Frequency dependent detection in a STED microscope using modulated excitation light

Emiliano Ronzitti,[1,3,4] Benjamin Harke,[1,4,*] and Alberto Diaspro[1,2]

[1]*Nanophysics, Istituto Italiano di Tecnologia, via Morego, 30, 16163 Genova, Italy*
[2]*Department of Physics, University of Genoa, 16146 Genoa, Italy*
[3] *Currently with the Wavefront Engineering Group, Laboratory of Neurophysiology and New Microscopies, (INSERM, CNRS), Université Paris Descartes, Sorbonne Paris Cité, Paris, France*
[4]*These authors contributed equally to this work*
[*]*Benjamin.harke@iit.it*

Abstract: We present a novel concept adaptable to any kind of STED microscope in order to expand the limited number of compatible dyes for performing super resolution imaging. The approach is based on an intensity modulated excitation beam in combination with a frequency dependent detection in the form of a standard lock-in amplifier. This enables to unmix fluorescence signal originated by the excitation beam from the fluorescence caused by the STED beam. The benefit of this concept is demonstrated by imaging biological samples as well as fluorescent spheres, whose spectrum does not allow STED imaging in the conventional way. Our concept is suitable with CW or pulsed STED microscope and can thereby be seen as a general improvement adaptable to any existing setup.

OCIS codes: (180.0180) Microscopy; (180.1790) Fluorescence microscopy.

References and links

1. S. W. Hell and J. Wichmann, "Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy," Opt. Lett. **19**(11), 780–782 (1994).
2. M. A. Lauterbach, "Finding, defining and breaking the diffraction barrier in microscopy - a historical perspective," Optical Nanoscopy **1**(1), 8 (2012).
3. E. Abbe, "Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung," Archiv Für Mikroskopische Anatomie **9**(1), 413–418 (1873).
4. S. W. Hell, "Toward fluorescence nanoscopy," Nat. Biotechnol. **21**(11), 1347–1355 (2003).
5. V. Westphal and S. W. Hell, "Nanoscale resolution in the focal plane of an optical microscope," Phys. Rev. Lett. **94**(14), 143903 (2005).
6. K. I. Willig, S. O. Rizzoli, V. Westphal, R. Jahn, and S. W. Hell, "STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis," Nature **440**(7086), 935–939 (2006).
7. J. Keller, A. Schönle, and S. W. Hell, "Efficient fluorescence inhibition patterns for RESOLFT microscopy," Opt. Express **15**(6), 3361–3371 (2007).
8. S. W. Hell, "Microscopy and its focal switch," Nat. Methods **6**(1), 24–32 (2009).
9. B. Harke, J. Keller, C. K. Ullal, V. Westphal, A. Schönle, and S. W. Hell, "Resolution scaling in STED microscopy," Opt. Express **16**(6), 4154–4162 (2008).
10. C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. von Middendorff, A. Schönle, and S. W. Hell, "Direct observation of the nanoscale dynamics of membrane lipids in a living cell," Nature **457**(7233), 1159–1162 (2009).
11. T. J. Gould, J. R. Myers, and J. Bewersdorf, "Total internal reflection STED microscopy," Opt. Express **19**(14), 13351–13357 (2011).
12. M. Leutenegger, C. Ringemann, T. Lasser, S. W. Hell, and C. Eggeling, "Fluorescence correlation spectroscopy with a total internal reflection fluorescence STED microscope (TIRF-STED-FCS)," Opt. Express **20**(5), 5243–5263 (2012).
13. E. Auksorius, B. R. Boruah, C. Dunsby, P. M. P. Lanigan, G. Kennedy, M. A. A. Neil, and P. M. W. French, "Stimulated emission depletion microscopy with a supercontinuum source and fluorescence lifetime imaging," Opt. Lett. **33**(2), 113–115 (2008).
14. J. B. Ding, K. T. Takasaki, and B. L. Sabatini, "Supraresolution imaging in brain slices using stimulated-emission depletion two-photon laser scanning microscopy," Neuron **63**(4), 429–437 (2009).
15. G. Moneron and S. W. Hell, "Two-photon excitation STED microscopy," Opt. Express **17**(17), 14567–14573 (2009).

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

16. P. Bianchini, B. Harke, S. Galiani, G. Vicidomini, and A. Diaspro, "Single-wavelength two-photon excitation-stimulated emission depletion (SW2PE-STED) superresolution imaging," Proc. Natl. Acad. Sci. U.S.A. **109**(17), 6390–6393 (2012).
17. B. Harke, J. Varghese Chacko, C. Canale, H. Haschke, and A. Diaspro, "A novel nanoscopic tool by combining AFM with STED microscopy," Optical Nanoscopy **1**, (2012).
18. S. Galiani, B. Harke, G. Vicidomini, G. Lignani, F. Benfenati, A. Diaspro, and P. Bianchini, "Strategies to maximize the performance of a STED microscope," Opt. Express **20**(7), 7362–7374 (2012).
19. C. A. Wurm, D. Neumann, M. A. Lauterbach, B. Harke, A. Egner, S. W. Hell, and S. Jakobs, "Nanoscale distribution of mitochondrial import receptor Tom20 is adjusted to cellular conditions and exhibits an inner-cellular gradient," Proc. Natl. Acad. Sci. U.S.A. **108**(33), 13546–13551 (2011).
20. B. Harke, "3D STED microscopy with pulsed and continuous wave lasers," Niedersächsische Staats-und Universitätsbibliothek Göttingen (2008).
21. D. Wildanger, E. Rittweger, L. Kastrup, and S. W. Hell, "STED microscopy with a supercontinuum laser source," Opt. Express **16**(13), 9614–9621 (2008).
22. V. Westphal, S. O. Rizzoli, M. A. Lauterbach, D. Kamin, R. Jahn, and S. W. Hell, "Video-rate far-field optical nanoscopy dissects synaptic vesicle movement," Science **320**(5873), 246–249 (2008).
23. N. Chen, C. H. Wong, and C. J. Sheppard, "Focal modulation microscopy," Opt. Express **16**(23), 18764–18769 (2008).
24. G. Vicidomini, G. Moneron, C. Eggeling, E. Rittweger, and S. W. Hell, "STED with wavelengths closer to the emission maximum," Opt. Express **20**(5), 5225–5236 (2012).
25. R. Kasper, B. Harke, C. Forthmann, P. Tinnefeld, S. W. Hell, and M. Sauer, "Single-Molecule STED Microscopy with Photostable Organic Fluorophores," Small **6**, 1379–1384 (2010).
26. T. Staudt, A. Engler, E. Rittweger, B. Harke, J. Engelhardt, and S. W. Hell, "Far-field optical nanoscopy with reduced number of state transition cycles," Opt. Express **19**(6), 5644–5657 (2011).
27. R. Schmidt, C. A. Wurm, S. Jakobs, J. Engelhardt, A. Egner, and S. W. Hell, "Spherical nanosized focal spot unravels the interior of cells," Nat. Methods **5**(6), 539–544 (2008).

1. Introduction

Stimulated Emission Depletion (STED) Microscopy [1] has been extensively established as a revolutionary fluorescent optical method to overcome the diffraction limit [2, 3] towards nanometer ranges [4, 5]. The STED concept, firstly conceived and realized by Hell's group [1], proposed to modify a traditional scanning microscope scheme by coupling the focused excitation beam to a spatially-structured depletion beam. In particular, a depletion intensity pattern forming a central zero in the focus (i.e. a doughnut shape) [6, 7] enforces a spatially selective de-excitation to the ground state of the peripheral fluorescent molecules and a confinement of the fluorescence emission only to the region close to the centre. This leads to a shrinking of the area of fluorescent molecules implying an improvement of the effective resolution of the system [8]. Remarkably, since the resolution scaling is defined by the efficiency of the depletion process which has no a priori theoretical photophysical constrictions, the achievable resolution is in principle unlimited [5, 9]. The use of standard fluorophores in identical experimental conditions as in confocal microscopy makes it straightforward to combine STED with known imaging and analysis techniques such as fluorescence correlation spectroscopy [10], total internal reflection fluorescence [11, 12], fluorescence lifetime microscopy [13], two-photon microscopy [14–16], atomic force microscopy [17], etc.

However, practically, the resolution of a STED microscope is still limited by some factors which hamper the efficiency of the excited state de-population of the molecules among the depletion region. Such inefficiency is driven by phenomena which either limit the efficiency of the STED effect (e.g., limitation in depletion laser power deliverable) [7] or favor a re-population of the excited state during the STED beam illumination (e.g., direct excitation of the fluorescent molecule induced by the depletion laser beam) [8]. The net consequence is a non-neglectable number of molecules populating the excited state in the region where the STED beam falls which thereby determines a residual of fluorescent signal in the vicinity of the focus centre. Clearly, especially in densely packed samples, the magnitude of such fluorescent residuals can compromise in practice the resolution scaling results, ranging from a hampering of the signal to noise ratio to a severe loss of any super-resolution capability.

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

Still, according to some experimental conditions [18], the STED effect can be easily obtained with the available depletion laser powers as demonstrated by several experimental applications [6, 9, 19] where an outstanding resolution improvement is obtained. In particular, in order to avoid the excitation of the fluorophores by the depletion beam, it is essential that the STED wavelength is chosen sufficiently far from the dye's excitation spectra, so that the direct excitation by the STED beam can be neglected and the excited state can be easily depopulated. Otherwise, the high power employed for the depletion laser, can induce massive STED excitation rates [10]even for low absorbance wavelengths, compromising any super-resolution achievements. This clearly represents a bothersome drawback which limits the applicability of the STED microscopy to just particular combinations of fluorophores and laser lines at the proper wavelength distances. Unfortunately, depletion wavelengths sufficiently far from the excitation range lie usually in the tail of the emission spectra where the cross-section for stimulated emission is low. Such a low stimulated emission cross-section has to be necessarily compensated by very high depletion laser powers. When the STED beam contributes significantly to the overall fluorescence the excitation intensity can be enhanced in order to ensure to some extend the required contrast for imaging. However, that strategy cannot be followed in the case of the fluorescence being already near the saturation regime and in any case a higher excitation intensity comes along with higher photobleaching and especially in the case of a CW STED laser combination, the final resolution suffers from a high excitation level.

It is clear that methods able to avoid the detection of fluorescence signal generated by the STED laser are highly desirable. In this article, we use temporal modulation of the excitation light in order to decouple the fluorescent emission induced by the STED laser from the fluorescence signal induced by the excitation light. The paper describes the theoretical concept and shows the experimental validation of such a proposed Modulated Stimulated Emission Depletion (ModSTED) microscopy.

Theory

In order to estimate the contribution of the excitation induced by the STED beam on the STED microscopy performances, we consider a population of fluorophores subjected to a CW excitation intensity I_{ex} and an CW intensity I_{sted}.

The fraction of molecules in the excited state N_1, linearly related to the fluorescent signal, is given by:

$$\frac{dN_1(t)}{dt} = -(k_f + k_{sted}) \cdot N_1(t) + (k_{ex} + k'_{sted}) \cdot N_0(t) \tag{1}$$

where k_f is the fluorescence rate inversely related to the fluorescence lifetime, while k_{sted} is the depletion rate; k'_{sted} and k_{ex} are the excitation rate induced by the STED laser beam and the excitation laser beam, respectively. N_0 is the fraction of molecules in the ground state.

The different rates depend on the excitation wavelength λ_{ex} and the depletion wavelength λ_{sted}, through:

$$\begin{cases} k_{sted} = \sigma_{em}(\lambda_{sted}) \cdot I_{sted} \\ k'_{sted} = \sigma_{ex}(\lambda_{sted}) \cdot I_{sted} \\ k_{ex} = \sigma_{ex}(\lambda_{ex}) \cdot I_{ex} \end{cases} \tag{2}$$

where σ_{ex} and σ_{em} represent the absorption and stimulated emission cross-section, respectively. Assuming the system in temporal equilibrium and $N_0 + N_1 = 1$, the fraction of molecules in the excited state is

$$N_1 = \frac{k_{ex} + k'_{sted}}{k_f + k_{sted} + k_{ex} + k'_{sted}} \tag{3}$$

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

Assuming $k_f >> k_{ex}$ and $k_{ex} >> k'_{sted}$ [20], thus in the hypothesis of being far away from any excitation saturation and using a STED wavelength far away from the excitation spectrum, by increasing the STED intensity, the complete depopulation of the excited state can be most efficiently obtained.

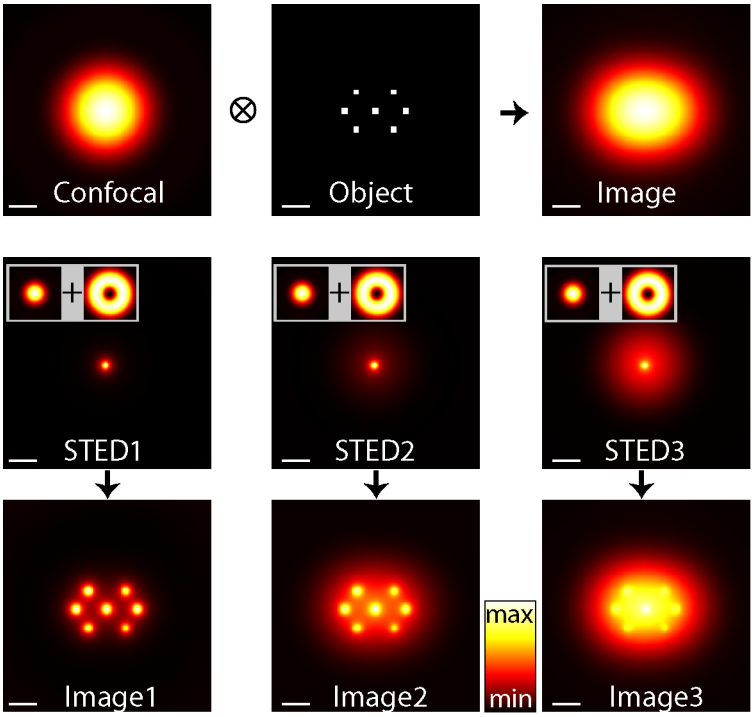

Fig. 1. Simulation on the effect of residuals of fluorescence on STED microscopy performances. Confocal image of a group of subdiffracted beads (upper row). Middle row: STED image of a single point emitter (effective point spread function) by assuming k_{ex} = 0.01*k_{sted} and k'_{sted} = 0 (left), k'_{sted} = 0.1*k_{sted} (middle) and k'_{sted} = 0.5*k_{sted} (right). Bottom row: STED image of a group of point emitters in sub-diffraction distance imaged by the above described effective point spread functions. Scale bars: 100 nm.

Clearly, the power of the STED laser requested to achieve a complete depopulation of the excited state is strictly related to the stimulated emission cross-section. A high σ_{em} is desirable, as in this case the complete depopulation of N_1 could be achieved using a STED laser at relatively lower power.

However, a high σ_{em} is obtained for wavelengths ranging around the emission peak, which unfortunately correspond to spectra regions where significant tails of the excitation spectrum lie. In this condition, the repopulation of the excited state driven by the STED laser can no more be neglected. Consequently, an increase of the STED power does not unequivocally induce a complete depopulation of the excited state, but depending on the wavelengths employed the competitive processes ruled by k_{sted} and k'_{sted} can only lead to a partial depopulation of the excited state in the equilibrium.

In Fig. 1 the effect of partial depopulation of the excited state in STED microscopy, thus of residual fluorescence along the doughnut depletion region, is illustrated by the imaging simulation of single point emitters (top row, middle panel). Imaging with a conventional confocal focus (top row, left) cannot resolve these points since they are placed in sub-diffraction distance (top row, right). The superposition of a Gaussian excitation spot with a STED beam shaped in a doughnut-like configuration forms an effective focal area whose dimensions are remarkably reduced (Fig. 1 middle row, left). When modelling a residual

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

fluorescence from the doughnut region, the Full Width Half Maximum (FWHM) of the effective focal area of a STED microscope is not directly influenced, but a fluorescent halo around the peak is formed (middle row; middle and right image). Without residual fluorescence (bottom row, left) the structure can be well resolved while the situation radically changes when imaging the spot structure with a residual fluorescence. In this case the superposition of the residual fluorescent halos given by an incomplete STED process dramatically decreases the imaging contrast, preventing any capability to distinguish the different objects.

In current STED microscopes, relatively long STED wavelengths lying in the far tail of the emission spectrum are employed and a high depletion laser power has to be delivered.

In this scenario, a method which allows extending the range of STED depletion laser wavelengths and avoiding the fluorescence induced by the same STED laser would be of obvious interest.In order to achieve this goal, let us remember the essential aim of the STED microscopy: suppress the fluorescence emission from the molecules in the vicinity of the focus. Hence, in order to avoid the limitation introduced to STED microscopy by a possible STED laser excitation, we don't need to eliminate the STED repopulation phenomenon, but simply eliminate the fluorescent signal induced strictly by the excitation of the depletion laser. Thus, from this perspective, we simply would need a system able to produce a controlled decoupling of the fluorescence emission induced by the excitation laser from the emission due to the STED depletion laser. In this way, we could be able to distinguish and thus reject the undesired fluorescence residuals emerging along the doughnut region as fluorescent halos around the central sub-diffraction sized effective PSF spot.

In order to reach this aim, we propose to temporally modulate the excitation beam. Assuming a temporal linearity between the excitation and emission, the distinction of the fluorescence emission due to the depletion laser and the excitation laser is possible discriminating the collected light on the basis of its temporal frequency. In the following paragraph, this concept will be experimentally described and validated.

Experimental setup

All experiments presented are made on a custom made STED microscope based on two continuous wave lasers. The excitation laser 532nm (Sapphire, Coherent, USA) is modulated by an externally placed acousto optical modulator (AOM, AA Optoelectronic, France) which is modulated by an analog voltage delivered by a frequency generator (81150A, Agilent Technologies, USA). Another CW laser with a fixed wavelength of 642nm (MPB Communications, Canada) delivers the light needed for the STED beam path. A vortex phase plate (PP, RPC photonics, USA) placed in the STED beam path generates the required phase retardation so that a doughnut shaped light distribution in the sample is formed. Excitation and STED beam are combined by a dichroic mirror (AHF Analysentechnik, Germany) and their foci are scanned through the sample by a pair of scanning galvanometric mirrors (Cambridge Technologies, USA).

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

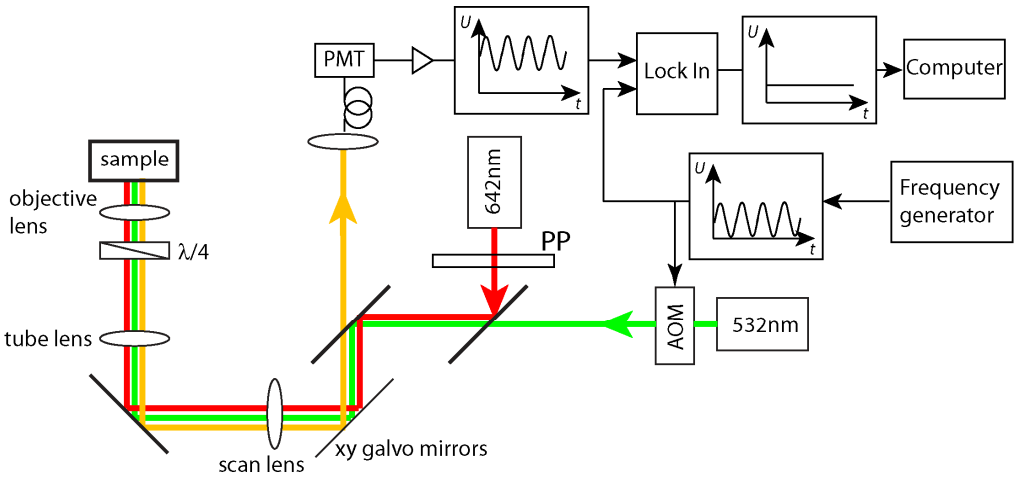

Fig. 2. Schematic of the custom made STED microscope. AOM: Acousto optical modulator, PMT: photo multiplier tube, PP: phase plate.

By a scan and tube lens combination (Leica Microsystems, Germany) the mirrors are placed into the conjugated plane of the back focal plane of the objective lens (OL, NA 1.4, Oil immersion, PLAN APO, Leica Microsystems). The fluorescence is descanned, separated from the other beams by a proper dichroic mirror (AHF Analysentechnik) and focused onto a multimode fiber (Thorlabs, Germany) acting as a pinhole corresponding to a size of 1 Airy unit. The output of the fiber is connected to a GaAsP photo multiplier (PMT, Hamamatsu, Japan) generating an output current corresponding to the fluorescence signal. A current amplifier (sim918, Stanford Research Systems, USA) converts the current into a voltage with a fixed ground level which is then read by a lock-in amplifier (sr844 rf, Stanford Research systems) set at the fundamental harmonic of the excitation laser ensuring that only the emission intensity produced by the modulated excitation laser is collected. The filtered signal is then sent to the setup PC.

Results

We tested our setup with fluorescent spheres (40nm red fluorescent, Invitrogen, USA) which have been published previously especially after imaging in a STED microscope based on a super continuum laser source [18, 21]. Usually for these kinds of sample, an excitation wavelength of around 570nm excites the molecules and fluorescence is detected around 610nm. For successful STED imaging, a STED wavelength of around 700nm is chosen in order to avoid fluorescence generated by the STED beam. The excitation and emission spectra are shown in Fig. 3(a). The use of a 642nm STED laser wavelength would be highly desirable to depopulate the singlet state as it corresponds to an emission spectrum range where the stimulated emission cross-section is relatively high. However, considering the high depletion power employed, a not negligible amount of molecules can be excited by the STED beam even if the excitation cross section is remarkably weak (see also inset of Fig. 3(a)).

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

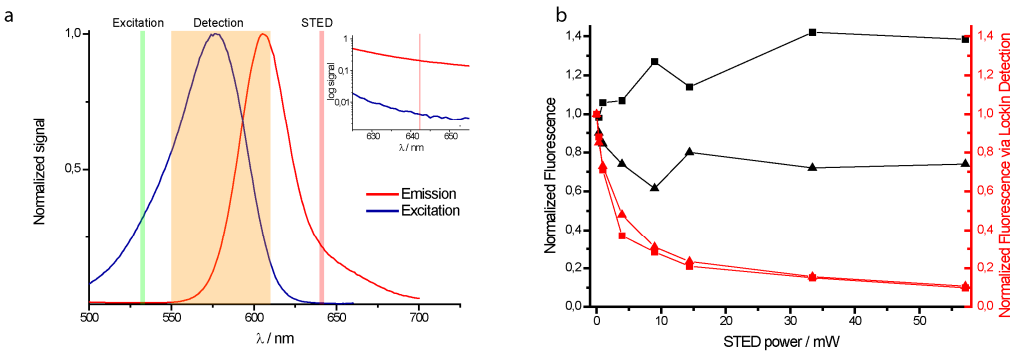

Fig. 3. (a) Excitation and Emission spectrum of red fluorescent spheres (Invitrogen). (b) fluorescence depletion curve measured in the classical mode (black) and in the modSTED configuration (red) for two different excitation powers of 7.1µW (squares) and 11.4µW (triangles). All power values are measured in front of the back aperture of the objective lens.

In order to understand whether this sample can be imaged also with our wavelength combination of 532nm and 642nm of excitation and STED wavelength, respectively, we tested the fluorescence reduction for various STED intensities measured on single fluorescent beads placed on a poly-L-lysine (Sigma Aldrich, Europe) covered glass slide. The fluorescence is measured while illuminating a single bead with excitation and STED light and then normalized to the fluorescence signal emitted when only the excitation light is switched on. All foci are Gaussian shaped, so the phase plate in the STED beam path has been removed. The excitation beam power is modulated with a frequency of 20kHz while the STED beam intensity is kept unmodulated.

The data directly measured by the PMT is presented in the black graphs of Fig. 3(b) for two different excitation powers (11.4µW, triangles and 7.1µW, squares). It shows the massive fluorescence signal generated by the STED beam and the fact that even exciting with higher excitation power cannot lead to a significant fluorescence decrease. Clearly, sub-

diffraction resolution STED imaging on such kind of sample is barely possible in the classical STED scheme. If we analyze the same data by means of a lock-in amplifier, we can separate the fluorescence generated by the excitation beam from the signal generated by the STED beam. This can be done in real time and the data are shown in the red graph of Fig. 3(b). In this modSTED configuration the fluorescence follows nicely the expected depletion curve which demonstrates the possibility to perform STED when the fluorescence generated by the STED beam is removed from the overall fluorescence signal. Notably, in the modSTED configuration the fluorescence can be effectively reduced independently from the excitation power. The capability to effectively switch off the fluorescence is a key experiment within the STED concept: once the fluorescence signal can be sufficiently reduced by the presence of a STED beam, sub-diffraction resolution imaging is possible. For this, we placed back the phase plate into the STED beam path in order to create a doughnut shaped focus. We performed imaging of the same sample used for the previous experiments. As above mentioned, we use for all experiments a modulation frequency of 20 kHz due to some constrains in bandwidth of one our hardware components, i.e. the current amplifier. Otherwise, higher frequencies are possible as long as they are not in the same regime as the fluorescence lifetime since this would change the sinusoidal behavior and by this the detection efficiency. For imaging fluorescent samples with modSTED the modulation frequency gives an upper limit for the imaging speed: the time constant of the lock-in amplifier has to be longer than one period of the modulation frequency. In order to avoid aliasing effects, the pixel dwell time has to be at least as long as this time constant which is set in our experiments to 100 µs.

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

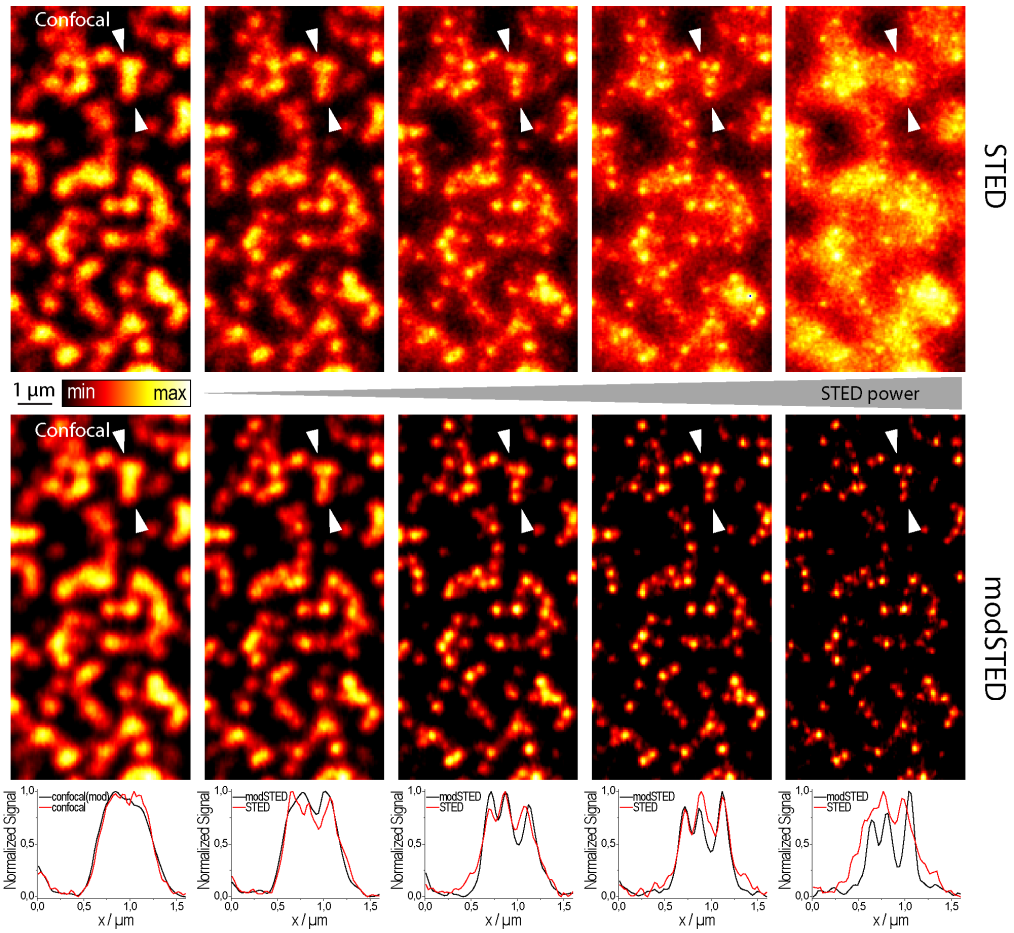

Fig. 4. Comparison of STED images in conventional STED (top row) and ModSTED configuration for different STED powers (4.4 mW; 16.1 mW ; 44.5 mW; 124.5 mW)from left to right) imaging Red Fluorescent 40nm beads with 532nm excitation (11.4 µW) and 642nm STED beam. All power values measured at the back aperture of the objective lens. Confocal standard images (top row, left column) and filtered by lock-in Amplifier (bottom row, left image). Pixel dwell time: 120 µs. Bottom line: line profiles for each STED power value along the path indicated in the image. For better visualization all data were smoothed by a standard Gaussian low pass filter.

Figure 4 shows different sets of measurements, performing conventional STED (top row) and modSTED (middle row) at different STED powers. As notable, a strong background appears in the conventional STED mode (top row) and only after filtering with the lock-in amplifier, the full data can be extracted (middle row). For better visualization, line profiles for each STED power setting along the indicated path are shown in the bottom row. For comparison of the techniques each profile has been normalized to its minimum and maximum value. The problem of increasing background in the conventional STED mode gets more significant while imaging with higher STED power. This is especially true for the case of densely packed areas, where fluorescence signal emitted by several spheres within the area where the STED light falls, gets more evident. In this case, it is impossible to extract information from the data. Filtering the fluorescence signal by means of a lock-in amplifier shows that the fluorescence induced by the STED beam can be effectively removed from overall fluorescence, thus super-resolution performances are completely maintained and the image

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

shows outstanding resolution. Notably, the filtering happens in real time and does not require any *a priori* knowledge of the sample.

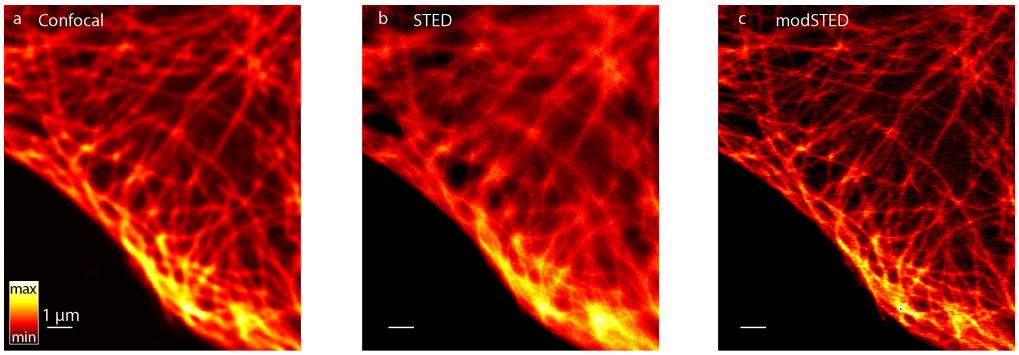

Fig. 5. Imaging of microtubules immunolabeld by the dye ATTO550 and imaged in Confocal mode (a), conventional STED mode (b) and in modSTED (c) configuration. Excitation power 1.8 µW, STED power 180 mW. Pixel dwell time: 100 µs. All data is raw data without any additional post-processing computation.

Finally we tested the modSTED concept also in biological samples, in which the microtubule are immunolabeled by the dye ATTO550 (Atto-Tec GmbH, Siegen, Germany). Notably this dye is basically compatible to the wavelength combination the setup can offer. However, in order to perform STED imaging with an acceptable performance, a high excitation power has to be applied in order to compensate for the background induced by the STED light. This high excitation power surely induces a higher amount of photo bleaching and in case of a living sample would increase photo toxicity. For our test we applied a comparatively low excitation power of 1,8 µW which is sufficient to obtain a confocal image (Fig. 5(a)). But in this case, the STED beam generates a strong fluorescent background which significantly reduces the image

quality in particular in areas where many filaments are located (Fig. 5(b)). When we apply the modSTED technique, we can recover the full image information and the massive background generated by the STED beam can be efficiently removed.

Conclusion

In this paper, we propose a new method, named ModSTED, to overcome one of the major limitations of traditional STED microscopy. By temporal modulation of the excitation beam intensity and by proper frequency dependent detection, the fluorescence signal generated by the STED beam can be completely removed from the overall fluorescence signal and by this, only the desired fluorescence generated by the excitation beam is recorded. This method is especially important for the flexibility of a STED microscope. Due to the STED beam excitation, the choice of excitation and STED wavelength is often very inflexible to small spectral shifts of the investigated dyes. In this work, we showed the capability to image a dye whose spectrum is red shifted relative to the proper dye in classical configuration. Additionally, we showed biological imaging of microtubules decorated by the dye ATTO550 which was excited with very low light level in order to reduce photobleaching and photo toxicity. ModSTED allowed imaging these samples without any significant loss in SNR, which has been impossible in the classical STED mode. Importantly, no *a priori* knowledge of the sample is required and the filtering performed by a Lock in amplifier happens in real time during the image acquisition. The image acquisition time was limited by the time constant of the lock-in amplifier and the modulation frequency. Both factors can be further optimized in practice in order to achieve fast imaging as it can be achieved by usual STED microscopy [22]. The efficiency of the modSTED method is strictly related to the performances of the frequency detection system which ultimately determines the SNR of the

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013

demodulated image. In particular, a high sensitivity of the lock-in amplifier is required in those situations where the constant fluorescence signal induced by the STED beam is notably high in comparison to the amplitude of the modulation of the fluorescence signal induced by the excitation beam. In other situations where the modSTED concept is still required but the amplitude of the modulated fluorescence signal is high in comparison to the constant fluorescence signal generated by the STED beam, the fairly expensive lock in amplifier can be also replaced by a more cost effective electronic frequency band pass or by a computer performing a Fast Fourier Transform in order to separate and filter the signal in the frequency space [23].

Alternatively, the fluorescence contribution by the STED beam can also be removed frame or line wise by simply recording the signal generated by the STED beam and subtract it from the overall image. However, this approach can introduce artifacts since photobleaching during a line or a frame scan changes the signal level. With modSTED the filtering happens pixel by pixel and thereby excludes any competing effects. A pixel by pixel subtraction can be also achieved when using a pulsed STED laser which has twice the repetition rate of the pulsed excitation laser [24] and a time gated detection. Still, this method is restricted to the use of fairly expensive pulsed laser systems and fast electronics to deal with high laser repetition rates.

It is worth mentioning that the modSTED strategy does not avoid the STED beam excitation of the fluorophore, but simply rejects this contribution from the overall image. Consequently, the STED beam can lead to an increase in photobleaching respect to more red-shifted depletion wavelengths just due to an enhanced number of cycling the fluorophore undergoes. In case of working with samples with a very limited photo stability, combining modSTED with approaches such as the use of appropriate chemical buffers [25] or concepts to reduce the cycling number of the fluorophore [26] can effectively reduce the photobleaching problem.

The concept modSTED is not restricted to specialized STED microscope, but can be added to any pulsed or CW STED configuration. Often, in a pulsed STED microscope a repetition rate of 80 MHz is used to image fast and since this frequency is more than three orders of magnitudes different from the modulation frequencies used here, it does not interfere and therefore modSTED can be successfully applied. Also in case of two photon excitation in combination with pulsed [16] or CW [14, 15] STED beam, the low excitation level has to compete with the background generated by the STED beam excitation which decreases thereby the SNR. Also here, modSTED should be able to filter the signal originally generated by the two photon excitation laser. In conclusion, modSTED can be adapted to any already existing STED microscope as long as the excitation laser source can be frequency modulated which can be done internally in the laser control or by applying an external low-cost AOM.

Since with modSTED, the excitation power can be set completely independently from the STED beam power, it can be set to low values reducing the overall stress in live cell experiments. Additionally, multi-color schemes such as the work with long stoke shift dyes [27] are inherently simplified and opens the way for modSTED to become a very standard detection scheme in STED microscopy.

Acknowledgments

We kindly want to acknowledge Dr. Bruno Torre for the support with the electronic equipment and Dr. Giuseppe Vicidomini for fruitful discussions.

#179631 - $15.00 USD Received 12 Nov 2012; revised 20 Dec 2012; accepted 21 Dec 2012; published 3 Jan 2013