



Revealing nanostructures in brain tissue via protein decrowding by iterative expansion microscopy

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Many crowded biomolecular structures in cells and tissues are inaccessible to labelling antibodies. To understand how proteins within these structures are arranged with nanoscale precision therefore requires that these structures be decrowded before labelling. Here we show that an iterative variant of expansion microscopy (the permeation of cells and tissues by a swellable hydrogel followed by isotropic hydrogel expansion, to allow for enhanced imaging resolution with ordinary microscopes) enables the imaging of nanostructures in expanded yet otherwise intact tissues at a resolution of about 20 nm. The method, which we named ‘expansion revealing’ and validated with DNA-probe-based super-resolution microscopy, involves gel-anchoring reagents and the embedding, expansion and re-embedding of the sample in homogeneous swellable hydrogels. Expansion revealing enabled us to use confocal microscopy to image the alignment of pre-synaptic calcium channels with post-synaptic scaffolding proteins in intact brain circuits, and to uncover periodic amyloid nanoclusters containing ion-channel proteins in brain tissue from a mouse model of Alzheimer’s disease. Expansion revealing will enable the further discovery of previously unseen nanostructures within cells and tissues.

There is great desire to understand how proteins are arranged, with nanoscale precision, within cells and tissues. Super-resolution microscopy has offered the capability of combining biomolecular recognition with nanoscale optical resolution through staining with target protein-specific antibodies¹. However, such techniques are limited by the accessibility of labels to the biomolecules to be imaged, because it is the labels that are visualized, not the biomolecules themselves. Many crowded, biomolecule-rich structures are at the core of biological functions and disease states, with inter-protein distances being smaller than the size of antibodies, which may thus prohibit access to biomolecules of interest by labels. Overcoming this issue requires a technology that can not only achieve super-resolution down to the low tens of nanometres, but also enable decrowding of biomolecules in cells and tissues.

We reasoned that to decrowd proteins from each other, we could leverage the spatial expansion property of expansion microscopy (ExM)^{2,3} in which cells and tissues are densely permeated by an even mesh of swellable hydrogel, and then expanded to obtain enhanced resolution on ordinary microscopes. To establish decrowding and simultaneously achieve resolution on par with the best classical super-resolution techniques (down to ~20 nm resolution),

we combined the expansion principle with a scheme for biomolecular preservation to build a technology that we term expansion revealing (ExR). By direct comparison of biomolecular structures where labelling was done in the normal crowded environment vs after the decrowding effect of ExR, with both being imaged at the same level of resolution, we demonstrate that ExR indeed reveals previously unknown biological information and can indeed lead to the discovery of nanostructures in brain tissue that would otherwise have remained invisible. We anticipate that ExR will enable the visualization of a variety of previously undescribed biological nanostructures. We illustrate the capabilities of ExR by unmasking key components of synaptic nanocolumns as well as periodic nanostructures potentially of relevance to Alzheimer’s disease, both in mouse brain tissue.

Results

ExR enables simultaneous super-resolution and decrowding. To achieve super-resolution down to the low tens of nanometres, two rounds of expansion (or 15–20X expansion factor) are required. One would ideally minimally alter the proteins during the expansion process while decrowding them from one another as much as

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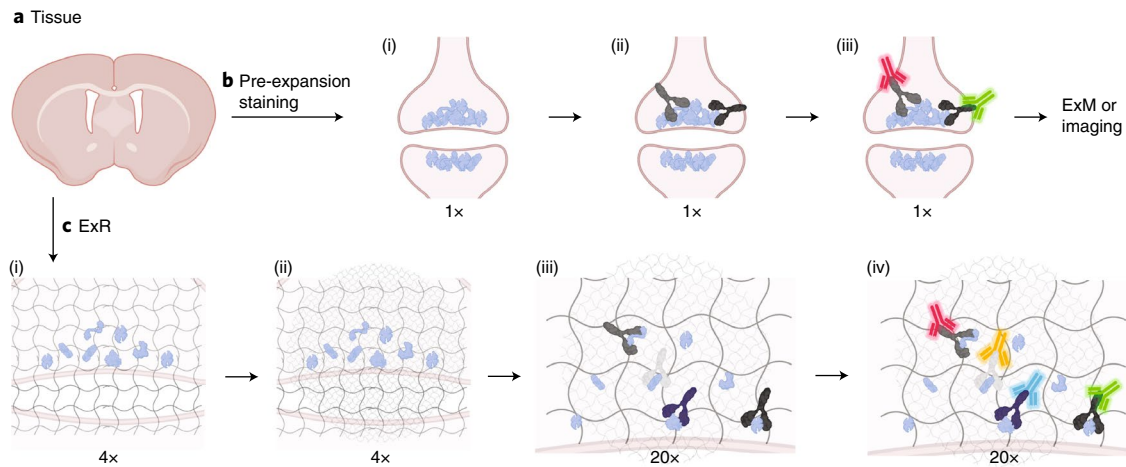


Fig. 1 | ExR, a technology for decrowding of proteins through isotropic protein separation. **a**, Schematic of coronal section of mouse brain before staining or expansion. **b**, Conventional antibody staining may not detect crowded biomolecules, shown here in pre- and post-synaptic terminals of cortical neurons. **(i)** Crowded biomolecules before antibody staining. **(ii)** Primary antibody (Y-shaped proteins) staining in non-expanded tissue. Antibodies cannot access interior biomolecules, or masked epitopes of exterior biomolecules. **(iii)** Secondary antibody (fluorescent green and red Y-shaped proteins) staining in non-expanded tissue. After staining, tissue can be imaged or expanded using earlier ExM protocols, but inaccessible biomolecules will not be detected. **c**, Post-expansion antibody staining with ExR. **(i)** Anchoring and first gelation step. Specimens are labelled with gel-anchoring reagents to retain endogenous proteins, with acrylamide included during fixation to serve as a polymer-incorporatable anchor, as in refs. ^{56,58}. Subsequently, the specimen is embedded in a swellable hydrogel that permeates densely throughout the sample (grey wavy lines), mechanically homogenized via detergent treatment and heat treatment, and expanded in water. **(ii)** Re-embedding and second swellable gel formation gelation. The fully expanded first gel (expanded 4X in linear extent) is re-embedded in a charge-neutral gel (not shown), followed by the formation of a second swellable hydrogel (light grey wavy lines). **(iii)** 20X expansion and primary antibody staining. The specimen is expanded by another factor of 4X via the addition of water, for a total expansion factor of ~20X, then incubated with conventional primary antibodies. Because expansion has decrowded the biomolecules, conventional antibodies can now access interior biomolecules and additional epitopes of exterior molecules. **(iv)** Post-expansion staining with conventional fluorescent secondary antibodies (fluorescent blue and yellow Y-shaped proteins, in addition to the aforementioned red and green ones) to visualize decrowded biomolecules. Schematic created with BioRender.com.

possible. To satisfy these conflicting requirements, ExR (Fig. 1a–c) achieves this with a new scheme. We reasoned that one could expand a brain specimen through one round of gelation and expansion, and that this first swellable hydrogel could then be further expanded if we formed a second swellable hydrogel in the space opened up by the first expansion, and then iteratively swelled the specimen a second time (see Methods for details). The proteins, being anchored to the first hydrogel throughout the entire process, would be retained because the original hydrogel would be further expanded by the second swellable hydrogel, and not cleaved or discarded. Thus, ExR imposes no extra processing steps on the proteins than is required for the first expansion. In the final step, the proteins can be antibody labelled (Fig. 1c) after decrowding and before imaging, enabling visualization of proteins that would have been missed if stained in the crowded state (Fig. 1b).

We first quantified the global isotropy of the expansion process in ExR and found a similar low distortion (that is, of a few percent over length scales of tens of microns, Supplementary Fig. 1) as we did for previous ExM protocols^{3–7}. To measure effective resolution, we focused on synapses, given both their importance for neural communication and utility as a super-resolution testbed, staining post-expansion cortical synapses (Fig. 2a) with antibodies against the pre-synaptic protein Bassoon and the post-synaptic proteins PSD95 and Homer1, among other synaptic proteins (Fig. 2b–d). We found that when we measured the mean distance between domains containing the proteins PSD95, Homer1 and Shank3, we obtained values (Fig. 2e) that matched classical results found using stochastic optical reconstruction microscopy (STORM)⁸ (note that our study focused on Shank3 and this earlier study focused on Shank1) in the low tens of nanometres. Thus, ExR exhibits effective resolution on

the order of ~20 nm comparable to our earlier iterative expansion microscopy (iExM) protocol, which did not retain proteins⁴.

To further validate the nanoscale precision of ExR, we compared pre-expansion DNA points accumulation for imaging in nanoscale topography (DNA-PAINT, a classical super-resolution method) and ExR, using the same specimen and the same field of view for comparison across the two technologies. We examined synaptic nanostructures stained with antibodies against synapsin using cultured neurons, which are amenable to classical high-precision super-resolution methods such as DNA-PAINT (see Methods). Both ExR and DNA-PAINT images show qualitatively similar staining patterns and resolution (Fig. 2f and Extended Data Fig. 1a–c). To quantitatively assess the nanoscale precision of ExR, we calculated, as a linearized distortion measure between ExR and PAINT, the shifts (in nm) between the half-maxima of the autocorrelation (ExR-ExR or PAINT-PAINT) and cross-correlation (ExR-PAINT) functions for single synaptic puncta imaged using both modalities (Extended Data Fig. 1d–f). Our analysis of 5 regions of interest (ROIs) from 3 wells of cultured neurons (1 culture batch) showed mean linearized distortion of 4.91 nm for the ExR-PAINT cross-correlation vs PAINT-PAINT autocorrelation (Fig. 2h, 95% CI of the mean (–29.93, 39.75)) and 18.78 nm for the ExR-PAINT cross-correlation vs ExR-ExR autocorrelation (Fig. 2i, 95% CI of the mean (–20.10, 57.66)). This analysis is a conservative one: ExR could in principle, through the decrowding effect (Fig. 1a–c), additionally alter the synapsin staining vs that of PAINT, but note that here, even if we conservatively lump all such decrowding-related changes into the category of distortion (as the above analysis does), we get an upper bound on the distortion in the low double-digit nanometres.

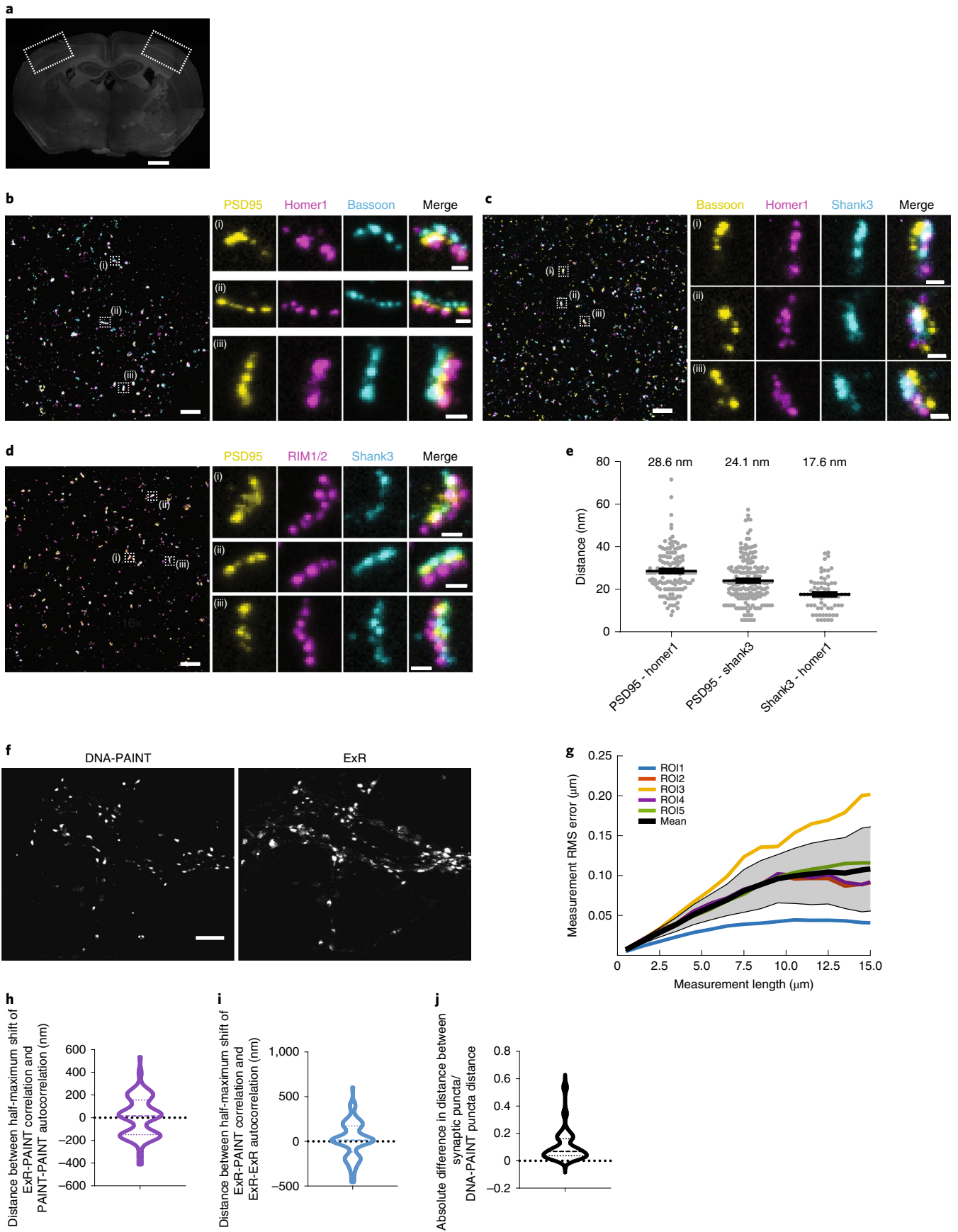
To compare the ability to distinguish between neighbouring nanostructures using DNA-PAINT and ExR, we examined pairs of neighbouring synaptic puncta (Extended Data Fig. 1g). We found that the absolute value of the difference in synaptic puncta distances as measured using ExR vs DNA-PAINT, normalized to the distance measured using DNA-PAINT, was $\sim 11\%$ (Fig. 2j, 95% CI of normalized absolute difference (0.06228, 0.1548); Extended Data Fig. 1i, 95% CI of difference between synaptic puncta centroid distance, PAINT-ExR: $(-0.05419, 0.03649)$; see Supplementary Table 1 for detailed statistics; $n = 27$ pairs from 5 ROIs from 3 wells of cultured neurons from 1 culture batch). The coefficient of variation for distance between synaptic puncta imaged using ExR, calculated as the standard deviation of the difference in distance between pairs of synaptic puncta (PAINT-ExR) divided by the mean distance of the same pairs from DNA-PAINT, was 0.1367 ($n = 27$ cropped synaptic puncta pairs, 5 ROIs from 3 wells of neurons from 1 culture batch). Furthermore, we found no significant difference in the number of unique puncta detected after thresholding (Extended Data Fig. 1h, 95% CI of difference between number of synaptic puncta, PAINT-ExR: $(-0.2936, 0.1336)$, $n = 50$ pairs, 5 ROIs from 3 wells of neurons from 1 culture batch). Similarly, we found no significant difference in the total number of synaptic puncta detected after thresholding across the entire imaging field of view (Extended Data Fig. 1j, paired *t*-test between ExR and DNA-PAINT images, $P = 0.9271$, $t = 0.09735$, d.f. = 4, $n = 5$ fields of view from 3 wells of neurons from 1 culture batch). Finally, analysis of the root mean square error (see Methods) between ExR and DNA-PAINT revealed low-percent error over a typical ROI size, comparable to previous expansion microscopy methods (that is, < 50 nm error for measurement scales of $\sim 4 \mu\text{m}$ and < 150 nm error for measurement scales of $\sim 15 \mu\text{m}$; Fig. 2g). Thus, ExR exhibits a high degree of precision and low distortion compared with a classical high-resolution super-resolution method, DNA-PAINT.

High-fidelity enhancement of synaptic protein visualization via ExR. To gauge whether ExR could reveal nanostructures in the brain that were not visible without decrowding, and to further probe whether it incurred any costs in terms of decreased resolution or added distortion relative to classical staining (that is, pre-expansion staining and thus no decrowding), we devised a strategy where we would stain brain slices pre-expansion with an antibody against a synaptic protein, in such a way that antibodies would be anchored to the expansion hydrogel for later visualization. We then perform

ExR and re-stain the same proteins with the same antibody a second time, thus enabling a within-sample comparison of a given protein across both conditions (with and without decrowding) at the same level of resolution to reveal any nanostructural differences. For pre-expansion staining (see Methods for details), we immunostained mouse brain slices containing somatosensory cortex (Fig. 3a) with primary antibodies followed by 6-((acryloyl)amino)hexanoic acid, succinimidyl ester (abbreviated AcX)-conjugated secondary antibodies, so that these antibodies could be attached to the swellable hydrogel for post-expansion tertiary antibody staining and visualization. This allowed us to compare pre-ExR staining to post-ExR staining at the same resolution level and for the same field of view, which are important for noticing any changes in nanostructural detail. We noted that Homer1 and Shank3 exhibited very similar visual appearances when we compared pre- vs post-ExR staining (quantified below), so we designated these two stains as ‘reference channels’, that is, co-stains that could help define synapses for the purposes of technological comparison, and that we could use to help us gauge whether other proteins were becoming more visible at synapses.

We chose 7 synaptic proteins important for neural architecture and transmission for this experiment—the pre-synaptic proteins Bassoon, RIM1/2 and the P/Q-type calcium channel $\text{Ca}_v2.1$ $\alpha 1A$ subunit, and the post-synaptic proteins Homer1, Shank3, SynGAP and PSD95 (Fig. 3b–h), staining for each protein along with a reference channel stain (note: when we imaged Homer1, we used Shank3 as the reference channel, and vice versa). These proteins were chosen on the basis of their frequent use as synaptic markers, their known interaction with one another and/or their functional importance at the synapse^{9–15}. Specifically, Shank3 is a key post-synaptic scaffolding protein that interacts with PSD95 and Homer1, among other proteins, in the post-synaptic density⁹, and Shank3 mutant mice display autistic-like behaviours and striatal changes¹⁶; RIM1/2 promotes calcium channel localization at the pre-synaptic active zone¹⁷, controls synaptic vesicle release¹⁸ and enhances the size of the readily releasable pool¹⁷; Bassoon and calcium channels contribute to vesicle release and promote vesicle reloading¹⁹; PSD95, another key post-synaptic scaffolding protein, drives maturation of excitatory synapses²⁰ and PSD95 mutant mice display enhanced long-term potentiation and impaired learning²¹; SynGAP regulates spine formation¹⁴; Homer and Shank form a higher-order complex that forms a binding platform for post-synaptic proteins²².

Fig. 2 | Validation of ExR using synapses and comparison with DNA-PAINT. **a**, Low-magnification widefield image of a mouse brain slice stained with DAPI, with dotted box highlighting the somatosensory cortex used in subsequent figures for synapse staining. Scale bar, 500 μm . **b–d**, Confocal images (maximum intensity projections) of representative fields of view (cortical L2/3) and specific synapses after ExR expansion and subsequent immunostaining using antibodies against PSD95, Homer1 and Bassoon (**b**); Bassoon, Homer1 and Shank3 (**c**); and PSD95, RIM1/2 and Shank3 (**d**). Scale bar, 1 μm , left image; 100 nm, right images; in biological units (that is, the physical size divided by the expansion factor, used throughout the paper unless otherwise indicated). Shown are images from one representative experiment from two independent replicates. **e**, Measured distance between centroids of protein densities of PSD95 and Homer1, PSD95 and Shank3, and Shank3 and Homer1, in synapses such as those in **b–d**. The mean distance (again, in biological units) between PSD95 and Homer1 is 28.6 nm ($n = 126$ synapses from 3 slices from 1 mouse), 24.1 nm between PSD95 and Shank3 ($n = 172$ synapses from 3 slices from 1 mouse), and 17.6 nm between Shank3 and Homer1 ($n = 70$ synapses from 3 slices from 1 mouse). Mean \pm s.e.m. are plotted; individual grey dots represent the measured distance for individual synapses. **f**, A pre-expansion DNA-PAINT image (left) and a registered confocal ExR image (maximum intensity projection, right) of the same field of cultured neurons after immunostaining with antibodies against Synapsin 1. Scale bar, 4 μm . Shown are images from one representative experiment from two independent replicates. **h**, Estimated population distribution (violin plot of density, with a dashed line at the median and dotted lines at the quartiles) of the shift (in nm) at which the correlation is half-maximal for PAINT-PAINT autocorrelation and ExR-PAINT correlation (calculated pixel-wise between intensity values normalized to the minimum and maximum of the image, see Methods; see Supplementary Table 1 for statistics. $n = 101$ synaptic ROIs from 5 fields of view from 3 wells of cultured neurons from 1 culture batch). **i**, Same as **h**, for ExR-ExR autocorrelation vs ExR-PAINT correlation. **j**, Estimated population distribution (violin plot of density, with a dashed line at the median and dotted lines at the quartiles) of the normalized absolute difference in radial distance between neighbouring synaptic puncta centroids (absolute value of PAINT-ExR, normalized to PAINT distance; see Supplementary Table 1 for statistics. $n = 27$ cropped synaptic pair ROIs from 5 fields of view of $30 \mu\text{m} \times 30 \mu\text{m}$ each, 1 culture batch). **g**, Root mean square error vs measurement length (in biological units), calculated via a non-rigid registration algorithm of DNA-PAINT vs ExR-processed cultured neurons ($n = 5$ fields of view from 3 wells from 1 culture batch). Black line, mean; grey shading, standard deviation.



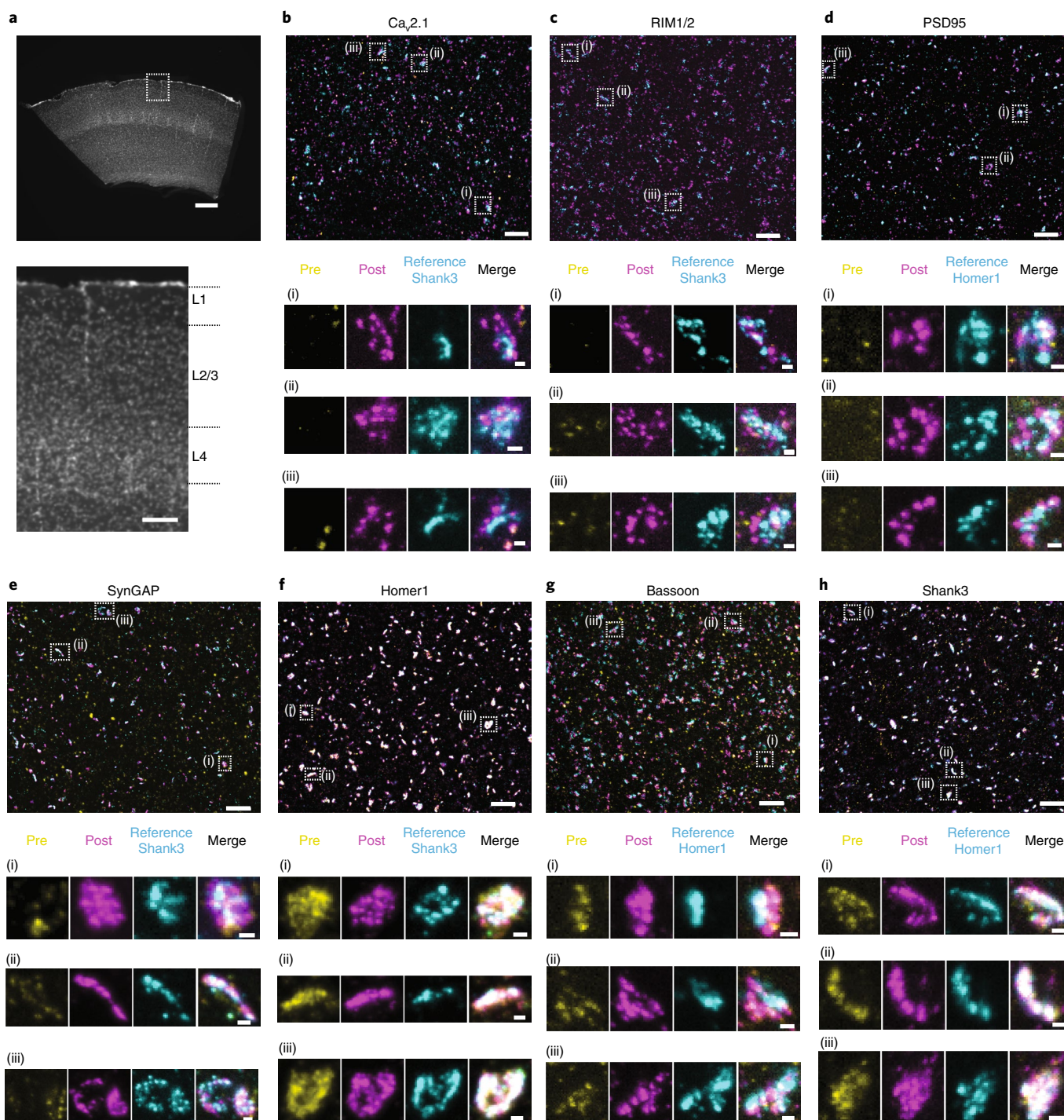


Fig. 3 | Validation of ExR enhancement and effective resolution in synapses of mouse cortex. **a**, Low-magnification widefield image of a mouse brain slice with DAPI staining showing somatosensory cortex (top) and zoomed-in image (bottom) of boxed region containing L1, L2/3 and L4, which are imaged and analysed after further expansion in **b–h**. Scale bar, 300 μm (top) and 100 μm (bottom). **b–h**, Confocal images of maximum intensity projections of specimen after immunostaining with antibodies against Ca_v2.1 (Ca²⁺ channel P/Q-type) (**b**), RIM1/2 (**c**), PSD95 (**d**), SynGAP (**e**), Homer1 (**f**), Bassoon (**g**) and Shank3 (**h**) in somatosensory cortex L2/3. For pre-expansion staining, primary and secondary antibodies were stained before expansion, the stained secondary antibodies anchored to the gel, and fluorescent tertiary antibodies applied after expansion to enable visualization of pre-expansion staining. For post-expansion staining, the same primary and secondary antibodies were applied after ExR. Antibodies against Shank3 (**b, c, e, f**) or Homer1 (**d, g, h**) were applied post-expansion as a reference channel. Confocal images of cortex L2/3 (top) show merged images of pre- and post-expansion staining, and the reference channel. Zoomed-in images of three regions boxed in the top image (**i–iii**, bottom) show separate channels of pre-expansion staining (yellow), post-expansion staining (magenta), reference staining (cyan) and merged channel. Scale bar, 1.5 μm (top); 150 nm (bottom of **i–iii**). Shown are images from one representative experiment from two independent replicates.

All seven proteins exhibited well-defined images when post-expansion stained, with the geometry reflecting characteristic synaptic shapes. For example, a pre-synaptic and a post-synaptic stain (Fig. 3b,c,g) revealed parallel regions with a putative synaptic cleft in between, although note that this was only visible if the synapse was being imaged from the side; if a synapse was being imaged with the axial direction of the microscope perpendicular to the synaptic cleft, then the image might look more disc-shaped^{4,8,23}. In many cases, however, post-ExR staining revealed more detailed structures of synapses compared with what was visualized with pre-expansion staining—for example, calcium channels (Fig. 3b), RIM1/2 (Fig. 3c), PSD95 (Fig. 3d), SynGAP (Fig. 3e) and Bassoon (Fig. 3g) appeared more prominent post-expansion than pre-expansion. Applying a conventional antigen retrieval protocol did not result in such improvements (Supplementary Fig. 2), suggesting that the decrowding effect observed via ExR was indeed due to expansion, and not simply due to denaturation or antigen retrieval-like effects associated with other aspects of the ExR process.

To quantitate the improvement in staining enabled by ExR, we measured the amplitude and volume of each synaptic protein stain, both within and just outside of identified synapses. First, we manually identified between 49–70 synapses (see Supplementary Table 2 for exact numbers) per $\sim 350 \times 350 \times 20 \mu\text{m}^3$ (in physical units, for example, what is actually seen through the microscope lens) field of view, choosing the largest and brightest synapses on the basis of reference channel staining (that is, Homer1 or Shank3). We developed an automated method to segment synaptic puncta from nearby background. Briefly, we created binary image stacks for each channel using a threshold equal to a multiple of the average measured standard deviation of five manually identified background regions (not blinded to condition), filtered three-dimensional (3D) connected components on the basis of size and used dilated reference channel ROIs to segment putative synaptic puncta (Supplementary Fig. 3 and Methods). We dilated reference channel-defined ROIs to relax the requirement of exact co-localization of pre-synaptic proteins with a post-synaptic reference. We found that post-expansion staining increased signal intensity and mean total volume of signal within dilated reference channel-defined ROIs, without meaningfully affecting background staining (that is, signal just outside the dilated reference channel-defined ROIs) (Extended Data Fig. 2a,b). In summary, all proteins except Homer1 and Ca_v2.1 showed significantly increased signal intensity of post-expansion staining within dilated reference ROIs, with minimal increase in the background (Extended Data Fig. 2a and Supplementary Table 3 for full statistical analysis). (We note that there was no change in signal intensity in the background for PSD95, but observed a bimodal distribution of signal intensity increases in the foreground and background due to abnormally high signal in one animal.) Similarly, all proteins except Homer1 exhibited increased total volume of post-expansion staining signals within dilated reference ROIs, and minimal or no increase in the volume of background ROIs (Extended Data Fig. 2b and Supplementary Table 4 for full statistical analysis).

Because Homer1 and Shank3 had the smallest changes in pre- vs post-expansion staining, they were chosen as reference channels to indicate the locations of putative synapses for pre- vs post-expansion test stain comparisons, as mentioned above. Different antibodies may bind to different sites on a target protein, and we found that a different antibody against PSD95 (from Cell Signaling Technology, CST3450S) from the one used in Fig. 3d (ThermoFisher, MA1-046) showed similar signal intensity and volume when compared pre- vs post-staining (Supplementary Fig. 4); perhaps, in future studies, multiple antibodies against different parts of a single protein in pre vs post-expansion comparisons could be used to gauge the density of the environment around different parts of that protein.

We further analysed synaptic protein signals pre- vs post-expansion in the context of different cortical layers. We quantified

volume and signal-to-noise ratio (SNR; signal intensity divided by standard deviation of the background) of each protein in 3D synaptic structures by binarizing signals over a threshold (a multiple of the standard deviation of the intensity within a manually selected background region; see Methods) and selecting putative synapses in each $\sim 350 \times 350 \times 20 \mu\text{m}^3$ field of view imaged above, comparing the values of pre- vs post-expansion staining in each layer of somatosensory cortex (L1, L2/3 and L4, respectively) (Extended Data Fig. 2c,d). Post-expansion images exhibited larger volumes and improved SNR in each layer for all synaptic proteins (see Supplementary Tables 6 and 7 for full statistics).

In previous work, we showed using the original iterative expansion microscopy (iExM) protocol, which uses pre-expansion staining, that we could achieve effective resolutions of ~ 20 nm with low distortion due to the expansion process⁴. As another independent way to gauge the potential distortion obtained by staining with antibodies post-expansion, we compared the shapes of synaptic puncta as seen with the pre-expansion stain, with the shapes as seen with the new post-expansion stain, using the within-sample dual staining method used in Fig. 3b–h. We compared various properties of synaptic puncta between pre- and post-expansion staining conditions, using the reference proteins Homer1 and Shank3 since they had similar intensities and volumes when comparing pre- and post-expansion datasets, and therefore might be appropriate for comparing shape features across these conditions (Extended Data Fig. 3). In summary, we did not see any substantial distortion being introduced by post-expansion staining; for example, we found no significant difference in the number of synaptic puncta when we compared pre- vs post-expansion staining in the same sample (Extended Data Fig. 2e), and when we measured the shift in puncta positions between pre- and post-stained conditions, we observed average shifts of <10 nm (in biological units, that is, physical size divided by the expansion factor) between post- and pre-expansion staining for both Homer1 and Shank3 (Extended Data Fig. 2f,g; see Supplementary Table 5 for full statistics). Thus, ExR preserves, relative to classical pre-expansion staining, the locations of proteins with high fidelity for the purposes of post-expansion staining.

Synaptic nanocolumns coordinated with calcium channel distributions. Coordinating pre- and post-synaptic protein arrangement in a nanocolumn structure which aligns molecules within the two neurons contributes to precision signalling from pre-synaptic release sites to post-synaptic receptor locations^{23,24}, as well as to the long-term plasticity of synaptic function²⁵. Given that ExR is capable of unmasking, with nanoscale precision, synaptic proteins that are otherwise not detectable, we next sought to explore the nanocolumnar architecture of pre- and post-synaptic proteins, with a focus on important molecules that have not yet been explored in this trans-synaptic alignment context. As noted above, ExR greatly helps with visualization of calcium channels, which are among the most important molecules governing the activation of synaptic release machinery, with nanometre-scale signalling contributing to the precision of synaptic vesicle fusion. However, the nanoscale mapping of calcium channels in the context of nanocolumnar alignment in brain tissue remains difficult^{26–28} (Fig. 3b). We thus applied ExR to investigate whether calcium channels occupy nanocolumns with other pre- and post-synaptic proteins, such as the critical pre- and post-synaptic proteins RIM1/2 and PSD95, respectively, across the layers of the cortex (Fig. 4a–d).

We first performed a 3D autocorrelation function ($g_s(r)$)-based test which provides information about the intensity distribution within a defined structure (see Methods). Any heterogeneity in the intensity distribution within the cluster will result in a $g_s(r) > 1$, and the distance at which the $g_s(r)$ crosses 1 can be used to estimate the size of the internal heterogeneity, here termed a nanodomain²³. In all cortical layers, our autocorrelation analysis shows that all

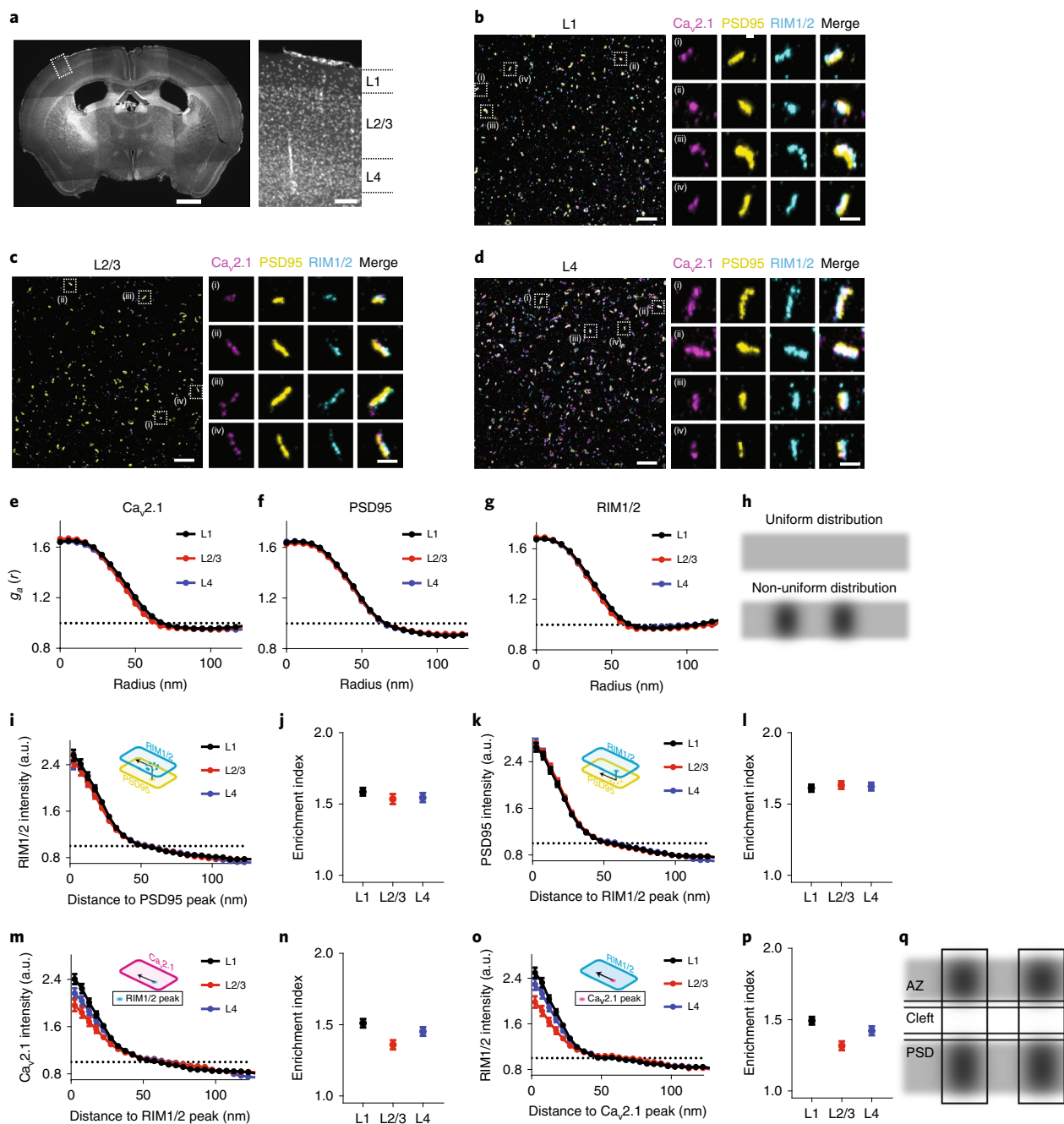


Fig. 4 | ExR reveals how calcium channel distributions participate in trans-synaptic nanoarchitecture. **a**, Low-magnification widefield image of DAPI-stained mouse brain slice (left) and zoomed-in view (right) of the boxed region showing layers 1–4 of the cortex. Scale bar, 1,000 μm (left) and 100 μm (right). **b–d**, Confocal images (maximum intensity projections) of layers 1, 2/3 and 4, respectively, after performing ExR and immunostaining with antibodies against $\text{Ca}_v2.1$ (calcium channel) (magenta), PSD95 (yellow) and RIM1/2 (cyan). Left: low-magnification images. Right: zoomed-in images of four regions (i–iv) with separated channels for each antibody along with the merged image. Scale bar, 1 μm (left) and 100 nm (right, i–iv). Shown are images from one representative experiment from two independent replicates. **e–g**, Autocorrelation analysis for $\text{Ca}_v2.1$ (**e**), PSD95 (**f**) and RIM1/2 (**g**) for different layers. **h**, Schematic illustration of protein distribution based on interpretation of autocorrelation results in **e–g**. A uniform distribution (top) would be predicted if $g_s(r) = 1$ at all radii (dotted lines in **e–g**), whereas a non-uniform distribution with one or more regions of high local intensity (bottom) would be predicted if $g_s(r) > 1$ at short radii and decayed as the radius is increased. **i–p**, Enrichment analysis that calculates the average molecular density for RIM1/2 to the PSD95 peak (**i**), PSD95 to the RIM1/2 peak (**k**), $\text{Ca}_v2.1$ to the RIM1/2 peak (**m**) and RIM1/2 to the $\text{Ca}_v2.1$ peak (**o**), with the corresponding mean enrichment indices shown in **j**, **l**, **n** and **p** (see Methods for exact n values). Error bars indicate s.e.m. **q**, Schematic illustration of protein distribution based on interpretation of enrichment analysis results in **i–p**. Enrichment values above 1 represent regions of high local intensity in the measured channel, so the enrichment profiles in **i**, **k**, **m** and **o** suggest that the peak of the reference channel closely aligns with regions of high intensity in the measured channel for each of the four comparisons. Therefore, this suggests that enriched regions of any two proteins (RIM1/2, PSD95 and $\text{Ca}_v2.1$) are aligned in nanoscale precision with each other. Calcium channels located close to vesicle fusion sites (dictated by RIM1/2) may enhance the calcium sensitivity of fusion. Additionally, post-synaptic receptors may be exposed to higher, faster peaks of neurotransmitter concentration when vesicle release sites are located directly opposite receptor nanoclusters (PSD95 being a receptor-anchoring protein). AZ, active zone; PSD, post-synaptic density.

3 proteins explored exhibited a non-uniform arrangement, forming nanodomains with average diameters of about 60–70 nm (in biological units; Fig. 4e–h). To analyse the spatial relationship between the two distributions and the average molecular density of RIM1/2, PSD95 and Ca_v2.1 relative to each other, we performed a protein enrichment analysis, which is a measure of volume-averaged intensity of one channel as a function of distance from the peak intensity of another channel (see Methods). To more easily compare the extent of the enrichment between these proteins in each layer, we also calculated the enrichment index, which is an average of all enrichment values within 60 nm (biological units) of the peak of a designated channel. Our analysis shows that the centres of nanodomains of RIM1/2, PSD95 and Ca_v2.1 are enriched with respect to each other (Fig. 4i–p).

Of particular interest, the nanoscale co-localization of Ca_v2.1 with RIM1/2 (and thus the vesicle site) probably minimizes the distance between the channels and the molecular Ca sensors that trigger vesicle fusion (Fig. 4q), consistent with the physiological concept of nanodomain coupling that tunes the efficacy and frequency-dependence of neurotransmission²⁹. Furthermore, the precise alignment between RIM1/2 and PSD95 may reduce the diffusion distance of the released neurotransmitter before reaching post-synaptic receptors (Fig. 4q). Thus, these nanoscale arrangements may help to optimize the speed, strength and plasticity of synaptic transmission. To the best of our knowledge, the 3D nanoarchitecture of the distribution of voltage-gated calcium channels within the trans-synaptic framework in brain tissue has not been previously probed.

Periodic amyloid nanostructures in Alzheimer's model mouse brain. In addition to densely crowded proteins in healthy functioning compartments like synapses, densely crowded proteins appear in pathological states like Alzheimer's disease. Protein aggregates known as β -amyloid are thought to play roles in synaptic dysfunction, neurodegeneration and neuroinflammation³⁰. However, the densely packed nature of these aggregates may make the nanoscale analysis of their ultrastructure within brain tissue difficult to understand. To understand the nanoarchitecture of β -amyloid in the cellular context of brain tissue, we applied ExR to the brains of 5xFAD Alzheimer's model mice (Fig. 5a), as this widely used animal model of Alzheimer's exhibits an aggressive amyloid pathology³¹. We employed two different commercially available antibodies for β -amyloid, 6E10 (which binds to amino acid residues 1–16 of human A β peptides) and 12F4 (which is reactive to the C terminus of human A β and has specificity towards A β 42). We were particularly interested in investigating the relationship between amyloid deposits and white matter tracts, as these regions have been implicated in human imaging data^{32–35} but are less investigated in mouse models. We previously reported the accumulation of A β aggregates along the fornix, the major white matter tract connecting the subiculum and mammillary body, early in disease progression³⁵.

We co-stained the amyloid antibodies along with the axonal marker SMI312 and compared pre-expansion staining with that obtained after ExR. Plaques appeared to be larger and to have finer-scaled features in post-expansion staining than in pre-expansion staining (Fig. 5b,c) when visualized by either 6E10 or 12F4 antibodies; thus, the post-expansion staining may unveil aspects of plaque geometry that are not easily visualized through traditional means. Additionally, post-ExR staining revealed detailed nanoclusters of β -amyloid that were not seen when staining was done pre-expansion (Fig. 5b,c). These nanodomains of β -amyloid appeared to occur in periodic structures (Fig. 5b(i–iv),c(i–iv)). Co-staining with two different β -amyloid antibodies, D54D2 (which binds to isoforms A β 37, A β 38, A β 40 and A β 42) along with 6E10 or 12F4 in ExR-processed 5xFAD fornix showed similar patterns of periodic nanostructures, which means the observed periodic nanostructures are probably

not composed of specific isoforms (Extended Data Fig. 4a,b). These nanodomains, and periodic structures thereof, were not visualized through pre-expansion staining, which was also confirmed using unexpanded tissue (Extended Data Fig. 4c).

To see whether the periodic nanostructures of β -amyloid revealed through ExR were just non-specific staining artefacts of ExR, we performed ExR on wild-type (WT) mice as a control, which should not have any labelling for human β -amyloid. No β -amyloid structures were observed in ExR-processed WT brain (Supplementary Fig. 5a). Our quantitative analysis examining the volume of amyloid in ExR-processed WT and 5xFAD mice confirms that there is indeed a large amount of amyloid volume occupied in 5xFAD samples, but essentially no such volume occupied in ExR-processed WT mice (Supplementary Fig. 5b). The lack of amyloid staining in ExR-processed WT mice makes it unlikely that the staining seen in ExR-processed 5xFAD mouse brain is non-specific. Applying a conventional antigen retrieval protocol recovered some amyloid staining, but less than by ExR (Supplementary Fig. 6), suggesting that the decrowding effect observed via ExR was indeed due to expansion and not simply due to denaturation or antigen retrieval-like effects associated with other aspects of the ExR process. The intact staining of axonal and myelin markers (Fig. 6 and Extended Data Fig. 5) adjacent to the amyloid puncta highlight how, within the same volume of tissue, some structures can exhibit periodicity while others remain smooth and continuous, highlighting the small length scales over which the architecture of nanostructures is regulated in cells and tissues.

Co-clustering of amyloid nanodomains and ion channels. To understand the biological context of these periodic A β nanostructures, we stained brain slices with antibodies against the ion channels Nav1.6 and Kv7.2. Alzheimer's disease is associated with altered neuronal excitability and alterations in ion channels³⁶. We stained ExR-processed 5xFAD fornix-containing brain slices with antibodies against potassium channels (Kv7.2) and against β -amyloid (12F4) (Fig. 6a and Supplementary Fig. 7a). The periodic β -amyloid nanostructures co-localized with periodic nanoclusters of potassium channels. In ExR-processed WT fornix, such β -amyloid clusters and frequent clusters of potassium or sodium channel staining were not found (Supplementary Fig. 7b,c). Co-staining of β -amyloid (12F4) and sodium channels (Nav1.6) showed co-localization as well (Fig. 6b and Supplementary Fig. 7d). Specifically, over half of β -amyloid nanoclusters co-localized with Nav1.6 (Supplementary Fig. 7d(i)), and within these co-localized regions, Nav1.6 puncta were much smaller than β -amyloid nanoclusters (Supplementary Fig. 7d(ii)). We also found other interesting amyloid patterns, such as helical structures along axons (Fig. 6b).

To probe the periodic amyloid/Kv7.2 patterns further, we obtained a cross-sectional profile of a stretch of axon, showing that A β 42 (magenta) and Kv7.2 (yellow) were highly overlapping and had similar periodicities (Fig. 6c). A further histogram analysis showed the periodicity of both repeated protein structures to be 500–1,000 nm (Fig. 6d), which we confirmed by Fourier analysis (Fig. 6e,f). This periodicity is notably higher in spatial frequency, by orders of magnitude, than the inter-node-of-Ranvier distances of brain white matter, which range from 20–100 μ m^{37–39}.

Our analysis along individual segments of axons showed that a high fraction of A β 42 clusters contained Kv7.2 clusters (Fig. 6g), a result that we confirmed by measuring the distance between the centroids of overlapping A β 42 and Kv7.2 nanoclusters, finding extremely tight co-localization within a few tens of nanometres (Fig. 6h). Median sizes of nanoclusters were around 150 nm for both A β 42 and Kv7.2 (Fig. 6i); indeed, overlapping nanodomains of A β 42 and Kv7.2 were highly correlated in size (Fig. 6j), suggesting a potential linkage between how they are formed and organized as multiprotein complexes.

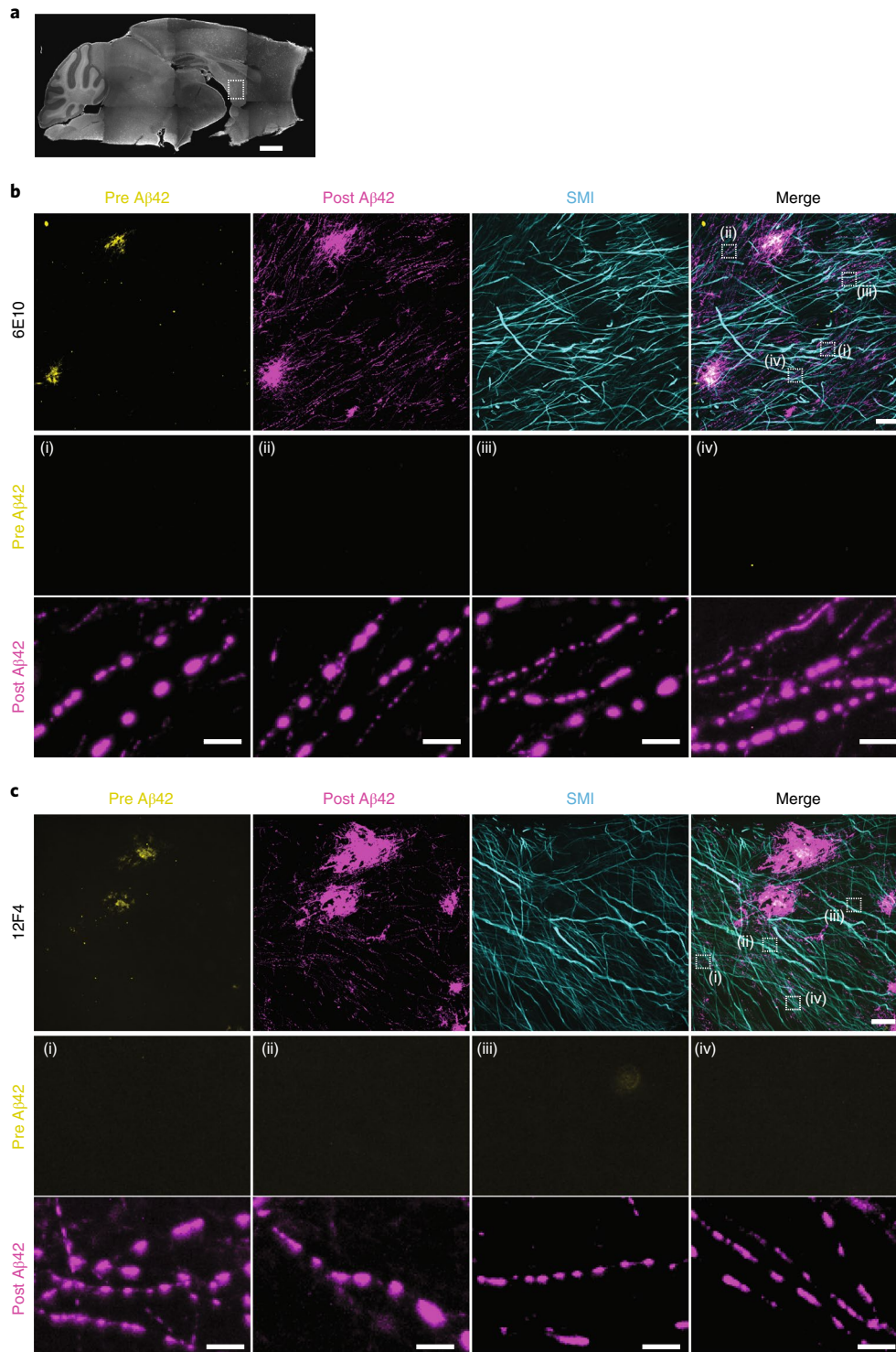


Fig. 5 | ExR reveals periodic nanoclusters of A β 42 peptide in the fornix of Alzheimer's model 5xFAD mice. **a**, Epifluorescence image showing a sagittal section of a 5xFAD mouse brain with the fornix highlighted (dotted box). Scale bar, 1,000 μ m. **b,c**, Top row: ExR confocal images (maximum intensity projections) showing immunolabelling against A β 42 peptide with two different monoclonal antibodies 6E10 (**b**) and 12F4 (**c**). From left to right: pre-expansion immunolabelling of A β 42 (yellow), post-expansion labelling of A β 42 (magenta), post-expansion SMI (neurofilament protein), and merged pre- and post-expansion staining of A β 42 with post-expansion staining of SMI. Middle and bottom rows: insets (**i–iv**) showing the regions of interest highlighted (dotted boxes) in the merged images in the top rightmost panels of **b** and **c**; middle row: pre-expansion A β 42 labelling; bottom row: post-expansion A β 42 labelling. Post-ExR staining reveals periodic nanostructures of β -amyloid, whereas pre-expansion staining can detect only large plaque centres. Scale bar, 10 μ m (top); 1 μ m (bottom, **i–iv**).

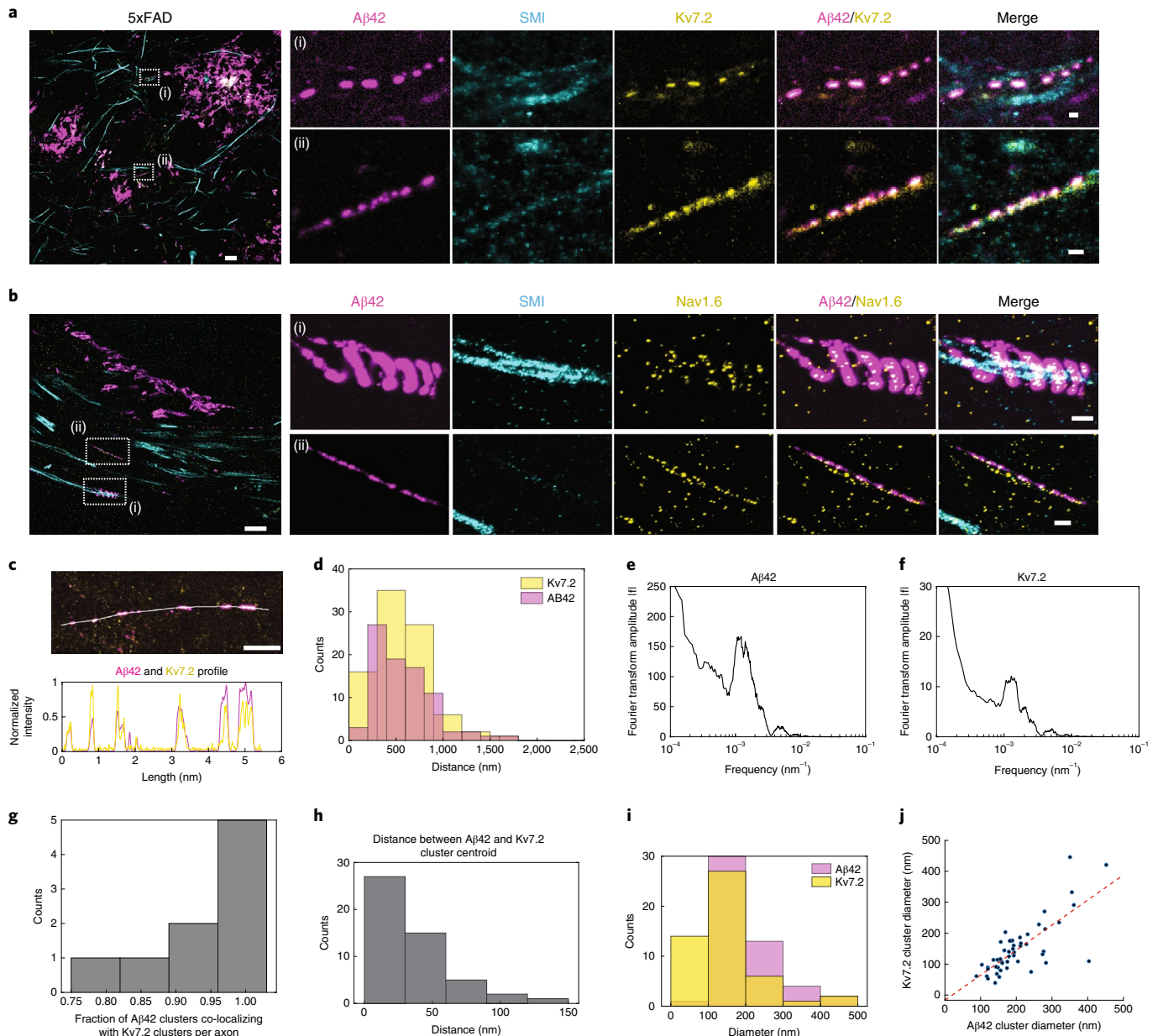


Fig. 6 | ExR reveals co-localized clusters of A β 42 peptide with potassium and sodium ion channels in the fornix of Alzheimer's model 5xFAD mice. **a, b**, ExR confocal image (maximum intensity projections) showing post-expansion A β 42 (magenta), SMI (cyan) and Kv7.2 (yellow) staining (**a**), and A β 42 (magenta), SMI (cyan) and Nav1.6 (yellow) staining (**b**) in the fornix of a 5xFAD mouse. Leftmost panel, merged low-magnification image. Scale bar, 4 μ m. Insets (**i, ii**) show close-up views of the boxed regions in the leftmost image for A β 42, SMI, Kv7.2, A β 42-Kv7.2 merged and A β 42-Kv7.2-SMI merged, respectively (**a**), and A β 42, SMI, Nav1.6, A β 42-Nav1.6 merged and A β 42-Nav1.6-SMI merged, respectively (**b**). Scale bar, 400 nm; $n = 5$ fields of view from two slices from two mice. **c**, ExR confocal image (maximum intensity projections) showing A β 42 (magenta) and Kv7.2 (yellow) clusters in a 5xFAD mouse (top) with the indicated cross-section profile shown (bottom). Scale bar, 1 μ m. Shown are images from one representative experiment from four independent replicates. **d**, Histograms showing distances between adjacent A β 42 (magenta) and Kv7.2 (yellow) clusters in 5xFAD mice along imaged segments of axons ($n = 97$ A β 42 clusters, 92 Kv7.2 clusters from 9 axonal segments from 2 mice). **e, f**, Fourier-transformed plots of A β 42 (**e**) and Kv7.2 (**f**) showing the same peak position (from the same dataset as in **d**). **g**, Histogram showing the fraction of A β 42 clusters co-localizing with Kv7.2 clusters along individual segments of axons ($n = 50$ cluster pairs from 9 axonal segments from 2 mice). **h**, Histogram showing the distance between the centroids of co-localized A β 42 and Kv7.2 clusters (same dataset as in **d**). **i**, Histograms showing the diameters of A β 42 clusters (magenta) and Kv7.2 clusters (yellow) ($n = 50$ cluster pairs from 9 axonal segments from 2 mice). **j**, Scatterplot showing the diameters of co-localized A β 42 and Kv7.2 clusters. Red line, $y = x$, for reference.

To facilitate visualization of the 3D shapes of these A β 42 and Kv7.2 puncta, we show three orthogonal slices in the x - y , x - z and y - z planes (x - and y -directions being transverse directions, and the z -direction being axial) that intersect the centre of each puncta. Qualitatively, we observed that the majority of these puncta are

oblong, with smooth continuous A β 42 ellipsoids and more punctate Kv7.2 puncta, with some (but not all) Kv7.2 puncta being found within A β 42 puncta. A larger volume of A β 42 (as a fraction of total A β 42 puncta volume) was found inside Kv7.2 puncta compared with the fraction of Kv7.2 volume found inside A β 42 puncta (Fig. 7 and

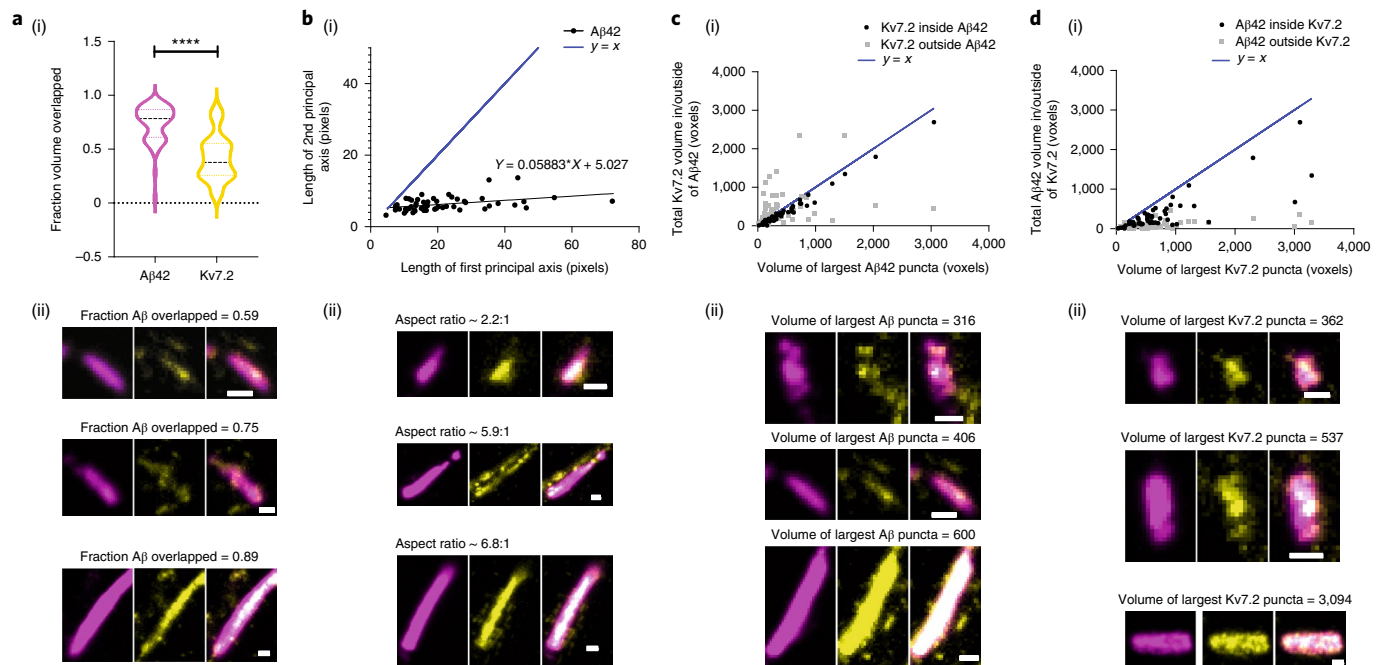


Fig. 7 | Analysis of A β 42 peptide and potassium ion channel nanoclusters in shapes and their relationships. **a**, (i) Fraction of total A β 42 and Kv7.2 puncta volume overlapped with one another in cropped A β 42 clusters ($n = 55$ clusters from 2.5x FAD mice; $P < 0.0001$, two-sided pairwise t -test). $****P < 0.0001$. (ii) Representative images illustrating the difference in the proportion of mutually overlapped volume between A β 42 and Kv7.2 as a fraction of total A β 42 or Kv7.2 volume. Scale bar, 100 nm. **b**, (i) Length of the second principal axis of the ellipsoid that has the same normalized second central moments as the largest A β 42 punctum in an ROI, vs the length of the first principal axis of this ellipsoid (in pixels, 1 pixel = 11.44 nm in x - and y -directions (transverse) and 26.67 nm in the z -direction (axial)); black points represent individual manually cropped ROIs; slope of best-fit line from simple linear regression = 0.05883, $P = 0.0020$, $F = 10.59$, $d.f. = 53$). Compare to the line $y = x$ (blue). (ii) Representative images illustrating the oblong shape of A β 42 puncta for three first principal axis lengths: shorter (top row), medium (middle row) and longer (bottom row). While the length of the first principal axis varies significantly between these examples, the length of the second principal axis remains similar among the three clusters. **c**, (i) Total volume (in voxels, 1 voxel = $11.44 \times 11.44 \times 26.67$ or $3,490 \text{ nm}^3$) of Kv7.2 puncta overlapped/inside (black, $R^2 = 0.980$, $P < 0.0001$) and outside (grey, $R^2 = 0.0582$, $P = 0.0979$) A β 42 puncta as a function of the volume of the largest A β 42 puncta within an ROI, compared to the line $y = x$ (blue). (ii) Representative images illustrating that the volume of Kv7.2 outside of A β 42 is relatively constant as A β 42 puncta size increases. As in **b(ii)**, the three clusters shown are ordered by increasing size. **d**, (i) The converse of **c**: total volume of A β 42 puncta overlapped/inside (black, $R^2 = 0.6531$, $P < 0.0001$) and outside (grey, $R^2 = 0.1876$, $P = 0.0010$) of Kv7.2 puncta as a function of the volume of the largest Kv7.2 puncta within an ROI, compared to the line $y = x$ (blue). (ii) Representative images illustrating that the volume of A β 42 outside of Kv7.2 is smaller than the volume of A β 42 co-localized with Kv7.2, and both values are positively correlated with the volume of the largest Kv7.2 puncta. Scale bar (**a(ii)**–**d(ii)**), 100 nm. Shown are images from one representative experiment from four independent replicates.

Supplementary Fig. 8a). On average, the mean volume of an A β 42 puncta was slightly larger than that of a Kv7.2 puncta (Supplementary Fig. 8b, paired t -test, $P = 0.0001$, $t = 4.116$, $d.f. = 54$). Quantification of shape characteristics confirmed these observations and revealed more subtle patterns. Representative images illustrating the observed trends are shown in Fig. 7a(ii)–d(ii). Despite the larger volume of A β 42 puncta, the fraction of A β 42 volume mutually overlapped with (inside of) Kv7.2 puncta was larger than the fraction of Kv7.2 mutually overlapped with (inside of) A β 42 puncta (Fig. 7a; $P < 0.0001$, $t = 10.94$, $d.f. = 54$). When considering the ellipsoidal shapes of these puncta, the relationship between the second and first principal axis lengths was highly sublinear (Fig. 7b), and the average aspect ratio (ratio of first to second principal axis length) was $\sim 3.5:1$ (mean 3.464, standard deviation 1.911, $n = 55$ puncta), indicating a highly oblong shape (slope of best-fit line from simple linear regression = 0.05883, $P = 0.0020$, $F = 10.59$, $d.f. = 53$). While the number of Kv7.2 present in each manually cropped ROI was not correlated with the volume of the largest A β 42 puncta within the cropped ROI (Supplementary Fig. 8c; simple linear regression, 95% CI of slope (-0.003940 , 0.005947)), the mean Kv7.2 puncta volume was significantly correlated with the mean A β 42 puncta volume (Supplementary Fig. 8d; simple linear regression, 95% CI of slope (0.09878 , 0.2437), $R^2 = 0.2977$, $P < 0.0001$, $F = 22.47$, $d.f. = 53$).

Thus, as the size of A β 42 aggregates increases, Kv7.2 aggregates also increase in size, but not in number. We found that the volume of Kv7.2 puncta inside A β 42 puncta is highly correlated with the volume of the A β 42 puncta (Fig. 7c; simple linear regression, 95% CI of slope (0.8353 , 9037), $R^2 = 0.98$, $P < 0.0001$, $F = 2,593$, $d.f. = 53$), but the volume of Kv7.2 puncta outside of A β 42 puncta is not correlated with A β 42 puncta volume (Fig. 7c; simple linear regression, 95% CI of slope (-0.03919 , 0.4503), $R^2 = 0.05082$, $P = 0.0929$, $F = 2.838$, $d.f. = 53$). Conversely, the total volume of A β 42 puncta inside of Kv7.2 puncta was not as strongly correlated with the volume of the largest Kv7.2 puncta (Fig. 7d; simple linear regression, 95% CI of slope (0.4198 , 0.6307), $R^2 = 0.6531$, $P < 0.0001$, $F = 99.29$, $d.f. = 53$). The volume of A β 42 puncta outside of Kv7.2 was weakly but significantly correlated with the size of the largest Kv7.2 puncta (Fig. 7d; 95% CI of slope (0.0250 , 0.09245), $R^2 = 0.1876$, $P = 0.0010$, $F = 12.24$, $d.f. = 53$). Finally, we found that the non-overlapped volume as a function of overlapped volume was much larger on average for Kv7.2 than for A β 42 (Supplementary Fig. 8e; paired t -test, $P < 0.0001$, $t = 5.985$, $d.f. = 54$). Taken together, these results show that Kv7.2 and A β 42 puncta are correlated in size only when physically co-localized in a tightly registered fashion, perhaps pointing to new potential hypothesized mechanisms of aggregation.

In myelinated axons, Kv7 potassium channels and voltage-gated sodium (Na_v) channels co-localize tightly with nodes of Ranvier periodically along axons⁴⁰. We investigated whether the distribution of β -amyloid is related to the myelination state of the axons. In 5xFAD mouse fornix, myelin proteolipid protein (PLP) staining was intact and partly overlapped with both SMI and A β 42 clusters (Extended Data Fig. 5a), with the PLP-SMI relationship being similar to that between PLP and SMI in WT animals (Extended Data Fig. 5b). Quantification of both PLP and SMI in A β 42+ axons did not show major changes in either SMI or PLP staining intensity as a function of A β 42 presence (Extended Data Fig. 5c).

While the biological relevance of this periodicity and co-localization of β -amyloid with Kv7.2 and Nav1.6 needs further investigation, it is interesting to note that Nav1.6 and Kv7.2 ion channels can regulate neural excitability^{41–43}, and A β peptides have also been known to influence excitability⁴⁴. As these A β structures were often, although not always, co-localized with SMI-positive axons and are highly reactive for A β 42, we interpret these structures as periodic amyloid depositions. In the future, it will be interesting to see whether these structures play direct roles in neural hyperexcitability in Alzheimer's. It is curious to reflect here that periodicity and order^{45–55} are often thought of as associated with healthy biological systems whose functionality may be supported by such crystallinity. On the other hand, disorder, misalignments and misfoldings are often tied to pathological states. Here we find a curious mixture of the two—a periodicity that seems to be associated with a pathological state and may have implications for new hypotheses related to Alzheimer's pathology. We are excited to see how ExR might reveal many kinds of previously invisible nanopatterns in healthy and disease states due to its ease of use and applicability to multiple contexts, as seen here.

Discussion

The ability to optically study the crowded and complex 3D molecular nanoarchitecture of cells and tissues, such as in brain tissue, is challenging because optical super-resolution methods visualize fluorescent tags (such as antibodies) that are bound to target biomolecules, and these tags may not be able to access all the biomolecules within a nanostructure. Here we present ExR, a new expansion microscopy method that reveals previously invisible biological nanostructures by enabling 20 nm resolution imaging and protein decrowding. ExR leverages the spatial expansion property of expansion microscopy^{2,3} and the 20 nm resolution of iterative expansion⁴ to physically decrowd densely packed biomolecules, providing conventional antibodies with better access to epitopes, revealing more detailed and even previously unseen structures when compared with pre-expansion staining, including in intact tissues.

While many expansion papers have mentioned or implied decrowding^{6,56–59}, previous claims have not been accompanied with systematic data demonstrating specificity of signal-to-noise increase in specific biological structures vs background, minimal distortion between pre- and post-expansion staining, and/or whether decrowding vs heat-mediated antigen retrieval is the dominant contributor to improved staining. Nevertheless, these pioneering efforts are exciting. For example, ultrastructure expansion microscopy (U-ExM)⁵⁸ combined with confocal microscopy showed higher labelling efficiency than in unexpanded direct stochastic optical reconstruction microscopy (dSTORM) images. Indeed, the authors noted inhomogeneous tubulin signal “probably because of epitope masking of antibodies” and in the discussion, stated that the relatively smaller antibody size in post-expansion labelling approaches “unveiled” the chirality of the centriole. As another example, a previously described method that combines post-expansion staining with single-molecule localization microscopy (Ex-SMLM)⁵⁹ also claimed that expansion of the sample increases epitope accessibility and labelling efficiency. As with U-ExM, the authors claim that

post-expansion staining “increases epitope accessibility and thus labelling efficiency”. Here we showed, in comparisons of pre- and post-expansion staining in the same samples of brain tissue, that epitopes were indeed unmasked using ExR. We showed that the decrowding effect is not due to increases in non-specific signal or antigen retrieval effects, and that ExR does not introduce any substantial distortion relative to the non-expansion super-resolution technique DNA-PAINT.

By comparing pre- and post-expansion staining, we found that enhanced post-expansion staining is observed within synaptic structures without a corresponding increase in non-specific background staining, and preserves protein labelling abundance and localization with low distortion (<10 nm). ExR revealed biological structures, such as previously undescribed nanoscale arrangements of calcium channels within trans-synaptic nanocolumns²³ and periodic amyloid nanostructures co-clustering with ion channels in a model of Alzheimer's disease. Pre-synaptic functional diversity is maintained in part by how calcium channels are organized with respect to vesicle-resident sensor proteins that mediate calcium-fusion coupling, yet dense active-zone complexes have been difficult to map accurately in mammalian brain. The clustering of calcium channels in nanocolumns as we have observed here probably represents just one of many unique organizations of these ion channels to be discovered. The discovery of a unique pattern of periodic depositions of ion channels in amyloid aggregates in Alzheimer's disease mouse model brains is another example of the unexpected observations that can be made using this tool. Observing the distribution of ion channels in axons *ex vivo* has been hindered by the crowded nature of axon tracts, and these results have implications for both saltatory conduction *in vivo* and the mechanisms of hyperexcitability in Alzheimer's disease. We thus expect ExR to open up opportunities to observe new, previously unidentifiable biological structures via decrowding, which cannot be obtained by other super-resolution techniques.

Going forward, because ExR is an iterative expansion technique, it may be possible to achieve 100 \times expansion with one more round of re-embedding and gelation to obtain even better resolution and potentially single-molecule localization. Furthermore, ExR could in principle be combined with RNA fluorescence *in situ* hybridization (FISH), as in expansion FISH (ExFISH)⁶⁰, to label both proteins and RNA molecules, enabling visualization of the precise geometry and spatial relationship between RNA and proteins. Finally, because ExR anchors endogenous proteins to the gel, repeated rounds of antibody staining and stripping could in principle be performed, facilitating highly multiplexed imaging of potentially hundreds of proteins in the same field of view. Multiplexed ExR could in principle be used to create high-dimensional protein-protein co-localization maps, potentially revealing complex putative interactions and biological states that cannot be captured with imaging of only a few proteins at once (for example, through conventional three-colour staining and imaging). Thus, we anticipate that ExR will facilitate discoveries in biology and medicine.

Methods

Brain-tissue preparation. All procedures involving animals were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Massachusetts Institute of Technology Committee on Animal Care. Both male and female wild-type mice (C57BL/6 or Thy1-YFP, 6–8 weeks of age, from JAX) and 5xFAD mice (12–13 months of age, from the Mutant Mouse Resource and Research Center) were used because of the current study's focus on developing and validating a technology. Mice were deeply anaesthetized using isoflurane in room air. Mice were transcardially perfused at room temperature with ice-cold 10 ml 2% (w/v) acrylamide in phosphate buffered saline (PBS), followed by ice-cold 10 ml 30% acrylamide (w/v) and 4% paraformaldehyde in PBS. Brains were collected and incubated in 20 ml of the same fixative solution (30% acrylamide and 4% paraformaldehyde in PBS) at 4 °C overnight. Fixed brains were transferred to 100 mM glycine at 4 °C for 6 h, then stored in PBS at 4 °C for long-term storage or sectioned to 50–100- μ m-thick slices with a vibrating microtome (Leica VT1000S).

Cultured-neuron preparation. Cultured mouse hippocampal neurons were prepared from postnatal ~day 0 Swiss Webster mice (Taconic) (both male and female mice were used) as previously described⁵¹. In summary, imaging chambers (112358 CS16-CultureWell removable chambered coverglass, Grace Bio-Lab) were pre-treated with diluted Matrigel, and 5,000–10,000 cells were plated in each well. Neurons were grown at 37°C and 5% CO₂ in a humidified atmosphere for 14 d before fixation. The cells were also briefly washed with 1x PBS warmed to 37°C before fixation. The cultured cells were then fixed with 4% paraformaldehyde (PFA) in 1x PBS for 10 min at room temperature. Immediately thereafter, the fixation solution was replaced with a solution of 0.7% PFA, 1% acrylamide in 1x PBS, and the samples were incubated at 37°C for 6 h. Once incubation was done, samples were washed with 1x PBS. Samples were stored at 4°C overnight. Before expansion, the coverglass from the cell culture well was separated using the coverglass removal tool (103259, Grace Bio-Lab).

Expansion of brain-tissue slices and cultured neurons. For the first gelling step, brain slices or cultured neurons were incubated in the first gelling solution (8.625% (w/v) sodium acrylate, 2.5% (w/v) acrylamide, 0.075% (w/v) N,N'-methylenebisacrylamide (Bis), 0.2% (w/v) ammonium persulfate initiator, 0.2% (w/v) tetramethylethylenediamine (TEMED) accelerator and 0.2% (w/v) 0.01% 4-Hydroxy-TEMPO) for 30 min at 4°C. Slices were embedded in 4-well dish gelling chambers on coverglasses surrounded by excess first gelling solution, and incubated at 37°C for 2 h. Cultured neurons were incubated in the first gelling solution with coverglass on top at 37°C for 2 h. After the incubation, gels containing the tissue or cultured neurons were cut out from the chamber and incubated with denaturation buffer (200 mM SDS, 200 mM NaCl and 50 mM Tris pH 9)⁵ for 1 h at 95°C. Denatured gels were fully expanded via 4 washes for 15 min each with 5 ml distilled (DI) water in a 6-well plate.

For the re-embedding step, expanded first gels were incubated in re-embedding solution (13.75% (w/v) acrylamide, 0.038% (w/v) Bis, 0.025% (w/v) ammonium persulfate, 0.025% (w/v) TEMED) twice, replacing the first solution with freshly made re-embedding solution for 1 h each time on a shaker at room temperature. The re-embedded gels were transferred to a 4-well dish gelling chamber on coverglasses with a coverglass on top. The gelling chambers were placed in a ziplock bag with nitrogen flushing for 5 min and incubated for 2 h at 45°C. The re-embedded gels were washed 3 times for 15 min each with 5 ml PBS in a 6-well plate.

For the third gelling step, the re-embedded gels were incubated in third gelling solution (8.625% (w/v) sodium acrylate, 2.5% (w/v) acrylamide, 0.038% (w/v) Bis, 0.025% ammonium persulfate, 0.025% (w/v) TEMED) twice, replacing the first solution with freshly made third gelling solution for 1 h each time on a shaker at room temperature. The third gels were placed in a 4-well dish gelling chamber on coverglasses with a coverglass on top. The gelling chambers were placed in a ziplock bag with nitrogen flushing for 5 min and incubated at 60°C for 1 h.

Gels were fully expanded in DI water by changing excess water five times for 2 h each and trimming axially to reduce thickness to 1 mm to facilitate subsequent immunostaining and imaging.

Immunostaining of expanded tissues and cultured neurons. Expanded gels were incubated in blocking solution (0.5% Triton X-100, 5% normal donkey serum (NDS) in PBS) for 2 h at room temperature. Gels were then incubated with primary antibodies (see Methods Antibody list) in '0.25T' blocking buffer (0.25% Triton X-100, 5% NDS in PBS) overnight at 4°C. Gels were washed in washing buffer (0.1% Triton X-100 in PBS) six times for 1 h each time on a shaker at room temperature. Gels were then incubated with secondary antibodies in blocking solution overnight at 4°C, and washed in washing buffer six times for 1 h each on a shaker at room temperature. ExR-processed sample images were acquired using a Nikon CSU-W1 confocal microscope with 100% laser power and 1 s exposure time. Z-steps are varied between 0.250 and 0.500 µm.

Super-resolution imaging (DNA-PAINT). *Antibody conjugation.* The antibody (Jackson ImmunoResearch Laboratories, 711-005-152) was conjugated with thiolated DNA following the published protocol⁶². Briefly, 250 µM 5' thiol-modified DNA oligonucleotides were reduced by 100 mM dithiothreitol in 1x PBS + 1 mM EDTA for 1 h at room temperature. Then, excess dithiothreitol was removed using NAP5 columns (GE Healthcare Life Sciences, 17-0853-02). Carrier-free antibody (without BSA or sodium azide) formulated in 1x PBS was concentrated to 2 mg ml⁻¹ using 50 kDa Amicon Ultra filters (EMDMillipore, UFC505096). Then, crosslinkers (PEGylated SMCC crosslinker, ThermoFisher, A35397) were added to an antibody tube at a 5:1 molar ratio (crosslinker:antibody) and reacted for 2 h at 4°C. Next, antibody was purified using 0.5 ml 7 kDa Zeba desalting columns (LifeTechnologies, 89883) to remove excess crosslinker. Reduced thiol-DNA oligonucleotide was then mixed with antibody at a 5:1 molar ratio (DNA:antibody) and reacted overnight at 4°C. Final conjugated antibodies were washed five times with 1x PBS (100 µg ml⁻¹) in Amicon Ultra filters (50k) to remove unreacted DNA oligonucleotides. Conjugated antibody was kept at 4°C until use.

DNA sequences (5' to 3'): /5ThioMC6-D/ATACATCT (ID, Docking; Integrated DNA Technologies); AGATGTAT /Atto-655/ (ID, Imager; Biosynthesis).

Immunostaining. Neuron cultures were blocked and permeabilized in 0.1% Triton X-100, 0.1% Tween20, 3% IgG-free BSA and 5% NDS for 2 h at room temperature. Cell cultures were incubated with primary antibodies (diluted in 0.1% Triton X-100, 0.1% Tween20, 3% IgG-free BSA) overnight at 4°C, and washed with 0.1% Tween20 and 1% IgG-free BSA five times (10 min each). Secondary antibodies were diluted in 0.1% Triton X-100, 0.1% Tween20 and 3% IgG-free BSA, incubated with samples for 2 h at room temperature and then washed as for primary antibodies. A 1:100 dilution of 100 nm gold nanoparticles (753688-25ML, Sigma-Aldrich) was then added to wells and gently spun down in a centrifuge (500g, 5 min). Finally, the cells were briefly washed three times with 1x PBS + 500 mM NaCl.

DNA-PAINT. Super-resolution imaging was accomplished on a Nikon Eclipse Ti microscope that was (1) operated by Nikon Elements software, (2) used a 1.49 NA CFI Apo ×100 objective, (3) had a perfect focus system and (4) had a total internal reflection fluorescence (TIRF) laser (488 nm, 561 nm, 647 nm). Lasers were operated in TIRF mode for all acquisitions. For image acquisition, an electron multiplying (EM) charge coupled device (iXon X3 DU-897, Andor Technologies) was used. Cameras were operated at 5 MHz refresh rate with 100 EM gain and a 50 ms exposure time. A ×1.5 lens was introduced into the optical path allowed for imaging, with a pixel size of 110 nm. Images were acquired using RAM capture via Nikon Fast Timelapse acquisition. Frames (15,000–20,000) of single-molecule image stacks were then acquired and analysed in Picasso. To correct for image drift, 100 nm gold nanoparticles were used as fiducial markers in the imaging process. DNA imager stock solution was diluted and used in 1x PBS + 500 mM NaCl at a working concentration of 2 nM.

Decrowding experiments. *Nanoscale-resolution imaging of synapses in somatosensory cortex.* For pre-expansion antibody staining (Fig. 3b–h), brain slices were incubated with primary antibodies in blocking solution overnight at 4°C. Stained tissues were washed in washing buffer six times for 1 h each time on a shaker at room temperature. Secondary antibodies (100 µl, 1 mg ml⁻¹) were incubated with 6-((acryloyl)amino)hexanoic acid and succinimidyl ester (AcX) (2 µl, 1 mg ml⁻¹) overnight at room temperature to prepare AcX-conjugated secondary antibodies. Primary antibody-stained tissues were then incubated with AcX-secondary antibodies in blocking solution overnight at 4°C and washed in washing buffer six times for 1 h each time on a shaker at room temperature. Tissue expansion was carried out as previously described. The tertiary antibodies were stained after expansion to bind against AcX-secondary antibodies to visualize the pre-expansion staining.

For post-expansion antibody staining, expanded gels were incubated with the same primary and secondary antibodies without AcX conjugation. Antibodies against Shank3 or Homer1 were provided as a reference channel after expansion.

Quantification and validation of the decrowding effect. *Comparison of ExR and DNA-PAINT synapsin staining in cultured neurons.* Two-dimensional DNA-PAINT images (Fig. 2h,i) were rendered using Picasso in Python⁶³ using a blur width of 0.0, oversampling of 4.74–5.42 (corresponding to a pixel resolution of 20.31–21.10 nm per /pixel), and maximum density of 350. The oversampling parameter was calculated on a per-well basis to match the pixel resolution given by the measured expansion factor of 7.7–8.0X for ExR images. The expansion factor for each well was calculated by measuring the distances between identical pairs of synapsin puncta in pre- and post-expansion images and dividing the physical units in the post-expansion image by the physical units in the pre-expansion image. The mean value over five such distances was calculated: 7.9X for well 1, 7.7X for well 2 and 8.0X for well 3. Post-expansion ExR stacks were background subtracted using Fiji's Rolling Ball algorithm with radius 50 pixels and collapsed to two dimensions using a maximum intensity projection. DNA-PAINT and ExR images were registered using custom MATLAB scripts employing automatic intensity-based rigid body registration. Images were first minimum-maximum normalized, passed through a 2D Gaussian filter (sigma = 5) and binarized using Otsu's thresholding method (MATLAB's 'graythresh'). The geometric transform was estimated using MATLAB's configurations for multimodal intensity-based registration, with the following optimizer parameters: initial radius, 0.004; epsilon, 1.5 × 10⁻⁴; growth factor, 1.01; maximum iterations, 300. An initial rigid body transformation was estimated before calculation of the final transform using the same parameters.

To assess the accuracy/resolution and distortion of ExR compared to DNA-PAINT, we calculated linear distortion of nanoscale synaptic puncta, the difference in pairwise distances between neighbouring synaptic puncta and the differences in mean synaptic puncta number in identical cultured-neuron samples imaged using the two technologies. To analyse nanoscale error introduced by ExR relative to DNA-PAINT, ROIs containing single synaptic puncta were manually segmented from DNA-PAINT and corresponding ExR images taken from the same fields of view in the same specimens. Pixel intensity was minimum-maximum normalized separately for ExR and DNA-PAINT channels. Manually cropped individual synaptic ROIs from ExR images were automatically registered to their DNA-PAINT counterparts (note: after the global registration) using a rigid body transformation calculated using custom MATLAB scripts as previously described, with MATLAB's default configurations for monomodal intensity-based registration and without initial transformation estimation. This automatic registration failed

for 6 out of 107 ROIs, which were then excluded from subsequent analyses. We calculated pixel-wise autocorrelation and cross-correlation as a function of distance as follows. Images were shifted by one voxel in each direction and padded using the intensity values of the pixels that were shifted out at that step. The pairwise linear correlation coefficient between pixel intensity values in DNA-PAINT and ExR staining channels, or PAINT-PAINT or ExR-ExR staining channels for autocorrelation, was calculated using MATLAB's 'corr' function. To calculate the half-maximal shift distance, we fit a third-degree polynomial (MATLAB's 'fit' with 'poly3') to the correlation or autocorrelation function, and used the best-fit curve to estimate the shift distance at which the correlation or autocorrelation reached 50% of its maximum value. Each of these calculations was repeated for each shift in the x - and y -dimensions. The mean differences in half-maximal shifts over x - and y -dimensions are plotted in Fig. 2h,i.

The total number of synaptic puncta for each image was calculated using custom MATLAB scripts (Extended Data Fig. 1j). Images were first passed through a 2D median filter of size (10, 10), binarized using Otsu's method and filtered to exclude puncta smaller than 150 nm in size. Synaptic puncta number was calculated from 2D connected components in the binary image with connectivity 26. Corresponding pairs of neighbouring synaptic puncta (within $\sim 1 \mu\text{m}$ from one another) and single synaptic puncta were manually cropped from DNA-PAINT and ExR images and binarized as described above ($n = 50$ ROIs from 5 fields of view, 6 ROIs each, 1 culture batch). Puncta centroids were calculated from 2D connected components in the binary image (connectivity, 26) using MATLAB's 'regionprops' function. For cropped ROIs in which two synaptic puncta were counted after thresholding for both ExR and DNA-PAINT (Extended Data Fig. 1g-i; 27/50), the radial distance between puncta centroids (x_1, y_1) and (x_2, y_2) was calculated as $\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$. In Fig. 2j, the absolute value of the difference in distances between neighbouring synaptic puncta was normalized to the DNA-PAINT image. The coefficient of variation for distance between synaptic puncta imaged using ExR was calculated as the standard deviation of the difference in distance between pairs of synaptic puncta (PAINT - ExR) divided by the mean distance of the same pairs from DNA-PAINT.

Root mean squared error (RMS) between ExR and DNA-PAINT images was calculated as previously described. Briefly, a custom MATLAB script was used to implement a B-spline-based non-rigid registration between pre- and post-expansion images, yielding vector fields for deformation within the images. These vector fields were then used to calculate root-mean-square length distortions across varying lengths. For plotting, RMS error values were binned into measurement length increments of $1 \mu\text{m}$ and the mean RMS error within each bin is shown.

Decrowding analysis of manually segmented synapses. We compared the amplitude of signal intensity in the foreground (putative synapses) and background (everything else). First, we manually identified, on the basis of brightness and size of reference channel staining, 47–70 of the largest, brightest synapses per $\sim 350 \times 350 \times 20 \mu\text{m}$ (physical units) field of view (see Supplementary Table 2 for exact numbers of synapses; one field of view per cortical layer, three cortical layers per sample, two mice per synaptic protein). We developed an automated method to segment putative synaptic puncta from background. First, background was subtracted from image stacks using ImageJ/Fiji's Rolling Ball algorithm with a radius of 50 pixels. Images were then binarized using a threshold calculated as seven times the standard deviation of the average intensity of manually identified background regions selected every tenth slice of the z -stack. Binary images were passed through a 3D median filter of radius $5 \times 5 \times 3$ pixels to remove small puncta of non-specific staining. We then identified 3D connected components from the filtered binary stack using MATLAB's 'bwconncomp' function, with a pixel connectivity of 26, meaning that pixels are connected if their faces, edges or corners touch. Connected components smaller than $100 \times 100 \times 100 \text{ nm}^3$ (biological units) were removed, as most synapses are larger than this volume. ROIs, or putative synaptic puncta, were defined as 3D connected components of the filtered binary reference stack dilated using a disk structuring element with a radius of six pixels. A radius of six pixels ($\sim 100 \text{ nm}$, in biological units) was chosen because both pre- and post-synaptic proteins of the same synapse, but not other synapses, fall within this range (the synaptic cleft is $\sim 12\text{--}20 \text{ nm}^3$), which we confirmed by manual inspection. Segmented synapses with zero filtered connected components (synaptic puncta) in the reference channel were excluded from further analysis. We calculated the average intensity of background-subtracted images either within or outside of these dilated reference ROIs (Extended Data Fig. 2a) to measure signal increase within putative synapses relative to signal increase in the background. All images were acquired under the same microscope conditions to allow for comparison of mean signal intensity. The total volume of pre- or post-expansion staining test puncta located within dilated reference channel ROIs (Extended Data Fig. 2b) was calculated from binarized stacks of pre- and post-expansion channels (thresholded and filtered as described for the reference channel) after multiplying the dilated binary reference stack (for inside dilated reference ROIs) or its inverse (for just outside dilated reference ROIs, but still within the manually cropped synaptic area) by the binary pre- or post-expansion stack and calculating the sum of non-zero voxels for each product. Data are shown as the mean of each measure across the ~ 50 synapses per field of view, and the deviation was calculated as the

standard error of these means across the nine fields of view (three fields of view for three animals) for each protein. We identified measured signal from one field of view from one animal as an outlier using the ROUT method⁴⁶ with $Q = 1\%$ in GraphPad Prism and excluded it from further analysis and visualization.

Quantification of synaptic properties. To compare volume and SNR of pre- and post-expansion staining (Extended Data Fig. 2c,d), we used the same dataset as for Extended Data Fig. 2a,b analysis. First, the background was subtracted from image stacks using ImageJ/Fiji's Rolling Ball algorithm with a radius of 50 pixels. Images were then binarized using a threshold calculated as seven times the standard deviation of the average intensity of manually identified background regions selected every tenth slice of the z -stack. We then identified and selected the biggest 3D connected components in pre- and post-staining test channels separately in each layer of somatosensory cortex (L1, L2/3 and L4), as these are most probably to be synapses. We calculated the voxel and signal intensity in the largest 3D connected components from 49–70 manually selected synapses (see Supplementary Table 2 for exact numbers for each layer, protein and mouse). The signal intensity was divided by the standard deviation of the background intensity to calculate the SNR.

Analysis of distortion introduced by ExR relative to pre-expansion staining. To calculate the number of synaptic puncta, background-subtracted images were first thresholded as described previously (on the basis of a multiple of the standard deviation of manually identified background regions) and passed through a $3 \times 3 \times 5$ voxel (1 voxel = $17.16 \times 17.16 \times 40 \text{ nm}^3$) median filter. MATLAB's 'bwconncomp' function was used to find connected components (putative synaptic puncta, connectivity of 26) and connected components with fewer than 30 voxels of volume were excluded from further analysis. Distortion between pre- and post-expansion images was calculated as was done for distortion between DNA-PAINT and ExR images. Specifically, for the plots shown in Extended Data Fig. 3g–l, images were shifted by one voxel in each direction and padded using the intensity values of the pixels that were shifted out at that step. To calculate pixel-wise correlations and autocorrelations (Extended Data Fig. 3g–h), images were first normalized to their minimum and maximum intensity values. From these, we calculated the pairwise linear correlation coefficient (MATLAB's 'corr') between pixel intensity values in the pre- and post-expansion staining channels, and the pre-/post-expansion staining channels for autocorrelation. To calculate the half-maximal shift distance (Extended Data Fig. 2f,g), we fit a third-degree polynomial (MATLAB's 'fit' with 'poly3') to the correlation or autocorrelation as a function of shift distance and used the best-fit curve to estimate the shift distance at which the correlation or autocorrelation reached 50% of its maximum value. For the plots shown in Extended Data Fig. 3i,j, the correlation was calculated with a slight modification to account for differences in puncta volume. First, background-subtracted images were masked on the basis of the corresponding binary image. Second, non-zero pixels were divided by the mean intensity value in the non-zero regions. Finally, the correlation was calculated as the pairwise linear correlation coefficient (MATLAB's 'corr') between masked mean-normalized intensity values in the pre- and post-expansion staining channels. Mutually overlapped volume was calculated as the sum of non-zero pixels in the intersection of the binary pre- and post-expansion staining z -stacks, and normalized to the total puncta volume (sum of non-zero pixels in the binary z -stack) in the pre-expansion staining channel (Extended Data Fig. 3k,l). Each of these calculations was repeated for each shift in the x -, y - and z -directions. Synapses with zero puncta in the pre- or post-expansion staining channels were excluded from analysis.

Comparison between antigen retrieval and decrowding effect. Confocal images were obtained after immunostaining with antibodies against Ca_v2.1, PSD95 and Homer1 (Supplementary Fig. 2) and 6E10, 12F4 and SMI (Supplementary Fig. 6) with or without antigen retrieval treatment to compare signal quality for antigen retrieval vs ExR treatment. To determine whether antigen retrieval by heat denaturation alone is the dominant factor underlying increased signal quality afforded by ExR, we treated one group of tissues with a standard antigen-retrieval step (placing tissues in 20 mM sodium citrate at pH 8 and incubating at 100 °C for 30 s and 60 °C for 30 min)⁴¹. Tissues with or without this antigen-retrieval step were processed by ExR. Then, we compared the amplitude of signal intensity in foreground (putative synapses) and background (everything else). First, we manually identified, on the basis of brightness and size of reference channel staining (Homer1 for Ca_v2.1, Shank3 for PSD95 and Homer1), 30 of the largest and brightest synapses per $\sim 350 \times 350 \times 20 \mu\text{m}$ (physical units) field of view ($n = 30$ synapses from 1 field of view from 1 mouse). We used the automated segmentation procedure and calculated mean signal intensity and volume as described above (see 'Decrowding analysis of manually segmented synapses').

Protein distance measurement and synaptic nanocolumn results analysis. For analysis, potential synapses were manually identified and selected on the basis of (1) the juxtaposition of pre-synaptic clusters and post-synaptic clusters and (2) the co-localization of clusters on the same side of the synapse (Figs. 2e and 4e–g,i–p). As camera pixel size was 167 nm (physical units) and the step size of the z -stack was 250 nm (physical units), the voxel size was not equivalent in all dimensions.

Because isometric voxels were necessary for subsequent analysis, each voxel was then subdivided into 12 smaller isometric voxels, each 83.3 nm (physical units) in all three dimensions. For comparisons of RIM1/2 and PSD95, one cluster was shifted in space to optimally overlap with the other cluster, as previously described^{13,64}. The vector of this shift was determined by cross-correlation of the two clusters and defined both the trans-synaptic axis and the distance between the two clusters. For comparisons of RIM1/2 and Ca_v2.1, the shift distance was set as 0, and for comparisons of RIM1/2 and PSD95, putative synapses with a RIM1/2 to PSD95 peak-to-peak distance of less than 20 or greater than 180 nm (biological units) were rejected from further analysis, consistent with the dimensions of the active zone and PSD. Any synapses that extended beyond the z-range of the imaged stack were also excluded.

Autocorrelation ($g_s(r)$) and protein enrichment analyses were adapted from previously described localization data-based analyses^{13,66}. The 3D autocorrelation function ($g_s(r)$) reports the increased probability of finding a similar signal at a distance (r) away from a given signal, and thus can be used to quantify the heterogeneity of the measured signal within a defined volume. The autocorrelation of each synaptic cluster was normalized by the autocorrelation of an object with the same shape and volume and that has a homogenous voxel intensity set to the average intensity of the synaptic cluster. Therefore, a synaptic cluster with a homogenous intensity will give a $g_s(r) = 1$ at all radii, and local intensity peaks within a synaptic cluster will result in a $g_s(r) > 1$ over a radius of the size of the region of high intensity. The cluster boundary was defined on the basis of fluorescent intensity after convolution with a spherical kernel ($r \approx 300$ nm).

The molecular distribution of two different protein clusters relative to one another was characterized using a cross-enrichment analysis. The cross-enrichment analysis was performed by measuring the angularly averaged voxel intensity of one protein cluster (measured cluster) as a function of the distance from the point of peak intensity in the other protein cluster (reference cluster, shifted in space as described above). This value was then normalized by the angularly averaged intensity (as a function of the distance from the point of peak intensity in the reference cluster) of an object of the same shape and volume as the measured cluster, with voxels set to the average intensity of the measured cluster. Regions of high local intensity in the measured cluster will result in values (normalized intensity) > 1 . The enrichment index was calculated by taking the average of the enrichment values within a radius of 60 nm from the peak of the reference cluster.

Synapse numbers (n) for the analysis from 2 mice:

Autocorrelations

Ca_v2.1 (Fig. 4e): $n = 144$ synapses (Layer 1), 101 synapses (Layer 23), 103 synapses (Layer 4)

PSD95 (Fig. 4f): $n = 144$ synapses (Layer 1), 101 synapses (Layer 23), 103 synapses (Layer 4)

RIM1/2 (Fig. 4g): $n = 144$ synapses (Layer 1), 101 synapses (Layer 23), 103 synapses (Layer 4)

Enrichment analysis

RIM1/2 enrichment to PSD95 peak (Fig. 4i,j): $n = 153$ synapses (Layer 1), 103 synapses (Layer 23), 108 synapses (Layer 4)

PSD95 enrichment to RIM1/2 peak (Fig. 4k,l): $n = 152$ synapses (Layer 1), 102 synapses (Layer 23), 108 synapses (Layer 4)

Ca_v2.1 enrichment to RIM1/2 peak (Fig. 4m,n): $n = 150$ synapses (Layer 1), 103 synapses (Layer 23), 107 synapses (Layer 4)

RIM1/2 enrichment to Ca_v2.1 peak (Fig. 4o,p): $n = 153$ synapses (Layer 1), 99 synapses (Layer 23), 108 synapses (Layer 4)

Enrichment index values (mean \pm s.d.):

RIM1/2 to PSD95 peak (Fig. 4j): 1.585 ± 0.330 (Layer 1), 1.535 ± 0.358 (Layer 23), 1.545 ± 0.332 (Layer 4)

PSD95 to RIM1/2 peak (Fig. 4l): 1.611 ± 0.308 (Layer 1), 1.632 ± 0.269 (Layer 23), 1.622 ± 0.285 (Layer 4)

Ca_v2.1 to RIM1/2 peak (Fig. 4n): 1.510 ± 0.364 (Layer 1), 1.359 ± 0.330 (Layer 23), 1.452 ± 0.314 (Layer 4)

RIM1/2 to Ca_v2.1 peak (Fig. 4p): 1.493 ± 0.330 (Layer 1), 1.317 ± 0.311 (Layer 23), 1.422 ± 0.322 (Layer 4)

Analysis of the Alzheimer's results. Comparison of A β 42 volume in WT vs 5xFAD. For Supplementary Fig. 5b, 3D image stacks of A β 42 and SMI312 staining were background subtracted via rolling-ball background subtraction with a 200 pixel radius using ImageJ/Fiji. For each colour channel, the standard deviation for the background was calculated using a 75×75 pixel window. Subsequently, each colour channel was binarized by applying a threshold of 28 times the standard deviation of the background. This value was determined by evaluating the amount of thresholding required to remove putative non-specific staining spots. Finally, after binarization, the volume of A β 42 and SMI312 for each field of view was determined by adding up the segmented pixels of each colour channel.

Distance measurement between clusters. To calculate the distance between adjacent clusters for either A β 42 or K ν 7.2 (Fig. 6d), clusters that line along SMI312 neurofilaments were manually cropped out in 3D. Then, after applying rolling-ball background subtraction with a 100 pixel radius, the centroid of each cluster was

annotated manually using ImageJ/Fiji in 3D. Given that the spacing between clusters is much larger than the size of each cluster, we reasoned that manual labelling of the centroids incurs minimal error. Finally, the distance between adjacent clusters was calculated in 3D.

Calculation of A β 42 and K ν 7.2 cluster diameter. After applying rolling-ball background subtraction with a 100 pixel radius to 3D fields of view of A β 42 and K ν 7.2 staining, overlapping K ν 7.2 and A β 42 clusters were manually cropped out. After calculating the standard deviation of the background of each channel, the cropped images were binarized by applying a threshold ten times the standard deviation of the background. The volume of each cluster was then identified via connected component analysis using MATLAB's 'bwconncomp' function. Finally, the centroid and principal axis length of each cluster were determined using the associated 'regionprops' function, which models each connected component region as an ellipsoid. The centroid values were then used to calculate the distance between overlapped A β 42 and K ν 7.2 clusters (Fig. 6g–i).

A β 42 and K ν 7.2 cluster shape analysis. ROIs containing single A β 42 puncta that were part of a periodic chain-like structure were manually identified ($n = 55$ ROIs, 5 ROIs per field of view, from 11 fields of view from 2 mice) from background-subtracted images (ImageJ/Fiji's Rolling Ball algorithm, radius of 50 pixels). To visualize the 3D shape of A β 42 and K ν 7.2 puncta within these ROIs, we resliced the image stack along both transverse dimensions at equal spacing to the axial dimension. We display the middle slice in each stack in the x - y plane in Fig. 7a(ii)–d(ii) and the middle slice in each stack in the x - y , y - z and x - z planes in Supplementary Fig. 8a (where x - and y -directions are transverse, and z -direction is axial). To quantify shape features, we used CellProfiler's⁶⁷ Watershed⁶⁸ segmentation module to segment puncta within manually extracted ROIs using a footprint of 30 pixels for each channel. A custom MATLAB script was deployed to calculate the number of puncta in each channel, mean and maximum volume and surface area of these puncta, length of the three principal axes of the ellipsoid that have the same normalized second central moments as the region for the largest puncta, and the total volume of puncta overlap between A β 42 and K ν 7.2 as the number of non-zero pixels in the intersection of the binary image stacks. To quantify the statistical significance of the relationships between these measures, either two-tailed paired t -tests or simple linear regression were used as described in the text.

Expansion factor and measurement of the root mean square error. A Thy1-YFP mouse was perfused as described above and 50 μ m coronal sections were prepared using a vibratome. Before expansion, yellow fluorescent protein (YFP) fluorescence was imaged in six fields of view from the cortex of three cortical slices. Subsequently, these slices were processed with the ExR protocol as described above. Expanded slices were then labelled with a primary antibody against green fluorescent protein (GFP; ThermoFisher, A-11122) and a secondary antibody (see Supplementary Table 10 for list of antibodies). The same fields of view imaged pre-expansion were identified and confocal images of the antibody staining were acquired. Pre- and post-ExR images were acquired on an Andor spinning disk (CSU-X1 Yokogawa) confocal microscope with a 40×1.15 numerical aperture water objective.

To determine distortion arising from the process of ExR, pre- and post-expansion images were aligned and deformations in images were determined as described previously. Briefly, pre- and post-ExR images were background subtracted with a Rolling Ball background-subtraction algorithm (ImageJ/Fiji) with a 200 pixel radius. Then, corresponding confocal planes from pre and post images were identified and registered using Fiji's Turboreg method, allowing for scaling and rigid rotation. Then, a custom MATLAB script was used to implement a B-spline-based non-rigid registration between pre- and post-expansion images, yielding vector fields for deformation within the images. These vector fields were then used to calculate root-mean-square length distortions across varying lengths.

To calculate the expansion factor, the physical distances between feature-containing YFP structures (for example, dendrites and axons) in fluorescent protein-containing specimens were measured in pre- and post-ExR images. In cases of 5x-FAD or C57BL/6 mice, DAPI staining of neighbouring cells was used to measure corresponding distances in pre- and post-ExR images, and the expansion factor was calculated by dividing the distances.

Tables listing the chemicals and antibodies used are available as Supplementary Information.

ExR protocol.

1. Mouse tissue slices

- Anaesthetize mice using isoflurane in oxygen and perfuse with 10 ml 2% acrylamide in PBS followed by 10 ml 30% acrylamide and 4% paraformaldehyde in PBS.
- Collect brains and incubate in 20 ml of the same fixative solution (30% acrylamide and 4% formaldehyde in PBS) at 4°C overnight.
- Transfer fixed brains to 100 mM glycine at 4°C for 6 h.
- Store tissues in PBS at 4°C for long-term storage.
- Slice tissues on a vibrating microtome to a thickness of 50–100 μ m.

2. Gelation

A. Gelling for first expansion

- i. Incubate brain slices in the first gelling solution for 30 min at 4°C.
- ii. Place brain slices with excess first gelling solution between two no. 1.5 coverglasses separated by two pieces of no. 1.5 coverglasses, and then incubate at 37°C for 2 h.
- iii. Cut out gels from the chamber and incubate with denaturation buffer (200 mM SDS, 200 mM NaCl and 50 mM Tris pH 9) for 1 h at 95°C.
- iv. Wash gels four times with DI water in shaker and expand gels in DI water at 4°C overnight.

B. Re-embedding

- i. Incubate expanded first gels in re-embedding solution twice for 1 h each time in shaker at room temperature.
- ii. Transfer gels between no. 1.5 coverglasses separated by slide glass and incubate with excess re-embedding solution at 45°C for 2 h.
- iii. Wash gels three times with PBS in shaker.

C. Third gelling

- i. Incubate the re-embedded gels in the third gelling solution twice for 1 h each time in shaker at room temperature.
- ii. Transfer gels between no. 1.5 coverglasses separated by slide glass and incubate at 60°C for 1 h.
- iii. Wash gels four times with DI water in shaker and expand gels in DI water at 4°C overnight.
- iv. Trim gels axially to 1 mm thickness.

3. Staining

- i. Incubate gels in blocking solution (0.5% Triton X-100, 5% NDS in PBS) for 2 h at room temperature.
- ii. Incubate gels with primary antibodies in '0.25T' blocking buffer (0.25% Triton X-100, 5% NDS in PBS) overnight at 4°C.
- iii. Wash gels with washing buffer (0.1% Triton X-100 in PBS) six times for 1 h each time.
- iv. Incubate gels with secondary antibodies in blocking solution at 4°C overnight.
- v. Wash gels with washing buffer (0.1% Triton X-100 in PBS) six times for 1 h each time and expand gels in DI water for 20X expansion or 0.05x PBS for 15X expansion.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw and analysed datasets generated during the study are too large to be publicly shared, yet they are available for research purposes from the corresponding authors on reasonable request. Source data are provided with this paper.

Code availability

The custom code used in this study is available on Zenodo at <https://doi.org/10.5281/zenodo.6383293> and on GitHub at <https://github.com/schroeme/ExR>.

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Author contributions

D.S. initiated work, developed the ExR technology, contributed key ideas, designed and performed experiments and interpreted data for all projects, and wrote and edited the manuscript. J.K. contributed key ideas, designed and performed experiments for all projects, performed analysis for decrowding, and wrote and edited the manuscript. A.T.W. co-developed approaches to measuring decrowding, contributed key ideas to ExR technology development and decrowding analysis, designed and performed experiments, analysed data for all projects, and wrote and edited the manuscript. M.E.S. contributed key ideas, designed and implemented analysis, visualization and statistical tests for all projects, created the schematic in Fig. 1 with input from others, and wrote and edited the manuscript. Z.P. contributed key ideas, designed and performed experiments, and conducted analysis for the Alzheimer's project. T.B.T. contributed key ideas, designed experiments, created software, analysed and interpreted data related to synapses, and helped in writing and editing the manuscript. A.-H.T. created software and analysed data related to synapses. E.D.N. designed experiments, interpreted experimental data for the Alzheimer's project, and helped in writing and editing the manuscript. J.Z.Y. oversaw the Alzheimer's project and designed experiments for it. H.S. performed DNA-PAINT validation experiments. D.P. prepared cultured neurons. P.Y. contributed key ideas and designed experiments for DNA-PAINT validation. L.-H.T. conceptualized and initiated the Alzheimer's project, provided oversight and funding, contributed key ideas and designed experiments, and helped in writing and editing the manuscript. T.A.B. contributed key ideas, designed experiments and interpreted data for the synapse project, and helped in writing and editing the manuscript. E.S.B. supervised the project, initiated work, contributed key ideas, designed experiments, helped with data analysis and interpretation, and wrote and edited the manuscript.

Competing interests

D.S., A.T.W., J.K. and E.S.B. are co-inventors on a patent application for ExR (US 2020/0271556 A1). E.S.B. is co-founder of a company seeking to deploy applications of ExM-related technologies. The other authors declare no competing interests.

Additional information

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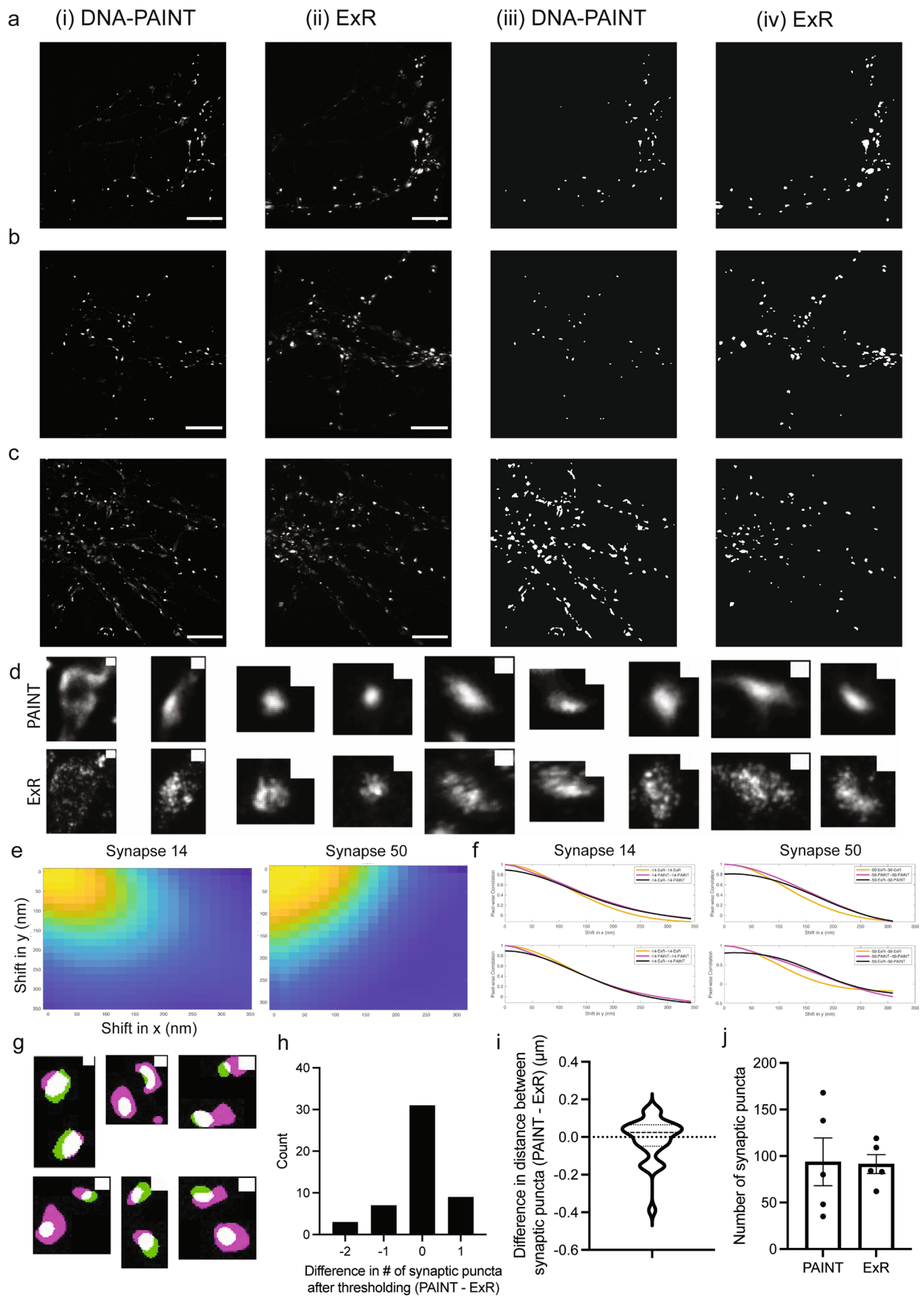
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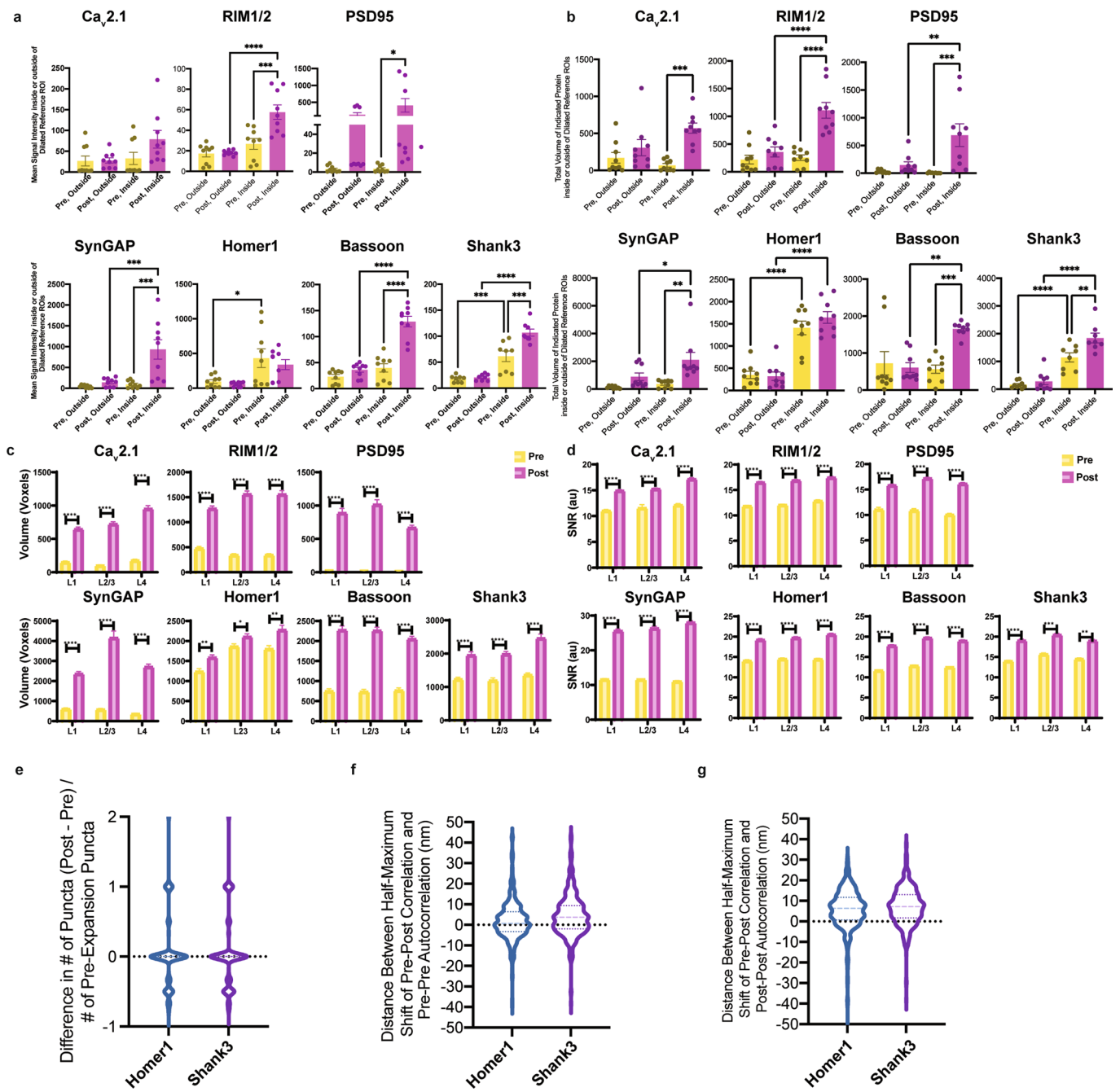
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Extended Data Fig. 1 | See next page for caption.

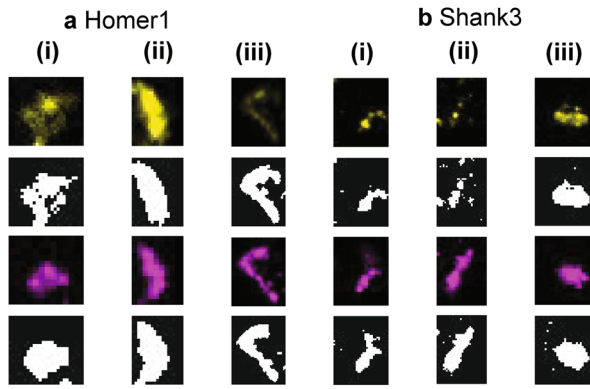
Extended Data Fig. 1 | Comparison of synaptic nanostructures imaged using DNA-PAINT and ExR in cultured neurons. (a-c) Three representative fields of view imaged using DNA-PAINT (i) and ExR (ii) after rigid body registration to DNA-PAINT images. Scale bar = 5 μm . (iii-iv) Processed, binary versions of (i) and (ii) used to automatically count synaptic puncta number and pairwise distances. Scale bar, 5 μm . **(d)** Representative manually-cropped matched synaptic ROIs for DNA-PAINT (top row) and ExR (bottom row), used for the distortion analysis shown in Fig. 2h,i (scale bar = 250 nm). **(e)** Pixel-wise correlation between min-max normalized ExR and DNA-PAINT channels as a function of shift distance in x- and y-directions for two randomly selected synaptic ROIs. **(f)** Pixel-wise autocorrelation between min-max normalized DNA-PAINT (PAINT-PAINT, magenta), ExR (ExR-ExR, yellow), and pixel-wise correlation between DNA-PAINT and ExR (PAINT-ExR, black) as a function of shift distance in x- and y-directions for the synaptic ROIs shown in **(e)**. **(g)** Representative manually-cropped pairs of synaptic puncta used to generate the data shown in Fig. 2j and panel i. Shown is an overlay of DNA-PAINT (green) and ExR (magenta) binary masks (scale bar = 250 nm). **(h)** Histogram of difference in number of synaptic puncta counted after thresholding pairs of synaptic puncta. **(i)** Difference in radial distance between pairs of synaptic puncta, DNA-PAINT - ExR (mean = -0.008854, 95% CI [-0.05419, 0.03649]). **(j)** Total number of synaptic puncta for the five fields of view imaged using DNA-PAINT and ExR (two-sided paired t-test, $p = 0.9271$, $t = 0.09735$, $df = 4$). All data are from 5 ROIs from 3 wells of one cultured neuron batch. Shown are images from one representative experiment from two independent replicates.



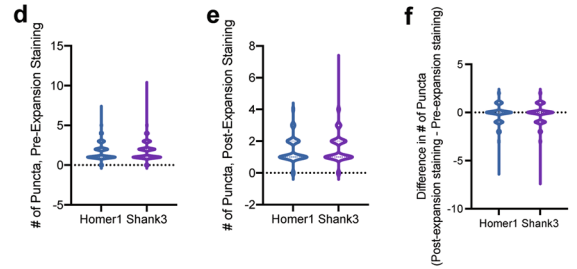
Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Analysis of the ExR decrowding effect. (a, b) Quantification of decrowding in a set of manually identified synapses. Statistical significance was determined using Sidak's multiple comparisons test on two-sided t-tests following ANOVA (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P < 0.001$, **** $P < 0.0001$, here and throughout the paper, and plotted is the mean, with error bars representing standard error of the mean (SEM), here and throughout the paper). **(a)** Mean signal intensity inside and outside of (that is, nearby to) dilated reference ROIs for pre- and post-expansion stained manually-identified synapses (Supplementary Table 2 for numbers of technical and biological replicates)). Data points represent the mean across all synapses from a single field of view. **(b)** Total volume (in voxels; 1 voxel = $17.16 \times 17.15 \times 40$, or $11,779$, nm^3) of signals inside and outside of dilated reference ROIs, in both cases within cropped images containing one visually identified synapse (Supplementary Fig. 3h), for pre- and post-expansion stained manually-identified synapses (Supplementary Table 2 for numbers of biological and technical replicates). Data points represent the mean across all synapses for 3 fields of view from 3 biological replicates ($n = 9$ fields of view total per protein). **(c)** Mean voxel size and **(d)** mean signal-to-noise (SNR) ratio of pre- and post-expansion immunostaining showing 7 proteins in somatosensory cortex regions L1, L2/3, and L4 of 3 mice. Plotted is mean and SEM. To compare the 3D voxel size and SNR of pre- and post-expansion stained synapses for each of the seven proteins, three two-sided t-tests (one for each layer) were run ($n = 49$ – 70 puncta per layer from 3 mice; Supplementary Table 2 for exact n values). Statistical significance was determined using multiple t-tests corrected using the Holm-Sidak method, with $\alpha = 0.05$. **(e)** Population distribution (violin plot of density, with a dashed line at the median and dotted lines at the quartiles) of the fractional difference in the number of synaptic puncta between post- and pre-expansion staining channels for Homer1 and Shank3 ($n = 480$ synapses from 3 mice). **(f)** Population distribution of the difference in distance (in nm) between the shift at which the correlation is half maximal half-maximal shift for pre-pre autocorrelation and post-pre correlation (calculated pixel-wise between intensity values normalized to the minimum and maximum of the image, see Methods) for x-, y-, and z-directions (x- and y-directions being transverse, z-direction being axial) for Homer1 and Shank3 ($n = 458$ synapses, 3 directions each, from 3 mice). **(g)** Same as **(f)**, for post-post autocorrelation and pre-post correlation.

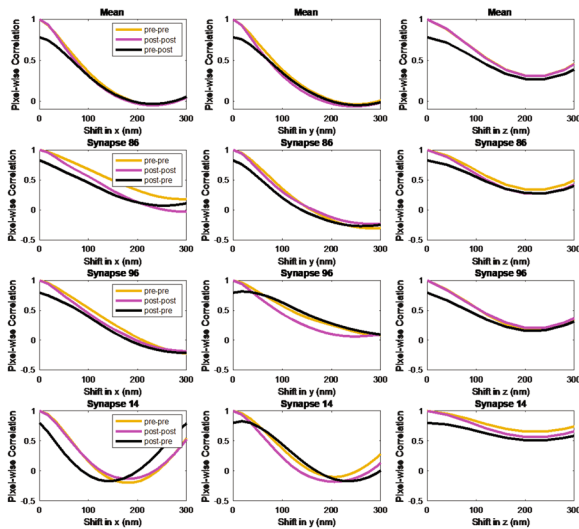
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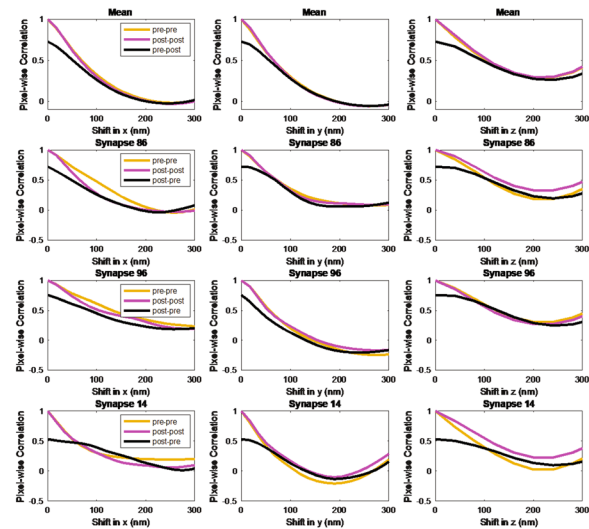
	Homer1			Shank3		
	a(i)	a(ii)	a(iii)	b(i)	b(ii)	b(iii)
# pre	1	1	2	3	1	1
# post	1	1	1	2	1	1



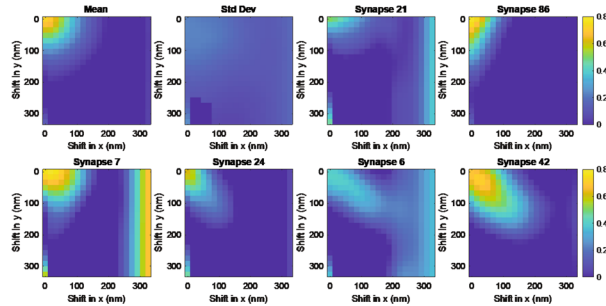
g Homer1 : Pixel-wise Correlation and Autocorrelation



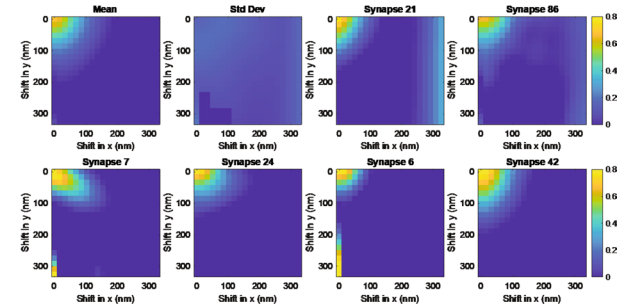
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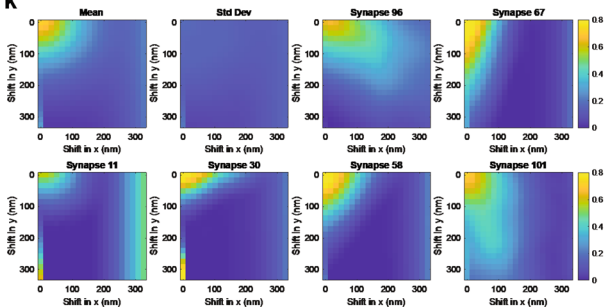
i Homer1 : Pixel-wise Correlation between Mean-Normalized, Masked Pre- and Post-expansion Channels



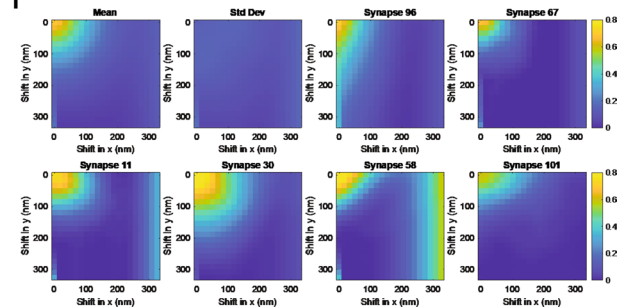
j Shank3 : Pixel-wise Correlation between Mean-Normalized, Masked Pre- and Post-expansion Channels



k Homer1 : Pre/Post Mutually Overlapped Volume Normalized to Pre-Expansion Volume



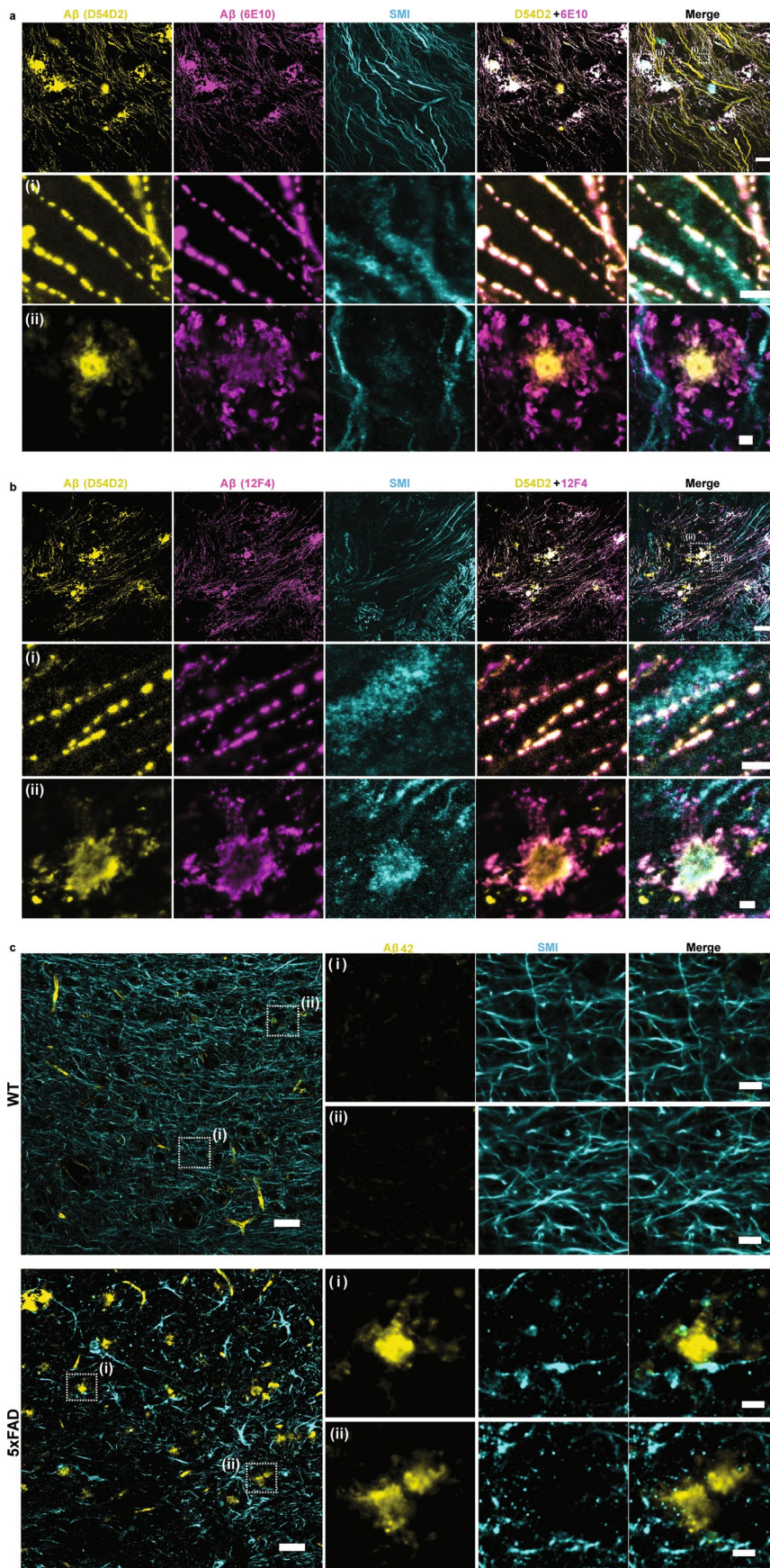
l Shank3 : Pre/Post Mutually Overlapped Volume Normalized to Pre-Expansion Volume



Extended Data Fig. 3 | See next page for caption.

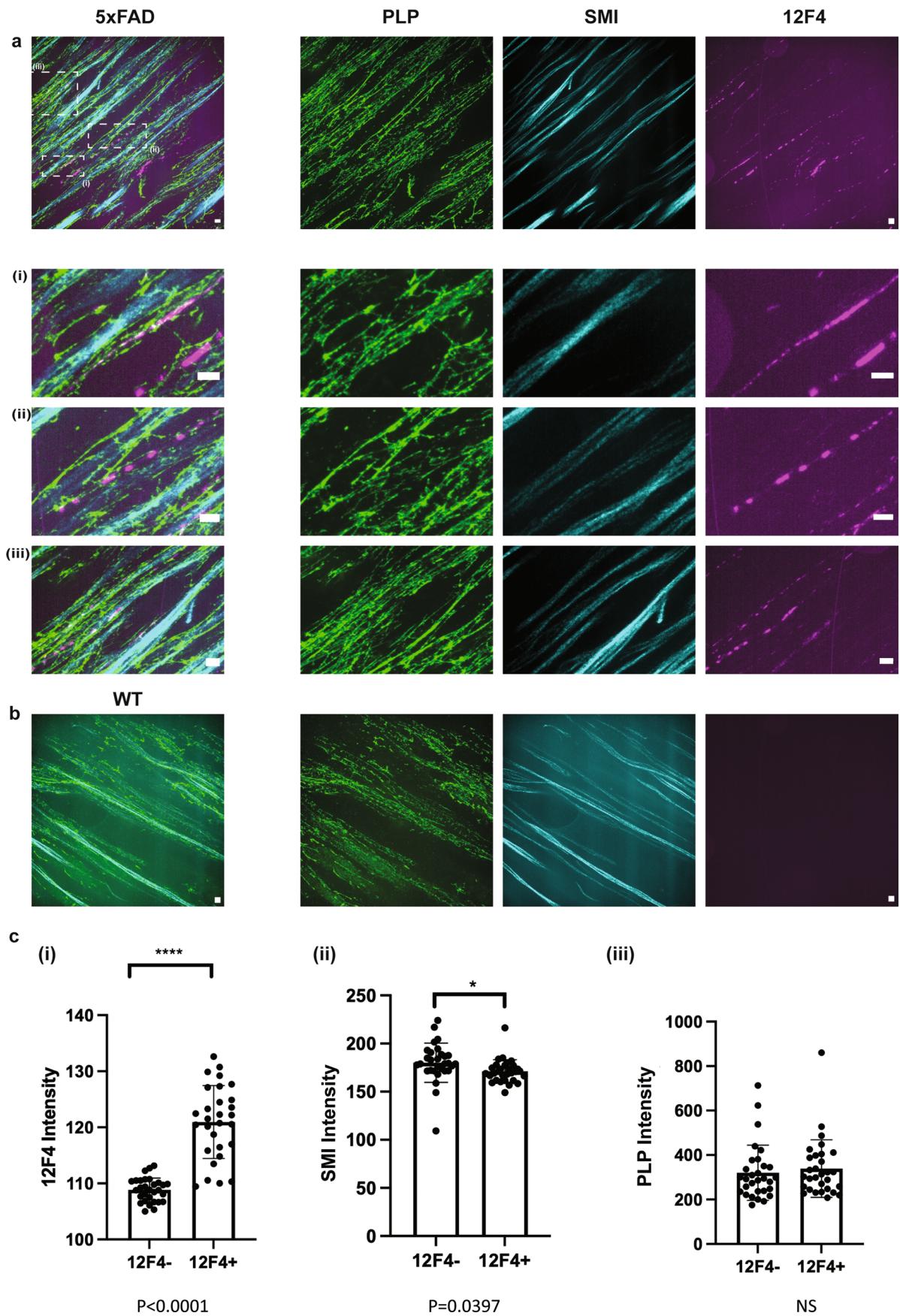
Extended Data Fig. 3 | Analysis of the distortion caused by post-expansion staining, as compared to classical pre-expansion staining. (a-b)

Representative background-subtracted and binary images of Homer1 **(a)** and Shank3 **(b)** in pre- and post-expansion staining channels (top row (yellow): pre-expansion channel, second row (black/white): binary pre-expansion channel, third row (magenta): post-expansion channel, bottom row (black/white): binary post-expansion channel). **(c)** Number of synaptic puncta for pre- and post-expansion staining channels, after filtration, for the images in (a-b). **(d)** Population distribution (violin plot of density, with a dashed line at the median and dotted lines at the quartiles) of the number of synaptic puncta in the pre-expansion staining channel for Homer1 and Shank3 (see Supplementary Table 5 for statistics for this figure). **(e)** Population distribution of the number of synaptic puncta in the post-expansion staining channel for Homer1 and Shank3. **(f)** Difference in the number of synaptic puncta between post- and pre-expansion staining channels normalized to the number of synaptic puncta in the pre-expansion staining channel. **(g-h)** Pixel-wise autocorrelation between pre-expansion (pre-pre, yellow), post-expansion (post-post, magenta), and pixel-wise correlation between pre- and post-expansion (pre-post, black) as a function of shift distance in x- (left column), y- (middle column), and z- (right column) directions for Homer1 **(g)** and Shank3 **(h)**. The mean across all synapses is shown in the top row, and representative synapses are shown in the second through fourth rows. These values were used to calculate the linearized error measure shown in Extended Data Fig. 2f, g. **(i-j)** Pixel-wise correlation between mean-normalized, masked pre- and post-expansion channels as a function of shift distance in x- and y-directions ($z=1$) for Homer1 **(i)** and Shank3 **(j)**. The mean across all synapses is shown in the top left, standard deviation across all synapses shown in second from the top left, and representative synapses are shown in the remaining plots. **(k-l)** Mutually overlapped volume between pre- and post-expansion stained synaptic puncta, normalized to total puncta volume in the pre-expansion staining channel, as a function of shift distance in x- and y-directions ($z=1$) for Homer1 **(k)** and Shank3 **(l)**. The mean across all synapses is shown in the top left, standard deviation across all synapses shown in second from the top left, and representative synapses are shown in the remaining plots. Analysis was conducted on 480 (before exclusion based on size) synapses for Shank3 and Homer1 from 3 mice (see Supplementary Table 5 for exact numbers).



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | ExR and unexpanded tissue confocal images showing immunolabeling of A β 42. ExR confocal images (single z-slices) showing immunolabeling of A β 42 with two different monoclonal antibodies **(a)** D54D2 + 6E10 and **(b)** D54D2 + 12F4 with SMI co-staining in the fornix of 5xFAD mouse (n = 3 fields of view of 2 slices from 2 mice). Scale bar, 10 μ m (top row), 1 μ m (i, ii panels). **(c)** Unexpanded tissue confocal image, a single z-slice, showing pre-expansion A β 42 (yellow) and SMI (cyan) staining in the fornix of WT (upper panel) and 5xFAD mice (lower panel) (n = 3 fields of view of 1 slice from 1 mouse per WT and 5xFAD, respectively). Scale bar, 30 μ m (left panel) and 6 μ m (panels i, ii)).



Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | ExR confocal images showing immunolabeling of PLP, SMI, and 12F4 in 5xFAD and WT fornix. ExR reveals relative localization of A β 42 peptide and myelin in the fornix of Alzheimer's model 5xFAD and WT mice (n=2 fields of view of 1 slice from 2 mice per WT and 5xFAD, respectively). **(a)** ExR confocal image (max intensity projections, 900–1000 nm thickness) showing post-expansion A β 42 (magenta), SMI (cyan) and PLP (green) staining in the fornix of 5xFAD mice. Leftmost panel, merged low-magnification image; right images show individual channels. Insets (i–iii) show close-up views of the boxed regions highlighted in the upper left image. **(b)** ExR confocal image (max intensity projections, 1.72 μ m thickness) showing post-expansion A β 42 (magenta), SMI (cyan) and PLP (green) staining in the fornix of wild-type mice. Leftmost panel, merged image. All images were subtracted background using Fiji's Rolling Ball algorithm with radius 50 pixels, and adjusted with auto-contrast. Scale bar = 500 nm. **(c)** Comparison of 12F4, SMI, and PLP intensity levels along axons in 5xFAD fornix with and without 12F4. To analyze axonal amyloid beta deposition with myelination and SMI intensity, we measure the (i) 12F4, (ii) SMI and (iii) PLP intensity levels along 10 axons with (12F4+) and without 12F4 (12F4-) from the same field of view (n=3 fields of view of 2 slices from 2 5xFAD). All images were subtracted background with 50 pixels, and adjusted with auto-contrast for analysis by ImageJ. On each axon, three lines were drawn cross-sectionally across each axon in Image J and averaged intensity levels of PLP, 12F4 and SMI from different channels were measured respectively along these lines. For 12F4+ axons, each line was drawn across the centroid of amyloid beta deposition. For 12F4- axons, lines were positioned randomly along the axon. We then compared PLP, 12F4 and SMI intensity levels between 12F4+ and 12F4- axons. Plotted is the mean, with error bars representing standard error of the mean (SEM). Two-sided paired t-test, (i) ****p < 0.0001, t = 6.112, df = 18, (ii) p = 0.0595, t = 2.012, df = 18, (iii) p = 0.6580, t = 0.4502, df = 18.

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| <input type="checkbox"/> | <input checked="" type="checkbox"/> | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
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<i>Give P values as exact values whenever suitable.</i> |
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| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection Raw image data were collected by a Nikon Eclipse Ti inverted microscope with NIS-Elements software.

Data analysis Fiji/ImageJ and custom MATLAB (versions R2014, R2019a-b, and R2020a-b) scripts were used to process images. Python 3 was used for some statistical analyses. The code is available on Zenodo at DOI:10.5281/zenodo.6383293 and on Github at <https://github.com/schroeme/ExR>.

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Data

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw and analysed datasets generated during the study are too large to be publicly shared, yet they are available for research purposes from the corresponding authors on reasonable request.

Field-specific reporting

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- Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The studies were done with 1 or more mouse specimens with 2 or more fields of view each.
Data exclusions	Some synapses were excluded from analysis. Criteria for exclusion are detailed in Methods. We identified measured signal from one field of view from one animal as an outlier using the ROUT method with Q=1% in GraphPad Prism, and excluded it from further analysis and visualization.
Replication	All attempts at replication were successful.
Randomization	Randomization was not relevant to the study because the synapse staining of the somatosensory cortex was obtained from one strain (C57BL/6), and the amyloid-beta staining was obtained from 5xFAD, with WT as a control.
Blinding	Blinding was not relevant to most of the study because the synapse staining of the somatosensory cortex (L1, L2/3, L4) was imaged as is, and simply analysed after taking images, and because there are obvious differences in WT and 5xFAD tissues regarding the presence of amyloid plaques. The investigators were blinded to the groups for analysis of the layer-specific synaptic nanocolumn dataset (Fig. 3).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involvement in the study
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
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<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involvement in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used	Antibody information, including supplier names and catalogue numbers, is provided in the Supplementary Information.
Validation	Information on the validation of each primary antibody was provided on the manufacturer's website. All antibodies used in the study are commercially available.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	Both male and female wild-type mice (C57BL/6 or Thy1-YFP, 6-8 weeks) and 5xFAD mice (12–13 months) were used.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.

Ethics oversight

All procedures involving animals were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Supplementary information

Revealing nanostructures in brain tissue via protein decrowding by iterative expansion microscopy

In the format provided by the authors and unedited

Contents

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Supplementary Fig. 8 | Additional exemplary images and analyses of A β 42 and Kv7.2 nanocluster shapes and their relationships.

Supplementary Table 1 | Statistical analysis for DNA-PAINT vs. ExR comparisons (Fig. 1j–l, Supplementary Fig. 4).

Supplementary Table 2 | Numbers of technical replicates (synapses) used in Fig. 2 analysis for each synaptic protein.

Supplementary Table 3 | Statistical analysis for Fig. 2i: synapse decrowding analysis statistics of mean signal amplitude within dilated reference channel ROIs.

Supplementary Table 4 | Statistical analysis for Fig. 2j: synapse decrowding analysis of mean puncta volume within dilated reference channel ROIs.

Supplementary Table 5 | Accompanying statistics for Fig. 2m–o and Supplementary Fig. 5: analysis of distortion caused by post-expansion staining.

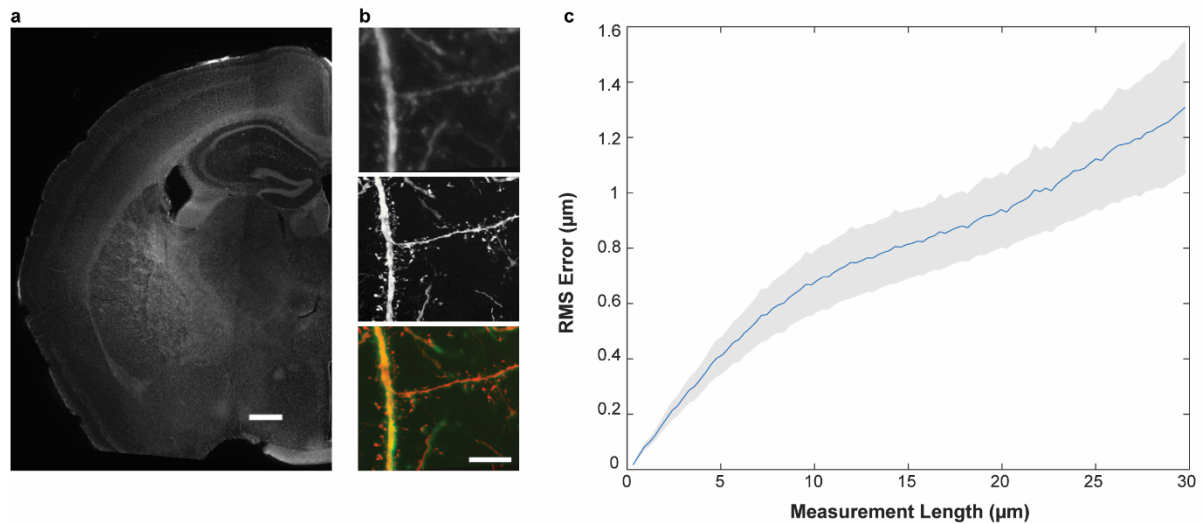
Supplementary Table 6 | Statistical analysis for Fig. 2k: synaptic puncta analysis for mean volume of the largest synaptic connected components.

Supplementary Table 7 | Statistical analysis for Fig. 2l: synaptic puncta analysis of mean signal-to-noise ratio within the largest synaptic connected components.

Supplementary Table 8 | Statistical analysis for Supplementary Fig. 2: comparison of the effects of antigen retrieval and ExR procedures on mean signal intensity within and outside of synaptic puncta.

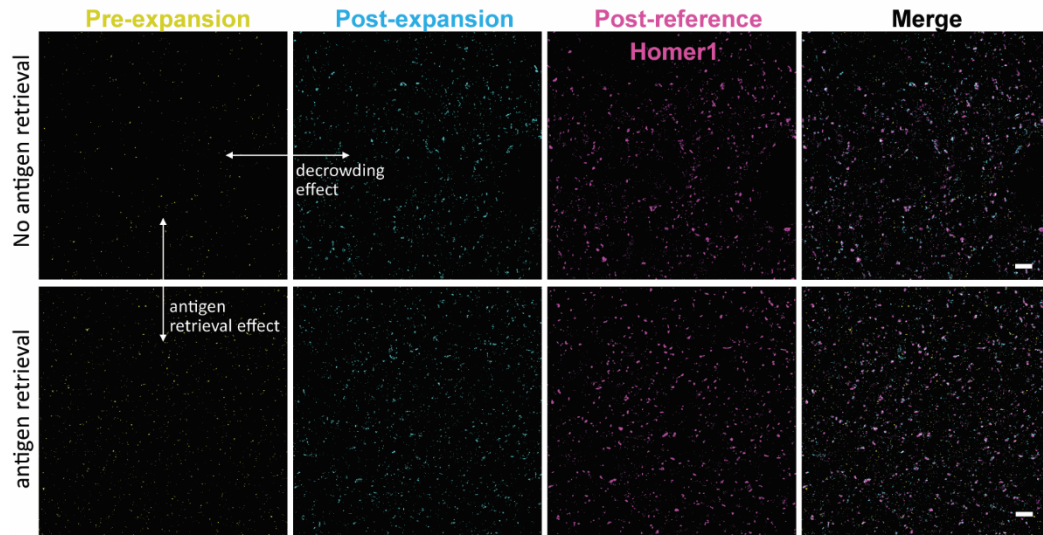
Supplementary Table 9 | Statistical analysis for Supplementary Fig. 2: comparison of the effects of antigen retrieval and ExR procedures on total signal volume within and outside of synaptic puncta.

Supplementary Table 10 | List of chemicals, antibodies and gel solution of ExR.

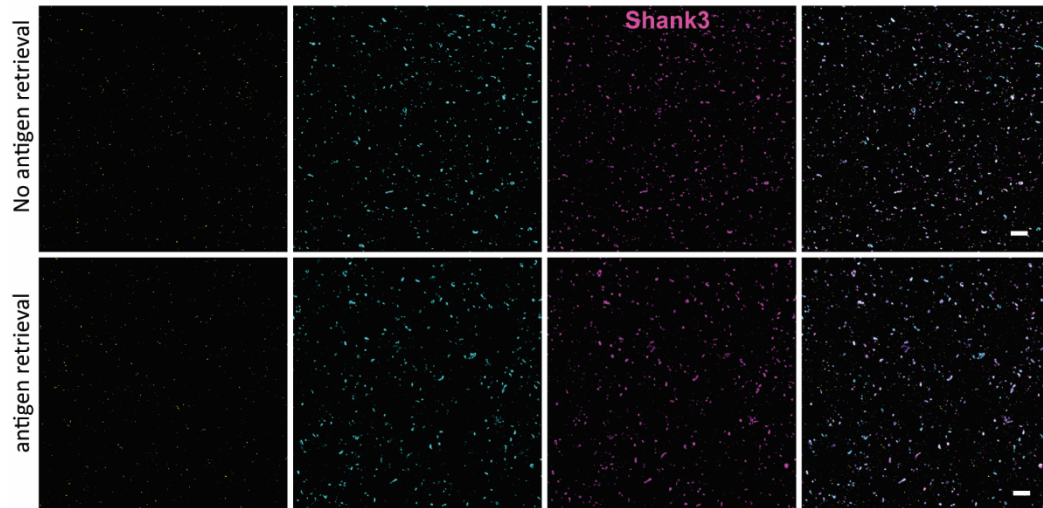


Supplementary Fig. 1. Measurement of distortion of ExR in single z-slice confocal images of intact brain tissue vs. ExR tissue, imaging the same field of view before and after expansion, show that the expansion process is globally isotropic. (a) Widefield image of Thy1-YFP brain slice before expansion. (Scale bar, 500 μm .) (b) Confocal images after immunostaining with antibodies against GFP before expansion (top), after expansion (middle) and merged (bottom). (Scale bar, 5 μm , in biological units, meaning physical size divided by the expansion factor, here and throughout the paper unless otherwise indicated) (c) Root mean square error, vs. measurement length (in biological units), calculated via a non-rigid registration algorithm of intact vs. ExR-processed brain ($n=6$ fields of view from 3 coronal tissue sections, from one mouse). Blue line, mean; gray shading, standard deviation. Shown are images from one experiment, the only replicate of this experiment.

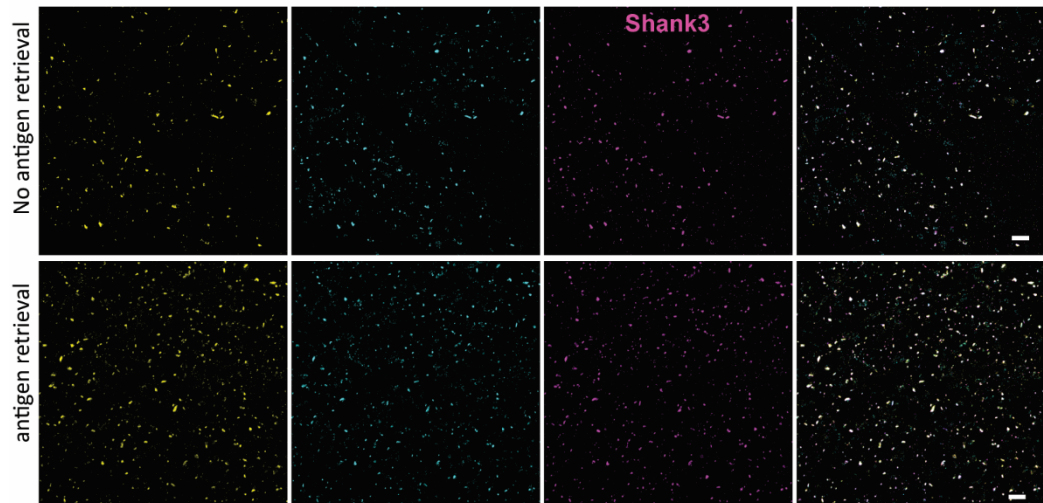
a Ca_v2.1

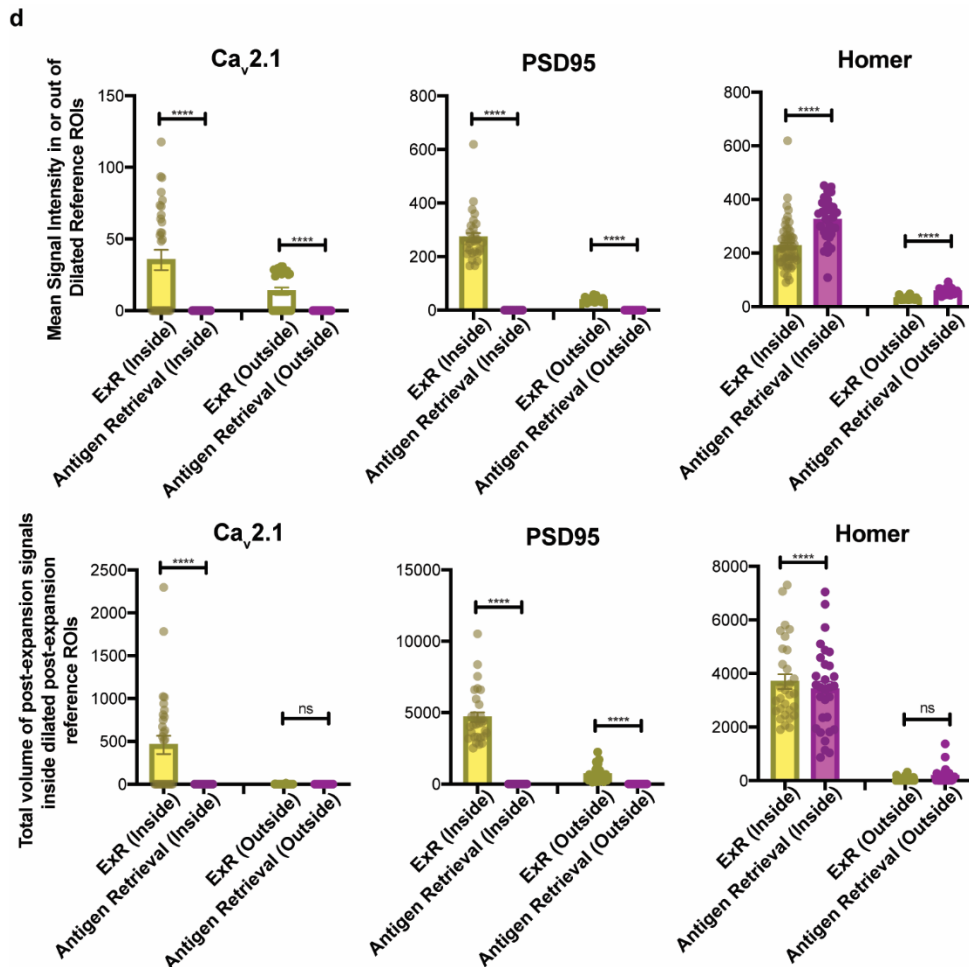


b PSD95

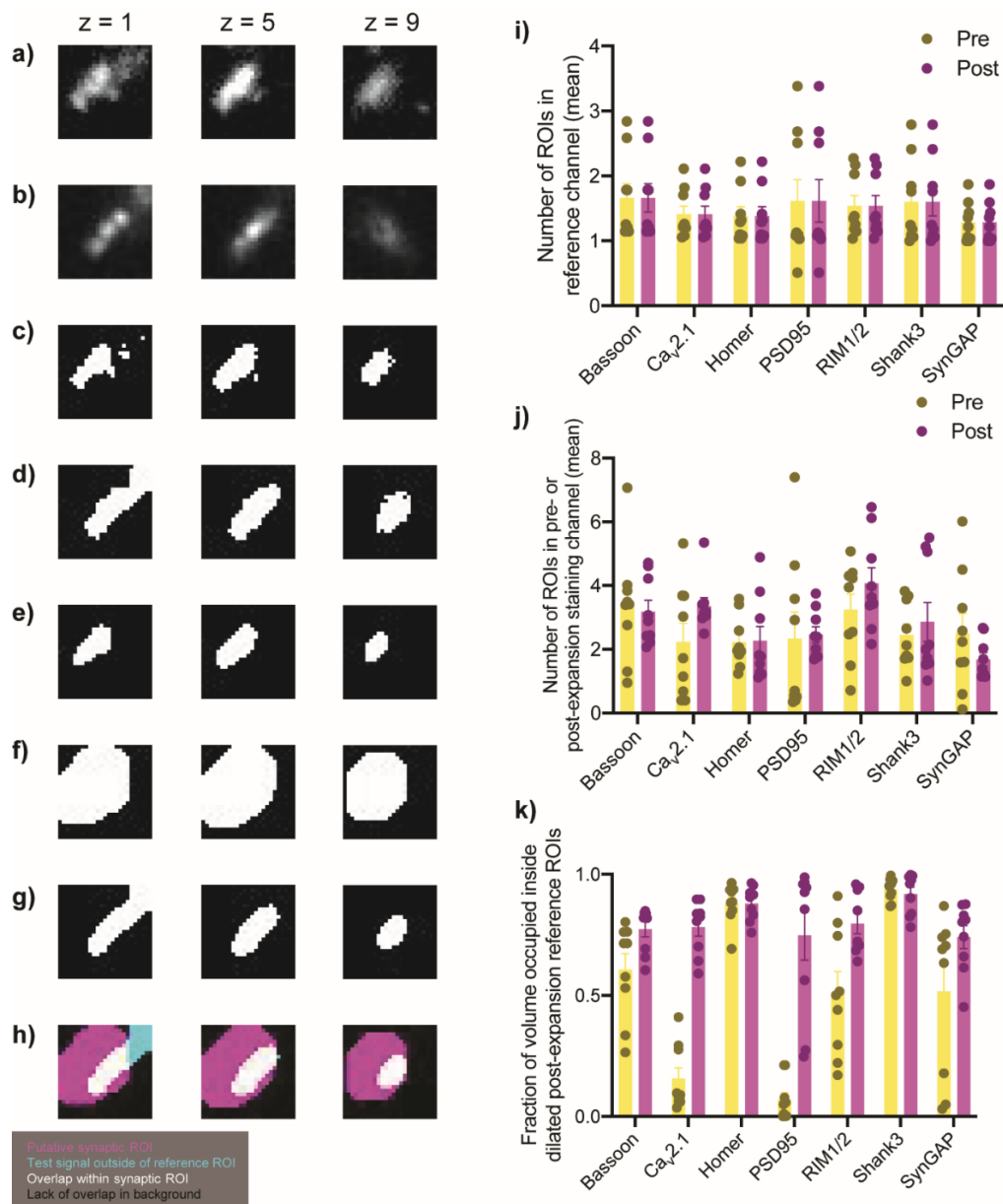


c Homer1

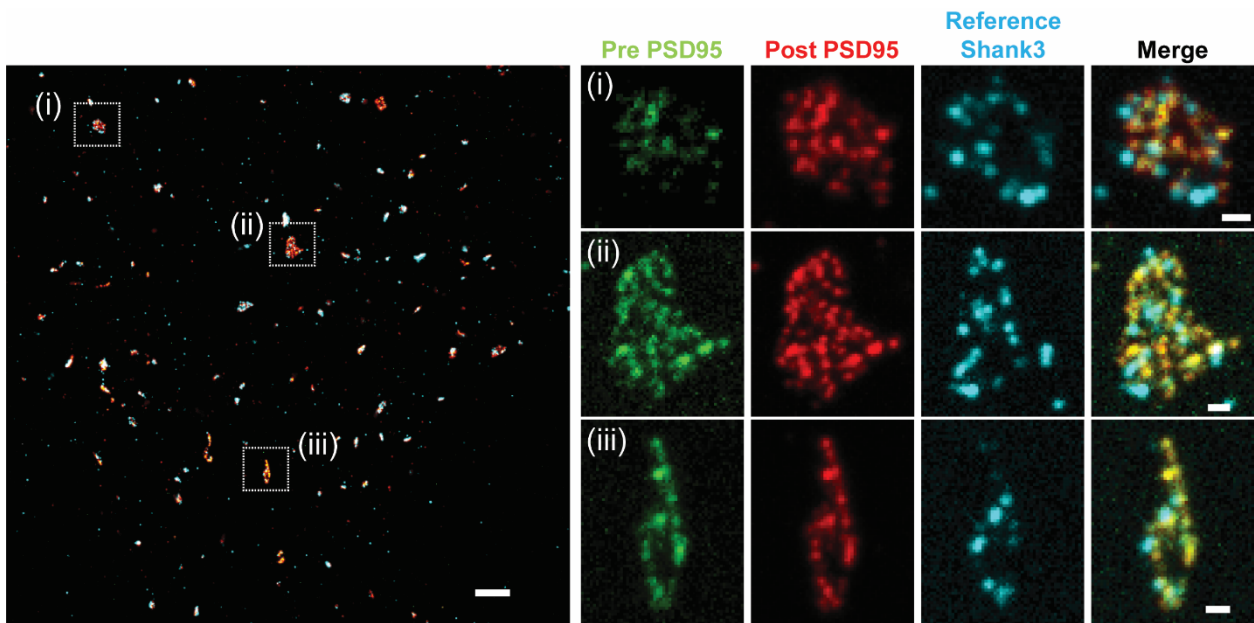




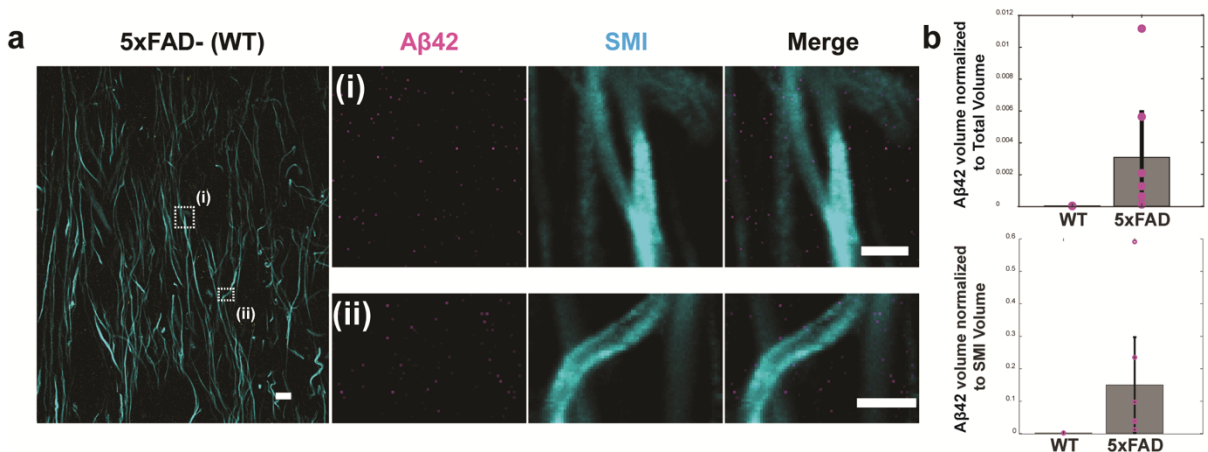
Supplementary Fig. 2. Comparison of antigen retrieval vs. ExR. **(a-c)** Confocal images (max intensity projection) of pre-expansion and post-expansion immunostaining using the same imaging setup with antibodies against **(a)** Ca_v2.1, **(b)** PSD95, and **(c)** Homer1 with or without antigen retrieval. Scale bar, 1 μ m. **(d)** Quantification of mean signal intensity (top row; all images were acquired under the same exposure and laser power) and total volume (bottom row) in or out of dilated reference ROIs with or without antigen retrieval steps (two-sided Sidak's multiple comparisons test following ANOVA; ****, $P < 0.0001$; ns, not significant; $n = 30$ synapses from 1 field of view from 1 mouse). Plotted is the mean, with error bars representing standard error of the mean (SEM). Antigen retrieval does not improve staining of proteins (Ca_v2.1 and PSD95) that cannot be stained without decrowding (ExR). In the case of Homer1, which shows good staining quality even without decrowding, antigen retrieval slightly increased overall mean signal intensity in both foreground and background, but did not increase volume. Thus, for some antibodies, the traditional antigen retrieval procedure may increase overall signal intensity relative to decrowding (ExR), potentially in a non-specific manner. We conclude that the decrowding effect due to expansion, not antigen retrieval, is the dominant factor contributing to increased staining quality with ExR. For full statistics, see **Supplementary Tables S8** and **S9**. Shown are images from one experiment, the only replicate of this experiment.



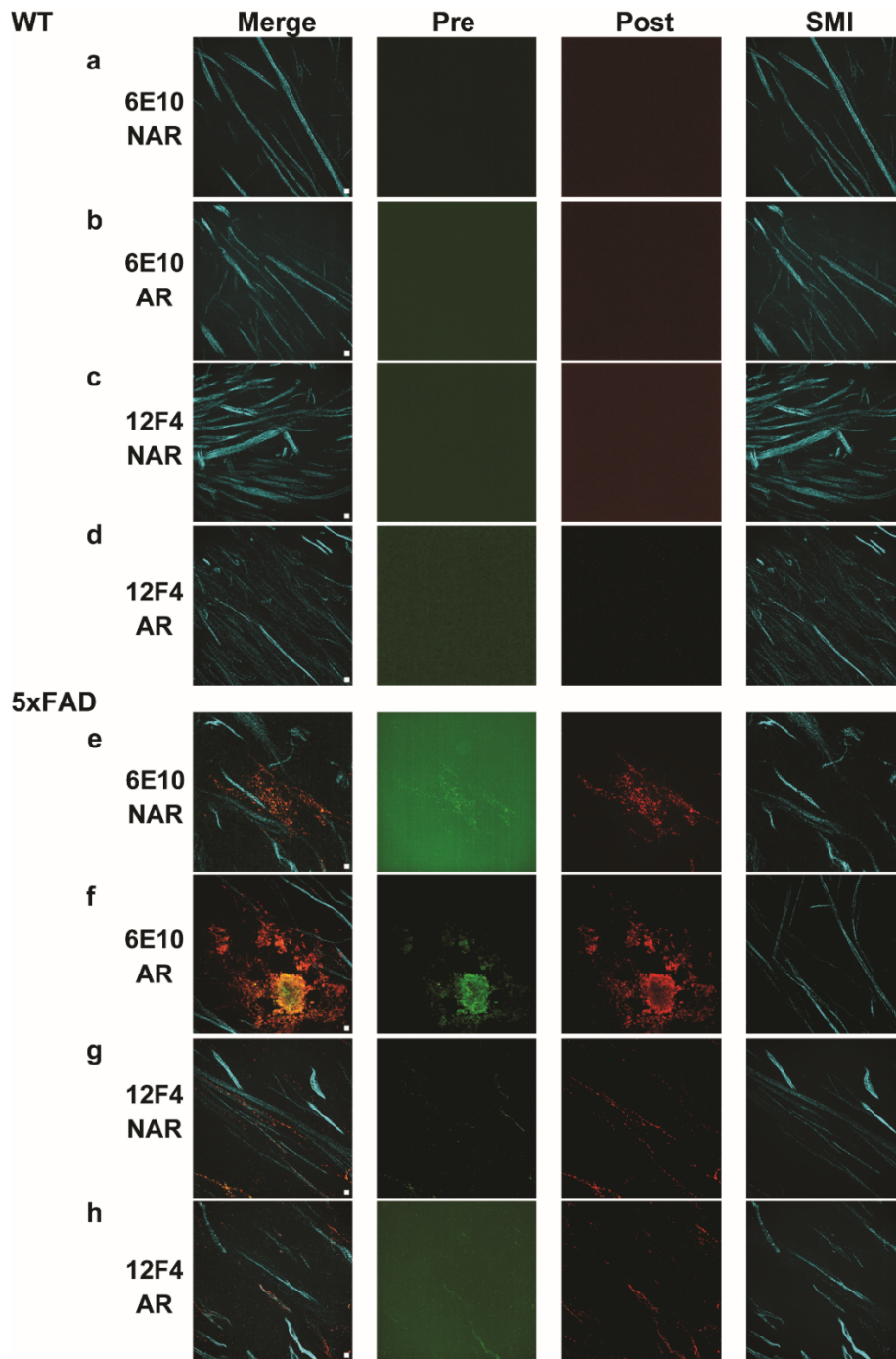
Supplementary Fig. 3. Illustration and further quantification of synaptic decrowding analysis. Synapse size = 25x25x11 voxels ~ 425x425x440nm. Test (post-expansion) stain is Bassoon, reference stain (also post-expansion) is Homer1. **(a)** Background subtracted reference, **(b)** Background subtracted test stain, **(c)** Binary reference, **(d)** Binary test, **(e)** Filtered binary reference, **(f)** Dilated reference, **(g)** Filtered binary test stain, and **(h)** Overlay of **(f)** and **(g)**. **(i)** Mean number of ROIs per synapse for reference channel, for various test channels, shown on the x-axis. **(j)** Mean number of ROIs per synapse for pre- or post-expansion test channels. **(k)** Fraction of test channel (g) volume occupied inside dilated post-expansion reference ROIs (f). For (i)-(k), plotted is the mean, with error bars representing standard error of the mean (SEM). This measure can be interpreted as a true positive rate, because it is the fraction of “true” synapses that we are able to detect with ExR. See **Supplementary Supplementary Table 2** for numbers of technical and biological replicates.



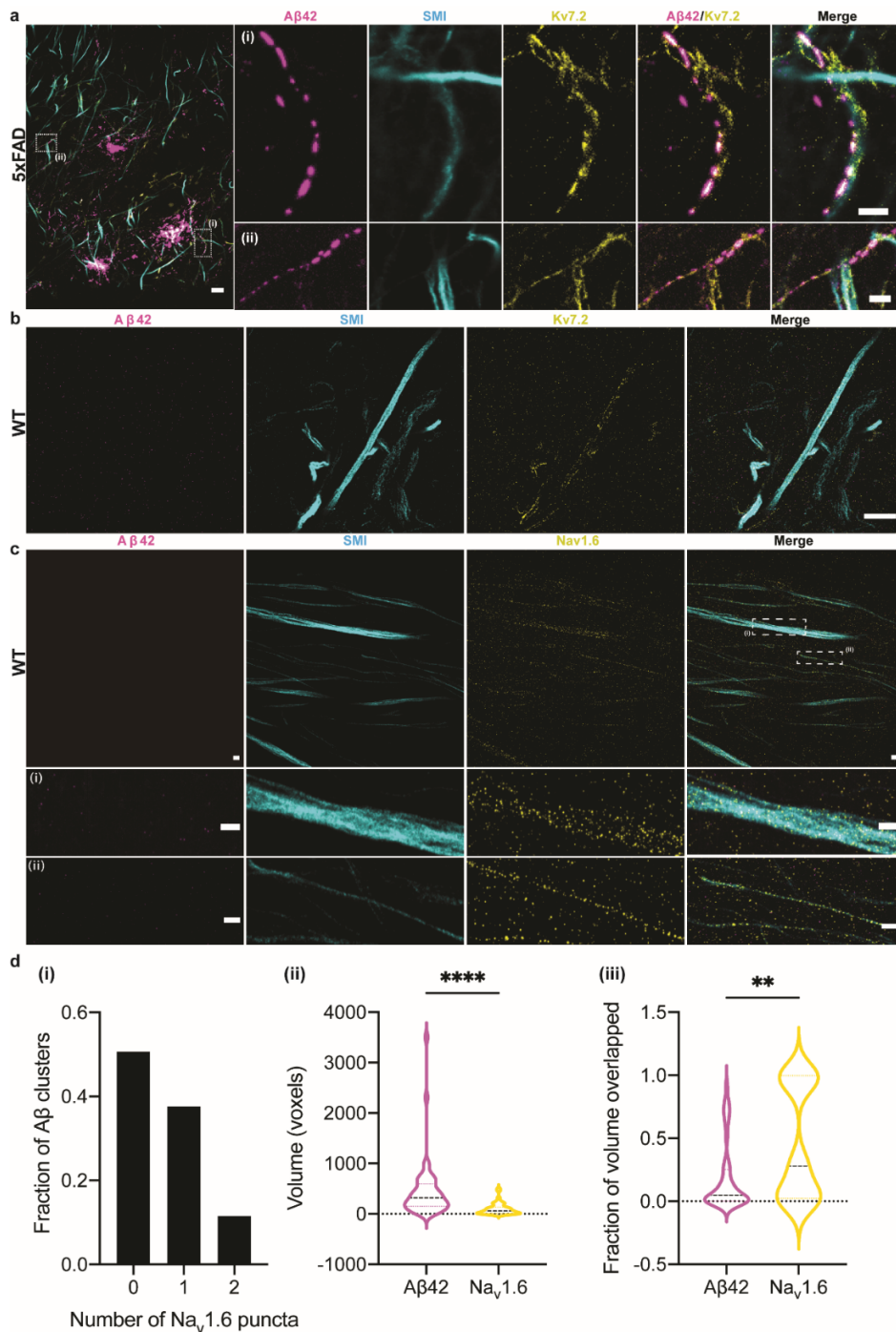
Supplementary Fig. 4. Confocal images (single z-slice) of somatosensory cortex layer 2/3, comparing pre-expansion and post-expansion immunostaining with antibodies against PSD95 (from Cell Signaling Technology, product number CST3450S) and post-expansion Shank3 for the reference ($n = 3$ fields of view of 2 slices from 1 mouse). Scale bar, 1 μm (left panel), 100 nm (right panel).



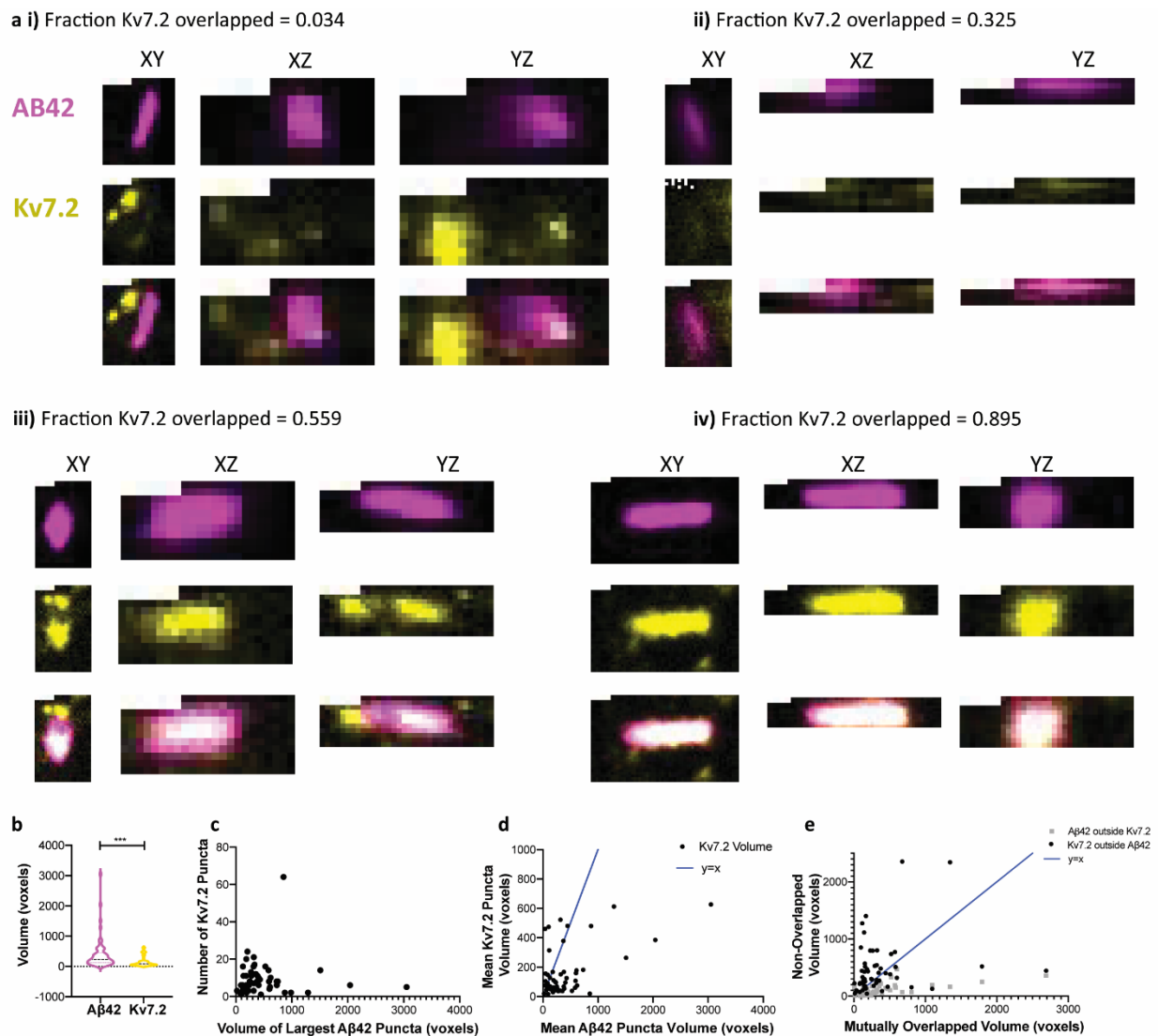
Supplementary Fig. 5. ExR confocal images showing (a) post-expansion A β 42 and SMI staining in wild-type (WT) mice. Left image, low magnification image in the fornix. Insets (i) and (ii), close-up views of A β 42 and SMI staining patterns from boxed regions in the left image. (Scale bar = 4 μm (left panel); 1 μm (right panels, i-ii)). Shown are images from one representative experiment from three independent replicates. (b) Histograms showing the volume of A β 42 clusters and aggregates in WT and 5xFAD specimens (Fig. 4) normalized to the total field of view (FOV) volume (top) or the volume of SMI (bottom). Two sample two-sided Kolmogorov-Smirnov test, p -values < 0.001, $N=14$ data points (7 per condition) from 14 FOVs from 4 mice (2 mice per condition). Error bars indicate SEM.



Supplementary Fig. 6. Comparison of antigen retrieval vs. ExR of **(a-d)** wildtype and **(e-h)** 5xFAD brain slices ($n=3$ fields of view of 2 slices from 2 mice per WT and 5xFAD, respectively). Confocal images (max intensity projections, 500 – 800 nm thickness) of pre-expansion (green) and post-expansion (red) immunostaining using the same imaging setup with antibodies against **(a, b, e, f)** 6E10 and **(c, d, g, h)** 12F4 with or without antigen retrieval with post-expansion immunostaining with antibodies against SMI (cyan). (AR: with antigen retrieval, NAR: non-antigen retrieval). All images were subtracted background using Fiji's Rolling Ball algorithm with radius 50 pixels, and adjusted with auto-contrast. Scale bar = 500 nm.



Supplementary Fig. 7. (a) Additional exemplar (i.e., with large puncta for easy visibility) confocal ExR images (single z-slice) showing post expansion Aβ42 (magenta), SMI (cyan) and Kv7.2 (yellow) staining in the fornix of 5xFAD mouse (n=3 fields of view of 2 slices from 2 mice). Scale bar = 4 μm (left panel), 400 nm (panels i, ii). **(b)** ExR confocal image (single z-slice) showing post expansion Aβ42 (magenta), SMI (cyan) and Kv7.2 (yellow) staining in the fornix of WT mouse (n=3 fields of view of 1 slice from 1 mouse). Scale bar = 2 μm. **(c)** ExR confocal images showing immunolabeling of Nav1.6, SMI, and 12F4 in WT fornix. ExR confocal image (max intensity projections, 640 nm thickness) showing post expansion Nav1.6 (yellow), SMI (cyan) and Aβ42 (magenta, 12F4) staining in the fornix of WT mouse (n=3 fields of view of 2 slices from 2 mice). Insets (i-ii) show close-up views of the boxed regions highlighted in the upper left image. All images were subtracted background using Fiji's Rolling Ball algorithm with radius 50 pixels, and adjusted with auto-contrast. Scale bar = 500 nm. **(d)** Distribution comparison of Nav1.6 within beta-amyloid nanoclusters in the fornix of 5xFAD mouse. (i) Normalized histogram of the number of Nav1.6 puncta in manually-selected ROIs containing individual Aβ nanoclusters. (ii) Mean puncta volume of Aβ (magenta) and Nav1.6 (yellow) within the ROIs containing 1 or more Nav1.6 puncta. (iii) Fraction of total Aβ42 (magenta) and Nav1.6 (yellow) puncta volume overlapped with Nav1.6 or Aβ42. N = 82 ROIs cropped from 8 fields of view from 2 mice. Asterisks indicate statistical significance from two-sided paired t-tests, ** p < 0.01, **** p < 0.001.



Supplementary Fig. 8. Additional exemplary (good quality, not necessary representative) images and analyses of A β 42 and K $_v$ 7.2 nanocluster shapes and their relationships. **(a)** 3D representation of an A β 42 and K $_v$ 7.2 nanocluster using orthogonal slices in the x-y, x-z, and y-z planes, at the center of each nanocluster for nanoclusters with 3.4% **(i)**, 32.5% **(ii)**, 55.9% **(iii)**, and 89.5% **(iv)** of total K $_v$ 7.2 volume inside the A β 42 puncta. The orthogonal projections illustrate the oblong shape of these nanoclusters. Scale bar = 100 nm. **(b)** Distribution of mean puncta volume for A β 42 and K $_v$ 7.2 nanoclusters (violin plot of density, with a dashed line at the median and solid lines at the quartiles). The mean of A β 42 puncta is significantly larger than that of K $_v$ 7.2, on average (two-tailed paired t-test, $p = 0.0001$, $t = 4.116$, $df = 54$). **(c)** Scatter plot of the number of K $_v$ 7.2 puncta vs. the volume of the largest A β 42 puncta in the cropped ROI, showing no significant correlation between the two (simple linear regression, 95% CI of slope $[-0.003940, 0.005947]$, $p = 0.6855$, $R^2 = 0.003118$). **(d)** Scatter plot of the mean K $_v$ 7.2 puncta volume vs. the mean A β 42 puncta volume, illustrating a sublinear relationship but significant positive correlation between the two (simple linear regression, $R^2 = 0.2977$, $p < 0.0001$, 95% CI of slope $[0.09878, 0.2437]$). Compare to the line $y = x$ (blue). **(e)** Scatter plot of the non-overlapped volume for A β 42 (gray squares) and K $_v$ 7.2 (black dots) vs. the mutually overlapped volume of the two proteins, compared to the line $y = x$ (blue). The volume of K $_v$ 7.2 outside of A β 42 is on average larger than the volume of A β 42 outside of K $_v$ 7.2 (two-tailed t-test on the difference between the ratio of non-overlapped volume to overlapped volume for K $_v$ 7.2 and A β 42, $p < 0.0001$). While the non-overlapped volume of A β 42 is correlated to the mutually overlapped volume (95% CI of slope $[0.07733, 0.1700]$, $R^2 = 0.3510$, $p < 0.0001$), the volume of K $_v$ 7.2 outside of A β 42 is not (95% CI of slope $[0.03205, 0.5237]$, $R^2 = 0.05608$, $p = 0.0817$).

Supplementary Table 1 | Statistical analysis for DNA-PAINT vs. ExR comparisons (**Fig. 2h-j, Extended data Fig. 1**).

i) Descriptive statistics for the population distribution of the difference between shift at the half-maximal correlation between ExR-PAINT correlation and PAINT-PAINT autocorrelation (nm) (**Fig. 2h**).

Number of values	101
Minimum	-412.3
Maximum	535.1
Range	947.4
Mean	4.913
Std. Deviation	176.5
Std. Error of Mean	17.56
Lower 95% CI of mean	-29.93
Upper 95% CI of mean	39.75

ii) Descriptive statistics for the population distribution of the difference between shift at the half-maximal correlation between ExR-PAINT correlation and ExR-ExR autocorrelation (nm) (**Fig. 2i**).

Number of values	101
Minimum	-458.5
Maximum	603.1
Range	1062
Mean	18.78
Std. Deviation	196.9
Std. Error of Mean	19.60
Lower 95% CI of mean	-20.10
Upper 95% CI of mean	57.66

iii) Descriptive statistics for the population distribution of the absolute difference in distance between synaptic puncta normalized to the DNA-PAINT puncta distance (**Fig. 2j**).

Number of values	27
Minimum	0.004659
Maximum	0.5383
Range	0.5337
Mean	0.1085
Std. Deviation	0.1169
Std. Error of Mean	0.02251
Lower 95% CI of mean	0.06228
Upper 95% CI of mean	0.1548

iv) Descriptive statistics for the population distribution of the difference in distance between synaptic puncta (PAINT-ExR, **Extended Data Fig. 1i**).

Number of values	27
Minimum	-0.3886
Maximum	0.1476
Range	0.5362
Mean	-0.008854
Std. Deviation	0.1146
Std. Error of Mean	0.02206
Lower 95% CI of mean	-0.05419
Upper 95% CI of mean	0.03649

Supplementary Table 2 | Numbers of technical replicates (synapses) used in **Extended Data Fig. 2** analysis for each synaptic protein, layer, and mouse.

Protein	Layer	Animal	Number of Synapses
Cav2.1	1	A	54
	2/3	A	55
	4	A	50
	1	B	51
	2/3	B	56
	4	B	52
	1	C	50
	2/3	C	63
	4	C	61
Bassoon	1	A	55
	2/3	A	55
	4	A	55
	1	B	54
	2/3	B	55
	4	B	55
	1	C	61
	2/3	C	63
	4	C	63
Homer1	1	A	50
	2/3	A	55
	4	A	50
	1	B	50
	2/3	B	49
	4	B	50
	1	C	63
	2/3	C	63
	4	C	63
PSD95	1	A	49
	2/3	A	52
	4	A	60
	1	B	70
	2/3	B	55
	4	B	54
	1	C	63
	2/3	C	63
	4	C	63
RIM1/2	1	A	55
	2/3	A	52
	4	A	55
	1	B	55
	2/3	B	58
	4	B	55
	1	C	64
	2/3	C	63
	4	C	63
Shank3	1	A	49
	2/3	A	50
	4	A	44
	1	B	55
	2/3	B	50
	4	B	55
	1	C	63
	2/3	C	62
	4	C	63
SynGAP	1	A	51
	2/3	A	50
	4	A	50
	1	B	49
	2/3	B	55
	4	B	50
	1	C	60
	2/3	C	61
	4	C	61

Supplementary Table 3 I Statistical analysis for **Extended Data Fig. 2a**: synapse decrowding analysis statistics of mean signal amplitude within dilated reference channel ROIs.

3-way analysis of variance (ANOVA) was used to test the statistical significance of the effect of multiple factors, including protein, pre- or post-expansion staining, and inside or outside of dilated reference ROIs as categorical variables, on the mean value of signal intensity inside ROIs. Further analysis was then conducted on selected significant factors. To determine which of the seven proteins had significantly different mean signal amplitude between pre- and post-expansion staining, multiple 2-way ANOVAs were run using linear models with inside/outside reference ROIs and pre/post staining as categorical variables. ANOVA p-values were corrected using the Holm-Sidak method. To compare the mean signal amplitude within dilated reference ROIs of pre- and post-expansion stained synapses, Sidak's multiple comparisons test, following one-way ANOVA, was run to compare the means of each pair of groups (pre/inside, pre/outside, post/inside, post/outside) in GraphPad Prism, only on those proteins that showed a significant effect of the pre/post expansion staining factor in 2-way ANOVA. For all ANOVA testing, the Python *statsmodels* package was used to run ordinary least squares models and calculate ANOVA tables with Type III sum of squares. We identified measured signal from one field of view from one animal as an outlier using the ROUT method⁶⁵ with Q=1% in GraphPad Prism and excluded it from further analysis and visualization.

i) 3-way ANOVA table

Factor	SSE	df	F	Pr(>F)
Intercept	994509	1	17.273	4.51E-05
Protein	2446111	6	7.081	5.90E-07
In/Out	1395065	1	24.230	1.59E-06
Pre/Post	803235	1	13.951	0.000234664
Residual	13760399	239		

ii) Multiple 2-way ANOVAs, with Holm-Sidak corrected p-values

Protein	Uncorrected: P(>F), Pre/Post Factor	Corrected: P(>F), Pre/Post Factor	Reject null?	Uncorrected: P(>F), Inside/Outside Factor	Corrected: P(>F), Inside/Outside Factor	Reject null?
Bassoon	0.0000	0.0001	True	0.0000	0.0000	True
Ca _v 2.1	0.1195	0.2248	False	0.0627	0.1215	False
Homer1	0.3965	0.3965	False	0.0003	0.0013	True
PSD95	0.0169	0.0499	True	0.1997	0.1997	False
RIM1/2	0.0054	0.0214	True	0.0001	0.0003	True
Shank3	0.0027	0.0132	True	0.0000	0.0000	True
SynGAP	0.0013	0.0077	True	0.0036	0.0107	True

iii) Sidak's multiple comparisons test following ordinary one-way ANOVA

Protein	Pre-Post Inside, Pr(>t)	Pre-Post Outside, Pr(>t)	Pre Inside-Outside, Pr(>t)	Post Inside-Outside, Pr(>t)
Bassoon	<0.0001	0.9995	0.4909	<0.0001
Ca _v 2.1	0.1228	0.9867	0.9867	0.0924
Homer1	0.6019	0.9582	0.0019	0.0104
PSD95	0.0474	0.8223	>0.9999	0.2612
RIM1/2	0.0003	0.9993	0.5007	<0.0001
Shank3	0.0001	0.9868	0.0002	<0.0001
SynGAP	0.0001	0.9319	0.9909	0.0003

Supplementary Table 4 I Statistical analysis for **Extended Data Fig. 2b**: synapse decrowding analysis of mean puncta volume within dilated reference channel ROIs.

3-way analysis of variance (ANOVA) was used to test the statistical significance of the effect of multiple factors, including protein, pre- or post-expansion staining, and inside or outside of dilated reference ROIs as categorical variables, on the mean value of ROI volume. Further analysis was then conducted on selected significant factors. To determine which of the seven proteins had significantly different mean ROI volume between pre- and post-expansion staining, multiple 2-way ANOVAs were run using linear models with inside/outside reference ROIs and pre/post staining as categorical variables. ANOVA p-values were corrected using the Holm-Sidak method. To compare the mean signal amplitude within dilated reference ROIs of pre- and post-expansion stained synapses, Sidak's multiple comparisons test, following one-way ANOVA, was run to compare the means of each pair of groups (pre/inside, pre/outside, post/inside, post/outside) in GraphPad Prism, only on those proteins that showed a significant effect of the pre/post expansion staining factor in 2-way ANOVA. For all ANOVA testing, the Python *statsmodels* package was used to run ordinary least squares models and calculate ANOVA tables with Type III sum of squares. We identified measured volume from one field of view from one animal as an outlier using the ROUT method⁶⁵ with Q=1% in GraphPad Prism and excluded it from further analysis and visualization.

i) 3-way ANOVA table

Factor	SSE	df	F	Pr(>F)
Intercept	58206948	1	177.797	1.08E-30
Protein	20486989	6	10.430	2.86E-10
In/Out	23573079	1	72.005	2.27E-15
Pre/Post	15401378	1	47.044	5.89E-11
Residual	78243638	239		

ii) Multiple 2-way ANOVAs, with Holm-Sidak corrected p-values

Protein	Uncorrected : P(>F), Pre/Post Factor	Corrected: P(>F), Pre/Post Factor	Reject null?	Uncorrected: P(>F), Inside/Outside Factor	Corrected: P(>F), Inside/Outside Factor	Reject null?
Bassoon	0.0253	0.0499	True	0.0409	0.0955	False
Ca _v 2.1	0.0003	0.0017	True	0.3200	0.3200	False
Homer1	0.3935	0.3935	False	0.0000	0.0000	True
PSD95	0.0016	0.0065	True	0.0329	0.0955	False
RIM1/2	0.0001	0.0008	True	0.0014	0.0070	True
Shank3	0.0075	0.0223	True	0.0000	0.0000	True
SynGAP	0.0003	0.0017	True	0.0240	0.0925	False

iii) Sidak's multiple comparisons test following ordinary one-way ANOVA

Protein	Pre-Post Inside, Pr(>t)	Pre-Post Outside, Pr(>t)	Pre Inside-Outside, Pr(>t)	Post Inside-Outside, Pr(>t)
Bassoon	0.0010	0.9690	0.9235	0.0016
Ca _v 2.1	0.0002	0.5706	0.7732	0.0807
Homer1	0.5069	0.9980	<0.0001	<0.0001
PSD95	0.0005	0.8602	0.9990	0.0062
RIM1/2	<0.0001	0.7407	0.9930	<0.0001
Shank3	0.0048	0.9029	<0.0001	<0.0001
SynGAP	0.0014	0.2981	0.9439	0.0343

Supplementary Table 5 I Accompanying statistics for **Extended Data Fig. 2e-g** and **Extended Data Fig. 3**: analysis of distortion caused by post-expansion staining.

(i) Descriptive statistics for the number of puncta in the pre-expansion staining channel for Homer1 and Shank3 (**Extended Data Fig. 3d**).

	Homer1	Shank3
Number of values	480	480
Minimum	0	0
Maximum	7	10
Range	7	10
Mean	1.623	1.742
Std. Deviation	1.014	1.147
Std. Error of Mean	0.04629	0.05234
Lower 95% CI of mean	1.532	1.639
Upper 95% CI of mean	1.714	1.845

(ii) Descriptive statistics for the number of puncta in the post-expansion staining channel for Homer1 and Shank3 (**Extended Data Fig. 3e**).

	Homer1	Shank3
Number of values	480	480
Minimum	0	0
Maximum	4	7
Range	4	7
Mean	1.438	1.552
Std. Deviation	0.7968	0.9305
Std. Error of Mean	0.03637	0.04247
Lower 95% CI of mean	1.366	1.469
Upper 95% CI of mean	1.509	1.636

(iii) Descriptive statistics for the difference in the number of puncta in the post-expansion staining channels for Homer1 and Shank3 (**Extended Data Fig. 3f**).

	Homer1	Shank3
Number of values	480	480
Minimum	-6.000	-7.000
Maximum	2.000	2.000
Range	8.000	9.000
Mean	-0.1854	-0.1896
Std. Deviation	0.9546	0.9945
Std. Error of Mean	0.04357	0.04539
Lower 95% CI of mean	-0.2710	-0.2788
Upper 95% CI of mean	-0.09980	-0.1004

(iv) Descriptive statistics for the difference in the number of puncta in the post-expansion staining channels, normalized to the number of puncta in the pre-expansion staining channel, for Homer1 and Shank3 (**Extended Data Fig. 2e**). Values with zero puncta in the pre-expansion staining channel were excluded.

	Homer1	Shank3
Number of values	480	480
Minimum	-0.8571	-0.8333
Maximum	2.000	2.000
Range	2.857	2.833
Mean	0.02939	0.03420
Std. Deviation	0.4978	0.5145
Std. Error of Mean	0.02272	0.02348

Lower 95% CI of mean	-0.01525	-0.01194
Upper 95% CI of mean	0.07404	0.08034

(v) Descriptive statistics for the difference in half-maximum correlation shift between pre-post pixel-wise correlation and pre-pre pixel-wise autocorrelation (**Extended Data Fig. 2f**).

	Homer1	Shank3
Number of values	1374	1374
Minimum	-99.00	-99.00
Maximum	66.00	64.00
Range	165.0	163.0
Mean	1.969	4.409
Std. Deviation	14.29	14.93
Std. Error of Mean	0.3856	0.4027
Lower 95% CI of mean	1.213	3.619
Upper 95% CI of mean	2.726	5.199

(vi) Descriptive statistics for the difference in half-maximum correlation shift between pre-post pixel-wise correlation and post-post pixel-wise autocorrelation (**Extended Data Fig. 2g**).

	Homer1	Shank3
Number of values	1374	1374
Minimum	-99.00	-99.00
Maximum	49.00	53.00
Range	148.0	152.0
Mean	5.623	7.124
Std. Deviation	15.75	14.78
Std. Error of Mean	0.4250	0.3987
Lower 95% CI of mean	4.789	6.342
Upper 95% CI of mean	6.457	7.907

Supplementary Table 6 I Statistical analysis for Extended Data Fig. 2c: synaptic puncta analysis for mean volume of the largest synaptic connected components.

3-way analysis of variance (ANOVA) was used to test the statistical significance of the effect of multiple factors, including protein, layer, and pre- or post-expansion staining as categorical variables, on the mean value of synapse volume. Categorical variables for interactions between protein and pre/post staining and layer and protein were included in the linear model based on biological feasibility of these interactions. Further analysis was then conducted on selected significant factors. To determine which of the seven proteins had significantly different volume between pre- and post-expansion staining, multiple 2-way ANOVAs were run using linear models with layer and pre/post staining as categorical variables. ANOVA p-values were corrected using the Holm-Sidak method. To compare the volume of pre- and post-expansion stained synapses for each of the seven proteins, three t-tests (one for each layer) were run (see **Supplementary Supplementary Table 2** for numbers of biological and technical replicates). Statistical significance was determined using the Holm-Sidak method, with $\alpha = 0.05$. Each layer was analyzed individually, without assuming a consistent standard deviation. For all ANOVA testing, the Python *statsmodels* package was used to run ordinary least squares models and calculate ANOVA tables with Type III sum of squares.

i) 3-way ANOVA table

Factor	SSE	df	F	Pr(>F)
Intercept	1.30E+09	1	1073.4	1.41E-219
Protein	8.25E+08	6	113.2	5.73E-137
Layer	2.09E+06	2	0.9	4.23E-01
Pre/Post	5.51E+08	1	453.4	1.31E-97
Protein-Pre/Post Interaction	7.98E+08	6	109.5	1.26E-132
Layer/Protein Interaction	2.25E+08	12	15.4	1.21E-32
Residual	8.74E+09	7196		

ii) Multiple 2-way ANOVAs, with Holm-Sidak corrected p-values

Protein	Uncorrected: P(>F), Pre/Post Factor	Corrected: P(>F), Pre/Post Factor	Reject null?
Bassoon	6.69E-103	0.00E+00	True
Cav2.1	3.91E-118	0.00E+00	True
Homer1	8.86E-08	8.86E-08	True
PSD95	1.04E-98	0.00E+00	True
RIM1/2	1.28E-112	0.00E+00	True
Shank3	6.53E-33	0.00E+00	True
SynGAP	3.63E-71	0.00E+00	True

iii) Multiple two-sided t-tests, with Holm-Sidak corrected p-values

Protein	Layer	Mean Difference	SE of difference	t ratio	df	Adjusted p value
Bassoon	L1	-1528	117.6	12.99	346.0	<0.000001
	L2/3	-1524	102.3	14.90	352.0	<0.000001
	L4	-1283	89.08	14.40	352.0	<0.000001
Cav2.1	L1	-495.0	34.63	14.29	314.0	<0.000001
	L2/3	-621.6	34.79	17.87	352.0	<0.000001
	L4	-775.0	50.89	15.23	330.0	<0.000001
Homer1	L1	-351.5	96.68	3.636	332.0	0.000963
	L2/3	-241.3	95.23	2.534	340.0	0.011737
	L4	-473.2	143.9	3.288	332.0	0.002230
PSD95	L1	-862.6	67.45	12.79	370.0	<0.000001
	L2/3	-989.2	72.70	13.61	348.0	<0.000001
	L4	-648.8	36.41	17.82	354.0	<0.000001
RIM1/2	L1	-795.4	65.49	12.14	354.0	<0.000001
	L2/3	-1216	71.79	16.93	352.0	<0.000001
	L4	-1212	78.45	15.45	352.0	<0.000001
Shank3	L1	-723.6	116.8	6.196	330.0	<0.000001
	L2/3	-787.4	108.1	7.282	330.0	<0.000001
	L4	-1102	137.6	8.009	352.0	<0.000001
SynGAP	L1	-1758	125.9	13.97	324.0	<0.000001
	L2/3	-3574	338.9	10.55	338.0	<0.000001
	L4	-2345	135.2	17.35	328.0	<0.000001

Supplementary Table 7 I Statistical analysis for Extended Data Fig. 2d: synaptic puncta analysis of mean signal-to-noise ratio within the largest synaptic connected components.

3-way analysis of variance (ANOVA) was used to test the statistical significance of the effect of multiple factors, including protein, layer, and pre- or post-expansion staining as categorical variables, on the mean value of synapse SNR. Categorical variables for interactions between protein and pre/post staining and layer and protein were included in the linear model based on biological feasibility of these interactions. Further analysis was then conducted on selected significant factors. To determine which of the seven proteins had significantly different SNR between pre- and post-expansion staining, multiple 2-way ANOVAs were run using linear models with layer and pre/post staining as categorical variables. ANOVA p-values were corrected using the Holm-Sidak method. To compare the SNR of pre- and post-expansion stained synapses for each of the seven proteins, three t-tests (one for each layer) were run ($n = 50$ puncta per layer, $n = 2$ mice). Statistical significance was determined using the Holm-Sidak method, with $\alpha = 0.05$. Each layer was analyzed individually, without assuming a consistent standard deviation. For all ANOVA testing, the Python *statsmodels* package was used to run ordinary least squares models and calculate ANOVA tables with Type III sum of squares.

i) 3-way ANOVA table

Factor	SSE	df	F	PR(>F)
Intercept	8.47E+04	1	5890.1	0.00E+00
Protein	2.01E+04	6	232.8	1.22E-272
Layer	4.24E+02	2	14.8	4.05E-07
Pre/Post	1.10E+04	1	767.6	1.27E-160
Protein-Pre/Post Interaction	2.23E+04	6	257.9	8.56E-300
Layer/Protein Interaction	1.09E+03	12	6.3	2.84E-11
Residual	1.04E+05	7196		

ii) Multiple 2-way ANOVAs, with Holm-Sidak corrected p-values

Protein	Uncorrected: P(>F), Pre/Post Factor	Corrected: P(>F), Pre/Post Factor	Reject null?
Bassoon	4.89E-207	0.00E+00	True
Cav2.1	1.03E-47	0.00E+00	True
Homer1	1.43E-104	0.00E+00	True
PSD95	1.94E-107	0.00E+00	True
RIM1/2	1.59E-158	0.00E+00	True
Shank3	4.40E-71	0.00E+00	True
SynGAP	2.21E-245	0.00E+00	True

iii) Multiple two-sided t-tests, with Holm-Sidak corrected p-values

Protein	Layer	Mean Difference	SE of difference	t ratio	df	Adjusted p value
Bassoon	L1	-6.098	0.2822	21.61	346.0	<0.000001
	L2/3	-6.833	0.3117	21.92	352.0	<0.000001
	L4	-6.465	0.2634	24.54	352.0	<0.000001
Ca _v 2.1	L1	-3.874	0.2798	13.85	314.0	<0.000001
	L2/3	-3.680	0.6483	5.677	352.0	<0.000001
	L4	-5.019	0.3597	13.95	330.0	<0.000001
Homer1	L1	-5.189	0.4036	12.86	332.0	<0.000001
	L2/3	-5.183	0.3768	13.76	340.0	<0.000001
	L4	-6.056	0.3778	16.03	332.0	<0.000001
PSD95	L1	-4.772	0.4618	10.33	370.0	<0.000001
	L2/3	-6.304	0.4015	15.70	348.0	<0.000001
	L4	-6.076	0.3070	19.80	354.0	<0.000001
RIM1/2	L1	-4.648	0.2251	20.65	354.0	<0.000001
	L2/3	-4.818	0.2397	20.10	352.0	<0.000001
	L4	-4.597	0.2901	15.84	352.0	<0.000001
Shank3	L1	-5.048	0.3913	12.90	330.0	<0.000001
	L2/3	-4.791	0.4970	9.640	330.0	<0.000001
	L4	-4.492	0.3907	11.50	352.0	<0.000001
SynGAP	L1	-13.94	0.5943	23.46	324.0	<0.000001
	L2/3	-14.72	0.5860	25.12	338.0	<0.000001
	L4	-16.95	0.5411	31.32	328.0	<0.000001

Supplementary Table 8 I Statistical analysis for Supplementary Fig. 2: comparison of the effects of antigen retrieval and ExR procedures on mean signal intensity within and outside of synaptic puncta.

To compare the mean signal amplitude within dilated reference ROIs of antigen retrieval- and non-antigen retrieval-treated synapses, Sidak's multiple comparisons test was run to compare the means of samples treated with ExR and samples treated with antigen retrieval followed by pre-expansion staining (AR) in the foreground or background, as defined by the reference channel, in GraphPad Prism. 2-way ANOVA was run in GraphPad Prism using Type III sum of squares.

i) 2-way ANOVA table for Ca_v2.1.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	4380	3	F (3, 232) = 35.20	P<0.0001
Antigen retrieval	2939	1	F (1, 232) = 70.85	P<0.0001
Pre/post-expansion staining	84306	3	F (3, 232) = 677.6	P<0.0001
Residual	9622	232		

ii) Sidak's multiple comparisons test (two-sided) on the difference between ExR (decrowding) and antigen retrieval followed by pre-expansion staining (AR) for Ca_v2.1, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	35.40	30.16 to 40.64	Yes	<0.0001
Background	13.66	8.420 to 18.91	Yes	<0.0001

iii) 2-way ANOVA table for Homer1.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	98791	3	F (3, 232) = 328.4	P<0.0001
Antigen retrieval	60058	1	F (1, 232) = 598.9	P<0.0001
Pre/post-expansion staining	2437902	3	F (3, 232) = 8104	P<0.0001
Residual	23264	232		

iv) Sidak's multiple comparisons test (two-sided) on the difference between decrowding (ExR) and antigen retrieval followed by pre-expansion staining (AR) for Homer1, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	-142.3	-150.5 to -134.2	Yes	<0.0001
Background	-26.61	-34.77 to -18.46	Yes	<0.0001

v) 2-way ANOVA table for PSD95.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	113634	3	F (3, 232) = 809.4	P<0.0001
Antigen retrieval	45778	1	F (1, 232) = 978.2	P<0.0001
Pre/post-expansion staining	2007684	3	F (3, 232) = 14301	P<0.0001
Residual	10857	232		

vi) Sidak's multiple comparisons test (two-sided) on the difference between decrowding (ExR) and antigen retrieval followed by pre-expansion staining (AR) for PSD95, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	271.5	265.9 to 277.1	Yes	<0.0001
Background	37.87	32.31 to 43.44	Yes	<0.0001

Supplementary Table 9 I Statistical analysis for Supplementary Fig. 2: comparison of the effects of antigen retrieval and ExR procedures on total signal volume within and outside of synaptic puncta.

To compare the total volume within dilated reference ROIs of antigen retrieval- and non antigen retrieval-treated synapses, Sidak's multiple comparisons test was run to compare the means samples treated with ExR and samples treated with antigen retrieval followed by pre-expansion staining (AR) in the foreground or background, as defined by the reference channel, in GraphPad Prism. 2-way ANOVA was run in GraphPad Prism using Type III sum of squares.

i) 2-way ANOVA table for Ca_v2.1.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	3664	3	F (3, 232) = 0.5364	P=0.6578
Antigen retrieval	3496	1	F (1, 232) = 1.536	P=0.2165
Pre/post-expansion staining	9616433	3	F (3, 232) = 1408	P<0.0001
Residual	528212	232		

ii) Sidak's multiple comparisons test (two-sided) on the difference between decrowding (ExR) and antigen retrieval followed by pre-expansion staining (AR) for Ca_v2.1, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	458.9	420.1 to 497.7	Yes	<0.0001
Background	0.6667	-38.18 to 39.51	No	>0.9999

iii) 2-way ANOVA table for Homer1.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	9272076	3	F (3, 232) = 68.37	P<0.0001
Antigen retrieval	2924938	1	F (1, 232) = 64.71	P<0.0001
Pre/post-expansion staining	635644935	3	F (3, 232) = 4687	P<0.0001
Residual	10487186	232		

iv) Sidak's multiple comparisons test (two-sided) on the difference between decrowding (ExR) and antigen retrieval followed by pre-expansion staining (AR) for Homer1, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	285.7	112.6 to 458.8	Yes	<0.0001
Background	-124.6	-297.7 to 48.48	No	0.4955

v) 2-way ANOVA table for PSD95.

Factor	SSE	DF	F (DFn, DFd)	Pr(>F)
Interaction	18508762	3	F (3, 232) = 266.9	P<0.0001
Antigen retrieval	8905439	1	F (1, 232) = 385.3	P<0.0001
Pre/post-expansion staining	667591009	3	F (3, 232) = 9628	P<0.0001
Residual	5362087	232		

vi) Sidak's multiple comparisons test (two-sided) on the difference between decrowding (ExR) and antigen retrieval followed by pre-expansion staining (AR) for PSD95, grouped by location inside dilated reference ROIs (foreground) or outside dilated reference ROIs (background).

Group	Mean Diff.	95.00% CI of diff.	Significant?	Adjusted P Value
Foreground	4676	4552 to 4800	Yes	<0.0001
Background	690.8	567.0 to 814.6	Yes	<0.0001

Supplementary Table 10 | List of chemicals, antibodies, and gel solution of ExR.

i) List of chemicals

Product Name	Vendor	Product Number
Sodium acrylate	Santa Cruz	CAS7446-81-3
Acrylamide	Sigma	A9099
N,N'-Methylenebisacrylamide (BIS)	Sigma	M7279
Ammonium persulfate (APS)	Sigma	A3678
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma	T7024
4-Hydroxy-TEMPO (HT)	Sigma	176141
6-((acryloyl)amino)hexanoic Acid, Succinimidyl Ester (AcX)	ThermoFisher	A20770
Sodium dodecyl sulfate (SDS)	Sigma	436143
Sodium Chloride (NaCl)	ThermoFisher	AM9760
Tris Buffer	Fisher scientific	77-86-1
Paraformaldehyde	Electron Microscopy Sciences	15710
Triton X-100	Sigma	X100
Glycine	Sigma	50046
PBS 10x	ThermoFisher	70011044
Normal Donkey Serum	Jackson ImmunoResearch	017-000-121
Sodium citrate dihydrate	Sigma	W302600

ii) List of antibodies

Primary / Secondary	Target	Host	Vendor	Product number	Dilution
Primary	Cav1.2	Guinea pig	Synaptic Systems	152 205	1:200
Primary	RIM1/2	Rabbit	Synaptic Systems	140 203	1:200
Primary	PSD95	Mouse	ThermoFisher	MA1-046	1:200
Primary	PSD95	Rabbit	Cell Signaling Technology	CST3450S	1:200
Primary	SynGAP	Rabbit	ThermoFisher	PA1-046	1:200
Primary	Homer1	Rabbit	Synaptic Systems	160 003	1:200
Primary	Bassoon	Rabbit	Synaptic Systems	141 003	1:200
Primary	Shank3	Guinea pig	Synaptic Systems	162 304	1:200
Primary	A β 42 (6E10)	Mouse	BioLegend	SIG39320	1:200
Primary	A β 42 (12F4)	Mouse	BioLegend	SIG39142	1:200
Primary	A β 42 (D54D2)	Rabbit	Cell Signaling Technology	CST8243S	1:200
Primary	SMI	Chicken	Abcam	ab4680	1:400
Primary	Kv7.2	Mouse	Santa Cruz	sc-271852	1:200
Primary	Nav1.6	Rabbit	Abcam	ab65166	1:200
Primary	SynapsinI	Rabbit	Abcam	ab8	1:200
Primary	GFP	Rabbit	ThermoFisher	A-11122	1:200
Secondary	Mouse	Goat	ThermoFisher	A28175 (Alexa Fluor 488 nm)	1:200
Secondary	Mouse	Goat	ThermoFisher	A11031 (Alexa Fluor 546 nm)	1:200
Secondary	Mouse	Donkey	Biotium	20124 (CF 633 nm)	1:200
Secondary	Mouse	Donkey	ThermoFisher	A10036 (Alexa Fluor 546 nm)	1:200
Secondary	Rabbit	Goat	ThermoFisher	A11034 (Alexa Fluor 488 nm)	1:200
Secondary	Rabbit	Goat	ThermoFisher	A11035 (Alexa Fluor 546 nm)	1:200
Secondary	Rabbit	Goat	ThermoFisher	A10520 (Cyanine3)	1:200
Secondary	Rabbit	Donkey	Biotium	20125 (CF 633 nm)	1:200
Secondary	Rabbit	Donkey	ThermoFisher	A10040 (Alexa Fluor 546 nm)	1:200
Secondary	Guinea pig	Donkey	Biotium	20171 (CF 633 nm)	1:200
Secondary	Chicken	Goat	ThermoFisher	A11039 (Alexa Fluor 488 nm)	1:200
Secondary	Chicken	Donkey	Biotium	20168 (CF 633 nm)	1:200

iii) Monomer solution of ExR

Component	Stock Concentration*	Amount (mL)	Final Concentration*
Sodium acrylate	38	9	8.6
Acrylamide	50	2	2.5
Sodium Chloride	29.2	16	11.7
PBS	10x	4	1x
Water	-	3.6	-
Total	-	34.6	-

* All concentrations in g/100 mL except PBS

iv) Gel solution of ExR

Chemical	Stock Concentration (g/100 mL)	1st gel solution (μL)	Re-embedding solution (μL)	3rd gel solution (μL)
Monomer	-	864	-	864
Acrylamide	50	-	275	-
Bis acrylamide	2	40	18.75	20
Water	-	36	701.25	111
4-HT	0.5	20	-	-
TEMED	10	20	2.5	2.5
APS	10	20	2.5	2.5
Total (mL)	-	1	1	1