Review article

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Nanoscale imaging of the functional anatomy of the brain

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Abstract: Progress in microscopy technology has a long history of triggering major advances in neuroscience. Super-resolution microscopy (SRM), famous for shattering the diffraction barrier of light microscopy, is no exception. SRM gives access to anatomical designs and dynamics of nanostructures, which are impossible to resolve using conventional light microscopy, from the elaborate anatomy of neurons and glial cells, to the organelles and molecules inside of them. In this review, we will mainly focus on a particular SRM technique (STED microscopy), and explain a series of technical developments we have made over the years to make it practical and viable in the field of neuroscience. We will also highlight several neurobiological findings on the dynamic structure-function relationship of neurons and glia cells, which illustrate the value of live-cell STED microscopy, especially when combined with other modern approaches to investigate the nanoscale behavior of brain cells.

Keywords: extracellular space; glial cells; SMLM; STED; super-resolution microscopy; synapse.

Zusammenfassung: Fortschritte in der Mikroskopie-Technik haben in der Vergangenheit immer wieder große Durchbrüche in den Neurowissenschaften ausgelöst. Die superauflösende Fluoreszenzmikroskopie, berühmt für die Durchbrechung der Beugungsgrenze der Lichtmikroskopie, bildet hier keine Ausnahme. Sie ermöglicht beispiellosen Zugang zum anatomischen Aufbau und der Dynamik von Nanostrukturen, die mit konventioneller Lichtmikroskopie nicht auflösbar sind, von der ausgefeilten Anatomie der Nerven- und Gliazellen bis hin zu den Organellen und Proteinen in ihrem Inneren. In diesem Überblicksartikel werden wir hauptsächlich auf die STED-Mikroskopie eingehen und eine Reihe von technischen Neuerungen erläutern, die wir im Laufe der Jahre anwendungsspezifisch dafür entwickelt haben. Wir werden dabei einige unserer neurobiologischen Untersuchungen und Resultate über Synapsen, Gliazellen und den Extrazellulär-Raum vorstellen, wo die ‚live-cell' STED-Mikroskopie in Kombination mit anderen modernen Ansätzen einen entscheidenden Beitrag leisten konnte.

Schlüsselwörter: Extrazellulärer Raum; Gliazellen; SMLM; STED; Super-Resolutions-Mikroskopie; Synapsen.

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Introduction

Progress in microscopy technology has a long history of triggering major advances in neuroscience, most prominently with Golgi's staining technique in the 1870s, electron microscopy (EM) in the 1950s, confocal microscopy, 2-photon microscopy, fluorescent proteins, and optogenetics in recent times. Synergistic and self-amplifying, these innovations have dramatically accelerated the symbiotic progress of technology and biology, the discovery of biological luminescence and the runaway success of GFP-based biosensors being a prime example.

In fact, one of the great controversies in neuroscience history exemplifies the role that new tools and technology play for scientific progress. Enabled by technical breakthroughs in staining and viewing samples of human

brain tissue, the Spanish neuroanatomist Ramon y Cajal made seminal observations about the shape and arrangement of brain cells (DeFelipe, 2009). Against the reigning 'reticular theory' promoted by Camillo Golgi, who thought of the brain as a diffuse network of anastomosing neurons, Cajal proposed the 'neuron doctrine' where discrete, physically separated cells (neurons) transmit electrical signals at special microscopic junctions (chemical synapses) formed by dendritic spines. Both of them were partly right in the end, sharing a Nobel Prize in 1906, because neurons have synapses and gap junctions that mediate stochastic-quantal and gradual-analogue electrical communication, respectively, as since proven by more modern cell biology techniques.

In the perpetual cycle of innovation and discovery, the advent of super-resolution microscopy (SRM) is a recent milestone. It refers to fluorescence imaging techniques that cleverly exploit the 'on–off' behaviour of fluorophores to resolve features in a sample that remain obscure to conventional light microscopy. Recognized by the Nobel Prize in 2014 (shared by Stefan W. Hell, William E. Moerner and Eric Betzig) for breaking the diffraction barrier of light microscopy (which is around 200 nm), SRM opens a new window on the "nanocosm" of biological life.

Indeed, SRM methods are on track to offer single-digit nanometer spatial resolution, unearthing ever more data on the molecular organization and dynamics of cells and tissues. SRM is a powerful tool, especially in neuroscience, because it can capture the extremely elaborate morphology of neurons and glial cells, as well as the crowded arrangement of organelles and molecules inside of them (Figure 1) (Tønnesen and Nägerl, 2013). As the anatomical designs and dynamics of these nanostructures are tightly linked to brain functions, there is a huge interest to develop and apply SRM. Accordingly, SRM is becoming a mainstay in biology departments and core facilities around the world only a few years after their principles and prototypes were developed by a handful of bio-photonics labs.

In this review article, we will explain the basic principles of SRM and highlight several methodological innovations and neurobiological applications we have driven over the last few years.

SRM basics

To understand how super-resolution can be achieved, we first need to appreciate why the resolution of conventional microscopy is limited. Photons that are emitted by a point source of light (such as a single fluorophore) are diffracted by the optics of the microscope, causing them to be projected into a blurry spot instead of a nice and crisp point on the retina, camera screen or other light detector. The size of this spot (Δr) corresponds to the 'diffraction limit of light microscopy' or the 'spatial resolution' of the microscope, which is incarnated by Abbe's simple formula $\Delta r = \lambda/2{\cdot}NA$, where λ denotes the wavelength of the light and NA the numerical aperture of the microscope objective.

Importantly, if there is just one fluorophore, it can be localized very precisely by calculating the geometric center of the spot. However, if there are too many fluorophores that are too close to each other (closer than Δr), this localization step becomes impossible and the fluorophores can no longer be distinguished as their images merge into a blurry whole. This is the case of conventional fluorescence microscopy where basically all fluorophores emit photons at the same time, making the image irredeemably fuzzy.

There are mainly two ways to get around this problem and achieve a resolution that is 10 times or even a hundred times better than Δr (Figure 2). In both cases, the fluorescent dye molecules, and the ability to control their photophysical state with light, take centre stage.

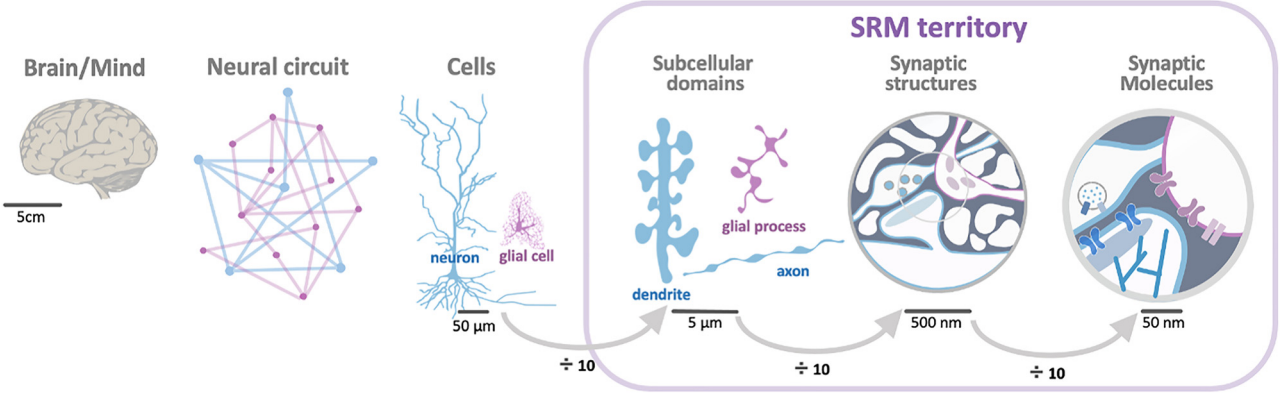

Figure 1: Super-resolution microscopy opens a window into the nanocosm of the brain.
SRM can capture the extremely elaborate nanoscale anatomy of neurons and glial cells, as well as the way organelles and proteins are arranged inside of them.

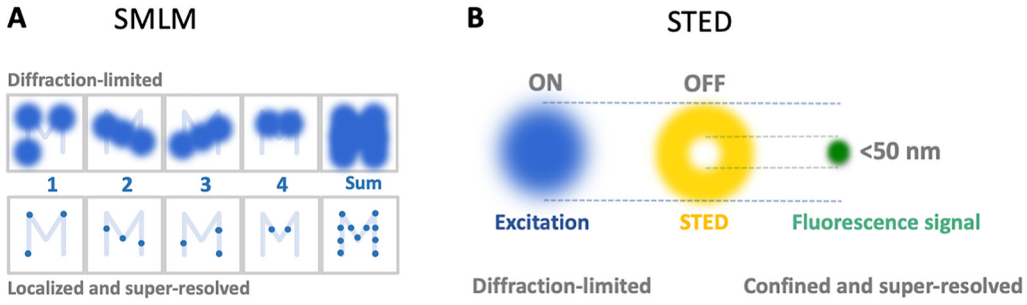

Figure 2: Two major 'tricks' to crack the diffraction limit of light microscopy.
(A) In SMLM, only a sparse subset of fluorophores is stochastically turned on at a given time, making it possible to localize each of them very precisely. By repeating this cycle many thousands of times, a super-resolved image can be constructed by merging all the individual localizations. (B) In addition to an excitation laser, a STED microscope is also equipped with a second laser with the purpose of de-exciting the molecules. By shaping this laser like a donut, it can strongly suppress the fluorescence on the edge of the excitation spot, thereby increasing the spatial resolution.

On the one side, one can find methods that are based on the detection of single molecules (such as PALM, STORM and uPaint) (Fürstenberg and Heilemann, 2013). Instead of exciting many fluorophores at the same time, only a sparse subset is stochastically turned on at a given time, using a special switching mechanism. Under such conditions, it is possible to determine very precisely the location of individual fluorophores, to 'localize' them, assigning each of them a spatial coordinate. By repeating this cycle many thousands of times, each time turning on a different subset of fluorophores, a super-resolved image can be constructed by merging all the individual localizations (Figure 2A).

By analogy, single-molecule localization microscopy (SMLM), as it is commonly referred to, is like drawing a picture in pointillist style, but only after shrinking down all the little dots that collectively produce the composite image. While SMLM is the method of choice for imaging nanoscale protein distributions in cells because of its unrivalled spatial resolution (<10 nm) and ability to provide quantitative information on the number and diffusion of single molecules, its overall temporal resolution is quite slow (on the order of minutes per image).

On the other side, there are methods that reduce the fluorescence spot in laser-scanning microscopes (such as STED and RESOLFT) (Hell, 2007). This reduction is achieved by a second laser (the STED laser), which can de-excite, or 'turn off', molecules by stimulated emission, well known from laser physics. By shaping the STED laser like a donut, it can suppress the fluorescence on the edge of the excitation spot, while leaving it untouched in the middle of the donut, effectively increasing the spatial resolution of the microscope (Figure 2B).

Acquiring a STED image then is like drawing with a pencil that is mounted in front of two closely spaced erasers so that the pencil marks left behind become much thinner. While STED imaging generally offers less spatial resolution (~20–30 nm) than SMLM, it can be relatively fast (on the order of seconds per image) and is well-suited for volumetric imaging of diffusible cytosolic fluorophores to reveal nanoscale cell morphology.

Not only does SRM cost extra time and money, it also comes at the price of technical challenges and setbacks due to its more stringent requirements and taxing conditions compared to conventional microscopy. With SRM, it is typically more challenging to obtain sufficient signal/noise ratios, depth penetration, sample stability. SRM techniques are also more prone to phototoxicity and photobleaching. Hence, for many years, its application was limited to imaging chemically fixed samples very close to the coverslip and remained disconnected from other experimental approaches or measurements. This immaturity was a far cry from the wide scope and effectiveness of well-established fluorescence techniques, such as 2-photon microscopy or simple wide-field imaging.

Fortunately, numerous incremental technical improvements cumulatively have made a big difference for SRM performance, from sample labelling (engineering tags that are brighter and more photostable, smaller, more specific and less invasive) to microscope instrumentation and optics (with tighter engineering tolerances, digital control and automation) to image processing and analysis (with more computing power and better algorithms). Having come of age, SRM is now an indispensable tool for cellular and molecular neuroscience.

In the following, we will describe our journey to making STED microscopy easier to use and more impactful, explaining a series of innovations and tweaks we have introduced over the years.

STED microscopy in living brain tissue

After the demonstration that STED microscopy can greatly improve live-cell imaging of neurons in brain slices (Nägerl et al., 2008), properly resolving their morphological details for the first time with photons instead of electrons, it became desirable to make STED a more useful tool for neurobiologists.

To image in multiple colours is essential for distinguishing different cellular structures (e.g. presynaptic axons, postsynaptic dendrites, glial processes). While this is a given for conventional fluorescence microscopy, it is more difficult to achieve for STED microscopy where the STED laser constrains the available spectral bandwidth. Sidestepping this problem, we came up with a robust method for two-colour STED imaging of neuronal morphology (Tønnesen et al., 2011). By using spectrally similar fluorophores (e.g. GFP and YFP), it is possible to get away with a single pair of excitation and STED lasers to simultaneously image both fluorophores at super-resolution. The two overlapping fluorescence signals can be spectrally separated by simple image processing ('linear unmixing'). This solution avoids the cost and complexity of adding lasers to address each fluorophore separately and rules out chromatic errors to boot.

Many neurobiological questions can only be addressed inside live brain tissue, which demands a certain amount of tissue depth penetration. Unfortunately, the quality of the STED donut degrades rapidly with imaging depth inside biological tissue, obliterating spatial resolution and image contrast. With early STED microscopes, which were designed for oil-immersion objectives, it was only possible to image a few microns into the tissue because of a large mismatch in refractive index between the oil–sample interface. This causes spherical aberrations, which blur out the excitation and STED laser spots. Switching to a glycerine-immersion objective (which has a smaller mismatch and a correction collar to reduce aberrations), proved to be a simple and effective remedy. It significantly extended the depth penetration of STED, making it possible to resolve cellular actin structures as thin as 70 nm at depths of 80 µm below tissue surface (Urban et al., 2011).

In another tack, we developed a new combination of 2-photon and STED microscopy, aiming to combine the unique benefits of both techniques. It enabled us to image in acute brain slices beyond the debris and damaged cells on the surface from cutting the slices (Bethge et al., 2013). We have also adopted an approach to preserve the STED donut by shaping the wavefront of the STED laser beam (Bancelin et al., 2021). It is based on 'adaptive optics', a technique originally developed by astronomers to counteract the optical aberrations from the Earth's atmosphere (Rodríguez and Ji, 2018). When combined with modern computational tools like machine learning, it is possible to optimize the adaptive parameters on the fly, which will greatly facilitate maintaining a good donut deep inside heterogenous tissue.

Another way to achieve higher depth penetration in fixed tissue samples is to modify the optical properties of the sample itself. Recently, we demonstrated that embedding brain slices in a refractive index-matching medium renders them transparent and suppresses the spherical aberrations at the oil–sample interface (Angibaud et al., 2020).

Armed with these technical improvements, we set out to address several interesting neurobiological questions about the morphological dynamics and plasticity of neurons and glial cells. In essence, STED microscopy could solve the classic impasse, where light microscopy can do live-cell imaging but doesn't have enough resolution, while EM has enough resolution but can't do live-cell imaging.

Experiments were usually—unless stated differently—performed in cultured organotypic hippocampal slices, which is a very stable and accessible experimental preparation that retains the main anatomical relationships and synapto-physiological properties of the intact hippocampus.

Spine neck plasticity regulates synapse compartmentalization

Dendritic spines, the tiny dendritic membrane protrusions that Cajal famously observed and drew, are the postsynaptic structural compartments of excitatory synapses in the brain. Despite intense investigations for more than a century, these beautiful structures are still quite enigmatic (Figure 3A). Conspicuously shaped, with a prominent head and elongated neck, spines transform synaptic signals through chemical and electrical compartmentalization (Adrian et al., 2014; Yuste, 2013). However, the impact of spine morphology on synapse compartmentalization remained difficult to assess, because spines, especially their necks, are so small and difficult to resolve. Enabled by a combination of STED and modern electrophysiological and biophysical techniques, we could illuminate the relationship between the nanoscale structure and function of spines (Tønnesen et al., 2014).

We demonstrated that spine necks become substantially wider and shorter after the induction of functional

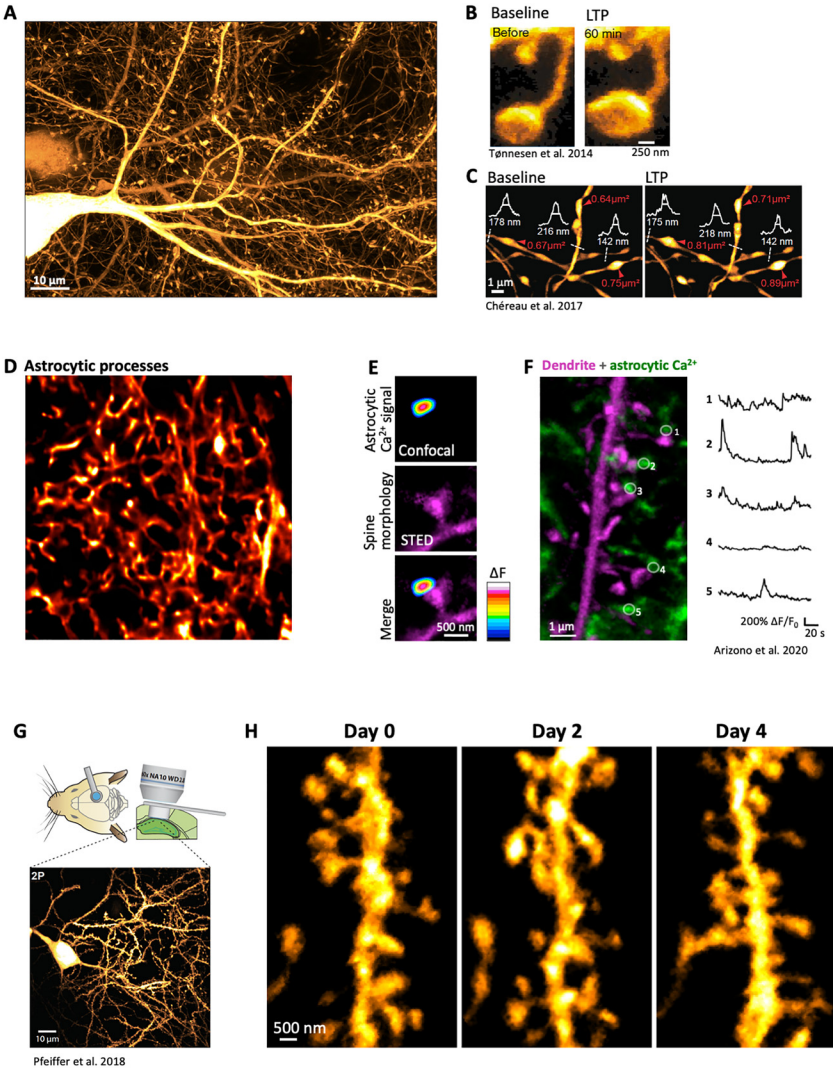

Figure 3: Dissecting the functional nano-anatomy of the brain using live-cell STED microscopy.
(A) A typical pyramidal neuron in the brain, which projects elaborate dendritic and axonal arbors. Experimental induction of synaptic plasticity (LTP) resulted in spine head enlargement/spine neck widening (B) and transient enlargement of axonal boutons (C). (D) Astrocytic processes exhibit a reticular organization in their hyperfine processes. (E) Mapping the Ca^{2+} signals onto the super-resolved morphology showed astrocytic Ca^{2+} activity associated with single synapses. (F) Unique astrocytic Ca^{2+} patterns associated with individual synapses. (G) The combined use of the hippocampal window preparation and a long working distance objective (top) gives access to CA1 hippocampal neurons *in vivo* (bottom). (H) *In vivo* chronic super-resolution imaging (2P-STED microscopy) reveals a high turnover rate of dendritic spines in the hippocampus.

synaptic plasticity induced by glutamate uncaging. These changes could reflect a structural mechanism to modify the strength of synapses (Figure 3B), because any changes that reduce the electrical resistance of the spine neck (which is what neck widening would be expected to do) will decrease the amplitude of excitatory synaptic potentials (EPSP) in the spine head.

Counterintuitively, this reduction in EPSP would actually potentiate the synapse by preventing spine voltages from reaching the Nernst (reversal) potential of the synaptic conductance, where the ionic current into the spine ceases to flow. For this disinhibition effect to be physiologically noticeable, the synaptic conductance and neck resistance would have to be high enough for spine EPSPs to approach the reversal potential, which is likely to be the case for spines with large heads and long or thin necks (Tønnesen and Nägerl, 2016).

Axon plasticity tunes conduction speed of action potentials

Several studies over the 20 years have punctured the classic view of axons as electro-anatomical cables that faithfully conduct action potentials (AP) in an all-or-none fashion to downstream synaptic targets (Debanne et al., 2011; Rama et al., 2018). It was shown that axons have a variety of sophisticated ways to regulate AP conduction (Sasaki et al., 2011) and synaptic transmission (Alle and Geiger, 2006), and thus the timing of signal processing in the brain. While AP conduction speed in unmyelinated axons depends strongly on axon diameter, it was unknown whether AP conduction speed could be dynamically regulated by activity-dependent changes in this biophysical parameter. Again, given the minute

dimensions of the structures involved, this question could not be addressed by conventional light microscopy.

Enabled by time-lapse STED imaging paired with electrophysiology in hippocampal brain slices, we showed that high-frequency AP firing induced physical enlargement of axons, where axonal boutons showed a transient increase, which was followed by a sustained widening of the intervening axonal shafts (Figure 3C). These structural dynamics were mirrored by bidirectional changes in AP conduction speed. A causal link between the nano-structural and functional changes was also supported by pharmacological experiments and mathematical modelling, which closely predicted the effects on AP conduction. Our findings revealed a novel structural plasticity mechanism that tunes the timing of fast electrical signalling (Chéreau et al., 2017).

Astrocytic nanostructures generate calcium signals at tripartite synapses

If conventional light microscopy struggles to resolve neuronal morphology, it totally fails for astrocytes, a type of glial cells in the brain, which has an even more elaborate morphology than neurons. Their nanoscale processes wrap around synapses, forming so-called tripartite synapses, whose elements are thought to be all in close communication to tune synaptic transmission and plasticity. Perisynaptic astrocytic processes are responsible for glutamate uptake from the synaptic cleft and appear to release neuroactive substances that can modulate synaptic transmission. But because everything looks so fuzzy under a normal microscope, it has been difficult to witness how astrocytes interact with synapses (Rusakov, 2015).

To overcome this problem, we turned to 3D-STED microscopy and combined it with confocal Ca^{2+} imaging and FRAP experiments to assess their signalling activity and biophysical properties. We observed that astrocytic processes form a reticular meshwork of nodes and shafts that were frequently arranged as rings (Figure 3D–F). The nodes gave rise to spontaneous Ca^{2+} signals, which tended to stay confined, but could also spread to neighbouring nodes via the shafts. FRAP experiments indicated that nodes can effectively compartmentalize diffusible signals by virtue of their structural design. Mapping the Ca^{2+} signals onto the STED-resolved morphology showed that astrocytic Ca^{2+} activity was associated mostly with single synapses, consistent with the idea that astrocytes can communicate 'privately' with many different synapses at the same time.

Altogether, our study shines new light on the nanoscale organization of astrocytes in live brain tissue, identifying astrocytic nodes as the elusive anatomical structure that may regulate neuronal communication at tripartite synapses (Arizono et al., 2020).

Two-photon STED microscopy reveals turnover of hippocampal spines *in vivo*

Rewiring neural circuits through synapse formation and elimination is thought to be a key mechanism of learning and memory. While experience-dependent spine plasticity has been extensively studied in superficial cortical areas using 2-photon microscopy, little is known about it in the hippocampus, despite its outsize importance for memory processing. This knowledge gap was mainly due to difficulties in gaining optical access to this deeply embedded brain structure, and the fact that 2-photon microscopy struggles to correctly report hippocampal spine density (Attardo et al., 2015; Gu et al., 2014), which can be 10 times higher for hippocampal than cortical neurons (Holtmaat et al., 2006).

In this context, we set out to develop a super-resolution approach to monitor spine plasticity over extended time periods in the hippocampus *in vivo*, developing further our 2-photon STED microscope. To gain optical access, we surgically removed a piece of cortex above the hippocampus and implanted a metal cylinder into the space created. We also retrofitted our 2-photon STED microscope with a long-working distance objective to optically reach the hippocampus below the cortical surface. Performing repeated 'chronic' imaging of fluorescently labelled hippocampal neurons in anesthetized mice, we monitored their spine density and spine turnover over a few days (Pfeiffer et al., 2018).

Because of the high acuity of our imaging approach, the measured spine density was more than twice as high as the values reported by previous 2-photon studies, in good agreement with the gold-standard EM literature. Enabled by this high spine detection efficiency, we could follow individual spines through time. We observed that a stunning 40% of them turned over (i.e. were lost or replaced, while their total number, or density, stayed constant) during four days of observation (Figure 3G and H). Our study provides direct evidence for a high level of circuit remodelling in the hippocampus, supporting the view that hippocampal

synapses serve as transient buffers and dynamic relays for newly formed memory traces.

Super-resolution shadow imaging (SUSHI) of the extracellular space

Despite all of its strong points, fluorescence microscopy has the distinct disadvantage that you only see what you label, leaving you literally in the dark about the rest.

To get a non-biased and truly comprehensive view, we have developed an 'inverted' strategy to label brain tissue. Instead of marking individual cells, we simply label and image the spaces between the cells, using a highly diffusible but membrane-impermeable fluorescent dye and a homemade 3D-STED microscope. Collectively, these in-between spaces are called the extracellular space of the brain (ECS), taking up roughly 20% of the volume of the brain and containing cerebrospinal fluid and the extracellular matrix.

We named the technique 'super-resolution shadow imaging' or SUSHI (Tønnesen et al., 2018) because all cells appear as dark shadows in a bright sea of fluorescence (Figure 4A). SUSHI generates a super-resolved negative imprint of the space occupied by membrane-bound

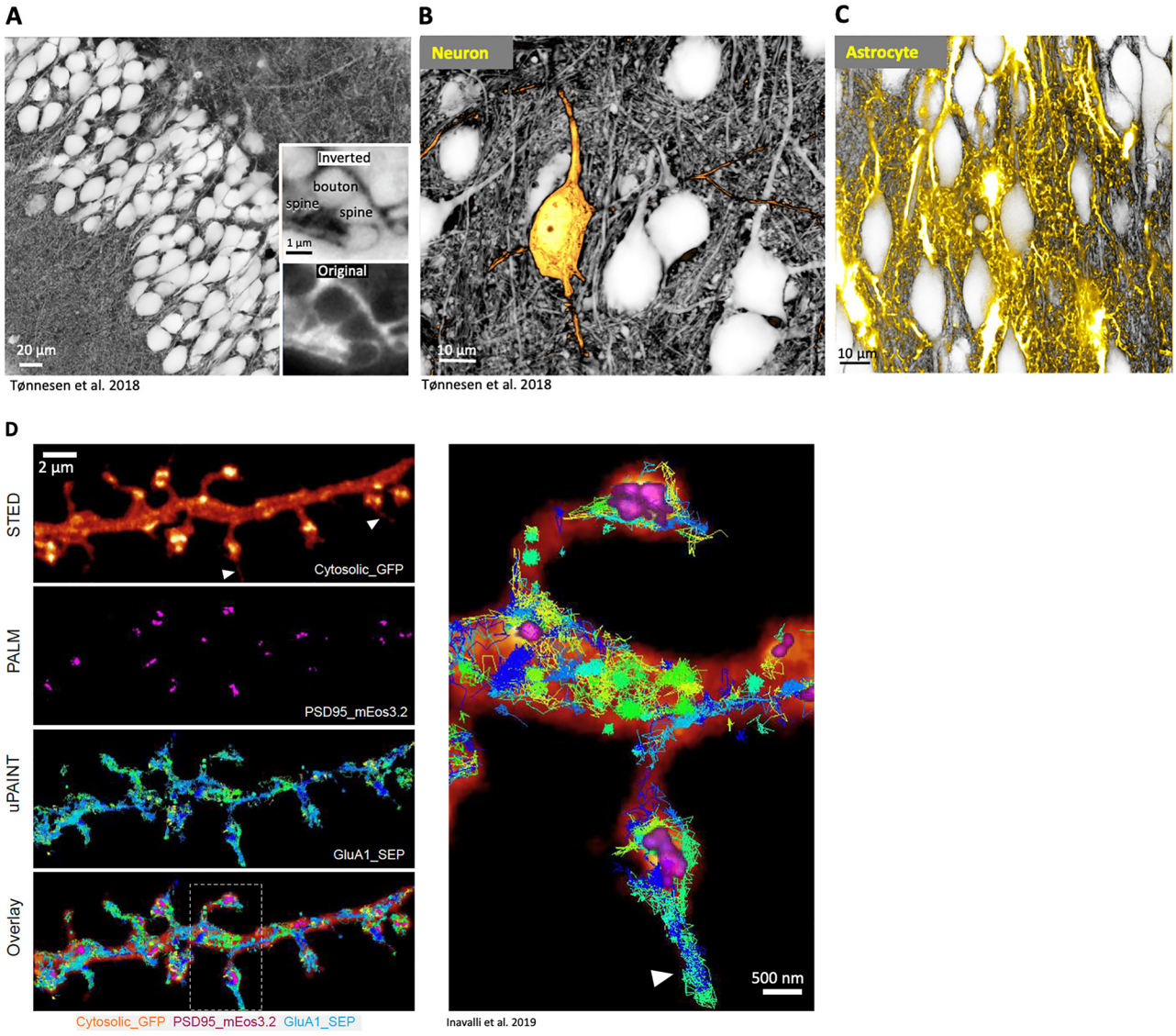

Figure 4: New applications for STED microscopy.
(A) An inverted SUSHI image showing imprints of all brain cells in the tissue. This can be combined with positively labelling of a particular cell type such as neurons (B) and astrocytes (C). (D) Combination of STED and PALM makes it possible to image nanoscale dendritic spine morphology (orange, STED) together with scaffolding proteins (pink, PALM) and receptor dynamics (blue, uPAINT) (left). White arrow indicates 'spinules', a type of 'mini-spine' extending from dendritic spines, where receptors are especially dynamic (right).

cellular structures. As in regular photography, the negative image holds the same structural information as the positive image, making it possible to view the anatomical organization of live brain tissue in a sweepingly panoramic, yet exquisitely detailed way.

Even though the labelling is inherently unspecific, different cell types can readily be distinguished based on their shape. It is even possible to discern synaptic clefts, owing to the nanoscale spatial resolution and favourable contrast conditions, where the super-thin but brightly labelled extracellular fissures stand out against the pitch-black cellular structures that Sandwich them (Figure 4A).

Labelling the ECS rather than individual cells comes with several practical advantages. It is easy to apply, and is much more resistant to photobleaching and phototoxicity. This is because bleached dye molecules get continuously replenished via diffusion and toxic photoreaction products don't build up inside the cells but wash away rapidly.

Not only is SUSHI—especially in combination with positively highlighted cells (Figure 4B and C)—useful for imaging cells and their anatomical relationships, it can also reveal the complex nano-architecture of the ECS. Surrounding all cells, the ECS is the obligatory transit station for extracellular signalling molecules and therapeutic substances, influencing neuronal communication and the efficiency of drug treatments.

EM and MRI have been used to reveal the ECS, but they provide only grossly distorted/static or imprecise/macroscale views, respectively. In fact, the ECS shrinks down to a uniformly thin layer after chemical fixation for EM, bearing little resemblance with its live version, which is actually very voluminous and heterogenous.

Physiologically regulated (Xie et al., 2013) and highly sensitive to experimental stimulations (Arizono et al., 2021; Tønnesen et al., 2018), the ECS provides a dynamic and biophysically influential three-dimensional stage, where neurons and glia cells perform in concert. As a versatile technique, SUSHI can be readily applied to other tissues and organs, from tumours to salivary glands (Stolp et al., 2020).

SRM^2 to image the molecular and morphological organization of live cells

Over the last few years, we have seen a growing diversification and cross-fertilization of SRM techniques, towards more quantitative, multiproperty and integrated analyses of ever more complex biological samples. While SRM offers wonderful opportunities to unravel nanoscale structures and events, current SR microscopes do not offer a good way to reveal both protein distributions and the morphological shape of cells. It meant either one or the other, but not both.

Recently, hybrid SRM modalities were introduced that incorporate optical motifs of STED to enhance the spatial precision of SMLM, such as MINFLUX (Balzarotti et al., 2017) and LocSTED (Puthukodan et al., 2020), but they remain essentially single-molecule imaging techniques.

We have recently overcome this problem by combining fully-fledged versions of SMLM and STED on a single platform, offering a 'best-of-both-worlds' solution (Inavalli et al., 2019). It places the molecular information in the context of the morphological organization of the cells, which is crucial for deciphering cellular nanobiology.

In essence, the new platform makes it possible to closely correlate, in space and time, the data from both imaging modalities. Based on an optimized workflow, one can rapidly and repeatedly switch back and forth between the PALM and STED modes without undue spectral crosstalk and bleaching of the fluorescent probes of the respective modalities.

Using this new approach, we could resolve the distribution and diffusional mobility of several prominent synaptic proteins, such as glutamate receptors (subunits of AMPA receptors, GluA1 and GluA2) and scaffolding proteins (PSD95), within distinct dendritic microstructures in dissociated neuronal cell cultures. We observed that GluA1 molecules are basically arrested inside PSD95 clusters in the spine head but diffuse around rapidly in spinules, which are tiny protrusions projecting from the spine heads (Inavalli et al., 2019), giving us intriguing new insights into the nanoscale dynamics of the molecular machinery of synapses.

Modular and flexible by design, the new platform also incorporates other SRM techniques, such as uPAINT and SUSHI, thereby opening a doorway to important discoveries in cellular biology and neuroscience (Figure 4D).

Outlook

Progress in neuroscience will continue to be shaped by advances in optical microscopy, coming from molecular biology, physics, chemistry, engineering and computer sciences. The new neurophotonics technologies will in time eclipse everything we currently know in terms of speed, precision and gentleness to record and manipulate the key players of brain function from genes and molecules to cells and circuits.

It's probably the right time to start dreaming about linking *in vivo* brain nano-structure, including the ECS, with

nano-physiology and even higher brain functions such as memory and sleep, breaking down the barriers between the different scales and concepts.

To achieve this ambitious goal, we will need more technology 'mash-ups', combining fluorescence SRM with more macroscale and label-free imaging modalities like photoacoustic imaging, MRI or Raman spectroscopy. This would produce complementary and correlative data for a given biological system or give each of the techniques a leg up, opening experimental access to more key parameters on wider temporal and spatial scales.

All this will require the creative imagination of stubborn pioneers and the swarm intelligence of the scientific community, who come up with stimulating controversies and keep the cycle of innovation and discovery turning, very much in the spirit of Cajal and Golgi!

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Dr. Misa Arizono studied neuroscience and received her PhD from the University of Tokyo in 2012 in the Mikoshiba lab. Since 2013, she has been a postdoc in the Nägerl team in Bordeaux. Using cutting-edge microscopy techniques, such as STED and lattice light-sheet calcium imaging, she has been deciphering the biophysical mechanisms that shape the communication between neurons and astrocytes. She has received numerous distinctions, including competitive postdoc fellowships from Japan, the Inoue Research Award *for Young Scientists* and poster prizes at international workshops and conferences. On weekends, she enjoys cooking while traveling around the world of ideas with podcasts. She loves graphic design and scientific communication. Go explore her website: https://arizono0202.wixsite.com/misa-arizono.

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Dr. Stéphane Bancelin studied physics at the École normale supérieure Paris-Saclay and received his PhD in 2013 from the École Polytechnique in Palaiseau, where he worked on SHG microscopy to image connective tissue. During his first postdoc at the University of Quebec in Canada, he developed interferometric approaches in nonlinear microscopy. For his second postdoc, he joined the Nägerl team in Bordeaux, where he is working on the development of super-resolution microscopy to study the cellular mechanisms of memory formation *in vivo*. He recently obtained a tenured position as CNRS researcher at the Interdisciplinary Institute for Neuroscience.

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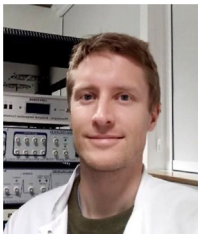
Agata Idziak studied biomedical science and cellular neuroscience at the Philipps-Universität Marburg in Germany. She pursued her master thesis in the team of Prof. Nägerl, studying perineuronal nets using super-resolution microscopy approaches developed by the team. Winning a highly competitive PhD fellowship from Bordeaux Neurocampus, she lives and learns under the supervision of Misa and Valentin, studying the structure & function of brain extracellular space with an array of cool techniques, including new biosensors, SUSHI, lattice light-sheet microscopy (and soon e-phys). In her free time, she likes to travel, experiment in the kitchen, play tennis and hit the twittersphere.



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Dr. Jan Tønnesen studied biology at the University of Copenhagen. He received his PhD from Lund University in Sweden, under the supervision of Prof. Kokaia, where he investigated cell-replacement therapy in Parkinson's disease and optogenetic control of epileptiform activity. From 2010 to 2016, he was a postdoc and researcher in the lab of Prof. Nägerl, using STED microscopy to study spine plasticity. He won the 'Great Advances in Biology' prize from the French Academy of Sciences (2018) for his work on the SUSHI technique. Since 2016, he is a group leader at the Achucarro Basque Center for Neuroscience in Bilbao, where he continues to address questions about synaptic signalling and neuronal excitability using electrophysiological and advanced optical microscopy approaches. When not performing cutting-edge experiments in darkened rooms, he enjoys angling and the outdoors.

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Dr. Ronan Chéreau studied biology at the *Ecole Pratique des Hautes Etudes* in Paris. He received his PhD from the Univ. of Bordeaux in 2014 under the supervision of Prof. Nägerl. Using STED microscopy, he studied the morpho-functional changes of hippocampal axons during plasticity in mouse brain slices. In 2014 he joined the lab of Prof. Holtmaat in Geneva where he has continued to study the physiology of axons. He is particularly interested in understanding the role of the thalamocortical feedback fibers activity during sensory perception and learning and how this activity is integrated in the cortex.

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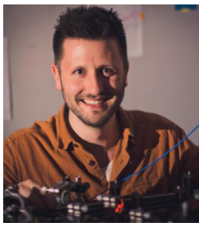
Dr. Philipp Bethge studied psychology and neurobiology at the University Landau and University of Magdeburg before joining the Nägerl lab for his PhD where he developed 2-photon STED microscopy with pulsed laser sources. He continued as a postdoc with an HFSP fellowship in the lab of Prof. Helmchen at the Univ. of Zurich, working on large field-of-view multiarea multiphoton microscopy. He has become the scientific coordinator of the Helmchen team, but also an avid bird photographer and paragliding dare-devil.



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Dr. Thomas Pfeiffer studied molecular biosciences and neuroscience at the University of Heidelberg, Germany. During his master thesis in the group of Prof. Draguhn, he combined calcium imaging and electrophysiological techniques to visualize the activity of hippocampal cell assemblies. Thomas joined the Nägerl lab in Bordeaux for his PhD, studying the dynamics of dendritic spines and microglia using STED and two-photon microscopy in mouse brain slices and *in vivo*. Thomas is currently a postdoctoral researcher with Prof. Attwell at the University College London in the UK, where he focuses on interactions between brain microvasculature and the immune system. In his next move, he wants to shake up the UK biotech industry.

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Dr. V.V.G. Krishna Inavalli obtained his PhD in physics from University of Hyderabad, India, where he developed a method to generate structured light beams with orbital angular momentum and isolated polarization singularities and their applications by demonstrating the rotational Doppler effect. During his first postdoc at the University of Illinois at Urbana Champaign, USA, he developed novel microscopy techniques to image biological tissues and plasmonic nanofilms and worked on techniques for complex wavefront shaping. For his second postdoc, he joined the teams of Valentin Nägerl and Jean-Baptiste Sibarita in Bordeaux, where he developed sharper correlative super-resolution microscopes for cellular neuroscience. Recently, he set sail for the UK, after getting appointed as head of the microscopy group at the Center for Cancer Immunology at the Univ. of Southampton in the UK.