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Review Article

The internal architecture of dendritic spines revealed by super-resolution imaging: What did we learn so far?

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ABSTRACT

The molecular architecture of dendritic spines defines the efficiency of signal transmission across excitatory synapses. It is therefore critical to understand the mechanisms that control the dynamic localization of the molecular constituents within spines. However, because of the small scale at which most processes within spines take place, conventional light microscopy techniques are not adequate to provide the necessary level of resolution. Recently, super-resolution imaging techniques have overcome the classical barrier imposed by the diffraction of light, and can now resolve the localization and dynamic behavior of proteins within small compartments with nanometer precision, revolutionizing the study of dendritic spine architecture. Here, we highlight exciting new findings from recent super-resolution studies on neuronal spines, and discuss how these studies revealed important new insights into how protein complexes are assembled and how their dynamic behavior shapes the efficiency of synaptic transmission.

Contents

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Introduction

Dendritic spines are specialized, micron-sized membrane protrusions on the dendritic shaft that contain the components of the postsynaptic machinery necessary for efficient excitatory synaptic transmission. Most notably, spines contain the postsynaptic density (PSD), the multi-molecular structure essential for the anchoring of glutamate receptors, and a dense, highly branched network of actin filaments. The morphology of individual dendritic spines is highly variable, even along a single dendrite, and found to strongly correlate with the strength of synaptic transmission. For instance, bigger spine heads contain larger PSDs and a higher number of AMPA-type glutamate receptors (AMPARs) [1,2]. Also, spine morphology is highly plastic, is continuously changing shape over time, and can be dramatically modified by plasticity-inducing stimuli such as long-term potentiation (LTP) or depression (LTD), which are associated with enlargement or shrinkage of the spine respectively [3]. However, although gross spine morphology is a broadly held correlate of synaptic maturity and strength, we have little information on the organization of the molecular machineries *within* the spine that mechanistically determine synaptic function.

Classically, insights in the internal structure and localization of proteins within spines originate from ultrastructural studies using electron microscopy (EM). Indeed, EM provides the highest resolution currently possible and exciting new EM-based technologies continue to be developed and improved, but the resolving nature of EM relies on invasive fixation procedures and is inherently incompatible with live-cell imaging. Fluorescent light microscopy on the other hand allows for specific, non-invasive labeling of cellular structures in living cells. However, because of the inherent limit in resolving power imposed by the diffraction of light, the spatial resolution of fluorescent imaging is restricted typically to ~250 nm. Fortunately, the development of a variety of super-resolution imaging techniques that can by-pass this physical limit now offer a combination of live-cell compatibility, high specificity and efficient labeling at superior resolution far below the classical diffraction limit. In a short period of time these techniques have revolutionized cell biology research by providing the resolving power to study sub-diffraction cellular structures such as neuronal synapses.

For the technical concepts of the different super-resolution imaging techniques such as stimulated emission depletion (STED) microscopy, structured illumination microscopy (SIM), photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), we would like to refer to a few of the excellent reviews that have recently appeared on this topic [4–6]. Here, we focus on recent new insights emanating from the most recent super-resolution studies on the spatial organization of protein complexes within dendritic spines, and discuss how these findings have contributed to our understanding of the mechanisms that control synaptic transmission and plasticity.

Internal organization of the PSD determines synaptic efficacy

The PSD is the central organizing unit in the spine head and is critical for the concentration of neurotransmitter receptors at the postsynaptic membrane to efficiently appose them to the presynaptic active zone. A variety of scaffolding molecules forms the core of the PSD and connects the receptors at the surface with cytosolic signaling molecules and actin regulators [7]. Alterations in the levels of synaptic AMPARs underlie long-term changes in synaptic strength associated with LTP and LTD, and the trafficking steps that underlie these processes have been studied extensively [8]. But, a less well understood aspect of receptor biology is how receptors are positioned once they have reached the synapse, even though it is predicted that the spatial pattern of AMPAR localization within the PSD directly impacts synaptic efficacy [9]. Because only a subset of AMPARs that is in the direct vicinity of the presynaptic site of release becomes activated, the local density of receptors apposing the release site is a critical determinant of synaptic strength [10–13]. Indeed, ultrastructural and live-cell imaging studies have indicated that AMPARs are preferentially immobilized within subsynaptic domains [14–18]. Moreover, the first study employing super-resolution STORM to map protein organization within individual synapses revealed a very high variability of lateral organization of receptor subtypes between individual synapses [19]. This study used STORM to measure the localization of a number of pre- and postsynaptic proteins in synapses of the main olfactory bulb and showed that within individual synapses the lateral distribution of AMPA and NMDA-type receptors is very heterogeneous and that this distribution varied greatly between different synapses. Importantly, this result clarified the apparent discrepancy in the EM literature on whether AMPA and NMDA receptors are preferentially localized centrally in the synapse or more peripherally, by showing that in fact both receptors could be found in either distribution type, and that this differentiation was not so clearly defined but varied substantially between synapses. Also, a study using a combination of live-cell single-molecule tracking PALM for AMPARs and elegant computational analyses, taking advantage of the high density of molecular tracks, provided clear evidence for a heterogeneous distribution of AMPARs within spines, with clear ~300-nm hotspots of stably retained AMPARs, likely organized by physical interactions with scaffolding molecules [20]. However, the mechanisms that determine the distribution of receptors as well as the scaffolds that retain them remained elusive.

Three independent, complementary super-resolution studies that appeared shortly after each other addressed the role of PSD-95 in positioning AMPARs [21–23]. All three studies consistently found that in the vast majority of synapses scaffolding molecules such as PSD-95 and AMPA receptors are organized in distinctive nanodomains, ~80 nm in diameter. Furthermore, two-color single-molecule imaging revealed that while AMPARs and PSD-95 nanodomains do not overlap completely in every synapse [22], in most synapses there is a significant 2-fold enrichment of synaptic AMPARs in PSD-95 domains that can be modified by changes in synaptic activity [21,23]. Also, shRNA-induced loss of PSD-95 disrupted the nanodomain organization of AMPARs, paralleled by a reduction in mEPSC amplitude [22]. Reversely, down-regulation of AMPARs disrupted the subsynaptic distribution of PSD-95 [21]. Using a recombinant antibody specifically recognizing the palmitoylated form of PSD-95 it was furthermore shown that ongoing palmitoylation of PSD-95 by the palmitoyltransferase DHHC2 is a critical determinant for the enrichment of PSD-95 in subsynaptic domains [23]. Together, these studies have thus demonstrated that both scaffolds and receptors are organized in distinct subsynaptic nanodomains, and that receptor-scaffold interactions underlie the formation and maintenance of these subsynaptic nanodomains (Fig. 1A).

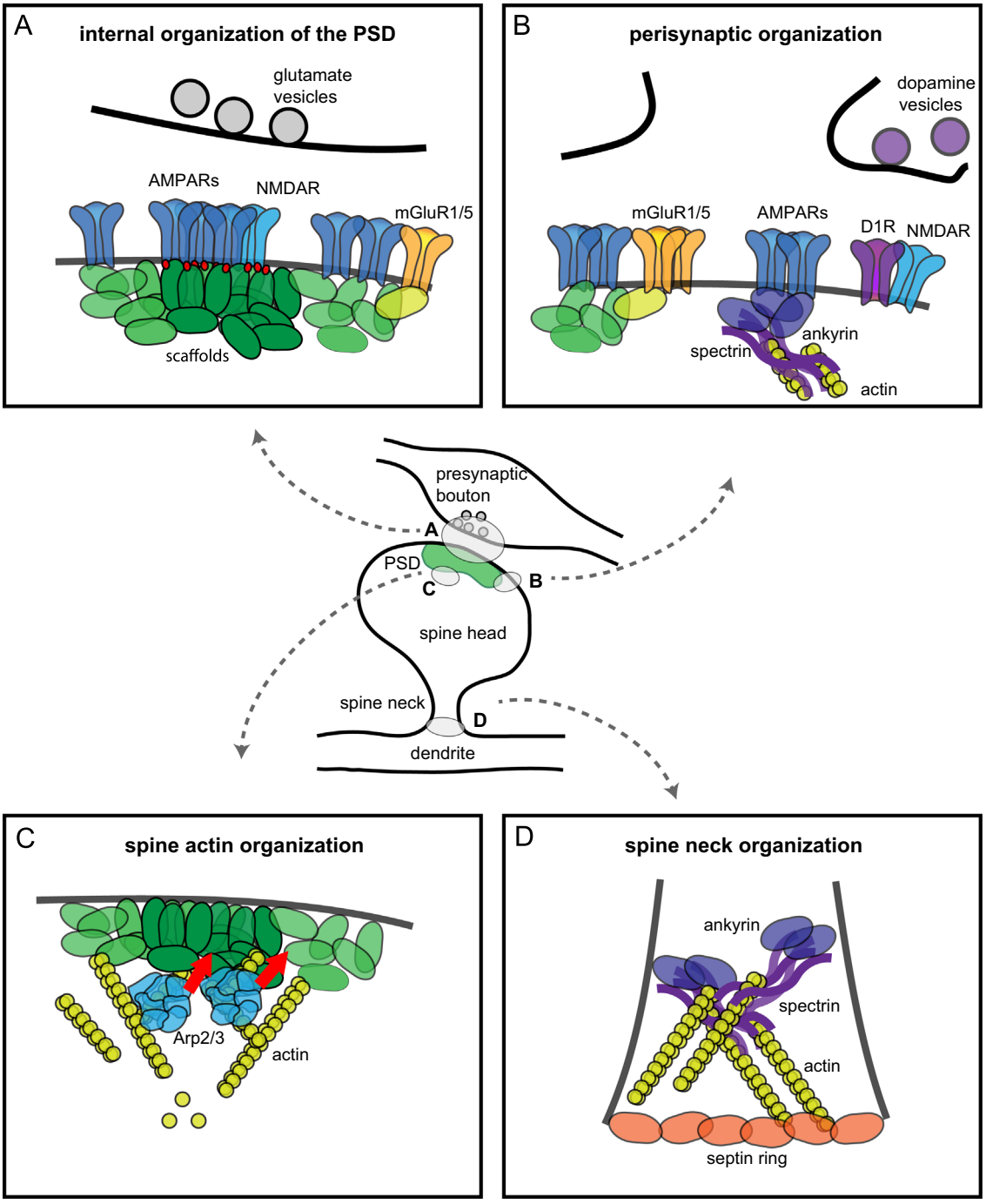

Fig. 1 – Schematic representation of the internal organization of spines revealed by super-resolution imaging. (A) Synaptic scaffolding molecules (green) are organized in subsynaptic nanodomains (darker green) enriched in AMPARs to increase the efficiency of synaptic transmission. Posttranslational modifications such as palmitoylation (red dots) might mediate the accumulation of scaffolds such as PSD-95 into these domains. (B) Specific receptor types including mGluR1/5 and D1Rs are enriched in the perisynaptic zone. AMPARs are transiently retained here to exchange with the synaptic pool, which could be facilitated by binding to perisynaptic ankyrin G nanodomains that are coupled to the actin cytoskeleton via spectrin. Perisynaptic D1R clusters provide a platform for extrasynaptic NMDAR. Activation by dopamine disrupts this interaction allowing NMDARs to diffuse into the synapse. (C) Actin nucleation factors such as the Arp2/3 complex preferentially accumulate close to the PSD to locally promote the formation and polymerization of new actin branches. The generated force (red arrows) is likely to impact the internal organization of the synapse. (D) Ankyrin G nanodomains within the spine neck might organize actin filaments to restrict the diffusion of molecules in and out of the spine. Septin based complexes at the base of the spine neck could form an additional diffusion barrier for transmembrane proteins.

Another consistent finding from these studies was that larger synapses contain more and larger subsynaptic nanodomains [21–23]. Also, chronic reduction of synaptic activity, known to induce a homeostatic increase in synaptic responses [24], increased PSD-95 nanodomain cluster number and area [21]. In contrast, a brief stimulation of synaptic activity with high K+ triggered a drastic decrease in the overlap of PSD-95 nanodomains and AMPARs [23]. Thus, the observed subsynaptic protein organization is modifiable by synaptic activity, and suggests that such alterations could underlie stable changes in synaptic strength. Indeed, computational simulations showed that there is a strong dependency of receptor activation on the distance between the release site and the receptor nanocluster [22], and that postsynaptic currents are significantly larger when release takes place on a non-random arrangement of AMPARs compared to a random distribution, with release events on a cluster of AMPARs evoking a two-fold higher amplitude compared to "off-cluster" events [21]. Thus, modifying the internal organization of synapses can directly potentiate or depress synaptic responses, even in the absence of altered receptor numbers. In the light of recent findings that challenged the idea that phosphorylation and protein-protein interactions in the intracellular tail of the AMPAR C-tail are required for LTP [25,26], two critical aspects of the molecular theory underlying the expression of LTP, it is tempting to speculate that activity-induced reorganization of the PSD interior can facilitate receptor retention and optimize receptor positioning to achieve efficient potentiation of synaptic currents independent of receptor phosphorylation or binding. Even further, simultaneous reorganization of post- and presynaptic elements, potentially via adhesion molecules or retrograde messengers, could facilitate alignment of the presynaptic release site with postsynaptic receptor nanodomains increasing the efficiency of synaptic transmission.

Perisynaptic molecular organization preserves a reservoir of glutamate receptors

The border of the PSD is well-defined by a steep drop in receptor and scaffold density, and is surrounded by a less dense, but compositionally distinct perisynaptic region, roughly defined as an annular ring of ~100–200 nm around the PSD. The functional significance of this domain for synaptic transmission is still unclear, but this region for instance contains the components of the endocytic machinery that together form a stable endocytic zone for synaptic receptors [27]. The perisynaptic region has also been suggested to control the exchange of glutamate receptors between synaptic and extrasynaptic regions. For instance, single-molecule tracking studies have shown that the perisynaptic population of AMPARs is highly mobile, but that this population increases in size upon stimuli that depress synaptic responses, while potentiating stimuli decrease the perisynaptic pool of AMPARs [28]. Moreover, unlike other glutamate receptors, metabotropic glutamate receptors (mGluRs) have been found to preferentially enrich at the edge of the synapse [29]. Also, a recent SIM study showed that the distribution of Norbin, a cytosolic mGluR interacting protein and important regulator of mGluR signaling, was excluded from the synapse, marked by PSD-95, but spatially overlapped with the spine actin pool [30]. Another modulatory receptor type, the dopamine D1 receptor (D1R) was recently shown to cluster in regions close to, but not in the PSD of hippocampal neurons [31]. Interestingly, this study found that perisynaptic D1R clusters retained a pool of NMDARs that upon stimulation by dopamine were released to become trapped in the synapse, effectively reducing the threshold for LTP induction (Fig. 1B). Specific interactions with scaffolds or the cytoskeleton that can explain the preferential perisynaptic accumulation of these receptors have not been described though; leaving the mechanisms that can control the exchange of perisynaptic receptors unclear.

Findings from a recent super-resolution study however might shed more light on such mechanisms. Using SIM this study found that the classic marker of the axon initial segment, ankyrin-G, is also present in dendritic spines of cortical neurons and clusters in dense patches surrounding the PSD and within the spine neck [32]. Interestingly, even though ankyrin-G nanodomains seemed to be only partially overlapping with the PSD, ankyrin-G was found in a molecular complex with AMPARs and the actin-binding protein β-spectrin. Moreover, ankyrin-G overexpression enhanced the levels and stability of spine AMPARs measured with FRAP, while knockdown reduced AMPAR levels and AMPAR-mediated currents. Thus, postsynaptic ankyrin-G significantly contributes to the retention of AMPARs in spines, and perhaps functions as a direct scaffold for the perisynaptic pool of AMPARs (Fig. 1B). In addition, it could also indirectly promote AMPAR stability by stabilizing the overall spine actin cytoskeleton via β-spectrin, or, as we will discuss in the paragraph below, it could contribute to the barrier function of the spine neck, hindering the diffusion of AMPARs out of the spine.

Heterogeneous organization of actin dynamics within spines

Spines contain a complex, highly branched actin cytoskeleton and a large diversity of signaling molecules and complexes that stabilize, depolymerize or seed new actin filaments. The complexity of actin organization and dynamics in spines sustains a wide variety of functions required for proper synaptic functioning [33,34]. However, because the cytoskeleton is prone to destruction by chemical fixation, it is important to use live-cell imaging techniques to probe the spatial dynamics of actin and actin-binding proteins. Time-lapse STED and PALM imaging using probes specific for F-actin revealed a very heterogeneous sub-spine distribution of actin filaments, with distinct hotspots in the spine head and neck, which rapidly reorganized on a minute time scale [35,36]. Moreover, even though indirect measurements of actin flow in live dendritic spines using targeted photoactivation have indicated a preferential flow of polymerization starting at the tip of the spine head directed to the base of the spine [37], measurements using single actin monomer tracking consistently showed a much more heterogeneous actin flow in mature, than in filopodia-like spines, supporting the notion that the distribution of actin dynamics within mature spines is highly heterogeneous [38–41]. Thus, while low resolution imaging techniques measured a preferential tip-to-base flow of actin, this phenomenon appears to emerge from a much more complex underlying organization of actin dynamics. Indeed, localized sites of high polymerization activity can be found throughout the spine, even in the spine neck, but are more often associated with the PSD [42]. Also, tomography studies have indicated a preferential accumulation of short, highly branched filaments close to the PSD [43]. Supporting this notion, the molecular components of the Arp2/3 (actin-related protein) complex, the

molecular machinery required for the formation and polymerization of new actin branches [44], were found to be concentrated and immobilized at sites immediately adjacent to the PSD (~400 nm from the center of the PSD) [38], consistent with immuno-EM data [45]. Moreover, components of the WAVE complex that promotes Arp2/3 activity, were even more closely associated with the PSD (~250 nm from the center of the PSD) and formed immobilized clusters largely overlapping with the PSD. In contrast, factors that generally promote elongation of actin, like VASP and formin-like protein-2, were found at sites more distal from the PSD [38]. Thus, the PSD seems to be a central docking site preferentially recruiting actin nucleation promoting factors. Scaffolding molecules such as members of the Shank family, can directly bind several actin nucleation promoting factors such as cortactin and Abp1, as well as components of the Arp2/3 complex [46–49], providing an efficient docking platform for actin branching close to the synapse (Fig. 1C). Under basal conditions the continuous force generated by this highly dynamic pool of perisynaptic actin is likely to support both the ongoing PSD shape changes that can be observed by time-lapse confocal imaging [16,50], and the subsynaptic distribution of AMPARs and scaffolds [16,21]. Furthermore, in this arrangement synaptic scaffolds provide a direct link between surface receptor activity and actin remodelers and can as such mediate the rapid reorganization of the actin cytoskeleton associated with synaptic plasticity.

The spine actin cytoskeleton undoubtedly has numerous other functions beyond its role at the PSD. For instance, using single-molecule tracking PALM the mobility of CaMKII, a kinase activated by NMDARs and critical for the expression of LTP, was shown to be regulated by the actin cytoskeleton [51]. The CaMKII holoenzyme can directly interact with actin filaments via its CaMKIIβ subunits, and co-expression of CaMKIIβ reduced the mobility of CaMKII. But, actin depolymerization specifically affected the pool that moved at intermediate velocities, while leaving the slow/immobile population of CaMKII, that would be more likely to interact with the slowly treadmilling actin monomers, unaffected [51]. Thus, these data rather imply that the crowded actin meshwork in spines serves as a molecular sieve to counteract the free diffusion of unbound signaling molecules, perhaps to allow them to interact with local substrates more efficiently. Indeed, immobilization of CaMKII triggered by NMDAR activation was not restricted to synaptic regions, but was also observed at distinct sites in the spine head further away from the PSD [51].

These super-resolution imaging studies revealed a heterogeneously distributed and dynamic actin network within spines, probably reflecting the diversity of functions the actin cytoskeleton has at distinct sites within the spine. It will be important to further delineate the contribution of the actin cytoskeleton to other processes that support synapse function such as receptor endocytosis and myosin-V based cargo transport into spines [52,53].

The spine neck functions as a diffusion barrier

The spine neck, which isolates the spine head from the dendritic shaft, can be up to 1 μm long, but is only ~75–300 nm in diameter [54], and is generally thought to compartmentalize signaling events within individual spines by restricting diffusion of molecules in and out of the spine head [55]. However, even though it is widely accepted that the spine neck restricts the exchange of molecules and determines synaptic functioning, many aspects of the mechanisms that control this diffusional barrier are largely unknown. For instance, what is the exact contribution of spine neck geometry to the extent of compartmentalization, and how are protein complexes within the spine neck composed and organized to form a physical barrier for diffusion? Super-resolution techniques allow more accurate measurements of spine morphology, and particularly measurements of the diameter of spine neck are considerably more precise than measured with confocal microscopy [32,56,57]. Especially live-cell STED imaging has enabled time-lapse imaging of spontaneous and plasticity-induced spine shape changes, even in hippocampal slices [56], and even more impressively, allowed the simultaneous measurement of spine neck geometry with the diffusional exchange of molecules in and out of the spine at both high spatial and temporal resolution [58,59]. These experiments uncovered the precise relationship between spine neck morphology and the diffusion of unbound cytosolic proteins, and found that neck width, much more than length, determines the flux of proteins into the spine.

Aside from spine neck geometry, what is the contribution of specific molecular assemblies within the spine neck to its compartmentalizing function? Only a few molecules are known to specifically localize to the spine neck, but a component of the septin complex, septin-7, has recently been found to stably localize at the base of the neck of most (~80%) spines [60]. In non-neuronal cells septin-based complexes have been described to form highly regulated and complex cytoskeletal structures involved in membrane remodeling and partitioning, and septins have been found to efficiently restrict diffusion between different cellular compartments. In neurons, septins may have a similar role in controlling diffusion between the spine and dendritic shaft. Using FRAP and single-molecule tracking it was found that the diffusion of transmembrane proteins, most notably AMPARs, but not cytosolic molecules, was significantly hampered in spines with a pronounced septin-7 based cluster at the base [60]. Furthermore, in septin-7 knockdown neurons, transmembrane proteins were less strictly confined to spines, and more frequently passed the spine neck, further indicating that septin-based structures contribute to the barrier function of the spine neck (Fig. 1D).

As described above, ankyrin-G expression was not restricted to the perisynaptic region, in fact, distinct ankyrin-G nanodomains were also found within the spine neck [32]. Although it is hard to specifically test the contribution of this subpopulation of ankyrin-G to synaptic function, the presence of an ankyrin-G nanodomain in the spine neck was clearly associated with a higher abundance of synaptic AMPARs. Also, the effects of ankyrin-G on spine morphology were much more pronounced when overexpressed ankyrin-G was present in the spine neck. Thus, this specific sub-spine localization of ankyrin-G might contribute to the barrier function of the spine neck, perhaps via spectrin-actin intermediates (Fig. 1D). Finally, as spine neck width has also been found to modulated by LTP-inducing stimuli [32,59], it becomes increasingly clear that regulation of spine neck morphology and the protein machineries that comprise the neck, significantly contribute to synaptic function.

Conclusions and outlook

In this review, we highlighted some of the major findings from recent super-resolution imaging studies that started to uncover the molecular organization within dendritic spines. In general,

super-resolution imaging has revealed an unprecedented complexity within the micron-sized confines of the spine, where different compartments can now be clearly distinguished spatially and compositionally. Even within the PSD, protein localization is highly heterogeneous, with AMPARs and scaffolds concentrating in distinct nano-scale domains. It is therefore critical to probe the functions of such specific sub-spine compartments and test their contribution to synaptic transmission and plasticity. However, this approach is certainly not trivial. For instance, how can we specifically target the actin-regulating proteins that are anchored at the synapse, or the molecular complexes in the spine neck, while leaving the other complexes intact? Also, while imaging of sub-spine structures and single-molecule tracking are now becoming versatile tools and enable the mapping of protein ensembles within individual spines at nanometer resolution, classic functional assays of synaptic transmission such as electrophysiological recordings are still mainly suitable for cell-wide measurements of synaptic function and are not always compatible with simultaneous super-resolution imaging. The integration of such techniques, ideally within a single experimental setup will be a great challenge for future studies. Nonetheless, the rapid pace at which optical imaging techniques are developing is very exciting, with continuing improvements in imaging resolution and applications to increasingly complex biological system such as thick brain tissue, and even living animals. Especially the development of fluorescent probes that increase specificity and precision, such as nano-bodies [61], aptamers [62] and DNA paint [63], or probes that recognize specific organelles [64,65] and specific protein modifications [23] will further diversify and advance the possibilities of super-resolution studies. Altogether, we believe super-resolution imaging has tremendously expanded our understanding of synapse biology, providing the field with new fundamental concepts to build on, and will continue to proof itself as an unrivaled method rapidly advancing progress in neuroscience.

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