Robust nanoscopy of a synaptic protein in living mice by organic-fluorophore labeling

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Extending superresolution fluorescence microscopy to living animals has remained a challenging frontier ever since the first demonstration of STED (stimulated emission depletion) nanoscopy in the mouse visual cortex. The use of fluorescent proteins (FPs) in in vivo STED analyses has been limiting available fluorescence photon budgets and attainable image contrasts, in particular for far-red FPs. This has so far precluded the definition of subtle details in protein arrangements at sufficient signal-to-noise ratio. Furthermore, imaging with longer wavelengths holds promise for reducing photostress. Here, we demonstrate that a strategy based on enzymatic self-labeling of the HaloTag fusion protein by high-performance synthetic fluorophore labels provides a robust avenue to superior in vivo analysis with STED nanoscopy in the far-red spectral range. We illustrate our approach by mapping the nanoscale distributions of the abundant scaffolding protein PSD95 at the postsynaptic membrane of excitatory synapses in living mice. With silicon-rhodamine as the reporter fluorophore, we present imaging with high contrast and low background down to ∼70-nm lateral resolution in the visual cortex at ≤25-μm depth. This approach allowed us to identify and characterize the diversity of PSD95 scaffolds in vivo. Besides small round/ovoid shapes, a substantial fraction of scaffolds exhibited a much more complex spatial organization. This highly inhomogeneous, spatially extended PSD95 distribution within the disk-like postsynaptic density, featuring intricate perforations, has not been highlighted in cell- or tissue-culture experiments. Importantly, covisualization of the corresponding spine morphologies enabled us to contextualize the diverse PSD95 patterns within synapses of different orientations and sizes.

in vivo fluorescence nanoscopy | superresolution imaging | synaptic architecture | PSD95 | protein labeling

Unraveling the structure and molecular composition of synapses is paramount to elucidating the mechanisms of synaptic transmission. Since many neurological and psychiatric disorders are associated with alterations in synapse morphology, understanding synapses also has wide implications in the pathophysiology of the brain. Accurate information about the organization of key proteins on the nanoscale is essential to characterizing synaptic molecular architecture and to discovering the principles of its organization.

For decades, EM has essentially been the only method to study the ultrastructural morphology of synapses and their internal molecular organization (1). EM visualization methods, however, are limited in their molecular specificity and labeling coverage, as well as by the number of synapses typically investigated. The time demands of both EM data acquisition and analysis lead to a lower throughput compared with optical methods. Most importantly, EM cannot be applied to living cells, tissue, or animals. Fluorescence microscopy, in contrast, offers these features but has long lacked the required spatial resolution necessary to identify the distribution of molecular entities in the confined space of the synapse. Nevertheless, fluorescence imaging, and especially two-photon excitation microscopy, has been one of the most powerful and widely used imaging techniques in the neurosciences due to its outstanding ability for live-cell and in vivo applications with specific molecular recognition (2–4).

The advent of lens-based subdiffraction (superresolution) fluorescence microscopy methods such as those called stimulated emission depletion (STED) (5, 6) and reversible saturable/switchable optical linear fluorescence transitions (RESOLFT) (7, 8), and later also photoactivated localization microscopy/stochastic optical reconstruction microscopy (PALM/STORM) (9–11), jointly referred to as fluorescence nanoscopy (12), has dramatically changed the outlook on resolution. These approaches provide minimally invasive access to the composition and function of synapses (13, 14). Live-cell nanoscopy in particular has begun to enable explorations across the neurosciences (15–20), opening up research directions which could not be addressed by electron or conventional optical microscopy. These studies, however, investigated neuronal morphology and synaptic protein organization and function in model cell or tissue systems, and hence may not have fully reflected the physiological applications with specific molecular recognition (2–4).

Significance

In vivo fluorescence microscopy with resolution well beyond the diffraction limit entails complexities that challenge the attainment of sufficient image brightness and contrast. These challenges have so far hampered investigations of the nanoscale distributions of synaptic proteins in the living mouse. Here, we describe a combination of stimulated emission depletion microscopy and endogenous protein labeling, providing high-quality in vivo data of the key scaffolding protein PSD95 at the postsynaptic membrane, which frequently appeared in extended distributions rather than as isolated nanoclusters. Operating in the far-red to near-IR wavelength range, this combination promises reduced photostress compared with prior in vivo nanoscopy at much shorter wavelengths.

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environment and complexity in a living animal. The ideal approach to characterizing the spatial distribution of a synaptic protein is therefore to map its appearance in vivo, excluding artifacts induced by culturing cells or tissues (21). However, in vivo nanoscopy comes with additional complexities of the experiment. Major challenges are the higher susceptibility to optical aberrations and to inherent motion of the animal due to heartbeat and breathing, which motivates the use of fast data acquisition. Additionally, light scattering reduces the signal collection efficiency with increasing imaging depth.

STED nanoscopy is so far the only superresolution fluorescence microscopy approach which has been demonstrated in living brain slices (16, 22) and in the brain of anesthetized live mice (23–26). These previous in vivo STED imaging studies examined dendritic structures based on overexpression of fluorescent proteins (FPs) either as volume labeling or to mark the actin network. A notable exception is the most recently reported application of in vivo STED imaging to a synaptic protein endogenously tagged with EGFP (27). The use of green or yellow FPs in STED nanoscopy, however, brings about increased hazards of tissue photodamage due to the higher photon energies of the required excitation and STED light in the green–yellow range. Indeed, previous in vivo STED imaging experiments using enhanced yellow FPs (EYFP) exhibited instances of phototoxic side effects in dendritic morphologies (23, 24). Considering that common natural absorbers in tissue have their highest absorption at visible wavelengths (28, 29), working in the far-red to near-IR spectral range is favorable. In addition, imaging with light at red-shifted wavelengths benefits from reduced scattering, better optical penetration, and improved image contrast due to lower auto-fluorescence (30). A recent report of in vivo STED nanoscopy in mice with far-red-emitting FPs (mNeptune2 and tagsRFP657) did not observe any obvious tissue photodamage (25). At the same time, red to far-red FPs show lower performance in terms of photostability and quantum yield compared with their green/yellow counterparts (31–35). These previous STED studies expressed FP constructs in cultured cells or tissues (21). However, in vivo nanoscopy requires excitation and STED light in the green – yellow range. For the latter class of labeling systems, the protein of interest or the fluorescent probe exhibit specific binding characteristics and are expressed in small molecular probes or enzymatic protein tags. Recently, several far-red and near-IR dyes with excellent STED compatibility have become available which label intracellular proteins with high specificity in living cells, for example small molecular probes (31–35) or by means of the self-labeling techniques of SNAP-, Halo-, or Clip-Tag fusion proteins (36–40). For the latter class of labeling systems, the protein of interest is genetically fused with an engineered enzyme tag which covalently binds the corresponding fluorescent probe substrate (41–43). A major benefit of this kind of fusion tag is that the fluorescent dye ligand can be freely chosen, depending on the requirements of the imaging application.

Exploiting this benefit, we now demonstrate a versatile approach for imaging the nanoscale distribution of a synaptic protein in vivo, specifically in dendritic spines of the neocortex of anesthetized live mice. To this end, we used a transgenic knock-in mouse line engineered to express endogenous postsynaptic density 95 (PSD95) protein in the HoloTag enzyme. The HoloTag sequence allowed us to covalently label PSD95 in vivo with the ligand silicon-rhodamine-Halo (SiR-Halo). We stereotactically injected SiR-Halo into layer I of the visual cortex during the surgical preparation and performed in vivo superresolution imaging on a home-built upright STED nanoscope with excitation of far-red fluorescence at 640 nm and near-IR 775-nm deexcitation. The utilization of the near-IR SiR-Halo ligand resulted in highly specific labeling of PSD95 and imaging with superior signal-to-noise ratio and increased photostability compared with recently described FP-based in vivo experiments. We found that endogenous PSD95 formed diverse nanoscale arrangements with large variations in shape and size among individual synapses, as revealed by confocal co-visualization of the dendritic morphology relative to the superresolved PSD95 scaffolds. In many synaptic sites, PSD95 resided in compact, round or ovoid assemblies with dimensions often considerably smaller than the diffraction limit. Frequently, we observed the PSD95 scaffolds as complex, oval or ring-like arrangements with irregular borders and internal perforations, which were not visible in the confocal mode and have not been reported in such detail in experiments with cultured neurons or brain slices. Our study underscores the importance of in vivo superresolution microscopy and introduces a powerful method for future investigations of the molecular organization and function of the brain in living animals.

Results

In Vivo Labeling of Endogenous PSD95. To address the signal and photostability limitations of FPs in in vivo superresolution STED imaging, we explored an approach that exploits the molecular specificity of a genetically encoded self-labeling enzyme tag fused to the protein of interest, combining it with the superior photophysical performance of organic fluorophores. We utilized a HaloTag enzyme, which is the highest reported binding rate among the widely used enzymatic self-labeling protein tags (SNAP, Halo, and Clip). PSD95 is the major constituent of the PSD in dendritic spine heads, where it is known to act as a scaffold for numerous other proteins (44, 45) and controls synaptic plasticity and learning (46). The HoloTag was fused to the C terminus of PSD95 using a previously described genome engineering approach (45). In this mouse line, PSD95-HaloTag is expressed under the control of the regulatory elements of the mNeptune2 gene (40, 32, 37, 47). By means of the self-labeling technique of SNAP-, Halo-, or Clip-Tag fusion proteins (36–40), the latter class of labeling systems, the protein of interest is genetically fused with an engineered enzyme tag which covalently binds the corresponding fluorescent probe substrate (41–43). A major benefit of this kind of fusion tag is that the fluorescent dye ligand can be freely chosen, depending on the requirements of the imaging application.

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emission only when bound to the target protein, while it is almost nonfluorescent in its unbound state (36). As expected, PSD95 showed the characteristic punctate distribution throughout the cortical dendritic network, with Lifeact-EYFP (Fig. 1D) or cytosolic volume-filling EGFP (SI Appendix, Fig. S2) expressed in a sufficient number of dendrites to enable morphological recognition of the spine or spine head, which is colocalized with an EYFP/EGFP-labeled spine head, which is expected as not all PSD95 puncta exhibited different dimensions ranging from small and round to larger ones with irregular shapes. Not all PSD95 puncta colocalized with an EYFP/EGFP-labeled spine head, which is expected as not all pyramidal neurons were transduced by the virus, whereas PSD95 was endogenously expressed at the excitatory synapses of all neuronal cells.

With the labeling in place, starting at ~1 h after dye injection, imaging was possible for hours, with our experiments typically completed within 4 h. Upon moving to a new region of interest (ROI), we encountered similar signal intensities as initially in prior ROIs, with no systematic signal reduction over the experiment. Local photobleaching at ROIs upon repeated imaging is discussed below. This persistence of signals is consistent with the finding in cultured neurons that endogenous PSD95 turnover is on the order of a few days (48), and that significant turnover leading to a partial turnover of the PSD95 ligand from the cortical surface to 100 μm with significant lateral broadening from the injection sites. Asterisks: superficial blood vessels. (Scale bar: 250 μm.)

**A Dedicated Platform for Far-Red in Vivo STED Nanoscopy.** Having established the labeling strategy, we aimed at resolving the native nanoarchitectures using STED nanoscopy. To this end, we developed a compact in vivo STED nanoscope (SI Appendix, Fig. S1) for the far-red spectrum with a main design focus on mechanical stability, implementing short optical beam paths with groups of components decoupled from each other on separate breadboards in a modular fashion. Fibers delivered the laser light for excitation and deexcitation to the core module of the system (STED unit) and guided the fluorescence to the detection module. The STED unit was directly attached to the microscope stand, without the need for redirecting beams once they were coaxially aligned. An independent translation stage for mouse positioning, which was mechanically decoupled from the microscope body and other optomechanics, ensured that the impact of vibrations or thermal fluctuations on the associated optics was minimal. Our imaging system allowed us to adjust the lateral 2D resolution down to 40–50 nm (as determined with ~20-nm fluorescent bead samples). It rendered SiR-Halo-labeled β-tubulin–HaloTag filaments in cultured living cells with a width of 66 nm FWHM for the maximally available STED average power of 177 mW (all powers stated in back aperture), suggesting an FWHM resolution of down to 50–60 nm (SI Appendix, Fig. S4).
In vivo STED Nanoscopy of PSD95 Scaffolds with Excellent Structural Definition. In vivo STED imaging was performed in ROIs identified from confocal overview images at 5- to 25-μm depth below the cortical surface, comparable to previous STED imaging in the mouse neocortex (24, 25). Whereas the confocal images showed PSD95 scaffolds as blurred puncta or elongated shapes of various dimensions and brightness, spanning from the diffraction limit up to ∼1.5 μm across, STED nanoscopy resolved the nanoscale architecture of individual PSD95 arrangements in unprecedented detail. We observed that PSD95 formed diverse nanoscale arrangements with large variations in size and shape among individual synapses (Figs. 2 and 3). These variations are putatively in part due to the differing orientations and axial positions with respect to the focal plane. The appearance of a PSD95 scaffold in a 2D recording can be drastically affected by its relative orientation to the imaging plane. In the 2D STED images, the PSD95 geometries typically appeared as small and round/ovoid, thin and elongated, or as larger structures with complex perforations (Fig. 3).

The in vivo STED imaging was carried out in regions largely devoid of prominent vascularization, to reduce motion artifacts induced by pulsing blood vessels. Furthermore, rigid fixation of the mouse head and the cranial window (SI Appendix, Fig. 5B) largely prevented artifacts due to respiration-induced motions. Only rarely, abrupt lateral displacements were observed during the scan in imaging regions where the pulsing of blood vessels was sufficiently strong (SI Appendix, Fig. 5C). Such instances were recognizable by inspection of thin features oriented along the slow scan axis, and we moved to a different field of view (FOV) whenever this was observed.

The SiR labeling facilitated STED recordings of PSD95 with robust signals of up to 100–120 counts per pixel in the brightest parts of the structure (typically exceeding 50–60 counts per pixel throughout), and a low background noise level of usually one to three counts. In a given FOV, the high photon budget and photostability of SiR under STED conditions allowed the recording of at least six sequential image scans with sufficient brightness and contrast to appreciate internal PSD95 scaffold morphologies (SI Appendix, Fig. S5). At the utilized average powers for deexcitation by STED of 53 or 66 mW in the in vivo experiments, a resolution of <90 nm was estimated based on the thinnest (∼80–95 nm) features in PSD95 scaffolds represented at robust signal intensities (compare examples in SI Appendix, Fig. S6). Further-enhanced resolution in vivo, at the level of ∼70 nm, was demonstrated for a higher STED power of 169 mW (SI Appendix, Fig. S6). Nevertheless, we typically limited the STED power to P_{STED} of 53 or 66 mW, except for occasional imaging at this higher STED power to explore current limits to the achievable resolution with our labeling and imaging approach. This allowed repeated imaging at still approximately fourfold improved resolution over confocal microscopy and with robust signal. Our resolution estimate of <90 nm is consistent with the experimentally determined STED-power dependence in our test experiments with cultured cells (SI Appendix, Fig. S4), indicating that the resolution performance of our setup can be fully harnessed in vivo.

Contrary to the SiR-Halo experiments, in vivo STED imaging of the far-red FPs mGarnet or mNeptune2 fused to PSD95 (virus-mediated expression) did not achieve sufficient resolution and image brightness to be able to resolve structural features to the same extent (SI Appendix, Fig. S7). Although the resolution enhancement between confocal and STED images of the far-red FPs was apparent, the overall performance did not reach that of SiR-based imaging.

![Fig. 2.](https://www.pnas.org/cgi/doi/10.1073/pnas.1807104115)
Fig. 3. Impressions of PSD95 scaffold nanoarchitectures in vivo. (A–C) Examples of the different appearances of PSD95 assemblies (SiR-Halo ligand, magenta) in 2D imaging in (Left) confocal mode, (Middle) STED mode, and (Right) the overlay of the STED image of PSD95 with the confocal image of the dendritic reference labeling (Lifeact-EYFP, green). (A) Examples of small round/ovoid PSD95 scaffolds, primarily located in small dendritic spine heads as revealed by the reference label. (B) Examples of thin elongated PSD95 scaffold side views in large spine heads. (C) Examples of complex PSD95 nanoarchitectures, with overall dimensions (white arrowheads) determined in the raw STED images from intensity profiles. Images represent magnified views extracted from full-field-of-view recordings. The fast scan axis for all images shown is indicated by an arrow in the bottom right. All images were smoothed for display with a one-pixel-wide Gaussian filter. Imaging parameters are listed in SI Appendix, Table S1. (All scale bars: 500 nm.)
A semiautomated analysis based on 2D STED images (n = 1,148 PSD95 scaffolds; Fig. 4 and SI Appendix, Fig. S8 and Semiautomated Analysis of PSD95 Scaffolds) revealed that the size of the arrangements spanned dimensions (minor × major axis, referred to as the short and the long axis) from 105 nm × 126 nm in the case of small puncta to 606 nm × 1,438 nm for the largest structures (Fig. 4A). The PSD95 arrangements featured mean dimensions of 237 nm × 458 nm, and a mean area of 0.12 μm² (scaffold area: the area enclosed by the outermost perimeter of the scaffold) (Fig. 4B). In the majority of analyzed assemblies (57%; Fig. 4A), we observed small round or ovoid PSD95 arrangements (Figs. 2B, I and 3A) and chose to designate scaffolds “small round/ovoid” if their long and short axes l and s were bounded by the condition l + s² ≤ 500 nm (in nanometers) (Fig. 4A). For these scaffolds of long-axis diameters up to 500 nm, short-axis diameters were in the range of 103–335 nm (mean: 189 nm; SI Appendix, Fig. S9 A and B), and thus often considerably smaller than the diffraction limit.

In addition to the small round/ovoid scaffolds, the confocal images often showed large elliptical PSD95 assemblies, whereas with STED imaging these shapes were revealed as thin elongated (Fig. 2B, II and Fig. 3B) and wider extended structures with complex perforations (Fig. 2B, III and Fig. 3C), as well as intermediate shapes. For this more heterogeneous category of scaffolds (43%), the mean dimensions were 299 nm × 657 nm on the short and long axis, respectively (Fig. 4A and SI Appendix, Fig. S9 E and F). The complex-shaped, oval or ring-like structures with irregular borders and perforations were highly diverse, and without an apparent common structural geometry (Fig. 3C and SI Appendix, Fig. S10). We observed mostly continuous nanoarchitectures, with largely uniform signal intensity within the occupied domains. Rarely, hotspots of signal (i.e., protein density) were found within the extended continuous irregular distribution.

Noting that the overall areas bounded by the outer perimeters of scaffolds were not always entirely filled with PSD95 protein (black composite examples in Figs. 2B, I and III, and 3C (in parts III) and 3C and SI Appendix, Fig. S10), our semiautomated analysis also allowed us to extract a filling-fraction parameter. This filling fraction (Fig. 4C) quantifies the relative area (as an approximation to the volume) within the scaffold occupied by PSD95 (SI Appendix, Semiautomated Analysis of PSD95 Scaffolds). The scaffolds in the small round/ovoid category featured almost complete (close to 100%) filling throughout. For the larger scaffolds, the filling fractions were typically in a range from 60 to 100%, with a nonnegligible fraction of scaffolds with filling fractions ranging from 20% to 40% and even as low as 5%.

In scaffolds taken from each of the most prominent geometric appearances within four large FOVs, the smallest measured widths (FWHM, intensity line profiles; SI Appendix, Fig. S6) were almost identical, with 88 ± 6 nm (mean ± 1 SD, N = 58; Pstead = 53 ± 66 mW) for the small round/oval-shaped, 92 ± 6 nm (N = 24; Pstead = 53 or 66 mW) for the thin elongated ones, and 91 ± 9 nm (N = 8; Pstead = 53 or 66 mW) for larger scaffolds of a more complex architecture. These sizes are approximately equal to the 90-nm in vivo resolution realized in this part of the imaging, suggesting the underlying protein distribution along the respective direction may thus be even more narrowly confined.

### Relating the PSD95 Nanomorphology to the Spine Shape

The appearance of PSD95 arrangements in STED recordings with lateral resolution enhancement can be highly dependent on the orientation and axial position of the synapse with respect to the focal plane. For small round/oval-shaped PSD95 distributions at or near the achieved resolution, the impact of orientation on appearance in the STED imaging is marginal. We speculated that thin elongated PSD95 patterns were mainly a result of synapses observed as side views, whereas complex-shaped morphologies could represent top views of the PSD. We thus examined the 2D appearance of PSD95, putting it in context of the Lifeact-EYFP/cytosolic EGFP imaging channel to ascertain spine-head orientation. Where this determination was possible, our data showed thin elongated PSD95 scaffold structures arranged along the postsynaptic membranes of dendritic spine heads in side views (N = 9 for the examples in Figs. 2B, II, 3B, and 5), as identified by the reference labeling showing the dendritic spine head connected to the neck and the shaft. Complex-shaped morphologies were observed in top or near-top views of synapses (N = 10 for the examples in Figs. 2B, III, 3C, and 5), imaged as elliptical disks of the EYFP/EGFP reference. The lack of resolution enhancement along the z direction complicated the unambiguous interpretation of observed 2D images. The disk-shaped PSD has been reported to have <50 to 50 nm extent in the direction perpendicular to its plane (49). The wide range of orientations in which the spine was encountered in our imaging led to different imaged projections of the PSD95 distribution located at the PSD. This explains the observation that examples of axial z-stack STED imaging with a step size of 400 nm showed a thin elongated scaffold appearance in one focal plane and revealed a complex and perforated nanoscale arrangement in the adjacent plane (Fig. S4). Such repeated imaging was not possible when utilizing far-red FPs instead of SiR (SI Appendix, Fig. S7) since, for Pstead almost equal to those of the SiR imaging, photobleaching prevented z-stack recordings with multiple frames at high resolution for these labels.

### Discussion and Outlook

In vivo nanoscale fluorescence imaging has been applied relatively rarely and still represents a frontier, not only due to the complexity of the experiments but also due to a lack of adequate labeling tools. Whereas previous in vivo STED studies employed FPs, we here introduced an approach to overcome the signal and photostability limitations of FPs by exploiting the self-labeling HaloTag in combination with the near-IR organic dye SiR, a high-performance synthetic fluorophore.
With a typical focal-plane resolution of ≤ 90 nm (or down to ≤ 70 nm) in living mice, in vivo STED imaging allowed us to identify the nanoscale architecture of PSD95 scaffolds in the molecular layer of the visual cortex, with appearances in various shapes and sizes among individual synapses (Figs. 2 and 3). Overall, the size of the PSD95 arrangements (Fig. 4A) spanned from dimensions of ~100 nm × 130 nm in the case of small puncta to ~600 nm × 1,400 nm for the largest structures and allowed us to visualize complex-perforated arrangements in great detail. In this aspect of a high diversity of PSD95 morphologies—from small to large—our observations are consistent with previous super-resolution studies on the organization of PSD95 (27, 52).

As a main challenge to the interpretation of observed structures, the relative orientation to the imaging plane drastically affected the appearance of a PSD95 scaffold. Although the identification of a single scaffold’s orientation was ambiguous, two classes of scaffolds were distinguished by introducing a bound on their size. We found that ~57% of the PSD95 assemblies were relatively small and round/ovoid (Figs. 3A and 3D and SI Appendix, Figs. S6A and S9). The other, larger structures (43%) appeared either thin and elongated or extended and complex with irregular borders and perforations (Figs. 3B and C and SI Appendix, Figs. S6 B and C, S9, and S10), or as intermediate shapes. The PSD95 scaffolds which appeared thin elongated are plausible side views of the (predominantly complex) assemblies, orientated perpendicular to the imaging plane. The morphological reference imaging allowed us to confirm this in several examples. For all three main classes of appearance, the width of smallest features was ~90 nm (or ~70 nm for the higher STED power; SI Appendix, Fig. S6). This width was indicative of the resolution achieved at the respective STED power. Importantly, the distinctive complex morphologies were resolved in such detail only in the STED imaging mode, whereas confocal imaging did not resolve the internal structure.

Contrary to recent reports (18, 19, 52–56), our data at 70- to 90-nm resolution showed no indications of sparse, isolated PSD95 clustering. Notably, the complex-shaped scaffolds appeared to be continuous (albeit convoluted) structures, not fragmented or clustered. PSD95 “nanoclusters” or “nano-domains” have been reported in several superresolution studies of living or fixed dissociated rat hippocampal or cortical neurons (18, 19, 53, 54, 56) and in fixed brain sections of the mouse hippocampus (52). All these studies based conclusions on the number of nanoclusters per synaptic site, identified as elevated density of localizations in PALM/STORM nanoscopy data, or as particularly bright image regions in STED images. In consensus, these studies reported spines containing typically one to two PSD95 clusters, much fewer with three or more clusters and rarely observed more extensive patterns (containing up to five individual clusters). In these works the structures were analyzed within the cluster paradigm, rather than considering the existence of distributed, continuous arrangements. The studies diverge in their conclusions on the spatial extent of clusters, with determinations ranging from 80 to 150 nm. Furthermore, the cluster terminology has also been used to include rather large domains of up to 400–600 nm in diameter (mean: ~300 nm) (53). As an important aspect, the studies concluded that clusters were separated by substantial distances of 100–300 nm, that is, farther apart than the resolution of the various fluorescence nanoscopy experiments of between 25 and 80 nm. The resolution down to ~70 nm in our PSD95 analysis in the living mouse would have readily resolved clusters at the reported spacings. The discrete clustering in all these reports, which included studies in living cell-culture systems (18, 19, 53, 56), with separations almost as large as the diffraction limit, is not supported by our observations in vivo. The different resolutions between the aforementioned reports and our present in vivo study cannot account for the qualitatively different observations, given that even a report claiming 25-nm resolution in live cells quantified nanoclusters to be ~80 nm in size individually (18).

Instead, possible explanations for the discrepancy include the effects of culturing neurons in a flat monolayer without the presence of other cell types such as astrocytes. A further difference is that our in vivo imaging explored dendritic spines in the molecular layer (layer I) of neurons from deeper cortical layers, rather than neurons from hippocampal regions. Furthermore, different rodent species (mouse vs. rat in the other reports except ref. 52) were used. On a technical level, the majority of past reports imaged transiently (over)expressed PSD95, whereas our present study examined the mouse brain with endogenous protein expression, that is, without possibly uncontrolled effects of the PSD95 overexpression on interacting proteins, as pointed out for AMPA receptors in one study (19). Choices of image processing and presentation, in particular thresholding, may additionally affect the PSD95 data and may lead to a clustered appearance by unduly emphasizing the hotspots which we also additionally affect the PSD95 data and may lead to a clustered appearance by unduly emphasizing the hotspots which we also
discussed in refs. 18, 19, and 54, but they may be interpreted as indicative of PSD95’s being present at more moderate levels also in the proximity of clusters. In contrast, our STED data suggest that PSD95 protein is distributed as continuous “bands” of varying length, as often seen within the complex, convoluted structures. STED nanoscopy revealed PSD95 scaffolds as heavily segregated into spatial domains either containing or entirely lacking the protein. In the PSD95-containing regions, its concentration appeared rather homogenous. Note that STED imaging renders protein concentration differences in the specimen linearly, from very low to highest encountered density. This is a strength of our approach, and the localization of PSD95 in well-separated clusters, as observed in refs. 18, 19, and 52-56, can be excluded for our in vivo conditions based on our results.

To summarize, while small round/ovoid scaffolds were commonly observed, our in vivo data also suggest the existence of many large PSD95 scaffolds with complex internal organization, rather than small numbers of isolated nanoclusters. The present resolution did not allow us to exclude the possibility that larger numbers of clusters are densely spaced within the highly convoluted scaffold, but certainly not just one to three nanoclusters. Therefore, it will be necessary to further scrutinize the smallest structural units of PSD95 in vivo with yet higher resolution, a challenge that will certainly be successfully met by STED nanoscopy or related concepts.

From our data we can further discern the relation of the PSD95 nanoscale morphology to the spine head shape. By utilizing information from a second imaging channel showing the spines’ morphologies, our results indicate that small round/ovoid PSD95 assemblies are located in relatively small dendritic spines (~600-nm mean spine-head diameter), whereas the complex-shaped PSD95 scaffolds and their (partial) side views tend to occur in larger spine heads. This is in agreement with previous EM data, which have shown that small spine heads harbor small round postsynaptic densities, while large (mushroom) spines contain complex-shaped and perforated PSDs, and that their appearance depends on the angle of view (57, 58).

Our results provide one of the first nanoscale views of protein distributions in living mice, and the quality of the image data demonstrates the strength of our chosen labeling and imaging approaches. Previous in vivo superresolution studies used the most common and conventional labeling method based on the expression of FPs. Compared with our data achieved with far-red FPs, we observed a substantial contrast and resolution improvement of the superresolved SiR-Halo-labeled PSD95 structures (compare Figs. 2 and 3 to SI Appendix, Fig. S7). For the far-red FPs, photobleaching limited the imaging to single frames (SI Appendix, Fig. S7), without the ability to record z-stacks or perform time-lapse analyses.

This pronounced difference can be largely attributed to the higher photostability of the organic dye compared with FPs in STED nanoscopy. SiR enabled z-stack acquisition (Fig. 5) and repeated imaging at STED resolution (SI Appendix, Fig. S5). These capabilities allowed us to better ascertain the 3D character of the PSD95 assemblies, which appeared static on the few-minutes timescale. Although the SiR-Halo ligand was directly injected into the brain and was most likely also present in an unbound fraction during STED imaging, the background in the images was exceptionally low. This can be ascribed to the high degree of fluorogenicity of SiR, which shows an increase in fluorescence only upon covalent binding to the biomolecular target structure (59), in our case to the HaloTag protein. A
comparison of our imaging results to the recent in vivo STED data of PSD95 labeled with EGFP (27) shows that our approach revealed the PSD95 organizations in finer detail and with improved image contrast, benefiting the visualization of the complex scaffolds. The data in ref. 27 are broadly consistent with our imaging parameters in both studies were chosen, out of practical considerations, with multiple image acquisitions in mind for performing time-lapse or z-stack recordings. In either study, the main focus was not set on obtaining merely one best image in a single-shot approach. Even so, the presented datasets let us conclude that the endogenous near-IR synthetic fluorophore labeling demonstrated herein enables superior and robust nanoscale imaging performance.

Another notable advantage of our approach is the option of attaching a synthetic fluorescent ligand of choice. This allows adapting the approach to any spectral window and affords greater flexibility with regard to, for example, pulse-chase analyses. We opted for SiR as a red label, since in the far-red to near-IR spectral range autofluorescence, absorbance, and scattering are reduced in tissue, aspects that are prominent drawbacks for blue-shifted wavelengths. While former in vivo STED studies of EYFP-labeled structures reported phototoxic effects, such as swellings in dendrites (23, 24), a recent study with the far-red FP mNeptune2 (560-nm excitation and 732-nm STED wavelengths) discussed no signs of photodamage (25). We confirmed this observation. The wavelengths utilized in the present work (640-nm excitation and 775-nm STED) are shifted even further to the near-IR. Based on the evaluation of confocal images of the Lifeact-EYFP reference labeling, we observed no ligand-related toxicity or effects from the intracortical injections on the dendritic morphology, or phototoxicity or tissue damage from the repeated overview, confocal, and STED imaging. Whereas we used SiR-Halo for our experiments, the HaloTag technology would also allow the utilization of other fluorescent ligands. The far-red components of new dyes for the far-red to the near-IR spectral range are of special interest for deep-tissue and in vivo optical imaging and might yield ligands with even better photophysical properties, including enhanced brightness and photostability, while also being cell-permeable. A step toward the near-IR spectrum was made by the design of the membrane-permeable silicon-rhodamine derivatives 680SiR (λabs = 679 nm, λex = 697 nm; ref. 34) and SiR700 (λabs = 715 nm; ref. 33), which were shown to work well in cultured cells and home-built upright STED microscope (SI Appendix, Fig. S1) with a 63× glycerol-immersion objective lens, scanning pair of laser spots for 640-nm fluorescence excitation and 775-nm STED deexcitation of SiR (as a plane doughnut), and 473-nm excitation for EGFP/EYFP (confocal). The far-red FPs, also explored for PSD95 labeling and STED imaging, were excited by a 594-nm laser. Image acquisition of 5 × 5 μm² to 20 × 20 μm² FOVs was typically performed with 20-μm pixel size and 30-μs dwell time, resulting in acquisition times of ~2–30 s for the large STED images and ~1.3–5.6 s for image portions containing the individual PSD95 assemblies (depending on the acquisition mode and width of the FOV). Further details on image acquisition parameters are in SI Appendix, Table S1.

All animal procedures were conducted in accordance with the Animal Welfare Law of the Federal Republic of Germany and the regulation about animals used in experiments and were approved and authorized by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.

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