ONLINE METHODS

Proteins

Expression and purification of SNAP-25, soluble syntaxin-1a fragment (residues 1-264, Sx) and synaptobrevin-2 soluble fragment (residues1-96, Sb) are described elsewhere[10,16-18]. Briefly, proteins were expressed in *E. Coli* BL21(DE3) with thrombin-cleavable, 6xHis tags and purified using combinations of Ni-NTA, ion exchange and gel filtration chromatographies. 6xHis tags were removed with thrombin. Previously described mutations were K76C-Q197C for SN25CC and Q20C-Q197C for SN25NC[18] in a SNAP-25 template with the 4 native cysteines mutated to serine. Quikchange (Stratagene) created mutations not previously described in SNAP-25 (G43D) and Sb (S28C-A72C in Sb-NC_28_72, A5C-A72C in Sb-NC_5_72).

A pet28c vector for 6xHis-tagged, human SNAP-29[19] was a gift from Zu-Hang Sheng (NIH)[20]. SNAP-29 contains no cysteines; therefore SN29NN was generated by mutations R51C-Y198C, which align with the SNAP-25NN label sites[18].
SNAP-29 was expressed in *E. Coli* BL21(DE3). Cultures (super broth, 50 μg ml^{-1} kanamycin sulfate) were grown to OD_{600} 1.0 at 37ºC while shaking. The temperature was decreased to 24ºC, 0.5 mM IPTG was added and cells were collected after 8-10 hrs. For denaturing purification, cells were resuspended at .2g ml^{-1} in lysis buffer (10 mM Tris, 100 mM NaH_2PO_4, 5 M Urea, pH 8.0), disrupted by sonication, and centrifuged at 12K RPM for 30 minutes at 4˚C. Supernatant was bound to Ni-NTA beads (1 hr, 4˚C). Beads were washed with lysis buffer at pH 8.0 and then at pH 6.8 followed by elution in pH 5.9 lysis buffer. SNAP-29 at 50 to100 μM aggregated unless urea was present above 2 M. SNAP-29 was dialyzed into buffer A (20 mM Tris, 2.5 M Urea, pH 7.3) and then purified

with a MonoQ column (GE Biosciences) on a linear gradient in buffer A from 0 to 1 M NaCl (peak elution ~225 mM NaCl). Molecular weight and purity were verified by SDS-PAGE (Phastgel, GE Biosciences). The 6xHis tag was not removed from SN29NN.

Dye Labeling

As described elsewhere[10,16-18,21], proteins were incubated with a 10-fold molar excess of maleimide-dyes at pH 7.4 for 4 hr, followed by gel filtration (PD10, GE Biosciences) and extensive dialysis to remove unbound dye. Double-labeled experiments used equimolar mixtures of donor and acceptor dye (either Alexa555-Alexa647 (Invitrogen) for SN25NC, SN29NN, Sb-NC_28_72, and Sb-NC_5_72 or Cy3-Cy5 (GE Healthcare) for SN25CC). The two cysteines were not selective for specific dyes so 50% of double-labeled molecules had one donor and one acceptor and the other double-labeled molecules had either two donors or two acceptors. Dye-labeling efficiencies of double-labeled proteins were SN25CC 53%-43%, SN29NN 50%-46%, SN25NC 48%-56%, Sb5_72 28%-26%, and Sb28_72 45%-40% (donor-acceptor). The second dye was omitted from reactions for singly-labeled control experiments.

Characterization of *in vitro* FRET from double-labeled proteins

We encapsulated single proteins inside 100 nm diameter, biotinylated liposomes[22], which were immobilized on a biotinylated-BSA and streptavidin coated surface in a chamber between a quartz microscope slide and a glass coverslip. We measured smFRET from the immobilized proteins using prism-type, total internal reflection (TIR) illumination[23,24] with the same filters and detection used for *in vivo* work (below). SNAP-29 was diluted in urea-free buffer so that final urea was < 7 mM before liposome encapsulation. FRET from isolated SN25NC is 0.3 (Choi and Weninger,

unpublished) and from SN29NN is 0.37 (**Supplementary Fig. 1a**). FRET from SN25NC and SN25CC in parallel, SNARE complex with Sx and Sb is reported elsewhere[18] as 0.3 and 0.9, respectively.

We verified that SNAP-29 forms SDS resistant SNARE complex with Sx and Sb. SN29NN, Sx and Sb were mixed in that order at 1:2:5 (molar ratio) with urea always present at 2.5 M. The mixture (<0.1 ml) was dialyzed against 1 L of buffer (20 mM Tris, 300 mM NaCl, pH 8.0, 2mM β-Mercaptoethanol, 5% v/v glycerol) with a Slide-A-Lyzer MINI Dialysis Unit (Pierce). The dialysis buffer was preheated to 37 º C before the sample was added. After the protein mixture in the dialysis unit had been in 37 º C buffer for 10 min, the dialysis flask was placed at 4 º C for 12 hr. with stirring. SDS-PAGE of the resulting complex (**Supplementary Fig. 1c**) shows the SN29NN-containing SNARE complex is SDS stable without boiling and dissociates upon boiling in SDS buffer. FRET from the SN29NN SNARE complex was measured by the liposome encapsulation method (above) as 0.82 (**Supplementary Fig. 1b**).

Cell culture and media.

BS-C-1 cells were grown to ~ 80 % confluency on coverslips in Modified Eagle Media (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % gentomycin. PC-12 cells were grown ~ 50 % confluent on coverslips coated with poly-l-lysine in DMEM with 10% horse serum, 5 % FBS and 1 % pen/strep. Before culture, coverslips were cleaned (sequential sonication in: soapy water (Alconox), acetone, ethanol, 1 M KOH and deionized water), autoclaved and stored in sterile deionized water. All cell culture reagents were purchased from HyClone.

We used Erie Scientific glass coverslips (part # 25CIR-1.5D, ThermoFisher Scientific) as they had the lowest autofluorescence among the glass coverslips we tested. To further reduce fluorescence backgrounds, we changed the cell culture media at least 2 hrs before imaging to fluorescence-free MEM (without phenol red, Gibco) with 20 mM HEPES, 1 % gentomycin, 2 mM Trolox (Sigma) and 200 μM Cyclooctatetraene (COT, Alfa Aesar). Trolox-containing media was stirred overnight before use.[25]

Microscopy

We built an objective-type TIR system[23,24] using an Olympus IX71 microscope with a 60x, 1.45 N.A. or a 100x, 1.4 N.A. (UIS2) oil immersion objective (Olympus). An expanded, 7 mW, 532 nm laser beam focused on the back focal plane of the objective lens traveled parallel the optic axis so that changing the distance from the optic axis controlled the beam's incident angle at the coverslip-media interface. We usually worked in full TIR although incident angles just below the threshold for TIR were occasionally used to generate a propagating beam that entered the sample at a steep angle. The critical angle for TIR at a glass-water interface is 61°. The maximum incident angles achievable with 1.4 and 1.45 N.A. lenses are near 67° and 73° respectively. In TIR with incident angles above the critical angle, an evanescent light wave penetrates beyond the glass toward the cell with an exponential decay constant of 100 to 200 nm depending on the precise angle. Thus TIR limits illumination to molecules near the membrane adhered to the coverslip. Alternately, the 'near-TIR' method imaged deeper objects while still providing lower backgrounds than normal illumination. FRET efficiency ratio is not affected by the intensity of the excitation light or position in an evanescent wave.

Supplementary Video 1 is 'near-TIR' and the microinjector tip is visible above the cell, whereas **Supplementary Video 2** is TIR and the microinjector tip is not visible.

Fluorescence emission collected by the objective was split into donor and acceptor spectral ranges with a Dualview (Photometrics, with 645dcxr mirror and filters: donor-HQ585/70m, acceptor-HQ700/75m, Chroma) and recorded with a Cascade 512B emCCD camera (Roper Scientific). 7 % leakage of donor signal into the acceptor channel was subtracted in analysis.

Differential interference contrast (DIC) optics were used to image cellular structures. The polarizer between the objective and the camera required for DIC was removed for fluorescence imaging. A heated sample stage maintained cells at 37ºC during imaging on the microscope. Imaging at room temperature increased cellular autofluorescence.

Microinjection

We microinjected proteins at concentrations ~10-100 nM using a Femtojet (Eppendorf) while observing cells in place on the microscope under DIC imaging. The injection volume was estimated at 1-10 % of the cell volume by comparing the total fluorescence intensity of cells injected with known dye concentrations to the intensity of single dyes. All proteins were injected in solutions containing 50 mM Tris, 150 NaCl, pH 7.4, 1 mM DTT, except for SN29NN, which was injected in the same buffer including ~50-75 mM urea (remaining after dilution from the stock containing high urea to maintain protein solubility).

Typically, a cell was selected based upon its flat, isolated appearance in a DIC image and its relatively low autofluorescence under green laser illumination. The initial

imaging to choose cells typically involved 10 to 60 s of laser exposure, which could bleach some autofluorescence background. This prebleaching was not required for smFRET measurements in many cells. An initial DIC image was acquired for the selected cell. Cells were then microinjected under DIC illumination to ensure proper filling and then the green laser was turned on 2-5 s after injection. Movies were recorded with 100ms exposures during and after injection. DIC images were reacquired at the end to verify cell morphology post-microinjection.

Success of injection by an experienced operator was highly probable using the Femtojet, so oftentimes injections were performed with only green laser light illuminating the cells. **Supplementary Videos 1-3** were performed with such green illumination active during injection.

Data analysis

We used custom software to identify localized peaks in either the donor or acceptor channels of the movies by requiring the mean intensity of 9 pixel areas to be 3 to 8 times above the standard deviation of the background. The threshold was adjusted on a cell-by-cell basis for reliable identification and verified by eye. The identified spots were mapped to the complementary donor or acceptor channel, background was subtracted (calculated pixel-by-pixel using the mean value of the 16 pixels surrounding a 3x3 region of interest), and intensity timecourses were extracted. A FRET histogram (**Fig. 1e**) for single molecule events occurring in **Supplementary Video 1** using SN25CC in BS-C-1 cells was assembled by accumulating the average FRET emission level for each molecule detected within 0.2 s (FRET<0.5) and 1.0 s (FRET>0.5) following the injection. The different duty cycles yielded sufficient populations in both peaks. This FRET efficiency

histogram is consistent with expectations from the structure of the SNARE complex[26,27] and in vitro smFRET measurements[18]. Correcting the areas under the two Gaussian-shaped peaks near FRET efficiencies of 0 and 1 by the duty cycle of detection yields %HighFRET from this cell to be 22 %, consistent with the values reported in **Fig. 2c** for many more cells.

Single molecules were confirmed by the emission levels as well as single step transitions at the beginning and end of events. The presence of anticorrelated intensity jumps of the donor and acceptor intensities (where the acceptor dye emission vanishes due to bleaching and the donor dye emission rises simultaneously) confirms smFRET interactions (**Fig. 1c-d, Supplementary Fig. 2**). However, many high smFRET events did not show this anticorrelation. Commonly, high acceptor emission would commence and end abruptly without donor emission (**Supplementary Fig. 2g-h**). Because the acceptor bleaches faster than the donor in our system, this observation suggests fast SNAP-25 and SNAP-29 binding and unbinding. Molecules with low FRET emission, indicative of membrane localization without SNARE complex formation, typically did not show such transient binding (see **Supplementary Videos 1-3**).

To compare the efficiency of generating high smFRET events across different proteins and different injections of multiple cells, we analyzed data as fractions (%HighFRET) of the number of high FRET events (#highFRET, FRET > 0.7) divided by the sum of #highFRET and low FRET (#donorOnly, FRET < 0.7) events (%HighFRET = 100*#highFRET/(#highFRET+#donorOnly)) for each of the first 10 frames following an injection during a 10 Hz movie. Those 10 values of %HighFRET accumulated for around ten cells were assembled into histograms (**Fig. 2c-f** and **Supplementary Fig. 3**)

for the different combinations of proteins and cells used. The averages of these data sets are compared in **Supplementary Table 1**. This quantity represents the amount of high FRET due to SNARE complex formation relative to the total amount of protein injected. Details of single particle tracking and residence time analyses are in **Supplementary Note**.

Control Experiments

Injections of protein-free buffer resulted in occasional "false positive" events in the donor channel, but no high smFRET events were observed as spots in the acceptor channel.

To test if high smFRET signals result from aggregation of dye-labeled proteins we prepared donor-only and acceptor-only SNAP-25 by labeling a construct containing only 1 label site (K76C) with Alexa 555 or Alexa 647 (SN25C). Equal concentrations of the donor-only and acceptor-only SNAP-25 were mixed and microinjected into BS-C-1 cells as described above. No high smFRET events were detected when we used these mixtures for experiments (N = 14 cells, **Supplementary Table 1** and **Supplementary Fig. 3g**).

To examine the effects of the illumination protocol or the injection on the cells, we conducted multiple microinjections of the same cell. Using the standard protocol above, BS-C-1 cells were injected with double-labeled SN25CC and a data set was acquired. Then 2 min of green laser illumination was used to bleach the injected dye. After bleaching, a second injection was performed on the same cell and a new data set was acquired. The two data sets were analyzed for %HighFRET and histograms of the first and second injection appear similar with no significant change in the average value

(**Supplementary Fig. 5**), which was also similar to the initial SN25CC study (**Fig. 2c**). %HighFRET from PC-12 cells under the same double injection and photobleaching protocol also did not change significantly. This result demonstrates that the laser exposure and microinjections did not significantly alter the behavior of SNAP-25 in the cells.

Assessing effectiveness of additives for improving dye lifetime *in vivo*

We used additives in our imaging solutions common in *in vitro* single molecule fluorescence studies to improve dye lifetime and stability. To quantitate their effect inside cells we used fluorescently labeled polystyrene beads. Amino-polystyrene microspheres (0.1 μm, Polysciences) were labeled with NHS-Alexa647 per manufacturer's instructions at densities of near 50 dyes per bead. Labeled microspheres were diluted in buffer (50 mM phosphate, 150 mM NaCl, pH 7.4), sonicated and microinjected into BS-C-1 cells. The beads were brighter than single dyes and diffused more slowly, which simplified tracking and analysis of photobleaching rates.

We measured photobleaching of microsphere-attached Alexa647 inside cells with media additives. Fluorescence-free media augmented with Trolox[25,28] (2mM) and COT (200 μM) was added to the cells 4 hours before imaging. Just before imaging, glucose oxidase (5000 units ml^{-1}, Sigma), catalase (500 units ml^{-1}, Sigma) and 2% glucose were also sometimes added to the media. Fluorescence of Alexa647 was directly excited using objective-type TIR with 1.5 mW of 635 nm laser light, observed through a 650 nm long pass filter and recorded with an emCCD. The beads were tracked in the 10 Hz movies by custom software and background subtracted intensity time courses were extracted (**Supplementary Fig. 6**, inset). The decay of the fluorescence intensity was fit to single

exponentials and the average of the time constants for multiple tracked beads in several cells is plotted in **Supplementary Fig. 6**. An improvement of a factor of 4 in lifetime of dyes in the cell interior was achieved with combinations of the additives in the external media.

Supplementary File	Title
Supplementary Figure 1	Characterization of SN29NN.
Supplementary Figure 2	Additional examples of smFRET in cells.
Supplementary Figure 3	Histograms of %HighFRET values.
Supplementary Figure 4	Residence times of individual SN25CC membrane binding events in BS-C-1 cells.
Supplementary Figure 5	%HighFRET is unchanged for two sequential injections of a single cell before and after extensive photobleaching.
Supplementary Figure 6	Effect of antioxidants and oxygen scavengers in media on *in vivo* microscopy.
Supplementary Table 1	%HighFRET values for combinations of proteins and cells.
Supplementary Note	Single molecule tracking and residence times.
Supplementary Video 1	Microinjection of Cy3-Cy5 labeled SN25CC into a BS-C-1 cell under 'near'-total internal reflection (online **methods**) with green illumination. The left half of the screen is the acceptor channel while the right half is the donor channel. Recorded at 10 frames sec^{-1}.
Supplementary Video 2	Microinjection of Cy3-Cy5 labeled SN25CC into a PC-12 cell under full total internal reflection with lower protein concentration injected than in video 1. Recorded at 10 frames sec^{-1}.
Supplementary Video 3	Microinjection of Alexa555-Alexa647 labeled G43D mutant of SN25CC into a BS-C-1 cell with the same conditions as video 1. Recorded at 10 frames sec^{-1}.

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