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Protocol extension

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Observing isolated synaptic vesicle association and fusion ex vivo

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Abstract

Here, we present a protocol for isolating functionally intact glutamatergic synaptic vesicles from whole-mouse brain tissue and using them in a single-vesicle assay to examine their association and fusion with plasma membrane mimic vesicles. This is a Protocol Extension, building on our previous protocol, which used a purely synthetic system comprised of reconstituted proteins in liposomes. We also describe the generation of a peptide based on the vesicular glutamate transporter, which is essential in the isolation process of glutamatergic synaptic vesicles. This method uses easily accessible reagents to generate fusion-competent glutamatergic synaptic vesicles through immunoisolation. The generation of the vGlut peptide can be accomplished in 6 d, while the isolation of the synaptic vesicles by using the peptide can be accomplished in 2 d, with an additional day to fluorescently label the synaptic vesicles for use in a single-vesicle hybrid fusion assay. The single-vesicle fusion assay can be accomplished in 1 d and can unambiguously delineate synaptic vesicle association, dissociation, Ca^{2+}-independent and Ca^{2+}-dependent fusion modalities. This assay grants control of the synaptic vesicle environment while retaining the complexity of the synaptic vesicles themselves. This protocol can be adapted to studies of other types of synaptic vesicles or, more generally, different secretory or transport vesicles. The workflow described here requires expertise in biochemistry techniques, in particular, protein purification and fluorescence imaging. We assume that the laboratory has protein-purification equipment, including chromatography systems.

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Key points

- This protocol describes an immunoisolation-based approach for isolating functionally intact glutamatergic synaptic vesicles from whole-mouse brain tissue and their use in a single-vesicle assay to examine their association and fusion with plasma membrane mimic vesicles.
- Isolated vesicles are amenable to a range of functional studies. In addition to the fusion assay described in this protocol, the vesicles can be used for cryo-electron microscopy and cryo-electron tomography.

Key references

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Introduction

Neurotransmitter-containing synaptic vesicles fuse with the plasma membrane at the active zone to release their contents into the synaptic cleft, thus propagating a signal from one neuron to the next[1,2]. Although many components of the core molecular machinery of this process have been identified, the molecular mechanism of triggered exocytosis and the role of ancillary components remain uncertain. Studying exocytosis in situ is complicated by multiple confounding variables, such as the crowded molecular environment in a neuron, unknown cooperative or combinatorial effects of surrounding proteins and the involvement of various up- and downstream regulators of synaptic vesicle fusion. A reductionist in vitro assay with the minimal components necessary for triggered fusion (i.e., synaptotagmin and SNAP receptor (SNARE) complex proteins) is helpful because additional components can be incorporated to build a more physiologically complex system[3]. The findings from such in vitro fusion experiments have provided new insights into the specific functions of presynaptic proteins such as Munc13. For instance, these experiments revealed Munc13's role in (i) opening the syntaxin-Munc18 complex and facilitating the transition of syntaxin into the ternary SNARE complex[4,5], (ii) positioning SNAP-25 correctly in relation to syntaxin and synaptobrevin[6,7] and (iii) connecting the synaptic vesicle and plasma membranes[8]. Moreover, a reductive in vitro approach can be used to identify and segregate the roles of different domains within an individual protein, such as the domains of complexin, which play distinct roles in regulating fusion[9–11].

However, overly reduced systems also have drawbacks, in that they may not behave appropriately unless more components are incorporated into the experiments. For example, early minimal reconstitutions of Ca^{2+}-triggered synaptic vesicle fusion require relatively high Ca^{2+} concentrations to trigger fusion[3,12]. Only after adding other components did the Ca^{2+} sensitivity reach a physiological range of ~10–100 μM[6]. Unregulated and incomplete dead-end fusion is also a concern in highly reduced systems[12]. Thus, fusion assays that combine synthetic and purified components offer a hybrid intermediate between purely in vitro and purely in vivo experiments. In one example of a hybrid fusion assay, synaptic vesicles isolated by size-exclusion chromatography (SEC) were fused to planar lipid bilayers containing reconstituted syntaxin–SNAP-25 SNARE proteins (also referred to as 't-SNARES') in a Ca^{2+}-dependent fashion[13,14]. Although this method had the advantage that the planar lipid bilayer mimicked the relatively flat character of the plasma membrane, proteins within the bilayer were free to move, and tracking vesicles for an extended period was difficult. Consequently, this required synaptic vesicles to be added in the presence of (100 μM–1 mM) Ca^{2+}, and therefore, synaptic vesicle fusion occurred immediately upon association. However, cryo-electron tomography studies[15–17], interferometric scattering microscopy[18] and fluorescent microscopy experiments[19] suggest that a stable associated state of isolated synaptic vesicles (ISVs) exists at resting Ca^{2+} concentrations. Here, we present the protocol for a single-vesicle fusion assay that uses ISVs and immobilized plasma membrane mimic vesicles and permits the examination of vesicle stability before triggering fusion by injection of Ca^{2+}.

Development of the Protocol

Here, we extend our previous single-vesicle fusion protocol[20] to use ISVs instead of synthetic proteoliposomes. This begins with extending a previously established synaptic vesicle isolation method[21,22] to yield free, functionally intact ISVs. Regarding the plasma membrane mimic (PM) vesicles[20], the modified fusion protocol described here uses the same equipment as our previous protocol, except that we include a content dye.

Vesicle isolation

For vesicle isolation, we used crude synaptic vesicles isolated from 6–12 approximately postnatal day 20 (P20) mice as a starting point, by using a protocol previously reported[21,22]. Glutamatergic synaptic vesicles were then enriched by immunoisolation using an antibody against the vesicular glutamate transporter 1 (vGlut1) followed by protein-G paramagnetic bead

immunoisolation, again like previously reported methods[21,22]. The ISV protocol described here differs from these previous methods in the elution step[23]. After binding to beads, the beads are washed once with vesicle buffer (or PBS) containing 0.5% (wt/vol) BSA and twice with PBS alone, followed by gentle competitive elution by the addition of a molar excess of a peptide corresponding to the antibody epitope, vGlut peptide.

Preparation of the vGlut peptide to high purity and yield for use in the ISV isolation is critical and, if done incorrectly, can lead to contaminants in the final peptide sample. Briefly, the peptide was prepared by over-expression in *Escherichia coli*, followed by affinity capture via the N-terminal GST tag of the expressed fusion protein and subsequent SEC. Although high peptide yields were achieved, we encountered contamination in the sample even after size-based separation. Heating the sample proved to be successful in separating the contaminants from the peptide. This step takes advantage of the lack of a tertiary structure of the peptide, which allows it to remain soluble while the denatured contaminants precipitate out of solution, allowing for easy removal. Furthermore, eluting the peptide by using reduced glutathione instead of cleaving it from the GST tag while attached to the beads increased the yield of the final product, because this ensures that most, if not all, of the peptide is recovered from the beads. Moreover, tag cleavage and removal efficiency were increased when the tagged peptide was in solution. Note the dialysis molecular weight cutoff (~2 kDa) is small enough to retain the peptide while permitting sufficient glutathione dilution.

Hybrid fusion assay

Our previous fusion assay used purely synthetic synaptic vesicle mimics[20]. The synthetic 'donor' vesicle (the synaptic vesicle mimic containing reconstituted synaptobrevin and synaptotagmin) encapsulated sulforhodamine dye and incorporated the lipid dye DiD (1,1'dioctadecyl-3, 3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) whereas the PM mimic 'acceptor' vesicles were left unlabeled. Content mixing (i.e., fusion) was reported by the dequenching of sulforhodamine B. One advantage to this method was that hemifusion could be distinguished from complete fusion. While PM vesicles contain syntaxin–SNAP-25 complexes, SM (for Sec1/Munc18-like) vesicles contain syntaxin-Munc18 complexes. However, direct generation of SM vesicles is not possible, because of the incompatible detergents used in syntaxin and munc18 purifications. Thus, as an option, the PM vesicles may be converted to these more physiologically analogous and active SM vesicles before tethering them to the imaging surface, as described in ref. 23.

Here, we use similar synthetic PM/SM vesicles that are tethered to the passivated imaging surface (via PEG-biotin-NeutrAvidin-biotin-phosphoethanolamine (PE) interaction) and contain reconstituted full-length syntaxin-1A and SNAP-25A binary complexes as a starting point. PM/SM vesicles contain a high concentration of self-quenched sulforhodamine B. At the same time, the ISVs are labeled with Alexa-647. This is a crucial difference from our previous protocol[20] for two reasons: (i) there is no simple method to incorporate sulforhodamine B into the ISV lumen, but more importantly, (ii) if one were to include the content dye as an indicator for the ISV-PM/SM vesicle association (as was previously done[6,24]), it is not possible to distinguish between ISV-PM/SM vesicle association and immediate ISV-PM/SM vesicle fusion, because both produce a sharp stepwise increase in fluorescence. Thus, in the configuration presented here, the association of an ISV can be unambiguously identified as a stable stepwise increase in fluorescence in the 'red' channel, which measures the fluorescence intensity of the Alexa-647 dye[23,25]. At the same time, ISV-PM/SM vesicle fusion is reported by a stable stepwise fluorescence increase in the 'green' channel, which measures the fluorescence intensity of the sulforhodamine dye.

Our assay distinguishes between ISV-PM/SM association, Ca^{2+}-independent fusion during association and Ca^{2+}-triggered fusion. Thus, our improved protocols allow not only the examination of ISV-PM/SM vesicle fusion but also the ability to assess the stability of ISV association on the time scale of minutes. Our protocol can also generate samples for cryo-electron microscopy (cryo-EM)[17], opening the possibility of directly investigating structural intermediates involved in synaptic vesicle fusion.

Applications of the method

Existing synaptic vesicle purification methods generate a molecularly heterogeneous population of synaptic vesicles as a starting point. The protocols described here may serve as a starting point for investigating functional differences in different populations of ISVs; only the elution peptide and antibody used for labeling need to be changed. We envision that the ISVs may be used in fusion assays, such as the one presented here. The ISVs can also be used for cryo-EM and cryo-electron tomography[17,26]. Moreover, our ISV isolation and purification technique is also relatively simple, and the crude synaptic vesicle starting material (LP2) can be stored for long periods; thus, it may prove attractive when there is limited space to maintain multiple transgenic mouse lines or when transgenic mice survivability is low. For example, eight CD1 P20 mice (~3 g (wet weight) of brain) provided enough starting material for ~5 isolations, each permitting 20 association and 20 fusion experiments. For cryo-EM, the same number of animals would yield enough ISVs for ~200 grids. The ISV fusion assay presented here uses a relatively slow acquisition rate, because we prioritized ISV number (i.e., frame size over speed). Moving to faster acquisition frequencies and fusion kinetics is possible by imaging smaller cropped regions or using more-advanced cameras with faster frame rates.

Comparison with other methods

Previous methods produced ISVs[21,22], but further purification was limited to biochemical analysis because the integrity of the ISVs was potentially affected by the particular elution conditions during their preparation and analysis (i.e., mass spectrometry). In another approach, the purified ISVs remained functionally intact but were purified only by size or density[13,27]. A different peptide-based approach was used to isolate a non-specific population of ISVs[28].

Here, we isolate an enriched population of specific ISVs (glutamatergic, in this example) on the basis of immunoisolation followed by competitive elution with a molar excess of a small 56-aa peptide comprising the C-terminus of mouse vGlut1 that includes the epitope of the antibody used in the immunoisolation. The basis for the isolation is competitive elution. Thus, by changing the antibody and corresponding elution peptide, alternate populations of ISVs could be targeted[29].

Limitations

In addition to the general limitations discussed above, our fusion assay has two specific limitations. First, in the current setting, this fusion assay does not distinguish between a hemi-fused dead-end state and no fusion[12], because we measure only content mixing but not lipid mixing. However, this assay could be easily modified by including a lipid-mixing marker[12] to address this limitation. Second, our assay uses tethered PM/SM vesicles rather than a planar lipid membrane, thus potentially introducing additional physical forces due to the acceptor membrane curvature (the diameter of PM/SM vesicles is ~50–100 nm).

Overview of the Procedure

This protocol is divided into two Procedures: Procedure 1 describes the expression and purification of the vGlut elution peptide, and Procedure 2 describes the use of the vGlut elution peptide in the immunoisolation of ISVs and a fluorescent hybrid fusion assay that may be used to examine the fusion of the ISVs with PM/SM vesicles. Procedure 1 consists of two stages: the expression of the vGlut peptide (Steps 1–25) and the peptide purification (Steps 26–36). Procedure 2 consists of multiple stages: the preparation of PM/SM vesicles and the isolation and elution of ISVs from lysis pellet 2 (LP2) (Steps 1–32). Both vesicle types are used in the hybrid fusion assay. The hybrid fusion assay is further divided into chamber preparation (Steps 33–40), the fusion assay (Steps 41–61) and data analysis (Steps 62 and 63). The new chamber glass or recycled glass cleaning was described previously[20] and may be performed earlier and stored at −20 °C. We note that the synaptic vesicle isolation begins from a crude synaptic vesicle mixture (LP2) produced by using an established protocol[21,22]. This LP2 fraction may be prepared before the synaptic vesicle isolation, so long as it is flash-frozen in liquid nitrogen and stored at −80 °C. Similarly, preparation of the vGlut elution peptide may be performed any time before the elution, and the peptide is stable for many months at −80 °C.

Experimental design

Peptide preparation and vesicle isolation

For peptide isolation, because the vGlut peptide lacks aromatic amino acids, it is challenging to track throughout the purification process and to calculate its concentration. Therefore, using a liquid handling system with a UV monitor capable of detecting multiple wavelengths is highly recommended for the final SEC purification step. The final concentration of peptide may be determined by using bicinchoninic acid (BCA) if desired because this lack of aromatic amino acids renders the peptide undetectable in a Bradford assay.

Generating larger volumes of the LP2 fraction (i.e., using more mouse brains than proposed here or using rats instead of mice) may require increasing the centrifugation speed. In particular, we found increasing the spins before and after osmotic lysis to 14,597*g* and 54,871*g*, respectively, was necessary to achieve a suitable pellet. Optimization may be required depending on the type of rotor and the number of mouse brains available.

Before performing the fusion assay described here, the labeling efficiency of ISVs (what percentage of ISVs are labeled) must be determined. We used immunogold negative-stain electron microscopy to assess labeling efficiency. ISVs were labeled first with primary fluorescent antibody, then added to a grid and incubated with 1:1,000 10-nm gold-conjugated secondary antibody for 2 h at 4 °C in an inverted Petri dish with ddH_2O included on the periphery to increase the humidity in the chamber and prevent sample drying. Other labeling efficiency methods may be used at the user's discretion.

PM vesicle generation

For PM vesicle generation, the molar amount of syntaxin-1A used should be kept constant with lipid mass in a 1:200 protein-to-lipid ratio. Therefore, the total volume of syntaxin-1A used is dependent on the concentration of syntaxin-1A. To limit syntaxin self-aggregation, we add an excess of SNAP-25 to encourage the formation of binary SNARE complexes. We typically add 3:1 SNAP-25/syntaxin-1A. Using these conditions, experimenters are free to adjust the volumes added according to the concentrations of the purified proteins. Two important points need to be considered. First, keep in mind the total volume of the tube in which the reconstitution occurs, because large volumes may result in spillage. Second, the buffers for the reconstituted proteins need to be compatible. Once vesicles are formed, they are amenable to subsequent modifications, such as SM conversion.

Hybrid fusion assay

The hybrid fusion assay described here produces fluorescence data in which vesicle association or fusion may be detected by small stepwise changes in the fluorescence intensity of dyes.

Here, we use a prism-based total internal reflection fluorescence (TIRF) microscopy technique. However, the protocol could be easily adapted to other imaging modalities. Our microscope consists of two excitation lasers of wavelengths 648 and 532 nm. We use a 60× water immersion objective with an optisplit two-beam splitter in the emission pathway. The details of our imaging setup were described previously[20], but we stress that our system is designed to be flexible, and the user may adjust the dyes and antibodies used here to accommodate their own capabilities.

Solutions in the imaging chamber can be exchanged by removing liquid from the inlet wells by pipetting. For example, we have used this to study Ca^{2+}-triggered fusion[23]. Care should be taken not to bump or move the stage during aspiration. A gel-loading pipette tip may be helpful.

Fusion assay analysis

We have developed custom MATLAB (Mathworks) scripts, complete with a graphical user interface, for detecting the time and amplitude of these stepwise changes. Using these scripts, each trace is corrected for background and baseline fluorescence by averaging the first user-defined number of frames (the default is 23 acquisition frames, ~4.6 s) and subtracting each time point by this baseline. Next, a moving average and standard deviation are calculated by using user-defined acquisition time bins (default: 10) to create a prior baseline value. A moving average of user-defined acquisition time bins (default: 8) is then compared to the preceding value. Any test value is considered a putative 'hit' if its value is at least a user-defined threshold greater than the preceding baseline (default: 1.4×) plus 1 s.d.

In addition, the average of fluorescence acquisition frames (default: 20) after the putative hit, as well as the final acquisition time bins of the entire trace, must be minimally greater than the beginning of the experiment (default: 1.4×). These two constraints ensure the collection of stepwise increases in fluorescence and eliminate events in which surface-tethered PM/SM vesicles might rupture, photobleach or otherwise disappear. A delay gap (default: 10 frames) may also be included to prevent multiple positives for the same event. The MATLAB program compiles a list of points that meet these criteria as putative hits, which may be manually inspected as true hits or false positives and optionally saved as exemplary traces. The analysis can also be performed without inspection, in which all putative hits are assumed true. By default, we intentionally made these constraints relatively generous to enrich true positives over false negatives and allow for manual curation. This analysis is repeated for both channels, and separate user-defined values may be applied to each channel.

Detecting Ca^{2+}-independent fusion. For Ca^{2+}-independent fusion analysis, a stepwise increase in the red channel must coincide with or precede a stepwise increase in the green channel. Any change in the green channel without a change in the red channel, while rare, should not be counted, because they may be artefactual. For the analysis presented here in Anticipated results, traces that included more than one increase in the red channel are counted as multiple association events, and any fusion occurring therein is not counted, because it cannot be determined which ISV fuses with the PM/SM vesicle. Thus, the fusion events here are derived only from single vesicle-vesicle associations. Association events should be normalized to the total number of observed PM/SM vesicles (also called 'total ROIs').

Detecting vesicle dissociation. For dissociation, the fluorescence trace must not only contain a stepwise increase as described above, but also a similar negative abrupt decrease (as opposed to a sloping decrease). A reduction in fluorescence is detected as described above, except the value must be lower than the prior fluorescence minus 1 s.d. of the preceding fluorescence intensity. Note that this does limit the minimum dwell time to ~4.6 s so that a prior baseline may be established. Dissociation events and dwell times are normalized to the total number of available surface-tethered PM/SM vesicles (total ROIs).

Detecting Ca^{2+}-triggered fusion. In the case of Ca^{2+}-triggered fusion experiments[23], the green channel is treated exactly as above. However, because free Alexa-647 dye was also used to indicate Ca^{2+} arrival, the Alexa-647 (red) fluorescence intensity values are not corrected for background fluorescence (doing so would obscure the global increase in fluorescence upon triggering). The traces are averaged together, and the first point above a user-defined noise level (as calculated above) is determined to be the time of Ca^{2+} arrival. Fusion times are normalized to the arrival of Ca^{2+} for each experiment. Fusion events are normalized to the total number of ISV-PM/SM vesicle pairs identified before fusion. If desired, the red traces can be treated individually for more precise timing of Ca^{2+} arrival per ROI. Here, our acquisition rate was slower than the arrival of Ca^{2+}, so for convenience, a single Ca^{2+} arrival time was used for all ROI.

Materials

Biological materials

- *Mice*. We have used 6–12 P20 or older mice with a total wet weight of <6 g for each LP2 generation[23] (any strain or age could be used (e.g., CD1 mice)).
  ▲ **CAUTION** All animal handling, euthanasia and tissue collection should be performed per institutional regulatory board guidelines. The work described in this protocol was performed in accordance with the guidelines of the National Institutes of Health and was approved by the Stanford Administrative Panel on Laboratory Animal Care (APLAC) Institutional Animal Care and Use Committee (protocol no. 29981).

Reagents

vGlut peptide expression

- vGLUT1 peptide GST plasmid (DNA 2.0; Addgene, cat. no. 218677)
- BL21 Star (DE3) chemically competent *E. coli* (Thermo Fisher, cat. no. C601003)
- S.O.C. medium (Thermo Fisher, cat. no. 15544034)
- Ampicillin agar Plates (carbenicilin agar plates can be used as well)
- LB medium, powder (MP Biomedicals, cat. no. 3002022)
- Ampicillin sodium salt (Sigma, cat. no. A9518)
- Ammonium sulfate (Sigma, cat. no. A4418)
- Potassium phosphate monobasic (Sigma, cat. no. PX1565)
- Sodium phosphate dibasic anhydrous (Fisher Scientific, cat. no. S379)
- Glycerol (VWR Chemicals BDH, cat. no. BDH1172)
- D(+) Glucose (Sigma, cat. no. G7528)
- α-Lactose monohydrate (Sigma, cat. no. L3625)
- Magnesium sulfate heptahydrate (Fisher Scientific, cat. no. BP213-1)
- Trace metals mixture (1,000×; Teknova, cat. no. T1001)

Peptide purification

- Lysozyme (Sigma, cat. no. L6876)
- Deoxyribonuclease I (Sigma, cat. no. DN25)
- EDTA-free protease inhibitor cocktail tablets (cOmplete Mini; Roche, cat. no. 11836170001)
- Tris pH 8 (Corning, cat. no. 46-031-CM)
- Tris pH 7.5 (Corning, cat. no. 46-030-CM)
- Sodium chloride (Fisher Scientific, cat. no. BP358)
- DTT (AmericanBio, cat. no. 3483-12-3)
- EDTA solution, 0.5 M, pH 8 (VWR, cat. no. BDH7830)
- Glutathione superflow agarose bead resin (Thermo Fisher, cat. no. 25238)
- Glutathione, reduced (Fisher Scientific, cat. no. BP252125)
- 3C protease (purchase from vendor of choice (e.g., EMD Millipore, cat. no. 71493))
- BCA protein assay kit (Pierce; Thermo Fisher, cat. no. 23227)

Hybrid fusion assay reagents

- LP2 starting material[21]
- vGlut1 antibody for vesicle isolation (SYSY, cat. no. 135 311, RRID: AB_887880)
- Alexa Fluor 647 anti-synaptophysin antibody (Abcam, cat. no. 196166)
- Goat-anti-rabbit IgG, 10 nm, gold (EMS, cat. no. 25108)
- Sepharose CL-4B (Millipore Sigma, cat. no. GE17-0150-01)
- Brain total lipid extract (Avanti, cat. no. 131101C)
- L-α-Phosphatidylinositol-4,5-bisphosphate (Avanti, cat. no. 840046)
- 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, cat. no. 850457C)
- 1-2-dioleoyl-*sn*-glycerol 18:1 diacylglycerol (DAG; Avanti Polar Lipids, cat. no. 800811C)
- 1-oleoyl-2-2(12-biotinyl(aminododecanoyl))-sn-glycero-3-phosphoethanolamine (sodium salt) (Biotin-PE; Avanti Polar Lipids, cat. no. 860562)
- Bio-Beads SM-2 adsorbent (Bio-Rad, cat. no. 152-3920)
- Sulfhorhodamine B (Thermo Fisher, cat. no. S1307)
- NeutrAvidin (ThermoFisher, cat. no. 31000)
- mPEG-SVA (molecular weight: 5,000 daltons; Laysan Bio, cat. no. mPEG-SVA-5000)
- Biotin-PEG-succinimidyl valerate (molecular weight: 5,000 daltons; Laysan Bio, cat. no. biotin-PEG-SVA-5000)
- Alexa Fluor 647 fluorescent dye (Alexa-647) carboxylic acid, tris(triethylammonium) salt (Thermo Fisher, cat. no. A33084)
- Protein G Dynabeads for immunoprecipitation (Thermo Fisher, cat. no. 10004D)
- Float-A-Lyzer G2 dialysis device, molecular weight cutoff (MWCO): 300 kDa (Spectra Por, cat. no. G235036)

- Pierce ECL western blotting substrate (Thermo Fisher, cat. no. 32106)
- Kimwipe (Fisher Scientific)
- vGlut 1 rabbit polyclonal antibody for western blot (Synaptic Systems, cat. no. 135 303; RRID: AB_887875)
- vGAT rabbit polyclonal antibody for western blot (Synaptic Systems, cat. no. 131 013; RRID: AB_2189938)
- Synaptophysin mouse monoclonal antibody for western blot (Abcam, cat. no. ab8049; RRID: AB_2198854)
- Goat anti-rabbit IgG H&L (HRP) (Abcam, cat. no. ab97051; RRID: AB_10679369)
- IRDye800CW goat anti-mouse IgG (Licor, cat. no. 926-32210; RRID: AB_621842)
- IRDye800CW goat anti-rabbit IgG (Licor, cat. no. 926-32211; RRID: AB_621843)
- Syntaxin-1A (70 µM minimum) (purified as in ref. 23)
- SNAP-25 (100 µM minimum) (purified as in ref. 23)
- Laser-cut double-sided sticky tape (Saunders UNI-3M, with dimensions 45 mm × 50 mm)

Equipment

- Bottle top filter, 0.22-µm pore size (Steritop; Millipore, cat. no. SCGPT10RE)
- Plate incubator
- Innova 40 benchtop orbital shaker (Eppendorf, cat. no. M1299-0080)
- Innova 44R incubator shaker (Eppendorf, cat. no. M1282-0004)
- AirOtop enhanced seal (Thomson, cat. no. 899425)
- Erlenmeyer flask
- Water bath
- Ultra-yield cell culture flasks (2.5 liters; Thomson, cat. no. 931136-B)
- Centrifuge (Avanti JXN-26; Beckman Coulter)
- Fixed-angle aluminum rotor (JLA-8.1000; Beckman Coulter, cat. no. 363688)
- Immersion blender
- Sonic dismembrator (Fisherbrand Model 505; Fisher Scientific, cat. no. FB505110)
- Ultracentrifuge (Optima XL-100K ultracentrifuge; Beckman Coulter)
- Ultracentrifuge rotor type Ti-45 (Beckman Coulter, cat. no. 339160)
- Polycarbonate bottle assembly, 6 pack (Beckman Coulter, cat. no. 355622)
- Gravity flow column (Kimble Kontes; Fisher Scientific, cat. no. k4204002505)
- AKTA START protein purification system (Cytiva)
- Dialysis cassettes (2-kDa MWCO, 12 ml; Thermo Scientific, cat. no. 66212)
- Mini tube rotator (Fisherbrand; Fisher Scientific, cat. no. 88-861-051)
- AKTA pure protein purification system (Cytiva)
- Ultra centrifugal filter unit (Amicon; Fisher Scientific, cat. no. UFC900324)
- HiLoad Superdex 75 16/60 preparative column (Cytiva)
- Standard gel electrophoresis chamber (BioRad)
- Invitrogen iBlot gel transfer station (no longer sold); may use any standard western blot transfer apparatus
- Quartz slide for imaging chamber (VWR or any producer; maybe include predrilled holes as appropriate for the user's experimental setup)
- Cover glass, 1 ounce (no. 1) 24 × 40 mm (VWR or any equivalent size and thickness subject to the user's specific optical setup)
- Double-sided tape (3M) or optionally laser-cut double-sided sticky tape (Saunders). For laser-cut double-sided sticky tape, the dimensions are 50 mm × 45 mm with five rectangles of 19 mm × 3.5 mm cut out spaced 3 mm apart (Saunders project reference name: JL_Fusion Chamber)
- Camera acquisition software (SmCamera)
- MATLAB with Signal Processing Toolbox installed (or other equivalent software may be used)
- Motorized syringe pump or manual syringe (any vendor)

Reagent setup

Solutions for vGlut peptide expression

20× NPS. Combine 0.5 M ammonium sulfate, 1 M potassium phosphate and 1 M sodium phosphate in a total volume of 2,000 ml. Filter the solution by using a sterile 0.22-µm filter. The solution can be stored at room temperature (21–23 °C) for 3 months.

50× 5052. Combine 500 g (weight by volume) of glycerol, 100 g of glucose, 210.6 g of α-lactose monohydrate in a total volume of 2,000 ml. Filter the solution by using a sterile 0.22-µm filter. The solution can be stored at room temperature for 3 months.

Expression medium. Supplement 900 ml of LB medium with 50 ml of 20× NPS, 20 ml of 50× 5052, 1 ml of 1 M magnesium sulfate, 1 ml of ampicillin (50 mg/ml stock) and 100 µl of trace metals mixture. The medium should be made fresh the same day or the night before, sterile sealed and stored at room temperature.

Solutions for peptide purification

Lysis buffer. Combine 50 mM Tris pH 8, 150 mM NaCl, 2 mM DTT and 1 mM EDTA in a total volume of 500 ml made fresh.

Wash buffer. Combine 50 mM Tris pH 8, 150 mM NaCl, 4 mM DTT and 0.5 mM EDTA in a total volume of 200 ml made fresh.

Elution buffer. Combine 50 mM Tris pH 8, 150 mM NaCl, 4 mM DTT and 10 mM reduced glutathione in a total volume of 50 ml made fresh. Adjust pH with NaOH if necessary.

Dialysis buffer. Combine 50 mM Tris pH 7.5, 150 mM NaCl and 1 mM DTT in a total volume of 1,000 ml made fresh.

Vesicle buffer (pH 7.4). Combine 20 mM HEPES and 90 mM NaCl in a total volume of 1,000 ml stored at room temperature. Filter before use. Adjust pH with NaOH if necessary.

3C protease cleavage. Add 500 µg of 3C protease into 100 µl of dialysis buffer (or use according to the manufacturer).

Solutions for purifying ISVs and performing the hybrid fusion assay

Immunoisolation blocking buffer (pH 7.4). Combine 20 mM HEPES, 90 mM NaCl and 0.5% (wt/vol) BSA in a total volume of 1 ml per 50 µl of paramagnetic beads. Alternatively, 0.5% (wt/vol) BSA in PBS may also be used. Store at 4 °C and filter by using a 0.22-µm filter before use. Discard after 1 month. Adjust pH with NaOH if necessary.

Vesicle buffer (pH 7.4). Combine 20 mM HEPES with 90 mM NaCl in a total volume of 2 liters. Make fresh and filter by using a 0.22-µm filter before use. Adjust pH with NaOH if necessary.

Western blot blocking buffer. Combine Tris-buffered saline, 1% (vol/vol) Tween-20 and 5% (wt/vol) BSA. Alternatively, any standard blocking buffer may be used. The buffer may be stored for 1 month at 4 °C.

Western blot primary antibody labeling buffer. Prepare a 1:2,500 dilution of vGlut antibody, a 1:2,500 dilution of vGAT antibody and a 1:500 dilution of synaptophysin antibody in blocking buffer, or use other manufacturer-recommended dilution in blocking buffer. Make fresh.

Western blot wash buffer. Prepare a standard wash buffer (e.g., Tris-buffered saline and 0.1% (wt/vol) Tween-20). The buffer may be stored for 1 month at 4 °C.

Western blot secondary labeling buffer. Prepare a 1:3,500 dilution or a supplier-recommended dilution of 800CW goat anti-rabbit or -mouse antibody in western blot blocking wash buffer. Make fresh.

PM-to-SM conversion buffer. Supplement vesicle buffer with final concentrations of 0.02 µM NSF (*N*-ethylmaleimide-sensitive factor), 0.1 µM α-SNAP, 2 µM Munc18, 1,000 µM ATP, 1,000 µM $MgCl_2$ and 500 µM TCEP (tris(2-carboxyethyl)phosphine). Take into account the volume of a 20× dilution of PM vesicles that will be added upon generation. See ref. 23. Make fresh.

5× soluble protein solution. This is a solution containing desired components that should be added simultaneously as the ISVs. We suggest concentrating this solution as much as possible and diluting it directly into the ISV sample to limit ISV dilution. For example, for a 5 µM complexin solution, combine 5 µM SNAP-25, 2.5 mM TCEP and 25 µM MUN domain of Munc13 in vesicle buffer added directly to ISVs such that the final concentration is 1 µM complexin, 1 µM SNAP-25, 500 µM TCEP and 5 µM MUN[23]. Make fresh.

LP2 (crude synaptic vesicle). Prepare the crude synaptic vesicle preparation from mouse brain material according to refs. 21,30 Use a BCA protein assay kit on the LP2 fraction to determine the total protein concentration. Distribute into 500-µg aliquots, snap-freeze in liquid nitrogen and store at −80 °C. LP2 may be stored for ≥1 year. We found that larger volumes of starting materials (i.e., more mouse brains) required increasing centrifugation steps.

NeutrAvidin. Prepare a stock solution of 1 mg/ml NeutrAvidin in filtered water. Protect from light and store at 4 °C. The stock solution may be stored for months at 4 °C. The 1:10 dilution used should be made fresh.

Procedure 1

vGlut peptide expression

● **TIMING 3 d**

Day 1: transform vGLUT1 peptide GST plasmid into *E. coli* cells

● **TIMING ~2 h**

1. Thaw competent cells (~2.66×10^8) on ice for ~10 min.
2. Mix 20 ng of plasmid with 50 µl of competent cells. Flick the tube two to three times to mix.
3. Incubate on ice for 30 min.
4. Remove the cells from ice and place them in a 42 °C water bath for exactly 30 s.
5. Remove the cells from the water bath and place them on ice for 2 min.
6. Add 700 µl of SOC medium and place the cells in an orbital shaker for 1 h at 37 °C.
7. After 1 h, dispense 50 µl and 100 µl onto two ampicillin LB agar plates that have been prewarmed to 37 °C. Spread the cells by using a cell spreader.
8. Incubate the plates overnight in an incubator at 37 °C.
9. (Optional) Prepare six ultra yield cell culture flasks containing 900 ml of LB medium each and autoclave. This can optionally be done on day 2.

Day 2: large-scale expression

● **TIMING ~30 h**

10. *Starter culture*. Pick one isolated colony from yesterday's transformation and mix with 150 ml of LB medium supplemented with ampicillin (50-µg/ml concentration). Use an Erlenmeyer flask for the starter culture.
11. Grow starter culture in an orbital shaker for 8–9 h at 37 °C and 225 rpm.
12. Prepare six ultra yield cell culture flasks containing 900 ml of LB medium each and autoclave. This can optionally be done on day 1 (see Step 9).
13. Prepare expression medium. Make sure that the LB medium temperature is close to room temperature.

14. Inoculate each flask of expression medium (made in the previous step) with 15 ml of starter culture.
15. Incubate in an incubator shaker at 225 rpm at 37°C for 6 h. After 6 h, lower the temperature to 30 °C and incubate for 16 h.

Day 3: large-scale expression (continued)

● **TIMING** ~15 min

16. Harvest the cells by centrifugation (4,700*g* for 10 min at 10 °C).
    ■ **PAUSE POINT** At this point, the cells can be flash-frozen in liquid nitrogen and stored at −80 °C, and the preparation can be continued at a later time.

Peptide purification

● **TIMING** 2 d

17. Resuspend the cell paste in 360 ml of lysis buffer supplemented with lysozyme, deoxyribonuclease I and six mini protease inhibitor tablets. Homogenize by using an immersion blender.
18. Lyse the cells by using a sonicator: 60% power (~300 W), 3 s on, 9 s off, 5 min total. Repeat two times. During sonication, the sonicator probe should be submerged ~10 cm into the lysate.
    ▲ **CRITICAL STEP** Perform sonication on ice, stirring between sonication rounds to dissipate heat.
19. Spin the lysed cells to remove debris (144,000*g* for 40 min at 4 °C). Discard the pellet and collect the supernatant.
20. Pour 30 ml of glutathione superflow agarose beads into a gravity flow column. Equilibrate the column with 2 column volumes of lysis buffer.
21. Mix the supernatant with beads. Incubate at 4 °C with stirring for 1–2 h.
22. Spin down the beads (870*g* for 10 min at 4 °C). Carefully remove the supernatant, avoiding disturbing the pelleted beads.
23. Resuspend the beads in wash buffer, transfer them to the gravity flow column and connect the column to the AKTA START system.
24. Wash with 200 ml of wash buffer by using a flow rate of 1 ml/min at 4 °C.
25. Elute the tagged peptide with elution buffer by using a flow rate of 0.5 ml/min at 4 °C. Collect 1-ml fractions.
    ▲ **CRITICAL STEP** The elution buffer should have a pH between 7.5 and 8.
26. Pool fractions with the highest absorbance at 280 nm. Dialyze overnight in 1,000 ml of dialysis buffer by using a 2-kDa MWCO dialysis cassette at 4 °C to remove the reduced glutathione.

Day 4: peptide purification (continued)

27. Remove the sample from the dialysis cassette, add 500 µg of 3C protease and incubate at room temperature for 2 h with end-over-end rotation with a rotation speed of 12 rpm.
28. Add another 250 µg of 3C protease and incubate at 30 °C for 2 h.
29. Heat the sample in a water bath set at 55 °C for 10 min to precipitate the cleaved GST tag.
30. Spin down the sample (4,500*g* for 5 min at 4 °C), discard the pellet and collect the supernatant.
31. Increase the water bath temperature to 62 °C and incubate the supernatant for 10 min.
32. Repeat Steps 29 and 30.
33. Filter the sample by using a 0.22-µm syringe filter and run the sample through a Superdex 75 16/600 column connected to the AKTA pure protein purification system and equilibrated with vesicle buffer. Use a flow rate of 1 ml/min and collect 2-ml fractions. Multiple AKTA runs will be required to purify the peptide. The runs should be monitored by using the 215-nm wavelength, because the cleaved peptide lacks aromatic amino acids and cannot be detected by absorbance at 280 nm.
    ▲ **CRITICAL STEP** Do not concentrate the sample before the AKTA run. Precipitation has been observed after concentration.
    ■ **PAUSE POINT** After SEC runs, the sample can be kept at 4 °C for ≤24 h.
34. Combine the fractions containing the peptide from all the runs and concentrate them by using a 3-kDa MWCO concentrator.

35. Measure peptide concentration by using the BCA protein assay. Peptides may be stored at any concentration. We typically store peptides at ~10 mg/ml.
    ▲ **CRITICAL STEP** Use a protein quantification assay that does not rely on absorbance at 280 nm.
36. Divide into aliquots and flash-freeze by using liquid nitrogen. Store at −80 °C.

Procedure 2

Preparation of vesicles

▲ **CRITICAL** Before day 1, prepare LP2 as described in Reagent setup.

Day 1: ISV preparation

● **TIMING ~30 min**

1. Thaw 500 µg of LP2 per 50 µl (1.5 mg) of Protein G Dynabeads.
2. Dilute 500 µg of LP2 to 1-ml final volume with blocking buffer (vesicle buffer + 0.5% (wt/vol) BSA).
3. Add 5 µl of vGlut1 monoclonal antibody to LP2.
4. Incubate overnight on a rotator at 4 °C or for a minimum of 2 h.

Day 1: PM vesicle preparation

● **TIMING ~20 min**

5. Combine brain total lipid extract, PIP2, biotin-PE, DAG, POPC and other lipids, if desired, in a chloroform-resistant tube (e.g., a glass tube). The specific concentration used may be altered if desired. As an example, we use the above lipids with final percentages (wt/vol) of 94% brain total lipid extract, 1% PIP2, 1% biotin-PE, 1% DAG and 3% POPC. If more PIP2 is desired, we exclude POPC.
6. Dry the combined lipids under inert gas (argon or nitrogen) to form a lipid film.
7. Place the lipid film in a vacuum overnight.
   ▲ **CRITICAL STEP** This step is to ensure that no chloroform remains. This step can be shortened to 2–4 h.

Day 2: ISV preparation

● **TIMING ~3.5 h + overnight**

8. Wash Protein G Dynabeads with 1 ml of blocking buffer three times.
9. Add 1 aliquot (500 µg of total protein) of LP2 to 50 µl of Protein G Dynabeads. Incubate for 2 h on a rotator.
10. Apply a magnet and aspirate the crude ISV solution. Resuspend the beads with 1 ml of blocking buffer for 5 min.
    ▲ **CRITICAL STEP** During resuspension, the beads should stick together slightly, similar to a clump of dirt or dried mud. Gently pipette until they disperse.
    ▲ **CRITICAL STEP** Avoid introducing bubbles during resuspension.
11. Apply a magnet, aspirate the blocking buffer and resuspend the beads with 1 ml of vesicle buffer (or PBS without BSA). Note that the beads should no longer stick together and behave like fine particulates.
12. Repeat Step 11 once more.
13. Immediately add 25 µl of elution peptide at a 1–5-mg/ml concentration (from Procedure 1). Incubate for 20 min, periodically gently resuspending by flicking or tapping the bottom of the tube.
    ▲ **CRITICAL STEP** When performing elution, lower concentrations require longer incubation times. Experimenters should empirically determine the concentration and incubation time that suit their downstream applications, with prioritizing lower concentrations.
14. Repeat elution two times for a total of 75 µl. Thus, the final volume of ISVs is 75 µl/50 µl of protein G Dynabeads.
    - To continue with the hybrid fusion assay, proceed to Step 15.
    - Alternatively, unlabeled ISVs are now ready for biochemical, biophysical or structural studies.
15. Add 1:1,000 anti-synaptophysin antibody labeled with Alexa-647 to the ISVs and incubate overnight.

Day 2: PM preparation

● **TIMING ~2–3 h**

16. Equilibrate a CL-4B column with vesicle buffer or another running buffer.
17. Add 2.5 mg of sulforhodamine powder per 100-µl volume of syntaxin-1A, SNAP-25 and vesicle buffer. The powder should be added to each separate tube containing syntaxin-1A, SNAP-25 and vesicle buffer to a final concentration of 20–50 mM each. From this point on, keep the proteins in the dark when possible. The exact concentration of sulforhodamine used should be empirically determined and depends on the sensitivity of the experimenter's microscope setup.
18. Add syntaxin-1A in 1:200 protein/lipid ratio while gently vortexing to resuspend the lipids (see Step 23 of ref. 20 for more details).
    - The vesicle buffer volume should be calculated as volume = (Stx × 3.5) − S25, where Stx is the volume of syntaxin-1A used and S25 is the volume of SNAP-25 used. (For example, if 200 µl of 83 µM syntaxin 1-A and 100 µl of 498 µM SNAP-25 is used, an additional 600 µl of vesicle buffer is required). The purpose of the vesicle buffer is to dilute the final OG (octyl-β-D-glucoside) concentration (present in the syntaxin-1A preparation) to <1%, near the critical micelle concentration of 23–25 mM.
    - Once protein containing sulforhodamine has been added, keep the lipid film protected from light. We wrap the tube in aluminum foil.
19. Allow the lipids to resuspend and periodically vortex to speed resuspension. This step takes ~20–30 min.

    ◆ **TROUBLESHOOTING**
20. Add vesicle buffer containing sulforhodamine.
21. Add SNAP-25A in 3× molar excess to syntaxin-1A.
22. Let the mixture equilibrate for 15 min.
23. Add the mixture to the CL-4B column.
24. While running on the column, the mixture should separate into two fractions: a light pink fraction and a darker fraction. Collect the first light pink fraction.

    ▲ **CRITICAL STEP** If the mixture is not separating into distinct populations, the column may contain residual detergent from cleaning after previous use. During equilibration, fill the column with buffer and add 1 ml of air to generate bubbles. If bubbles form and do not immediately burst, wipe the surface of the buffer with a Kimwipe to remove any detergent that might reside at the surface of the buffer.

    ◆ **TROUBLESHOOTING**
25. Collect ~1.2 ml of PM vesicles (from a 5-ml bed-volume column).
26. Dialyze PM vesicles overnight in a 10,000-MWCO dialysis cassette.

    ▲ **CRITICAL STEP** Protect PM vesicles from light.

Day 3: ISV preparation

● **TIMING ~2 h**

27. Dialyze ISVs in a 300-kDa MWCO dialysis cassette for 2 h. ISVs are now labeled and ready for use. Note that the ISV volume should approximately double after dialysis.
28. (Optional) For experiments using additional soluble proteins (e.g., complexin), we directly dilute a 5× solution of those proteins into ISVs. This minimizes further dilution of the ISV sample and allows smaller amounts of protein to be used instead of including these proteins in the dialysis (Step 26).

    ▲ **CRITICAL STEP** Efforts should be taken to minimize further dilution of ISVs.

Day 3: PM preparation

● **TIMING ~5 min**

29. Collect the PM vesicles from the dialysis. The vesicles can now be used.
30. Store the vesicles on ice protected from light (if fluorescently labeled) until required.

(Optional) Day 3: conversion of PM vesicles to SM vesicles

● **TIMING ~1 h**

▲ **CRITICAL** For PM-to-SM vesicle conversion, follow Steps 31 and 32. Alternatively, proceed to Step 33.

31. During ISV dialysis (Step 27), incubate the PM vesicles in a 20× dilution of PM-to-SM conversion buffer[23]. Note that further dilution may be necessary, depending on PM vesicle reconstitution.
32. Incubate for a minimum of 30 min.

Hybrid fusion assay

▲ **CRITICAL** Microscope alignment and calibration should be carried out with attention to specific fluorophores and optics in the instrument and according to the specific fluorophores being used. Here, we used the procedures and software described in Steps 18 and 19 of ref. 20. We have made minor modifications to our previous protocol to speed up and simplify imaging chamber assembly.

▲ **CRITICAL** Microscope chambers may be prepared during ISV dialysis. Caution should be taken when preparing them too far in advance (days), because contamination and loss of chamber integrity may occur with prolonged storage.

Chamber assembly

● **TIMING ~ 20 min–1 h depending on level of experience**

▲ **CRITICAL** Here, we provide instructions on how to prepare an imaging chamber[23]. However, any equivalent preparation, including purchasing pre-constructed and passivated microfluidic chambers, is acceptable.

33. Prepare an imaging chamber from cleaned glass (as in Fig. 1). For glass cleaning and PEGylation, follow Steps 1–14 of ref. 20. At variance to ref. 20, here we used Biotin-PEG-SVA and mPEG-SVA since the SCM compounds used in ref. 20 are no longer available from the vendor. Cleaned, passivated slides and cover glass may be stored for several months at −20 °C. Avoid touching the passivated surface of the storage container. We store slide and cover glass pairs together in 50-ml conical tubes such that the passivated surfaces face away from each other.

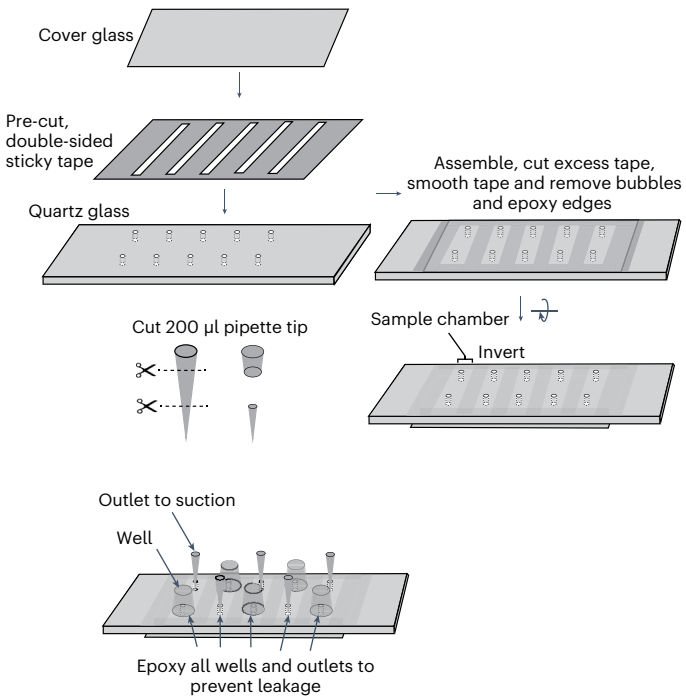

Fig. 1 | Slide preparation. Pre-cut, double-sided sticky tape is sandwiched between a quartz sample slide and a glass coverslip. Pipette tips and bottoms are cut and used as sample wells (inlet) and suction ports (outlets). Depending on the fusion quartz hole distribution, wells and suction ports may need to be alternated to accommodate all the channels.

34. Apply laser-cut, double-sided tape to the quartz slide. The cutout areas will form the imaging chambers. Thus, the tape should be cut according to the specific dimensions of the experimenter's slides. We use a 50 × 45 × 0.075-mm double-sided tape with five 19 × 3.5-mm cutout areas with 3 mm spaces between cut areas. The center of the third cutout is the geometric center of the tape (Fig. 1).
35. Add a PEG-coated cover glass to the double-sided tape.
▲ **CRITICAL STEP** Be careful to provide sufficient space for the microscope objective to image the channels without hitting the edge of the cover glass.
36. Press the cover glass onto the double-sided tape by using the wide end of a pipette tip.
▲ **CRITICAL STEP** Take care not to apply too much pressure to the glass and avoid applying any pressure to the chamber sections of the glass, because it may cause cracking. Avoid using cracked channels, because fluid may leak onto the microscope.
37. Apply epoxy to the edges of the imaging chamber.
38. Allow the epoxy to dry for 5 min.
39. Turn the chamber over, add cut pipette tips and wells and secure with epoxy. The precise size is user defined.
▲ **CRITICAL STEP** Take care to avoid strings of epoxy from landing in the center of the chamber, because this is the side where the TIRF prism will sit (for TIRF microscopy experiments) and must be flat and clean. In addition, consider the width of the prism when placing the wells.
40. Allow the epoxy to dry for 5 min. The chamber may now be used.

Performing the fusion assay

● **TIMING ~5 min or longer depending on the exact experimental design**

41. After chamber assembly, inject ~5 µl of NeutrAvidin (0.1-mg/ml final concentration) directly into the sample chamber by using a pipette, injecting into the hole in the inlet well. Note that more or less NeutrAvidin volume may be required on the basis of the precise volume of the sample chamber. In addition, the NeutrAvidin concentration may be altered depending on the ratio of biotin-PEG used. Here, we are assuming a biotin-PEG concentration of 0.01% (wt/vol). The biotin-PEG concentration may be altered according to the experimenter's needs.
42. Incubate for 5 min.
43. Wash each imaging chamber with a minimum of 200 µl of vesicle buffer by directly pipetting into the slide holes. Buffer leaving the chamber via the pipette tip outlet should be discarded. Add one additional drop of buffer directly to the inlet well, covering the hole, to prevent drying.
▲ **CRITICAL STEP** From this step on, do not allow the sample chamber to dry out or allow the formation of bubbles within the sample chamber.
44. Dilute PM/SM vesicles 1:20–1:50 and inject an ~2× excess volume into the chamber. For a 5-µl volume chamber (for the dimensions given in Step 34), 8–10 µl is sufficient. This injection volume need not be precise but should be enough to ensure that the entire channel volume has been exchanged. Note that when injecting a small (1×) volume, the liquid will flow up the outlet pipette-tip side of the chamber and then, because of gravity, flow back into the chamber, effectively back-flushing and removing what has been added. Thus, to prevent this, we recommend an ~2× volume or enough to expel a droplet from the chamber to ensure that all the chamber liquid is now sample.
45. Incubate for 5 min. Note that the incubation time may be varied to adjust the amount of PM/SM vesicles as the user desires.
46. Wash again with 200 µl of buffer.
▲ **CRITICAL STEP** Any bubbles that pass through the chamber will destroy bound PM/SM vesicles. Therefore, avoid bubbles.
47. Wipe the top and bottom exterior of the sample chamber with a Kimwipe to remove any dirt, fingerprints or accumulated liquid.
48. Mount the sample chamber onto the microscope and find a suitable acquisition area.
49. Attach a suction line from a syringe or syringe pump to the outlet (Fig. 1). We find that using a motorized syringe pump to induce suction over injection of sample is easier to control. But either configuration will work. A manual syringe system may also be used.

▲ **CRITICAL STEP** Take care that there is sufficient slack in the suction line or that the suction line is otherwise vibrationally stable, because injecting solution may cause stage shifts.

50. Assess the distribution of PM vesicles. PM vesicle distribution should be punctate and sparse.
◆ **TROUBLESHOOTING**
51. Add ISVs to the inlet well but do not draw them into the imaging chamber.
◆ **TROUBLESHOOTING**
52. Begin image acquisition by using imaging software compatible with the experimenter's microscope setup. Collect ~30 frames of baseline fluorescence images, which will be used to identify putative association sites in data analysis. For the microscope setup, we use a system similar to the one presented in ref. 20.
    - Note: At variance to ref. 20, here we imaged with only 1 EMCCD (electron-multiplying charged-coupled device) camera with two colors.
    - Note: Other image-acquisition software may be used. We use the SmCamera software.
53. While continuing to record images, draw ~12 µl of the ISVs into the imaging chamber from the sample well by using a motorized syringe connected to the outlet tip (Fig. 1).
    - Note: The exact volume is subject to the experimenter's specific microfluidic chamber.
    - Note: The motorized syringe settings must be empirically determined.
    - Note: This step is analogous to Step 68 in ref. 20, only now the well ensures that liquid remains contained and away from the imaging area/optical oil.
54. Acquire as many images as desired.
55. During or after recording ISV association, carefully remove the ISVs from the inlet well. These samples may be returned to ice and saved for later use or discarded.
56. Add wash buffer to the wells with a pipette. Washes and triggering solution volumes may be empirically determined. We typically use 200 µl (~50× the chamber volume) for washes. Care should be taken to avoid injecting a bubble, and avoid bumping the imaging chamber.
◆ **TROUBLESHOOTING**
57. After washing, aspirate the remaining wash solution and add triggering solutions; we use 50–100 µl for the Ca^{2+}-triggering solution. Care should be taken to avoid injecting a bubble.
◆ **TROUBLESHOOTING**
58. If recording was stopped during washes, resume recording. Again collect ≥30 frames of baseline fluorescence.
59. While continuing to record images, draw ~12 µl of the triggering solution into the imaging chamber from the sample well by using a motorized syringe connected to the outlet tip (Fig. 1).
60. Record as many images as desired.
61. When ready to move to the next imaging chamber, remove the suction tube from the current outlet and move it to the next chamber's outlet. Repeat Steps 50–60 for the next chamber.

Hybrid fusion assay data collection and analysis

● **TIMING ~2–10 min per movie, depending on the number of ROIs present**

62. Convert data to text files by using the smt2txtFiles_JL.m MATLAB custom script (available at https://github.com/brungerlab/single_molecule_matlab_scripts). Note that for subsequent processing using the Fusion_Detector_2_0_7_1JL.m, data for each ROI should be stored in a separate text file containing five columns. The columns should denote time, channel 1 ('green') raw signal, channel 1 background, channel 2 ('red') raw signal and channel 2 background. A single line header of the text files is expected.
63. Identify ISV-PM/SM vesicle association and fusion events or Ca^{2+}-triggered fusion events by using the Fusion_Detector_2_0_7_1JL.m MATLAB custom script. To manually curate putative hits, leave the 'plot traces' box checked. To perform analysis automatically, deselect this box. Note that the fusion_Detector_2_0_7_1JL.m MATLAB custom script contains a graphical user interface that allows users to select the type of analysis to be performed and permits users to either manually curate putative hits or automatically identify them, as well as plot histograms and save hits for easy example image generation.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step (Procedure 2)	Problem	Possible reason	Solution
19	PM lipids do not dissolve	Incomplete drying	Repeat Steps 7 and 8
24	The mixture does not separate into two distinct fractions on the column	Some detergent may have been present on the column	With pure water in the column reservoir, inject air and monitor the surface for bubbles. Wipe the water off the surface
50	Too many PM/SM vesicles	There may be too many attachment sites, too many PM/SM vesicles or both	Dilute biotin-mPEG or dilute PM/SM vesicles
	PM/SM vesicles appear on islands	The imaging quartz slide is dirty	Use a clean slide
	Too few PM/SM vesicles; dim	PM/SM vesicles may have photobleached	Keep PM/SM vesicles in the dark as much as possible
		PM/SM vesicles may burst	Ensure that the CL-4B column is entirely devoid of detergents. Avoid air whenever pipetting. Use clean, washed dialysis equipment
51	Too many ISVs	ISVs (Step 26) may not have dialyzed sufficiently	Repeat dialysis with a larger dialysis volume
	Nonspecific ISV association	The imaging chamber may be dirty	Repeat the experiment with a clean imaging chamber
51, 56, 57	All ROIs report a fluorescence change at the 30th frame	Vibration from the motorized syringe moves the sample chamber	Secure the tubing to the microscope stage or otherwise stabilize the injection tubing

Timing

Procedure 1

vGlut peptide expression: 3 d

Steps 1–9, transforming the plasmid-containing vGlut peptide construct into *E. coli* cells: ~2 h
Steps 10–15, large-scale expression of the vGlut peptide: ~30 h
Step 16, harvesting *E. coli* cells: ~15 min

Peptide purification: 2 d

Steps 17–26, resuspending, lysing, purifying and pooling the tagged vGlut peptide from cells: ~8 h
Steps 27–33, removing the GST tag from the vGlut peptide: ~7 h minimum (might be longer, depending on how many SEC runs are required to purify the total volume of the sample)
Steps 34–36, combining fractions, measuring concentration and freezing purified vGlut peptide: ~2 h (this can vary depending on how much volume needs to be concentrated, as well as the desired concentration)

Procedure 2

Preparation of LP2: 1 d (performed before the steps here)

Day 1

Steps 1–4, binding ISVs in LP2 with monoclonal vGlut-1 antibody: ~30 min
Steps 5–7, preparing the lipid film for PM vesicles: ~20 min

Day 2

Step 8, washing Protein G Dynabeads: ~5 min
Step 9, incubating Dynabeads with LP2: ~2 h

Steps 10–14, isolating, washing and eluting ISVs from Protein G Dynabeads: ~1.5 h
Step 15, labeling ISVs with fluorescent antibody: overnight
Step 16, (performed in parallel with steps 17–22) equilibrating the CL-4B column with vesicle buffer: ~1 h
Steps 17–22, reconstituting syntaxin-1A and SNAP-25 into PM vesicles: ~1 h
Steps 23–26, generating PM vesicles by running on a CL-4B column: ~5–20 min, depending on the column flow rate

Day 3
Steps 27–30, dialyzing unbound fluorescent antibody from ISVs and collecting PM vesicles: ~2 h
Steps 31–32, optionally converting PM vesicles to SM vesicles: ~1 h
Steps 33–40, making the imaging chambers: ~ 20 min–1 h
Steps 41–61, imaging ISV fusion with PM/SM vesicles: ~5 min or longer per channel, depending on the precise experimental design
Step 62–63, fusion assay data analysis: ~2–10 min per movie collected depending on the number of ROIs present

Anticipated results

vGlut peptide preparation

Upon completing the protocol described here, one should expect yields of purified vGlut peptide between 2 and 2.5 mg of ~10 mg/ml per liter of cells (a representative SEC trace and SDS gel are shown in Fig. 2a,b). With the use of our imaging chambers, this protocol should produce

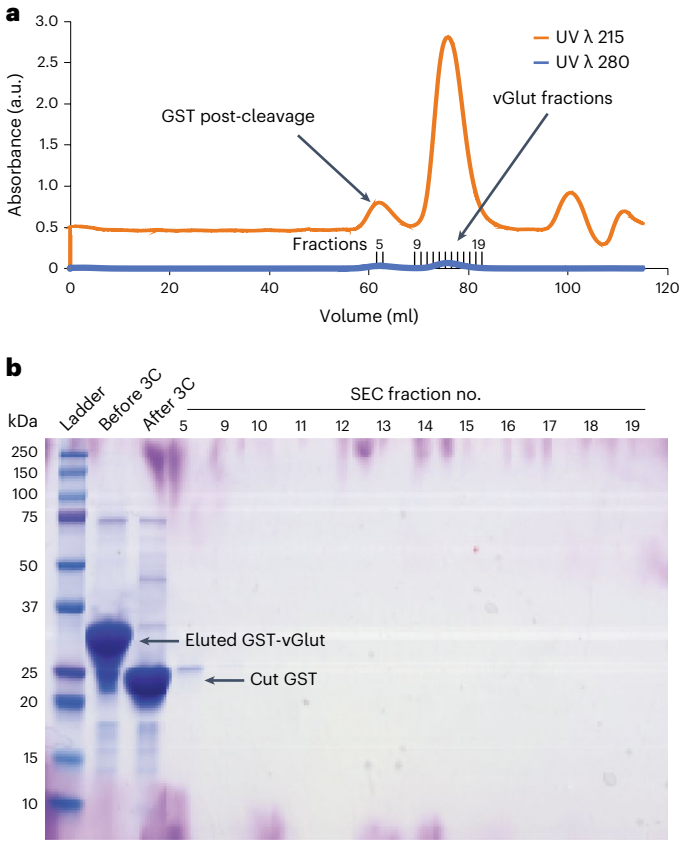

Fig. 2 | Example SEC chromatogram of a vGlut peptide preparation. a, As a final purification step, the sample was loaded onto a Superdex 75 16/600 size exclusion column and eluted at a flow rate of 1 ml/min. 2-ml fractions were collected at the indicated volume. Peptide elution was monitored at $\underline{A}$ = 215 nm, suggesting that the peptide elutes at 70–90 ml. Fractions 9–19 were collected for this experiment and checked for purity by using SDS–PAGE. **b**, SDS–PAGE of relevant fractions from the example SEC chromatogram of a vGlut peptide preparation. No observable contaminants are present, and the vGlut peptide does not stain well and is not obvious; nevertheless, when fractions 9–19 are pooled and assessed by BCA protein assay, substantial protein is present.

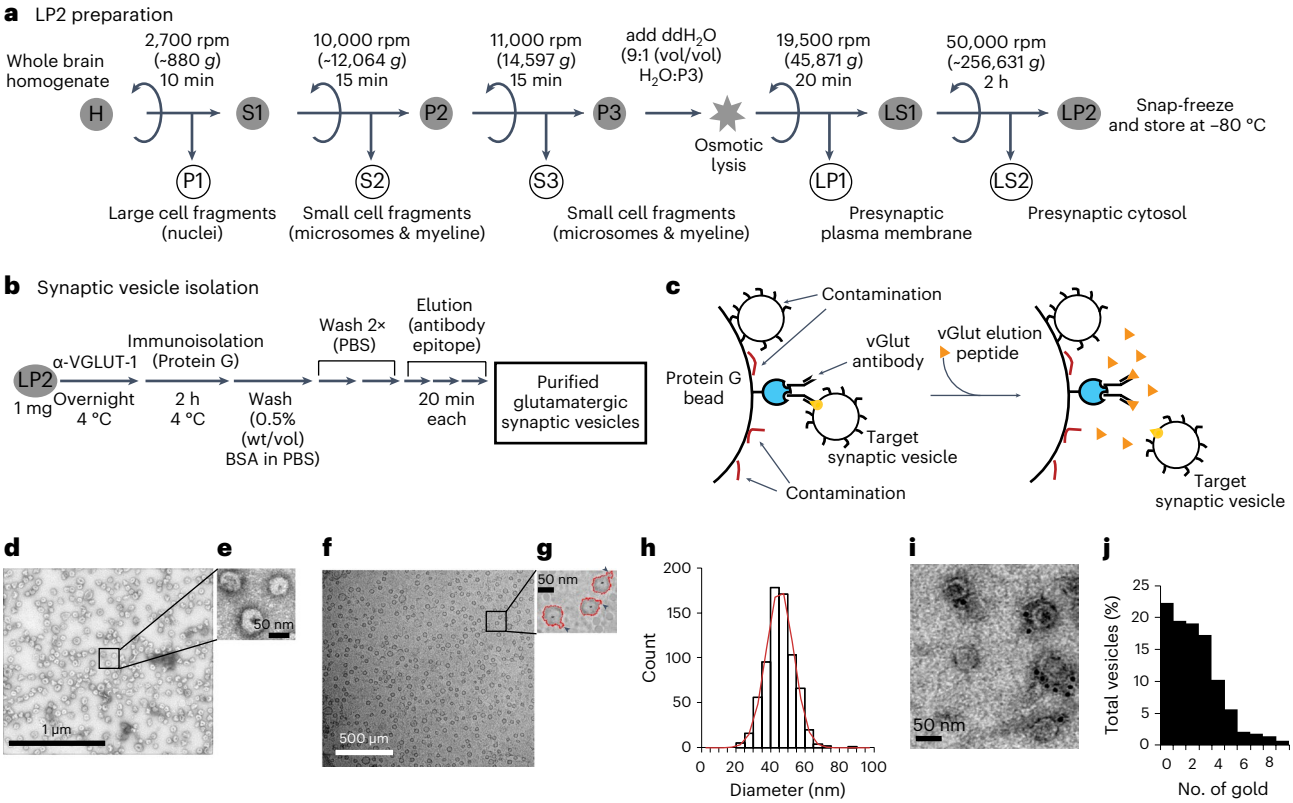

Fig. 3 | Isolation scheme of ISVs and example verification by electron microscopy. a, Differential centrifugation scheme to prepare crude LP2 containing synaptic vesicles. **b**, Synaptic vesicle isolation from LP2. **c**, Cartoon demonstrating the theory behind the peptide-based elution. **d** and **e**, Example negative-stain electron micrographs from a typical isolation. ISVs had a mean diameter of 49.7 ± 6.3 nm (from 268 ISVs collected from two grids, from ref. 23). **f** and **g**, Example cryo-EM micrographs of an ISV preparation. Note the appearance of putative V-ATPase V1 domains protruding from ISVs (arrowheads). Data reproduced from ref. 23. **h**, ISVs observed in cryo-EM micrographs had a mean diameter of 42.8 ± 7.9 nm (from 717 ISVs observed in two cryo-EM grids, from ref. 23), as expected, slightly smaller than the diameters measured by negative-stain electron microscopy. Data reproduced from ref. 23. **i**, Example immunogold negative-stain electron micrograph using an anti-synaptophysin antibody. **j**, Quantification of immunogold negative-stain electron micrographs. We observed only very few unbound gold secondary antibody instances, suggesting that primary labeling may be slightly higher. Images and analysis shown in **d**–**j** are reproduced from ref. 23 under a Creative Commons licence CC BY 4.0.

enough undiluted ISVs for ~35–40 independent channels per vesicle isolation or 100–150 independent channels per vGlut peptide preparation.

Isolation of glutamatergic synaptic vesicles

The isolation scheme and resulting elution (Fig. 3a–c) should produce many ISVs that can be visualized by negative-stain electron microscopy (Fig. 3d,e) and cryo-EM (Fig. 3f,g). ISV diameters can be measured by using the cryo-EM images. They should have a mean diameter of 45.4 ± 8.4 nm (Fig. 3h). Most ISVs have one to two large protrusions from the ISV (Fig. 3g, black arrowheads) corresponding to synaptic vesicle V-ATPases. Moreover, the ISV population should be enriched for vGlut over the vesicular GABA transporter (vGAT) as assessed by western blotting (Fig. 4) at levels similar to those in ref. 21. Synaptophysin can be used as a loading control (Fig. 4).

Expected yields of ISVs after the isolation are 8.5 ± 0.5 µg of SV protein/mg of magnetic beads. For example, for one LP2 preparation, we used eight wild-type CD1 P20 mice (~3 g (wet weight) of brain), which provided enough starting material for approximately five different synaptic vesicle isolations. For each isolation, we used 3 mg of magnetic beads, yielding enough ISVs to permit 20 association and triggered fusion experiments. For cryo-EM, the same number of animals would yield enough ISVs for ~250 cryo-EM grids, assuming 4 µl of sample per grid.

As described in the hybrid fusion assay protocol above, to monitor ISVs, we incubate purified ISVs with an anti-synaptophysin antibody labeled with Alexa-647 (synaptophysin is a ubiquitous synaptic vesicle protein) overnight. After labeling, we dialyze ISVs in a 300-kDa-cutoff cassette to remove excess, unbound antibodies. Negative-stain immuno-EM (immuno-electron microscopy) using 10-nm gold particles conjugated to a goat-anti-rabbit secondary antibody can be used to confirm labeling efficiency. Typically, in our experiments, >77.7% of ISVs are labeled with at least one antibody (Fig. 3i,j). Moreover, we observe virtually no free secondary antibodies, suggesting that the labeling efficiency may, in fact, be higher.

Measurement of ISV-PM/SM vesicle association

We present the following experiments as an example dataset of what might be expected from the hybrid fusion assay described in the above protocol (data reproduced from ref. 23). The general flow of the protocol is depicted in Fig. 5a and is adaptable to the specific preparation of the user. This example uses only ISVs and PM vesicles with no added soluble proteins; complexin was not included. As mentioned above and in ref. 23, PM vesicles can be converted to SM vesicles, and other synaptic vesicle fusion machinery components can be included.

The fusion assay can be divided into steps of association and fusion; that is, Ca^{2+} does not need to be premixed with the ISVs and PM/SM vesicles, and these two processes can be examined independently (allowing for the removal or exchange of components between association and fusion). To measure ISV-PM/SM association, we first establish a baseline of 30 frames and then inject ISVs and continue imaging for 1 min (a final frame of an example movie is shown in Fig. 5b, with example traces derived from associations marked in the image). We can identify five distinct association modalities: stable ISV association (Fig. 5c), in which an ISV arrives, and there is no change in the fusion indicator; ISV association concomitant with the fusion of the vesicle pairs, indicating immediate fusion upon association (Fig. 5d); stable ISV association followed by a delayed fusion (Fig. 5e); association of multiple ISVs to a single fluorescent spot (Fig. 5f); and finally, in this configuration, we rarely observed an ISV arriving, remaining at the PM/SM vesicle for some time, followed by a decrease only in the ISV fluorescence. We interpret these transiently associating events as dissociation events (Fig. 5g), in which the ISV arrives and remains for a dwell period and then leaves the ROI (Fig. 5g). We note that these dissociation events do not preclude future association events (as shown in the example fluorescence trace). We also note that the ISV-PM/SM vesicle association and subsequent fusion analysis performed here are dependent on the labeling efficiency of the fluorescent antibody used (here, anti-synaptophysin antibody labeled with Alexa-647) as well as the specific experimental conditions (such as accessory proteins and PM/SM vesicle composition[23]).

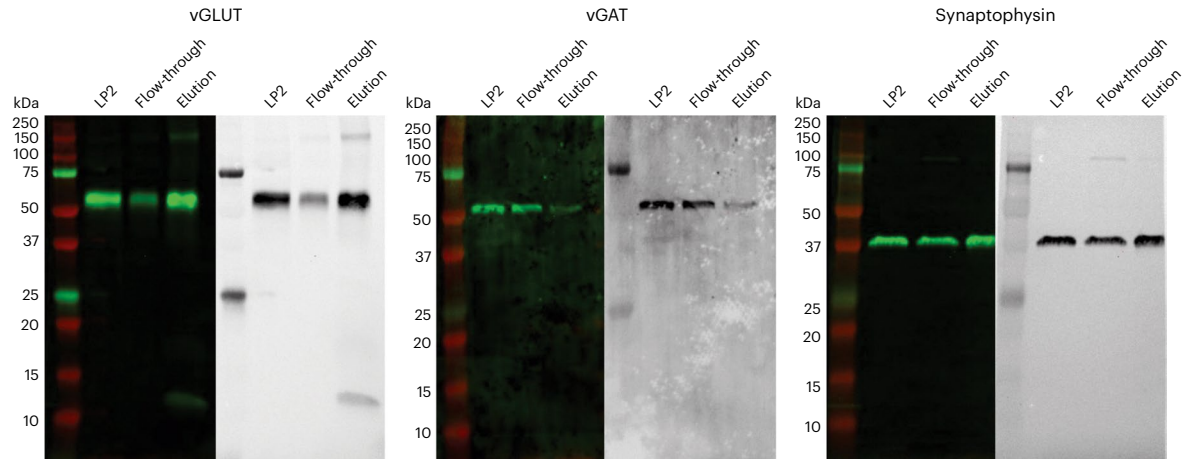

Fig. 4 | Example western blot analysis of the isolation probed with antibodies against vGlut, vGAT and synaptophysin. The starting LP2 (1 µl), flow-through (10 µl) and vGlut (10 µl) elution samples were run on an SDS–PAGE, subsequently transferred to nitrocellulose and probed by using rabbit anti-vGlut (left) and rabbit-anti-vGAT (middle) antibodies followed by secondary IRDye800CW-goat anti-rabbit antibody and mouse-anti-synaptophysin (right) followed by IRDye800CW-goat anti-mouse secondary. In the elution, vGlut is present, whereas vGAT is present only in small amounts relative to the starting LP2.

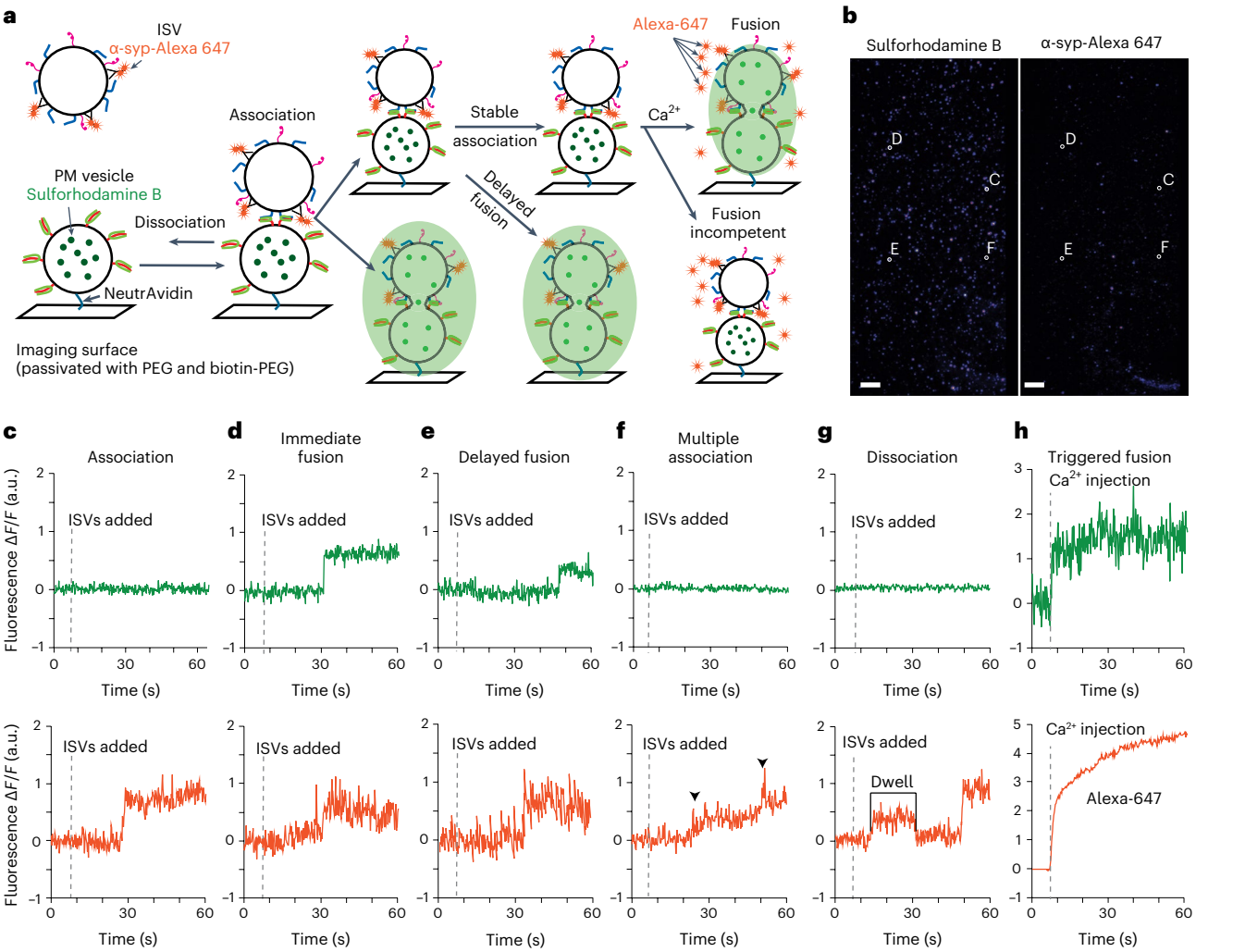

Fig. 5 | Example of ISV association with PM vesicles that mimic the plasma membrane. a, The experimental scheme: association of labeled ISVs with imaging-surface tethered synthetic ('PM') vesicles that mimic the plasma membrane. During association, ISVs may or may not fuse as the imaging continues; ISVs may remain associated (stable association) or fuse (delayed fusion). **b**, Representative fields of view. Left is the sulforhodamine B (fusion indicator) detection ('green') channel; right is the Alexa-647 (ISV-PM vesicle association) detection ('red') channel. Scale bars represent 10 µm. Example ROIs marked 'C–F' are shown below. **c**, Stable ISV-PM vesicle association. A stable stepwise increase in the red channel indicates ISV arrival, and no change in the green channel indicates no fusion has occurred. **d**, A stepwise increase in the red channel concurrent with a stable stepwise increase in the green channel indicates that an ISV has arrived and, within 1 s, fused with the PM vesicle. **e**, A stepwise increase in the red channel followed after some delay by a stepwise increase in the green channel indicates delayed fusion. **f**, Multiple ISVs could be seen associating with some PM vesicles (steps indicated by arrowheads). **g**, Some ISVs associate with PM vesicles for a period followed by dissociation, a stepwise decrease in fluorescence. Note that the corresponding PM vesicles were still available for a second round of association. **h**, Starting from stable ISV-PM vesicle associations, Ca^{2+} solutions together with free Alexa-647 are injected, and ISV-PM vesicle fusion is monitored. Example normalized trace at a single ROI showing fusion as a stepwise increase in the green sulforhodamine channel and Ca^{2+} arrival as indicated by fluorescence by the free Alexa-647 monitored in the red channel. Images in **b** and traces in **c**–**g** reproduced from ref. 23 under a Creative Commons licence CC BY 4.0.

After ISV association, we typically wash the imaging chamber with ~50 channel volumes of vesicle buffer to remove any unbound ISVs. The remaining pairs may be imaged again for 1 min to monitor longer-latency Ca^{2+}-independent fusion, if desired. For example, in the analysis of 2,066 ISV-PM vesicle pairs, only 55 pairs fused without Ca^{2+}, making the Ca^{2+}-independent fusion rate in our system ~0.028 ± 0.007 vesicle pairs/min. After washing, Ca^{2+} or other triggering solutions may be injected to induce fusion (Fig. 5h). Here, as an example, we injected a solution containing 500 mM $CaCl_2$ with free Alexa-647 as an indicator of Ca^{2+} arrival. Identification of

association, dissociation and fusion events from each movie can be processed and analyzed in the accompanying MATLAB scripts.

Our single vesicle fusion assay allows multiple forms of fusion activity to be monitored. For example, by normalizing the fusion to the number of ISV-PM/SM vesicle pairs identified in the first ~30 frames before triggering (Fig. 5b, h), one can determine the gross fusion competence of the ISV population. Alternatively, normalizing the fusion that occurs in the first second (five acquisition frames) to the total fusion that occurred yields the degree of synchronization of fusion to the triggering stimulus, here, the arrival of Ca^{2+}. Note, however, that in previous work, we normalized fusion in the first bin to the total fusion[6].

Data availability

All imaging data and post-processed files are available in the Stanford Digital Repository at https://doi.org/10.25740/wc823yz5804.

Code availability

MATLAB analysis scripts for the single-vesicle fusion experiments are available on GitHub (https://github.com/brungerlab/single_molecule_matlab_scripts)[31]. The smCamera software (Taekjip Ha's laboratory, Johns Hopkins University, Baltimore, MD) was used for acquisition and is available at https://github.com/Ha-SingleMoleculeLab (ref. 32).

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Author contributions

J.L. performed the experiments and designed the hybrid fusion assay with input from A.T.B. J.L. and R.A.P. designed the vGlut peptide. J.L. and J.J.P. developed the custom Matlab software to analyze the fusion assay data. J.L., J.J.P. and C.W. generated LP2. A.L.W., L.E. and

C.W. optimized the purification protocol for the vGlut peptide. L.E., A.L.W. and R.A.P. produced the proteins used in the hybrid fusion assay. N.G., C.W. and J.L. performed western blots. J.L., L.E. and A.T.B. wrote the paper.

Competing interests
The authors declare no competing interests.

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