

STED microscopy: increased resolution for medical research?

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Optical imaging is crucial for addressing fundamental problems in all areas of life science. With the use of confocal and two-photon fluorescence microscopy, complex dynamic structures and functions in a plethora of tissue and cell types have been visualized. However, the resolution of 'classical' optical imaging methods is poor due to the diffraction limit and does not allow resolution of the cellular microcosmos. On the other hand, the novel stimulated emission depletion (STED)

microscopy technique, because of its targeted on/off-switching of fluorescence, is not hampered by a diffraction-limited resolution barrier. STED microscopy can therefore provide much sharper images, permitting nanoscale visualization by sequential imaging of individual-labelled biomolecules, which should allow previous findings to be reinvestigated and provide novel information. The aim of this review is to highlight promising developments in and applications of STED microscopy and their impact on unresolved issues in biomedical science.

Keywords: fluorescence, nanoscale imaging, STED, stimulated emission depletion microscopy, super-resolution.

Challenges in medical optical imaging

Fluorescence microscopy is a tool with superb sensitivity that allows individual-labelled biomolecules to be detected by the absorption of light and the reemission of fluorescent photons. In the last decade, the technique has provided new experimental possibilities to decipher several biochemical questions related to the heterogeneous cellular world [1]. Why is this 'impressive' optical tool not applied in the routine clinical setting, in the same way as other imaging methods such as X-ray radiography, magnetic resonance imaging, ultrasonography and positron emission tomography? The main physical disadvantage of this method, which hampers clinical applications, is light scattering, which generates 'poor' penetration depth of human tissue. A diffuse image is often the final result with fluorescence microscopy when trying to visualize deep within dense cellular material whilst attempting to enable both excitation light to enter and emission light to exit the sample and achieving targeted labelling of the area of interest. Internal imaging by microscopy avoids the problem of optical penetration by placing the tool in the vicinity of the studied objects as in endoscopy. The only opportunities for human optical microscopy are

dermatological surface layer investigations (e.g. glucose measurement) and ophthalmological investigations of the visual sensory system (e.g. assessment of a damaged cornea), to specify two clinical examples. However, longer wavelength (near-infrared) microscopy has been developed and applied since the early 1990s to allow a better penetration depth into cellular structures, thus enabling imaging of several hundred micrometres into the highly scattering medium of tissue and in living systems [2]. For example, employing near-infrared wavelengths, and interferometry as in optical coherence tomography, allows topographically deeper imaging in ophthalmology to obtain detailed images within the retina and in interventional cardiology to aid the diagnosis of coronary artery disease.

The main methods for studying human diseases, to provide effective treatment, are as follows: (i) tissue biopsy, followed by optical histology; (ii) cellular diagnostics of cultivated infectious diseases, for example with specific fluorescent probes to highlight infectious agents; and (iii) collection of body fluids (blood, saliva, urine or spinal fluid) with subsequent molecular diagnostics using sensitive fluorescence detection of well-characterized

biomarkers [3]. The scientific tool used in the latter case is most often simply a pair of optical lenses and a sensitive detector connected to a computer with appropriate software and a coloured probe that can be detected at very low concentrations when specifically attached to the biomolecules of interest. In other words, this tool that is found in almost every clinical environment is an optical microscope that uses fluorescence (or absorption in the case of histology investigations) for profiling the expression of a set of cellular biomarkers already known or suspected to be related to a disease. As an indication of the importance of this field, this tool also represents the hallmark of cutting edge intellectual quality; many photographs of scientists are taken sitting in front of an optical microscope.

Why has this century-old tool become so important in biomedical science, as it cannot penetrate even 1 mm into the human body? The two most important qualities of optical microscopy are first the ultimate sensitivity of individual fluorescent molecules (highlighted above) and secondly the resolution power of visible light for live-cell imaging. The resolution of the above medical imaging tools ranges from submillimetre to tens of micrometres. The resolution of optical microscopy is 100- to 1000-fold higher, meaning that it may achieve impressive resolutions of a few hundred nanometres in sharp optical focus. Thus, being able to view the 'microcosmos' of the cellular world and its topological structure, as well as interactions over time, in several colours and in three dimensions, enables the generation of images carrying information about biological mechanisms. However, no tool can (or should) be used under conditions in which it physically cannot provide accurate information. According to basic optical principles, the resolution of an optical microscope is limited by the wave-like nature of light (i.e. diffraction broadening). Thus, optical microscopy fuses individual-labelled images of fluorescent molecules into a single blurred image if the structural extent or separation in the sample (d) is closer than the Abbe limit [4]:

$$d = \lambda / (2n \sin \alpha) = \lambda / 2NA \quad (1)$$

where n describes the refractive index of the specimen, α measures the solid half-angle from which light is gathered by an objective, λ is the wavelength of light used to excite the specimen, and NA is the numerical aperture. To obtain high

resolution (i.e. small d values), short wavelengths and high NA values ($NA = n \sin \alpha$) are optimal. However, this is technically difficult in optical microscopy. The typical light gathering capacity of immersion oil objectives is about $NA = 1.4$, so that the lowest resolution is given by $d \approx \lambda/3$. Typically, the shortest optical wavelength that is used is above 340 nm, where glass in normal objective lenses is transparent. Using quartz lenses instead allows ultraviolet wavelengths to be used; however, this may have pronounced phototoxic effects, for example in live-cell imaging, due to energetic photons. The use of longer visible wavelengths to preserve live-cell capabilities may only resolve subcellular features with structural extent or separation of maximally 200 nm in the focal plane of a fluorescence microscope (Fig. 1). Attempting to systematically image and visualize the heterogeneous cellular world and decipher the bimolecular microcosmos is therefore futile. One is basically blind to the scale mechanistically governing the structure and function of the cell, shown for example with classical imaging of the postsynaptic protein topology of the membrane-bound sodium pump in neurons [5].

New optical opportunities with sharper images

It is worth considering whether the resolution of an optical microscope could be tuned to the macromolecular scale of cell constituents to better investigate a biomedical hypothesis. Is this possible given that eqn 1 has been assumed to be insurmountable for at least a century? Fortunately, to gain a better biological understanding of the cell, novel imaging modalities have 'shattered' the diffraction limit of light and revolutionized fluorescence microscopy in the last two decades. Twenty years ago, there was an important novel development in this field: stimulated emission was (theoretically) employed to turn off the spontaneous fluorescence process in selected regions of the image [6]. Targeted 'switching' was then experimentally pursued [7, 8] and developed to separate fluorescently labelled biomolecules in the cellular environment in the range of tens of nanometres, to answer biological questions through nanoscale imaging [9, 10]. This pioneering far-field super-resolution imaging technique was termed stimulated emission depletion (STED) microscopy, as it was realized that switching (i.e. selected stimulated depletion of fluorescence) should produce much sharper images. Today, a custom-built or commercial STED microscope typically generates a

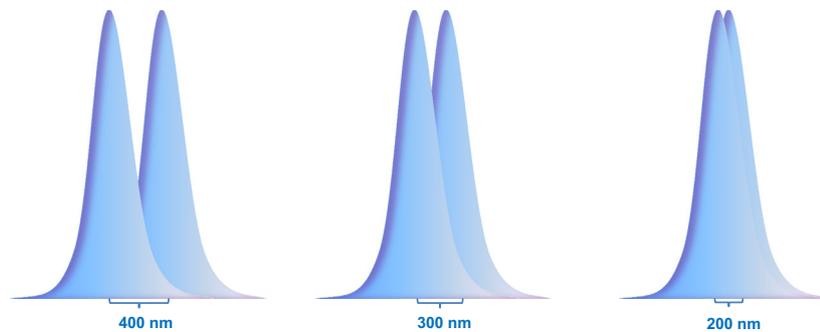


Fig. 1 The sharpness of the diffraction-limited focus only allows entities to be resolved and separated simultaneously if they are at a distance of at least the width of the generated optical blur, that is, d in eqn 1. The physical separation of labelled fluorescent molecules thus needs to be considerably >200 nm to be resolved using conventional microscopy at visible wavelengths.

maximum resolution of 20–50 nm, which has allowed the nanoscale topology of the cellular microcosmos to be imaged.

What is the underlying mechanism of this fluorescence microscopy technique and how does it generate sharp images? Targeted switching allows fluorescence to occur only within a nanoscale area, thus separating and resolving neighbouring fluorescently labelled molecules (Fig. 2). Thus, super-resolution imaging requires only the addition of a (red-shifted) STED laser to a scanning confocal fluorescence microscope with the beam profile modified to produce no light in some area(s) of the focus. Most often, the STED profile is optically sculpted to generate a ‘doughnut-shaped’ light distribution in the focal plane (i.e. a ring of high laser intensity surrounding an area of zero intensity). Finally, overlapping the STED profile onto a Gaussian-shaped excitation laser beam profile allows targeted switching. Fluorescent molecules are thus either switched off by stimulated emission in the outer areas (induced via the STED beam) or allowed to generate a detectable fluorescence signal from the few central molecules that are optically ‘un-switched’. Scanning the coaligned beams over the fluorescent sample and detecting sequentially each nanoscale pixel builds up a super-resolution image; no data processing is necessary as the raw detection of nanoscale-separated positions is induced via a purely physical (optical switching by stimulated emission) process. Separating individual fluorescent molecules and sequentially building up the targeted imaged positions via the switching profile are thus the novel principle underlying the high resolution of STED microscopy.

To further understand STED microscopy and its intrinsic physical principles, it is necessary to understand Einstein’s quantum theory [11]. Essentially, optical fluorescence microscopy involves three processes: absorption, spontaneous emission and stimulated emission. These processes and the effect they may have are shown schematically in Fig. 2c. Initially, when a fluorescent probe molecule absorbs light (Fig. 2c, blue photon), the electronic organization of the absorbing probe is changed by exciting an electron from the ground state to a higher energy level. Within a relatively short time (typically a few nanoseconds), the absorbed energy can then be spontaneously reemitted as fluorescence (Fig. 2c, green photon) relaxing the fluorescent probe to its ground state. Because of internal energy losses in the probe molecule, due, for example, to vibrational or rotational movements, the emitted fluorescence has less energy than the absorbed photon. This difference is utilized in fluorescence microscopy to separate spectrally excitation light from spontaneous emission (i.e. separate the background from the fluorescent signal). It is also possible to actively force the excited fluorescent molecule to reemit the absorbed energy. After the molecule has been excited to a higher state and internally relaxed, interaction with another photon (Fig. 2c, red photon) can stimulate the excited molecule back to the ground state. Excess energy can then be removed via an exact copy of the stimulating light (Fig. 2c, additional red photon), which can again be separated spectrally from the fluorescence or excitation light. This latter duplication process is stimulated emission, which in STED microscopy basically operates as an off switch for fluorescence, instantaneously removing absorbed energy in selected

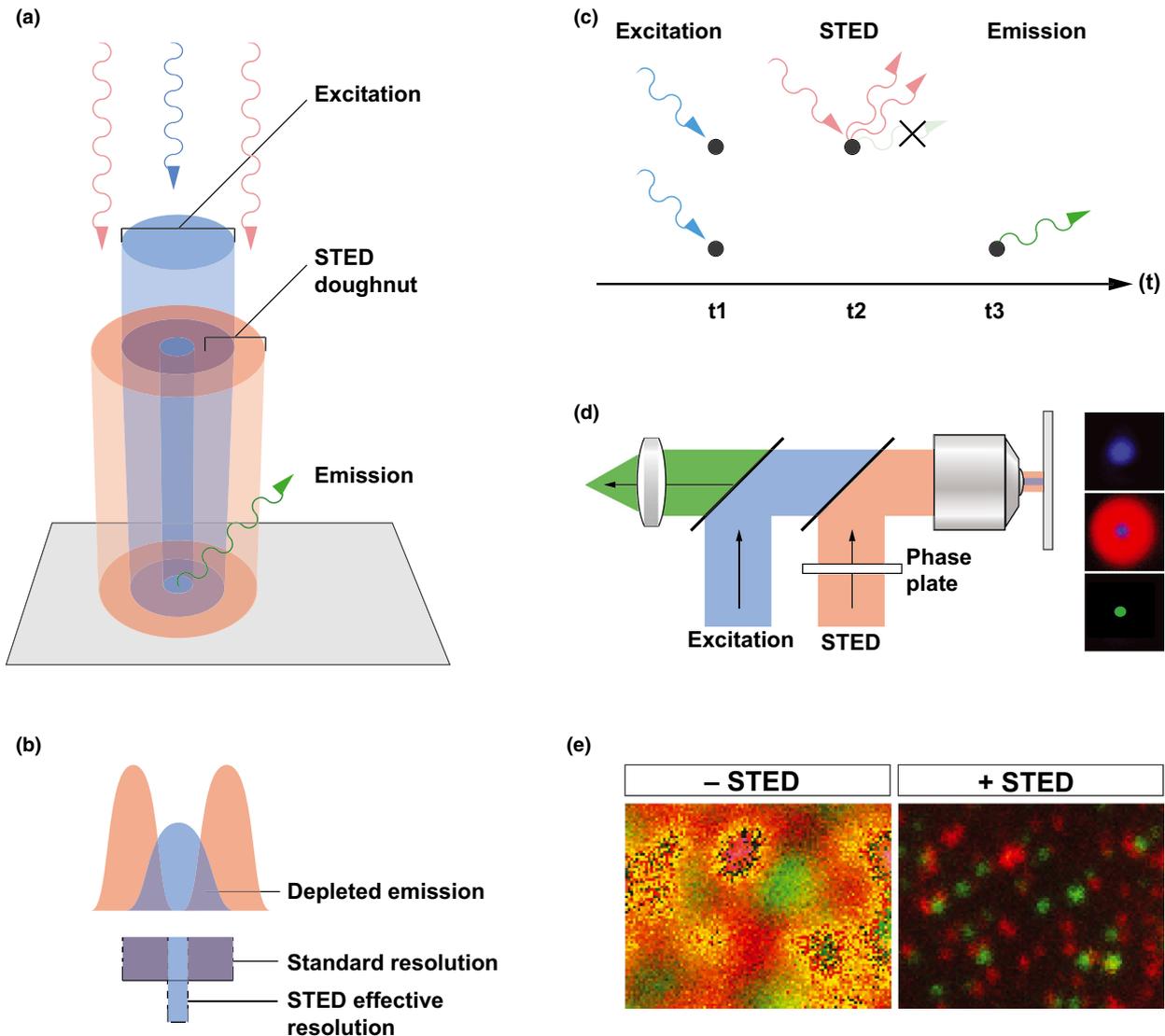


Fig. 2 (a) Laser light is used to excite (blue) fluorescence emission (green) in the area of focus. A sculptured STED focus (red) is used to selectively turn off emission in the outer rim allowing better separation of fluorescent entities. (b) Increased depletion power (red) reduces the common area of emission and generates a sharper effective focus with nanoscale resolution power. (c) A fluorescent molecule (black circle) following absorption of excitation light (blue photon) spontaneously emits fluorescence (green photon) after a short time (usually a few nanoseconds) to return to its ground state. Addition of STED light (red photon) can rapidly stimulate the excited molecule to emit the absorbed energy (additional red photon) and return to the ground state, thus turning off fluorescence (green). (d) Schematic diagram of an optical STED microscope with a phase plate sculpturing the depletion beam (red) into a 'doughnut-shape' in the focal plane, overlapped with the excitation beam (blue), and the resulting effective detected fluorescence emission (green). (e) Standard fluorescence microscopy image of a mixture of 40 nm fluorescence beads (red and green colour coded) and the super-resolved STED image allowing nanoscale separation and visualization.

parts of the focal volume. Furthermore, the wavelength of the stimulated emission depletion beam is selected to be sufficiently long (red-shifted compared to the excitation beam) to avoid secondary

excitation of fluorescent probe molecules. This selection also simplifies spectral separation of the signal and the background. Increasing the intensity of the STED beam to saturate the depletion

profile finally confines the fluorescence focal spot several-fold, in all directions [10]. In other words, STED allows nanoscale imaging by ensuring that narrowly spaced fluorescent probe molecules are switched to different states; they are either stimulated not to fluoresce (off) or left undepleted and allowed to fluoresce (on). It is noteworthy that if the duplication process occurs simultaneously for many molecules placed within a cavity (e.g. between two mirrors bouncing light back and forth), stimulated emission will amplify light as it oscillates and produce a bright laser (i.e. light amplification by stimulated emission radiation). Thus, the same fundamental stimulation process used to create a bright laser beam has advanced the route to diffraction-unlimited imaging in STED microscopy, by selectively switching fluorescent molecules off and on, which directly generates a raw nanoscale image [12].

Fluorescence is the common signal in STED microscopy, but its probability of occurring can be controlled with the stimulated emission depletion beam. To effectively deplete or quench the fluorescent state, the rate of stimulated emission depletion must outperform the spontaneous decay rate. Mathematically, a modified Abbe equation expressing the resolution improvement can be approximated as:

$$d = \lambda/2NA(1 + \zeta)^{1/2} \quad (2)$$

where ζ is the saturation factor expressing the ratio of the applied (maximum) STED intensity to the saturation intensity, $\zeta = I_{\max}/I_s$. The saturation intensity (corresponding to the value of half the emission signal) is dependent on the orientational and rotational behaviour of the fluorescent probe, as well as on the wavelength, temporal structure and polarization of the STED light [13]. Thus, with increasing intensity of stimulated emission depletion, increasingly smaller fluorescence focal widths can be generated, which in turn allows smaller and more narrowly spaced fluorescent objects to be visualized. With sufficiently intense depletion intensities, the fluorescent focal spot can thus be scaled down to molecular sizes in a STED microscope with saturation factors above 100 and maximum focal widths of below 20 nm as reported using common fluorescent dyes at the single-molecule level [14]. The achievable resolution is thus increased by 5- to 15-fold with STED microscopy, compared to conventional light microscopy.

In the last decade, complementing methods in super-resolution microscopy have also pursued switching to generate sharper fluorescent images. Methods for localization of stochastically switched individual molecules [e.g. PALM, photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM)] or spatially nonuniform illumination and targeted (nonlinear) switching [e.g. structured illumination microscopy (SIM)] have been developed. The multiple choices available are similar in many respects, but distinct in others (data generation, speed, multiplexing, resolution and analysis), which allows the most suitable technique to be selected for the particular research purpose [15–17]. The main characteristics of STED are (i) the possibility of nanoscale optical sectioning (i.e. reduced background in thicker samples), (ii) fast imaging rates (i.e. tracking of dynamic processes with a low level of artifacts due to imaging drift), (iii) use of standard fluorescent proteins or suitable bright dyes and (iv) direct generation of a raw super-resolution image (with optional mathematical processing to enhance image quality before data analysis) [18].

In the following sections, we will review the technological and methodical developments in STED microscopy in recent years. Thereafter, we will discuss considerations of the technique. Finally, we will consider the application possibilities of STED microscopy. All sections can be read independently and in no particular order.

Technological developments in STED microscopy

A typical STED microscope uses a pair of coaligned laser beams, one for excitation and a second (high power) beam for the depletion of fluorescence (Fig. 2d). The spatial shape of the depletion beam is such that it produces an annular pattern, with a node of zero intensity in the middle that allows fluorescence to be switched off in the periphery of the focal volume. Combining lateral and axial depletion patterns allows nanoscale resolution in three dimensions, where the ‘doughnut-shaped’ profile enhances lateral resolution (X,Y) and an axial depletion profile (see Fig. 3) improves the Z-resolution. As targeted switching using STED microscopy requires light to selectively ‘turn off’ molecules sequentially, developments in laser technology have been linked to a simpler, user-friendly and turnkey STED approach. Historically, an expensive and somewhat complex pulsed laser system has been used for stimulated emission

depletion microscopy [7]. Applications to ‘simplify’ the method by introduction of continuous-wave (CW)-STED have generated resolution down to 30 nm laterally (approximately 100 nm axially) using scientific lasers. Thus, adding a bright CW laser for STED can convert a regular scanning confocal fluorescence microscope into a three-dimensional super-resolution system [19], applicable even for nanoscale imaging of organelles in living cells [20]. A summary of common STED modalities is shown in Table 1.

In parallel to the technological approach of applying nonpulsed lasers as in CW-STED, the implementation of spectrally broad laser systems (e.g. white light laser sources or stimulated Raman scattering light sources), where excitation and depletion pulses are generated from the same light source, has been shown to be a straightforward

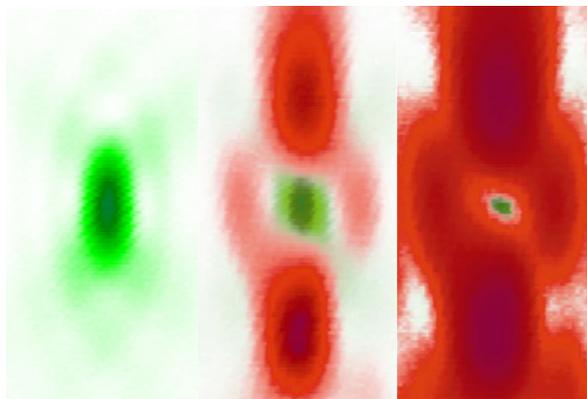


Fig. 3 A conventional focal volume of fluorescence (green) has an axial extent of several hundred nanometres. By applying an axial STED profile (red) and increasing its laser power, selective depletion of the fluorescence effectively sharpens the focal depth (and additional lateral resolution improvement can be achieved) to about 100 nm.

way to simplify the STED approach and conveniently provide multicolour imaging [21–24]. Furthermore, even live-cell imaging of *Caenorhabditis elegans* labelled with enhanced green fluorescence protein (eGFP) has been achieved with a similar compact and flexible laser system [25]. As the biological cell is a densely packed microcosm, imaging at the nanoscale in three dimensions is often necessary to be able to reveal its internal components and unravel the mechanistic processes. Several approaches to spatially sculpture in three dimensions, the distribution of the STED light with a central minimum (see Figs 2a and 3), allow nanoscale imaging for biological as well as material science applications [22, 26]. Moreover, more technologically complex STED systems using dual-opposing high-numerical objectives to generate interfering focal patterns, which suppress the spontaneous fluorescence process isotropically, have been applied for biological nanoscale imaging inside cells (maximum resolution approximately $30 \times 30 \times 30$ nm) [27, 28].

The improved image resolution in stimulated emission depletion microscopy is encoded spatially by the STED beam which optically targets fluorescent molecules to be switched off or remain on. As the STED beam has a spatially varying strength around its focal minimum, the (stimulated and spontaneous) lifetime of neighbouring fluorophores will vary. This selective information can be applied to further improve the nanoscale localization of individual-labelled molecules by filtering the detected fluorescence signal in time. Theoretical and experimental applications of this time-gated approach have been applied in STED microscopy to provide higher targeted resolution [29, 30] (i.e. temporal filtering to detect fluorescence signals occurring only from the centre of the nondepleted focus; see Fig. 2b,c). Given the often challenging and laborious preparation of biological samples for

Table 1 STED modalities

Modality	Laser type	Probes	Excitation and depletion
CW-STED	CW excitation	Blue/green dyes	Blue/green excitation
	CW depletion	Yellow (red) fluorescent proteins	Orange/red depletion
Gated CW-STED	Pulsed excitation	Blue/green dyes	Blue/green excitation
	CW depletion	Yellow/red fluorescent proteins	Orange/red depletion
Pulsed (NIR) STED	Pulsed excitation	Red/far-red dyes	Green/orange excitation
	Pulsed depletion		Red/far-red depletion

CW, continuous wave; STED, stimulated emission depletion; NIR, near-infrared.

super-resolution microscopy, there is a need to simplify the technical aspects of STED and make the method more robust to broaden its accessibility to the research community. This has been the goal of several developments regarding the focal profile used for targeted switching [31, 32]. As the system essentially contains only one extra optical component, it is possible that this microscope could be implemented as a nanoscopic endoscope, thus simplifying *in vivo* imaging at high resolution.

To further allow better penetration depth into cellular structures, several groups have also recently extended STED microscopy to two-photon excitation, using different optical systems and modalities [33–36]. This has allowed deeper imaging into tissues, such as brain slices [37–39]. Switching from the surface-based high-numerical oil objectives (index matched to the coverslip) commonly used for STED to more biocompatible glycerol-based optics (index matched to the specimens) has also advanced the possibility of penetrating deeper into living tissue and allowed nanoscale images even within the brain of living animals [40]. For dynamic nanoscale investigation of fluorescently labelled biomolecules, STED microscopy has, after initial proof-of-concept studies [41], also been applied to study membrane-anchored lipids in cellular systems using STED combined with fluorescence correlation spectroscopy FCS [42, 43]. This single-molecule analysis approach complements video-rate imaging (using a small region of interest) achieved by fast scanning technology that has been applied, for example, to dynamically follow labelled synaptic vesicles in axons [44].

Methodical developments to improve STED imaging

What are (i) the best probes, (ii) the best way of measuring, (iii) the maximally achievable resolution and (iv) the most suitable analytical approach to extract data to investigate nanoscale biomedical hypotheses? The answers to these questions depend on the biological system being studied; how well the fluorescent sample is prepared and the extent to which the nanoscale being imaged can be correlated with the biomedical function of interest. This suggests that several inherent (measurement) problems may be present when attempting to visualize the nanoscale.

The size of a bulky fluorescent probe such as an antibody–dye complex might perturb the molecular

system being studied [45]. Even the physical properties of the fluorescent probe and its linker must be considered for optimal measurement and data extraction [46]. Thus, as always in fluorescence microscopy, the microenvironment of the probe may influence the outcome of the measurement, and vice versa the way of labelling may affect the achievable outcomes. For example, not all epitopes are available because steric hindrance blocks ‘true’ labelling using antibodies; in addition, application of fluorescent proteins being transfected into the biological system may severely perturb expression levels, and proteins might be guided to incorrect cellular positions. In the (new) eyes of a nanoscopic imager, all the considered questions asked above are thus entangled and need consideration to allow seeing the truth.

Thus size is important when it comes to super-resolution; not only the labelling density needs to be high to achieve (or claim) a resolution of tens of nanometre, the ‘disturbance’ on the nanoscale must also allow higher densities to be deduced so that true biological topology can be accurately dissected. Advances in methods for site-specific targeting of small-molecule probes to cellular proteins are thus important for nanoscale imaging [47–50] as shown by live-cell compatible imaging in HeLa cells and fibroblasts. Approaches to make smaller ‘click chemistry’ linkers have also been shown to be suitable for STED imaging [51], with combinations of SNAP-tag fusion proteins allowing resolution of the cytoskeleton structure in living mammalian cells. Moreover, switching to imaging of thicker samples places strong demands on optically matched samples. Advances in imaging matching material applicable for fixed groups of cells or tissue slices have allowed nanoscale imaging with STED with performance maintained when optically penetrating the sample [52–54].

Pessimistically speaking, is there no easy gain in using super-resolution STED microscopy? Optimistically, the STED system is a basic fluorescence microscope with one laser for excitation and the same or another for targeted off-switching; STED microscopy might thus be used to generate unblurred images of any fluorescent sample. Visualization of such unblurred nanoscale images might, however, generate evidence that does not support currently accepted hypotheses. Figure 2e shows an example of nanosized beads (mixture of red and green; diameter 40 nm) first imaged by conventional confocal fluorescence microscopy, which is

performed with the STED beam turned off. When interpreting such an image (Fig. 2e, left), it is often erroneously assumed that 'yellow' overlap reveals nanoscale colocalization in the sample. Knowing that the confocal microscope, in the best case, can resolve entities separated by around 200 nm, essentially no separation below this scale is possible. Switching on the STED beam in the microscope allows visualizing of the nanoscale and dissecting individual nanobeads being separated, pointing towards that with increased resolution, no colocalization at all is present in the sample. This loss of information is, however, overcome by the now available much more complex description of the topological distribution of the nanobeads (assuming this could have a physiological meaning). The insight available from super-resolution thus demands methodical development of suitable analytical approaches for information extraction [55]. With knowledge on super-resolution STED demands and possibilities, rethinking of cell biological mechanisms might also be necessary, prompted by the biomedical imaged nanoscale being resolved or shown to be perturbed [56]. In the case of colocalization, STED microscopy has a topological benefit (as well as the improved resolution) as the sculptured profile defines the origin of the signals. In multicolour imaging, where some misalignment of the two excitation foci becomes 'realigned' by the central targeting zero of the depletion beam, it is beneficial to apply STED microscopy for 'true' colocalization [24].

Improving the resolution of a fluorescent microscope in all spatial directions allows increasingly better resolution of the nanoscale. However, if the preparation of the biological sample (and its labelling density) is not simultaneously improved (i.e. 2-fold to keep the required resolution in a bottom-up perspective), resolved images may not be limited by the performance of the STED microscope but by the poor sample preparation. As in any fluorescent microscope, the quality of the image and its contrast and resolution are ultimately determined by the fluorescent reporter molecule; this is especially the case in STED where the intrinsic states of the molecules are selectively targeted in the optical switching process. Demands on fluorescent reporter molecules are high (photostable, bright etc.), but with a plethora of suitable fluorescent reporters, advances in STED applications are shown with almost any type of probe. It has been shown several times that genetically expressed fluorescent proteins are suitable probes for STED microscopy. The

most commonly used are clones of eGFP, yellow fluorescent protein (YFPs) and some red fluorescent proteins [7–61], which have been applied, for example, to image the dynamics of the endoplasmic reticulum in living mammalian cells and the morphology of filled dendritic spines in hippocampal neurons. Both pulsed and CW depletion lasers allow resolution of <50 nm in living cells. Therefore, studies that are currently performed in cells using confocal microscopes may be carried out in future with substantially improved spatial resolution using STED microscopy.

Bright and photostable dye molecules have been used since the introduction of this technique to visualize the nanoscale in cell biology; more than 20 are suitable for nanoscale imaging (see www.nanoscopy.de). Additional developments to generate dedicated reporters with superior performance as well as excellent biocompatibility are ongoing [62, 63]. This is expanding STED microscopy to all areas covered by 'classical' optical imaging, thus allowing it to be combined with any microscopy modality, fluorescent probes or fluorescence analysis techniques. New 'exotic' reporters consisting of so-called *Farbenzentra* in diamond are also being considered as fluorescent probes. Trapped atomic vacancies inside the rock solid crystal act as fluorescent emitters that can be optically switched by STED [64]. Manufacturing advances are presently being pursued (such as the development in the last decade of quantum dots) to produce biomodified nanocrystals with single vacancies for biological nanoscale imaging. A summary of the common advantages and disadvantages of STED microscopy is shown in Table 2.

Considerations in STED microscopy

The performance of STED microscopy is dependent on several associated factors. First, the perfect minimum of the STED profile needs consideration as residual depletion intensity will dim the signal in the nanoscale image, and, in the worst case, it may not be visible above the background. Secondly, the spatial overlap of the excitation and depletion beams is important as this selects centrally located fluorescent molecules to generate the sequentially detected signal by the targeted switching principle. For 'perfect' depletion performance, the overlap should not be off more than 10–20 nm and this should be stable over time so as not to deplete the signal focus [13]. It is thus crucial to minimize drift between the two laser beams; this has been the

Table 2 Advantages and disadvantages of STED modalities

Parameter	Advantages Disadvantages
Fluorescent dye (immunolabelling)	Bright, more photostable compared to fluorescent proteins below Cell-penetration protocol needed
Fluorescent protein (transfection)	Less bright, less photostable compared to dye molecules above Genetically expressible in cells
Cell cultures (not confluent)	Thin structures, lower background Less physiological
Tissue (<30 µm)	Thick structure, higher background More physiological
Resolution	Setup dependent: typically XY: 40–50 nm; Z: ~100 nm
Imaging speed	ROI dependent: typically seconds; Video-rate possible (~20 fps)
Image processing	Not mandatory (raw image); optional to enhance contrast

STED, stimulated emission depletion; ROI, region of interest; fps, frames per second.

driving force for implementation of some of the reported easy STED approaches [31, 32]. Sample drift must also be minimized to image true nanoscale topology, even though STED is a fast scanning approach that is able to generate a nanoscale image of a whole cell within a few seconds. However, movements of tens of nanometres over the acquired image might then be deceiving when conclusions are being drawn. Stable optical systems with feedback loops to prevent (severe) drift are thus always needed for the application of super-resolution microscopy. The climate of the room, the presence of any vibration and the selection of the optimum coverslip thickness and immersion mixture can all severely affect the possibility of achieving maximal resolution. Thirdly, assuming all environmental and physical parameters are well controlled, the interplay between the STED depletion intensity and the fluorescent probe in the sample is an important consideration. It is also crucial to selecting the 'best' powerful STED laser that matches the highest possible depletion wavelength, without inducing any reexcitation, photodestruction or sample damage (e.g. induced by dirty mounting medium, use of lung cells from a smoker, or reaching the

maximum absorption of haemoglobin in blood cells or chlorophyll in plant cells). On the other hand, it is also possible to select an optimal fluorescent probe (a dye molecule or fluorescent protein) for a STED laser as the spectrum of the probe and the laser need to be simultaneously 'tuned'. In essence, the STED wavelength needs to be far-red-shifted to avoid reexciting the probe. As the microenvironment of the fluorescent probe might shift the excitation and emission spectra (sometimes even abolishing the fluorescence in unfavourable conditions), the tuning of the STED laser must often be reinvestigated before the final nanoscale imaging can be optimally obtained. Different optimal conditions might thus be found in different biological systems and with different probes. As STED microscopy relies on switching the fluorescent probes (i.e. manipulating which photophysical state is populated), this is not an unforeseen effect, but it may be beneficial or may be a quantum mechanical problem that needs to be resolved. Practically, the STED depletion wavelength operates equally well within a range of 20–30 nm, thus finding suitable working conditions is not difficult, especially using tunable lasers or with knowledge of suitable fluorescent probes acquired in the last decade.

Fourthly, the question of whether high laser intensity can affect the sample should be addressed. The only possible 'drawback' of including an additional STED laser in fluorescence microscopy is a small decrease in the detected signal, due to residual intensity in the centre of the annular depletion pattern, and a slight increase in photobleaching of the fluorescent molecules. The latter is not a new finding within the field of fluorescence microscopy and has so far not prevented the novel STED technique from showing very prominent resolution enhancement of 5- to 15-fold in each spatial dimension. Selection of photostable fluorescent probe molecules and/or adaptation of their microenvironments (e.g. addition of antifading agents) minimizes photobleaching in STED microscopy [46]. Addition of a red-shifted (depletion) laser may sometimes also prevent photobleaching and increase the fluorescence signal as this laser depopulates additional electronic energy levels of the fluorescent probe molecules [65, 66]. This process is not entirely beneficial in STED microscopy as the repopulation of the fluorescent state adds a signal which is often not depleted. This means that there may be an additional or residual fluorescence over a diffraction-limited volume,

which will weaken the degree of resolution enhancement that can be achieved.

Fifthly, the selection of pulsed versus CW modes should be considered: which is the best technique and which will produce the best resolution? In simplistic terms, most pulsed STED microscopes use high power near-infrared light for depletion, whereas the wavelength for the depletion using CW lasers is in the visible range. Already, a tuning towards shorter wavelengths allows a higher resolution, as eqn 2 is scalable in regard to this parameter. However, the way depletion is achieved must also be considered and is sensed by the saturation factor (ζ) in the latter equation. The effectiveness of the fluorescence depletion differs in pulsed and CW-based STED microscopy. In the case of a pulsed STED system, the intensity of the depletion beam is concentrated during one pulse, totally depleting the fluorescence immediately after an excitation pulse. In the CW approach, the STED intensity is distributed equally over time and thus an excited molecule only senses a fraction of the depletion intensity. CW mode STED thus needs an average of about 3- to 4-fold increased laser power for the same resolution enhancement [19]. However, a benefit of using CW excitation is that the fluorescence signal can be increased by a similar factor due to the continuous excitation, which makes fast imaging possible [20]. In addition, fast scanning modulation decreases photobleaching of the fluorescent probe molecules by not allowing the probe to enter reactive sensitive states such as the triplet or any radical states. As the probe in general only switches between the ground and excited states (i.e. absorbing and emitting photons), the effective fluorescence per unit time is actually increased. Similar signal enhancement can be gained with a pulsed STED system, in which a lower repetition rate of excitation and stimulated emission depletion maintains the fluorescent probe molecule in the absorption–emission cycle [67]. In other words, before fluorescent molecules ‘sense’ an additional excitation and depletion event (CW or pulsed), they can relax to the ground state and thereby avoid potential ‘photoexecution’. A combination with pulsed excitation and CW depletion basically merges the two laser protocols and allows additional flexibility of signal gating and thus temporally filters out a higher spatial resolution [30]. In addition to filtering the signal, the repetition rate of the excitation may also be tuned to allow the fluorescent probes to relax (i.e. cool) between imaging events. It is also worth noting that

as biological material commonly has an autofluorescent background in the blue/green part of the spectrum, selection of red/far-red fluorescent probes, and suitable longer wavelength STED microscopy, might be beneficial to achieve higher signal-to-noise ratio (i.e. better contrast of the nanoscale image). The practical conclusion is therefore that an infinite degree of resolution without a detectable specific signal will (unfortunately) provide a dull image.

Having considered the additional characteristics of the STED technique compared to confocal fluorescence microscopy (i.e. the STED laser and its intrinsic profile), the final two important considerations are as follows: (i) how samples are prepared and (ii) which biological issues can be addressed. As the STED microscope uses fluorescence as a signal generator for producing a nanoscale image, sample protocols used previously for immunolabelling or live-cell transfection fluorescence microscopy can be applied with largely unchanged. However, they need to be superbly prepared as the super-resolution power of the STED microscope will identify the slightest artefact [18]. The following conditions are recommended for optimum sample preparation to achieve the highest STED resolution: use of high-precision 170 μm coverslips (No. 1.5H) matching the objectives carrier correction; use of index-matched mounting medium that does not absorb any STED light; addition of antifading reagents suitable for the fluorescent probes; and stable temperature and degree of vibration. The major goal is to be able to visualize the topology of interest with a resolution of 20–50 nm in a crowded cellular environment in three dimensions with several colours and a fast time scale in dynamic live-cell settings. However, as stated above, the effectiveness of the STED microscopy usually depends on the system being studied, the question being asked, how well the fluorescent sample is prepared and how well the image can be correlated with the function under investigation. Thus, increasingly higher resolution is sought. However, as the resolution increases, the sample also needs to be improved. Labelling strategies to identify the nanoscale landscape and density of the biomolecule of interest demand suitable small probe carriers such as directly conjugated antibodies (or fragments thereof), fluorescent proteins, aptamers or nanobodies [45, 57, 68]. Such methodical endeavours are highly related to the future development of super-resolution microscopy, including

approaches better applicable to analysing discretely resolved nanoscale images.

Possible applications of STED microscopy

Advances in optical fluorescence microscopy most often follow the route of improvements in single-colour two-dimensional imaging, multicolour two-dimensional imaging, three-dimensional imaging and finally live-cell applications (isolated tissue, whole organism animal and lastly human live imaging). STED microscopy has achieved all these steps (except imaging in humans) and been used to visualize previously unseen details with superb resolution in many areas of life science. To visualize further details beyond the 250–400 nm resolution afforded by one-photon confocal or two-photon fluorescence microscopy, it can be beneficial to use STED microscopy (as well as complementary super-resolution techniques). One example of a scientific field in which nanoscale resolution provided by STED has been used to investigate previously inaccessible areas is neuroscience. The cellular constituents of the brain such as neurons and the synaptic machinery are attractive for nanoscale imaging as the physical size of pre- and postsynaptic areas can be considerably less than a few hundred nanometres. Video-rate imaging of synaptic vesicle (diameter 40–80 nm) trafficking in live neurons [44] as well as topological imaging of protein architecture has been reported [69–72]. The accurate determination of the spatial distribution of a protein inside a cell is often intimately related to its function. In dendritic spines (the excitatory contact points between neurons), topological findings of super-resolved images have allowed additional neuronal proteins to be quantitatively mapped [5, 55, 73]. Dynamic morphology of dendritic spines and their inner protein distributions have also been visualized [59], even in living tissue and in the brain of living animals [39, 40], as mentioned above. Such activity-dependent synaptic changes (often termed plasticity) are considered to be essential in the brain for memory storage and learning.

Understanding the molecular mechanisms by which neurons process and integrate synaptic inputs, as well as how these mechanisms are modified by activity, is a central challenge in neuroscience. Of particular interest are neuronal mechanisms that may be responsible for regulating signal localization and controlling the spatiotemporal regulation of biological functions in the brain.

Figure 4 shows an early outlook of what STED could achieve and now actually, only a few years later, basically have achieved in neurobiology, where it is now possible to visualize the protein topology in the synaptic area [5, 55, 73, 74]. To further advance the understanding of molecular (protein) localization and dynamics, STED has been applied to image, for example, the protein synaptotagmin in individual synaptic vesicles showing that it remains concentrated in small clusters after exocytosis instead of being dispersed across the plasma membrane [75]. Additionally, STED has been used to visualize the interaction between the SNARE motif and syntaxin clusters at plasma membrane sites where secretory granules and caveolae fuse [76, 77]. The distribution of the Bruchpilot protein centred at synaptic zones in *Drosophila* neuromuscular junction has also been revealed [78]. Furthermore, with macromolecular resolution, STED has been able to resolve the protein patterns on endosomes, the punctuated structure of intermediate filaments in neurons and nuclear protein speckles in mammalian cells [79]. The organization of the amyloid precursor protein in neuroblastoma and the nicotinic acetylcholine receptor in ovary cells has also been imaged by STED [80, 81]. Such images have previously been accessible only by electron microscopy, highlighting the usefulness of STED as a means of studying a myriad of protein complexes.

Many fundamental functions of the human body are also regulated by receptors that respond uniquely to specific ligands. In other words, receptors in the cell membrane or in the cytoplasm form the signalling interface of the cell and play a crucial integrative function in cellular communication and regulation; the importance of the discovery of these receptors has been recognized by several Nobel Prizes [82]. A malfunction of a G protein-coupled receptor or its signalling chain is often the cause of a wide range of mood disorders and related diseases such as Parkinsonism, schizophrenia, attention deficit hyperactivity disorder, anxiety and depression. Therefore, substances that selectively bind to and alter the function of receptors and thereby influence behavioural disorders are attractive targets for the development of new pharmacological strategies. Application of STED microscopy has already identified mechanisms of transport, localization and interaction of receptors in neurons on the nanoscale; in addition, use of this technique in the near future is likely to lead to further elucidation of the nature of the physiological

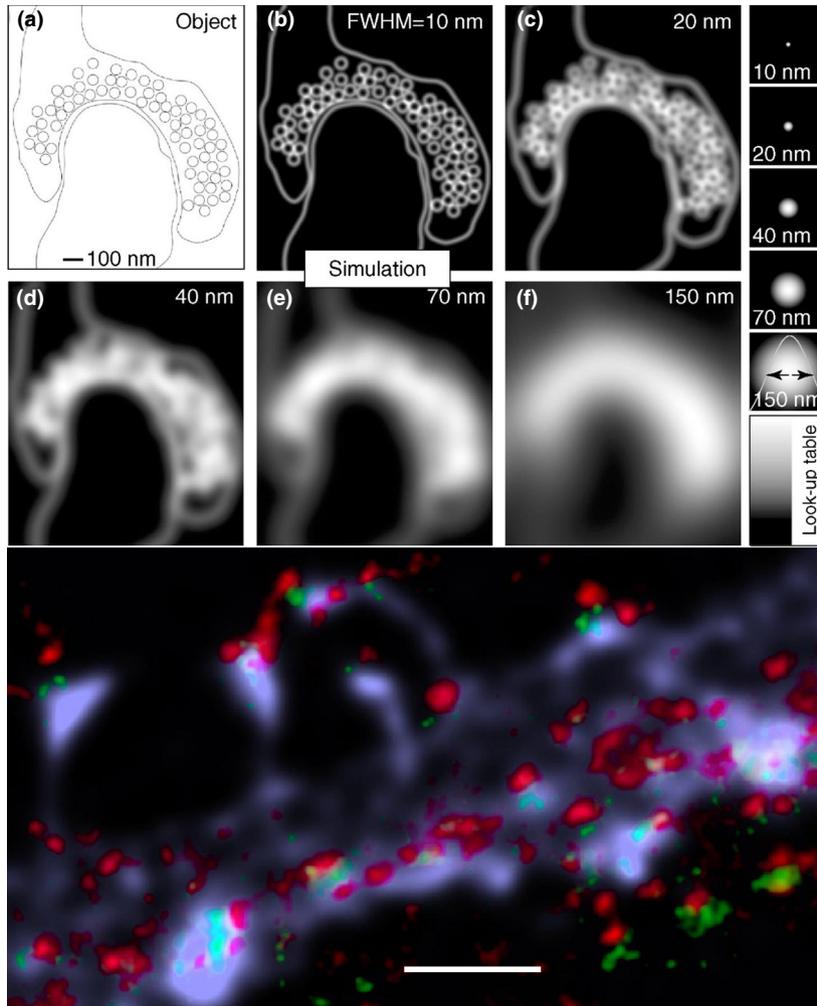


Fig. 4 (Top) (a) Schematic representation of a presynaptic site with synaptic vesicles and the postsynaptic side in an excitatory synapse, drawn to scale from an electron micrograph. (b–f) Simulated images with STED resolution of 10, 20, 40, 70 and 150 nm. At present, resolution of around 40 nm is commonly achieved in STED neuronal studies. Reprinted with the permission from the licensed publisher Elsevier Ltd. (Bottom) STED microscope images taken from hippocampal primary cultures transfected with eGFP (blue) to outline cellular borders and subsequently stained with antibodies against the presynaptic scaffold protein Bassoon (red) and the postsynaptic protein Homer (green). Scale bar indicates 1 μ m. The image has been post-processed using deconvolution software (Huygens, SVI, Hilversum, The Netherlands).

function, regulation and signalling roles of these receptors [55, 73].

The super-resolution STED method holds great promise for the investigation of dimensions on the scale of tens of nanometres, as all the above examples show. Moreover, use of dual-colour imaging allows nanoscale colocalization studies [83, 84], which provides the possibility of investigating (transient) cooccurrence of protein nanoassemblies. In a recent study using STED microscopy, in conjunction with quantitative colocalization analysis, nanoscale analysis was applied to investigate the complex distribution of membrane proteins in mitochondria [85]. As noted above, one of the biggest disadvantages of colocalization studies using 'classical' fluorescence imaging techniques is that the smallest achievable resolution is an

order of magnitude larger than typical interaction-relevant interprotein distances. Thus, macromolecular protein–protein interactions cannot be positively identified at present, using confocal or two-photon microscopy. With the STED technique, however, the necessary resolution can be reached, and therefore, this type of positive identification may be possible (see Fig. 2e).

To improve the resolution of STED imaging even further, increased laser depletion intensity and/or smaller fluorescent probe complexes might be useful. In live-cell STED, fluorescent proteins with masses of tens of kilodalton are applied [57, 58, 60], whereas in immunofluorescence applications, antibodies with larger masses (sizes) are used [9]. The much larger size of the antibodies (10–12 nm) compared to the fluorescent proteins (3–4 nm)

slightly widens the range of physical size of the cellular object that can be investigated. This may, in part, prevent the most accurate image localization that present STED setups typically allow from being reached [14] and might require selection of smaller probe molecules. However, the photostability and photoemission of the dye molecules used in immunochemistry are higher than fluorescent proteins rendering better imaging contrast. Furthermore, the achievable resolution for STED microscopy is influenced by the movement of the fluorescent probe. Therefore, sophisticated labeling strategies with smaller fluorescent labels are being developed and applied [48–51]. At present, the typical STED fluorescent probe molecule is a small dye (~1 nm); it does not need to be reduced in size until the required resolution starts to reach the nanometre range [64]. Of note, coupling of fluorescent probes to a biologically interesting molecule always introduces additional freedom of movement, which may alter the resolution in STED microscopy due to jitter. However, the nanoscale resolution of STED actually allows probing influences from this dynamics [46] making it possible to potentially use this ‘disturbance’ to further understand the nanoscale.

In addition to the studies discussed above, STED microscopy has been used to characterize the protein distribution of synapses at different tonotopic regions of the cochlea [86]. STED microscopy could be applied for investigation of such physiological function in the ear [87], as well as in the eye [88] and internally via endoscopic developments [89], to generate and enhance understanding of these and other biological systems in the human body. For example, understanding of the physiological function of pancreatic beta-cells from the islets of Langerhans could be enhanced considerably by the use of STED microscopy [90]. In particular, the elucidation of insulin granule exocytosis would be readily achievable, as has already been shown with granules in natural killer (NK) cells and the immune synapse [91]. The immune synapse is involved in the transfer of information across the mediated junction formed between T cells and/or NK cells and foreign agents (such as viruses). Diffraction-limited fluorescence imaging has been applied to investigate this connection between NK cells and target cells. Using STED microscopy, the immune synaptic junction has been even further resolved, which has led to a better understanding of its structure and function [91]. Furthermore, STED has been used to study

the internal nucleosome in cardiomyocytes to improve knowledge of the chromatin structure [92]. The centriole, a key component of the cellular control and division machinery, has also been investigated with STED to provide previously unobtainable details [93].

The primary cilium, the organelle that serves as a signalling centre of the cell, has been imaged by STED, revealing a differential protein topology [94]. In bacteriology, for example, STED microscopy has been applied to determine which factors control cytoplasmic translocation and activation of lytic proteins in *Streptococcus pneumoniae*, as only a small fraction was found to be attached to the extracellular cell wall [95]. Furthermore, using super-resolution STED fluorescence microscopy, nanoscale visualization of the viral envelope on the surface of individual HIV-1 particles has revealed a correlation between surface clustering and the efficiency of viral entry [96].

During the last decade, it has become clear from findings of the human genome project that a large fraction of all genes encode plasma membrane proteins. To understand the molecular mechanisms of proteins complexes, it is of fundamental importance to understand their structure (topology) and assembly dynamics [97, 98]. Of note, many membrane proteins are only active in protein complexes, so-called molecular machines, and it is not possible to determine their subcellular assembly topology using confocal or two-photon fluorescence microscopy. On the other hand, STED microscopy may be used to resolve several membrane proteins and their cooccurrence [5, 55]. It is also noteworthy that the total number of human proteins is (only) about 20 000, and their localization is currently being imaged by ‘classical’ fluorescence microscopy [99]. The application of STED microscopy to this project would enable unprecedented detail of the localization of essentially all these proteins, which should further increase understanding of the cellular protein microcosmos.

Moreover, the super-resolution properties of STED have been identified as essential in the field of translational medicine, to resolve cellular functions (with additional complementary imaging techniques) ‘from molecule to man’. The latest initiative is part of a project by the European Research Infrastructure for Imaging Technologies in Biological and Biomedical Sciences, in which super-resolution flagship centres will provide open

access to service and training in nanoscale imaging for European scientists [100, 101]. Additionally, in a recently completed European collaborative project, STED nanoscopy was used to monitor the molecular distributions within tumour-specific cells for detection of breast and prostate cancer [102]. The nanoscopic resolution power of STED was used to image morphological markers that correlate with malignant transformation and clinical tumour aggressiveness. The profound reorganization of the cytoskeleton of cells, driven by a deregulation of signal transduction pathways as a response to changes in the extracellular environment, suggests the possibility of visualizing a correlation with tumourigenesis using STED [103]. Additionally, the nanoscale topology of protein expression in blood platelets was imaged with STED microscopy, providing a means for clinical cancer diagnostics [104].

What advances in STED microscopy can be expected in the next 20 years? It is clear that with the technology being made commercially available more life scientists will gain access to STED microscopy [18]. This will mean rethinking how imaging of the nanoscale can be pursued along with understanding the associated problems and possibilities [56]. In addition, most scientific fields in which confocal fluorescence microscopy is currently used to view the intracellular environment (tissue, animals, etc.) will be able to benefit from STED microscopy by 2020. There will certainly be further developments in technology (e.g. optics, lasers, detectors), fluorescent probes and probe carriers (e.g. dyes, nanobodies, aptamers), but as always predicting the future with accuracy is difficult. The greatest advantage of STED imaging, as noted above, is that it operates at the scale of the key players and building blocks of the cell. This makes it possible to localize macromolecular assemblies, colocalize assembly complexes to reveal possible connections, and investigate dynamics to understand mobility, interactions and densities via spectroscopic (STED-FCS) analyses in living cells with nanoscale resolution. A summary of STED applications is provided in Table 3.

Concluding remarks

There have been 20 years of advances since the initial development of this novel super-resolution STED microscopy technique [6]. During this time, this nanoscale imaging tool has shown tremendous

potential for visualizing cellular biology, including details of cellular and even macromolecular structure, has been made compatible with live-cell imaging in several spectral channels and has been applied to measure fast nanoscale dynamics even on the single-molecule level; in the meantime, STED was also selected as the life science method of the year in 2008 [12]. After more than 100 years of being limited in terms of resolution, fluorescence microscopy has thus entered a totally new paradigm. In summary, nanoscale imaging of protein assemblies allows initial quantification of topological distributions (i.e. the precise location of an assembly and its coordinated connections to other assemblies) and estimations of the amount of proteins (i.e. the number of protein assemblies and their individual intensities thus possibly determining protein count) [5, 24, 55, 73, 75]. As the resolution of STED microscopy is technically limited by the hollow depletion beam minima and the surrounding peak intensity for off-switching (controlled by the photokinetics of the fluorescent probe molecules being manipulated), further advances are likely to be in the field of chemistry. Suitable and flexible laser sources are needed to allow optical manipulation of the fluorescent molecules, switching them to different states to sequentially separate individual entities. This suggests that optical engineering and physics holds the key to further developments. It is necessary to develop data analysis methods that approach the noncontinuous super-resolution of separated and distinct nanoclusters. Thus, information theorists and computer scientist can examine a myriad of possible hypotheses to provide accurate information from STED imaging. However, as this is a biomedical review, any advancement in this field is basically 'owned' by the production of the perfect biological sample (if the question being asked is

Table 3 Applications of STED

Scientific area	References
Neuroscience	[5–40, 44, 53–55, 59, 69–78, 81]
Cell biology	[9, 19, 20, 25, 27, 28, 42, 43, 51, 57, 58, 60, 85, 86, 92–94, 101–104]
Immunology, bacteriology and virology	[91, 95, 96]

STED, stimulated emission depletion.

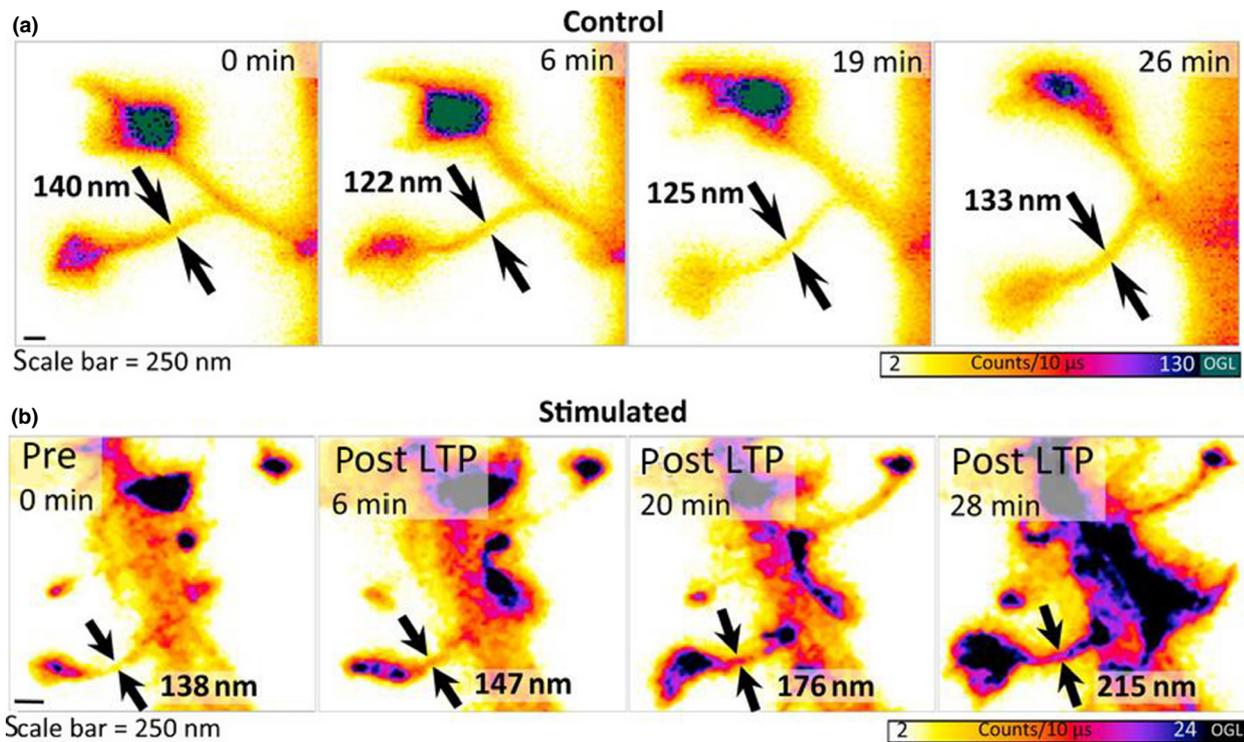


Fig. 5 STED microscopy of intramorphological actin processes in dendritic spines in organotypical hippocampal slice cultures from mice (imaging depth 10–80 μ m). Comparison between control and chemically stimulated neurons, transfected with actin-binding Lifeact-YFP, reveals dynamic morphological changes in the confined spine structures. Reprinted with permission from the licensed publisher Elsevier Ltd.

suitable), if one is to select only one field of improvement. Fruitful collaboration between different scientific areas is likely to advance the field as a whole.

How accessible is this novel STED technique? At present, there are only a few research facilities with either custom-built or commercial STED systems; however, this number is likely to continue growing in the near future, stimulated by the drive to better visualize complex biological structures and dynamic functions in a plethora of cell and tissue types on the nanoscale, possibly even in living organisms. This advance in technology (interplay between advances in chemistry, physics, biology, etc.) must also be accompanied by a greater number of skilled scientist to be able to fully exploit this technique for biomedical research. A complete understanding of molecular functions within cells is only achievable if all interactions are considered and visualized. Therefore, all investigations using confocal or two-photon fluorescence microscopy must be reexamined with

nanoscale imaging using STED (or other super-resolution techniques). In other words, nanoscale imaging will build on the knowledge gained during the last decades, but allow previous findings to be resolved with greater clarity. In particular, analysis of (complex) distributions of proteins via colocalization will in the future benefit, and indeed to some extent have already benefited, from the nanoscale resolution of STED microscopy [85]. It will thus be possible to 'correct' conclusion drawn from diffraction-limited confocal imaging (see Fig. 2e); this could lead to the development of better models and increased understanding of structural and functional interactions. For example, neuronal investigations of controlled and stimulated situations (Fig. 5), using super-resolution fluorescence STED microscopy in living systems, have dramatically increased understanding of how regulation can be influenced on the morphological nanoscale. Such resolved finding may allow a solid handle to pinpoint differences in the biomedical hypothesis of normal and abnormal (sick) situations. With increased understanding of

the cellular nanomachinery – how it works, how it is regulated and the signals involved – the development of novel and more effective pharmacological strategies can be expected, promoted in part by higher-resolution STED imaging.

STED is in fact one of a number of fluorescence switching techniques that allows the nanoscale to be viewed with optical microscopy. Switching in STED is, as discussed above, accomplished by turning the fluorescence off via stimulated emission depletion. It is also possible to switch fluorescence off by inducing conformational changes in fluorescent probe molecules, or ‘photoswitching’ them into a different (triplet or radical) state. Applications of several switching techniques, including the initial STED technique, have emerged in recent years and shown that the diffraction limit of fluorescence microscopy is becoming dated [10]. This ‘switch’ has provided much sharper focus, which has led to initiation of the exposure of the cellular microcosmos. The generalized modality for targeted super-resolution imaging, including STED, has been termed reversible saturable optical fluorescence transition (RESOLFT); such techniques must be driven by light and aim to separate and resolve (sequentially) neighbouring molecules. Recently, specially engineered fluorescent proteins (optically switched between long-lived conformational states) have allowed targeted live-cell imaging under low-light levels of photoswitching, and this has technologically been extended to highly parallel large field of views [105, 106]. Further advances in targeted nanoscale optical switching seem possible.

In conclusion, from the perspective of cellular biology and biomedicine, a fundamental goal is to understand the mechanisms operating in living cells by investigating interactions that elicit and direct molecular events. To date, the sheer complexity of cellular systems has meant that most optical experimental investigations have focused on single systems using relatively crude bulk ensemble-average measurements. Thus, to gain a better experimental understanding of cellular functions, the use of more powerful and precise nanoscopic techniques is required, allowing (at a bottom-up molecular level) the resolution of all functions and connections in different biological systems. It is hoped that an experimentally resolved cellular microcosmos would allow better understanding of the molecular world, and via preclinical and clinical research entangle this into

the possibility of making the ‘macroworld’ of our whole bodies healthier. STED microscopy has in this process begun to allow nanoscale resolution of cellular biology and its connections to disease processes.

Conflict of interest statement

The authors have no conflict of interest to declare.

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