Symposium

Super-Resolution Microscopy Opens New Doors to Life at the Nanoscale

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Super-resolution fluorescence microscopy holds tremendous potential for discovery in neuroscience. Much of the molecular machinery and anatomic specializations that give rise to the unique and bewildering electrochemical activity of neurons are nanoscale by design, ranging somewhere between 1 nm and 1 μm. It is at this scale where most of the unknown and exciting action is and where cell biologists flock to in their dreams, but it was off limits for light microscopy until recently. While the optical principles of super-resolution microscopy are firmly established by now, the technology continues to advance rapidly in many crucial areas, enhancing its performance and reliability, and making it more accessible and user-friendly, which is sorely needed. Indeed, super-resolution microscopy techniques are nowadays widely used for visualizing immunolabeled protein distributions in fixed or living cells. However, a great potential of super-resolution microscopy for neuroscience lies in shining light on the nanoscale structures and biochemical activities in live-tissue settings, which should be developed and harnessed much more fully. In this review, we will present several vivid examples based on STED and RESOLFT super-resolution microscopy, illustrating the possibilities and challenges of nano-imaging *in vivo* to pique the interest of tech-developers and neurobiologists alike. We will cover recent technical progress that is facilitating *in vivo* applications, and share new biological insights into the nanoscale mechanisms of cellular communication between neurons and glia.

Key words: **super-resolution; STED; RESOLFT; SUSHI; two-photon; in vivo; imaging; dendritic spines; microglia; synapse; actin**

Introduction

Nanoscale, dense, dynamic, and fragile are just some of the pesky properties of brain tissue that make it so hard to study the molecular and cellular mechanisms of thought and memory or the pathogenesis of brain diseases, such as Alzheimer's disease. The advent of intravital brain imaging with cellular resolution dates back to the invention of two-photon microscopy (Denk et al., 1990). While two-photon technology has progressed in many ways since its inception, and has come to be widely available and used, it has not progressed in one important aspect, and that is spatial resolution, which is limited to ~200–300 nm by the diffraction of light.

This long-standing limit, imposed on all modalities of light microscopy, not just two-photon microscopy, was overcome when a new class of principles and techniques were conceived, creating the field of super-resolution microscopy (including STED, RESOLFT, STORM, PALM, PAINT, MINFLUX). These techniques all have in common that fluorescent molecules are switched between fluorescent and nonfluorescent states in a way that two molecules, which are closer together than the diffraction limit, become discernible (Schermelleh et al., 2019). However, imaging with light in tissue is challenging, and not all super-resolution techniques are equally applicable there. For example, small variations in refractive index between cellular constituents, such as membranes, cytosol, or large protein aggregates result in distortions and scattering of the wavefront of the penetrating light and thus hamper image quality. Moreover, imaging in thick 3D tissue requires methods capable of acquiring images at different depths inside the tissue with high contrast (optical sectioning). This can be achieved by a pinhole in front of the light detector in confocal microscopy or by two-photon excitation, which confines the fluorescence to the focal region. Thus, super-resolution methods that are most easily applied to tissue are those that can provide a sufficient level of optical sectioning. Moreover, imaging in the living brain with its inherent movements typically requires fast recording speed and a compatible fluorescence labeling strategy.

In this review, we present recent advances of reversible saturable optical fluorescent transition (RESOLFT) and stimulated

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emission depletion (STED) microscopy concerning spatial resolution, depth penetration, color contrast, and unbiased labeling. Both techniques are based on the same fundamental idea: by switching off the fluorescence in the outer region of the excitation spot, spontaneous fluorescence becomes restricted to the central region. In this way, the fluorescent region can be reduced to a size greatly below the diffraction barrier, which scales inversely with the off-switching light intensity. In STED microscopy, such off-switching is performed by the process of stimulated emission that depletes the excited (fluorescent) state and requires light of a wavelength matching the emission spectrum of the fluorescent dye and a shaped beam featuring an intensity minimum in the focal plane (Sahl et al., 2019). Since stimulated emission depletion is fast (~1 ns), STED microscopy can offer a higher temporal resolution than other super-resolution techniques. RESOLFT microscopy utilizes reversibly switchable fluorescent proteins (rsFPs), which can be switched from a fluorescent state to a nonfluorescent (dark) state via changes in molecular conformation. The benefit of this technique is that the switching can be performed with very low light levels (W – kW/cm^2): much lower than the light intensities required for STED. However, this is achieved at the expense of temporal resolution, since on/off-switching with rsFPs is a much slower process than stimulated emission, which happens nearly instantly. Recently, this problem was mitigated by an approach termed Molecular Nanoscale Live Imaging with Sectioning Ability (MoNaLISA), which is based on a parallelized scanning scheme (Masullo et al., 2018). This has increased the imaging speed of the RESOLFT technique, making it a valuable alternative for super-resolution imaging in tissue and possibly *in vivo* in the future.

STED microscopy of living brain microstructure

As a point-scanning technique with high intrinsic optical sectioning, STED microscopy has attracted the attention of neurobiologists, who want to study nanoscale phenomena in a macroscale 3D context, such as thick brain tissue sections or the intact brain *in vivo*, and not just in cultured cells in a Petri dish. However, the application of super-resolution microscopy to neuroscience is still facing serious challenges concerning depth penetration, signal-to-noise, and phototoxicity, among many other practical problems related to live imaging of brain tissue.

STED microscopy has been used *in vivo* to visualize dendritic spines with superior spatial resolution compared with 2-photon microscopy, initially in proof-of-principle studies (Berning et al., 2012), but recently also in more biologically oriented work. By using 2-photon excitation STED (2P-STED) microscopy (Bethge et al., 2013) in combination with a special cranial window technique, it became possible to resolve and track dendritic spines over several days *in vivo* in the hippocampus (Pfeiffer et al., 2018). The study provided time-lapse imaging evidence that hippocampal spines are highly dynamic structures, with >40% of them turning over within just a few days under baseline conditions, raising important questions about the ephemeral nature of the morphologic substrates of learning and memory. The use of spatial light modulators facilitates shaping and sharpening the STED light in all three spatial directions, achieving a more isotropic enhancement of the spatial resolution of more deeply embedded target structures (Bancelin et al., 2021), which is important for *in vivo* imaging, where a certain depth penetration is required. The flexible scan-speeds of STED imaging fulfills an important prerequisite for *in vivo* imaging, since living organisms and their organs are in motion, hampering the use of super-resolution techniques based on single-molecule localization, which are much slower.

If conventional light microscopy struggles to resolve dendritic morphology, it is even more inadequate for imaging astrocytes. Their nanoscale processes wrap around synapses, forming the third element in "tripartite" synapses, whose dynamic properties may tune circuit function and animal behavior. They are responsible for mopping up the neurotransmitter glutamate and "neurophysiological" ions like K^+ from the synaptic cleft and releasing neuroactive substances that modulate synaptic transmission. However, because these structures look so fuzzy under a normal microscope, it has been difficult to decipher how they interact with synapses.

This problem was mitigated by a combination of STED microscopy and confocal Ca^{2+} imaging to reveal the nanoscale structure and signaling activity of astrocytes. The study revealed that astrocytic processes form a reticular meshwork of nodes and shafts that are frequently arranged as micron-sized rings (Arizono et al., 2020). The nodes give rise to spontaneous Ca^{2+} signals, which can spread to neighboring nodes via the shafts. Mapping the Ca^{2+} signals onto the STED-resolved morphology showed that astrocytic Ca^{2+} signals are associated mostly with single synapses. Interestingly, the ring-like structures frequently enclose dendritic spines and appear to constrict physically on hypoosmotic conditions (Arizono et al., 2021). Together, these studies pioneered the application of STED microscopy to astrocytes, identifying astrocytic nodes as the elusive anatomic structure that may enable astrocytes to communicate "privately" with many different synapses in parallel.

Despite all of its strong points, fluorescence microscopy has the disadvantage of visualizing only structures that are *a priori* fluorescently labeled, leaving you literally in the dark about the rest. To get a more complete picture, we developed a simple but compelling "inverted" strategy to image brain tissue. Instead of marking individual cells, the spaces between the cells are visualized (the extracellular space [ECS]), using a diffusible but membrane-impermeable fluorescent dye and a homemade 3D-STED microscope.

We dubbed the technique "super-resolution shadow imaging" (SUSHI) (Tønnesen et al., 2018), because all cells appear as dark shadows in a bright sea of fluorescence. SUSHI generates a super-resolved negative imprint of the space occupied by membrane-bound cellular structures, making it possible to view the anatomic organization of live brain tissue in a panoramic, yet detailed way. Although the labeling is by itself unspecific, different cells and their subcellular structures can readily be distinguished based on their morphology. Labeling the ECS rather than individual cells comes with several practical advantages. It is easy to apply and much more resistant to photobleaching and phototoxicity because the dye is on the outside of the cells, thus overcoming two key challenges of STED microscopy. Developed initially for organotypic brain slices, the shadow imaging concept can also be applied to other tissue preparations, from neuronal cell cultures (Inavalli et al., 2019) and acute slices of salivary glands (Stolp et al., 2020) to the intact brain *in vivo*, opening a new window on the anatomic organization of tissue (Fig. 1), and revealing neurons, glia cells, and blood vessels. Moreover, there is a growing interest to study the ECS in its own right, which collectively takes up ~20% of the volume of the brain. The ECS is the obligatory transit station for extracellular signaling molecules and therapeutic substances, influencing neuronal communication and the efficiency of drug treatments. Super-resolution approaches based on 2P-STED/SUSHI (Tønnesen et al., 2018)

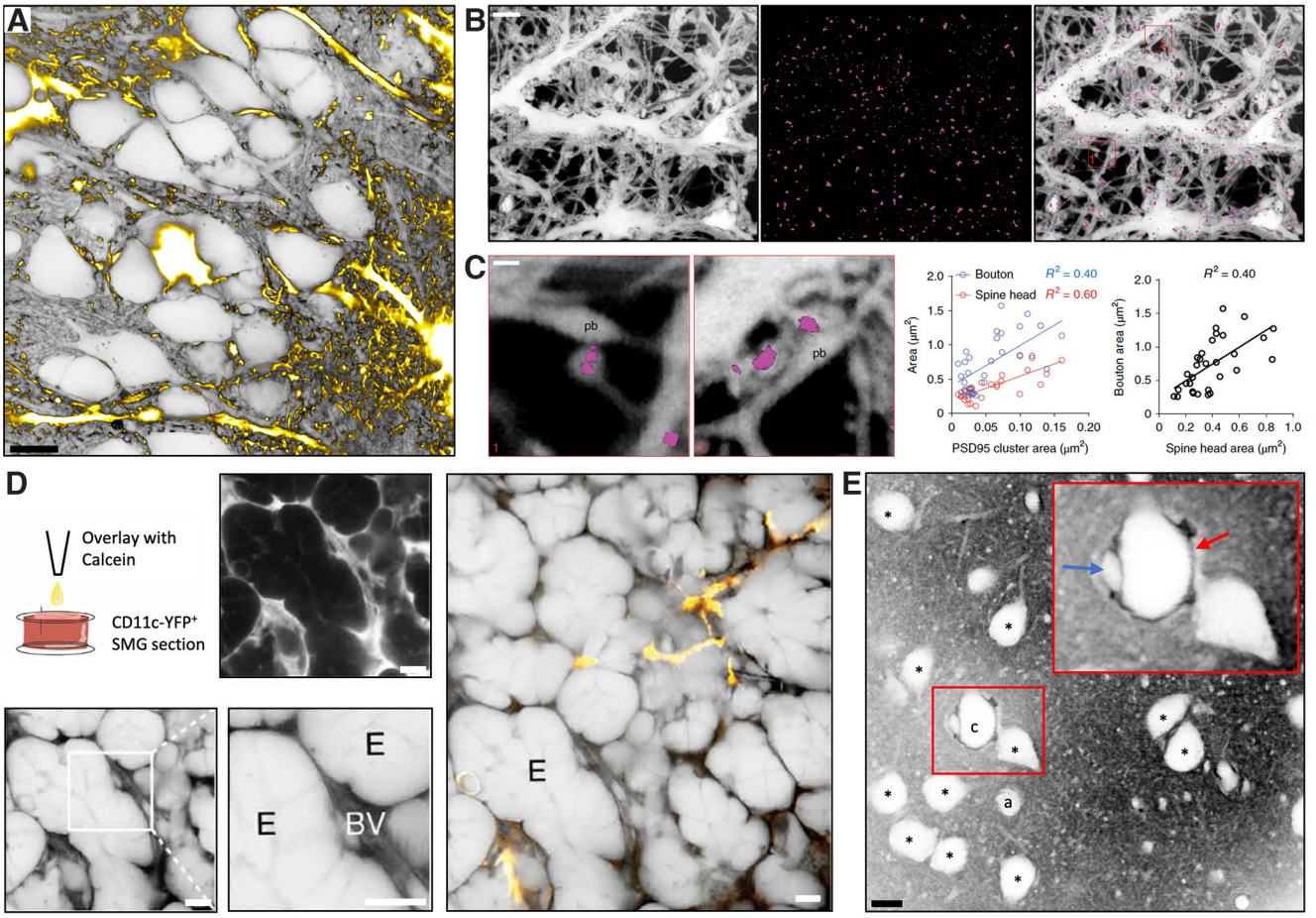

Figure 1. (Super-resolution) shadow imaging in cell cultures, tissue slices, and intact brain *in vivo*. ***A***, Super-resolution shadow imaging based on 3D-STED combined with genetic labeling of astrocytes (fluorescent protein ZsGreen). Scale bar, 10 µm. ***B***, Super-resolution shadow imaging (left) combined with the single-molecule localization microscopy technique PALM (middle) in neuronal cell culture to reveal the morphology and molecular organization of neurons (overlay, right). Punctate stain reveals distribution of endogenous PSD95, a prominent postsynaptic scaffolding protein in excitatory synapses, labeled with FingR_mEos3.2. Scale bar, 2 µm. ***C***, Zoom into areas of putative synaptic contacts between inversely labeled presynaptic and postsynaptic structures, revealing a correlation between PSD95 cluster and presynaptic bouton sizes. Scale bar, 1 µm. ***D***, Super-resolution shadow imaging of epithelium in salivary glands combined with genetic labeling of tissue macrophages (CD11-c with YFP). Scale bars, 10 µm. ***E***, Intravital shadow imaging based on 2-photon microscopy through a cranial window and augmented by adaptive optics using a spatial light modulator. The fluorescent dye (Alexa-488) was stereotaxically injected into the lateral ventricle. The inversely labeled neuropil reveals neuronal cell bodies (*), blood vessel/capillary (c) surrounded by pericyte (blue arrow) and perivascular space (red arrow), as well as glia cells (a). Scale bar, 10 µm.

and single-molecule imaging (Godin et al., 2017) are likely to be instrumental for revealing new key aspects of this important, if understudied, compartment of the brain in the years ahead.

Triple-label *in vivo* STED microscopy of presynaptic and postsynaptic structures

A fundamental ability of fluorescence microscopy is to simultaneously visualize several proteins with different fluorescent tags. Such parallel imaging typically uses fluorophores that are excited or emit in different spectral ranges and therefore can be separated by the excitation and/or detection wavelengths. However, this is a challenge for STED microscopy because a spectrally matched excitation and depletion laser is required for each color of the fluorescent label. Current multicolor STED implementations are mostly based on a single depletion beam; this approach bears the advantage that all images are co-aligned; however, it requires that all labels emit fluorescence in a spectral range close to that of the STED laser. For two labels, this is often achieved by combining two closely emitting dyes: either organic molecules (Göttfert et al., 2013; Bottanelli et al., 2016) or fluorescent proteins (Tønnesen et al., 2011; Willig et al., 2021). For example, the excitation and emission of EGFP and Citrine are only 20 nm apart but by selective excitation with a blue and green laser and by using two different detection channels (Fig. 2*A*), images of either label can be detected with <9% cross talk (Wegner et al., 2022). In addition to that, a third fluorescence marker can be added, for example, if it can be switched on and off and thus imaged sequentially in time. This is possible with rsFPs, such as rsEGFP2 (Fig. 2*B*); rsEGFP2 shows similar spectral properties as EGFP but can be switched from a fluorescent ON state to an OFF state with light (see also paragraph on RESOLFT microscopy). When subjected to a depletion beam in the ON state, stimulation depletion can be performed to achieve super-resolution STED imaging as with standard (nonswitchable) fluorescent proteins. Fluorescence readout with STED super resolution of the three fluorescent proteins can thus be performed as follows: For quasi-simultaneous imaging, each line scan of an image recording is repeated 5 times as depicted in Figure 2*C*. First, rsEGFP2 is switched to the OFF state with blue light; second, EGFP is read out by blue excitation and Citrine with green excitation light. Thereafter, the rsEGFP2 is switched to the ON state by UV light and read out with blue light. The EGFP signal, which is included in the readout of rsEGFP2, is subtracted by subsequent image processing. Since the on/off switching of rsEGFP2 is

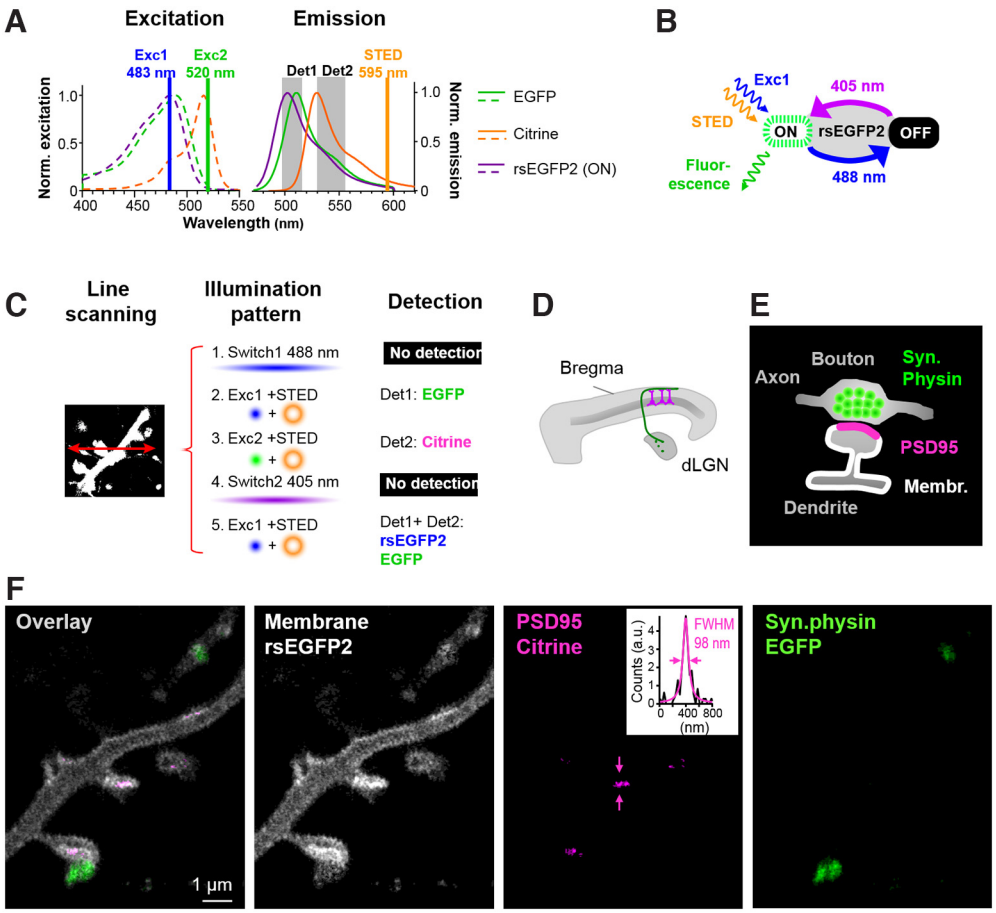

Figure 2. Triple-label *in vivo* STED microscopy of presynaptic and postsynaptic elements. ***A***, ***B***, Spectrally separated excitation and detection of EGFP and Citrine (***A***) are combined with temporal sequential imaging of the reversibly switchable fluorescent protein rsEGFP2 (***B***) for triple-label STED microscopy. ***C***, For quasi-simultaneous imaging, each line scan is repeated 5 times with the indicated illumination pattern and detection channel. Switching time in lines 1 and 4 is reduced by illumination with a line pattern. ***D***, Labeling strategy: thalamocortical projections (green) and apical dendrites of layer 5 pyramidal neurons (magenta) are labeled via viral vectors. ***E***, Presynaptic boutons of thalamocortical projections accumulate synaptic vesicles, which are marked by synaptophysin-EGFP (green); pyramidal neurons express the membrane marker myr-rsEGFP2-LDLR(Ct) (white) and postsynaptic marker PSD95.FingR-Citrine (magenta). ***F***, *In vivo* STED microscopy in layer 1 of the visual cortex of an anesthetized mouse reveals the synaptic nanostructure. Line profile at the marked position indicates sizes not measurable with conventional imaging. Images are smoothed and maximum intensity projected. Adapted from Willig et al. (2021).

much slower than the fluorescence readout, the switching was parallelized by illumination with line patterns (Fig. 2*C*) that enhanced the switching speed (Willig et al., 2021).

Since this method is based on genetically encoded fluorescent proteins, it is directly applicable *in vivo*. This was shown by labeling presynaptic and postsynaptic structures in the visual cortex of mice (Fig. 2*D*). Recombinant adeno-associated virus (rAAV) encoding the synaptic vesicle protein synaptophysin was injected into the thalamus and rAAV encoding a myristoylation tag to label the membrane (to reveal dendritic morphology) and the transcriptionally regulated antibody-like protein FingR.PSD95 (to label PSD95, a postsynaptic scaffolding protein), was injected into layer 5 of the visual cortex (Gross et al., 2013). About 3 weeks after transduction, a cranial window was implanted over the visual cortex. Subsequently, *in vivo* STED microscopy super-resolved presynaptic and postsynaptic elements (Fig. 2*E*,*F*). Accumulation of synaptic vesicles in presynaptic axonal boutons were sometimes in close contact with PSD95, suggesting the presence of a synapse (Fig. 2*F*). This shows that STED microscopy with its superior temporal resolution is capable of super resolving three different labels quasi-simultaneously with a spatial resolution of 60-80 nm (Willig et al., 2021); as such, it can be used to unravel the synaptic nano organization and presynaptic and postsynaptic alignment in the most natural environment: the living brain.

Longitudinal *in vivo* STED microscopy reveals the structural plasticity of stable spines in the cortex

Synapses, through their strengthening or weakening, are a primary locus for neuronal network rewiring in the brain. Synapses need to change dynamically during learning and experience, but they also need to be stable to maintain information over long periods of time (Holtmaat et al., 2005; Hofer et al., 2009; Fu et al., 2012). Excitatory synapses are primarily formed on dendritic spines: minute, highly dynamic processing units whose size is linked to synaptic strength. Because of the limited spatial resolution of classical two-photon excitation microscopy, it has been difficult to assess the substructure and plasticity of these delicate structures on the nanoscale; their bulbous head, which rests on a thin neck, could not be resolved. Therefore, spine heads and necks were often treated as an entity, by analyzing their total brightness changes, or they were treated as binary entities that are present or absent. It requires super-resolution methods to accurately image the shape of spines, and thus to precisely measure neck and head sizes. Furthermore, fast STED microscopy is superior to slower super-resolution techniques because the fast

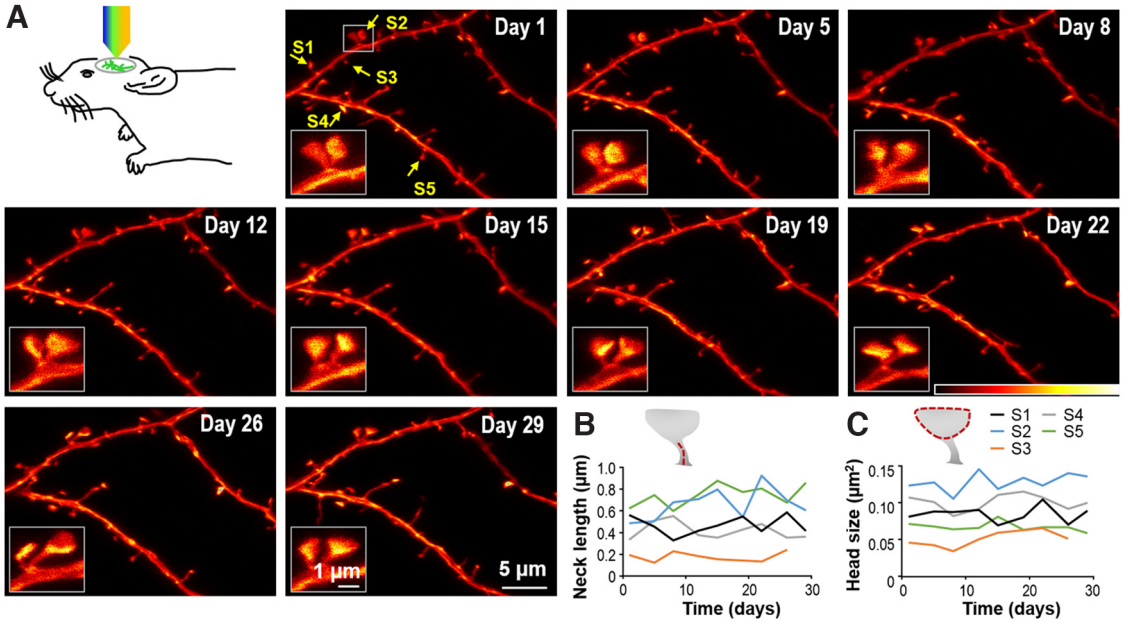

Figure 3. Longitudinal STED imaging in the mouse cortex reveals a delicate balance of stability and volatility. ***A***, STED microscopy through a cranial window over the motor cortex; repeated imaging of a Thy1-GFP mouse twice per week for up to 1 month reveals morphologic changes of dendritic spines in layer 1. ***B***, ***C***, Trajectories of neck length and head size of spines marked in ***A*** show fluctuations between two consecutive time points and persistence over the whole imaging period of 1 month. Adapted from Steffens et al. (2021).

scan-speed makes it more resistant to motion artifacts of living specimens. By optimizing the optical properties of the cranial window and refining the mounting of the anesthetized mouse, it became possible to super-resolve the delicate spine morphology in the mouse cortex over several weeks (Steffens et al., 2021). STED microscopy through a cranial window revealed spine morphology in an anesthetized mouse (Fig. 3*A*,*B*). Repeated imaging twice a week over a period of up to 28 d reported fine dynamic changes in spine morphology. All spine parameters changed between two consecutive imaging time points (Fig. 3*C*); these changes were similar in magnitude to those caused by LTP. Over a period of several weeks, the spine parameters fluctuated around a mean value; thus, average values were fairly constant. In summary, STED microscopy reveals a delicate balance of stability and volatility of dendritic spines *in vivo*.

Hippocampal 2P-STED *in vivo* imaging of postsynapses and microglia

The hippocampus is an important brain region for learning and memory. A subset of CA1 pyramidal neurons fires at specific positions in space and are called place cells (O'Keefe, 1979). Dendrites of hippocampal pyramidal neurons integrate input from CA3 and the entorhinal cortex in stratum oriens, radiatum and stratum lacunosum moleculare (Rogerson et al., 2014). This integration takes place at dendritic spines in the different hippocampal layers. In hippocampal slices, dendritic spines undergo structural plasticity in conjunction with long-lasting functional changes (LTP and LTD) (Engert and Bonhoeffer, 1999; Nägerl et al., 2004). Whether such morphologic changes also occur under physiological conditions in the intact brain, and whether they are associated with learning and memory, remains an open question. Structural plasticity of dendritic spines in the cortex was discovered in 2002, independently in two different laboratories using open-skull or thinned-skull cranial windows for repeated two-photon imaging (Grutzendler et al., 2002; Trachtenberg et al., 2002). Since the dorsal hippocampus is located ~1 mm below the cortical surface, it remained inaccessible for chronic *in vivo* imaging. Two early studies aspirated cortical tissue and implanted lenses and managed to acquire images of hippocampal dendritic spines over restricted periods of a few hours (Mizrahi et al., 2004; Barretto et al., 2009). Recording of spine morphology in the hippocampus *in vivo* over a few days was first achieved by a method that combined cortical aspiration, installation of a metal tube sealed with a coverslip at the bottom (Dombeck et al., 2010), and two-photon imaging with a long-working-distance objective (Gu et al., 2014). Application of the same technique enabled researchers to correlate changes of dendritic spine density with memory acquisition for the first time in a hippocampus-dependent memory test, contextual fear conditioning (Schmid et al., 2016).

In addition to the tube technique, implanting a gradient index (GRIN) lens to access the hippocampus also enabled long-term *in vivo* imaging over days with subcellular resolution of dendritic spines (Attardo et al., 2015). Gu et al. (2014) and Attardo et al. (2015) measured the kinetics of dendritic spine turnover in the hippocampus, but dendritic spine density of hippocampal CA1 neurons was lower in these studies (1-1.2 per micrometer) than in classical electron microscopy studies that measured a density of ~3 per micrometer (Harris et al., 1992). Attardo et al. (2015) used a model based on dendritic spine densities in fixed brain sections to correct for this underestimation that was related to insufficient spatial resolution of two-photon imaging through GRIN lenses or with long-working distance microscope objectives. STED in combination with two-photon excitation was used to improve the lateral (x,y) spatial resolution up to 70 nm (Pfeiffer et al., 2018). Dendritic spines and spine necks are clearly resolvable using 2P-STED *in vivo* imaging in the hippocampus at a depth of 50 µm (Fig. 4*A*–*C*). Furthermore, a high NA objective with a sufficiently long working distance was used to reach the hippocampus (Fig. 4*A*).

Although several new long-working distance objectives have been developed in the past years, an objective that fulfills all the requirements has yet to become available; coverslip correction, NA > 1.0, water immersion, working distance >2.5 mm, and

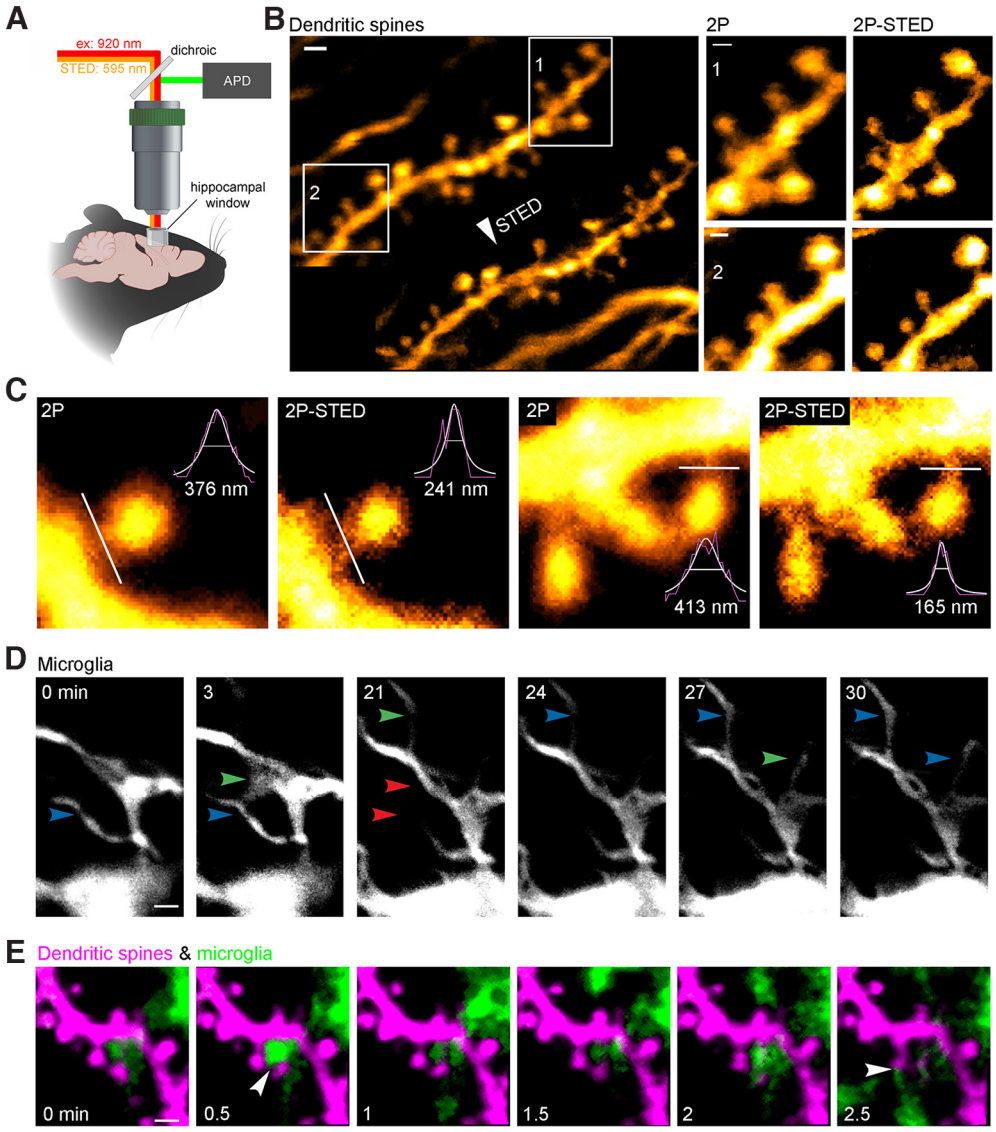

Figure 4. Hippocampal 2P-STED *in vivo* imaging of dendritic spines and microglia. ***A***, Schematic illustrating hippocampal window implantation and 2P-STED imaging in dorsal CA1. ***B***, Exemplary images of dendritic spines of CA1 pyramidal neurons comparing 2P and 2P-STED *in vivo* imaging at 50 µm depth (Thy1-GFP-M mice). Adapted from Pfeiffer et al. (2018). Parts of the figure have been created with www.BioRender.com. ***C***, Exemplary spine neck measurements comparing 2P and 2P-STED mode. ***D***, 2P-STED *in vivo* imaging of microglial processes in the hippocampal CA1 region. Cx3cr1-GFP mice were used to express GFP in microglia. Blue arrows indicate stable microglial processes. Red arrows indicate retracted processes. Green arrows indicate newly emerging microglia processes. ***E***, Simultaneous 2P-STED *in vivo* imaging of microglia (Cx3cr1-GFP mice) and dendritic spines (Thy1-YFP-H mice) over a period of 2.5 min in dorsal CA1. Arrows indicate contacts of microglia and spines over a short period of time. Scale bars: ***B***, 2 µm, 500 nm; ***C***, 2 µm; ***D***, 1 µm.

pupil diameter <7 mm are currently not available. Pfeiffer et al. (2018) used a custom-made microscope to measure spine densities in CA1 pyramidal neurons with 2P-STED imaging and confirmed a high turnover rate of dendritic spines on hippocampal CA1 neurons, previously suggested by Attardo et al. (2015). Long-term 2P-STED *in vivo* imaging data over several days and weeks are not yet available for the hippocampus. But 2P-STED imaging has become easier, since 2P-STED microscopes can now be purchased from companies and do not have to be custom-made. The unprecedented temporal resolution of STED imaging, which is necessary for imaging in moving living specimens, makes it an ideal technique for *in vivo* approaches. Integration of spatial light modulators to position and shape the STED doughnut in the *x*,*y* direction and the bottle-shaped STED beam in *z* direction has become easier now, decreasing the alignment time significantly. These improvements have made it easier to acquire 2P-STED images *in vivo*, enabling the imaging of other cell types, such as microglia at super-resolution. Indeed, applying two-color 2P-STED imaging in the hippocampus *in vivo* enables analysis of the movements of fine microglial processes (Fig. 4*C*), as well as simultaneous recording of microglia processes and dendritic spines showing their interaction (Fig. 4*D*). The latter is currently only possible with conventional two-photon imaging (Nebeling et al., 2019). In the future, 2P-STED imaging will also enable faster imaging, which is necessary to monitor changes of fluorescent indicators for Ca^{2+}, neurotransmitters, or neuromodulators and cannot be conducted with slower super-resolution imaging techniques. In summary, applying STED imaging *in vivo* is a great way to record with unprecedented spatial resolution at time scales ranging from milliseconds to days and weeks from the same brain region, which would be next to impossible to achieve with other existing super-resolution methods.

Parallelized RESOLFT for neuroscience

Microscopy methods capable of monitoring biological processes occurring in parallel, over time in compartments that are micrometers apart are essential for imaging neurons, with their polarized geometry organized in somata, axons, and dendrites. Yet, a neuron is in contact with its surroundings via nanometric structures: the synapses. The dynamics of such multiprotein structures are made possible by the synchronous interaction of cytosolic proteins and membrane organelles within the submicrometric compartment of neurites. Nanoscopes that access resolution below the diffraction limit are required to resolve the nanoscale organization and dynamics of synapses, ideally providing information in extended field of view to not miss the overall neuronal cell complexity.

Among the super-resolution microscopy techniques currently available, STED and RESOLFT nanoscopy (Hell et al., 2003; Hofmann et al., 2005; Grotjohann et al., 2011; Testa et al., 2012) have the spatiotemporal resolution to resolve fine structures <70 nm at 1–30 Hz even in light-scattering tissues, such as nematodes, flies, rodent brain slices, and intact brains. This is because their working principle enables faster recording (1–30 Hz) and it is quite robust to background. The super-resolution information is generated by nano-volumes containing an ensemble of molecules rather than one at the time, resulting in higher signal-to-background. Additionally, they are often implemented in a confocal geometry, which facilitates imaging in 3D samples.

A recently developed parallelized version of the RESOLFT concept leverages its ability for live imaging (Fig. 5*A*). Because of the modest illumination intensities required for rsFP switching, RESOLFT can be implemented not only in a single point-scanning modality, but also "in parallel," enabling recording of larger sample areas without increasing the recording time.

MoNaLISA (Masullo et al., 2018) was specifically developed for parallelized RESOLFT imaging in 3D samples, such as brain slices, to monitor multiple neuronal processes at the same time and in different axial positions (Fig. 5*A*). To do so, multifoci illumination, which effectively blocks out-of-focus light, was introduced in combination with a sinusoidal illumination pattern to achieve, in parallel, subdiffraction spatial information. The sectioning ability of MoNaLISA is crucial to getting access to highly 3D samples, such as the 3D organization of actin filaments expressed in brain cells imaged at 15 µm depth in live organotypic slices (Fig. 5*B*,*C*, adapted from Masullo et al., 2018).

The parallelization speeds up the recording, unveiling dynamics at 1-2 Hz frame rates. This combined spatiotemporal resolution is crucial in investigating the neuronal endoplasmic reticulum (ER), which with its nanometric tubular structures, allocates resources to the extreme periphery of protruding neurites (Fig. 5*D*). In MoNaLISA, compared with confocal microscopy, the structural complexity and fast dynamic reorganization of the tubular endoplasmic reticular network could be followed over time (~40 frames) in different neurites with the reduced photodamage possible with RESOLFT (Fig. 5*E*,*F*) (Damenti et al., 2021). Furthermore, the rearrangement of tubular ER contacting mitochondria could be recorded for different time intervals to show both fast (Fig. 5*E*) and slow (Fig. 5*F*) mitochondria–ER dynamics.

The ER network extends in the axial dimension, especially in soma and large neurites. We developed a system named 3D pRESOLFT (Bodén et al., 2021) featuring isotropic spatial resolution in x-y-z <80 nm and in a parallel geometry to allow volumetric imaging in a few tens of seconds. This new trade-off in spatiotemporal resolution allowed recording the rearrangement of neuronal organelles volumetrically within living neurons. In particular, ER tubules can be visualized while surrounding mitochondria, above and below, revealing a spatial organization otherwise lost in 2D projections (Fig. 5*G*).

Another fundamental aspect of imaging in neuroscience is the ability to observe rearrangements of neurons in response to stimuli. In particular, the strength of synaptic contacts is determined in part by the dimension of the dendritic spine head and the extent and alignment of presynaptic and postsynaptic compartments. These features are modified as part of the process underlying LTP (Fig. 5*H*) (Caroni et al., 2012; Haas et al., 2018; Hruska et al., 2018). To access the packed compartment of the dendritic spine with its complex 3D morphology, axial super resolution is crucial. The conventional microscopy approaches lack the spatial resolution to reveal the axial reorganization of synaptic proteins within dendritic spines at different time points during synaptic potentiation. The isotropic nanoscale spatial resolution of 3D pRESOLFT allowed us to explore the 3D reorganization of the actin cytoskeleton and the scaffolding protein Homer within dendritic spines before and after chemical LTP induction (Fig. 5*H*). The parallelized geometry enabled visualizing hundreds of synapses simultaneously, to pin-point their heterogeneous ultrastructural changes and to correlate the movements in different axial positions of the large neuronal area.

Overall, the parallelized RESOLFT imaging approach is a powerful tool for studying neurons; it is able to integrate spatiotemporal information over multiple scales, minimally perturbing the system of interest, and is compatible with functional studies.

Discussion and Outlook

The examples presented here show that super-resolution microscopy applied to *in vivo* settings is becoming a powerful tool for uncovering the nanoscale mechanisms of brain function. The required technology is now commercially available and has gained a certain degree of maturity as well as user-friendliness. Since microscope objectives with high numerical apertures (NA > 1.0 necessary for super-resolution microscopy) in combination with long-working distances are now available, even imaging in the hippocampus is now feasible (Pfeiffer et al., 2018). Technical advances have improved the microscopes suitable for super-resolution microscopy, but surgical and head fixation techniques have to keep pace. For example, implantation of cranial windows has become a more widely and well-established technique in many laboratories, but fine-tuning of the method is required for super-resolution imaging (Steffens et al., 2020). These advances indicate that super-resolution microscopy is ready for *in vivo* applications. Although brain tissue remains the main area of application of super-resolution microscopy *in vivo*, other organ systems are increasingly being investigated as well.

The development of parallelized RESOLFT imaging in 3D samples made it possible to monitor multiple neuronal processes at the same time and in different axial positions at low light levels. This technique has already been proven to work in brain slices *in situ* and is ripe for *in vivo*. It will be a great advantage to visualize the kinetics of proteins within subcellular structures, such as dendritic spines, astrocytic filopodia, or microglia fine processes with much lower light levels than what is currently used in STED microscopy. The superior temporal resolution of parallelized RESOLFT or STED microscopy will be instrumental in visualizing the kinetics of proteins within these structures. Labeling the interstitial fluid with membrane-impermeant

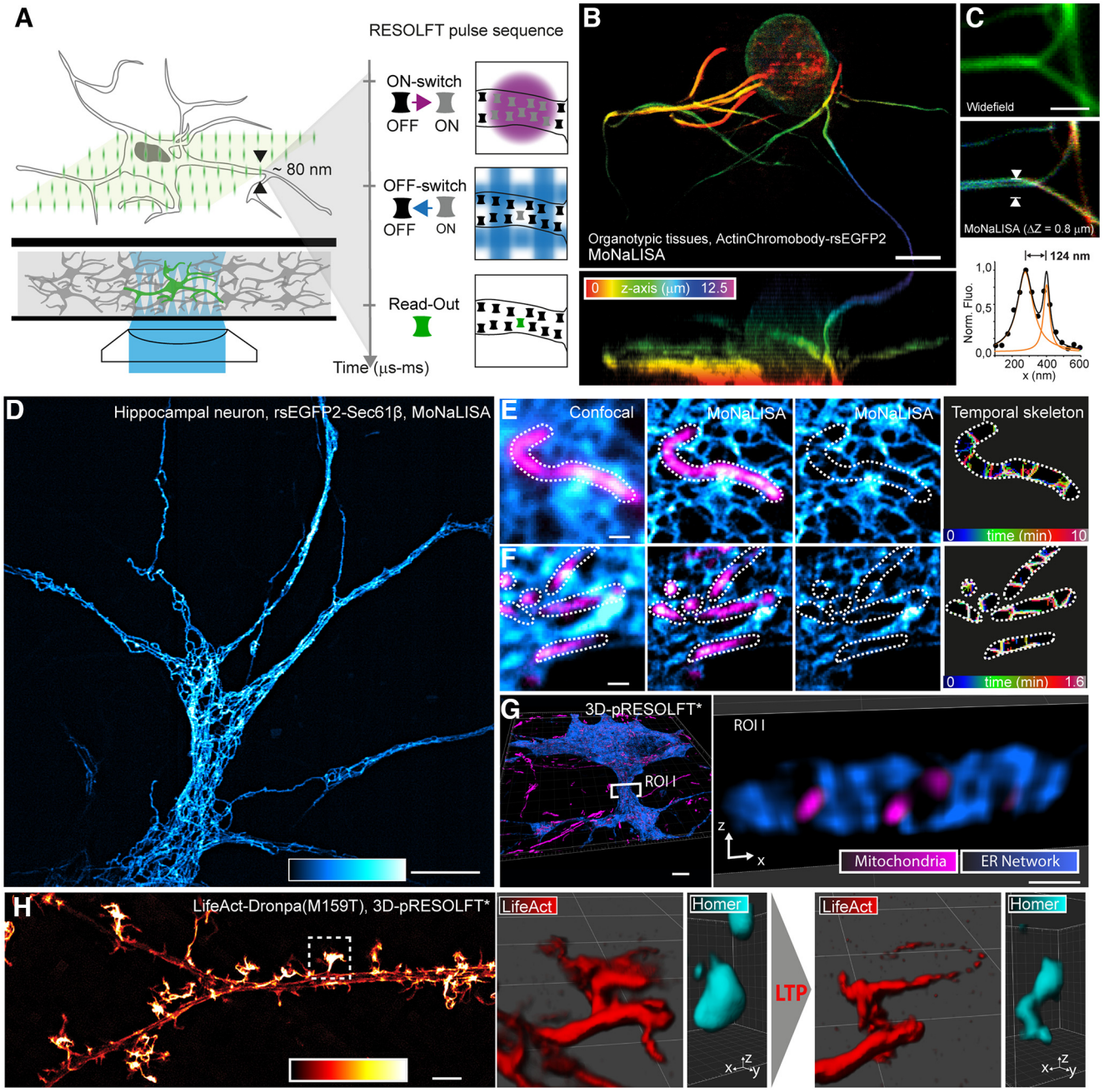

Figure 5. Parallelized RESOLFT in neurons. ***A***, Illustration of the principle of parallelized RESOLFT. ***B***, Young brain cells recorded in organotypic hippocampal rodent brain tissue and represented in an *x-y* and *x-z* maximum intensity projection. Scale bars, 5 µm. ***C***, Comparison between wide-field and MoNaLISA imaging of a neuron expressing LifeAct-rsEGFP2, where the sectioning ability helps resolve the ambiguity of the 3D spatial organization. The line profiles measured in the MoNaLISA image were averaged over 100 nm and fitted with a Gaussian profile. Scale bar, 500 nm. ***D***, ER in primary hippocampal neurons labeled via rsEGFP2-Sec61β. ***E***, ***F***, Dynamic rearrangement of the ER network with respect to mitochondria (labeled with MitoTracker Red) in a hippocampal neuron at 5 d in vitro (DIV5) for different time intervals, seconds (***E***) and minutes (***F***). The first panel reports both channels in confocal modality, while the second and third panels show the MoNaLISA image. In the last panel, the color code projection of the ER skeleton visualizes the different extent of movements that characterize the ER filaments that overlap the mitochondria. A high degree of movement results in well-defined color crossing the mitochondria, whereas a lower degree of movement translates into overlapping filaments and, therefore, white lines. Scale bars: 5 µm, 500 nm. ***G***, Volumetric imaging of the neuronal tubular ER with 3D pRESOLFT∗ in blue and the mitochondria in magenta within a neuron. The ROI I is a magnified *x,z* section in which single tubules of ER can also be disentangled along the axial direction. Scale bar: ~2.5 nm, 500 nm. ***H***, Three-dimensional reorganization on long-term chemical potentiation (LTP). 3D pRESOLFT∗ of actin structure in dendritic spines of mature hippocampal neurons (maximum intensity projection over 1.5 µm axial depth). Representative ROIs for the effect of the LTP, either on the dendritic spines' cytoskeleton (LifeAct- Dronpa(M159T) in red) or the scaffolding protein "Homer" (Homer-1C-Dronpa(M159T) in cyan). Panels adapted from Masullo et al., (2018); Bodén et al. (2021); and Damenti et al. (2021).

fluorescent dyes enables comprehensive and impartial visualization of the anatomic organization of living brain tissue. The shadow imaging approach bears a great potential for tissue imaging in slices or the living brain, ideally with the benefit of super-resolution, but it can be profitably used even without it for visualizing the tissue context in classical fluorescent intravital microscopy approaches. As shown here, it can be used to image dendritic spines for up to 1 month in the cortex and over several days in the hippocampus. Furthermore, more cell types, such as astrocytes and microglia, as well as

subcellular structures can be visualized at the nanoscale. The quest for *in vivo* STED imaging of multilabels has been overcome by combining standard fluorescent proteins with rsFPs. By adding a laser for switching, a two-color STED microscope can be turned into a triple-label super-resolution microscopy. In the future, this technique might be used to unravel the role of the nanostructural alignment of presynaptic and postsynaptic proteins or the plasticity of the "tripartite synapse" during behavioral learning. As for RESOLFT 3D and SUSHI, 2P-STED and STED microscopy could be more widely applied to improve spatial resolution in intravital imaging. It is up to the research community to start integrating these novel developments into their experiments.

A drawback of these techniques is certainly the limited penetration depth, which is a general challenge for all light microscopy techniques. However, recent progress has come from the use of adaptive optics (Booth, 2014) to improve the penetration depth of STED microscopy to 76 µm in the living mouse brain (Velasco et al., 2021). Adaptive optics and wavefront shaping will surely be developed much further in the future to improve the resolution of STED and RESOLFT microscopy deep inside light-scattering tissue.

Up to now, most super-resolution *in vivo* studies involve STED microscopy, probably owing to its inherent sectioning capability. Indeed, it is more difficult to use single-molecule localization-based techniques in densely labeled tissue. Here, the signal is typically detected with a camera; therefore, out-of-focus fluorescence cannot be rejected. However, *in vivo* functional labeling techniques can still be combined, for example, with *ex vivo* STORM measurements in slices (Prokop et al., 2021).

Another research area awaiting the application of super-resolution microscopy is neurodegenerative diseases. Many of these diseases are characterized by aggregating proteins whose structure and localization are usually hard to determine by classical confocal microscopy techniques. Furthermore, neurodegenerative diseases are characterized by synaptic failure, which predestines them for the application of super-resolution techniques (Padmanabhan et al., 2021). There are many more fascinating applications of *in vivo* super-resolution microscopy. If only researchers become more aware of the technical feasibility and biological possibilities, it will not be long before *in vivo* super-resolution microscopy will deliver on its promise and reveal the secrets of life at the nanoscale.

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