

# Recent applications of superresolution microscopy in neurobiology

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Chemical synapses in brain are structural differentiations where excitatory or inhibitory signals are vectorially transmitted between two neurons. Excitatory synapses occur mostly on dendritic spines, submicron sized protrusions of the neuronal dendritic arborizations. Axons establish contacts with these tiny specializations purported to be the smallest functional processing units in the central nervous system. The minute size of synapses and their macromolecular constituents creates an inherent difficulty for imaging but makes them an ideal object for superresolution microscopy. Here we discuss some representative examples of nanoscopy studies, ranging from quantification of receptors and scaffolding proteins in postsynaptic densities and their dynamic behavior, to imaging of synaptic vesicle proteins and dendritic spines in living neurons or even live animals.

## Addresses

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## Introduction

The new methodologies encompassed under the general terms ‘superresolution microscopy’ and ‘nanoscopy’ (e.g. stimulated emission depletion microscopy (STED), reversible saturable optical fluorescent transition microscopy (RESOLFT), stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), fluorescent photoactivation localization microscopy (FPALM), structured-illumination microscopy (SIM), and others) are currently one of the most rapidly developing research fields, having revolutionized optical microscopy at large. They combine the strength of light microscopy, i.e. the very

specific labeling of immunohistochemistry or genetically encoded tags, the live cell and tissue compatibility and the relative simple use with a resolution below the diffraction limit which is usually reserved for electron microscopy. The reader is referred to other chapters in this Volume for the physical principles behind super-resolution techniques, and for a more comprehensive treatment of the methodological aspects see Ref. [1]). Their applications in the biological sciences continue to grow and the Neurosciences in particular constitute one of the most fertile and challenging grounds for progress on this turf. Contemporary to these developments, unprecedented advances are also being made in unraveling the intricacies of brain organization. One of the avenues opened up by this expansive progress is connectomics, a topic which could be described as the attempt to decipher the connections of different regions of the brain at the mesoscale in structural and, hopefully, functional terms (for a couple of general overviews see [2,3]). Because synapses are the ultimate link in the connectivity meshwork of neurons, and because their substructure cannot be resolved by conventional light microscopy, in our view they constitute one of the critical test objects for superresolution microscopy in the Neurosciences.

Most excitatory synapses in the central nervous system occur on dendritic spines, the postsynaptic specialization envisaged as the smallest known processing unit in brain, and as reviewed in this work, the subject of intense scrutiny by superresolution microscopy. Spines exist in an assortment of morphologies and such structural variants appear to be directly associated with synaptic function. Briefly, the spine possesses a head region connected to the dendritic shaft through a neck region [4]. The head of the spine contains the postsynaptic density (PSD), the most prominent spine microdomain, which in turn contains a dense concentration of neurotransmitter receptors, largely glutamatergic receptors (AMPA and NMDA types), the predominant chemical substances responsible for excitatory transmission in brain [5]. Spine morphology has been shown to change along different functional processes — e.g. in long-term potentiation (LTP), the paradigmatic correlate of synaptic plasticity and learning (see e.g. [6•] for a review) — or under disease conditions (reviewed by Penzes et al. [7•]). In the following section we briefly discuss recent work in which superresolution microscopy has been used to address an important topic in the Neurosciences or played a key role in the experimental approach.

### Imaging presynaptic proteins

The presynaptic terminal harbors a complex secretory machinery, of which the densely packed synaptic vesicles form a key part. Upon binding and fusing with the postsynaptic membrane, synaptic vesicles release their cargo — neurotransmitter — in the synaptic cleft, an exocytic process which occurs with high temporal precision. Electron microscopy has provided snapshots of this highly dynamic process, but given the average size of synaptic vesicles — a few tens of nanometers — super-resolution microscopy is a welcome tool to decipher synaptic vesicle organization and to unravel presynaptic function at large under more physiological conditions than those met by electron microscopy.

One of the first biologically relevant experiments where superresolution was applied to this region of the synapse was the imaging of the clustering of the synaptic vesicle protein synaptotagmin [8]. Denker and co-workers continued work along these lines and studied the reserve pool of synaptic vesicles [9]. After imaging synaptic proteins involved in endocytosis, exocytosis and active zone function with STED microscopy ([9], Fig. S1) they proposed that the reserve pool constitutes a buffer zone for soluble accessory proteins. Another presynaptic protein extensively studied with STED microscopy is the *Drosophila melanogaster* ELKS family protein Bruchpilot (BRP), which forms the electron-dense active zone cytomatrix. Following an early study where the doughnut-like structure was shown for the first time [10], two-color STED microscopy was applied, making apparent a substructure of two different isoforms appearing in an alternating array of clusters [11]. After studying isoform-specific mutants these authors proposed that the size of the Bruchpilot cytomatrix determines the size of the readily releasable pool of synaptic vesicles. Bruchpilot was also studied in a *Drosophila* model for amyotrophic lateral sclerosis (ALS) where the fly homolog of one gene associated with ALS was ablated [12]; interestingly, STED microscopy revealed an aberrant organization of Bruchpilot.

### Imaging postsynaptic proteins

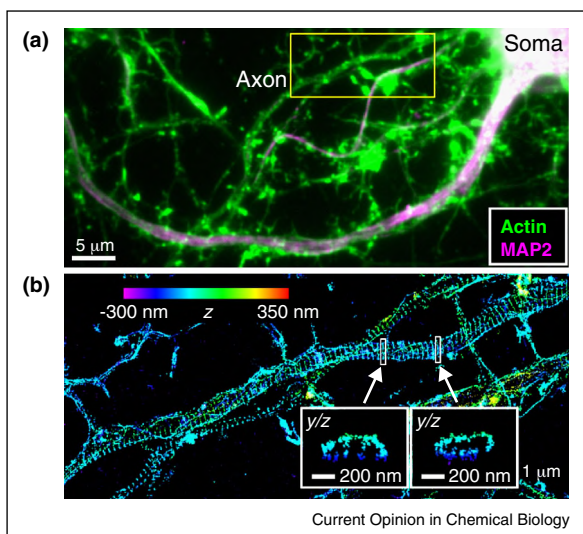
Postsynaptic protein assemblies have also been studied using superresolution microscopy. The first characterization of the supramolecular organization of a neurotransmitter receptor was addressed in an early STED study showing the size distribution, association into nanoclusters and long-range interactions of these nicotinic acetylcholine receptor nanoclusters, and their cholesterol dependence [13]. One-color and later two-color STED microscopy revealed the clustered distribution of another synaptic protein, the  $\alpha 3$  isoform of the  $\text{Na}^+, \text{K}^+$ -ATPase, which is responsible for the active transport of  $\text{Na}^+$  and  $\text{K}^+$  ions across the plasma membrane and is purported to play a role in excitatory synapses [14]. Subsequently, these authors studied the distribution of  $\text{Na}^+, \text{K}^+$ -ATPase in relation to the dopamine D1 receptor in dendritic spines

and applied nearest neighbor analysis to support the finding of both a co-localized and a non co-localized confinement of the receptor and the ionic pump macromolecules in dendritic spines of cultures striatal neurons [15]. A weak overlap albeit clustered distribution was found for the low-abundance phosphoprotein DARPP-32 with the dopamine D1 receptor in spines of striatal neurons [16]. DARPP-32 modulates synaptic conductance by acting as a substrate for several kinases and phosphatases.

An exhaustive work used three-color 3D STORM to map the organization of 10 presynaptic and postsynaptic proteins in cryosections from different brain regions [17<sup>\*</sup>]. By averaging hundreds of synapses, the distance of the presynaptic protein Bassoon and the postsynaptic protein Homer1 was determined to be 153.8 nm. With a third color the axial position of synaptic proteins was mapped relative to Bassoon and Homer1 with nanometer precision. This was accomplished for various proteins of the postsynaptic density, i.e. PSD-95 and Shank1, the calcium/calmodulin-dependent protein kinase-II (CaMKII), subunits of the NMDA and AMPA receptors, the metabotropic GABA receptor, Piccolo — a scaffolding protein related to Bassoon — and RIM1. Moreover, by tagging N-terminal and C-terminal for Bassoon and Piccolo it was found that in both cases the C-terminal region was located closer to the synaptic cleft, thus demonstrating the highly vectorial disposition of these two proteins in the synapse. The aforementioned group increased the resolution by resorting to astigmatism 3D STORM imaging with a dual-objective setup, yielding a resolution of <10 nm in the lateral and <20 nm in the axial direction [18]. More recently, also applying the 3D STORM technique, Zhuang and co-workers [19<sup>\*</sup>] disclosed the beautiful, ring-like structure of actin molecules wrapping around the circumference of axons, but not dendrites, in fixed hippocampal neurons (Figure 1); actin rings were evenly spaced with a periodicity of ~180–190 nm. Two-color images showed that 150–250 nm long rod-like shaped spectrin tetramers also exhibited a periodical distribution, alternating between the actin rings and bridging the gap between them. Adducin, an actin-capping protein, was shown to co-localize with the actin rings. Axons thus display a hitherto unknown ladder-like long-range order of some key cytoskeletal constituents.

For a full understanding of physiologically relevant processes it is highly desirable to have access to the dynamic changes of the structures revealed by superresolution microscopy. Progress has also been made in this direction. For instance, Frost *et al.* [20] tracked motion of single mEos2-tagged actin molecules in spines, revealing discrete points of polymerization near the PSD. To examine actin filaments, Izeddin *et al.* [21] used a filament-binding probe having as fluorescent moiety the photoconvertible tdEos fluorescent protein. They first imaged the actin

Figure 1



STORM imaging of actin filaments in the axon. **(A)** Conventional wide-field fluorescence image of actin (green) and the dendritic marker MAP2 (magenta) in a fixed neuron. **(B)** Three-dimensional STORM image corresponding to the inset outlined by the yellow rectangle in **(A)**, containing axons and devoid of dendrites. The periodic ring-like structure of actin wrapped around the circumference of the axon is clearly apparent. The white boxed insets display the  $y/z$  cross sections. Adapted from [19<sup>\*</sup>] with permission from AAAS.

cytoskeleton in spines of fixed hippocampal neuron cultures with a spatial resolution of  $\sim 25$  nm. Long-term PALM imaging of dynamic changes in the living neuron was also possible because of the replenishment of the low-affinity actin label at a slightly lower resolution ( $\sim 65$  nm) [21].

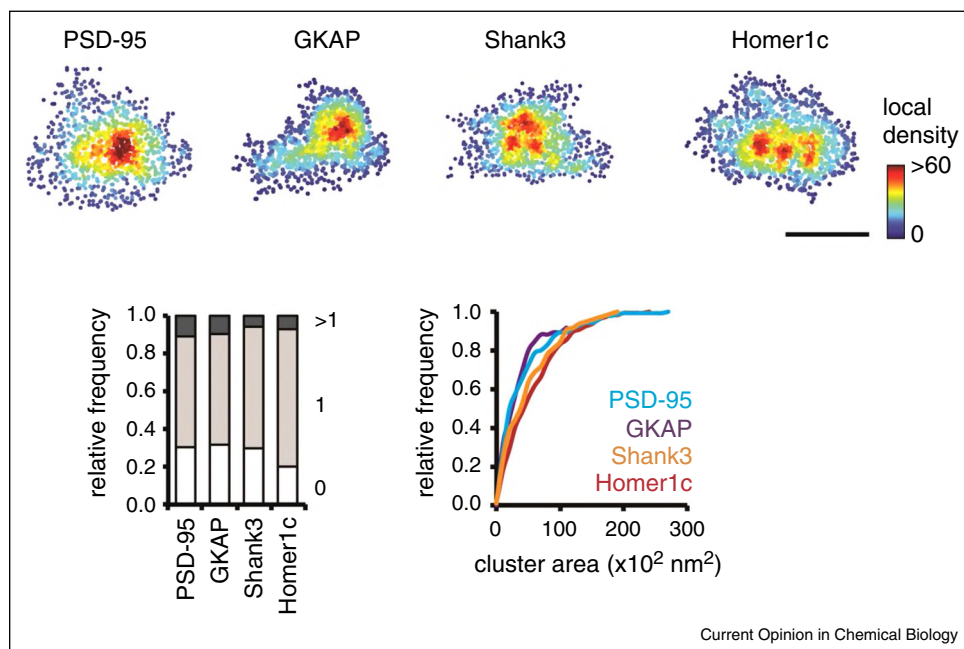
### Counting molecules in the synapse with stochastic superresolution microscopy

In addition to the subdiffraction topographical information that superresolution techniques are able to provide, they also offer some unique insights into functionally important issues in the Neurosciences. Thus the single-molecule information provided by stochastic switching methods such as PALM and STORM microscopies can be used to obtain valuable quantitative information on the protein of interest. In principle, in the case of an ideal photoactivatable probe emitting continuously until bleaching, the superresolution image reconstruction procedure becomes intrinsically a counting experiment. Gauging the absolute number and placement of proteins in a synapse, for instance, is of interest in the study of neurotransmission, since the efficiency of this process is highly correlated with the available amount of neurotransmitter receptors and changes in the number of molecules as a function of time. In this context, using PALM imaging, MacGillavry *et al.* [22<sup>\*</sup>] determined the distribution of immunostained NMDA receptors and

AMPA receptors tagged with the fluorescent protein mEos2 in the PSD, and established the non-random, nanocluster organization of four PSD scaffolding proteins in live neurons at 25 nm resolution (Figure 2). Receptor nanoclusters colocalized with the protein PSD-95, and the clustered architecture was found to control the amplitude and variance of simulated synaptic currents. They could also follow dynamic changes undergone by the nanoclusters. Similarly, Specht *et al.* [23] used a combination of PALM/STORM microscopies to quantify the abundance of the peripheral, non-receptor protein gephyrin, labeled with the fluorescent proteins Dendra2 and mEos2, in inhibitory synapses of a knock-in mouse expressing mRFP-gephyrin [23]. On average, 200 copies of gephyrin molecules were found per synapse, though the absolute figures and cluster size were highly dependent on the type of sample. These authors also showed that the other major inhibitory receptors in brain (GABA<sub>A</sub> receptors) colocalized with gephyrin [23]. In a very recent extension of that study, gephyrin was shown to form scaffolds which act as shallow energy traps ( $\sim 3$  kBT) for glycine neurotransmitter receptors [24]. The depth of these traps appears to be modulated by the biochemical properties of the receptor–gephyrin interaction loop.

When using a bright enough label and living cells, the stochastic switching techniques can also deliver single-molecule diffusion maps at very high particle densities. An approach termed universal point-accumulation-for-imaging-in-nanoscale-topography (uPAINT) implemented by Giannone *et al.* [25<sup>\*</sup>] used an anti-GluR2-ATTO647N antibody to map trajectories of AMPA receptors in cultured hippocampal neurons. These authors were able to map 189 trajectories of AMPA receptors in a single spine [25<sup>\*</sup>]. The combination of single-molecule subdiffraction imaging capabilities (i.e. PALM microscopy) with time-resolved single-particle tracking (SPT) can be used to follow the trajectories of individual molecules in the synapse. Thus, sptPALM of the glutamate subunit-1 AMPA receptor labeled with monomeric Eos2 allowed recording of a large number of individual AMPA receptor trajectories on hippocampal dendrites [26]. Combining the measured local diffusion maps with a mathematical model, the authors found that AMPA receptor accumulation is generated by interactions of the receptor with the membrane rather than molecular aggregation. Interestingly, in a third AMPA receptor superresolution study undertaken in part by the same authors, both uPAINT and sptPALM data of AMPA receptors were recorded and compared (Figure 3) [27<sup>\*</sup>]. Although in this study sptPALM visualized over-expressed AMPA subunits and uPAINT monitored endogenous AMPA receptors tagged with an antibody that did not label all surface receptors, both techniques yielded a comparable distribution of receptors. This close agreement was further confirmed with STED and electron microscopy, showing that AMPA receptors are not uniformly organized in the synapse. AMPA receptor

Figure 2

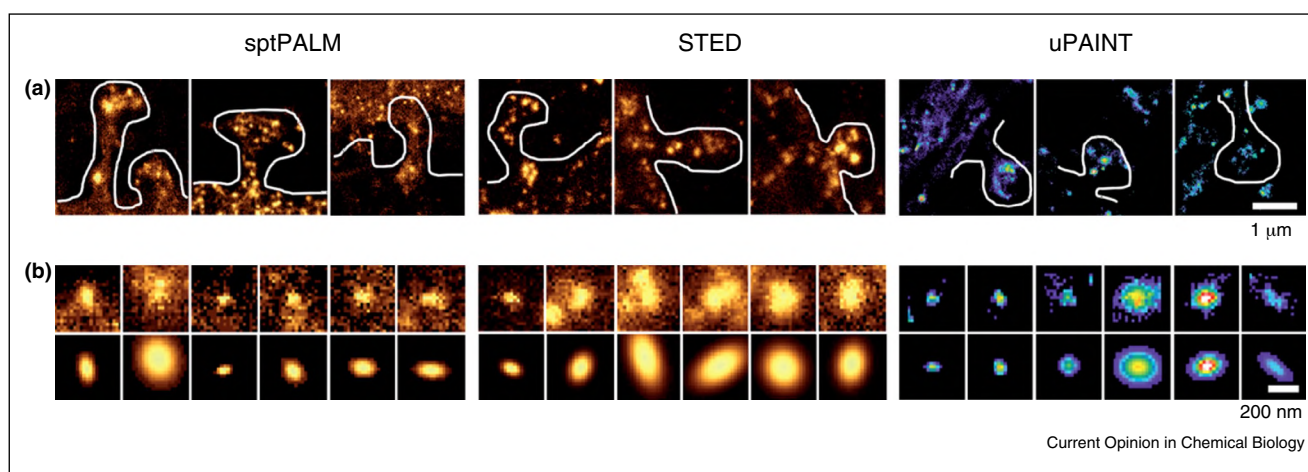


Nanocluster organization of scaffolding proteins in postsynaptic areas containing AMPA receptors. The top row provides examples of PSDs resolved with PALM for mEos2-tagged PSD-95, GKAP, Shank3, and Homer1c. The color-coded representation depicts the local density of the proteins in PSDs, highlighting 'hot spot' areas. Scale bar, 200 nm. In most synapses, these scaffold molecules were enriched in one or two nanoclusters, as shown in the bottom left bar diagram displaying the relative frequency of PSDs with 0, 1, or more clusters. The bottom right figure displays the cumulative frequency distribution and the similar mean of cluster area for the different scaffold molecules. Reprinted from [22] with permission from Elsevier.

nanodomains were also shown to change in shape in a highly dynamic fashion, often colocalizing with the scaffold protein PSD95 [27]. Another recent work applied similar experimental approaches to the adenosine triphosphate

(ATP)-gated P2X7 receptors, members of the purinergic receptor family, labeled with Dendra2 and studied with sptPALM [28]. P2X7 receptors hardly diffuse in the synaptic region, and two populations of receptors were found in

Figure 3



AMPA-R nanodomain size and density by multiple superresolution light imaging techniques. (A) Collection of superresolution images of spines imaged by sptPALM (mEos2-GluA1), STED and uPAINT (Surf-GluA2-ATTO647N). (B) Examples of AMPA-R nanodomains imaged by these techniques (B, upper row) and the corresponding fits with 2D anisotropic Gaussian functions (B, lower row). Most AMPA-R nanoclusters exhibited an elliptical shape, with a median between 63 and 78 nm in fixed neurons. Reprinted from [27] with permission from Elsevier.

extra-synaptic regions: a rapidly diffusing population and one stabilized within nanoclusters of  $\sim 100$  nm diameter.

### Imaging dendritic spines in acute brain slices and in the living animal

The function and dynamics of dendritic spines is most naturally addressed in the intact tissue, e.g. brain slices or even better in the living animal. Because of its penetration depth (down to several hundred  $\mu\text{m}$ ) and its lesser degree of scattering, two-photon microscopy became established in the last two decades as a most efficient method to study thick specimens, and most advantageously, the brain in the live animal [29,30]. However, hampered by a diffraction-limited resolution of 250–500 nm it cannot resolve spine details, thus calling for nanoscopy methods. Up to now dynamic superresolution imaging in the intact tissue has only been demonstrated with the coordinate-targeted superresolution techniques STED and RESOLFT. STED microscopy in combination with YFP was employed to reveal actin dynamics in dendritic spines with a resolution of 60–80 nm at a depth of 120  $\mu\text{m}$  in organotypic hippocampal brain slices [31]. Exchanging the fluorescent protein for Dronpa-M159T, a fast-switching variant of Dronpa, enabled the recording of similar images of actin distribution in slices at much lower light levels using RESOLFT microscopy [32]. STED microscopy of acute brain slices and imaging of dendritic spines has been used by two groups, who implemented a two-photon excitation in combination with pulsed stimulated emission depletion [33,34], one using cytosolic fluorescent protein labels and

establishing also a second color to image protrusions of microglia [35\*\*], the other injecting Alexa Fluor 594 into individual neurons [34].

It has also been possible to observe the fine details of the tiny dendritic spines *in vivo*, in a live mouse. STED microscopy revealed the dynamics of dendritic spines in the visual cortex of YFP-transgenic mice [35\*\*] and most recently of the actin cytoskeleton with a resolution of 50–70 nm (Figure 4) [36\*]. Thus, although the penetration depth of (one-photon) STED microscopy is still limited it can play out its strength in superficial.

### Conclusions and future prospects

Applications of superresolution microscopy in Neurobiology have advanced extremely rapidly in the last few years, providing new details on known neuronal organelles and hitherto unexplored details of synaptic components. Interestingly, in a few cases the new insights have led to revisiting our past views, confirming early hypotheses or challenging current interpretations of synaptic structure and function. These new experimental resources promise to remain major players in the field in years to come, especially in the study of the fine structure of synapses and their molecular constituents. The development and current availability of superresolution techniques affording temporal resolution to record the dynamics of subcellular organelles, single molecules in individual synapses or dendritic spines, and/or interrogate synaptic structure in the live animal, offer an extra twist. Additional but as yet unforeseeable new opportunities to correlate quantitative nanoscale structural data with functional information in the Neurosciences, for example by combining superresolution with functional imaging such as optogenetics or electrophysiology, lie around the corner.

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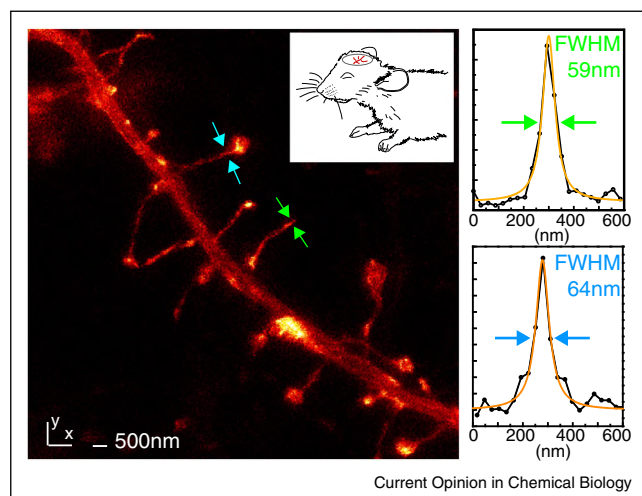
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**Figure 4**



*In vivo* STED microscopy of F-actin along the dendritic shaft and in dendritic spines. The image is the maximum intensity projection of the raw data, and it corresponds to a dendrite in the molecular layer of the visual cortex recorded in a live mouse. The blue and green arrows point to two narrow parts in the spine. Right panels: Line profiles of the blue and green-labeled positions and their corresponding Lorentzian fits (FWHM, full-width half-maximum). Adapted from [36\*] with permission from Elsevier.

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