 mW. However, it rapidly decreases with the increase of enhancement coefficient at the depletion power of 2.9 mW, leading to DE-STED image with no signal if the enhancement coefficient is big enough. When depletion power increases to 3.6 mW, the resolution can be improved to 30 ± 8 nm at enhancement coefficient of 4. The resolution improvement comes at the cost of the fluorescence signal; therefore, the image intensity becomes zero as the value of K further increases (e.g. the last two DE-STED images in the last row of Figure 4B).

We further evaluated the performance of DE-STED for imaging biological cells. The microtubules of monkey kidney cells (BSC-1) were immunolabeled with

Figure 4: Digital enhancement for resolution improvement when imaging 23-nm fluorescent beads.

(A) Confocal images at four imaging positions, and STED images at four depletion powers, as well as the corresponding donut images. FOV: $4.8 \times 4.8 \mu\text{m}^2$. (B) Digitally enhanced STED images at increased enhancement coefficient. Note: The number in each subimage denotes the peak intensity. (C) The dependence of DE-STED imaging resolution on the enhancement coefficient for four depletion powers.

Atto647N dye. For additional information on the sample preparation, one can refer to the published protocols [25, 26]. The traditional confocal and STED images were collected with the home-built STED system. With depletion wavelength of 750 nm, spatial resolutions of 105 and 85 nm in STED images can be achieved at depletion powers of 10 and 17 mW, respectively (Figure S7). The resolution of ~ 80 nm is available for many studies in biomedicine; however, the focused laser power density reaches up to $5.67 \text{ MW}/\text{cm}^2$ (typical donut area: $3 \times 10^{-9} \text{ cm}^2$) at a laser power of 17 mW, which induces severe sample damage and bleaching of the fluorophore. According to a series of tests, the optimum depletion power is in the range of 1–1.5 mW at the wavelength of 750 nm. For comparison, confocal, STED, and DE-STED images are shown in Figure 5. With depletion powers of 1.1 and 1.4 mW, the resolution of the STED images has not improved much (Figure 5A, D). A donut image with the photons from stimulated emission was obtained by subtracting the STED intensity from a confocal image. Images with improved resolution and low background can be obtained via DE-STED (Figure 5B, E). From the intensity profiles of microtubules, the background in the STED image is completely suppressed by DE-STED. At a depletion power of 1.1 mW, the mean FWHM value of the microtubules decreased from 192 nm for the STED image to 96 nm ($< \lambda/6$) for DE-STED image at a K value of 3 (Figure 5C). When depletion power increased to 1.4 mW, it decreased from 180 nm for STED image to 82 nm ($\sim \lambda/8$) for DE-STED image at a K value of

3 (Figure 5F). The images were deconvoluted to further improve the image quality.

With a lower depletion power of 0.8 mW, the resolution of the DE-STED image increases substantially with the increase of the K value. However, it does not further improve when the K value is greater than 3 and is eventually limited to approximately one-fifth of the excitation wavelength (Figure S8). Although the resolution of the DE-STED image can likely be further improved by applying a higher depletion power, the signal intensity will rapidly reduce with the increase of the enhancement coefficient, leading to a superresolution image with poor quality.

5 Conclusions

Digitally enhanced STED nanoscopy can achieve a spatial resolution of $\sim \lambda/8$ at a depletion power of 1.4 mW. Depletion power at this order of magnitude is 10 times lower than that in traditional STED imaging, and it will substantially reduce the damage to biological samples and the bleaching of fluorophores. Consequently, this method relaxes the constraints on fluorophores with lower photobleaching resistance, e.g. fluorescent protein with higher cell affinity, and on the lasers with lower peak power for achieving the same resolution. Most importantly, DE-STED can be applied to any type of STED microscope without any modification to the imaging system. As an easy-to-use method, DE-STED nanoscopy is highly

Figure 5: Digitally enhanced STED imaging of fixed BSC-1 cells.

The comparison of confocal and STED images at a depletion power of 1.1 mW. Pixel size: 10 nm; FOV: $20 \times 20 \mu\text{m}^2$. Scale bar, 3 μm . (B) A DE-STED image of the same area in (A) with a K value of 3. (C) The mean cross-section of the microtubules in the STED and DE-STED images in (A) and (B). (D) The comparison of confocal and STED images at a depletion power of 1.4 mW. Pixel size: 15 nm; FOV: $13 \times 13 \mu\text{m}^2$. Scale bar, 2 μm . (E) A DE-STED image of the same area in (D) with a K value of 3. (F) The mean cross-section of microtubules in the STED and DE-STED images in (D) and (E).

suitable for noninvasive live cell imaging for revealing more biological information.

However, the process of obtaining applicable donut images is very critical. Indeed, the pixel mismatch between two collected images can lead to a nonuniform distribution of PSF intensity in the donut image. The mismatch in one or two pixels can be considered negligible in most instances because it is usually much smaller than the sample size. However, it must be seriously considered if realizing a resolution that is comparable to the pixel size. In addition, the sample drift in imaging live cells may also cause the pixel mismatch. To overcome this problem, pixel-by-pixel subtraction between the confocal and the STED PSFs is required. The field programmable gate array-based data acquisition card provides a fully automatic pixel-by-pixel synchronization in fluorescent signal registration and the beam interruption, which can be considered as a solution [27].

Although DE-STED is expected to achieve unlimited resolution, its performance is still restricted by many factors, e.g. the wavefront quality of the depletion beam in the focal plane and the imaging speed and depth. To fully take the advantages of DE-STED, adaptive optics techniques can be utilized to reshape beam wavefront to realize superresolution imaging of thick samples at an ultralow depletion power.

Acknowledgments: This work was supported in part by the National Key R&D Program of China (2017YFA0700500), National Natural Science Foundation of China (61975127/61525503/61620106016/61835009/81727804, Funder Id: <http://dx.doi.org/10.13039/501100001809>), China Post-doctoral Science Foundation (2019M663050), (Key) Project of Department of Education of Guangdong Province (2015KGJHZ002/2016KCXTD007), Guangdong Natural Science Foundation Innovation Team (2014A030312008), and Shenzhen Basic Research Project (JCYJ20180305125304883/JCYJ20170818100153423/JCYJ20170412105003520).

References

- [1] Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt Lett* 1994;19:780–2.
- [2] Gustafsson MGL. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J Microsc* 2000;198:82–7.
- [3] Betzig E, Patterson GH, Sougrat R, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 2006;313:1642–5.

- [4] Rust MJ, Bates M, Zhuang X. Stochastic optical reconstruction microscopy (STORM) provides sub-diffraction-limit image resolution. *Nat Methods* 2006;3:793–5.
- [5] Klar TA, Jakobs S, Dyba M, et al. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proc Natl Acad Sci USA* 2000;97:8206–10.
- [6] Hell SW. Microscopy and its focal switch. *Nat Methods* 2009;6:24–32.
- [7] Blom H, Widengren J. Stimulated emission depletion microscopy. *Chem Rev* 2017;117:7377–427.
- [8] Vicidomini G, Moneron G, Han KY, et al. Sharper low-power STED nanoscopy by time gating. *Nat Methods* 2011;8:571–573.
- [9] Vicidomini G, Schönle A, Ta H, et al. STED Nanoscopy with time-gated detection: theoretical and experimental aspects. *PLoS One* 2013;8:e54421.
- [10] Wang Y, Kuang C, Gu Z, et al. Time-gated stimulated emission depletion nanoscopy. *Opt Eng* 2013;52:093107.
- [11] Vicidomini G, Moneron G, Eggeling C, et al. STED with wavelengths closer to the emission maximum. *Opt Express* 2012;20:5225–36.
- [12] Bordenave MD, Balzarotti F, Stefani FD, Hell SW. STED nanoscopy with wavelengths at the emission maximum. *J Phys D: Appl Phys* 2016;49:365102.
- [13] Gao P, Prunsche B, Zhou L, et al. Background suppression in fluorescence nanoscopy with stimulated emission double depletion. *Nat Photonics* 2017;11:163–9.
- [14] Nienhaus GU, Gao P. Precise background subtraction in stimulated emission double depletion nanoscopy. *Opt Lett* 2017;42:831–4.
- [15] Lanzañò L, Hernández IC, Castello M, et al. Encoding and decoding spatio-temporal information for super-resolution microscopy. *Nat Commun* 2015;6:6701.
- [16] Wang L, Chen B, Yan W, et al. Resolution improvement in STED super resolution microscopy at low power using a phasor plot approach. *Nanoscale* 2018;10:16252–60.
- [17] Tortarolo G, Sun Y, Teng KW, et al. Photon-separation to enhance the spatial resolution of pulsed STED microscopy. *Nanoscale* 2019;11:1754–61.
- [18] Liu Y, Lu Y, Yang X, et al. Amplified stimulated emission in upconversion nanoparticles for super-resolution nanoscopy. *Nature* 2017;543:229–33.
- [19] Ye S, Yan W, Zhao M, et al. Low-saturation-intensity, high-photostability, and high-resolution STED nanoscopy assisted by CsPbBr₃ quantum dots. *Adv Mater* 2018;30:1800167.
- [20] Chen C, Wang F, Wen S, et al. Multi-photon near-infrared emission saturation nanoscopy using upconversion nanoparticles. *Nat Commun* 2018;9:3290.
- [21] Kuang C, Li S, Liu W, et al. Breaking the diffraction barrier using fluorescence emission difference microscopy. *Sci Rep* 2013;3:1441.
- [22] Schoonderwoert V, Dijkstra R, Luckinavicius G, et al. Huygens STED deconvolution increases signal-to-noise and image resolution towards 22 nm. *Microsc Today* 2013;21:38–44.
- [23] Tanchenko A. Visual-PSNR measure of image quality. *J Vis Commun Image R* 2014;25:874–8.
- [24] Zhang C, Guo S, Cao J, et al. Object reconstitution using pseudo-inverse for ghost imaging. *Opt Express* 2014;22:30063.
- [25] Lampe M, Fouquet W. Requirements for samples in super-resolution fluorescence microscopy. *Neuromethods* 2014;86:343–67.
- [26] Göttfert F, Wurm CA, Mueller V, et al. Coaligned dual-channel STED nanoscopy and molecular diffusion analysis at 20 nm resolution. *Biophys J* 2013;105:L1–3.
- [27] Castello M, Tortarolo G, Coto Hernández I, et al. Removal of anti-Stokes emission background in STED microscopy by FPGA-based synchronous detection. *Rev Sci Instrum* 2017;88:053701.

Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/nanoph-2019-0475>).