Stimulated-emission-depletion microscopy with a multicolor stimulated-Raman-scattering light source

Brian R. Rankin, Robert R. Kellner, and Stefan W. Hell*

Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany
*Corresponding author: shell@gwdg.de

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We describe a subdiffraction-resolution far-field fluorescence microscope employing stimulated emission depletion (STED) with a light source consisting of a microchip laser coupled into a standard single-mode fiber, which, via stimulated Raman scattering (SRS), yields a comb-like spectrum of seven discrete peaks extending from the fundamental wavelength at 532 nm to 620 nm. Each of the spectral peaks can be used as STED light for overcoming the diffraction barrier. This SRS light source enables the simple implementation of multicolor STED and provides a spectral output with multiple available wavelengths from green to red with potential for further expansion. © 2008 Optical Society of America

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Stimulated-emission-depletion (STED) microscopy has proven to be a powerful tool for far-field, noninvasive, subdiffraction visualization of fluorescently labeled structures [1]. For example, it has achieved resolution on the order of tens of nanometers inside fixed cells [2], imaged the vesicle movement of live neurons at subdiffraction resolution [3], and visualized the three-dimensional stacking patterns of densely packed colloidal nanostructures [4]. The diffraction limit is overcome by interrogating a known position in the sample with a subdiffraction-sized detection volume, created by preventing the excited state at the periphery of the excitation focal spot via stimulated emission. The focal intensity distribution of the quenching light is engineered to have zero intensity in the center, and this, together with the saturable nature of the de-excitation mechanism, allows the confinement of the excited state to a spot many times smaller than that of a diffraction-limited focus.

However, the use of STED microscopy is limited by the availability, complexity, and cost of suitable light sources capable of switching the fluorescence ability of the dye off, i.e., saturating the stimulated-emission transition within the fluorescence lifetime. A thrust of current research is to identify and apply new light sources to simplify and broaden the implementation of STED [5–7]. Furthermore, light sources that provide a spectrum of STED wavelengths enable the user to freely choose fluorophores for sample labeling and open the possibility of multicolor imaging, invaluable for colocalization applications that benefit from resolution not limited by diffraction.

Here we demonstrate the application of a light source attractive for its inherent simplicity, compactness, broad spectral output, and low cost, utilizing stimulated Raman scattering (SRS) within a standard, single-mode, polarization-maintaining fiber. Unlike a previously reported implementation of STED employing a commercial supercontinuum source [7], the optical power is preserved within narrow spectral peaks within a comb-like spectrum, the output light is linearly polarized, and light suitable for STED is produced below 630 nm.

The phenomenon of SRS in optical fibers is well known [8–11]. When a threshold optical intensity is reached in a fiber, the number of photons with energy \( h\omega_p \) in the pump beam that scatter inelastically in the core becomes significant, yielding lower-energy, Stokes-shifted photons of energy \( h\omega_s \). The degree of the Stokes shift, \( \Delta\omega = \omega_p - \omega_s \), is determined by the Raman gain spectrum for the core material, \( g_P(\Omega) \), which for fused silica exhibits a maximum near 13 THz. Four-wave-mixing and dopants can modify the characteristic spectrum. Longer fibers increase the gain at the Stokes wavelengths quasi-exponentially until the pump wavelength is depleted, the gain is balanced by fiber losses at the pump and Stokes wavelengths, or both. The optimal choice of fiber length balances Stokes gain and fiber losses to yield the widest comb spectrum with the highest power for a given pump intensity.

A simple STED microscope was constructed to utilize the SRS light source and test its viability for gaining subdiffraction resolution. A schematic of the setup is shown in Fig. 1(a). Light with a wavelength of 532 nm was emitted by a passively Q-switched, 60 kHz microchip laser with 1 ns pulses, 0.5 \( \mu J \) average pulse energy, and a 0.3 nm spectral bandwidth (Alphalas GmbH, Göttlingen, Germany) and was collimated before being coupled into the fiber where SRS occurred, which had a 4 \( \mu m \) core diameter and nominal 410 nm cutoff wavelength (Fujikura, Tokyo, Japan). With a coupling efficiency of 50%, peak optical intensities on the order of 500 MW/cm\(^2\) were reached in the fiber at 532 nm. Six Stokes lines were generated using a 50 m fiber, extending from the pump wavelength at 532 to 620 nm. The spectrum of...
light exiting the fiber is shown in Fig. 1(b). The spectral width of each Stokes line increased gradually beyond that of the 532 nm fundamental to ~3.5 nm for the 620 nm line.

Light from the fiber was collimated, and the appropriate STED wavelength for the fluorophore marking the sample was selected with a bandpass filter. This light was coupled into a 30 m single-mode fiber to delay pulses sufficiently for the electronic triggering of excitation pulses for each corresponding STED pulse, a requirement due to both the pulse-to-pulse temporal jitter of the laser (on the order of 1 μs) and the different repetition rates for each Stokes wavelength. Owing to pulse energy fluctuations caused by passive Q switching of the pump laser (energies were bimodally distributed, with approximately half the pulses possessing twice the energy of the remaining half), not all pulses had the requisite energy to produce the most redshifted lines of the comb spectrum, and the repetition rate fell for each subsequent Stokes line, down to 18 kHz for 620 nm. Light exiting the delay fiber was then recollimated and directed through a helical phase delay plate (RPC Photonics, Rochester, N.Y.) and was reflected by a dichroic mirror through the two dichroic mirrors, and focused into a multimode fiber, which acted as a confocal pinhole that was fed into an avalanche photodiode (Perkin Elmer, Vaudreuil, Canada) for fluorescence detection. During data acquisition the sample was scanned through the stationary focus via a piezo stage (Melles Griot, Albuquerqu, N. Mex.), and the image was assembled with in-house image processing software.

To demonstrate the viability of light from the entire comb spectrum for STED resolution enhancement, from the fundamental wavelength to the last Stokes peaks, we performed STED using 532 nm and the last three Stokes lines, at 588, 604, and 620 nm. Typical measurements are shown in Fig. 2. Self-made silica beads resolved at 532 nm were marked with the fluorophore Atto 425 (Atto Tec, Siegen, Germany) and treated with DABCO (1,4-Diazabicyclo[2.2.2]octan) (Roth, Karlsruhe, Germany) to reduce photobleaching. For the remaining three STED wavelengths yellow–green fluorescent beads (Invitrogen, Carlsbad, Calif.) were imaged that contain a fluorophore unspecified by the manufacturer. Neurofilaments labeled with Atto 532 were imaged to demonstrate biological imaging. Additional screening of the dyes Dyomics 485 XL (Dyomics GmbH, Jena, Germany), Rhodamine Green (Invitrogen), and 5-Carboxyrhodamine 6G (Invitrogen) indicated that they are also well suited for STED quenching in the spectral range of 588 to 620 nm, though this list is by no means exhaustive. Average pulse energies at the back aperture of the objective lens for the employed STED wavelengths varied from 0.4 to 2.7 nJ. Because of the 60 kHz repetition rate of the pump laser and the reduction of the repetition rate for redshifted Stokes lines, pixel dwell times for image acquisition were typically between 15 and 30 ms. Pixel sizes of 10 to 20 nm were used. To obtain a reliable estimate of resolution, the images of 30–60 isolated beads from a single measurement were superimposed by aligning the positions of maximum signal from each bead image using in-house software, effectively averaging over the photon noise associated with each bead image. Assuming a Gaussian functional dependence for the effective point-spread function, FWHM values of 70–80 nm were obtained, averaging over the x, y, and both diagonal orientations in the image. FWHM values were typically larger in the x direction by 4% (STED at 532 nm) to 50% (STED at 620 nm) due to residual astigmatism in the STED point-spread function. Depending on the sample, bead sizes ranged from 24 to 45 nm. Using the bead size together with the measured FWHM values from the bead images yielded effective point-spread function values varying from 58 nm (588 and
604 nm STED) to 78 nm (620 nm STED). The lower resolution at 620 nm can be explained by the smaller cross section for stimulated emission of the fluorophore at this wavelength, making STED quenching less efficient. This simple method of implementing STED lends itself to several possibilities for improvement. First, the repetition rate of the pump laser at 532 nm should be brought into the megahertz range while preserving the requisite pulse energies, to facilitate faster image acquisition. To eliminate the variability of the repetition rate of different Stokes lines, a laser with minimal interpulse power fluctuations should be used. This would also enable the SRS source to provide both excitation and STED light for the microscope for suitable dyes, eliminating the need for a separate excitation laser. To ease the peak-power requirement of the SRS pump laser we are investigating the use of small-core optical fibers to increase the intensity of pump light in the fiber core. Further, with the same fiber that was used to produce the spectrum shown in Fig. 1(b), initially eight Stokes-shifted lines were observed, extending to 656 nm, but in the first hours of operation the two most redshifted lines disappeared. At 532 nm (at low power, insufficient for SRS) a reduction fiber transmission was also measured. We believe color-center formation in the fiber caused by the high optical intensities reduced the fiber transmission, an effect observed in other studies [12]. After this initial drop in transmission, however, the fiber characteristics remained stable for many (>6) months. The elimination of dopants in the fiber core should minimize color-center formation and the concomitant drop in fiber transmission, extending the SRS spectrum and increasing the available STED power during long-term operation.

Further development of the multicolor SRS light source holds encouraging potential for STED. It offers conceptual advantages over a supercontinuum source, as it should be possible to tailor the comb spectrum to a desired spectral range for a particular application (for example, by employing different pump wavelengths) while maximizing the pulse energies within each Stokes peak and minimizing the pump power wasted in generating light at unwanted wavelengths, enabling higher resolution. With a megahertz repetition rate and higher powers within each Stokes line, the light source would become an extremely attractive option for spectrally flexible STED imaging. In summary, we demonstrated the viability of a simple, multicolor SRS light source, consisting of a microchip laser coupled into standard optical fiber, for attaining multicolor subdiffraction resolution with a STED microscope.

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References