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Optical super-resolution microscopy in neurobiology

Stephan J Sigrist[1] and Bernardo L Sabatini[2]

Understanding the highly plastic nature of neurons requires the dynamic visualization of their molecular and cellular organization in a native context. However, due to the limited resolution of standard light microscopy, many of the structural specializations of neurons cannot be resolved. A recent revolution in light microscopy has given rise to several super-resolution light microscopy methods yielding 2–10-fold higher resolution than conventional microscopy. We here describe the principles behind these techniques as well as their application to the analysis of the molecular architecture of the synapse. Furthermore, we discuss the potential for continued development of super-resolution microscopy as necessary for live imaging of neuronal structure and function in the brain.

Addresses
[1] Cluster of Excellence NeuroCure, Institute for Biology, Freie Universität Berlin, Germany
[2] Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, MA, USA

Corresponding authors: Sigrist, Stephan J (stephan.sigrist@fu-berlin.de) and Sabatini, Bernardo L (bsabatini@hms.harvard.edu)

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Introduction — a need for super-resolution light/fluorescence microscopy in neurobiology

To achieve a deeper understanding of neuronal function, it is necessary to understand the underlying cellular processes, including the localization, interactions, and modifications of the constituent proteins and lipids. Great progress toward this aim has been achieved by tagging proteins of interest with fluorescent probes and measuring the resulting fluorescence distribution with high-resolution microscopy. However, even with highest numerical aperture objectives available, the optical resolution of conventional visible light microscopy is limited by diffraction to roughly half the wavelength of light (150–350 nm). Furthermore, for 2-photon laser-scanning microscopy (2PLSM) applied to biological samples, the diffraction limited resolution is typically even worse (400–500 nm). Cellular and subcellular structures central to neurobiology, most importantly synapses including presynaptic (active zones, presynaptic dense bodies, and neurotransmitter-containing vesicles) and postsynaptic specializations (postsynaptic densities, dendritic spines, and neurotransmitter receptor clusters) are typically smaller than a few hundred nm and are separated by only tens of nm. Thus, the diffraction limit leaves many structures in the crowded cellular space of neurons unresolved.

Available technologies for super-resolution light/fluorescence microscopy

Recent technological breakthroughs have given rise to several super-resolution light microscopy (SRLM) (or 'nanoscopy') methods that yield 2–10-fold higher resolution than conventional microscopy [1–3]. Currently, there are three main methodologies that are available and gaining widespread implementation.

Structured illumination microscopy (Figure 1a)

In *SIM*, the sample is illuminated with a series of sinusoidal striped patterns of high spatial frequency applied at several orientations [4,5]. Subsequent processing of the acquired images and mathematical reconstruction yields a high-resolution image of the underlying structure and a resolution improvement of ~2 ($xy \sim 100$ nm) in the focal plane (Figure 1) [2]. To reconstruct one plane, ~15 images with different illumination patterns are required, resulting in an acquisition time of many seconds to a few minutes for a 3D image stack. Three color 3D-SIM has successfully resolved features of the nuclear envelope that could not be discerned by confocal microscopy [6••]. The main attractive features of SIM for cell biological applications are that standard dyes and staining protocols can be used, and that multicolor 3D is possible with twofold isotropic improvement in resolution.

Stimulated emission depletion (STED) microscopy (Figure 1b)

In STED, resolution enhancement is achieved by shrinking the point-spread function (PSF) of the microscope by depleting the fluorescence emission in the outer areas of the diffraction limited spot via a process called stimulated emission [7]. This requires a doughnut-shaped STED laser beam of high intensity with a zero center. The aligned excitation and STED beams are scanned across the sample and the emission is collected in a confocal manner using sensitive detectors (Photo-Multiplier-Tube (PMTs) and Avalanche-Photodiode (APDs)). The resolution of such a scanning microscope is basically given by the spot size of remaining excited fluorophores and resolutions of 30–50 nm have been achieved with organic fluorophores [8,9]. STED microscopy has been successfully applied to biological

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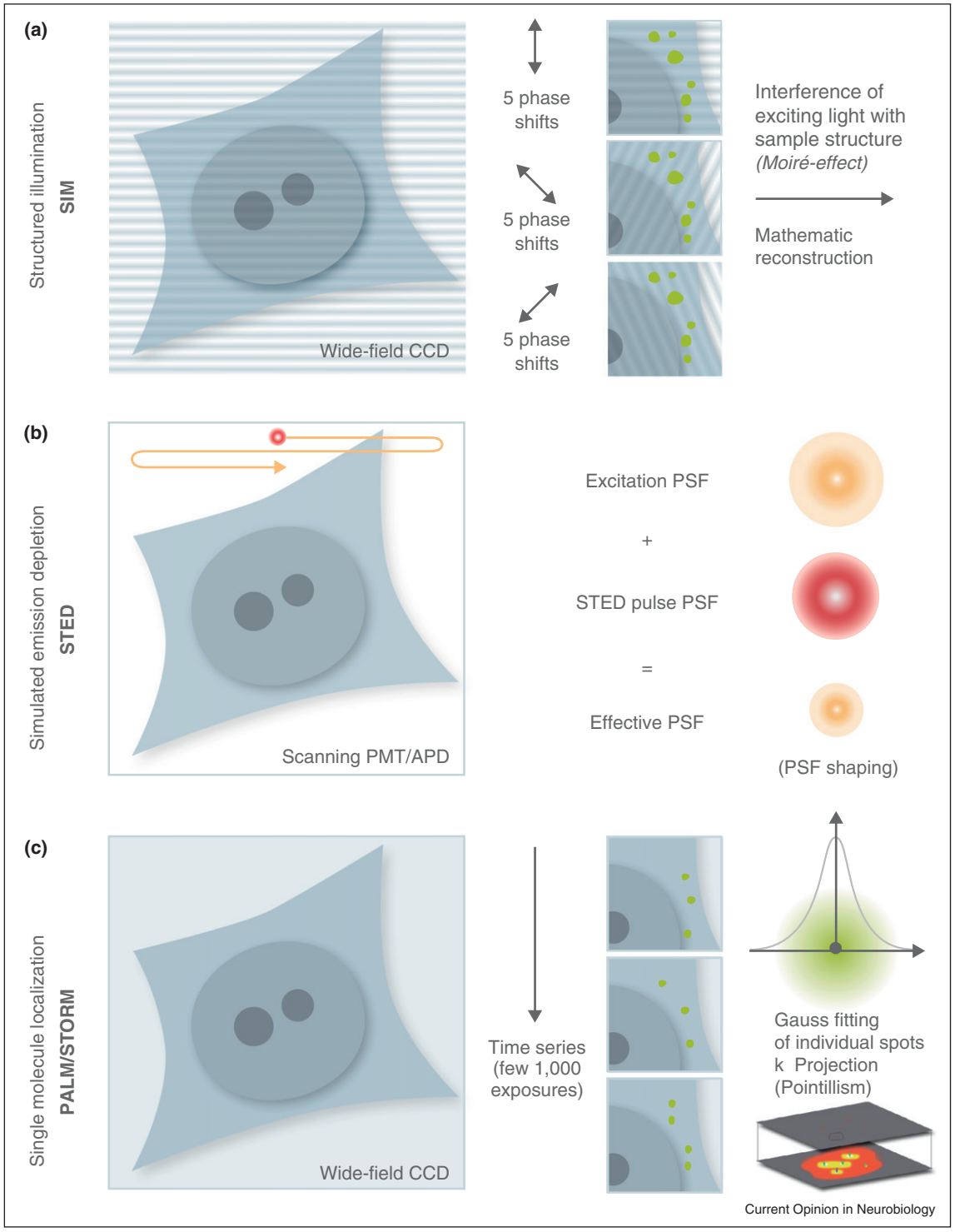
Figure 1



Principles of super-resolution light microscopy approaches. **(a)** Structured illumination microscopy (SIM), **(b)** stimulated emission depletion (STED), and **(c)** single molecule localization microscopy (photo-activatable localization microscopy, PALM and stochastic optical reconstruction microscopy, STORM). After [2].

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samples to visualize the nanoscale organization of proteins and lipids [10–12].

Single molecule localization microscopy (STORM, PALM; Figure 1c)

With most microscopy approaches, the fluorescent molecules are too densely packed to be individually resolved. In photo-activatable localization microscopy (PALM) [13,14] and stochastic optical reconstruction microscopy (STORM) [15], a small subset of the fluorescent molecules are sequentially switched on and imaged with a camera. If the fraction of activated fluorophores is kept low such that the interflurophore distance is large compared to the conventional resolution of the microscope, the spatial location of the active fluorophores can be calculated from the center of mass of the local fluorescence distributions. The final image is constructed in a cumulative manner by adding up all the single molecule localizations [13–16]. The accuracy of these pointilistic approaches is theoretically limited only by the number of photons detected from per activated fluorescent molecule, but in practice is also influenced by sample drift. To allow activation of single fluorescence molecules, PALM and STORM make use of photo-activatable fluorescent proteins and the photo-switching of pairs of cyanine dyes, respectively (for review see [9,17]). Several variations of this method have been published, including PALMIRA (PALM with independently running acquisition; [18]), GSDIM (ground state depletion and individual molecule return; [19]), or dSTORM (direct STORM [20]).

Super-resolution light microscopy for the analysis of synaptic architecture

Chemical synapses are specialized for rapid directional signaling and utilize elaborate signaling machineries. At synaptic active zone (AZ) membranes, action potentials lead to the formation of Ca^{2+}-microdomains at strategically localized clusters of voltage-gated Ca^{2+}-channels. These Ca^{2+}-microdomains trigger exocytosis of synaptic vesicles (SVs), a process that requires tight physical coupling between vesicles and Ca^{2+}-channels in order to operate efficiently. In addition to the major core machinery such as Ca^{2+}-channels and snare complexes that mediate the fusion process, recent proteomic and genomic studies have suggested additional protein species that localize to synapses [21,22]. Highly ordered synaptic protein architectures are clearly seen by electron microscopy, which reveals electron-dense specializations (cytomatrix at the AZ or CAZ) that cover the presynaptic plasma membrane at places where SV fusion occurs. By interactions with the core fusion machinery, the CAZ might confer speed and controllability to the SV fusion process. Moreover, the remarkable differences between CAZs might underlie the differences in SV fusion present between different synapse types [23]. The available evidence suggests that these protein-based architectures evolved to define synapse-specific differences in the spatio-temporal profile of SV fusion (short term plasticity). However, correlating synaptic structure with function requires a detailed characterization of the organization and concerted action of synaptic proteins. In turn, the dissection of the protein architecture of synapses demands imaging with nanometer scale resolution as well as highly specific and efficient molecular identification, difficult tasks to accomplish by conventional imaging techniques.

The last years have provided evidence that a rather small but conserved set of large, typically multidomain proteins (RIM, ELKS, and Liprin family) provide building blocks for the CAZs of both vertebrate and invertebrate synapses. Previous studies identified the ELKS-family member Bruchpilot (BRP), a coiled-coil rich protein of nearly 200 kDa, to localize to AZs in *Drosophila*. Mutants of *brp* were astonishing in that they completely lacked CAZs. Furthermore, Ca^{2+}-channels were mislocalized at AZs, SV release became very inefficient, and short-term synaptic plasticity was drastically altered [10,24].

STED super-resolution microscopy proved an efficient technique to determine why BRP is that critical for CAZ formation [25••] (Figure 2a–c). The analysis of BRP distribution using STED provided an entry point into the protein architecture of active zones, in that BRP was shown to adopt an elongated conformation. Thereby, the N-terminus of BRP was found in a distance of about 100 nm from the C-terminus, with the N-terminus superimposed on the Ca^{2+}-channel clusters centered the AZ membrane center (Figure 2b; for a schematic model see Figure 2c). STED analysis further showed that another AZ organizing protein — DLiprin-α — localizes to a distinct subcompartment of the AZ (Figure 2c, model). In these studies, STED provided ~80 nm *xy* resolution and the rather simple topology of *Drosophila* neuromuscular synaptic terminals allowed identification of whether individual AZs were imaged in a planar or vertical fashion.

In a more recent study [26••], a pointilistic approach (STORM, see above) was shown to be an effective tool to analyze the molecular architecture of subcellular structures in tissue samples. A systematic 3-color, 3D STORM imaging approach was used to map out the protein organization of synapses. The spatial relationships of 10 presynaptic and postsynaptic proteins were determined, revealing a highly oriented organization of presynaptic scaffolding proteins and a differential compartmental distribution of PSD components. Significant variations in the neurotransmitter receptor composition and distribution were observed from synapse to synapse as well as across brain regions (Figure 2).

STED and pointilistic methods: perspectives and combination with electron microscopy

As shown in the examples above, STED and STORM can successfully elaborate protein architectures at the synapse

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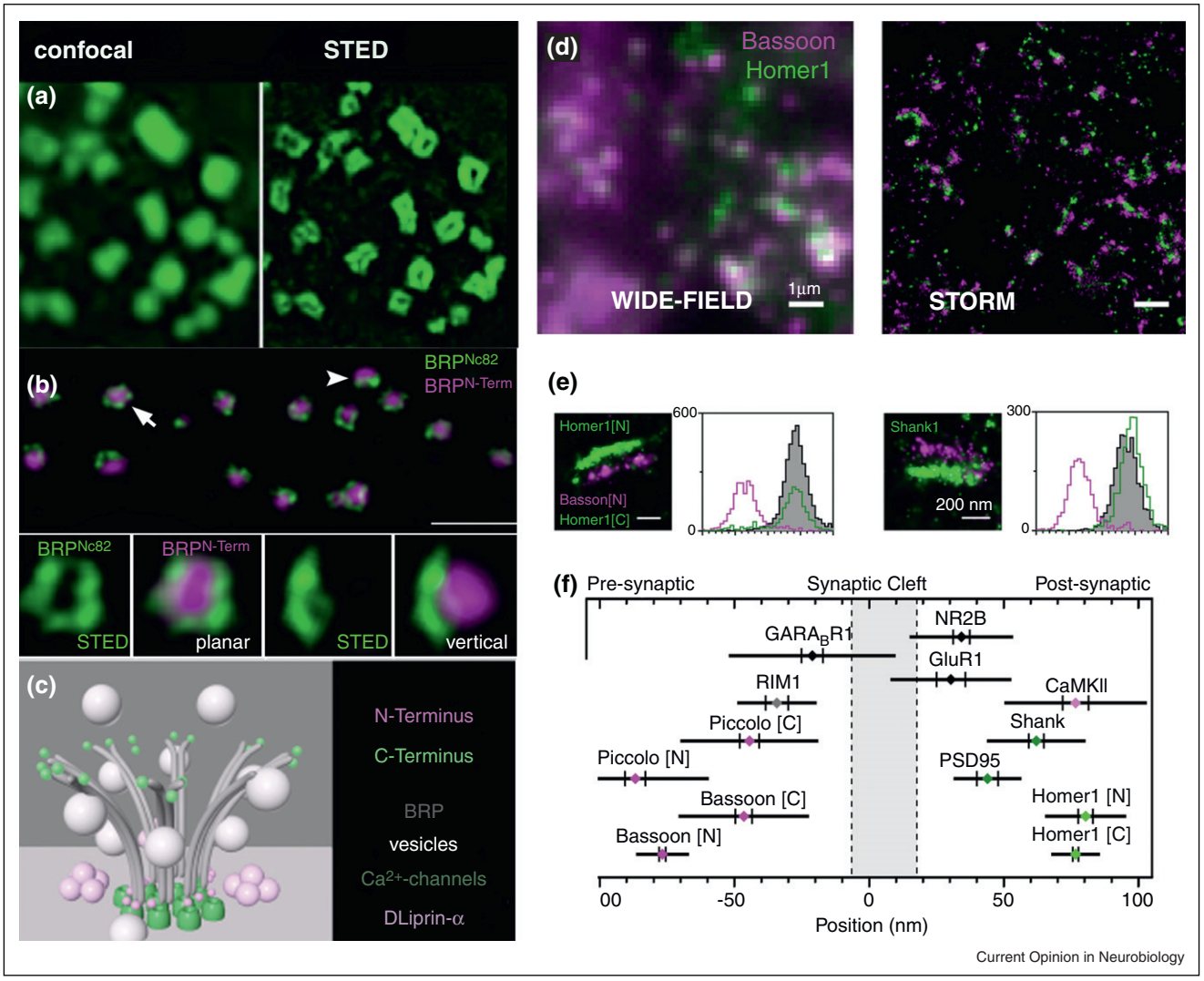
Figure 2



STED and STORM analysis of synaptic architecture. **(a–c)** STED imaging of *Drosophila* neuromuscular synapses. (a) STED microscopy reveals donut-shaped structures recognized by the monoclonal antibody Nc82 against the protein Bruchpilot that are not resolvable by confocal microscopy. (b) Upper panel: Boutons stained for BRP-N-Terminus (confocal; magenta) and BRP-Nc82 (STED; green) showing planar (arrow) and vertical (arrowhead) active zones. Lower panel: Magnifications of individual planar (left) and vertical (right) active zones stained for BRP-Nc82 (STED) and BRP-N-Terminus (confocal; b) and BRP-N-Terminus imaged with (STED). (c) Model of active zone organization at *Drosophila* neuromuscular synapses. **(d–f)** STORM imaging of presynaptic and postsynaptic scaffolding proteins. Presynaptic protein Bassoon and postsynaptic protein Homer1 in glomeruli of the mouse olfactory bulb were identified by immunohistochemistry using Cy3-A647 and A405-A647 conjugated antibodies, respectively. The conventional fluorescence image (d) shows punctate patterns that are partially overlapping, whereas the STORM image of the same area clearly resolves distinct synaptic structures. Further enlargement of the conventional images (e) does not reveal detailed structure of the synapses whereas the corresponding STORM images clearly distinguish the presynaptic Bassoon and postsynaptic Homer1 clusters. (f) Imaging of multiple proteins via STORM reveals their differential localizations in the synapse.
Taken with permission from [26••].

and beyond. Nevertheless, there still is substantial room for improvement and optimization for both techniques. Particularly, both methods pose challenges when it comes to multichannel super-resolution imaging and to improving imaging resolution in the *z*-axis. For STORM and PALM, multiple photo-switchable fluorophores of different colors have been described and recently used to produce multicolor maps of, for example, PSD composition [26••]. In addition, incorporation of cylindrical lenses and interferometric measurements allow for improvement in *z*-axis (axial) resolution, possibly to levels even beyond the *xy* place (transverse) resolution [27•,28•]. However, these approaches have not yet seen widespread use and the need for high precision chromatic aberration

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correction and other complex optics may slow their dissemination. For STED, multichannel systems that can sample from a wide spectrum of fluorophores are complicated due to the requirement of multiple depletion lasers [29••]. Alternatively, multicolor STED can be implemented more simply with a single depletion laser using multiple fluorophores with separable excitation or emission spectra that are depleted at a common wavelength [29••,30] (Leica). Axial resolution is improved in STED by extending the donut used a hollow shell in order that squeezes the PSF in the axial dimension as well. However, once again, the complexity and high power requirements of this system likely contribute to the lack of widespread dissemination of *xyz* super-resolution STED microscopes.

Fluorescence microscopy has the advantage that it can label near to all copies of a particular protein in a sample, whereas in electron microscopy immunocytochemical labeling density is typically low. Nevertheless, the ability to reveal information about the structure of organelles including their membranous aspects is a strength of electron microscopy. Recently, correlative fluorescence and electron microscopy permitted the nanoscopic localization of identified proteins in electron micrographs [31]. Proteins were tagged with the fluorescent proteins Citrine or tdEos and expressed in *Caenorhabditis elegans*, followed by fixation and plastic embedding. The tagged proteins in ultrathin sections were imaged using STED or PALM and the fluorescence distribution was aligned with organelles in the same sections imaged by electron microscopy. In this manner, a presynaptic CAZ protein, Liprin-α, was associated with the synaptic CAZ. Although further optimization of this approach is needed to produce higher quality electron micrographs, correlative fluorescence electron microscopy is a promising way of merging the advantages of electron and super-resolution light microscopy into one picture.

Toward live cell and in-tissue super-resolution imaging

The challenges of performing super-resolution imaging of living cells and in particular of living tissue are substantial. This is particularly the case for fluorescence imaging deep within scattering and light-absorbing tissue such as the mammalian brain or the fly central nervous system [32]. The principal challenges are as follows:

1. Prolonged or intense illumination can photo-damage the specimen, leading to distortion of the structure, death of the cells, or bleaching of fluorophores.
   By definition improving the resolution of a microscope while maintaining the signal-to-noise of each pixel in the image requires collecting more photons. The number of photons required per cubic micron of tissue is inversely proportional to the volume of the effective point-spread function — therefore total photon requirements increase with the cube of the resolution. For this reason, all super-resolution approaches require the use of stable and bright fluorophores that withstand intense or prolonged illumination. STORM and PALM also require these fluorophores to be switchable between dark and light states whereas STED requires that the fluorophore withstand multiple cycles of GHz excitation and depletion [9,33]. In contrast, SIM poses no requirement on the fluorophore beyond stability and brightness [34]. The importance of properties of the fluorophore are made clear when one considers the tremendous improvements in imaging that have been made possible in confocal and 2-photon microscopy due to improvements in genetically encoded and synthetic fluorophores [35].
2. Degradation of excitation or fluorescence wavefronts due to scattering, optically inhomogeneous specimens, and mismatched indices of refraction of the specimen and immersion media.
   As implied by the name, SIM requires that the excitation light achieve a specific spatial distribution in the imaging plane and that the spatial distribution of fluorescence be maintained en route to the imaging camera [34]. Thus, any optical aberrations that degrade the wavefronts of either the excitation or emitted light will degrade the reconstruction and reduce resolution. For these reasons, SIM is limited to thin sections (10–15 μm) that are optically well-matched to the immersion media, precluding its use in thick tissue sections but permitting high-resolution imaging of dispersed cells [34].

In contrast, for generic PALM and STORM, the properties of the excitation light and preservation of a spherical wavefront are largely irrelevant since it is the only the spatial information carried by the emitted photons that is used to determine the location of the activated fluorophore (but see [27•,28•,36] for more complex examples of STORM/PALM based 3D super-resolution imaging that requires a precise illumination wavefront). However, these approaches use the spatial location of emitted photons to reconstruct the image and hence suffer with distortion of the fluorescence wavefront. As described above, the spatial centroid of photons emitted from a single fluorophore are used to pinpoint the location of the fluorophore in the tissue. Therefore, if emitted (fluoresced) photons are highly scattered by the tissue, the spatial information is lost and the ability to localize the fluorophore is degraded. If the tissue is optically heterogeneous and the scattering is anisotropic, the image can be severely and irrevocably distorted. Thus, localization-based super-resolution approaches are limited to use in thin or at the surface of thick sections.

In STED microscopy, the depletion light needs to be precisely patterned and oriented in three dimensions

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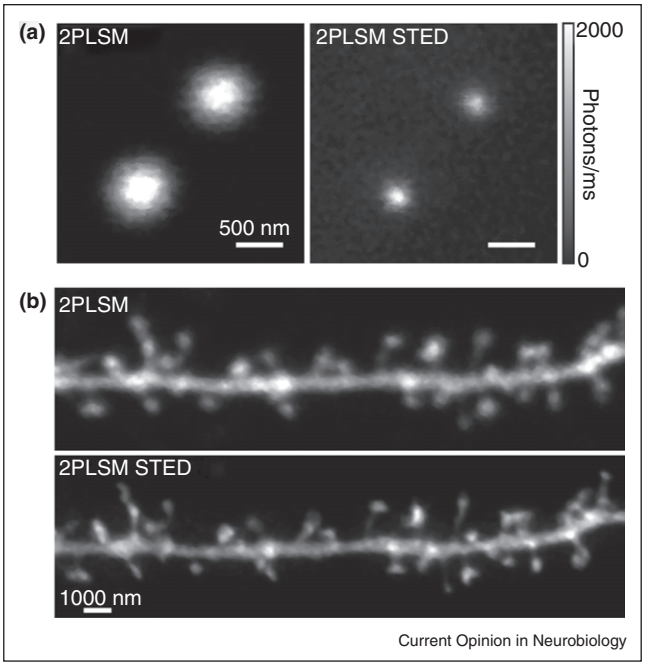
relative to the excitation beam [7]. Index of refraction mismatches between the specimen and the immersion media induce spherical aberrations but these, in theory and due to the rotational symmetry, have relatively little effect on STED beyond a less efficient use of power [37]. However, STED is highly sensitive to inhomogeneities in the tissue which degrade the shape of the annular depletion beam. These effects can be overcome with adaptive optics which corrects for distortions via closed-loop control of adjustable optics (e.g. [38••]). Alternatively, the use of near-infrared photons for excitation and depletion may improve the deep tissue preservation of the focal volume [39,40•]. The goal of these approaches is to maintain the zero intensity of the center of the depletion donut, as any depletion of the centrally located fluorophores quickly degrades the resolution of STED. As for 2-photon laser-scanning microscope, the resolution of STED is insensitive to scattering of the emitted photons because the image is reconstructed based solely on knowledge of the positioning of the excitation laser. Thus, no information is extracted from the location of fluoresced photons and fully scattered emitted photons can be used to form the image. However, thus far high-resolution 1-photon STED imaging has been performed on thin samples using a confocal pinhole in the detection path, presumably to reject out-of-focus and scattered photons. The pinhole rejects image-corrupting out-of-focus fluorescence resulting from diminished efficiency of the depletion beam away from the focal plane, autofluorescence of the tissue, forward-scattering of excitation photons, and fluorescence of the sample induced by the depletion beam. When imaging in scattering thick sections, the presence of such a pinhole will also reject potential signal-carrying fluorophores arising from the focal volume. In theory, the rejection of such photons simply reduces image intensity, but, as is true for traditional confocal microscopy, it is difficult to restore the photon counts necessary to achieve acceptable images. Nevertheless, pinhole-based 1-photon STED microscopy has been used to image exceptionally bright fluorescent neurons below the surface of the brain at resolutions beyond the diffraction limit [41,42•].

Recently STED 2-photon laser-scanning microscopy (2PLSM) using 2-photon excitation of fluorophores and 1-photon depletion has been demonstrated [39,40•,43] and used to image living neurons deep within mammalian brain tissue [40•]. This approach uses near-infrared photons for both excitation and depletion and exploits the natural optical sectioning of 2-photon excitation to form a super-resolution image without the need of a confocal pinhole. The deep-tissue resolution achieved with this approach is limited by deformation of the depletion annulus; nevertheless, the resolution of 2PLSM was improved to beyond the diffraction limit at depths of greater than 100 μm. STED 2PLSM will likely greatly benefit from the application of adaptive optics to compensate for tissue-induced optical aberrations [38••] (Figure 3).

Figure 3



STED 2PLSM imaging of a living neuron in an acute mouse brain slice. **(a)** Images of 200 nm diameter Crimson red fluorescence beads collected with 2PLSM (left) and STED 2PLSM (right) using a pulsed annular depletion beam to achieve resolutions below the diffraction limit. The images were collected near simultaneously by turning the depletion beam on and off on alternating lines. Analyses of the images reveal resolutions of 450 nm (left) and 90 nm (right), indicating that the STED resolution is well below the diffraction limit imposed by the 840 nm imaging laser. **(b)** Images of a spiny dendrite of a hippocampal CA1 pyramidal neuron filled with Alexa Fluor-594 collected with 2PLSM (top) and STED 2PLSM (bottom). The STED image shows a clear improvement in resolution allowing the detection of gaps between neighboring spines, the visualization of the nonspherical structure of many spine heads, and enhancing the signal of small structures relative to the thick dendrite. The dendrite was located approximately 100 μm below the surface of a 300 μm thick brain slice.

Future

The advent and dissemination of super-resolution imaging approaches is revolutionizing biological research, allowing the nanometer localization of proteins within the cell without the need for electron microscopy. Although these approaches have not yet been widely used for biological discovery, this is largely because the technologies are just becoming available beyond the pioneering laboratories that invented them. Nevertheless, the ability to localize proteins within cells at nanometer resolution will allow the composition and organization of macromolecular complexes to be deduced. The extension of these approaches to living cells and complex thick tissues will allow the real time observation of intracellular and morphological dynamics. Key future developments are the crossmodal integration of technologies. Seamless transitions from light to electron microscopy will allow the direct identification of the organelle localization of proteins as well as for the

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analysis of protein composition of organelles, mRNA granules, and other trafficking complexes. Similar localization will also be made possible with pure light microscopy once multicolor super-resolution approaches become standard.

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- •• of outstanding interest

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