Super-resolution Microscopy of Clickable Amino Acids Reveals the Effects of Fluorescent Protein Tagging on Protein Assemblies

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SUPPORTING INFORMATION

Materials

The chemicals and reagents used in this study were provided by Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany) or VWR (Hannover, Germany), unless stated otherwise. Propargyl-L-lysine (PRK) was prepared in-house in the Lemke laboratory, or purchased from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany). Star635P-azide[1] was a gift from Dr. Vladimir Belov, Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. Alexa Fluor® 647-azide (called hereafter AlexaFluor647-azide) was purchased from Molecular Probes (Eugene, USA).

Cells

Baby hamster kidney (BHK) fibroblasts and COS-7 monkey fibroblasts were used in this study. BHK cells were used for the expression of the majority of the constructs employed herein. COS-7 cells were used for the expression of syntaxin 1 in stimulated emission depletion microscopy (STED). BHK cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% tryptose phosphate, 5% fetal calf serum (FCS), 2 mM L-glutamine, 60 U/mL penicillin and 60 U/mL streptomycin. COS-7 cells were cultured in DMEM supplemented with 10% FCS, 4 mM L-glutamine, 60 U/mL penicillin and 60 U/mL streptomycin.

Constructs

The constructs used in this study were either purchased from Addgene or were gifts from Prof. Dr. Reinhard Jahn and Dr. John Chua (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), from Prof. Dr. Thorsten Lang (LIMES Institute, University of Bonn, Germany), Dr. Marcus Niebert (Department of Neuro- and Sensory Physiology, University Medical Center Göttingen, Germany). The vector pCMV tRNA-PylRS WT was used as previously described.[2] Refer to Table S1 for a list of the constructs used here, their origin and their reference sequences.

Table S1 List of plasmids, their providers and reference sequences

Protein name	Original vector	Plasmid source	Organism of origin	RefSeq
β-actin	pEGFP modified	Addgene 34839	*Homo sapiens*	NM_001101.3 NP_001092.1
amphiphysin	pADTet	Addgene 27692	*Mus*	NM_175007.2

Protein name	Original vector	Plasmid source	Organism of origin	RefSeq
			musculus	NP_778172.1
AP-2 μ	pcDNA3	Addgene 32752	*Rattus norvegicus*	NM_053837.1 NP_446289.1
complexin 1	pET28a	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_022864.3 NP_074055.1
Doc2α	pET-Doc2a	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_022937.2 NP_075226.1
5HT1a	pcDNA3.1 HA-5HT1a K101TAG	Dr. Marcus Niebert	*Mus musculus*	NM_008308.4 NP_032334.2
	pcDNA3.1 5HT1a-GFP K101TAG			
insulin receptor (IR)[3]	pEGFP-N1 INSR-GFP K676TAG	reference[3]	*Homo sapiens*	NM_000208 NP_000199
	pCI INSR K676TAG IRES-CFP			
Munc18-1	pcDNA3.1r	Dr. John Chua	*Rattus norvegicus*	NM_013038.3 NP_037170.1
PIPKIγ	pEGFP-C2	Addgene 22299	*Homo sapiens*	NM_012398.2 NP_036530.1
Rab3a	pET11d	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_013018.2 NP_037150.2
Rab5a	pGEX-2T	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_022692.1 NP_073183.1
Rab7a	pEGFP-C1	Addgene 12261	*Homo sapiens*	NM_004637.5 NP_004628.4
SNAP-23	pGEX3	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_022689.2 NP_073180.1
SNAP-25	pEYFP-C1	Prof. Dr. Thorsten Lang	*Rattus norvegicus*	NM_030991.3 NP_112253.1
SNAP-29	pET28a	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_053810.3 NP_446262.3
synapsin Ia	pEGFP	Ospedale San Raffaele S.r.l	*Rattus norvegicus*	NM_019133.2 NP_062006.1
synaptophysin	pGEX-KG	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_012664.2 NP_036796.1
syntaptotagmin I	pCMV5	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_001033680.2 NP_001028852.2
syntaxin 1a	pEYFP-N1	Prof. Dr. Thorsten Lang	*Rattus norvegicus*	NM_053788.2 NP_446240.2
syntaxin 6	pQTEV-STX6	Addgene 31581	*Homo sapiens*	NM_005819.5 NP_005810.1
syntaxin 7	pcDNA3.1	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_021869.2 NP_068641.2
syntaxin 13	pET28a	Prof. Dr.	*Rattus*	NM_022939.2

Protein name	Original vector	Plasmid source	Organism of origin	RefSeq
		Reinhard Jahn	*norvegicus*	NP_075228.2
α-synuclein	pEGFP-N3	Prof. Dr. Tiago Outeiro	*Homo sapiens*	NM_000345.3 NP_000336.1
VAMP2	pET-28a	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_012663.2 NP_036795.1
VAMP4	pEGFP-C3	Addgene 42313	*Homo sapiens*	NP_003753.2 NM_003762.4
Vti1a-*β*	pET28a	Prof. Dr. Reinhard Jahn	*Rattus norvegicus*	NM_023101.1 NP_075589.1

Amber stop codon mutants

SNAP-25, syntaxin 1, and VAMP2 (cloned in pEGFP-N1) and α-synuclein (pEGFP-N3) were subjected to site-directed mutagenesis. Amber stop codons (TAG) were inserted in the coding sequences of these proteins using specifically designed site-directed mutagenesis primers (see Figure S1 and Table S2 for details). To obtain the constructs lacking the fluorescent protein moieties, the FP sequences were removed using appropriate restriction enzymes (*AgeI* and *NotI* for SNAP-25 and syntaxin 1; *SmaI* and *NotI* for α-synuclein and VAMP2). Subsequently Ochre stop codons (TAA) were introduced immediately after the coding sequence of the proteins of interest (for SNAP-25 and syntaxin 1) or after the Amber stop codon (for α-synuclein and VAMP2). The resulting vectors were named pN1 and pN3, respectively.

Table S2 Amber mutants obtained by site-directed mutagenesis

	Amber position	Site-directed mutagenesis primers
SNAP-25	F84TAG TTC→TAG	TGACGGACCTAGGAAAATAGTGCGGGCTTTGTGTGTG CACACACAAAGCCCGCACTATTTTCCTAGGTCCGTCA
syntaxin 1	V255TAG GTC→TAG	GTCTGACACCAAGAAGGCCTAGAAGTACCAGAGCAAGGCAC GTGCCTTGCTCTGGTACTTCTAGGCCTTCTTGGTGTCAGAC
α-synuclein	G141TAG GGT→TAG	GACTACGAACCTGAAGCCTAGACCGCGGGCCCGGGATCC GGATCCCGGGCCCGCGGTCTAGGCTTCAGGTTCGTAGTC
	T142TAG AAC→TAG	GAACCTGAAGCCGGTTAGGCGGGCCCGGGATCC GGATCCCGGGCCCGCCTAACCGGCTTCAGGTTC
VAMP2	R125TAG CGG→TAG	GTCGACGGTACCGTAGGCCCGGGATCCAC GTGGATCCCGGGCCTACGGTACCGTCGAC

For 18 other proteins of interest (refer to Table S3), primers were designed to clone the rest of the constructs into vector backbones with or without GFP. The vector backbones of pEGFP-

N1 and pN1 VAMP2 R125TAG were employed for this purpose. They were enzymatically restricted in such a manner as to excise the VAMP2 sequence (see Table S3 for details on restriction enzyme pairs), but leaving behind the Amber mutation and GFP in the pEGFP-N1 vector, respectively the Amber and Ochre mutations in pN1. The PCR product for the coding sequence of the protein of interest was cut with the same enzymes as the destination vector. Subsequently both of them were ligated.

Table S3 Amber mutants obtained by PCR cloning

Construct	**Amber position**	**PCR cloning primers**	**Restriction sites**
β-actin	R457TAG	attaGCTAGCATGGATGATGATATCGCC attaGGTACCccGAAGCATTTGCG	*NheI* *KpnI*
amphiphysin	R692TAG	attaGCTAGCATGGCCGACATCAAG attaGTCGACccCTCCAGGCG	*NheI* *SalI*
AP-2 μ	R441TAG	attaGCTAGCATGATCGGAGGCTTATTC attaGTCGACccGCAGCGG	*NheI* *SalI*
complexin 1	R140TAG	attaGCTAGCATGGAGTTCGTGATGAAAC attaGTCGACccCTTCTTGAACATGTCC	*NheI* *SalI*
Doc2a	R409TAG	attaGCTAGCATGAGGGGCCGC attaGTCGACccGGCCAACGG	*NheI* *SalI*
Munc18-1	R600TAG	attaGCTAGCATGGCCCCCATTGG attaGTCGACccACTGCTTATTTCTTCGTC	*NheI* *SalI*
PIPKIγ	R674TAG	attaAGATCTATGGAGCTGGAGGTACCG attaGTCGACccTGTGTCGCTCTC	*BglII* *SalI*
Rab3a	R226TAG	attaGCTAGCATGGCCTCAGCCACAG attaGTCGACccGCAGGCGCAATC	*NheI* *SalI*
Rab5a	R134TAG	attaGCTAGCATGTACTACCGAGGAGCAC attaGTCGACccGTTACTACAACACTGGC	*NheI* *SalI*
Rab7a	R125TAG	attaGCTAGCATGACCTCTAGGAAGAAAGTG attaGTCGACccGCAACTGCAG	*NheI* *SalI*
SNAP-23	R216TAG	attaGCTAGCATGGATGATCTATCACCAGAAG attaGTCGACccGCTGTCAATGAGTTTC	*NheI* *SalI*
SNAP-29	R263TAG	attaGCTAGCATGTCTGGCTATCCTAAAAGC attaGTCGACccGAGTTGCCGC	*NheI* *SalI*
synapsin Ia	R710TAG	attaGCTAGCATGAACTACCTGCGG attaGTCGACccGTCGGAGAAGAG	*NheI* *SalI*
synaptophysin	R313TAG	attaGCTAGCATGGACGTGGTGAATC	*NheI*

Construct	Amber position	PCR cloning primers	Restriction sites
		attaGTCGACccCATCTGATTGGAGAAG	*SalI*
syntaptotagmin I	R427TAG	attaGCTAGCATGGTGAGTGCCAGTCATC attaGTCGACccCTTCTTGACAGCCAG	*NheI* *SalI*
syntaxin 6	R261TAG	attaGCTAGCATGTCCATGGAGGAC attaGTCGACccCAGCACTAAGAAG	*NheI* *SalI*
syntaxin 7	R125TAG	attaGCTAGCATGTCTTACACTCCGGG attaGTCGACccGCCTTTCAGACC	*NheI* *SalI*
syntaxin 13	R280TAG	attaGCTAGCATGTCCTACGGTCCC attaGTCGACccCTTAGAAGCAACCC	*NheI* *SalI*
VAMP4	R147TAG	attaGCTAGCATGCCTCCCAAGTTTAAG attaGTCGACccAGTACGGTATTTCATG	*NheI* *SalI*
Vti1a-β	R230TAG	attaGCTAGCATGTCAGCCGACTTCGAAG attaGTCGACccGTGTCCTCTGACAAAAAAAG	*NheI* *SalI*

Epifluorescence microscopy

For epifluorescence imaging the samples were prepared as above, but only optionally subjected to click reaction. The cell nuclei were stained for 5 min with 1 μg/mL DAPI (Molecular Probes), followed by three additional PBS washes, before mounting in Mowiol medium.

Epifluorescence imaging was achieved using an Olympus IX 71 inverted fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 20× objective (0.50 N.A., Olympus) and a 100 W mercury lamp (Olympus) which provided sample illumination. Images of were acquired by a 12-bit charge-coupled device (CCD) camera (6.45 μm pixel size; FView II, Olympus) operated by the CellF software (Olympus).

Data analysis

The analysis was performed based on Matlab routines (the Mathworks Inc, Natick, MA). GSDIM protein cluster analyses (Figure 4) were performed as follows. The images were scanned for local maxima, using an automatic thresholding procedure. The height of the local maxima was determined, and all maxima higher than the mean + one standard deviation of single-molecule spots were selected. The single-molecule spots were determined in parallel experiments, for each protein, by imaging cells exposed to the entire procedure, including the click reactions, in the absence of the UAA. These cells do not express the proteins of interest, and only capture the individual fluorophores by non-specific interactions. The selected spots

were then Gaussian-fitted, using automatically performed linescans on the spots, which were fitted by Gaussian functions, with the Curve Fitting Toolbox of Matlab. The fits provided both the size and the intensity of the clusters. A similar procedure was used for the STED images.

For Figure 3 (epifluorescence), fluorescence intensities were obtained as follows: regions of interest were generated by automatically thresholding the DAPI images (using an empirically-derived threshold, which was high enough to remove all background DAPI staining), and the fluorescence in the green and red channels was determined in each of these regions. The fluorescence intensity was corrected for background, by subtracting the average intensity of the extracellular regions.

Statistics

To assess the significance spot size measurements (Figure 4 and Figure 5), the Student's *t*-test was used.

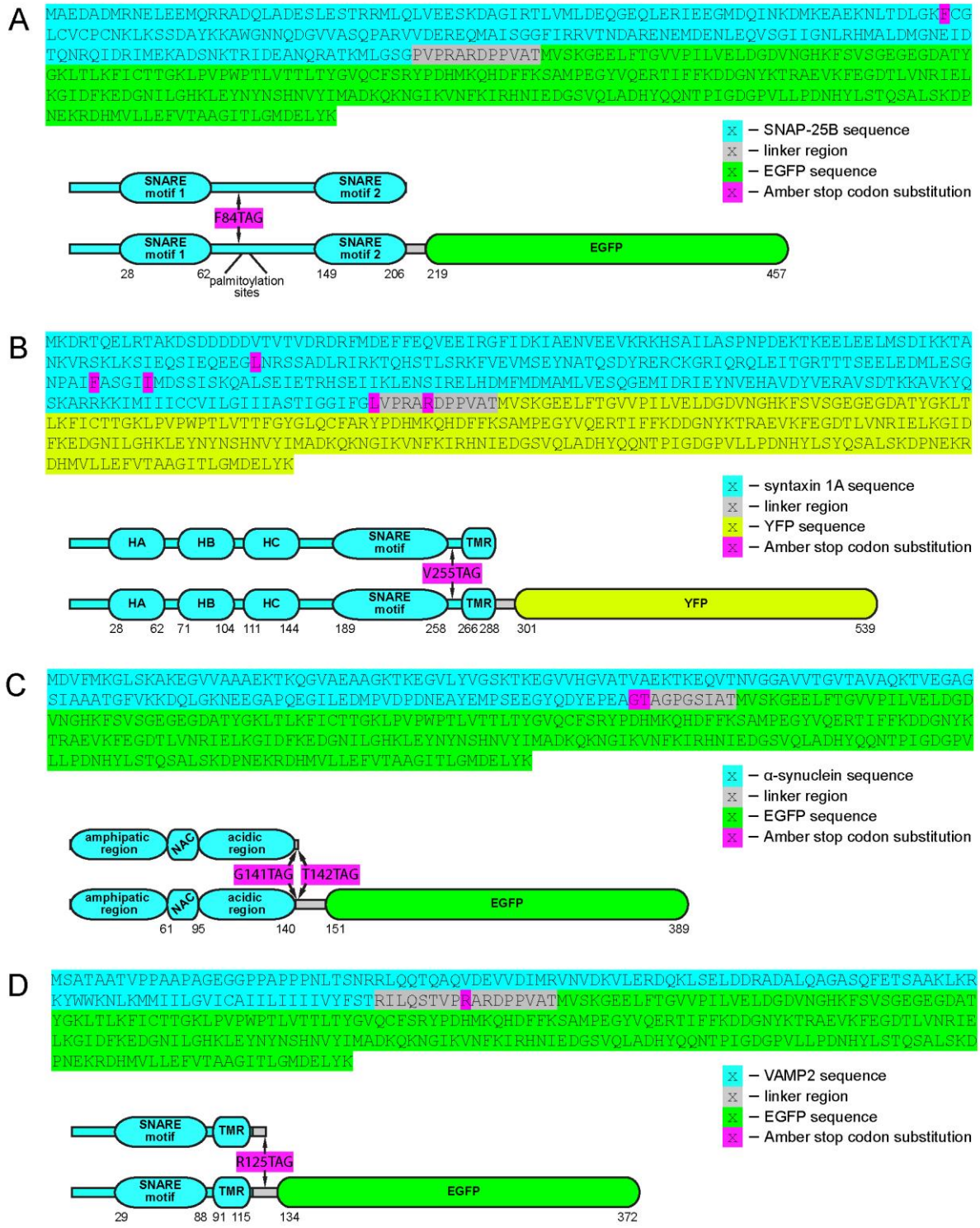

Figure S1. Constructs. Amino acid sequences and schematic depictions of SNAP-25-GFP (**A**), syntaxin 1-YFP (**B**), α-synuclein-GFP (**C**), and VAMP2-GFP (**D**). The colors used depicts as follows: light blue for the sequences of proteins, grey for linker regions, green for GFP, and yellow for YFP sequences. Purple boxes highlight positions where Amber stop codons were introduced by site-directed mutagenesis. For α-synuclein-GFP (**B**), codons encoding for the linker region residues G141 and T142 where substituted with the Amber (TAG) stop codon. In the same manner, F84TAG, V255TAG, and R125TAG replacements were carried out for SNAP-25-GFP (**A**), syntaxin 1-YFP (**C**), and VAMP2-GFP (**D**). Amber stop codons direct the incorporation of the unnatural amino acid PRK, if the cells are co-

transfected with a plasmid encoding for the suitable tRNA/RS pair. For each protein, GFP-free constructs were generated by enzymatic restriction, religation and site-directed mutagenesis of the products to encode for an Ochre (TAA) stop codon after the full-length coding sequence of the protein (see Constructs for further details on protein constructs).

Protocol for testing whether the spatial organization of the protein of interest is affected by FP tagging

I. Cloning the protein of interest into plasmids with or without GFP containing Amber stop codons (duration: 2-3 days)

To enable the rapid testing of the effects of FP tagging, we deposited two plasmids containing Amber stop codons, along with maps and sequences, in the Addgene repository (see Figure S2 for vector maps). The two vectors contain VAMP2, an Amber codon in the linker region, and a GFP at the C-terminus (+GFP plasmid; ID 69876 in the Addgene repository; see Figure S2 for details), or VAMP2 and the Amber codon alone (-GFP plasmid; Addgene ID 69877). Both plasmids are derived from the pEGFP-N1 vector (cat. no. 6085-1, Clontech Laboratories Inc., Saint-Germain-en-Laye, France). The pEGFP-N1 VAMP2-GFP R125TAG (+GFP) plasmid contains an in-frame Amber stop codon at position 125 in the linker region between the VAMP2 and the GFP coding sequences. This vector links GFP to the protein of interest. For easy testing purposes, the pN1 VAMP2 R125TAG A126TAA (-GFP) plasmid lacks the C-terminal GFP gene, and its Amber codon is followed by an Ochre stop codon. To test whether the protein of interest is affected by FP tagging, one only needs to clone the full-length protein into these two vectors. Appropriate pairs of restriction enzymes should be employed to excise the coding sequence of VAMP2 from the +GFP and -GFP vectors (see Figure S2 for detailed vector maps containing the restriction enzymes that can be used for cloning), and to insert the protein of interest in its place.

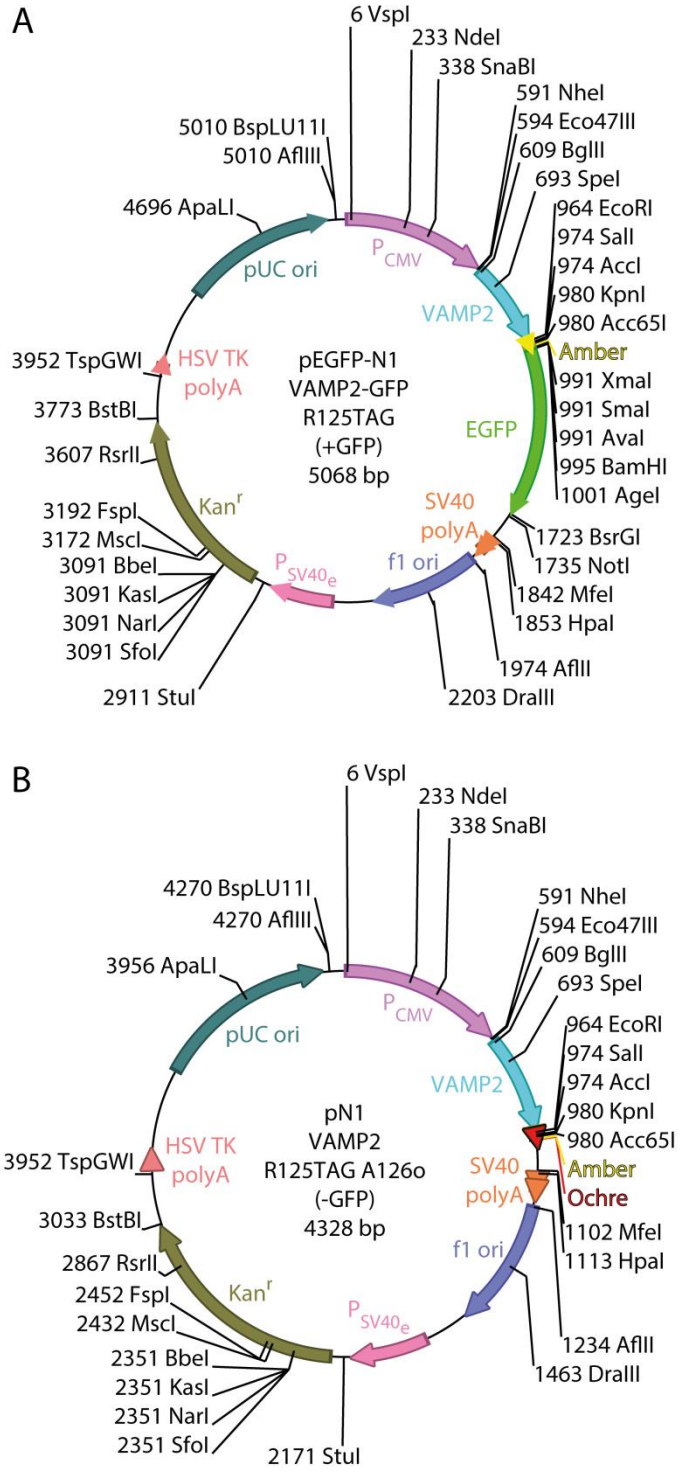

Figure S2. Vector maps for plasmids +GFP (pEGFP-N1 VAMP2-GFP R125TAG) and -GFP (pN1 VAMP2 R125TAG A126o). Both the pEGFP-N1 plasmid (**A**) and its pN1 counterpart (**B**) contain the VAMP2 sequence and an Amber stop codon at positions 987-989 of the vector (according to the vector annotation; shown in yellow). The pN1 vector is endowed with an Ochre stop codon (in red) immediately after the Amber one, and it lacks the GFP coding sequence, which was excised using the *SmaI* and *NotI* restriction enzymes. The rest of the vector backbone is identical for both plasmids: a human cytomegalovirus promoter

(P_{CMV}, in purple), a Simian virus 40 mRNA polyadenylation signals (SV40 polyA, in orange), the f1 phage origin of replication (f1 ori, in dark purple), an SV40 early promoter and origin of replication (P_{SV40e}, in pink), a gene for kanamycin/neomycin resistance (Kan^R/Neo^R, in olive color), a Herpes simplex virus thymidine kinase polyadenylation signals (HSV TK polyA; in peach color), and a pUC origin of replication (pUC ori, in turquoise). Aside from this, the unique restriction enzyme sites are indicated.

II. Cell transfection and UAA incorporation (1day)

To test the expression of the protein of interest and the incorporation of the unnatural amino acid (UAA) of choice, we recommend using either the BHK cell line (available at American Type Culture Collection, ATCC number CCL-10), or other cell lines that have high transfection efficiencies, such as the HEK293T cells (ATCC CRL-3216).[4] We recommend plating the cells either the evening before, or in the morning of the day when the transfection is performed. In this study we have employed 12 mm coverslips coated with a solution of 20 μg/mL poly-L-lysine hydrochloride (PLL; cat. no. P2658, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), in water. The PLL coating should be performed for at least 1 h (at room temperature). If the cells are plated in the morning, they should be allowed to attach to the substrate for at least 4 h before performing the transfection. The amount of cells plated should be adjusted such that the cells are about 70% confluent when transfection is performed. Approximately 1 h before transfection, the medium should be replaced to medium supplemented with the UAA (in our case, 250 μM of propargyl-L-lysine, PRK, 1 M stock in DMSO, cat. no. SC-8002, Sirius Fine Chemicals GmbH, Bremen, Germany). The corresponding controls would be wells where only normal medium is added. To reduce toxicity during transfection, a medium without antibiotics can be used.

Throughout this study we have employed Lipofectamine 2000 (cat. no. 11668-019, Invitrogen, Carlsbad, CA, USA) for transfecting the cells. Yet any transfection agent could be employed as long as it has good transfection efficiency, and the transfection parameters are optimized for UAA incorporation. For lipofection, we recommend mixing 3 μL Lipofectamine with 50 μL Opti-MEM (Gibco, cat. no. 31985070), and incubating this solution for up to 5 min (this incubation step is optional). Separately, the two plasmids required for UAA incorporation are added in a 1:1 ratio:

i) the plasmid encoding for the tRNA/tRNA synthetase from *Methanosarcina mazei*, and ii) the plasmid containing the protein of interest, +GFP or –GFP.

The maximum amount of DNA added in a well of a 12-well plate should be 2 μg. It is advisable to have controls in which the transfection mix was added in the absence of the UAA, to test for spurious GFP expression, for the toxicity of the UAA in the cell line used, or for the nonspecific binding of the dye during the click reaction (step III, below). We recommend allowing the cells to express the protein of interest for 12-18 h.

The plasmid system we have employed is based on *Methanosarcina mazei* tRNA and the wild-type pyrrolysyl-tRNA synthetase for the incorporation of PRK.[5,6] This plasmid, termed pCMV PylsRS WT, is available from the Lemke laboratory (Structural and Computational Biology Unit, European Molecular Biology, Laboratory, Heidelberg, Germany). However, other systems have been used to incorporate other amino acids amenable to click chemistry (see for example reviews by Lang and Chin, 2014, or Nikić and Lemke, 2015).[7,8]

III. Cell fixation and labeling using click chemistry (0.5 days)

After the desired time for protein expression and UAA incorporation has elapsed, remove the medium from the cells, wash them once with PBS, and incubate them in normal medium for 2-3 h to remove excess UAA. Briefly wash the cells in PBS and then perform fixation with 0.2% glutaraldehyde and 4% paraformaldehyde (PFA), for at least 30 min. Note that using glutaraldehyde in the fixation step helps better preserve the sample, as well as the multi-molecular arrangements formed by the protein of interest. However, this stronger fixation is more difficult to combine with immunostaining (if such a step is desired), since it has the drawback that many antibodies may not properly detect their targets, as they are unable to access the epitopes after glutaraldehyde fixation. The samples should be then briefly washed in PBS, and the free aldehyde groups should be quenched using 100 mM NH_4Cl and 100 mM glycine, in PBS. Afterwards, the samples should be washed 2-3 times with PBS, before proceeding to permeabilization. For this step we recommend using a solution of 0.1% Triton X-100 in PBS, with which the cells are incubated for 15 min, with 3 solution exchanges. Before proceeding to the click reaction, it is advisable to perform a strong blocking procedure that minimizes unspecific binding of the clickable dye. We have obtained best results with a solution of 5% bovine serum albumin (BSA, Albumin Fraction V, pH 7.0, cat. no. A1391, AppliChem GmbH, Darmstadt, Germany) and 5% peptone (tryptone/peptone ex casein, cat.

no. 8952.3, Carl Roth, GmbH + CoKG, Karlsruhe, Germany), in PBS containing 0.1% Triton X-100. This solution should be replaced by a less strong blocker, such as 3% BSA in PBS, for up to 5 min before the click reaction mix is added to the cells.

The click reaction is afterwards performed for 30 min in a dark humidified chamber, at room temperature. The instructions of the manufacturer (Click-iT Cell Reaction Buffer Kit, Life Technologies) suggest preparing the click solution immediately before adding it to the cells. This should contain 3 μM AlexaFluor647-azide (for STORM or GSDIM imaging, cat. no. A10277, Molecular Probes Inc., Eugene, OR, USA) or 25-50 μM Star635P-azide (for STED imaging, available on special order at Abberior GmbH, Göttingen, Germany), 1× component A, 2 mM component B ($CuSO_4$), and 1:10 dilution of component C, all mixed in water. The samples are then washed for 15 min with 5% BSA and 5% peptone in PBS, with 3 solution exchanges, and then are washed in the same fashion with PBS alone. The cells are now ready for embedding in melamine.

IV. Melamine embedding and thin sectioning (over 4 days; 2-3 actual working hours)

The melamine mix should be prepared 2 h in advance, because melamine requires a long time to dissolve. This protocol has been described in detail by Revelo and Rizzoli.[9] For two coverslips, dissolve 48 mg *p*-toluenesulfonic acid monohydrate in a 15 mL Falcon tube with 0.576 mL distilled water. Add 1.344 g melamine (2,4,6-Tris[bis(methoxymethyl)amino]-1,3,5-triazine, cat. no. T2059, TCI Europe, Zwijndrecht, Belgium) and vortex the resulting mix thoroughly to bring all the melamine in contact with the solution. Then allow it to get dissolved by shaking it for 2 h on a horizontal shaker, at 250 rpm.

Take the 12 mm coverslips (after the final PBS wash) and remove the buffer. Use BEEM capsules (Beem Inc., West Chester, PA, USA) with cutout bottoms to delineate the region(s) on the coverslip that you want to embed, and place the capsules upside down on the coverslips (with the opening in contact with the coverslip). Then add ~200 μL of melamine solution slowly on the capsule margin. Place the coverslip on a flat plastic support and then dessicate it in a box with silica gel (Sigma Chemical Company, St. Louis, MO, USA) for 24 h at room temperature, in the dark. This allows the melamine to penetrate the sample, and ensures a slow dehydration. Subsequently, the sample should be incubated on silica gel for a further 24 h at 40°C, for the melamine to polymerize. Then the BEEM tube can be filled with Epon resin

(Epofix kit, cat. no. 40200029, Struers, Ballerup, Denmark) and the sample should be dried incubated for approximately 48 h at 60°C.

After the melamine has hardened, the BEEM tube can be removed. The resulting embedding block can be now trimmed and cut, using an ultramicrotome, into thin sections (we recommend around 100 nm). Note that for GSDIM or STORM imaging the sections should be placed in the middle of a 12 mm coverslip. The sections adhere strongly to the coverslip while they dry. These coverslips can then be stored in 12-well plates until imaging is performed. Storing the coverslips for several weeks is possible.

V. *Sample mounting for super-resolution imaging (1 day)*

To identify differences between protein clusters with or without GFP, it advisable to use nanoscopy setups that reach a resolution of 20-30 nm. If only a resolution >30 nm is attained, the differences might not be discernible. To separate real signals from single flurophores non-specifically linked to the preparation, it is advisable to analyze in parallel transfected and clicked samples that were not exposed to the UAA, and which therefore only show non-specific labeling.

For STED measurements, the coverslips can be mounted in Mowiol (24% w/v glycerol, 0.1 M Tris-HCl, pH 8.5, 9.6% w/v Mowiol 4-88, cat. no. 475904, Merck Millipore, Darmstadt, Germany).

For GSDIM or STORM imaging, use the following imaging buffer recipe (previously described by Zhuang and collaborators):[10] 1% enzymatic scavenger system for oxygen in 50 mM Tris-Cl (pH 8.0), 10 mM NaCl, 10% glucose (w/v), and 10 mM β-mercaptoethyl-amine (pH 8.5, cat. no. 30070 or M9768, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). The enzymatic scavenger system comprises a mixture of 10 mg glucose oxidase (cat. no. G2133, Sigma, Saint Louis, MO, USA) and 50 μL of a 20 mg/mL solution of catalase (cat. no. C-40, Sigma, Saint Louis, MO,USA) in 200 μL PBS, which should be centrifuged for 1 minute at 13 000 rpm, and should then be stored at 4°C. Add a drop (~100 μL) of imaging buffer on a microscope slide, and then place the coverslip on it. Remove buffer excess and use a silicone-based polymer mix (twinsil speed, cat. no. 1300 100,

Picodent, Wipperfürth, Germany) to seal around the margins of the coverslip. Image the sample within the next few hours.

Different super-resolution microscopes can be used, according to the manufacturers' instructions. For details on the imaging parameters that we employed, please consult the Materials and Methods section.

Image processing may be performed. It is especially relevant for GSDIM/STORM imaging, to reconstruct the super-resolution images from the raw data files. In our case, the best GSDIM parameters consisted of a detection threshold of 25 photons/pixel, a center-of-mass fitting algorithm, and a 10 nm pixel size, using the manufacturer's software (Leica Application Suite, Advanced Fluorescence, version 3.2). STED is more straightforward in the sense that it does not require imaging in a specific buffer, nor does it require post-processing to obtain a final image, albeit filtering can be used to reduce the noise in these images. However, it should be noted that in this work we have used the STED images without any noise reduction procedure.

The data analysis routine should consist of the following: a thresholding procedure to select only clusters of multiple proteins, as opposed to single fluorophores, and a fitting procedure to determine the size and the intensity of individual protein clusters.

References

(1) Kolmakov, K.; Belov, V. N.; Bierwagen, J.; Ringemann, C.; Müller, V.; Eggeling, C.; Hell, S. W. Red-Emitting Rhodamine Dyes for Fluorescence Microscopy and Nanoscopy. *Chemistry* **2010**, *16*, 158–166.

(2) Plass, T.; Milles, S.; Koehler, C.; Szymański, J.; Mueller, R.; Wiessler, M.; Schultz, C.; Lemke, E. A. Amino Acids for Diels-Alder Reactions in Living Cells. *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 4166–4170.

(3) Nikić, I.; Plass, T.; Schraidt, O.; Szymański, J.; Briggs, J. A. G.; Schultz, C.; Lemke, E. A. Minimal Tags for Rapid Dual-Color Live-Cell Labeling and Super-Resolution Microscopy. *Angew. Chemie - Int. Ed.* **2014**, *53*, 2245–2249.

(4) Nikić, I.; Kang, J. H.; Girona, G. E.; Aramburu, I. V.; Lemke, E. a. Labeling Proteins on Live Mammalian Cells Using Click Chemistry. *Nat. Protoc.* **2015**, *10*, 780–791.

(5) Plass, T.; Milles, S.; Koehler, C.; Schultz, C.; Lemke, E. A. Genetically Encoded Copper-Free Click Chemistry. *Angew. Chem. Int. Ed. Engl.* **2011**, *50*, 3878–3881.

(6) Milles, S.; Tyagi, S.; Banterle, N.; Koehler, C.; VanDelinder, V.; Plass, T.; Neal, A. P.; Lemke, E. A. Click Strategies for Single-Molecule Protein Fluorescence. *J. Am. Chem. Soc.* **2012**, *134*, 5187–5195.

(7) Lang, K.; Chin, J. W. Cellular Incorporation of Unnatural Amino Acids and Bioorthogonal Labeling of Proteins. *Chem. Rev.* **2014**, *114*, 4764–4806.

(8) Nikić, I.; Lemke, E. a. Genetic Code Expansion Enabled Site-Specific Dual-Color Protein Labeling: Superresolution Microscopy and beyond. *Curr. Opin. Chem. Biol.* **2015**, *28*, 164–173.

(9) Revelo, N. H. . R. S. O. Advanced Fluorescence Microscopy. *Methods Mol. Biol.* **2015**, *1251*, 213–230.

(10) Dempsey, G. T.; Vaughan, J. C.; Chen, K. H.; Bates, M.; Zhuang, X. Evaluation of Fluorophores for Optimal Performance in Localization-Based Super-Resolution Imaging. *Nat. Methods* **2011**, *8*, 1027–1036.