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STED microscopy – easier instruments broadens the use

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Abstract

Stimulated Emission Depletion (STED) microscopy has been convincingly demonstrated for fundamental studies in cells, living tissue and organisms. Today, a major trend in the STED technique development is to make the instruments simpler and more user-friendly, without compromising performance. This has become possible by new low-cost, turn-key laser technology and by implementing specifically designed phase plates and birefringent segmented polarization elements. With these simpler and cheaper realizations of STED now becoming more broadly available, and with a parallel development of sample preparation and fluorophore reporter molecules ultimately setting the limit of the image quality, contrast and resolution, we can expect a significant increase in the use of STED, in science as well as for clinical and drug development purposes.

Highlights

- STED imaging of cells, living tissue and organisms has been convincingly demonstrated
- The STED technology has in the last 6-7 years become far easier to realize
- This largely relies on new laser technology and customized optical elements
- New fluorescent markers and sample preparation procedures support the development
- By increased accessibility, a strong increase in the use of STED can be expected

Introduction

Light microscopy has remained one of the most central tools in cellular and molecular biology ever since its invention four centuries ago. By fluorescence markers and labeling tools it is possible to non-invasively image specific components and processes in cells, tissues and whole organisms with utmost sensitivity and specificity. 20 years ago, light microscopy seemed in many aspects to be close to its maximum theoretical abilities. However, following the original work of Hell and Wichmann in 1994 [1] new diffraction-unlimited far-field optical imaging techniques, more widely referred to as super-resolution microscopy techniques, have launched a new era in cellular and molecular biology. In these techniques the diffraction limit of resolution, i.e. the fact that no sample can be imaged with a resolution beyond the wavelength of light by which the sample is observed, can be overcome by switching the emission of light from imaged objects within the sample on and off. Fluorescent objects or molecules in an imaged sample that are less than 200nm apart can now be resolved from each other with far-field light microscopy, if they can be observed separately in time.

In their work [1], Hell and Wichmann suggested laser excitation to be combined with stimulated emission of fluorescent molecules to turn the spontaneous fluorescence process on and off in a spatially targeted manner, thereby providing the means to resolve molecules separated in space by down to tens of nanometer. This far-field super-resolution imaging technique known as STED (stimulated emission depletion microscopy) has since then been followed by several other super-resolution imaging techniques, offering selective advantages, but all relying on the same basic on and off switching principle to resolve objects that are closer together than the resolution limit.

In this review, we first briefly describe the principle of STED, then mainly focus on methodical developments of STED that has occurred in the last 6-7 years (*since Hell "Far-Field Optical Nanoscopy" Science 2007, **316**:1153-1158*). We end by highlighting a few application areas and with a brief outlook on how the technique may develop in the next years.

Principle

In STED, targeted switching can confine fluorescence to exclusively occur within a nanoscale area, thus separating and resolving neighboring fluorescently labeled

molecules. This concept of super-resolution imaging only requires adding a (red-shifted) STED laser to the excitation beam of a scanning confocal microscope, with the STED beam profile modified to have no light in some area(s) of the excitation beam focus. Most often the profile is optically sculptured to be doughnut-shaped in the focal plane (i.e. a ring of high laser intensity surrounding a 'hole' of zero intensity, see figure 1).

FIGURE 1: Overlapping the STED doughnut (red) onto a Gaussian shaped excitation laser beam profile (blue) allows spatially targeted switching, where fluorescent molecules in the periphery of the excitation beam are 'switched off' by stimulated emission. The few molecules in the central part of the excitation beam on the other hand are allowed to generate fluorescence signal (green), in the absence of stimulated emission in the 'hole' of the STED beam. By scanning the co-aligned beams over the fluorescent sample, detecting sequentially the fluorescence from neighboring nanoscale areas, a super-resolution image can be generated. No data processing is necessary as the raw detection of nanoscale separated positions is the result of a pure physical (optical switching by stimulated emission) process. Mathematical processing to enhance the image quality and even further separate labeled fluorescent molecules are however possible to apply as an extra step. Lower right: Images of fluorescent beads acquired with a dual color STED instrument with the STED beam off (left) and on (right).

Apart from STED, several other complementing fluorescence super-resolution microscopy techniques are also at hand today, primarily based on localization of optically switched individual fluorescent molecules (PALM, photoactivated localization microscopy; STORM, stochastic optical reconstruction microscopy), or on the application of spatially non-uniform illumination and targeted (nonlinear) switching (SIM, structured illumination

microscopy). The principles, applications and recent advances of these techniques have been discussed elsewhere [2,3], and goes beyond the scope of this review. However, it can be noted that while all techniques share many features, they are also distinct in several ways (data generation, speed, multiplexing, resolution and analysis). This allows life scientists to select the most suitable super-resolution microscopy technique for their particular application [4]. Hallmarks of STED include the possibility of: nanoscale optical sectioning (due to the relatively low background in thicker samples), high imaging rates (allowing e.g. tracking of dynamical processes and suppressing artifacts due to imaging drift), direct generation of (raw) super-resolution images (i.e. data processing is optional), tuning ability of the resolution (if a lower resolution enhancement is required, fewer molecules need to be switched off, and higher fluorescence rates and imaging speeds are possible). Finally, a relatively large selection of standard organic fluorophores and fluorescent proteins has been found suitable for STED.

Major technological progress since 2007

Supported not the least by strong progress in laser technology, the STED technology has in the last 6-7 years become far easier to realize. Expensive and somewhat complex pulsed laser system previously required can now be replaced by simpler, user-friendly turn-key lasers. One example is the introduction of continuous wave laser sources for stimulated emission (CW-STED), abolishing the need for sub-nanosecond overlap of excitation and depletion pulses [5]. Confinement of the focal spot by CW-STED generated resolution down to 30 nm laterally and about 150 nm axially, showing that adding a bright doughnut-shaped CW laser beam for STED can convert a regular scanning (confocal) fluorescence microscope into a 3D nanoscale resolving system. Originally realized with scientific CW lasers, CW-STED has also been proven to be well realizable with more compact and low-cost CW semiconductor lasers [6]. Further, as the STED beam has a spatially-varying strength around its minimum the (stimulated and spontaneous) lifetimes of neighboring fluorophores will also vary. By time-gating the detected fluorescence signal this spatially encoded fluorescence lifetime information can be exploited to further improve the nanoscale

separation, which has been found especially beneficial for CW-STED systems [7,8].

Another benefit of the laser development is the implementation of spectrally broad laser systems (white light laser sources or stimulated Raman scattering light sources) into STED, which has proven to be a straightforward way to strongly simplify the first generation of STED instruments. Now, excitation and depletion pulses in several spectral intervals from one and the same light source can be generated, thereby offering a convenient means for multicolor STED [9,10,11,12]. Lateral resolution of down to 20-30 nm and a 3-dimensional volumetric resolution of 45 x 45 x 100 nm have been established using a single white light laser for both excitation and depletion, enabling even in vivo imaging of the fluorescent protein eGFP in *C. elegans* [13]. Further, by exploiting also fluorescence lifetime properties to separate labels, three-color super-resolution biological imaging has been demonstrated [14]. A simplifying step in multi-color co-localization studies can also be offered if only one STED beam can be used for several dye emissions, as exploited e.g. in the commercial STED systems offered by Leica. Since the doughnut defines from where the signal may originate, misalignments of the two different excitation foci can be simultaneously compensated by the central zero of the single STED beam.

Apart from 3D imaging there is a strong demand for STED imaging deeper into cellular and histological samples. For this reason, several groups have extended STED microscopy to two-photon excitation using different optical systems and modalities, allowing imaging deeper into e.g. brain tissue slices [15,16,17,18]. Switching from high-numerical aperture oil objectives (index matched to $NA \sim 1.51$) commonly used for STED to more biocompatible glycerol-optics (index matched to $NA \sim 1.46$) [19], implementation of adaptive optics in STED microscopy to cope with strongly aberrated imaging conditions [20], as well as advances in optically matching materials applicable for fixed groups of cells or

tissue slices [21] have also advanced the possibility to image deeper into biological tissue samples.

Given the often challenging and laborious preparation of biological samples for super-resolution microscopy, there is a need to make the technical aspects of STED even more simple and rugged in order to more easily bring it within reach of a broader community of life scientists. To meet this need several developments beyond implementation of new laser technology have been undertaken, not the least regarding the focal profile used for targeted switching. In particular, the application of a common phase-filter for the excitation and STED beam and the use of birefringent segmented polarization elements have been designed and applied [22,23]. Addition of such an optical element to an existing fluorescence confocal microscope, equipped with suitable lasers, turns the system momentarily into an “easy-STED” system.

As the biological cell is a densely packed microcosmic world, imaging at the nanoscale in three dimensions is often necessary to be able to reveal its inner secrets. This has provided a strong incentive to develop 3D STED approaches, with targeted switching also along the focal direction (z-axis). Practically, the axial beam-profile has been modified by phase-filters to spatially sculpture a light distribution with a central (z-axis) minimum, and in combination with a 2D STED-beam profile (improving the resolution along the x,y-axes) three dimensional nanoscale imaging has been achieved with a z-resolution approaching 100 nm [10,24]. Even higher resolution (40 x 40 x 40 nm) can be reached by technologically complex 4PiSTED systems using dual-opposing high-numerical objectives to generate interfering focal patterns, which suppresses the spontaneous fluorescence process isotropically everywhere except in the small central region [25,26].

One of the major strengths of STED as a super-resolution imaging technique is its imaging speed. Even video-rate imaging (albeit for smaller regions of interest) has been achieved using fast scanning technology, and has been applied to dynamically follow for example labeled synaptic vesicles in axons [27]. As a complementary approach, Fluorescence Correlation Spectroscopy (FCS) can be readily realized together with STED, opening for molecular dynamic studies on spatial scales not within reach by other techniques. Following initial proof-of-concept studies (*Kastrup et al. Phys Rev Lett* 2005, **94**, 178104), STED-FCS has enabled direct observation of the nanoscale dynamics of membrane lipids and proteins in live cells [28,29] and can provide a unique angle of view of the functional role of lipid-lipid and lipid-protein interactions in cellular signaling and regulation.

With an increased resolution follows also correspondingly higher demands on the biological sample preparation and on the labeling density. Ultimately, the quality of an image, its contrast and resolution, is determined by the performance of the fluorescent reporter molecules. To get the same per-pixel signal-to-noise ratio in STED as in conventional microscopy the typically fewer fluorescent molecules within each of the significantly smaller pixels have to deliver a similar fluorescence dose, and typically within shorter interrogation times. This is in principle a requirement STED has in common with all other fluorescence-base super-resolution imaging techniques. Moreover, to deliver performance in STED intrinsic states of the molecules need to be selectively targetable (by wavelength, time and location) in the optical switching process. Taken together, the demands set on the reporter molecules are high in STED microscopy, but since a broad range of fluorescent reporters has been found to meet the requirements, advances in applications are not limited by a lack of reporter molecules. There are viable alternatives over a broad spectral range, and for several categories of reporters:

Several genetically expressed fluorescent proteins have proven to be suitable reporters for STED microscopy. Most commonly used are clones of GFP, YFP's and some red fluorescent proteins [30,31,32]. This opens for a manifold of live

cell imaging studies to be carried out with substantially improved spatial resolution by STED in the future.

Bright and photostable organic fluorophores have since the dawn of STED microscopy been used, and more than twenty dyes are listed as suitable for nanoscale imaging (see *www.nanoscopy.de*). By ongoing development, new dyes are continuously added to the list, combining appropriate photophysical properties with good biocompatibility [33,34].

Nanocrystals is an emerging category of probes that have recently been found to possess excellent photo-switching properties for STED [35,36]. Trapped atomic vacancies inside the rock solid crystal function as luminescent emitters that can be optically switched by STED. Manufacturing advances are being pursued (like the development done in the last decade regarding Quantum Dots) to produce biocompatible nanocrystals with single vacancies for biological nanoscale imaging.

Obviously, size matters when it comes to super-resolution. The high labeling densities needed to claim tens of nanometers in resolution raises issues regarding dye-dye interactions and possible perturbations from the labels and their linker molecules onto the sample. Further, steric effects also set an upper limit for how high labeling densities that can be reached. Development and advances in methods for site-specific targeting of small-molecule probes to cellular proteins thus fits well into the needed toolbox to allow accurate nanoscale dissection of true biological topology. Along this line, several approaches to make smaller affinity and linker molecules developed in fluorescence microscopy have also been shown to be suitable for STED imaging [37,38,39].

Applications

Previous advancements in fluorescence microscopy have typically followed a route of improvement, including single color 2D-imaging, multi-color 2D-imaging, 3D-imaging, live-cell applications, studies in (live) tissue, organisms and finally in live animals. STED microscopy has followed a similar route. Over the last years, the ability of STED has been convincingly demonstrated for fundamental molecular studies in cells, living tissue and in organisms. Not the least in neurobiology, STED has advanced our understanding of neurons, their synaptic machinery and the related protein localization patterns and dynamics. Video-rate imaging of synaptic vesicle trafficking in living neurons, as well as topological mapping of protein architectures both on the pre- and post-synaptic side have been achieved [27,40,41,42]. In dendritic spines (the excitatory contact points between neurons) super-resolved images have allowed quantitative topological mapping of central regulating proteins [43,44,45]. Dynamic investigations of the morphology of dendritic spines and their inner protein distributions have also been visualized [19], even in live tissue and in the brain of live animals [46,47].

In general, resolving the fine details of protein distribution patterns within cells is a key to understand a range of fundamental cellular mechanisms. Here, STED has a very important role to fulfill and is only at the very starting point to explore this source of information. Several studies using STED for this purpose have already been undertaken, for instance to investigate cellular adhesion and migration processes [48] and bacterial autolysis and survival mechanisms [49]. It is also of high interest to exploit STED, and the information contained in the distribution patterns of proteins within cells for diagnostic purposes, in a similar way as up- and down-regulation of certain protein markers are used today as indicators of various diseases. As first examples along this line of applications, STED has recently been used to reveal distinct differences in the spatial distribution of cytoskeletal proteins in metastatic competent cells [50], reflecting

also the different elastic properties seen for many cancer cells compared to those of their normal counterparts. STED has also been applied to explore storage and release mechanisms of angiogenesis-regulating proteins in platelets [51], believed to be involved in the role platelets play in early malignancies, providing the proteins needed to stimulate local formation of blood vessels and tumor growth. Hypothetically, protein distribution patterns dissected by STED may provide diagnostic fingerprints of activation states of the platelets specific for malignant disease, and may also provide clues for how to manipulate the platelet mechanisms of protein storage and release to counteract early tumor development.

FIGURE 2: *Confocal (left) and STED (right) images of platelets from an ovarian cancer patient. In contrast to confocal imaging, STED allows analyses of numbers, sizes and localization patterns of the sub-granular clusters in which the proteins (here, pro-angiogenic VEGF and anti-angiogenic PF-4) are stored as well as of their possible co-localization. Procedures are described in [51]. Image taken by Daniel Rönnlund, KTH, Stockholm (collaboration with Marta Lomnytska and Gert Auer, Karolinska Univ Hospital, Stockholm).*

Conclusion/Outlook

Twenty years of advances in STED microscopy has passed since its invention. In the meantime STED has been appointed the life science method of the year (*Nature Meth* 2009, **6**:24-32) and has shown its tremendous potential for visualizing cellular biology, and its ability to see details of cellular and even macromolecular structures. What

advances in STED microscopy do we expect to see in the next twenty years? With the technology made commercially available an increasing number of scientists will get access to it. In cell biology there is certainly a strong demand to perform fluorescence imaging beyond the classical resolution limits. In the near future, we can thus expect a significant increase in the use of STED, in science as well as for clinical and drug development purposes. This will be reinforced by the fact that the original STED patent will expire very soon, whereby an increased number of instruments will be offered, by several manufacturers and to lower prices. An increase in the number of STED users will also be promoted by a major trend in the technology development in STED, towards more robust and cheap turn-key systems. For basically all fields of life science today using confocal microscopy it will be relevant to ask the question: why not use STED instead, offering an order of magnitude higher resolution?

Instrument hardware development will also lead to improved imaging performance of the STED instruments. However, it is evident that the major bottle-necks today are primarily related to the sample preparation and labels used. Therefore, it is likely that major leaps in performance will be related to the introduction of new labels, offering better photo-stability, biocompatibility, and for which targeted switching may be achieved also by other means than by stimulated emission. In fluorescence imaging it is difficult to achieve high resolution, speed and sensitivity at the same time. Fluorescent marker molecules only tolerate limited doses of excitation light, before they are photobleached, and the photobleaching rate increases with the excitation intensity in a non-linear manner. The light doses tolerated by live cells are often even lower than those tolerated by the fluorophore markers [52]. Both excitation and switching light doses need to be considered, and the detected fluorescence emission and number of switching events in proportion to these light doses are critical parameters. In this context, one niche of targeted switching microscopy has shown recent progress: REversibleSaturable Optical Fluorescence Transition (RESOLFT) is a generalization of STED microscopy that includes basically any molecular transition driven by light, implemented to sequentially separate and resolve neighboring fluorescently labeled molecules. Lately, specially engineered fluorescent proteins (optically switched between

long lived conformational states) have allowed live-cell imaging under low-light levels and has technologically also been extended to highly parallel large fields of view [53,54].

In cell biology, super-resolution imaging will open new avenues of information regarding cellular mechanisms and processes, but will also challenge old models based on diffraction limited imaging data [55]. Indeed, to take full benefit from super-resolution techniques and the continuous improvement of these techniques also prompts re-thinking of many cell biology mechanisms, where we eventually will have found ourselves to have lifted the lid of yet another layer of the Matryoshka doll of Mother Nature.

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