Laser-diode-stimulated emission depletion microscopy

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We report subdiffraction resolution in far-field fluorescence microscopy through laser-diode-stimulated emission depletion of molecular markers. The diode-generated focal intensities lead to a resolution improvement by ~45% in both lateral directions. Excitation and stimulated emission are performed with electronically synchronized diode pulses of 50–70 ps and 300–400 ps duration, respectively. The subdiffraction resolution is utilized to resolve neighboring individual molecules. © 2003 American Institute of Physics.

Breaking the diffraction barrier in far-field light microscopy is a challenging research goal. Stimulated emission depletion (STED) microscopy breaks this barrier by inhibiting fluorescence molecules at the outer region of a scanning excitation spot1,2 in a saturated manner. For this purpose, excitation and STED are performed by synchronized laser pulses, with STED pulses arranged in a doughnut spatial mode. As a result, fluorescence is inhibited at the spot periphery, but not in the center. This measure has the potential to fundamentally reduce the fluorescence spot size, with a theoretical limit at the molecular scale.

Calculations show that the STED pulses have to be significantly longer than the lifetime of the higher vibrational level of the ground state into which the molecule is stimulated, but shorter than the typical lifetime of the fluorescent state, that is from a few picoseconds to a nanosecond. Likewise, the number of stimulating photons per pulse and focal area, \( h_{\text{STED}}(r) \), has to be high enough to outperform spontaneous emission.\(^1\) This is the case if \( h_{\text{STED}}(r) \times \sigma \geq 1 \) with \( \sigma \approx 10^{-16} \text{cm}^2 \) denoting the cross section for stimulated emission. With a focal area of \( \sim 10^{-9} \text{cm}^2 \), typically \( > 10^7 \) photons per pulse are required, which, according to the theory, may be stretched over the whole picosecond time span.

Here, we reveal that conditions for the virtually complete depletion of near-infrared dyes can be met by modern high-repetition rate pulsed laser diodes of 250–450 ps pulses. As a result, we demonstrate laser-diode induced breaking of the diffraction barrier and imaging of single fluorescent molecules with subdiffraction resolution.

Laser diodes greatly facilitate the implementation of STED. So far, this type of microscopy utilized stretched femtosecond pulses of a mode-locked Ti:Sapphire laser and a synchronously pumped optic-parametric oscillator.\(^2\) Although laser diodes have been employed for modulating fluorescence in lifetime imaging,\(^3\) the efficiency of modulation could not be inferred from this data. Here, we report laser-diode induced depletion by 90%–95% of single dye molecules, thin layers, and bulk fluorescence solutions with suitable emission spectra. Our experiment reveals that laser diodes can even saturate depletion which is the key ingredient for breaking the diffraction barrier.

The experiment [Fig. 1(a)] utilizes a stage scanning setup with an oil immersion lens of 1.4 numerical aperture. Excitation was performed either by a 440 nm or by a 637 nm laser diode (LDH-440 and -635, PicoQuant GmbH, Berlin, Germany) with pulses of 68 and 54 ps, respectively. Fluorescence was backprojected onto a confocal detector featuring a diameter of half the Airy disk. To obtain the highest possible \( h_{\text{STED}}(r) \), two cross-polarized laser diodes (LDH-780, PicoQuant GmbH) of 303 ps pulse duration were combined by a polarizing beam splitter. All beams were spatially cleaned and expanded to illuminate the back aperture of the objective lens in full. The resulting point-spread functions (PSFs) were measured by imaging 100 nm gold beads nonconfocally [Figs. 1(b)–1(d)].

FIG. 1. (a) Laser-diode STED microscope. Whereas pulsed excitation is performed at 440 nm or 635 nm, STED is performed with two cross-polarized diodes, A and B, emitting at around 780 nm. Measured PSF in the focal plane \((x,y)\) as created by the (b) excitation diode, (c) STED diodes, and (d) STED diodes with phase plates inserted in the beams. The PSF (d) is required for attaining subdiffraction lateral resolution. Pol: polarization direction, PBS: polarizing beam splitter, \( \lambda/2 \): rotatable half-wavelength plate, DM: dichroic mirrors, BP: band pass filter, APD: avalanche photodiode, and P: pinhole.
recorded multichannel scaler traces with a temporal resolution of
quasicontinuous power ensured that, within the confocal
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bin 125 ns. The inset shows a trace of longer
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FIG. 2. (a) Multichannel-scaler traces of the STED diode and the concomitant behavior of fluorescence: Shut down of the STED diode (circles) causes equally fast recovery of fluorescence (dots) initially quenched by 73%; time bin 125 ns. The inset shows a trace of longer (50 μs) bins and reveals a 1% overshoot as compared to the medium fluorescence level. Note the zero suppression and the typical error bar in the plot; STED turn off was again at time point zero. (b) STED efficiencies ɛ > 90% achieved with laser diodes, both for single JA 26 fluorescent molecules and thin films of P2. At this pulse length, a STED beam intensity > 0.5 MW/cm² leads to saturation of the depletion.

Besides the small size, minimal maintenance, and low costs, the benefit of laser diodes is the electronic pulse timing. The timing of the diode drivers (PDL 808, PicoQuant) was derived from a common 40 MHz clock through a four-channel electronic delay line, with a precision of 10 ps and a range of 20 ns. Each channel could be disabled by an electrical input, acting as an individual shutter for each diode. The relative timing of the pulses was optimized for maximal STED which occurred for a delay of the STED pulse by 300 ps.

Fluorescence reduction was readily observed upon switching on the STED pulses [Fig. 2(a)] that were, in this case, 10–15 times longer than the stretched Ti:Sapphire pulses used so far. To rule out photobleaching as a cause of the fluorescence drop, we investigated the recovery time after the sudden switch off of the STED diode. A hallmark of photobleaching would have been a recovery time of > 10 μs, which is the typical time required by the diffusion of fresh molecules into the confocal volume. Hence, we recorded multichannel scaler traces with a temporal resolution of 125 ns. The sample was a fluorescent solution of 2 μM Pyridine 2 (P2) in ethanol. The employed 31 μW of quasicontinuous power ensured that, within the confocal volume > 50% of the molecules were excited after each pulse, minimizing the pool of potential “fresh bystander molecules.” To record the recovery speed, the fluorescence was collected for subsequent sets of 256 bins with the STED diodes switched on and off. This experiment was repeated until sufficient cumulative counts were collected per bin. Figure 2(a) shows a trace of 8 μs and a STED efficiency ɛ = 0.73. To observe potential variations on a millisecond time scale, the experiment was repeated with longer bin sizes of 10 μs, as displayed in the inset in Fig. 2(a). The data clearly demonstrate the fast recovery of the fluorescence signal.

The last pulse of the STED pulse train arrived shortly before the end of a 125 ns collection bin, so that this bin witnessed on average about 10% fewer STED pulses [Fig. 2(a)]. If this reduction is taken into account mathematically a virtually instant (< 50 ns) recovery is found. Careful analysis of the longer time trace [Fig. 2(a), inset] discloses a “beneficial” increase by 1% of the fluorescence right after the STED beam; this minor but interesting phenomenon will be a subject for future investigations.

Next the STED efficiency, ɛ was measured as a function of the diode power, both for single molecules of the Rhodamine derivative JA 26 and in a ~ 200–400 nm thick film produced from a 20 μM P2 solution. The lower power range was covered by using only diode B and by varying the angles of the Λ/2 plate. For individual molecules, ɛ strongly depends on the orientation between the transition dipole moment of the molecule and the STED pulse polarization. In a film, all orientations occur and therefore an average will be measured. In a solution, ɛ is further reduced by a volume effect: Molecules outside the excitation focus are excited as well, but due to the reduced intensity in these areas, they are depleted less efficiently, thus reducing the average ɛ. The differences in maximum efficiency—P2 solution 73%, P2 film 93%, JA 26 single molecule 97%—can be largely attributed to these effects. Hence, an important outcome is that, although an even higher pulse energy would still be advantageous, the diodes deliver intensities that reach saturation of the depletion. It is also interesting to note that due to the ten times longer pulses, the required focal peak intensity of the STED beam is lowered by the same factor. Therefore, parasitic detrimental nonlinear processes are significantly reduced with these STED pulses.

Finally, we investigated the attainable resolution improvement. We elected a linear excitation angle of 45° with respect to x and y and modified our setup with binary phase plates in each of the STED arms. Each plate introduced a phase shift of π in one of their halves with their dividing lines oriented parallel to the respective polarization. The combination of the two beams formed a nearly circular depletion doughnut [Fig. 1(d)]. To prove the sharpening of the fluorescence spot, we prepared a single molecule sample by spin coating a 10 nM solution of JA 26 dissolved in an acidified aqueous buffer containing 0.1% polyvinyl alcohol. The image in Fig. 3(a) is a confocal recording illustrating its resolution. The arrowhead highlights “blinking,” which, along with sudden bleaching, is a characteristic of single molecules.

Improved resolution is apparent in the STED-counterpart image [Fig. 3(b)] and in the enlarged insets showing a molecule...
lecular triad, indistinguishable by confocal imaging, but re-
solved with STED [Fig. 3(c)]. In Fig. 3(d), line plots through
the molecular peaks depict a separation of 156 and 160 nm
as well as a 40% reduction in total fluorescence. Because of
the random orientation of the transition dipoles, the resolu-
tion is established in a histogram of full width at half maxi-
mum (FWHM) values rendered by the individual molecules.
To negate artifacts induced by blinking, two data sets with
scan axes oriented in the x and y direction were evaluated
and the FWHM was measured along the fast scan axis.
Gaussian-fitted plots revealed 120 nm [Fig. 3(e)] and 139 nm
[Fig. 3(f)] resolution, shrinking the cross-sectional area of
the confocal PSF by a significant factor of 3.3.

In conclusion, we have demonstrated laser-diode in-
duced saturated depletion by stimulated emission and the
concomitant breaking of the diffraction barrier in far-field
fluorescence microscopy. Since more powerful diodes and
more wavelengths will be available within the next years, we
anticipate that pulsed laser diodes will be the sources of
choice for STED microscopy.

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