



REVIEW

Super resolution microscopy is poised to reveal new insights into the formation and maturation of dendritic spines [version 1; referees: 2 approved]

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Abstract

Dendritic spines and synapses are critical for neuronal communication, and they are perturbed in many neurological disorders; however, the study of these structures in living cells has been hindered by their small size. Super resolution microscopy, unlike conventional light microscopy, is diffraction unlimited and thus is well suited for imaging small structures, such as dendritic spines and synapses. Super resolution microscopy has already revealed important new information about spine and synapse morphology, actin remodeling, and nanodomain composition in both healthy cells and diseased states. In this review, we highlight the advancements in probes that make super resolution more amenable to live-cell imaging of spines and synapses. We also discuss recent data obtained by super resolution microscopy that has advanced our knowledge of dendritic spine and synapse structure, organization, and dynamics in both healthy and diseased contexts. Finally, we propose a series of critical questions for understanding spine and synapse formation and maturation that super resolution microscopy is poised to answer.



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Introduction

Dendritic spines are actin-rich protrusions on neurons that are critical for neurotransmission, as they are sites for the majority of excitatory postsynapses¹⁻³. Abnormal spines are found in a wide range of neuropsychiatric, neurodegenerative, and neurodevelopmental disorders⁴⁻⁶, further highlighting the importance of these structures in cognition. Spines typically consist of a thin neck and a bulbous head, which is 0.5 to 1 μm in diameter. Therefore, analyzing spine and synapse organization in detail was previously difficult owing to their small sizes, which are near the diffraction limit for conventional light microscopy^{7,8}. The advent of super resolution imaging has revolutionized the study of spines and synapses. Whereas conventional light microscopy has an effective limit of resolution at ~ 200 nm due to the diffraction of light, super resolution fluorescence microscopy can bypass this limit, increasing the resolving power to tens of nanometers. In terms of resolving power, super resolution microscopy is limited by the brightness and photostability of the probes used^{9,10}; the principles underlying super resolution microscopy have been discussed in detail in previous reviews^{7,9,11}. This enhanced resolving power enables more detailed examination of protein mobility in living cells. The live-cell application of super resolution microscopy is what currently sets it apart from electron microscopy, which can achieve a somewhat higher resolution (picometer) but is not compatible with live-cell imaging and requires stringent fixation conditions⁹. Because super resolution microscopy is compatible with live-cell imaging, dynamic changes in spine and synapse morphology can be readily observed¹²⁻¹⁴. Particularly exciting is the possibility of imaging the very early stages of spine formation and subsequent maturation, which has not been possible to study with conventional light microscopy. Additionally, the enhanced resolving power of super resolution microscopy permits a more precise analysis of protein localization and the organization of protein nanodomains within individual spines and synapses^{15,16}. This type of microscopy will be critical for detailing the organization and dynamics of the hundreds of proteins that are packed together in submicron structures, such as dendritic spines. Consequently, super resolution microscopy will enhance our knowledge of dendritic spine and synapse architecture to possibly reveal nanoscale abnormalities in diseased states and lend further insight into the mechanisms underlying neurodevelopmental disorders.

Super resolution probes

New probes created in the last few years, as discussed below, have made super resolution microscopy even more conducive to visualizing neurons, especially fine neuronal structures (i.e. dendritic spines), because these probes are optimized for live-cell imaging. Super resolution studies are primarily performed using small molecule fluorophores and photoactivatable and photoswitchable fluorescent proteins¹⁷⁻¹⁹. Although small molecule fluorophores are less bulky, brighter, and more photostable compared to fluorescent protein tags, they can fail to bind to their intended targets and/or bind to undesired targets. However, by fusing a protein of interest directly to a fluorescent tag (i.e. green fluorescent protein [GFP]), this limitation can be overcome, but these fluorescently tagged proteins tend to be bulky and display weaker photostability and brightness than small molecule fluorophores. Over the past few

years, researchers have focused on developing probes for super resolution microscopy that overcome the limitations of traditional fluorescent proteins and synthetic fluorophores. For example, quantum dots (QDs)²⁰, which are semiconductor nanoparticles, and nanobodies²¹, which are composed of the smallest fragment of an antibody that will still bind to antigens, have also been used to label proteins for super resolution microscopy. The advantage of QDs is that they are highly photostable and therefore amenable to live-cell, single-molecule fluorescence microscopy. The major shortcoming for using QDs to visualize small neuronal structures is their size. QDs have an average diameter of 15–35 nm²², making them difficult to utilize in spatially confined areas such as the synaptic cleft²³. To address this problem, Cai *et al.* developed small QDs (sQDs), which are about 7 nm in size and can easily label proteins in neuronal synapses²². As a proof of concept, Wang *et al.* used sQDs conjugated to a GFP nanobody to label the exogenously expressed α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) subunit GluR2, which is fused to pH-sensitive GFP (pHluorin), to track the lateral diffusion of AMPARs in synapses²⁴. As an alternative approach to traditional fluorescent proteins, Viswanathan *et al.* created “spaghetti monster” fluorescent proteins (smFPs)²⁵ that contain multiple copies of commonly used tags such as Myc, FLAG, or HA on their surface. These tags create additional antibody binding sites on smFPs, which leads to high antibody labeling density, making these probes brighter than traditional fluorescence proteins or individual antibodies and nanobodies. Probe brightness is a critical determinant of spatial resolution in single-molecule super resolution microscopy. To test the ability of smFPs to reveal submicron structures, smFPs were expressed as a filler to visualize a major class of spines in CA3 neurons, called “thorny excrescence” spines. These spines have small protrusions from their spine neck, which are difficult to label with conventional fluorescent proteins and dyes. Even at low expression levels, smFPs labeled these protrusions significantly better than enhanced GFP (EGFP) or lucifer yellow²⁵. Furthermore, the epitope tags on smFPs allow for strong labeling of proteins for which suitable, specific antibodies and nanobodies are not available. Finally, smFPs are also especially attractive for investigating the early stages of spine and synapse formation because their brightness makes them well suited to imaging proteins that are present at low levels.

Actin is the main cytoskeletal element in dendritic spines and underlies spine morphology and plasticity. Despite its importance, until recently, super resolution live-cell imaging of actin remodeling in spines was limited to the use of low-affinity actin probes, such as ABP-tEosFP²⁶ or exogenous expression of actin fused to a fluorescent protein¹⁹. To address this, Lukinavičius *et al.* developed probes for live-cell imaging of actin and tubulin using a silicon-rhodamine derivative conjugated to ligands that bind to these cytoskeletal elements²⁷. The high specificity, enhanced fluorescence, and low phototoxicity of these probes make them invaluable for super resolution imaging of cytoskeletal remodeling in dendritic spines.

Collectively, the creation of these new probes has made super resolution microscopy even more amenable to studying small structures in neurons, such as dendritic spines and synapses, with

unprecedented detail compared to conventional light microscopy. Although these probes overcome some of the weaknesses of older probes, newer probes are still needed that have all the characteristics of an ideal probe for imaging dendritic spines, including high specificity, brightness, and photostability, as well as small size.

Novel insights from super resolution microscopy A new view of the actin cytoskeleton in spines

Although actin remodeling, which is critical for dendritic spine morphology and structure, has been studied in spines using conventional light microscopy, super resolution microscopy is providing important new information regarding the actin cytoskeleton in spines. Tataavarty *et al.* showed that the incorporation of individual actin monomers into actin filaments is more complex and heterogeneous than originally demonstrated with confocal microscopy¹⁹. In spines, single actin filaments were found to undergo retrograde flow, while other individual filaments displayed anterograde flow, random motion, or no net movement. This heterogeneity of actin polymerization in spines was confirmed by Frost *et al.*²⁸. Furthermore, they demonstrated that certain subdomains in spines, such as the postsynaptic density (PSD) and spine neck, exhibit enhanced actin polymerization²⁸. Super resolution microscopy also revealed that approximately 70% of spines that appear globular or cup-shaped by confocal microscopy display finger-like membrane extensions, which were driven by filamentous-actin (F-actin) dynamics¹². Intriguingly, the nucleation of these extensions may not occur at the tip of the extension, as previously thought for other membrane protrusions²⁹. Instead, Abi1 and Nap1, which are components of the actin-nucleating WAVE complex, localized at a single, central domain at the PSD¹², suggesting that the extensions are initially nucleated at the PSD. This raises the interesting possibility that these extensions play a role in spine maturation by sensing changes in the local environment and relaying this information back to the PSD. In addition, when the actin cytoskeleton was disrupted by treatment with cytochalasin D, synaptopodin, which localizes to the spine neck³⁰, no longer regulated diffusion of the metabotropic glutamate receptor 5 (mGluR5)³¹. These results suggest that components of the actin cytoskeleton are critical for the synaptopodin-mediated effect on diffusion. Super resolution microscopy has already provided new insight into actin remodeling in stable spines, and it has the potential to reveal critical new information about actin structure and function in dendritic spine and synapse assembly and maturation.

The importance of nanodomains

Super resolution microscopy has been used to visualize protein nanodomains within both the PSD and other areas of the spine. The importance of these nanodomains in neuronal function is also beginning to become evident (reviewed by MacGillavry and Hoogenraad³²). Different individual nanodomains of the same protein display different life times and changes in morphology over time. For example, while 40% of AMPAR nanodomains do not remain stable for longer than 5 minutes, 20% persist for at least 1 hour¹⁵. Additionally, the morphology of PSD95 nanodomains has also been found to change with time³³. Intriguingly, when neurons were treated with tetrodotoxin, which blocks sodium channels to prevent neural signaling, the area of the PSD was increased³³, suggesting that the nanodomain composition within the PSD changes in response to neural activity. Less clear, though, is how protein

nanodomains are established during neuronal development and how they change over time in response to synaptic plasticity. A few studies have analyzed the changes in nanodomains in response to glutamate receptor activation or chemical long-term potentiation (LTP)^{34,35}. For example, Lu *et al.* examined the mobility of calcium/calmodulin-dependent kinase II (CamKII), a protein consisting of α and β subunits which is necessary for inducing LTP and plays a role in trafficking AMPARs into synapses³⁶. CamKII α was found to exist in three kinetic populations: slow, intermediate, and fast³⁴. Each population was associated with different binding partners, where the fast population was found to be the CamKII α subunit alone, the intermediate population consisted of the α subunit bound to the β subunit and F-actin, and the slow population was thought to be CamKII bound to immobile substrates. Interestingly, stimulation of N-methyl-D-aspartate receptors (NMDARs) by glutamate and glycine significantly decreased CamKII mobility both at the PSD and elsewhere in spines, suggesting that CamKII is important for not only modulating AMPAR density in synapses but functions elsewhere in spines. Moreover, nanodomains of ankyrin-G, an adaptor protein that is a risk factor for schizophrenia, autism, and bipolar disorder³⁷⁻³⁹, accumulate in spines in response to chemical LTP³⁵. Knockdown of ankyrin-G prevents increases in spine head enlargement, a correlate for spine maturity and synapse size^{40,41}, following chemical LTP stimulation³⁵. Intriguingly, there was no difference in spine head size between spines that contained ankyrin-G in the spine neck prior to LTP and those which contained ankyrin-G in the spine neck after LTP. This suggests that the presence of ankyrin-G in the spine neck is a marker for spines that have already fully matured. Interestingly, another protein involved in synapse organization, synaptic cell adhesion molecule 1 (SynCAM 1), displayed an increase in nanodomain size in response to long-term depression⁴². Collectively, these data indicate that changes to nanodomain composition and characteristics are key for altering synaptic strength and suggest that changes to nanodomain composition occur during different stages of spine development.

To date, super resolution microscopy has not been used to examine the formation of protein nanodomains in developing spines. However, data obtained from stable spines could be applicable to forming spines as well. For instance, Hruska *et al.* used super resolution microscopy to show that the neuronal adhesion protein ephrin B3 regulates the localization of PSD95 to stable synapses and that ephrin B3, but not other, related ephrins, is critical for stabilizing PSD95 nanodomains in spines⁴³. Interestingly, neuronal activity stimulated the phosphorylation of ephrin B3 at serine 332 (S332), which decreased ephrin B3 localization to synapses and impaired its interaction with PSD95⁴³. Thus, ephrin B3, when not phosphorylated at S332, may be critical for recruiting PSD95 to sites where new synapses are forming⁴³. Indeed, knockdown of either PSD95 or ephrin B3 decreases spine density^{44,45}; however, whether this effect is due to decreased spine maintenance or formation is not currently known.

Abnormal spines in diseased states

Using conventional light microscopy, alterations in dendritic spine size, number, and morphology have been found in neurological disorders such as Alzheimer's disease^{46,47}, schizophrenia^{48,49}, and Fragile X syndrome (FXS)^{50,51}. While confocal microscopy is

limited to 200 nm resolution, super resolution microscopy can potentially provide detailed insights into the structural changes and nanodomain composition of dendritic spines seen in these disorders. Presently, a few studies have examined the structural changes to dendritic spines in neurological and neurodegenerative disorders. Using super resolution microscopy, Šišková *et al.* observed that dendritic branching, dendritic length, and dendritic surface area in CA1 pyramidal neurons from an Alzheimer's mouse model were significantly reduced compared to those from wild-type (WT) mice⁵². Classically, confocal microscopy has shown that FXS is associated with an increase in long, thin, filopodia-like, immature spines. However, Wijetunge *et al.* found unexpected results when examining changes in spine density and morphology between WT mice and FXS model mice (Fragile X mental retardation protein knockout mice)⁵³. The spine densities in hippocampal and cortical brain regions from FXS mice were comparable to those observed with WT mice when imaged via super resolution microscopy. However, subtle changes in fine morphological structures such as neck length, neck width, and head size were observed during different developmental stages. Moreover, Barnes *et al.* also showed that animals from another mouse model for intellectual disability (SynGAP^{+/-}) display no significant change in spine density but instead show increased spine neck length and decreased neck width, leading to increased compartmentalization, compared to WT mice⁵⁴. Intriguingly, they demonstrated that common physiological pathways are disrupted in the SynGAP heterozygous model and the FXS model, leading to similar morphological changes in dendritic spines in both. Together, these findings suggest that abnormalities observed in dendritic spine morphology and density in diseased states are both developmental stage and brain region specific and that these changes are the result of disruptions in pathways shared by multiple diseases. Further research is needed to better understand the functional implications of structural abnormalities in dendritic spines in these and other neurological disorders.

Conclusions and future directions

Although the proper development of dendritic spines and synapses is critical for normal cognitive function, their small size has limited the acquisition of detailed images of their nanoscopic substructures via conventional light microscopy. Super resolution microscopy overcomes the diffraction barrier, which allows for the imaging of these structures. While electron microscopy can achieve even higher resolution, it is limited because it cannot currently be performed in living cells. In contrast, super resolution microscopy is amenable to live-cell imaging. Moreover, super resolution microscopy will be critical for visualizing interactions between actin and actin-binding proteins during the early stages of dendritic spine formation and their subsequent maturation. The recent development of probes that are smaller, brighter, more specific, and more conducive to live-cell imaging are turning super resolution microscopy into a vital new tool to better understand dendritic spine morphology,

organization, function, and plasticity. Indeed, super resolution microscopy has already revealed fascinating and important new information about both the gross anatomical structure of spines and the protein nanodomain composition as well as actin remodeling within them in both healthy tissue and in diseased states. Super resolution microscopy will be invaluable to many applications in neuroscience, but it specifically offers the potential to examine spine and synapse development at a level of detail in living cells which was previously not possible but is necessary to understand the underlying mechanisms that regulate this process.

Super resolution microscopy can now be used to address a number of intriguing questions about the development of dendritic spines. For example, when are synaptic nanodomains established during spine formation, and how do they affect filopodia and spine morphology? Do all synaptic proteins enter a forming spine simultaneously, or are they recruited sequentially? Which protein nanodomains assemble independently, and which domains require synaptic scaffolding proteins to assemble appropriate nanodomains? How does nanodomain composition correlate with overall spine morphology? For instance, are the properties of nanodomains in a developing filopodium the same as what is seen in mature synapses, or are there immature stages, where nanodomains show different properties in immature synapses? The answers to these and other interesting questions would lend insight into novel functions for synaptic proteins. It will be critical to not only assess the normal development of dendritic spines but also evaluate how spine formation and maturation are perturbed in neurological disorders, such as Alzheimer's disease, schizophrenia, and FXS. Super resolution imaging has the potential to reveal the mechanisms that underlie these abnormalities and allow for the generation of new treatments for these disorders.

Author contributions

C.M.R., M.R.P., and D.J.W. chose the topic and scope of this review. C.M.R. and M.R.P. performed the literature search and wrote the initial draft of the manuscript. C.M.R., M.R.P., and D.J.W. contributed to the writing and editing of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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