2000-fold parallelized dual-color STED fluorescence nanoscopy

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Abstract: Stimulated Emission Depletion (STED) nanoscopy enables multi-color fluorescence imaging at the nanometer scale. Its typical single-point scanning implementation can lead to long acquisition times. In order to unleash the full spatiotemporal resolution potential of STED nanoscopy, parallelized scanning is mandatory. Here we present a dual-color STED nanoscope utilizing two orthogonally crossed standing light waves as a fluorescence switch-off pattern, and providing a resolving power down to 30 nm. We demonstrate the imaging capabilities in a biological context for immunostained vimentin fibers in a circular field of view of 20 µm diameter at 2000-fold parallelization (i.e. 2000 “intensity minima”). The technical feasibility of massively parallelizing STED without significant compromises in resolution heralds video-rate STED nanoscopy of large fields of view, pending the availability of suitable high-speed detectors.

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References and links

15. RESOLFT: Reversible Saturable/Switchable Optically Linear (Fluorescence) Transitions.

1. Introduction

Fluorescence nanoscopy has pushed far-field optical imaging to nanometric resolutions [1–9]. STimulated Emission Depletion (STED) nanoscopy [1–3] in particular offers an appealing combination of live-cell capabilities, utilization of a wide variety of established fluorescent markers and direct, model-free image generation [2,10–12]. For large fields of view, single-point scanning STED implementations can require long image recording times of up to tens of minutes, due to the fine sampling step size which goes along with high spatial resolution and the fluorescence on/off switching requirement inherent to the STED and other superresolution concepts. Since the rate of emitted photons is limited, image recording time cannot be sped up much further without sacrificing either field of view, signal quality or resolution [13] unless parallel acquisition is introduced.

All fluorescence nanoscopy (or superresolution) methods to date which improve resolution far beyond the diffraction limit separate molecules by temporarily establishing distinguishable molecular states, a fluorescent “ON” state and a dark “OFF” state, within sub-diffraction sized sample regions and switching between the states in a targeted or stochastic fashion, to enable time-sequential readout of neighboring molecules in sub-diffraction regions [14]. While stochastic methods including (f)PALM/STORM/GSDIM [4–7] are parallelized by design owing to widefield acquisition, coordinate-targeted approaches of the STED [10–
RESOLFT [15,8,16] family attain parallelization via specific spatial patterning of the state-switching light field.

As an important case, a switch-off pattern consisting of two orthogonally crossed standing light waves can feature exactly one intensity minimum (ideally a true intensity zero) per diffraction-limited area. Such an optical pattern can therefore virtually reach the maximum degree of parallelization: scanning the unit cell of the two-dimensional periodic pattern merely once yields the full superresolved image. If \( m \)-fold parallelization is realized (i.e., \( m \) copies of the unit cell exist in parallel), sampling an \( m \) times larger field of view can be accomplished just as fast as scanning the unit cell alone. For identical imaging parameters and parallelization of order \( m \), previous limits of total recording time for large fields of view can be reduced \( m \)-fold. Notably, based on the results of the present article (\( m \geq 2000 \)), we expect that these developments will before long lead to video-rate capabilities (> 30 fps) for fields enlarged by four orders of magnitude or more.

Recently, parallelization of RESOLFT nanoscopy employing reversibly switchable fluorescent proteins (RSFPs) achieved ~70 nm resolution [17]. Since pixel dwell times in STED applications are typically two orders of magnitude shorter than for present RSFP-based RESOLFT (e.g., 50 µs vs. 10 ms) and since STED has been shown to perform well with a wide variety of bright and photostable synthetic fluorophores [12,18], it still is the method of choice for attaining highest spatiotemporal resolution. The demonstrated ability of STED nanoscopy to capture highly dynamic processes (e.g. [13]) can be extended to arbitrarily large fields of view as the challenges of parallelization are met.

A first approach to parallelize STED nanoscopy implemented four cloned excitation and fluorescence inhibition (STED) beams and four separate point detectors as detection unit [19], but upscaling of this design may prove difficult due to system complexity. The above concept of two orthogonal standing waves for switch-off [17] was recently adopted to attain 100-fold parallelization [20] of STED using a camera as parallelized detector. The available STED pulse energy limited the resolution to ~70 nm and the field of view to ~3 µm × 3 µm.

In this study we employ a laser with low repetition rate and high pulse energy. We attain a 30-fold larger field of view and introduce a second color, but most importantly, we show that STED nanoscopy based on a massively parallel design of two orthogonally crossed standing waves as STED light pattern can produce spatial resolution and image quality virtually on a par with state-of-the-art single-doughnut STED systems. We note that camera frame rates are still the major bottleneck in the quest for highest imaging speeds. But camera technology is progressing rapidly, and this may become of lesser relevance within a few years.

2. Methods

2.1 Interferometric fringe pattern

To parallelize STED, we built a pair of two-beam interferometers which projects an orthogonal superposition of two sets of interference fringes onto the sample plane of a standard fluorescence microscope (Fig. 1). Each of the one-dimensional sinusoidal fringe patterns arises from the plane wave interference of two beams crossing each other under a certain half-angle \( \theta \) (Fig. 1(a)). In contrast to similar approaches, where gratings [17] or a pair of Wollaston prisms [20] were used, we prepared beams for interference in two separately implemented sets of combined Michelson and Mach-Zehnder-like arrangements (Fig. 1(d)). Besides high transmission efficiency and full control and flexibility of all four beams, this approach allows to scan the fringe pattern instead of moving the sample.

For full modulation depth, the beams must be polarized linearly in direction of the interference fringes (compare sketch of the pupil plane in Fig. 1). Unlike in structured illumination schemes [21–23], no rotation of the fringe pattern is required. In fact, the created off-switching pattern provides rotationally uniform resolution (disregarding polarization effects) since the leading term of the Taylor series around the intensity nodes is of quadratic order in both lateral dimensions (see section 4.3).
Fig. 1. Widefield excitation (green) and patterned off-switching (red) of fluorophores realize the STED concept on a massively parallelized scale. (a) STED off-switching pattern produced as standing wave by interference of crossed beams. (b) Confined fluorescence signals (yellow) stemming from the targeted sample regions are imaged onto the EMCCD camera and (c) assigned to their corresponding pixels in the high-resolution image. The STED pattern is scanned across the sample on a slight diagonal path via piezo-controlled optical path length changes ("piezo scan") in the arms of two Michelson interferometers. The second orthogonal interferometer combination is not shown for simplicity. (d) Details of STED beam path before coupling into the microscope, including both interferometer arrangements. (e) Example of raw data detail, scale bar is 2 µm (in sample plane). PBS: polarizing beam splitter, DC: dichroic beamsplitter, TL: tube lens, OL: objective lens, λ/2: half-wave plate, λ/4: quarter-wave plate, SP: short pass filter, EMCCD: electron-multiplying charge-coupled device, EXC: Excitation.

The half-angle $\theta$ under which the STED beams impinge onto the sample plane governs the fringe period $w$ (i.e. the separation of adjacent zeros) given by $w = \lambda/(2n \sin \theta)$, for a STED wavelength $\lambda$ and refractive index $n$. Choosing $\theta$ close to the aperture angle $\alpha$ of the objective, $w$ matches the (Abbe-) diffraction limit $\Delta r = \lambda/(2n \sin \alpha)$ of the widefield microscope.
Therefore, this fringe pattern can yield nearly maximum diffraction-limited spatial node density. It also provides a very efficient means to generate steeply confined intensity minima beneficial for STED/RESOLFT resolution enhancement (see section 4.3).

2.2 Setup components

As STED laser we use a frequency-doubled fiber laser (Onefive, Katana HP (prototype)) with 775 nm wavelength, a pulse length of 0.7 ns and up to 2.2 W average power at 1 MHz repetition rate. The beam path is sketched in Fig. 1: A Keplerian telescope expands the beam to the desired size. The energy is balanced between the two interferometers and again between both interferometer arms with a half-wave plate and a polarizing beam splitter in each case (Fig. 1(d)). The deflected beams each pass a quarter-wave plate and are retro-reflected through it and through the polarizing beam splitter. The retro-reflecting mirrors are mounted on piezoelectric actuators (Physik Instrumente, P-753.11C with PZT-Servo Controller E-503.3C), which in turn sit on manual linear stages (Newport, 462-X-M). The linear stages are used to balance the optical path lengths; the piezo actuators scan the respective phase, thereby displacing the fringe pattern. All four beams are merged with one polarizing beam splitter, reflected off a dichroic filter (AHF Analysentechnik, F73-746, zt 625-745 rcp) and guided to the microscope (Leica Microsystems, DMI-3000B, APO 100x/1.44 OIL; custom-built sample mount [24] clamped directly onto the objective). Note that the final polarizing beam splitter enforces the correct linear beam polarizations required for full modulation depths.

As excitation source we use either a 635 nm laser diode (PicoQuant, LDH-D-C-635) or a supercontinuum laser (Fianium, Femto Power 1060) with two wavelengths (600 nm and 650 nm), selectable via a filter wheel. The excitation light is coupled into the common path towards the microscope by reflection off a dichroic beamsplitter (Semrock, RazorEdge LP 671) and merged with the switch-off beams at the dichroic bandpass. The pulse trains are coarsely synchronized by cables, fine tuning is realized via a custom-made electronic delay line. The STED laser is trigger master when used with the laser diode, and trigger slave when paired with the supercontinuum laser source for excitation. The fluorescence signals are imaged onto an electron-multiplying (EM) charge-coupled device (CCD) camera (Andor Technology, iXonEM + 897). Backscattered STED light is blocked with a shortpass filter (Semrock, BrightLine 750/SP). A camera pixel projects back to a 43 nm × 43 nm square in the sample plane.

2.3 Beam scanning and image generation

To scan the intensity zeros over the sample, phase differences between the interfering beams are introduced by shifting the retro-reflecting mirrors in the Michelson interferometers (piezo scan). We sample the unit cell with \( n \times n \) grid points. Since we move the first piezo \( n \) times faster than the second one, the intensity zeros follow a slightly diagonal path (slope \( 1/n \) relative to the pattern symmetry) (Fig. 1(c), exemplified for \( n = 3 \)). By the time the fast-moving piezo has scanned \( n \) pixels along the fast axis, the slow-moving piezo has moved one pixel and the next (slightly diagonal) pixel column is scanned. This beam-scanning scheme allows for efficient and continuous scanning without dead time such as the flyback in galvanometric or object scanners.

At each scanning step, the fluorescence signals from highly confined, fringe-period-spaced sample regions are imaged onto the camera (cf. Figures 1(b), 1(c) and 1(e)). Since the period comes close to the diffraction limit \( \Delta r \), the blurred, diffraction-broadened fluorescence spots overlap moderately in the image plane (Fig. 1(e)). (The diagram in Fig. 1(c) does not show this overlap for clarity of display of the concept). Three conceivable strategies to address this spatial “crosstalk” were examined: First, only the central camera pixels around each emission origin might be considered at the expense of reduced signals. Second, a multiple linear regression should yield the most accurate result: Since the location and the approximate shape of the fluorescence signal distributions can be determined beforehand, only their amplitudes must be fit. Therefore, the problem is reduced to a system of linear
equations with a unique least-squares-optimal solution. However, the memory requirement of this approach is demanding and scales up unfavorably with the degree of parallelization. Therefore, a third strategy as a compromise between the first two is proposed and used: A larger weighted pixel mask collects a bigger portion of the signal. Afterwards, the “crosstalk” is removed in a linear matrix operation that contains the relative crosstalk in the eight nearest neighbors. Tests on synthetic data have shown only minor differences between strategy two and three, with strategy three minimizing the computational processing cost. No knowledge of the exact microscope PSF is required to implement strategy three.

Many STED implementations with serial sampling with a fast point detector now use time-gated detection [25] to suppress spontaneous fluorescence that occurs before the STED pulse has left. Such time-gated detection to reduce background haze due to early photons is not yet adequately possible with current camera technology. In the present study, we merely subtract a corresponding scaled widefield background image from every pixel.

3. Results

![Resolution quantification. (a-c) Parallelized STED vs. widefield image of 20 nm Crimson beads with magnified inset. (d) The bead images were fit with a 2D Gaussian model function and full width at half maximum (FWHM) fit values are displayed color-coded in a scatter plot (corresponding to full region of (a)). (e) Histogram of the FWHM fit values.](image)

3.1 Resolution

To examine the highest possible resolution with the available STED laser, we concentrate the STED pulse energy in a narrow Gaussian envelope of 6.3 µm diameter (full width at half maximum, FWHM). We scan the 2D fringe pattern over the unit cell on 30 × 30 grid points of 12.5 nm pixel size. At a unit cell edge size of 374 nm, this yields ~223-fold parallelization within the FWHM region and 1250-fold parallelization across the full image. Figure 2(a) shows the superresolved image of dispersed 20 nm-diameter Crimson fluorescent microspheres (Life Technologies) in comparison to the corresponding widefield microscope image.
A magnified view of the boxed region (Figs. 2(b) and 2(c)) reveals the ~10-fold resolution increase.

To determine the resolution, we fit the beads with a two-dimensional Gaussian model function. The fit results are displayed color-coded as a scatter plot in Fig. 2(d). The resolving power decreases towards the periphery since it scales inversely with the square root of the STED intensity [26] and the STED pattern features a Gaussian intensity envelope. The histogram of the fit values in the center region (Fig. 2(e)) peaks at ~30 nm. A model calculation treating the bead images as a convolution of spatial resolution with the physical bead size of 20 nm thus estimates the technical resolving power to ~27 nm. The approximate STED energy in the sample plane is 1.5 nJ per unit cell in the center and > 0.75 nJ per unit cell within the FWHM region.

Fig. 3. (a) 2000-fold parallelized dual-color STED imaging of 200 nm fluorescent beads via excitation multiplexing: Crimson beads (intensity scales from black to green color) excited at 600 nm; Dark Red beads (black to red color) at 650 nm. Considerable cross-excitation at 650 nm is largely removed via linear unmixing. Note the increased image brightness near the periphery as a consequence of lower resolution due to fall-off of the STED intensity envelope.
(b,c) Magnified view of boxed region in (a).

3.2 Dual-color parallelized STED on large field of view

A massively parallelized STED nanoscope places high demands on the pulse energy output of the STED laser source as the STED light must illuminate a large area at once for parallel sampling. In this feasibility study, the STED pulse energy is a limiting factor to achieve highest resolution and large superresolved fields of view at the same time. Given the Gaussian envelope of STED intensity, the diameter of the highly-resolved field of view scales inversely to the resolution performance as a consequence of the square root law. If a lower resolving power of, say ~55 nm is sufficient, we can double the STED spot diameter accordingly. Figure 3(a) shows Crimson (in green color) and Dark Red (in red color) fluorescent beads (Life Technologies) with nominal diameters of 200 nm dispersed on the cover glass surface. The pixel size is 20 nm and the (slightly enlarged) unit cell is scanned on a 20 × 20 grid. The 20 µm wide circular superresolved region contains ~2000 STED zeros. Note that the
resolving power gradually decreases to ~110 nm towards the edges, still well below the diffraction resolution limit. To separate the two bead-labeling dyes, we record consecutively with excitation at 600 nm and 650 nm. Since the detection path is unaltered between both consecutive scans, a certain level of cross-excitation of the two dyes in both channels is inevitable. We note that spectral separation as well as colocalization performance could be improved in a straightforward manner by pulse-wise excitation multiplexing and two spectrally filtered detection channels. Both images are merged and colored using a linear unmixing algorithm similar to [27]. The magnified view (Figs. 3(b) and 3(c)) clearly illustrates the information gain of increased resolution: While the widefield image merely distinguishes the presence of both fluorophore colors, the STED image resolves the detailed shape of the beads. We see for example that the Dark Red beads are somewhat smaller than specified. We assume that this imaged size difference indeed traces back to a differing “real” bead size since five (and not six) (green) Crimson beads surround one (red) Dark Red bead.

3.3 Vimentin sample

We demonstrate the capabilities of parallelized STED imaging in a biomedical context on the larger field of view of 20 µm diameter. An image of a dense Abberior STAR 635P-stained vimentin filament structure in Vero cells is shown in Fig. 4(a). The magnified insets (Figs. 4(b) and 4(c)) and the intensity profile plots taken through the dashed line (Figs. 4(d) and 4(e)) show that the underlying filament structure is obscured in the widefield image, whereas it is clearly discerned in the STED counterpart. A fit of Gaussian model functions to the profile plot in the STED image (Fig. 4(e)) sets an upper bound for the resolution to ~68 nm (FWHM). In conjunction with the antibody staining (primary and secondary antibodies), the decorated vimentin fibers have a real diameter of ~45 nm [24]. Therefore the measured profile across the filament is consistent with an estimated instrument resolving power of ~55 nm by convolution arguments. The STED energy in this recording was 0.42 nJ per unit cell near the center of the sample plane.
The main focus of this study was to examine whether parallelized STED nanoscopy using the fringe pattern approach can attain resolutions comparable to doughnut-based single-beam STED systems. While implementation of axial superresolution remains to be addressed, we demonstrate that the massively parallelized approach delivers lateral sub-30 nm resolving power. Furthermore, sub-60 nm resolution in the center of a 20 µm-sized superresolved field of view and the dual-color capability already make the present instrument suitable for biomedical applications.

4.2 STED efficiency

Parallelized STED can reduce photobleaching due to its increased power efficiency [20]: As compared to doughnut-shaped STED foci, parallelized STED based on two orthogonally crossed standing waves can feature steeper intensity gradients around the locations of the zeros. In addition, the energy utilized in defining one of the zeros to demarcate one target coordinate is inherently shared with neighboring unit cells. Our calculations (section 4.3) yield a STED energy efficiency advantage of 15.4 \((\sin \theta / \sin \alpha)^4\). This means that up to 15.4 times less laser energy per STED zero is required to achieve the same resolution. At presently \(\theta \sim 40^\circ\), we indeed observe an efficiency increase close to the predicted factor of three in our experimental data when comparing to reported resolutions and STED pulse energies [11,24].

Previous studies suggest that the rather low repetition rate of 1 MHz we use might reduce photobleaching and increase the fluorescence yield per excitation cycle eight-fold to 30-fold due to a reduced triplet or dark state build-up [28,29]. This is known as triplet relaxation (T-rex) or dark-state relaxation (D-rex). A high degree of parallelization in general requires a high STED pulse energy. A low repetition rate keeps the average laser power reasonably low, avoids unnecessary damage of the sample and at the same time allows for T/D-rex conditions.

We note that although the applied STED pattern is an overlay of linearly polarized standing waves, we do not observe any polarization bias in the STED efficiencies. It appears that sufficient rotational fluorescence depolarization takes place during the comparably long STED pulse length of 0.7 ns [30].

4.3 STED efficiency – detailed considerations

In this section, we revisit the well-established expression for resolution enhancement beyond the Abbe limit in STED/RESOLFT-type nanoscopy on a conceptual basis, and compare it for the cases of classical doughnut-based STED (dSTED) and parallelized STED (pSTED).

The key enabling element of STED/RESOLFT nanoscopy is to make use of internal fluorophore state transitions and to drive these transitions in a targeted manner in space (see introduction and Fig. 5), enabling sequential readout of molecules at precisely defined coordinates without signal interference from their immediate neighbors. In the STED concept [1], a dedicated light distribution defines locations in space where the excited fluorescent state remains allowed but enforces return to the non-fluorescent ground state by stimulated photon emission everywhere except in close proximity of these locations. Confinement of the fluorescent state to a high degree by efficient switch-off allows to probe signals in substantially sub-diffraction regions and register an image by scanning. An intriguing consequence is that the fluorescent state can be confined down to arbitrarily small regions [3].

The STED beam (or, generally, the STED pattern, Fig. 5(a)) decreases the occupation probability of the fluorescent state according to an exponential decay law [31] (Fig. 5(d)): The probability \(p\) that a fluorophore is able to reside in the “ON” state is \(p_{ON} = \exp(-\ln(2) \cdot s) \cdot p_{exp}\). Here \(s = I/I_s\), where \(I\) is the intensity of the light field and \(I_s\) the intensity value at which \(p_{ON}\) is 0.5. The fluorophore excitation probability \(p_{exp}\) is set to 1 for the time being. Note that in general notation with the saturation parameter we could equivalently define \(s = h/h_s\) with \(h\) being the number of photons per unit area per pulse, \(h_s = \ln(2)/\sigma\) is then readily related to the cross-section of stimulated emission \(\sigma\) of order \(10^{-16}\) cm\(^2\) [31].

To attain spatially targeted inhibition of the fluorescent state occupation (switch-off), the saturation value is varied across \(x\): \(S(x) = s \cdot h(x)\), where \(h\) is a spatial intensity function of the lateral coordinate \(x\) (Fig. 5(a)). Appropriate normalization of \(h\) (to the \([0,1]\) interval) ensures
that $S(x)$ assumes values between 0 and $s$. In the case of dSTED, $h(x)$ represents the well-known doughnut-shaped STED light distribution. The “ON”-state probability becomes $p_{\text{ON}}(x) = \exp(-\ln(2) \cdot s \cdot h(x))$ (Fig. 5(c)). If we disregard for instance the effects of excitation and detection point spread functions, the resolution can be defined via the FWHM $d = 2 \cdot x_0$ of $p_{\text{ON}}$, with $x_0$ given by $p_{\text{ON}}(x_0) = 0.5$ or, equivalently, $s \cdot h(x_0) = 1$.

For relevant values of $s$, say $s > 4$, $h(x)$ is well approximated around the intensity zero at $x = 0$ by the quadratic approximation of the Taylor expansion: $h(x) \sim a \cdot x^2$. Then it follows from $s \cdot a \cdot x_0^2 = 1$ that $d$ must scale as $d \sim 1/\sqrt{s}$, the well-established inverse square-root dependence [26]. It is worth noting that the square-root scaling solely originates from the quadratic expansion of the intensity node. Since $h \sim I$ and $I = EE^* \sim x^{2k}$ for any physical zero-crossing of a diffraction-limited electric field $E$, any even power $2k$ of $x$ could in principle be aimed for in the design of $h(x)$ as the leading term dominating the curvature at the node. But e.g. $h(x) \sim a \cdot x^4$ implies $d \sim 1/s^{1/4}$, which would be a less favorable scaling.

![Figure 5](image_url)

**Fig. 5.** Resolution in coordinate-targeted nanoscopy. (a) $s \cdot h(x)$ for doughnut STED (dSTED) and parallelized STED (pSTED) for the case $\theta$ equal to the aperture angle $\alpha$. Same curvature near the zero (i.e. same quadratic approximation) yields the same resolution, cf. close-up shown in (b). The resolution enhancement (given by the FWHM) scales with $1/\sqrt{s}$. (c) “ON”-state probability $p_{\text{ON}} = p_{\text{exc}}(f(s \cdot h(x)))$. (d) Exponential decay $p_{\text{ON}} = f$ of population of the excited fluorescent state versus saturation parameter $s$. $p_{\text{ON}}$ is normalized to 1.

**Interpretation of p, h, s**

For the general case, the switching probability can be modeled as $p(x) = f(s \cdot h(x))$, with the probability $p$ that the molecules remain in their original (e.g. “ON”) state, a generic decay law $f(S)$ that fulfills $f(S = 1) = 0.5$ and a spatial distribution $h(x)$. The decay law $f$ contains in a broader sense the physics of the system, e.g. rate equations, timing, background photons, etc. The parameter $s = I_{\text{max}}/I_0$ comprises the physical chemistry through $I_0$ and the associated state transition cross-section. $h$ is the function of the spatial intensity or photon fluence distribution containing the (diffraction-limited) optics.
We see that the particular shape of $p(s)$ is not crucial. The resolution $d = 2 \cdot x_0$ is readily given by the defining equation $x_0$: $h(x_0) = 1/s$. The curvature of $h$ around the intensity zero is limited by diffraction. Therefore, it needs to be $s$ which is increased to obtain higher spatial resolution, as depicted in Fig. 5(c).

Resolution formula for dSTED

In order to derive the full resolution formula using the above framework, we consider realistic excitation and STED beam point spread functions that we obtain from numerical calculations incorporating polarization effects [32]. The circularly-polarized doughnut-shaped STED beam closely approximates the Laguerre-Gaussian mode $h(x) = x^2 \cdot e((w_d/2)^2 \cdot \exp(-\ln(2) \cdot x^2/(w_d/2)^2)$, compare Fig. 5(a). This definition fulfills max $\{h(x)\} = 1$ and the “crest width” is $w_d = 1.68 \cdot w_{\text{ste}}$. Here we use the characteristic parameter $w_{\text{ste}} = \lambda_i/(2 \sin a_i)$ for $i$ being one of \{exc, fl, STED\}, corresponding to the Abbe limit. The numerical factor 1.68 is inferred from the calculation. It indicates that the doughnut does not exhibit the “tightest” (i.e. $w_d > w_g$ as in Fig. 5(a)) intensity valley (around $x = 0$) allowed by diffraction because it concurrently confines the photon fluence to a quite localized overall lateral extent. The Gaussian excitation is well described by $h_{\text{exc}}(x) = \exp(-\ln(2) \cdot x^2/(w_{\text{exc}}/2)^2)$, with $w_{\text{exc}} = 1.15 \cdot w_{\text{exc,ste}}$. If we assume linear fluorophore excitation, i.e. $p_{\text{exc}} = \varepsilon \cdot h_{\text{exc}}$, the probability $p_{\text{ON}}$ that a fluorophore is switched on after application of excitation and STED beam is the product $p_{\text{ON}} = p_{\text{exc}} \cdot p_{\text{STED}}$ with $p_{\text{ON}}(0) = \varepsilon$. The resolution is defined via $p_{\text{ON}}(x_0) = \varepsilon/2$ which yields

$$p_{\text{on}}(x) = \frac{p_{\text{on}}(x_0)}{p_{\text{on}}(0)} \cdot \frac{1}{2} \quad \text{or, explicitly,}$$

$$\exp\left[-\ln(2) \cdot \left(\frac{1}{(w_{\text{on}}/2)} + \frac{s \cdot e}{w_d/2}\right) x_0^2\right] = \frac{1}{2}$$

where we have used the Taylor expansion of $h_{\text{STED}}(x) \sim x^2 \cdot e((w_d/2)^2$. It follows that

$$\left(1 + \frac{s \cdot e}{1.86} \cdot \frac{(2x_0)^i}{w_d^i}\right) = 1$$

where we assumed $w_{\text{ste}} \sim w_{\text{exc}} = 1.15 \cdot w_{\text{exc,ste}}$ and $w_d = 1.68 \cdot w_{\text{ste}} \sim 1.86 \cdot w_{\text{ste}}$ as common ratios of excitation, fluorescence and STED wavelengths. Solving for $2x_0$ yields the resolution formula

$$d = \frac{\lambda_i}{2NA} \frac{1}{\sqrt{1 + 0.8s}}$$

Note that in earlier derivations $\lambda_{\text{ste,STED}} = \lambda_i$ was assumed. Then follows the conceptually easiest form $d = \lambda_i/(2NA) \cdot 1/(1 + s)$.

Resolution formula for pSTED

In a similar manner, we consider the standing-wave light field for pSTED and its Taylor expansion to quadratic order: $h_{p}(x,y) = \sin(x \cdot \pi/w_d) + \sin(y \cdot \pi/w_g) - (x/w_d)^2 \cdot y^2$, where $r^2 = (x^2 + y^2)$ immediately yields the rotational symmetry of the intensity node. Therefore, we suppress the coordinate $y = 0$ and write $h_{p}(x,y) = h_{p}(x,0)$. The period $w_0 = \lambda_{\text{pSTED}}/(2 \sin \theta)$ at half angle $\theta$ generates steeper intensity nodes for $w_0 < w_d$. Alternatively, the intensity can be reduced if the same curvature (respectively, the same resolution) is aimed for (cf. Figures 5(a) and 5(b)). The excitation can in principle be structured in the same way [20]: $h_{\text{exc}}(x) = \cos(x \cdot \pi/w_d) \sim \exp(-\ln(2) \cdot x^2/(w_d/4)^2)$. We approximate $h_{\text{exc}}(x)$ for convenience with the exp-function and again formulate $p_{\text{STED}}(x) = s \cdot h(x)$. By analogy with the above argument, we find:
Comparison of pSTED and dSTED

As discussed, the curvature of \( p(x) \) determines the resolution, equal Taylor coefficients of quadratic order lead to the same resolution:

\[
\exp \left[ -\ln(2) \left( \frac{1}{(w_x/4)^2} + s \left( \frac{\pi}{w_x} \right)^2 \right) \right] = \frac{1}{2}
\]

\[
\Rightarrow \left( 4 + s \left( \frac{\pi}{2} \right)^2 \right) (2x)^2 \cdot \frac{1}{w_x, \sin \theta} = 1\]

\[
\Rightarrow d = \frac{\lambda_{\text{min}}}{2N\lambda} \frac{1}{\sqrt{4 + (\pi/2)^2} \cdot s \cdot \sin \theta / \sin \alpha} = \frac{\lambda_{\text{min}}}{2N\lambda} \frac{1}{\sqrt{3.25 + 2s} \cdot \sin \theta / \sin \alpha}
\]  

(5)

Therefore, the peak intensity can be \( \sim 2.5 \) times lower for pSTED. On an intuitive level it is worth noting that excess local switch-off intensities further away from the node add no additional benefit as long as \( S \) of the required level (say \( S > 6 \)) is ensured throughout. The 2D fringe pattern is more efficient in this regard. Note that the second, orthogonal fringe pattern adds intensity so that the intensity doubles in the unit cell “corners” of the resulting 2D pattern.

Next, we compare the STED power (or number of photons) needed to acquire one superresolved pixel. For pSTED, this is the power for one unit cell \( P_p = I_p \cdot w_{\theta}^2 \), for dSTED this is the full power required for the doughnut; \( P_d = I_d \cdot \pi \cdot e \cdot (w_d/2)^2 \) and therefore:

\[
\frac{P_p}{P_d} = \frac{I_p}{I_d} \cdot \frac{\pi \cdot e \cdot (w_d/2)^2}{w_{\theta}^2} = \pi \left( \frac{1.68}{2} \right) \left( \frac{\sin \theta}{\sin \alpha} \right)^2 = 15.4 \frac{\sin \theta}{\sin \alpha}
\]

(6)

(7)

This result has two implications if the angle \( \theta \) comes close to the aperture angle \( \alpha \). First, the up to 15-fold energy efficiency advantage of parallelized STED over doughnut-STED translates directly into a reduced photon dose that hits the sample. Second, if the transmission efficiency of the objective lens is not drastically reduced for large \( \theta \), a given laser photon budget (laser power) can be spread over a 15-fold larger field of view. Note that the Gaussian beams retain a circularly symmetric beam waist irrespective of the angle \( \theta \) if imaged through the microscope due to the Abbe sine condition.

The discussion of this section assumes that the STED pulse duration \( t_{\text{STED}} \) is short enough compared to the fluorescence lifetime \( t_{\phi} \) (e.g. 0.7 ns vs. 3 ns) so that the STED action can be considered an instantaneous process. Polarization effects are not considered.

### 4.4 Speed considerations

Highest imaging speeds are not further investigated in this context since we are limited by camera frame rates to 30 ms per full-frame sensor readout, where we expect significant technical progress within the next years. Substantial improvement is for example being made in the field of single-photon avalanche diode (SPAD) arrays [33]. Use of SPADs might additionally enable time-gated parallelized STED in the near future. Besides camera speed, the number of scanning steps to sample the unit cell determines the recording speed. The size of the STED pattern unit cell has as lower limit the (Abbe-) resolution \( \Delta r \) of the microscope of \( \sim 250 \) nm. For 25 nm resolution, we need – according to the Nyquist criterion – at least \((250/12.5)^2 = 400\) scanning steps, or 100 steps for 50 nm resolution. Thus, resolution can be traded quadratically against imaging speed (and field of view; see above).
In a recent publication on doughnut-STED, a 10 MHz repetition rate (per color) with 30 µs dwell time achieved 20 nm resolution [11]. If we presume in our case a moderate D-Rex gain of factor three, and thus a dwell time of 100 µs, we see the potential (if camera capability permits, i.e. full-frame readout at 0.1 ms per frame) to record 50 nm-resolution images with 100 frames per second (fps) and 25 nm-resolution images with 25 fps. As compared to doughnut-STED, the effective recording speed increase for comparable signal elicited from the sample is a factor of \( \frac{f_p}{f_d} \cdot (\text{T-Rex gain}) \cdot m \) (for \( m \)-fold parallelization), where \( f_p \) and \( f_d \) are the laser pulse repetition rates of the parallelized and doughnut modality respectively. As described, the superresolved field of view is just limited by the STED pulse energy.

5. Conclusion

While STED laser energy governs the field of view, camera frame rates still limit recording speed. Both domains have seen promising progress in recent years, and additional advances should be forthcoming.

We have shown that massively (several thousand-fold) parallelized STED nanoscopy is technically feasible without sacrificing resolution. The results pave the way for massively parallel video-rate nanoscopy recordings once the limitations in readout speed of present sensitive camera technology for large fields of view have been overcome.

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