Ultrahigh-resolution imaging reveals formation of neuronal SNARE/Munc18 complexes in situ

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Membrane fusion is mediated by complexes formed by SNAP-receptor (SNARE) and Secretory 1 (Sec1)/mammalian uncoordinated-18 (Munc18)-like (SM) proteins, but it is unclear when and how these complexes assemble. Here we describe an improved two-color fluorescence nanoscopy technique that can achieve effective resolutions of up to 7.5-nm full width at half maximum (3.2-nm localization precision), limited only by stochastic photon emission from single molecules. We use this technique to dissect the spatial relationships between the neuronal SM protein Munc18-1 and SNARE proteins syntaxin-1 and SNAP-25 (25 kDa synaptosome-associated protein). Strikingly, we observed nanoscale clusters consisting of syntaxin-1 and SNAP-25 that contained associated Munc18-1. Rescue experiments with syntaxin-1 mutants revealed that Munc18-1 recruitment to the plasma membrane depends on the Munc18-1 binding to the N-terminal peptide of syntaxin-1. Our results suggest that in a primary neuron, SNARE/SM protein complexes containing syntaxin-1, SNAP-25, and Munc18-1 are preassembled in microdomains on the presynaptic plasma membrane. Our superresolution imaging method provides a framework for investigating interactions between the synaptic vesicle fusion machinery and other subcellular systems in situ.

Intracellular trafficking as well as transmission of signals across cell membranes, such as release of neurotransmitters at neuronal synapses, is mediated by fusion of vesicles with target membranes. The energy required for membrane juxtaposition and fusion is provided by folding of cognate vesicular- and target-membrane SNAP (soluble NSF attachment protein) receptors (SNAREs) into tight helical bundles that bring the two lipid bilayers into close apposition (1). However, in vitro and in vivo neuronal SNAREs do not efficiently overcome transition barriers to proceed to full fusion, requiring the action of Secretory 1 (Sec1)/mammalian uncoordinated-18 (Munc18)-like (SM) proteins (2), as deletion of SM protein genes results in severe fusion defects in yeast (3), flies (4), worms (5, 6), and mice (7). Moreover, at a synapse the SNARE/SM protein fusion machinery is regulated by the Ca++ sensor synaptotagmin-1 (8) in coordination with complexin (9) to efficiently trigger neurotransmitter release (10, 11). Despite their central role, the exact mechanisms by which SM and SNARE proteins mediate fusion in vivo have been the subject of considerable debate.

At least four modes of interactions between SM proteins and SNAREs have been reported: (i) Many SM proteins bind to their cognate syntaxins via a conserved N-terminal peptide that is exposed in the SNARE complex, resulting in SM protein/SNARE complex assemblies (12–14); note that this interaction plays an important role when syntaxin is in either binary or tertiary SNARE complexes. (ii) In addition to engaging in the first mode, SM proteins involved in exocytosis (Munc18-1, -2, and -3) also bind to their cognate syntaxins in a closed configuration that kinetically impedes binary or ternary SNARE complex formation (15, 16) and that is largely independent of the interaction with the syntaxin N-peptide; note that this interaction also occurs for the L165A, E166A LE mutant of syntaxin that is predominantly open in solution (15). (iii) Munc18-1 binds to the neuronal ternary SNARE four-helix bundle, although the interaction with the syntaxin-1 N-peptide is essential for tight binding (17, 18), whereas Sec1p in yeast (but not the corresponding SM proteins in other organisms) also binds to assembled SNARE complexes, but independent of the syntaxin N terminus (19). (iv) The homotypic fusion and vacuole protein sorting (HOPS) complex containing the SM protein vacuolar protein sorting-associated protein 33 (VPS33) binds to its cognate SNARE complexes by an unknown mechanism that may involve interactions of other components of the HOPS complex with the SNARE. Of these interactions, only (i) is shared among several diverse fusion reactions, and all SM proteins are known to bind to assembled SNARE complexes.

Although these interactions are well characterized in vitro and are likely to occur in vivo, it is unknown especially for synaptic exocytosis when during fusion Munc18-1 associates with SNARE complexes and when and how the initial association with the closed syntaxin-1 conformation allows SNARE complex formation. Results from coinmunoprecipitation or biochemical fractionation assays, often used to characterize protein–protein interactions, can be skewed due to formation of inappropriate complexes after detergent solubilization, dissociation of unstable SM complexes, and other artifacts which may occur when assays are performed under detergent conditions.

Significance

Synaptic vesicle fusion is catalyzed by multiprotein complexes that bring two lipid bilayers into close opposition. Several assembly mechanisms have been proposed for the synaptic vesicle fusion machinery, but exactly how these proteins interact in vivo remains unclear. We developed two-color fluorescence nanoscopy to directly visualize molecular interactions in situ and discovered that syntaxin-1, SNAP-25, and Munc18-1 (mammalian uncoordinated-18), three essential components for neurotransmission, closely colocalize on the plasma membrane, suggesting possible pathways for SNARE-mediated membrane fusion. Our superresolution method provides a framework for delineating the molecular underpinnings of the synaptic vesicle fusion machinery.


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complexes, and other artifacts, whereas electron-microscopic analysis of immunogold particle distributions is cumbersome and not always easy to interpret.

Modern subdiffraction fluorescence imaging techniques (20) could overcome such caveats to gain insights into the native organization of SM and SNARE proteins in situ. Measurements of relative positions of fluorescent probes used for labeling the sample (21) can provide structural information accurate down to the molecular scale, achieving subnanometer precision and accuracy, significantly below the few-nanometer size of a protein molecule. Imaging extended structures by localizing multiple closely spaced fluorophores typically can achieve a remarkable ~10-fold improvement over conventional microscopy—with an effective full-width at half-maximum (FWHM) resolution of \(d \approx 20-40\) nm in the focal plane of a single lens (22–24) and \(d \approx 10-20\) nm for the combined focal plane of two opposed objective lenses (25–27). Despite these notable advances, however, the ultimate potential of such techniques in deciphering molecular structures and interactions inside a cell has yet to be fully realized.

Although single photoswitchable dyes are bright enough to be theoretically localized to a few nanometers, systematic effects such as apparatus drift (22–24) often result in a significantly worse experimentally obtainable resolution. Additionally, several multicolor proof-of-concept experiments have been described in systems with well-defined spatial organization (28–33), in which interpreting images of a priori known structures has been straightforward. However, due to experimental inaccuracies in registering multicolor single-molecule localizations and the lack of an analytical framework to extract biologically meaningful information from such data, colocalizing two irregularly distributed protein species on length scales below ~100 nm, down to the length scales associated with formation of distinct molecular complexes, has proved technically challenging.

Here we extend a previously reported (21) dual-color super-resolution imaging approach and demonstrate two-color subdiffraction imaging based on photoswitchable probes with photon-noise–limited \(d \approx 7.5\) nm FWHM resolution and with ~3-nm registration accuracy extended over cellular length scales (~15 \(\mu\)m). With these unique capabilities we assay nanometer-scale correlations between two endogenous intracellular protein complexes, has proved technically challenging. Under stochastic switching conditions, each Cy5 created a Lorentzian distribution of \(x\) points of FWHM \(2\sqrt{2}\sigma_x \approx 2.82\sigma_x = 9\) nm (Fig. 1L), as expected from the total signal and background counts (SI Methods and Fig. S2), demonstrating that our active stabilization approach successfully eliminates systematic errors due to drift. For standard photoactivated localization microscopy (PALM)/stochastic optical reconstruction microscopy experiments without feedback stabilization, the achieved ddsDNA and E-cadherin dimers. For cellular imaging, the long-term stability achieved, as evidenced by tracking an out-of-loop fiducial as reference, is 1.5 nm in \(xy\) and <4.0 nm in \(z\) (rms) over \(>1\) h (Fig. S1). Notably, fluorescent spheres or gold nanoparticles that are imaged on the same CCD used for single-molecule detection can also serve as fiducials (Fig. S1), achieving similar long-term stability (1.5 nm in \(xy\) and 8 nm in \(z\), rms) while allowing flexible feedback stabilization strategies.

Two popular subdiffraction imaging benchmarks demonstrate the improved resolution afforded by our actively stabilized system. To characterize the effective point-spread function we imaged short (20-bp) DNA duplexes containing a Cy5 fluorescent probe at the 3’ end of one strand, whereas the other end of the duplex was attached to a coverslip through biotin–streptavidin interactions. Under stochastic switching conditions, each Cy5 molecule had a Lorentzian distribution of \(x\) points of FWHM \(2\sqrt{2}\sigma_x \approx 2.82\sigma_x = 9\) nm (Fig. L4), as expected from the total signal and background counts (SI Methods and Fig. S2), demonstrating that our active stabilization approach successfully eliminates systematic errors due to drift. For standard photoactivated localization microscopy (PALM)/stochastic optical reconstruction microscopy experiments without feedback stabilization, the achieved

![Image](https://via.placeholder.com/150)

**Fig. 1.** Superresolution imaging of point-like (20 bp dsDNA) and one-dimensional (actin filaments) objects. (A) \(xy\) clusters of localization points for individual Cy5 molecules attached to the surface-tethered DNA. The clusters for each dye molecule were aligned by their respective center of mass and superimposed. Lorentzian fits through the distributions show \(2\sqrt{2}\sigma_x = 8.8\) nm and 9.3 nm FWHM resolution in \(x\) and \(y\), respectively. (B) Refinement of the \(xy\) distribution by selecting progressively better-localized molecules increases the effective resolution down to \(d_{eff} = 2.35\) ~ 7.5-nm FWHM (\(\sim\) 3.2 nm). Selecting random subsets of the localization points does not result in increased resolution (gray curve). (C) (Top) Diffraction-limited image of an Alexa 647-phalloidin decorated filament. (Scale bar: 1 \(\mu\)m) (Middle) Localization points (centers of observed spots) for the filament in Top (note different scales in \(x\) and \(y\)). (Bottom) Close-up of red rectangle in Top. The red line is a fifth-order polynomial fit. (D) Refinement of \(xy\) distributions by keeping progressively better-localized dye molecules decreases the measured filament width down to \(d \approx 2.35\) ~ 11-nm FWHM (\(\sim\) 4.5 nm).

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**Results**

**Actively Stabilized Superresolution Fluorescence Imaging at the Photon-Noise Limit.** We used active feedback systems that stabilize the position of the microscope stage in 3D during super-resolution data acquisition (21), thus eliminating systematic localization errors due to long-term drift (Fig. S1). Microspheres (0.5 \(\mu\)m) attached on the coverslip or approximately circular submicrometer features of cellular structures were used to obtain an accurate position of the sample by real-time processing their bright-field images on a sensitive CCD camera (Fig. S1). Such high-contrast fiduciary marks were tracked to a few nanometers rms at 5 Hz (Fig. S1) whereas a digital feedback loop implemented in the software controlled a three-axis piezoelectric stage to lock the \(xyz\) coordinates at the desired set point. Using a 0.5-\(\mu\)m bead as reference, we demonstrated (21) subnanometer localization precisions for Cyanine-5 (Cy5)/Alexa 647 attached to...
resolution is significantly worse (23, 29, 34) than with our stabilization system (see also Fig. S1, showing how Cy5-DNA positions cannot be corrected as well if the microscope is allowed to drift).

We note that because each Cy5 molecule undergoes multiple switching cycles, further improvement in the resolution is possible by taking into account only those cycles during which a dye emitted enough photons to be localized better than a certain error cutoff (SI Methods), improving the precision to $s = 3.2$ nm [corresponding to $d = 2 \sqrt{2\ln 2} \times 7.5$-nm FWHM resolution for a Gaussian peak, Fig. 1B]. Although decreasing the precision cutoff yields more accurate localization of the dye molecules, there is a trade-off because some dye molecules and hence some features of the reconstructed image may be missed with a too stringent precision cutoff (Fig. S3).

As a further test, we imaged 8-nm-thick actin filaments that were decorated by Alexa 647-labeled phalloidin. At length scales of 50–500 nm, each actin filament appeared slightly curved (Fig. 1C), consistent with an expected persistence length of ~10–20 μm (35). We fitted the localization points for contiguous 1- to 2-μm-long segments to a low-degree polynomial. The apparent rms width of each such segment (deviation of points from the fitted curve) was $\sigma_0 \sim 6$ nm, due to the smaller photon count in each on cycle for Alexa 647-phalloidin vs. DNA-attached Cy5. However, the elimination of systematic errors in our apparatus allows us to obtain a measurement of the filament width down to $s = 4.6$-nm rms ($d \sim 11$-nm FWHM, Gaussian peak) by refining the distributions on the basis of 20% of the points that were localized better than 3 nm (Fig. 1D).

**SNAP-25 and Syntaxin-1 Form Clusters of <100 nm on the Plasma Membrane.** The ~10-nm FWHM focal-plane optical resolution afforded by our technique can be extended to imaging of cellular ultrastructure in situ. We imaged the distribution of the endogenous SNARE proteins SNAP-25 and syntaxin-1 in cultured neurons by direct immunofluorescence (Figs. 2 and 3). In our measurements, individual primary antibodies labeled with Alexa 647 appeared as distinct clusters of localization points with rms widths of $\sigma_0 = 4.5$ nm (13-nm FWHM).

Strikingly, the distribution of antibodies along axons was not uniform; often we observed clusters of antibodies in close proximity (Fig. 2C and Fig. S4), suggestive of organization of SNAP-25s in discrete \( \leq 100\)-nm-sized domains. We verified that the clustered pattern of localization points was not due to incomplete data acquisition by repeatedly imaging the same region of interest and comparing successive datasets (Fig. S4). Also, saturation of available epitopes rules out incomplete staining (Fig. S4). Finally, in our experiments syntaxin-1 and SNAP-25 appeared clustered irrespective of the exact fixation protocols used (formaldehyde vs. -20 °C methanol).

Previous subdiffraction optical studies in neurons (31, 36) and rat adrenal gland pheochromocytoma (PC12) cells (37, 38), using stimulated emission depletion (STED) imaging in both fixed and live specimens, have also indicated the presence of nanometer-sized clusters for a variety of membrane or membrane-associated proteins, including syntaxin-1 and SNAP-25. Notably, although sequestration of such proteins seems to be a general paradigm, the resolution (30- to 80-nm FWHM) of those experiments was inadequate to directly resolve multiple copies of a protein in each nanodomain or to directly reveal details of the architecture of such domains. Rather, clustering was exclusively inferred by the apparent increase (less than twofold vs. isolated antibodies) in the size (37) and/or intensity (36) of resolution-limited spots in the STED images. In contrast, our observations at fivefold higher spatial resolution, akin to electron microscopy of direct or silver-enhanced immunogold staining (39), resolve individual antigens in close proximity. A recent localization-based imaging approach (40) also provided evidence for syntaxin-1 and SNAP-25 clustering in PC12 cells; however, the effects of multiple fluorophore localizations, the use of secondary vs. primary antibodies, and the actual experimentally obtainable resolution were not quantitatively characterized. Thus, the positions of individual molecules could not be unambiguously resolved.

We determined the SNAP-25 and syntaxin-1 distributions in cultured neurons, using our superresolution approach. The pair-distribution function $g(x) = \langle \rho(X)\rho(x-X)\rangle/\rho^2$, where $\rho(x) = \sum_\delta(x-x_\delta)$ and $\rho = \langle \rho(x) \rangle$, reports the density profile of the localization points centered on each point $x = (x, y)$. For a random distribution of points, $g(x) = 1$, whereas $g(x) > 1$ if clustering occurs (41, 42). For both SNAP-25 and syntaxin-1, the radial profile $g(r)$, based of the estimated $(x, y)$ coordinates of each single antibody, decays over a short distance from the origin, indicating a characteristic cluster size of radius <100 nm (Fig. S5). We note that the ability to localize individual molecules within clusters could enable a more thorough analysis of the physicochemical forces that drive membrane protein inhomogeneities. Here we focus on the development of two-color imaging capabilities and the characterization of the associations of distinct molecular species.

**A Fraction of Munc18-1 Is Associated with SNAP-25/Syntaxin-1 Membrane Clusters.** We probed interactions between two intracellular proteins at nanometer scales, using simultaneous two-color
superresolution imaging of Alexa 647 and Atto 532. Using two spectrally distinct fluorescent probes ensures negligible crosstalk between detection channels, enabling unambiguous identification of each detected molecule. We extended our previous two-color registration method (21) to calibrate the whole field of view of the CCD with an accuracy of ~3 nm (Fig. S6). Achieving accurate registration between the two detection channels was essential to measure the relative distribution of Munc18-1 and t-SNAREs at nanometer scales.

In conventional diffraction-limited confocal images, both SNAP-25 and syntaxin-1 as well as Munc18-1 appeared along the length of axons, following almost identical distributions (Fig. 3 A and E). In two-color superresolution images, the two proteins form small clusters, with a fraction of the clusters containing localization points from both species (Fig. 3 B and F). We performed a statistical analysis to measure the degree of overlap between SNAP-25 and syntaxin-1. Similarly to $g(x)$ for a single species, the cross-species pair-distribution function $g_{ij}(r)$, with $r = \rho(x)\rho(X - x)/\rho(x)\rho(X - x)$, reports the density profile of species $i$ centered on molecules of species $j$. For two completely randomly intermixed species we expect $g_{ij}(r) = 1$, whereas if the two species associate at short scales, $g_{ij}(r) > 1$ toward the origin.

The cross-correlation function for pairwise combinations of Munc18-1, SNAP-25, and syntaxin-1 showed a peak at the origin, indicating that these three proteins are associated at length scales <100 nm (Fig. 3 C and G). The radial profile $g_{ij}(r)$ has a roughly exponential decay $g_{ij}(r) \sim \exp(-r/\xi)$, with $\xi \sim 80$–100 nm for the various combinations (Fig. 3 D and H).

The pair-distribution analysis provides only a statistical measure of correlations averaged over the whole dataset. Inspection of the two-color superresolution images suggests that the syntaxin-1, Munc18-1, and SNAP-25 clusters overlap. We performed a local-density–based analysis (43, 44), calculating for each SNAP-25 localization point $i$ the radii (core distances) $cdist_{GG}$ and $cdist_{GR}$ of neighborhoods centered on $i$ that contain SNAP-25 or syntaxin-1 clusters of size $Minpts$ (minimum number of points required forming a cluster). An ordered plot of $cdist_{GG}$ and $cdist_{GR}$ reveals very similar density-based underlying clustering structures. Also, $cdist_{GG}$ and $cdist_{GR}$ are significantly correlated for a range of cluster sizes $Minpts \in [2, 19]$ (Pearson’s $r = 0.3-0.5$, $P$ value negligible) (Fig. S5).

The density-based analysis allows further quantification of the degree of clustering and colocalization between syntaxin-1, SNAP-25, and Munc18-1. The majority (67–75%) of syntaxin-1, SNAP-25, or Munc18-1 is assigned to clusters that contain on average $N \sim 10$ detected molecules within an area of less than 100 nm, whereas 49% of syntaxin-1 and 37% of Munc18-1 molecules overlap with the nearest SNAP-25 cluster, and 62% and 34% of SNAP-25 overlap with the nearest syntaxin-1 and Munc18-1 cluster, respectively (Fig. S7). The observed cross-correlation originates from spatial overlap of clusters that contain multiple copies of syntaxin-1, Munc18-1, and SNAP-25. Munc18-1 is thus likely recruited to plasma membrane SNAP-25 clusters through interactions with syntaxin-1.

The ability to measure spatial correlations between two proteins at nanometer scales allows further analysis of the Munc18-1–syntaxin-1–SNAP-25 interactions, using mutagenesis. Syntaxin-1, though to be the main binding partner of Munc18-1, exists in two isoforms, syntaxin-1A and -1B. In neurons from syntaxin-1A knockout mice (45), the level of Munc18-1 immunostaining was slightly reduced (approximately twofold), but the overall distribution and the relationship to SNAP-25 remained similar to the WT case (Fig. 4A). Observed with two-color superresolution imaging, the cross-correlation between Munc18-1 and SNAP-25 persisted (Fig. 4 D and E), indicating a redundant role for the two syntaxin-1 isoforms.

Because direct interactions between Munc18-1 and SNAP-25 do not occur in vitro (46), we used the observed nanometer correlation between Munc18-1 and SNAP-25 as a reference for dissecting the finer details of the tripartite Munc18-1, syntaxin-1, and SNAP-25 association. Knockdown of syntaxin-1B on the syntaxin-1A knockout background (47) resulted in a cross-correlation function for Munc18-1–SNAP-25 that showed no peak at the origin, indicating a complete loss of association (Fig. 4B).
Additionally, the overall pattern of Munc18-1 in confocal images as well as in superresolution images often showed reduced overlap with SNAP-25 staining and proportionally higher cell-body vs. axonal Munc18-1 localization (Fig. 4 B and C). As shown in the cross-correlation maps \( g(x, y) \) (D and F) and the radial pair distribution functions \( g(r) \) (E and G), the red line is an exponential fit with \( \xi = 99 \) nm. The Munc18-1–SNAP-25 correlation persists in the syntaxin-1A-null neurons (D and E; control shRNA), but is lost upon additional knockdown of syntaxin-1B (F and G).}

**Fig. 4.** Loss of Munc18-1–SNAP-25 colocalization upon deletion of both syntaxin-1 isoforms. (A and B) Overlaid confocal images of Munc18-1 (red) and SNAP-25 (green) from syntaxin-1A-null neurons infected with control (A) and syntaxin-1B shRNA (B) viruses. Significant Munc18–SNAP-25 overlap remains in the control (A) but is lost upon syntaxin-1B knockdown (B). (C) Xy-scatter plots of the Munc18-1 (red dots) and SNAP-25 (green dots) superresolution localizations show that upon deletion of both syntaxin-1 isoforms, SNAP-25 remains localized on the membrane whereas Munc18-1 is mostly distributed in the cytoplasm. The Munc18-1–SNAP-25 correlation persists in the syntaxin-1A-null neurons (D and E; control shRNA), but is lost upon additional knockdown of syntaxin-1B (F and G). As shown in the cross-correlation maps \( g(x, y) \) (D and F), and the radial pair distribution functions \( g(r) \) (E and G), the red line is an exponential fit with \( \xi = 99 \) nm. (H) Relative Munc18-1–SNAP-25 correlation amplitude \( g(r = 0) \) for measurements from syntaxin-1A-null neurons obtained from \( n = 2 \) independent mouse litters (\( n = 19 \) and \( n = 18 \) regions of interest for control and knockdown, respectively). Boxes, SE; whiskers, 10th–90th percentile; crosses, min–max. The correlation is significantly reduced upon additional syntaxin-1B knockdown (\( P = 0.006, \) one-way ANOVA). (Scale bars: 32 \( \mu m \) (A and B), 1 \( \mu m \) (C), and 100 nm (D and F).)

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Fig. 5. Rescue of Munc18-1–SNAP-25 cross-correlation by syntaxin-1A overexpression. (A) $xy$-scatter plots of superresolution localization of Munc18-1 (red) and SNAP-25 (green) in syntaxin-1A-null neurons that were infected with a virus encoding shRNA for mouse syntaxin-1 and overexpressing a rat syntaxin-1A. Overexpression of rat syntaxin-1A rescues the Munc18-1–SNAP-25 cross-correlation at nanometer scales, as shown in the cross-correlation map $g(x, y)$ (B) and pair-distribution function $g(r)$ (C). The red line is an exponential fit with $r = 56$ nm. (D) The syntaxin-1 N-peptide is important for recovering Munc18-1–SNAP-25 cross-correlation. Shown is the relative Munc18-1–SNAP-25 correlation amplitude $g(r = 0)$ for measurements from syntaxin-1A-null neurons obtained from $n = 2$ independent mouse litters ($n = 28, 14, 18$, and $21$ regions of interest for N-terminal deletion rescue, wild-type rescue, LE rescue, and knockdown respectively). Boxes, SE; whiskers, 10th–90th percentile; crosses, min–max. The correlation is significantly higher for WT rescue compared with the knockdown and the NTD rescue and for LE rescue compared with the knockdown ($P = 0.018, P = 0.044, and P = 0.022$, respectively, one-way ANOVA). (Scale bars: 1 μm (A) and 100 nm (B).) (E) A dynamic equilibrium model for Munc18-1, SNAP-25, and syntaxin-1 associations on the plasma membrane. Wild-type syntaxin-1 (yellow) can interconvert between (Top Left) an open conformation that can associate with SNAP-25 (green) and can bind Munc18-1 (gray) in an N-peptide-dependent configuration outside the sites of fusion but rather that the N-peptide-dependent tripartite open-syntaxin-1/SNAP-25/Munc18-1 state is significantly populated. After Munc18-1 recruitment, a fusion-competent complex can be formed with the addition of synaptobrevin-2 (red). Munc18-1 can participate in fusion pore opening by interacting with the ternary SNARE complex.

Discussion

Fluorescence Nanoscopy with Photon-Limited Spatial Resolution.

Dissecting the complex molecular assemblies and interactions that regulate neurotransmitter release has been hindered by the unavailability of high-resolution/high-sensitivity in situ imaging tools. Here we demonstrate fluorescence nanoscopy of biological specimens at ambient conditions down to the photon-noise limit of $d \sim 7.5$-nm FWHM resolution (3-nm localization precision). The performance achieved is an approximately two- to threefold improvement compared with conventional single-marker switching approaches (22) that demonstrated $\sim 20$-nm FWHM resolution benchmarks using similar fluorophores, photoswitching schemes, and photon efficiencies to those used here (23, 29) and that represent a widely adopted opto-mechanical platform for superresolution microscopy implementations. Our results are a unique example of using superresolution cross-correlation analysis and mutagenesis to characterize details of protein–protein interactions in vivo.

Higher resolution requires minimizing systematic effects while maximizing the photon budget. Three recent implementations that collect twice as many photons by imaging the sample using two opposed objective lenses have shown focal plane FWHM resolutions $\sim 10$ nm (27) and $\sim 20$ nm (25, 26), respectively. Faster acquisition reduces the effect of long-term instabilities and ref. 26 reports an $\sim 2$-nm short-term stability; however, the faster on–off dye cycling might have limited the photon budget and thus the obtainable resolution. Brighter fluorophores enable higher resolution but require correspondingly longer acquisition times, thus resulting in correspondingly higher susceptibility to instrument drift, and although the extra improvement in ref. 27 is...
noteworthy, this performance is significantly worse than the theoretical limit. Our active stabilization scheme is simpler and more efficient than these alternative approaches, while, more importantly, minimizing systematic errors due to long-term drift and achieving a currently unique to our knowledge performance very close to the theoretical resolution limit (Table S1).

In addition to our active feedback system, further increases in the raw resolution is possible. We note that with additional optimization of fluorescent probes (50) and/or switching kinetics (51), for \( \sim 10^5 \) collected photons in each on cycle, localization accuracy down to \( \sim 0.5 \text{ nm} \) (full width at half maximum) is possible in our experimental apparatus (21), although statistical errors of \( \sim 0.2 \text{ nm} \) in the interprobe distances could be achieved by repetitive measurements over multiple (e.g., \( \sim 10 \)) successive on cycles. Further refinement of the sample preparation procedures to achieve dense labeling, possibly via genetically encoded (50), enzymatically incorporated (52), or chemically attached (53) fluorescent tags, would further use the potential of our approach to reveal the exact molecular-scale architecture of subcellular structures.

We also developed optimized calibration standards and algorithms for two-color subdiffraction imaging, using spectrally separable fluorescent probes. Featuring negligible crosstalk and \( \sim 3 \text{-nm spatial registration accuracy over the size of a cell} \sim (15 \text{ μm}) \), our approach enables measuring the relative intracellular distributions of two distinct protein species. This result establishes a protocol for the unambiguous classification of structural elements and provides a general tool to analyze, in situ, interactions between protein components that form macromolecular assemblies. Our work significantly extends the scope of the early proof-of-principle demonstrations of well-characterized and spatially defined structures, like the cytoskeleton or clathrin cages, and discerns the unknown organization of irregularly distributed intracellular molecules.

Due to the demonstrated enhanced imaging performance, we foresee that refined approaches to carefully eliminate extraneous errors, such as the active feedback-stabilization and the two-color mapping calibration procedures we report here, will be used to improve the resolution of fluorescence “nanoscopy.” Importantly, deciphering molecular interactions on the basis of diffraction-limited imaging is often not possible as proteins that appear colocalized with a conventional microscope can be well separated on the nanoscale. The improved spatial resolution and multicolor registration accuracy afforded by our approach opens up the possibility for further applications to characterize intracellular protein–protein interactions and formation of defined molecular complexes in situ.

Association of Syntaxin-1, SNAP-25, and Munc18 at the Plasma Membrane. Here we show at 13-nm FWHM resolution that syntaxin-1, SNAP-25, and Munc18-1 are present in clusters on the plasma membrane, and we demonstrate in pairwise double-labeling experiments that these three proteins colocalize. This is to the best of our knowledge a unique two-color experiment demonstrating that syntaxin-1, SNAP-25, and SM proteins can be found in the same nanometer-sized clusters. The clusters have a size of \( 50–100 \text{ nm} \) and contain up to \( \sim 10 \) detected copies of each molecule (note that the actual number of molecules present may be higher, because steric hindrance can preclude efficient binding of multiple antibodies on closely packed complexes). We observed a striking colocalization of syntaxin-1, SNAP-25, and Munc18-1.

Assembly of the neurotransmitter release machinery during membrane fusion reactions is thought to proceed in several steps. Often, subsets of SNARE proteins can bind to each other in kinetically trapped, “dead-end” configurations, vivid examples being the 2:1 syntaxin-1–SNAP-25 (54, 55) complexes. Munc18-1 binds to closed syntaxin-1 in a binary complex that is thought to control the beginning of SNARE-complex assembly (45). Reconstitution experiments with Munc18-1, neuronal SNAREs, NSF, SNAP, and Munc13 suggest that the binary syntaxin-1/SNAP-25 complex is readily dissociated by NSF and SNAP, allowing Munc18-1 to capture the closed state of syntaxin, kinetically blocking assembly of binary or ternary SNARE complex (56). Upon action of Munc13 (57), presumably in conjunction with an approaching synaptic vesicle, binary or ternary SNARE complex formation is enabled, setting the stage for \( \text{Ca}^{2+} \)-triggered fusion. Specifically, when syntaxin opens up, the node of Munc18 binding to syntaxin is dependent on the syntaxin-1 N terminus for tight binding but does not require the autonomously folded three-helix “abc” bundle (Habc) domain anymore (14). Of these two binding modes, the first is observed only for SM proteins in exocytosis, whereas the second is generally present in many SM-protein/SNARE interactions. The second binding mode also involves a direct interaction of Munc18 with the four-helix bundle of the SNARE complex (17, 18). Munc18’s most important role, however, is probably in fusion directly by an as yet unknown mechanism because Munc18 and other SM proteins are generally essential for the fusion reactions in which they participate, more so often than synaptobrevin and SNAP-25 (7, 58), suggesting that their function is not that of a chaperone or SNARE booster, but truly intrinsic to the fusion process.

Because we observe a tripartite association of syntaxin-1, SNAP-25, and Munc18-1 in neurons, in principle, this suggests three possibilities: a close association of the syntaxin–Munc18-1 complex with SNAP-25, a complex involving the open conformation of syntaxin with both Munc18-1 and SNAP-25, or a dynamic equilibrium between these states. In either case, the Munc18-1 interaction with the N terminus of syntaxin would play a role: This interaction is essential for tight binding between Munc18 and the ternary SNARE complex (17, 48), whereas it strengthens the interaction between the closed conformation of Munc18 and syntaxin (48). This conclusion is further supported by our finding that deletion of the N terminus of syntaxin-1 largely abrogates the colocalization of Munc18-1 with SNAP-25 (Fig. 5D).

The observation that the Habc domain of syntaxin is not essential for exocytosis but that the N terminus is essential for both spontaneous and evoked release (47) does not directly rule out either state (Munc18–closed syntaxin-1 complex with SNAP-25 nearby or Munc18-1/syntaxin/SNAP-25 complex). However, our findings rescue the data with the Neuronal peptide deletion (NTD) mutant of syntaxin, which prevents tight binding of Munc18-1 to syntaxin-1 in a binary or ternary SNARE complex but not to closed monomeric syntaxin-1 (14), does not fully recover the Munc18/SNAP-25 cross-correlation (Fig. 5D), suggests that the observed tripartite Munc18, syntaxin-1, SNAP-25 associations involve binary or ternary SNARE complexes or a dynamic equilibrium involving these states.

Our results support the notion that in neurons, SNARE proteins are normally arranged in membrane patches, consistent with the overall subcellular organization of the neuronal plasma membrane, where channels and receptors are arranged into particular subdomains. This notion is surprising given that SNAREs effectively primarily function in presynaptic active zones, which coincide only with a tiny percentage if any of the patches containing SNAREs. Even though a single SNARE complex is sufficient to dock liposomes to membranes and promote spontaneous lipid and content mixing in vitro (59), conferring fast exocytosis in combination with the \( \text{Ca}^{2+} \)-sensor synaptotagmin requires more than one synaptobrevin molecule (60) and more than three SNAP-25 molecules (61). The organization of SNAREs in \( \leq 100 \text{-nm membrane domains}, \) similar to the size corresponding to individual presynaptic active zones, may enhance the efficiency and speed of synaptic vesicle fusion reaction.

A certain fraction of the clusters of Munc18-1, syntaxin-1, and SNAP-25 observed here likely contain binary (syntaxin-1/SNAP-25) or ternary (syntaxin-1/SNAP-25/synaptobrevin) SNARE complexes.
or they represent averages over dynamic states involving these complexes. It has been known for some time that SNARE and SM proteins involved in synaptic vesicle exocytosis are not actually enriched at sites of exocytosis (62), but the existence of structured clusters of SM/SNARE protein complexes throughout neurons is nevertheless surprising. It suggests that these complexes may operate in other fusion reactions, but more importantly indicates that the specificity and regulation of synaptic vesicle fusion operate at a level different from that of SNARE and SM proteins. How these clusters are formed and why SNARE and SM protein complexes are not randomly distributed in membranes remain unknown. A plausible hypothesis is that the biophysical properties of the phospholipid membrane with lipid subdomains containing enrichment of cholesterol may contribute to the formation of these clusters, but it seems unlikely that these clusters are only a consequence of such physicochemical forces and more likely that they represent the result of organizing proteins that are generally involved in controlling membrane fusion.

Given these previous observations, our finding that syntaxin-1 and SNAP-25 are abundantly present in a tripartite association with Munc18-1 is unexpected and raises a number of questions. It is unexpected because—as mentioned above—Munc18-1 also binds to syntaxin-1 in a different, independent mode, namely the closed conformation of syntaxin-1 before it assembles into any complex with another SNARE protein. Thus, it is surprising that our observations suggest that this complex might not be the only predominant complex outside of the sites of synaptic fusion—instead, we detected recruitment of Munc18-1 through a tripartite association with SNAP-25 and syntaxin that is N-peptide dependent (Fig. 5E). At the same time, our results independently validate the notion that Munc18-1 binding to the N-peptide of syntaxin-1 plays a central role in fusion as proposed previously (14, 17, 47, 49, 63–65).

Methods

Actively Stabilized Microscope. The two-color superresolution imaging setup is shown in Fig. 51. A feedback loop that tracked the bright-field image of a fiduciary in real time actuated a three-axis nanopositioning stage (Physik Instrumente; 561-3DD, E-710 controller) and locked the sample at a fixed x, y, z set point during acquisition data. For cellular imaging, in cases where no fiducials of high-enough contrast for z tracking existed in the field of view, we used an alternative focus-stabilization scheme (21), based on a dedicated stabilized near-infrared (NIR) laser beam that undergoes internal reflection (TIR) on the sample and is projected on a position-sensitive detector (Quadrant Photo-Detector, QPD).

Stochastic Single-Molecule Switching Imaging. We used ~5-kW/cm² and ~20-kW/mm² continuous-wave (CW) ~640-nm and 532-nm laser illumination for Cy5/Syntaxin 647 and Atto 532, respectively. We found that simultaneous illumination with both lasers resulted in irreversibly bleaching of the Alexa 647, as well as higher autofluorescence background in the red channel. Therefore, during cellular imaging, Alexa 647 was measured first for ~30 min, followed by ~40–50 min for Atto 532. The presence of an enzymatic oxygen scavenging system and a millimolar concentration of thiol enabled photoswitching (23, 66), each molecule undergoing a rapid transition to a dark state upon emitting a distribution of N0 mean collected photons (Fig. 52). To achieve the highest possible contrast ratio (molecules on: molecules off) we imaged Cy5/Syntaxin 647 without the presence of an activator dye (23, 29) or a high-power shorter-wavelength laser (34), allowing slow, spontaneous recovery of each molecule from the dark state (rate ~10⁷ s⁻¹). This cycle could be repeated several times, providing a cluster of localization points (centers of observed spots) from each on state for each molecule. Aligning such localization clusters from multiple molecules created a Lorentzian distribution of xy points with FWHM 2√2νσ ~ 2.828νσ (Fig. 5A, C) we used to parameterize the FWHM, as the SD for a Lorentzian distribution is infinite.)

Resolution Refinement. To determine the increased resolution afforded by keeping progressively more precisely localized molecules, only points in Fig. 1 A and C were kept for which the calculated precision ν was better than a certain cutoff νcut-off. The xy distributions (Fig. 1A) or the deviations from the fitted line (Fig. 1C) for the subset of points were fitted to a Gaussian peak. The refined resolution vs. νcut-off was determined from the fitted peak widths as d ~ 2.35. We note that in previous refinement attempts (22, 69) similar fractions of the data were kept (1–10%); however, the accuracy for the refined set of points was not quantitatively validated against a known structure. Practically, this procedure is expected to improve the resolution by approximately two- to threefold (SI Methods and Fig. 53) whereas in contrast, selecting a random subset of the original data does not improve the resolution.

Identification of Individual SNAP-25 Antibodies. To independently determine the superresolution image of individual Alexa 647-labeled SNAP-25 antibodies, we imaged a neuronal sample at dilute staining conditions, ensuring well-spaced antibodies. Because a single Alexa 647 dye can undergo multiple switching cycles, each SNAP-25 antibody appears as a cluster of xy localization points (approximately five points per hour of acquisition time). The FWHM of the distribution of points in each cluster is d = 13 nm, determined by aligning individual clusters by their center of mass (Fig. 2B).

Individual antibodies could also be identified as distinct clusters of Alexa 647 xy localization points in densely stained specimens (Fig. 2A). New antibodies could be readily resolved from the xy scatter plots, at minimum separations of ~30–50 nm, with <15-nm FWHM resolution (Fig. 54). To estimate a molecular probability density function ρ(x, y) we rendered each xy Alexa 647 localization point (Fig. 2C) as a normalized 2D Gaussian (22) of rms size given by Eq. 51a. Individual SNAP-25 antibodies were identified as local maxima of the resulting 2D ρ(x, y) surface (Fig. 2C), using a peak search algorithm. The xy coordinates of each SNAP-25 antibody were then determined from the centroid of ρ(x, y) around each peak.

Two-Color Registration over Extended Field of View. Two types of reference objects were used to register the coordinates between the two CCD channels: (i) 20–bp Cy3-Cy5 DNA duplexes, randomly distributed in the field of view, and (ii) a regular nanofabricated pattern of subwavelength holes on an Al-coated quartz wafer (SI Methods and Fig. 56). A set of reference coordinates obtained from multiple such objects and from sampling the whole field of view was used to obtain a mapping transformation (21), using a low-order polynomial or spline interpolation (SI Methods and Fig. 56).

Sample Preparation and Imaging Conditions. The preparations of DNA constructs, F-actin, dye-labeled antibodies, and neuronal cultures, as well as procedures and conditions for confocal immunofluorescence imaging, are detailed in SI Methods.

Quantitative Colocalization and Clustering Structure Analysis. We used the OPTICS algorithm (44) to perform a hierarchical ordering of the antibody localization points. For each point i we calculated core-distances (43) δcdist GA and δdist GA with respect to points of the same and opposite colors, respectively (Fig. 55). Clusters were identified using the DBSCAN algorithm (43), with Minpts = 3 and ε ~ 30–40 nm. The selected values for ε correspond to roughly the average core distances for the particular dataset; approximately twofold smaller ε values failed to identify all but the densest clusters whereas approximately twofold larger ε values resulted in merging all of the points into just a few large ones.

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Supporting Information

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SI Methods

Microscope Setup. The setup was built around an inverted optical microscope (Olympus IX-71) with an oil immersion (Nikon 60x N.A. = 1.49) objective lens. For fluorescence excitation we used an ~640-nm diode laser (Opnext) and a 532-nm solid-state laser (Melles-Griot), coupled into a single-mode optical fiber. For the separate focus-lock laser system we used a fiber-coupled 785-nm NIR diode pigtial (Thorlabs; LPS-PM785-FC). The diode was driven by a precision current source (Thorlabs; LDC201CU) and its temperature regulated with a thermoelectric cooler (Thorlabs; TED200C) to achieve stable emission.

In this implementation special care has to be exercised to ensure that the two separate detection systems (feedback stabilization CCD1 and superresolution imaging CCD2) remain registered during the data acquisition, e.g., as demonstrated with active optics for two-color imaging (1). In practice, we successfully used an open-loop feedback by relying on tight regulation of the room temperature ($\Delta T_{room} < 0.5$ °C) to passively maintain registration between the two detectors at $<3$ nm peak-to-peak over 1 h (Fig. S1). If present during prolonged acquisitions, the 1- to 2-nm residual drift in the data can be corrected in post-processing.

To achieve passive stability of the apparatus, we tuned the building temperature control feedback loop, regulating the temperature of the air entering the room with a reheat coil, to achieve less than 0.5 °C peak-to-peak variation over 1 d, using knowledge of feedback systems gained during the development of the microscope feedback controls. In addition, enclosing the microscope and all associated optical components further dampened fast temperature fluctuations (to $<0.1$ °C on the timescale of ~1 h) as well as mechanical disturbances from air currents.

The laser beams were combined using dichroic mirrors (Chroma) and delivered to the microscope with a polychroic mirror (Chroma) that reflected all laser beams but allowed transmission of emitted fluorescence. Either a blue (Thorlabs; M455L1, 455-nm emission peak) or a white light-emitting diode (Thorlabs; MCWHL1) facilitated simultaneous bright-field fluorescence imaging. The image on the side port of the microscope was split with a long-pass dichroic (Omega; 505DCLP) and the blue light was projected with relay lenses on a fast CCD (Andor; DV860DCS-BV, CCD1). The fluorescence image was further magnified, split by a long-pass dichroic with extended reflection (Chroma; 645DCXR), band-pass filtered to select cyanine-3 (Cy3) or Cy5 emission (Chroma; HQ570/50, HQ700/100), and projected onto two regions of the CCD (Andor; DV897CSO-BV, CCD2).

We note that real-time tracking of fiducials like luminescent gold nanoparticles or fluorescent microspheres imaged directly on CCD2 used for the single-molecule detection can also be used for feedback stabilization (Fig. S1A), significantly simplifying the instrument design, with the additional advantage that the feedback loop and the single-molecule localizations are inherently registered. The advantage of a separate position feedback system is higher loop bandwidth and thus higher stability, as well as the flexibility of separately optimizing the servo-loop and the fluorescence imaging parameters. Furthermore, by stabilizing the microscope and imaging all nearby molecules on the same CCD regions one can minimize the effect of CCD nonuniformity.

Single-Molecule Tracking. In each CCD frame, a 13 x 13 (pixels)$^2$ region of interest (ROI) centered around each molecule was fitted to a 2D Gaussian function to identify the center coordinates $\{x, y\}$, the width of the Point Spread Function in two orthogonal directions $\{s_x, s_y\}$, the peak height $A_0$, and background level $B$. Spots tracked over consecutive frames that appeared centered within a radius of 1 pixel were regarded as the same molecule and assigned the average values of $\{x, y\}$, $\{s_x, s_y\}$, $A_0$, and $B$ over all of the frames.

We estimated the number of photons $N$ collected from each molecule per switching cycle by the total counts over the 13 x 13 ROI (from all frames in that molecule’s stack, after background subtraction). Alternatively (for Fig. 2C only), we estimated $N$ from the 2D Gaussian fit as $n = 2\pi s^2 A_0$, where $s = \sqrt{\delta x \delta y}$. The precision $\sigma$ in the coordinates for each molecule was calculated on the basis of Eq. S1A, using an effective background term $n_{frame} b^2$ for a molecule tracked over $n_{frames}$ total frames and including a contribution of $b_0^2 = (5.2)^2$ photons/pixel from the CCD read-out electronic noise. Histograms for $N$ and $\sigma$ are shown in Fig. S2.

Derivation of the Photon-Noise-Limited Superresolved Point-Spread Function. Fluorescence photons encode information about the position of the emission source, with a single photon providing a spatial uncertainty of order the wavelength $\lambda$ (2). For fluorescence photons collected through the aperture of a microscope objective lens and imaged on a pixelated detector, with pixel size $a$ (in units of nanometers) and background rm noise in each pixel $b$ (number of photon counts), the statistical uncertainty $\sigma$ in the position of the fluorescence emitter is (3)

$$\sigma^2 = \frac{s^2 + a^2/12}{N} + \frac{8\pi s^2 b^2}{a^2 N^2}, \quad [S1A]$$

where $s$ (in nanometers) is the SD of the image point-spread function and $N$ is the total number of collected photons. The pixelation noise assumes a top-hat distribution of size $a$ and is valid when $s > a$ (3). We note that a more theoretical accurate treatment yields (4)

$$\sigma^2 = \frac{16 s^2 + a^2/12}{9 N} + \frac{8\pi(s^2 + a^2/12)^2 b^2}{a^2 N^2}, \quad [S1B]$$

For our experimental conditions the predictions of Eqs. S1A and S1B differ by <15% (Table S1). In each cycle, each dye molecule emits an exponentially distributed number of collected photons $N$,

$$D(N) = \frac{1}{N_0} e^{-\frac{N}{N_0}}, \quad [S2]$$

where $N_0$ is the mean number of collected photons. The distribution of the position uncertainty $\sigma$ in each cycle is $D(\sigma)d\sigma = -D(N)\frac{dN}{d\sigma} = -D(N)\frac{\sigma}{\sigma^2}$, thus assuming for simplicity no background and pixelation noise,

$$\frac{dN}{d\sigma} = -\frac{2\sigma^2}{\sigma^2} = -\frac{2N_0}{\sigma} \quad \text{and} \quad D(\sigma) = \frac{2\sigma_0}{\sigma} e^{-\frac{\sigma^2}{\sigma_0^2}}, \quad [S3]$$

where $\sigma_0 = \frac{\sigma}{\sqrt{N_0}}$. 

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The distribution of the measured \( x \) positions takes the form
\[
D(x) \propto \int_{-\infty}^{x} e^{-\frac{4}{\sigma^2} (x-x')^2} e^{-\frac{\sigma^2}{2}} dx = \int_{-\infty}^{x} e^{-\frac{4}{\sigma^2} x'^2} e^{-\frac{\sigma^2}{2}} dx = e^{-\frac{\sigma^2}{2} - \frac{4}{\sigma^2} x^2},
\]
and thus \( D(x) \) has a Lorentzian peak shape
\[
D(x) \propto \begin{cases} 
\frac{2\sigma^2}{x^2 + 2\sigma^2}, & \text{if } x < \sigma, \\
\frac{2\sigma^2}{x^2 + 2\sigma^2}, & \text{if } x > \sigma,
\end{cases}
\]
with full width at half maximum (FWHM) equal to \( 2\sqrt{2}\sigma_0 \approx 2.828\sigma_0 \).

**Mathematical Basis for Resolution Enhancement by Statistical Refinement of Localization Data.** By enforcing a cutoff \( \sigma_{\text{cutoff}} \) in the precision, the distribution of \( x \) positions becomes
\[
D(x) \propto \frac{2\sigma^2}{x^2 + 2\sigma^2} e^{-\frac{4}{\sigma^2} x'^2} e^{-\frac{\sigma^2}{2}} dx = e^{-\frac{\sigma^2}{2} - \frac{4}{\sigma^2} x^2},
\]
the product of a Lorentzian with FWHM equal to \( 2\sqrt{2}\sigma_0 \approx 2.828\sigma_0 \) and a Gaussian with FWHM equal to \( 2/\ln 2 \sigma_{\text{cutoff}} \approx 3.5\sigma_{\text{cutoff}} \). The FWHM can be expressed as
\[
\gamma(\nu) \equiv \text{FWHM}(D) / \sigma_{\text{cutoff}} = 2\sqrt{2}(2\nu^2\nu^2 - 2\nu^2),
\]
where the Lambert \( W \) function is defined as \( W(z)e^{W(z)} = z \) and the cutoff ratio \( \nu = \sigma_{\text{cutoff}} / \sigma_0 \). The refinement process when \( \sigma_{\text{cutoff}} < \sigma_0 \) results in final FWHM resolution of roughly \( \sigma_{\text{cutoff}} \gamma(\nu) \rightarrow 2.355 \) for \( \nu \gg 1 \), Fig. S3).

The ratio of the refined FWHM resolution over \( \sigma_0 \), \( \beta(\nu) \equiv \text{FWHM}(D) / \sigma_0 \gamma(\nu) / \nu \), decreases when a smaller \( \sigma_{\text{cutoff}} \) is chosen (Fig. S3). Meanwhile, the fraction \( \alpha \) of localizations kept,
\[
\alpha \equiv \int_{-\infty}^{x} D(\sigma) d\sigma \approx \int_{-\infty}^{x} \frac{2\sigma^2}{\sigma^2} e^{-\frac{4}{\sigma^2} x'^2} e^{-\frac{\sigma^2}{2}} dx = e^{-\frac{\sigma^2}{2}},
\]
vanishes rapidly with increasing \( \nu \) (Fig. S3). Thus, for a practical limit of \( \alpha \sim 0.1 - 1 \), two- to threefold resolution enhancement can be achieved over the initial Lorentzian FWHM of \( 2\sqrt{2}\sigma_0 \approx 2.828\sigma_0 \) can be obtained (Fig. S3).

**DNA Construct Preparation.** Modified DNA oligonucleotides (5’-Cy3-TTCCGGGCAAGCTCTCGGGGGGGA-Cy5-3’, 5’-biotin-AAAAAAAAACCGATCCCCCGAGCTGCCGGAATTC-CGG-3’) were synthesized and HPLC purified by Integrated DNA Technologies. To prepare duplexes we annealed the two complementary strands at 2 \( \mu \)M in 20 mM Tris-HCl (pH 7.9), 50 \( \mu \)M NaCl buffer at 95 °C for ~15 min following by cooling to room temperature over ~2–3 h. Note that the presence of Cy3 at one end of the ~7-nm-long DNA duplex does not affect Cy5 photoswitching at the other end.

**Modified Microspheres.** Biotin-PEG (NEKTAR; Biotin-PEG-mSBA, 5 kDa) was grafted at 10% wt/vol to 0.5-\( \mu \)m diameter aliphatic amine polystyrene spheres (IDC) in a 0.1-M sodium bicarbonate solution (pH ~ 9). After PEGylation, the beads were washed and stored in PBS at 4 °C.

**F-actin Polymerization.** G-actin (1:12 biotinylated G-actin; Cytoskeleton Inc.) was diluted to 0.4 mg/mL in 5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl2, 1 mM DTT, and 0.2 mM adenosine triphosphate (ATP). F-actin polymerization was initiated by addition of 6 \( \mu \)L of 500 mM KCl/20 mM MgCl2 and 5 \( \mu \)L of 100 mM ATP to 50 \( \mu \)L G-actin solution. After incubation at room temperature for ~2–3 h, 0.5 \( \mu \)L of 200 \( \mu \)M Alexa 647-Phalloidin (Invitrogen) was added and the formed filaments were stored at 4 °C until use.

**Sample Cells.** Grafiting of “cloudy-point” PEG on glass slides and coverslips, assembly into sandwich sample cells, and attachment of biotinylated beads and Cy5 duplexes were prepared as described in ref. 1. For F-actin attachment, after incubation of the PEG-biotin surface with streptavidin, formed actin filaments were diluted 1:100 in 1x PBS (pH 7.4) 1 mM MgCl2/0.2 mM ATP and bound to the surface for 5 min.

**Imaging Buffer.** For Cy5-DNA and SNAP-25 Alexa 647-immuno staining experiments, the oxygen-scavenging imaging buffer was 100 mM Hepes-KOH (pH 7.5), 160 mM KCl, 0.9% wt/vol glucose, 144 mM β-ME (Sigma), 1x glucose-oxidase/catalase enzyme stock, and 0.2% wt/vol BSA. β-ME appears to be stable under these conditions throughout the experiment (a few hours) as evidenced by efficient switching of the dyes. The composition of the imaging buffer was essential for achieving stable, oxygen-free conditions in the sample cell and efficient Cy5/Alexa 647 photoswitching. For the F-actin A647-phalloidin experiments the imaging buffer contained additionally 2 mM MgCl2 and 0.2 mM ATP.

**Wild-Type Primary Mouse Neuronal Cultures.** Animal protocols used in this study, as well as the overall mouse husbandry practices, were approved by the Institutional Animal Care and Use Committee at Stanford University. Cortices from newborn mouse pups were dissected in ice-cold Hank’s Balanced Salt Solution (HBSS), dissociated by trypsinization (0.05% trypsin-EDTA, for 10 min at 37 °C), triturated with a siliconized pipette in plating medium [MEM (Gibco) supplemented with 5 g/L glucose, 0.2 g/L NaHCO3 (Sigma), 0.1 g/L transferrin (Calbiochem), 0.25 g/L insulin (Sigma), 0.3 g/L glycosylated (Gibco), and 10% (vol/vol) FBS]. Dissociated cells were plated (100 \( \mu \)L) onto 12-mm coverslips, which were coated for ~30 min with Matrigel at 37 °C (BD Biosciences). Plating medium was replaced with growth medium [MEM (Gibco) containing 5 g/L glucose, 0.2 g/L NaHCO3 (Sigma), 0.1 g/L transferrin (Calbiochem), 0.3 g/L L-glutamine (Gibco), 5% FBS, 2% B27 supplement (Gibco), and 2 \( \mu \)M cytosine arabinoside (Sigma)] 24–48 h after plating. Neuronal cultures were used for immunofluorescence or immune-blotting/precipitation experiments at 14 d in vitro.

**Syntaxin-1A KO Neuronal Cultures.** Hippocampal neurons were cultured from neonatal syntaxin-1A KO mice, infected at 5–7 d in vitro (DIV5–7) with various lentiviruses, and analyzed at DIV13–16 essentially as described in ref. 5. Briefly, the mouse hippocampus was dissociated by papain digestion and plated on Matrigel-coated glass coverslips, and neurons were cultured for 13–16 d in MEM (Gibco) supplemented with B27 (Gibco), glucose, transferrin, FBS, and Ara-C (Sigma).

**Plasmid Construction and Production of Lentiviruses.** The packaging of lentiviruses and the infection of neurons with lentiviruses have been described in ref. 6. Briefly, the lentiviral expression vector and three helper plasmids, the pRSV-REV [plasmid expressing Human Immunodeficiency Virus 1 (HIV1) REV (Regulator of Expression of Virion Proteins) protein under control of the Rous sarcoma virus (RSV) U3 promoter], pMDL/pRRE [packaging plasmid that includes the gag (group-specific antigen) and pol

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(polymerase) HIV genes and an RRE (REV Response Element), and vesicular stomatitis virus G protein (VSVG) were cotransfected into human embryonic kidney (HEK) 293T cells (ATCC) at 6, 2, 2, and 2 μg of DNA per 25-cm² culture area, respectively. The transient transfections were performed with FuGENE 6 transfection reagent (Roche) following the manufacturer’s instructions. Supernatant with viruses was collected 48 h after transfection. Cortical neuronal cultures were infected at 5 DIV and used for biochemical or physiological analysis at 14–16 DIV. All steps were performed under level II biosafety conditions.

Development and Validation of Syntaxin-1 shRNAs. Syntaxin-1A and -1B cDNA sequences were chosen as targets of shRNAs. The sequences of the two regions are AGA GCC AGC TGG AGA TCA C and GAT CAT CAT TTT GTG TGT G. The shRNA oligos were synthesized (the sense strands of the oligos were TCG ACC AGC GCC AGC TGG AGA TCA CITT CAA GA GAG TGA TCT CCA GCT GCC TCT GTT TTT TTG GAA AT, and GCC GCC CGA TCA TCA TTT GTG TGT CCA GAC ACA CAG CAA ATG ATG ATCATTITTTGTGGAA A), annealed, and inserted into L309, using XhoI-XbaI and AscI and RsrII sites, respectively (6). The two shRNAs were driven by human H1 and U6 promoters, respectively. The effects of the syntaxin-1A and -1B were validated in wild-type cortical neuronal culture by Western blotting and quantitative RT-PCR. From Western blotting (6). The two shRNAs were driven by human H1 and U6 promoters, respectively. The transient transfections were performed with FuGENE 6 transfection reagent following the manufacturer’s instructions. Supernatant with viruses was collected 48 h after transfection. Cortical neuronal cultures were infected at 5 DIV and used for biochemical or physiological analysis at 14–16 DIV. All steps were performed under level II biosafety conditions.

Antibodies. Monoclonal antibodies against SNAP-25 (SMI-81; Sternberger Monoclonals) and syntaxin-1 (HPC-1; Synaptic Systems), as well as secondary anti-mouse and anti-rabbit (Invitrogen), were labeled with Alexa 647-NHS (Invitrogen) or Atto 532-NHS (ATTOTEC) amine-reactive dyes in a 0.1-M Na₂HCO₃ solution (pH ~ 9) for 30 min at room temperature. Unreacted dye was removed by five to six washes with PBS, using a Microcon concentrator (30-kDa cutoff; Millipore). Rabbit polyclonal antibody against mammalian uncoordinated-18 (Munc18-1) (P9529) (7) was used unconjugated.

Immunofluorescence Experiments. Cultured neurons were washed (three times) with PBS and fixed for 20 min at room temperature in PBS/4% paraformaldehyde (PFA). Following three washes with PBS, the fixed cultures were permeabilized for 15 min in blocking solution [PBS/3% BSA (Sigma)/0.1% Triton X-100 (Sigma)]. Permeabilized neuronal cultures were incubated with conjugated, dye-labeled primary antibodies for 1 h in blocking solution at room temperature. Alternatively, 1 h incubation with unlabeled primary antibodies was followed by 1 h incubation with dye-labeled secondary antibodies. The coverslips postfixed in 4% PFA were rinsed (six times) with PBS and stored at 4 °C. Laser scanning confocal microscopy at diffraction-limited resolution (Leica TCS SP-2) was performed on coverslips mounted in Vectashield (Vector Labs). Titration of the primary antibody concentration determined staining conditions at which the available SNAP-25 and syntaxin-1 epitopes are saturated and confirmed axon-specific staining (Fig. S4). Experiments were performed close to saturation to ensure efficient staining.

Immunoblotting and Immunoprecipitations. Either brain homogenates or cultured neurons were solubilized in 50 mM Tris-Cl buffer (pH 7.4) containing 150 mM NaCl and 0.1% Triton X-100. Following centrifugation at 16,000 g for 10 min at 4 °C, the clarified lysate was used for immunoblotting (after addition of 2× SDS sample buffer containing 10% β-mercaptoethanol) or subjected to immunoprecipitation. Immunoprecipitation was performed with the indicated primary antibodies and 30 μL of a 50% slurry of protein-G Sepharose beads (Amersham) for monoclonal IgG or protein-A Sepharose beads (GE Healthcare) for polyclonal rabbit sera, for 2 h at 4 °C. Control immunoprecipitations were performed with brain lysates with no antibody (for monoclonal antibodies) or with preimmune serum (for polyclonal rabbit sera). Following five washes with 1 mL of the extraction buffer, bound proteins were eluted with 2× SDS sample buffer containing 10% β-mercaptoethanol and boiled for 15 min at 100 °C. Coprecipitated proteins were separated by SDS-PAGE, with 5–10% of the input in the indicated lane.

Electron-Beam Lithography of Calibration Arrays. An ~1,000-Å Al film was deposited on quartz wafers, followed by spin-coating an ~2,500-Å layer of e-beam resist (ZEP-520). The nanohole array was written using a Raith-150 e-beam lithography tool as single-shot dots. The resist was developed in Xylene, followed by Methyliosobutylketone:Isopropyl-alcohol (MIBK:IPA 1:3), with a final IPA rinse and blow drying. The hole pattern was transferred to the Al film, using reactive-ion etching (p5000: Applied Materials). Characterization was done with a scanning-electron microscope (Hitachi). The final hole diameter depended on the dose during e-beam exposure and varied between ~90 nm and 250 nm.

Two-Color Mapping Transformations. We imaged the nanohole pattern simultaneously in the green and red channels of the CCD array (Fig. S6), obtaining a set of data {x, y} composed of the apparent coordinates of each nanohole i, x = (x, y) (Fig. S6). Translating the sample with the piezoelectric nanopositioning stage enabled us to sample the CCD array with finer resolution than the pitch of the hole pattern. We used two approaches to calibrate mapping transformations between coordinates in the green and red color images. In the simplest implementation we performed polynomial warping of degree N, to obtain matrices P, Q that can be used to obtain the transformed coordinates [e.g., from red (R) to green (G)]:

\[ x_{RG} = \sum_{k=0}^{N} P_k x_k^{G}, \quad y_{RG} = \sum_{k=0}^{N} Q_k y_k^{G}. \]  

We found that the typical registration accuracies achieved (Fig. S6) with such “global” transformations could not be significantly improved beyond ~8 nm by increasing the order of the polynomial or by increasing the spatial sampling frequency. Previously we had shown that the photoreponse nonuniformity of the CCD array gives rise to high-spatial-frequency “noise” in such mapping transformations (1). Because the CCD array can be sampled very finely (down to subpixel level, ref. 1), a “local” approach that calibrates the mapping by spline interpolation can be used to achieve higher accuracy. According to this scheme, any point in, e.g., the red color, \( \tilde{x}_R \) can be mapped to a point \( \tilde{x}_{RG} \) in the green color by

\[ \tilde{x}_{RG} = \sum_{i=0}^{N_p} c_i (\tilde{x}_R - \tilde{x}_i) + c_0 + c_1 \tilde{x}_R, \]

with \( \phi(r) \) being an appropriately chosen radial basis function (8), centered on each calibration point i. Thin-plate splines \( \phi(r) = r^2 \ln(r) \) have been used in the past for image warping and registration. In practice we find that various choices of radial basis function, including thin-plate splines or multiquadratic interpolation \( \phi(r) = \sqrt{1 + (er)^2} \), with e the tunable parameter, give similar results. The coefficients \( c_i \) are estimated by least-squares minimization and a regularization parameter (8, 9) λ is
introduced to take into account the localization errors by weakening the interpolation conditions. For \( \lambda = 0 \), we obtain a perfect interpolation through the experimental points, whereas for increasing \( \lambda \) the resulting mapping transformation is smoother. The limit \( \lambda \to \infty \) corresponds to a first-order linear mapping. The most accurate transformation is thus obtained by optimizing the level of smoothing to filter out noise in the calibration data, without losing information on the underlying pattern of spatial distortions (Fig. S6).

To validate the mapping transformation obtained using spline interpolation, we imaged Cy3 and Cy5 attached to 20-bp dsDNA duplexes (1). Several areas of the coverslip were imaged to obtain up to 100 pairs of Cy3-Cy5 positions that were randomly dispersed over the whole field of view of the CCD. The registration accuracy obtained for Cy3-Cy5 using the spline interpolation algorithm was \( \sim 3 \) nm (Fig. S6). We note that the set of coordinates from Cy3 and Cy5 on the dsDNA molecules can also be used to directly calibrate the mapping transformations, providing additional experimental flexibility.

**Density-Based Analysis of Clustering Structure.** To analyze the structure of the clustering patterns of SNAP-25 and syntaxin-1 molecules we used the OPTICS algorithm, to perform a hierarchical ordering of the SNAP-25 or syntaxin-1 localizations. We also calculated suitable “core distances” \( \{c_{\text{dist}}^{\text{GR}}, c_{\text{dist}}^{\text{GG}}\} \) for each, e.g., green localization point \( i \), indicating the radius of an area centered on point \( i \) that encompasses exactly \( \text{Minpts} \) points (minimum number of points required forming a cluster) of the same or opposite color (Fig. S5). Thus, \( \{c_{\text{dist}}^{\text{GR}}, c_{\text{dist}}^{\text{GG}}\} \) indicate the spatial extent of clusters of \( \text{Minpts} \) localizations centered on point \( i \). For \( \text{Minpts} \) in the range 2–19, we calculated the Pearson’s correlation coefficient between \( \{c_{\text{dist}}^{\text{GR}}, c_{\text{dist}}^{\text{GG}}\} \) to find out whether the spatial extent of green and red clusters is correlated.

Fig. S1. Loss of position information due to drift and active microscope stabilization. (A) Trajectories of Cy5 molecules attached to the coverslip and imaged on CCD camera CCD2 in a 15 μm × 15 μm region of interest. To simulate “drift” in a controlled manner, the sample was translated using the piezoelectric 3D stage, in the x direction only and under closed-loop control (by tracking a 0.5-μm bead on CCD1) while the y and z coordinates are locked by the feedback system. (B) Residual deviations of the x coordinate of each Cy5 molecule after the mean drift has been subtracted, vs. amplitude of the drift. Similar results were obtained for the y coordinates. (C) rms deviations of the x positions of the Cy5 molecules after drift subtraction. Note that each Cy5 could be tracked to ~2.7-nm rms in each frame, whereas 500 nm drift increased the uncertainty in the position of each Cy5 to ~10 nm. (D) Experimental setup. The bright-field image was projected on a CCD camera (labeled CCD1) and fluorescence of the Cy3 and Cy5 dyes was imaged with a second CCD camera (labeled CCD2). For the laser-based focus-lock system, the combination of quarter-wave-plate (QWP) and polarizing beam splitter (PBS) creates an optical isolator that directs the back-reflected total internal reflection (TIR) beam on a position-sensitive detector (QPD). The position of the beam on the QPD encodes axial position of the coverslip surface. The real-time coordinates x,y,z of fiduciary marks (or x,y from fiducials and z from the QPD) were fed to a digital servo-loop that controlled a three-axis piezoelectric stage to stabilize the microscope. (E) Bright-field image of neurons on CCD1, showing fiducials that were tracked by the feedback system. One fiducial is used to lock the 3D position of the microscope and the second serves as an out-of-loop reference. (F) The long-term rms stability of the microscope is ~1.5 nm in x,y and ~4.0 nm in z. (G) Trajectory of the x,y coordinates of the out-of-loop reference fiducial at 200-ms exposures. Red lines are 128-frame smoothed trajectories. (H) Relative registration between CCD cameras CCD1 and CCD2. We stabilized the microscope by tracking in real time a 0.5-μm bead, using CCD camera CCD1 and locking its x,y position with the piezoelectric stage. Simultaneously we imaged a separate 0.5-μm bead on CCD2 (fluorescence detection channel). We tracked this out-of-loop bead in CCD2 in x,y to 1.1-nm rms at 144-ms exposures, while its position remained stable to <3 nm (peak-to-peak) over 1 h. (I) Alternative microscope stabilization scheme using fiducial tracking directly on CCD camera CCD2. Forty-nanometer fluorescent nanospheres (Transfluospheres 488/645; Invitrogen) were attached to the coverslip surface and imaged on the same fluorescence channel as Cy5/Alexa 647. The apparent width of the nanosphere image s, as the focus was varied, was used to determine the axial position of the sample, with sensitivity ds/dz = 0.1 nm/nm. One nanosphere was used for stabilizing the stage and a second nanosphere served as an out-of-loop reference to monitor the performance of the feedback loop. The out-of-loop reference traces show σx = 3.5 nm and σz = 17 nm 3D tracking at 1 Hz (open symbols) whereas the long-term stability is 1.5 nm in x,y and 8.5 nm in z rms, respectively (solid lines, 64-frame average).
Fig. S2. Precision and photon-count histograms for Cy5, Alexa 647, and Atto532. (A) (Top) Cy5-DNA, (Middle) Alexa 647-PHF F-actin, and (Bottom) Alexa 647-antibody superresolution imaging. (Left) Histograms of localization precision $\sigma = \sqrt{\sigma_x^2 + \sigma_y^2}$; $\sigma$ was calculated according to Eq. S1a. Insets show cumulative distributions. (Right) Histograms of number of photons collected from each Cy5/Alexa 647 molecule in each cycle. Red lines are linear semilog fits, indicating an exponential distribution $D(N) \sim \exp(-N/N_0)$. (B) Atto 532-labeled antibodies. Inset shows cumulative distribution. Red solid line: double-exponential fit. The mean number of collected photons for Atto 532 is 13,216 in this case.
Resolution refinement based on photon counts. (A) Theoretical enhancement of FWHM resolution vs. cutoff ratio $\nu \equiv \frac{\sigma}{\sigma_{\text{cutoff}}}$ in the background-free case. See SI Methods for details. Starting at an initial FWHM(D) = 2.828$\sigma_0$ ($\beta(0) = 2.828$), the resolution is improving as a smaller $\sigma_{\text{cutoff}}$ is chosen (blue curve), and the ratio $\beta(\nu) \equiv \text{FWHM(D)}/\sigma_0$ drops with increasing $\nu$. For sufficiently small $\sigma_{\text{cutoff}}$, the resulting distribution will have a FWHM(D) $\sim 2.355\sigma_{\text{cutoff}}$ (red curve, showing a plateau in the ratio). (B) Fraction $\alpha$ of localization data kept in the statistical refinement process vs. cutoff ratio $\nu$. As a progressively more stringent localization precision cutoff is enforced (increasing ratio $\nu \equiv \sigma/\sigma_{\text{cutoff}}$), the fraction of localization points that survives drops as $\exp\left(-\nu^2\right)$. (C) Theoretical enhancement of FWHM resolution vs. fraction $\alpha$ of localization data kept. The blue curve show the ratio of the FWHM resolution over $\sigma_0$, starting at $\beta(1) = 2.828$ for the initial Lorentzian distribution. As a more stringent cutoff is enforced, the resolution improves ($\beta$ decreases) but also the number of points decreases. If only $\alpha \sim 10\%$ of the localization data are kept, an improvement of approximately twofold can be expected ($\beta(0.1) \sim 1.4$). For a practical limit of $\alpha \sim 1\%$ the expected enhancement is $\sim 3\times$ ($\beta(0.01) \sim 0.9$). In the asymptotic regime [red curve plateau $\gamma(\alpha \rightarrow 0) = 2.355$], further improvement in the resolution is sub-logarithmic in $\alpha$, $\beta(\alpha) \propto \frac{2.355}{\sqrt{\log(\alpha)}}$. 

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Fig. S4. Resolving individual antigens and epitope saturation/spatial sampling controls. (A–C) Resolving individual SNAP-25 antigens in close proximity. (A) Black dots are Alexa 647 localization points (Fig. 3C). Red circles indicate the locations of identified individual SNAP-25 antibodies. (Scale bar: 100 nm.) B and C show profiles of the density of the localization points across cuts indicated in A. Blue lines show fits to Gaussian peaks. The 13-nm FWHM of the clusters of localization points allows us to clearly resolve individual Alexa 647-labeled SNAP-25 antibodies at distances 30–50 nm. (D) Successive imaging of the same region verifies adequate spatial sampling. Syntaxin-1 Alexa 647-labeled primary antibody localizations are shown. Red symbols are the first 1,500 frames during the acquisition; black symbols are the last 1,500 frames. (Scale bar: 320 nm.) (E–P) Demonstration of saturation of SNAP-25 and syntaxin-1 epitopes and adequate spatial sampling. A first round of staining with Atto 532 syntaxin-1 primary antibody (E and F) was followed by a second round with Alexa 647 syntaxin-1 (G) or SNAP-25 (H) primary antibody. Similarly, Alexa 647-SNAP-25 (K and L) primary antibody was followed by Atto 532 SNAP-25 (M) or syntaxin-1 (N) primary antibody. Both syntaxin-1 and SNAP-25 epitopes can be saturated in the first round of staining, resulting in negligible signal from the second antibody (G and M). If epitopes are available, the second antibody gives robust signal (H and N). (I, J, O, and P) MAP-2 counterstaining using a rabbit polyclonal and Alexa 488 secondary antibody.
Fig. S5. Density-based clustering structure and correlation analysis for syntaxin-1 and SNAP-25 localizations. (A) Syntaxin-1 (red) and SNAP-25 (green) localizations. Inset illustrates the definitions of cdist_{GR} and cdist_{GG} for Minpts = 7. (B) Ordered plots (index calculated using the OPTICS algorithm) of the core distances cdist_{GR} and cdist_{GG} for data points in A for cluster size Minpts = 10. (C and D) Pearson’s correlation coefficient (C) for (cdist_{GR}, cdist_{GG}) and mean core distance (D) (cdist_{GR} × cdist_{GG})^{0.5} for Minpts in the range 2–19. (E) Pair-distribution functions for syntaxin-1 Alexa 647 and SNAP-25 Atto 532 antibody localizations (Fig. 3B). The solid curves are exponential fitting to the data with decay lengths ξ_{stx-stx} = 42 nm and ξ_{SN25-SN25} = 48 nm for syntaxin-1 and SNAP-25, respectively. The decay to zero at r < 15 nm is due to the fact that overlapping antibodies at about the resolution limit are not resolved.
Fig. 56. Two-color nanometer colocalization calibration: (A) Nano-fabricated hole array. SEM image of an array with 1-μm spacing is shown. (Scale bar: 1 μm.) (B) Diameter of holes increases with e-beam dose. Two-color mapping calibration: (C) Nanohole array image. (Upper) Cy3 channel; (Lower) Cy5 channel. (Scale bar: 5.12 μm.) (D) Registration using a polynomial transformation. (Scale bar: 5.12 μm.) (E) Residuals of polynomial transformation. (Scale bar: 20 nm.) (F) Registration accuracy for Cy3-Cy5 DNA molecules using spline-interpolation of the nanohole array coordinate data. (G) Residuals of the Cy3-Cy5 DNA mapping over the whole field of view.
Fig. S7. Quantitative colocalization of SNAP-25, syntaxin-1, and Munc18-1. (A and F) Clusters of SNAP-25/syntaxin-1 (A) and SNAP-25/Munc18-1 (F) localizations were identified using the DBSCAN algorithm with Minpts = 3 and \( \varepsilon_{SNAP-25/syntaxin-1} = 32.0 \) nm and \( \varepsilon_{SNAP-25/Munc18-1} = 28.8 \) nm, \( \varepsilon_{Munc18-1/SNAP-25} = 38.4 \) nm in A and F, respectively. About 67–75% of all of the molecules are assigned to clusters and the solid circles in each scatter plot represent the centroid of each identified cluster. (B, C, H, and G) The average profiles of syntaxin-1 (B), SNAP-25 (C and H), and Munc18-1 (G) clusters were obtained by centering each cluster at the origin \((x, y) = 0\), by subtracting for each cluster \((x_i, y_i)\) the mean coordinates \((x'_c, y'_c)\). For each cluster centered at the origin the nearest cluster of the opposite species is identified and overlaid in the scatter plots. (D, E, I, and J) Corresponding radial distributions of the first species centered clusters and overlaid second species nearest-neighbor clusters. Ninety-five percent of all of the clustered molecules are contained within a radius indicated by the vertical dashed lines. The fraction of the second species’ clustered molecules that are contained within the 95% radius quantifies the colocalization between the two molecules and is 62%, 49%, 34%, and 37% for (D) syntaxin-1/SNAP-25, (E) SNAP-25/syntaxin-1, (I) Munc18-1/SNAP-25, and (J) SNAP-25/Munc18-1, respectively.
Fig. S8. Interaction of Munc18-1 and SNAP-25 via syntaxin-1. (A) Wild-type mouse brain lysate (P50) was subjected to immunoprecipitation of SNAP-25, syntaxin-1 (Synt-1), synaptobrevin-2 (Syb-2), and Munc18-1 (Munc18), as well as a control immunoprecipitation without antibody (−IgG). To detect coimmunoprecipitated proteins, the immunoprecipitate samples and 5% of the input were separated by SDS/PAGE and immunoblotted (IB) with indicated antibodies (Cplxn, complexin-1,2). (B) Wild-type mouse brain lysates were used for immunoprecipitation as in A, using an antibody specific to monomeric SNAP-25 (SNAP-25mono) as well as control immunoprecipitation using preimmune serum (Pre-Imm). Coimmunoprecipitated proteins were detected by immunoblotting as indicated. (C) Lysates were prepared from syntaxin-1A KO mouse cortical cultures infected with syntaxin-1B KD lentiviruses (Synt-1A KO/B KD) without or with rescue expression of the wild type (+Synt-1AWT), N-terminal deletion (+Synt-1AΔN), or LE mutant (+Synt-1ALE) variants of Syntaxin-1A. Lysates were used for immunoprecipitation of SNAP-25 and Munc18, as well as control immunoprecipitation with preimmune serum (Pre-Imm). Input and immunoprecipitates were separated as in A and immunoblotted using Synt-1, SNAP-25, and Munc18 antibodies (Upper). Extent of coimmunoprecipitation was quantitated using 125I-labeled secondary antibody and phosphorimager analysis (Lower). (D) Level of coimmunoprecipitated protein was first normalized to immunoprecipitated protein and then to expression level in the input and is shown as percentage of wild-type rescue. Data shown are means ± SEMs; n = 3 cultures; *P < 0.05, **P < 0.01, ***P < 0.001, using Student’s t test.
Table S1. Numerical parameters

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<th>N\textsubscript{frames}</th>
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*\( b^2 \) in units of (photons\textsuperscript{2}/pixel).

\(^{\dagger}b^2_{\text{eff}} = (N_{\text{frames}}b^2)\).

\(^{\ddagger}\)Calculated from Eq. S1a.

\(^{§}\)Calculated from Eq. S1b.

\(^{\ast}\)Expected FWHM is equal to \(2\sqrt{2\sigma_0} \approx 2.828\sigma_0\).

\(^{\parallel}\)Peak fits of the data in Figs. 1 A and C and 2B.