Preparation and characterization of SNARE-containing nanodiscs and direct study of cargo release through fusion pores

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This protocol describes an assay that uses suspended nanomembranes called nanodiscs to analyze fusion events. A nanodisc is a lipid bilayer wrapped by membrane scaffold proteins. Fluorescent lipids and a protein that is part of a fusion machinery, VAMP2 in the example detailed herein, are included in the nanodiscs. Upon fusion of a nanodisc with a nonfluorescent liposome containing cognate proteins (for instance, the VAMP2 cognate syntaxin1/SNAP-25 complex), the fluorescent lipids are dispersed in the liposome and the increase in fluorescence, initially quenched in the nanodisc, is monitored on a plate reader. Because the scaffold proteins restrain pore expansion, the fusion pore eventually reseals. A reducing agent, such as dithionite, which can quench the fluorescence of accessible lipids, can then be used to determine the number of fusion events. A fluorescence-based approach can also be used to monitor the release of encapsulated cargo. From data on the total cargo release and the number of the much faster lipid-mixing events, the researcher may determine the amount of cargo released per fusion event. This assay requires 3 d for preparation and 4 h for data acquisition and analysis.

INTRODUCTION

Transport and release of proteins and various other molecules within the cell and between the cells often require the entrapment of these cargos in vesicles[1]. Transmembrane proteins are embedded in the membrane of the transport vesicle, whereas soluble cargos are encapsulated within it. To release cargos in a new environment, the vesicle must fuse with a target membrane, a process usually driven by protein machineries, for instance by soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins in intracellular trafficking and exocytosis[2–4], by transmembrane glycoproteins in viral fusion[5] or by other protein fusogen–mediating cell-to-cell fusion or organelle fusion[6,7]. During the fusion process, the continuity of the fused membranes is achieved by the opening of a pore through which soluble cargos will also be released. The size of the pore and the kinetics of its formation determine the timescale over which membranes are shared and their content released[8].

In general, studying a multifactor complex that includes transmembrane proteins, such as one that directs fusion-pore opening, is hampered by an uncertain stoichiometry and by the transient character of the complex. Reductionist biology, in which the variability of some parameters is restricted (or attempts are made to this end) so as to focus on the effects of the variation of a smaller number of parameters on the system studied, enables one to circumvent this issue. The purpose of this protocol is to describe a new approach for the reconstitution of isolated SNARE-mediated fusion pores. As we will describe below, the study of these nanopores enables us to establish general principles of pore lifetime, which probably limit the rate and extent of neurotransmitter release. The use of these systems also enables us to test how specific sequences in the SNARE proteins contribute to these pore characteristics.

Why study of pore biology has been limited in existing *in vitro* SNARE systems

Most existing approaches to reconstituting membrane fusion events are based on the classical 'liposome/liposome' fusion assay initially designed in 1981 and now routinely used by several groups to study fusion *in vitro*[2,9–12]. Two sets of liposomes, carrying cognate SNAREs, are required to conduct this assay[13]: the first set contains, among others, two types of fluorescent lipids, the 'donors' and 'acceptors', which carry dyes able to interact with each other through Förster resonance energy transfer (FRET)—for instance, nitro-2-1,3-benzoxadiazol-4-yl (NBD) as donor and rhodamine as acceptor. Each fluorescent lipid represents ~1.5% of the total lipid content of the first set of liposomes. At such concentrations, because of FRET, the donor emission signal is quenched by the proximity of the acceptor. The second set of liposomes does not contain any fluorescent lipids. When the two sets of liposomes are incubated together, they can fuse, at which point the fluorescent lipids are diluted within the resulting membrane and the donor fluorescence signal is enhanced. Monitoring this increase provides a direct readout of the rate of fusion between liposomes[2].

The liposome/liposome assay is very versatile and has greatly contributed to our understanding of the fusion process[2,14–16]. However, this assay presents several limitations when one is trying to investigate the late stages of fusion, including the collection of data on the expansion and stability of the pore. First, the rate of fusion in this assay is limited by the rate of docking[17], such that it is extremely difficult to follow the fusion step itself in the fusion process. Another limitation, indirectly related to the fact that docking is the limiting step, is that at low SNARE numbers on each liposome, fluorescence from fusion events approaches the background, nonspecific fluorescence signal obtained by removing or blocking

proteins on one set of liposomes[18]. Indeed, the rate of liposome encounters can be estimated from diffusion coefficients as well as the size and concentration of the liposomes.

Under various published measures of SNARE-mediated fusions, in which individual liposomes may carry ~20 SNAREs, we can estimate that several millions of liposome-liposome collisions occur for every successful fusion event. When SNARE density is further reduced to only a couple of SNAREs per liposome, the fusion rate quickly collapses close to the rate of nonspecific fusion (characteristic time of several hours), probably because the likelihood of two cognate SNAREs facing each other during a collision becomes very low. In other words, the rate of productive docking, and thus of liposome-liposome fusion, is inevitably highly dependent on the surface density of SNARE proteins and the probability of a successful collision. *In vivo*, docking is not the limiting factor because it is facilitated by other factors, so that fusion (or regulation of fusion) becomes rate limiting. *In vitro*, we can capture fusion at a dock-like state using stratagems to slow the subsequent fusion reaction, including reducing the temperature or choosing unfavorable lipid combinations[2]; however, even in these cases, the pore remains largely refractory to kinetic and maturation studies. The complexity of these studies descends from a third limitation of the liposome/liposome assay: the pore forms between two inaccessible compartments (the liposomes), and thus interrogation of the pore structure can only be conducted via compounds preloaded into one or both liposomes. As a consequence, stepwise maturation events are not detectable. Indeed, because pore formation is so fast and essentially irreversible in liposome-liposome fusion, currently available readouts only indicate the extent of membrane and cargo exchange when pore expansion is complete and thus tell us essentially nothing about cargo release through the short-lived initial nanopore itself. Finally, even an accurate quantification of the overall cargo release is difficult. This difficulty arises because when two liposomes fuse to form a larger liposome, the resulting species has an excess of membrane that seems to be compensated by an intake of fluids from the outer medium, as suggested by electron microscopy images in which the fusion liposome also assumes a spherical shape[19]. Hence, systematic exchanges probably occur between the outer medium and the fused liposomes, which affect cargo release measurements.

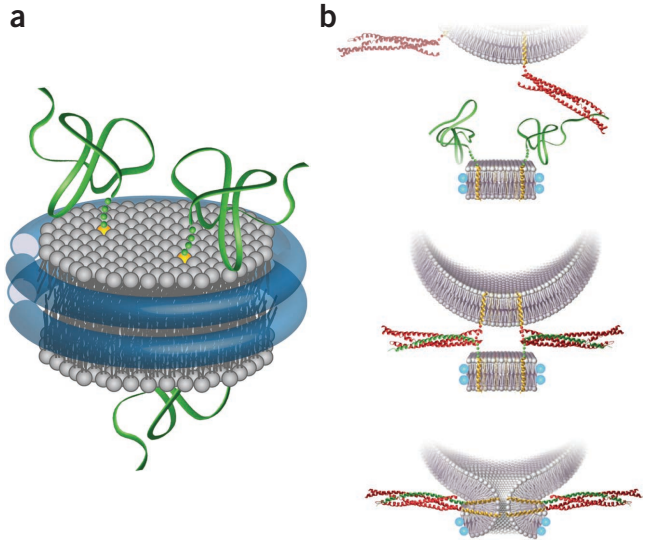

Figure 1 | A nanodisc and a liposome-nanodisc fusion assay. (**a**) The structure of a VAMP2-nanodisc (v-disc). Nanodiscs consist of a lipid-bilayer (gray) wrapped in two copies of an MSP (blue). VAMP2 (green) can be incorporated in the bilayer via its transmembrane domain (yellow) to form a v-disc that can be used for SNARE-mediated fusion assay. VAMP2 was generated using PyMOL software. (**b**) The fusion process between a v-disc and a t-liposome; top, VAMP2 and t-SNARE (syntaxin and SNAP-25) form a trans-SNARE complex; middle, zippering of proteins to form the SNARE complex brings the liposome and the nanodisc together. This interaction enables the fusion between the two bilayers to occur; bottom, formation of a fusion pore between the liposome and the nanodisc. The liposome content is released in the outer medium through the fusion nanopore.

Nanodiscs enable the analysis of SNARE-dependent initial-fusion nanopores

We recently described an assay inspired by the liposome/liposome approach, in which one of the two sets of liposomes is replaced by a nanometric, flat, suspended membrane called a nanodisc[20]. Nanodiscs are obtained by wrapping two copies of a membrane scaffold protein (MSP)[21–24] derived from human apolipoprotein A-1 around a lipid bilayer (**Fig. 1a**). The typical diameter of nanodiscs is 9–17 nm. The main advantage of the nanodisc/liposome assay is that it does not have the three limitations of the liposome/liposome assay described above. First, there are only 200–500 lipid molecules in one nanodisc[21]. Hence, even in situations where only one copy of the fusion-directing protein is present in each nanodisc, the protein/lipid ratio remains sufficiently high for the fusion rate to be markedly higher than that of the background, nonspecific fusion. Second, as the MSP is nanometric in size, the fusion pore itself must be nanometric-sized and cannot expand. Thus, any cargo release or any transfer of matter between the membranes must occur through this narrow fusion nanopore, which mimics the initial fusion pore in physiological fusion events. In addition, because of the small size of the nanodisc, the rate of membrane mixing will be at least ten times faster than that of complete cargo release. This difference in timescales enables us to make comparisons between them in the assay. For instance, a complete membrane mixing occurring while only a fraction of the liposome content is released indicates that the pore remained open long enough for lipids to be exchanged but resealed too quickly for the release of the full content of the liposome to occur. Hence, a direct study of the size and lifetime of the pore becomes possible by varying the cargo. Third, because a fusion pore opens up directly between the liposome and the exterior buffer, there is no surface/volume ratio constraint during a fusion event in the nanodisc/liposome assay and the liposome content is directly released into the outer medium (rather than another liposome as in the liposome/liposome assay). This feature greatly facilitates the quantification of the released content.

In addition to these crucial advantages, this assay provides other major improvements compared with the liposome/liposome assay. For example, because the pore directly opens to the exterior medium, it is much easier to probe whether the pore remains open at the end of the fusion by testing whether molecules added in the solution can enter the liposome. In addition, because the disc is smaller and more stable than the liposome, which is sensitive to osmotic pressure and shear stress, and because of the higher relative concentration of the protein on the nanodisc surface (than that on the liposome), it is much easier to separate protein-free nanodiscs

from those containing proteins. This feature greatly facilitates the preparation of samples in which each nanodisc possesses a single copy of the protein. Furthermore, as the surface of a nanodisc is ~50 times smaller than that of a liposome, during lipid mixing, the dyes contained in the nanodisc experience a 50-fold dilution instead of the twofold dilution that is typical of the liposome/liposome assay. This feature increases the sensitivity of the system for the detection of membrane mixing. Finally, the well-defined size of the MSP makes the nanodisc samples perfectly monodisperse in size, which increases the reliability of experimental results.

The nanodisc/liposome assay is described here for the case of SNARE proteins, but it could also be used to study other fusion machineries, such as those implicated in other intracellular processes or in virus fusions. In the latter case, influenza hemagglutinin (HA) seems to be a potential candidate because a mere change in pH (to a more acidic environment) will trigger fusion. If one HA trimer is incorporated in nanodiscs, it may be possible to study the fusion induced by a single trimer[25]. Indeed, the nanodiscs will primarily fuse with the surrounding liposomes when the pH is changed. The difference in size between the liposomes and the nanodiscs guarantees that a negligible fraction of fusion events will occur between two nanodiscs.

Limitations of the nanodisc/liposome assay

This assay has its own limitations. Even though it is ideal for studying the effect of a low number of protein molecules, the small size of the nanodiscs makes it difficult to put a large number of proteins or protein complexes in each nanodisc. In some instances, under the pressure of a large number or size of proteins, the nanodiscs may be polydisperse in size, which makes the interpretation of the results much more complicated. Another drawback of the nanodisc/liposome assay is that, unlike a liposome, both sides of the nanodisc are exposed to the solution. Hence, it is difficult to perform protease protection assays with nanodiscs.

Experimental design

Here we describe the complete protocol to prepare and to characterize nanodiscs that contain SNARE proteins and to measure the fusion of these nanodiscs with liposomes containing the cognate SNARE (**Fig. 1b**). Two versions of this nanodiscs/liposome assay can be envisioned. In the first, quenched fluorescent lipids are present in the nanodiscs, which enable the direct observation of the lipid mixing during fusion. In the second, a cargo is encapsulated in the liposome and its release in the outer medium through the fusion pore can be directly observed. For both versions, fluorescent measurements are performed in a spectrofluorometer. SNARE-containing nanodiscs are prepared and characterized using standard purification methods.

Nanodiscs. To study the function and structure of transmembrane proteins *in vitro*, lipid membrane–based reconstitution systems are often necessary. Lipid micelles, liposomes, giant vesicles, supported bilayers and lamellar or sponge phases are among the substrates commonly used to incorporate the proteins. Lipid nanodiscs have been developed over the past 10 years or so as an additional bilayer system[26]. Unlike traditional lipid micelles or liposomes formed only by lipid, nanodiscs contain both lipids and MSPs. Each nanodisc is wrapped by two copies of an MSP[27] so that the length of the α-helix of a monomeric MSP determines the size of the nanodisc. Depending on the MSP used, the diameter of nanodiscs currently generated ranges from 9 nm to 17 nm.

During preparation, the MSP:lipid ratio can be varied, and which ratio is the most useful depends on the protein to be incorporated. In the case of VAMP2 and MSP1E3D1, a MSP:lipid ratio of 2:120 is satisfactory[20]. The number of lipid molecules per nanodisc is predetermined by the size of the MSP. In the case of MSP1E3D1 (diameter of the nanodisc = 12 nm), ~300 lipid molecules are necessary for making the nanodisc. Thus, in theory, an experimenter will have to start with a 2:300 MSP1E3D1:lipid ratio. However, having an excess of MSP prevents the formation of other lipid structures (such as liposomes); which justifies the earlier advice to use a ratio of 2:120 instead. Free MSPs are removed during the protocol (see Step 9 of the PROCEDURE). Hence, nanodisc systems provide relatively small, homogenous, flat lipid membrane platform with a perfectly controlled size. Because of these unique properties, nanodiscs have rapidly become popular and are now frequently used for structural, functional or single-molecule studies of membrane proteins[22,28–31].

SNAREs can be incorporated into nanodiscs[20,24]. Hence, it is possible to use nanodiscs to study membrane rearrangements *in vitro*, as we demonstrated in the case of SNARE-induced fusion[20]. As the identity of the MSP determines the circumference of the nanodisc, a series of nanodiscs with different sizes may be obtained using different MSPs. In our studies, we used MSP1E3D1, a long MSP, to obtain nanodiscs of increased size, so that more SNAREs could be incorporated in them. By varying the input ratio of MSP to SNARE (or other target protein[31]), series of nanodiscs with different copy numbers of SNARE per nanodisc can be obtained (**Table 1**). Chimeric SNAREs (i.e., SNAREs having the transmembrane domain of a different protein) can also be incorporated in the nanodiscs. Alternatively, proteins can also be anchored to the nanodisc by chemical cross-linking.

By placing a tag on the SNARE (a His-tag, for instance), protein-free nanodiscs can easily be removed during preparation by performing an affinity purification (via a Ni-NTA column in the case of His-tag). Thus, samples can be prepared in which each nanodisc contains at least one SNARE. Preparations with different SNARE contents per nanodisc can then be used in fusion and content-release assays to study the influence of the number of SNARE pins and of the native transmembrane domain on the lifetime/size of

TABLE 1 | Correspondence between the input MSP:SUMO-VAMP2 ratio and the average number of VAMP2 per nanodisc.

Initial MSP:SUMO-VAMP2 ratio	Average number of VAMP2 per nanodisc
2:0.2	1.2
2:0.5	2.2
2:1	3.15
2:2	4.3
2:4	5.5
2:6	7.4
2:8	9.3

the fusion pore. For the rigorous characterization of the nanodisc, the combination of SDS-PAGE gel and electron microscopy is necessary. We have shown that, in the case of SNAREs, proteins are distributed in the nanodisc according to a Poisson distribution. However, for other proteins, it may be useful to check the validity of this result using single-molecule total internal reflection fluorescence (TIRF) microscopy[20]. We have confirmed that SNAREs are distributed on both sides of the nanodiscs by electron microscopy but we have not proven that this distribution is completely random[20].

In synaptic transmission, VAMP2 (or synaptobrevin-2) is found on the synaptic vesicle. VAMP2's cognate SNARE (t-SNARE for target SNARE), a heterodimer composed of syntaxin1 and SNAP-25, is on the presynaptic plasma membrane. We successfully and reproducibly obtained nanodisc preparations containing on an average one to nine VAMP2 molecules[20]. However, when the syntaxin1/SNAP-25 complex, which is larger and tends to aggregate, is incorporated in the nanodiscs, the nanodiscs become polydisperse and consequently the distribution of SNAREs among them cannot be easily determined. This occurrence is probably caused by steric perturbations due to the aggregation and the large size of t-SNARE compared with VAMP2, and it emphasizes the requirement for a characterization of each nanodisc sample by electron microscopy.

Lipid-mixing assay. The FRET-based lipid-mixing assay is similar to the liposome/liposome assay. The main difference is that the concentration of the nanodisc is equal to that of the liposome. In the liposome/liposome assay, about ten nonfluorescent liposomes per fluorescent liposome are mixed together. In this case, because of the 50-fold difference between the lipid amount in nanodiscs and liposomes, there is no need for an excess of nonfluorescent liposome. The very small number of SNAREs present on the nanodiscs with respect to liposomes also means that SNAREs from a fused nanodisc that were not engaged during the fusion process will probably associate with free cognate SNAREs from the liposome after fusion has occurred. Hence, in theory, each nanodisc can only go through a single round of fusion. In the liposome/liposome assay, in contrast, several rounds of fusion can be observed[13,32].

To monitor lipid mixing, we use a plate reader and follow the increase in the fluorescence of the donor. Our usual FRET pair is NBD/rhodamine but other pairs can work as well. NBD can be reduced and irreversibly quenched by sodium dithionite[33,34], a property that can be exploited in order to quantify full-fusion events (i.e., with pore opening) in which the pore has resealed. Indeed, if the NBD is not completely quenched, it means some NBD is trapped inside the liposome, where it cannot be reached by the dithionite. As NBD lipids were initially present only on the nanodisc, such observations indicate that there was an exchange of lipids between the nanodiscs and the liposomes and that some lipids reached the inner leaflet of the liposome bilayer, meaning full fusion with sharing of both leaflets occurred[33].

Because of the small size of the nanodisc, lipids will diffuse away through nanometric pore to the liposome in ~10 μs. This means that as soon as a fusion pore opens, lipid mixing is ultrafast. In the case of NBD, the sodium dithionite provides a direct measurement of the fraction of nanodiscs that underwent full fusion.

Content-release assay. To rigorously probe full-fusion events, we developed a content-release assay in nanodisc-liposome fusion. Liposomes are loaded with a cargo that, when released, triggers a signal—for instance, a change in fluorescence. For this purpose, we use the calcium ion, because it is very small and will pass even through tiny nanometric pores. In addition, its presence can easily be revealed by a variety of fluorophores. During fusion, calcium release can, therefore, be monitored in real time on a plate reader, just as for the lipid mixing, by recording the fluorescence increase of a calcium sensor. Other ways to observe cargo release can be envisioned (for instance, a self-quenching cargo that fluoresces when released, or a FRET pair that does not interact after dilution in the outer medium or a fluorescent dye that is reduced upon release and thus stops fluorescing). In any case, however, the size of the cargo is a very important factor, because it must be small enough to go through the nanometric pore that forms between the liposome and nanodisc.

Just as in the lipid-mixing assay, to quantify the amount of cargo released per fusion event, it is crucial for a fusion to occur between one liposome and one nanodisc. In this situation, the ratio between the total fraction of cargo released and the fraction of nanodiscs that experienced full fusion corresponds to the fraction of cargo released on average during one fusion event. This assumption is valid as long as liposomes do not fuse to two nanodiscs or more. Indeed, a liposome will release less and less cargo molecules as it undergoes more fusion events. Hence, as in the liposome/liposome assay, the initial fusion events correspond to nanodiscs and liposomes undergoing their first round of fusion. As the reaction goes on, some liposomes will start fusing with a second partner. In order to minimize the error in the quantification of content release per fusion event, we suggest avoiding going beyond 30% fluorescence change in the lipid-mixing assay for the analysis. This ensures that, at the very most, 15% of the fusion events involved more than one round of fusion.

MATERIALS

REAGENTS

- Chloroform (American Bioanalytical, cat. no. AB00350) **! CAUTION** Chloroform liquid and vapors are toxic. Wear a mask, gloves and chemical safety goggles while handling it.
- HEPES (Fisher Scientific, cat. no. BP310)
- KCl (Fisher Scientific, cat. no. BP366)
- $CaCl_2$ (Sigma-Aldrich, cat. no. C5080)
- NaCl (Sigma-Aldrich, cat. no. S9888)
- EDTA (Sigma-Aldrich, cat. no. EDS)
- *n*-Octyl-β-D-glucopyranoside (OG; Calbiochem, cat. no. 494459) **! CAUTION** OG may cause eye, skin and respiratory tract irritation. We recommend wearing protective gloves while handling it. Wear safety goggles and a lab coat or other long-sleeved garment to limit skin exposure.
- Nycodenz (Accurate Chemical and Scientific, cat. no. AN1002424)
- Bio-Beads SM-2 adsorbents (Bio-Rad, cat. no. 152-3920)
- Ni-NTA agarose (Qiagen, cat. no. 30230)
- Imidazole (Sigma-Aldrich, cat. no. I5513)
- Mag-Fluo-4 (Life Technologies, cat. no. M-14205)

- Sodium dithionite (EMD Millipore, cat. no. 1065070500) ! CAUTION Avoid contact with the skin, eyes and clothing. We recommend wearing protective gloves while handling it. Wear safety goggles, a lab coat or any other long-sleeved garment to limit skin exposure.
- Glycerol (J.T. Baker, cat. no. 2136-03)
- 1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC; Avanti Polar Lipids, cat. no. 850375)
- DTT (Roche, cat. no. 10708992001)
- V-lipid blend: palmitoyl-2-oleoyl phosphatidylcholine (POPC): 1,2-dioleoyl phosphatidylserine (DOPS): *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1, 2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE): *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE) = 82:15:1.5:1.5 (premixed in chloroform, custom order, 3 mM; Avanti Polar Lipids)
- Full-length t-SNARE (Reagent Setup)
- 6×-His-SUMO-VAMP2 (Reagent Setup)
- Membrane scaffold protein (MSP1E3D1; Addgene, plasmid 20066)
- TEV protease (Sigma-Aldrich, cat. no. T4455)
- SUMO protease (Life Technologies, cat. no. 12588-018)
- Bio-Rad protein assay kit (Bio-Rad, cat. no. 500-0001)
- *n*-Dodecyl-β-maltoside (Thermo Scientific, cat. no. 89903)
- Uranyl acetate (Ted Pella, cat. no. 6159-44-0) ! CAUTION Uranium is radioactive and a heavy metal. Wear gloves while handling it and do not swallow it.
- NuPAGE LDS sample buffer, 4× (Life Technologies, cat. no. NP0007)
- NuPAGE sample-reducing agent, 10× (Life Technologies, cat. no. NP0009)
- Novex Tris-glycine SDS running buffer, 10× (Life Technologies, cat. no. LC2675)
- PageRuler plus prestained protein ladder (Fermentas, cat. no. 26619)
- Bio-Safe Coomassie stain (Bio-Rad, cat. no. 161-0786)
- ImageJ free software (US National Institutes of Health)
- Milli-Q water

EQUIPMENT

- D-tube dialyzer, 6–8 kDa MWCO (Novagen, cat. no. 71509-3)
- Greiner centrifuge tube, 50 ml (Sigma-Aldrich, cat. no. T2318)
- Centrifuge tubes, 4 ml, 11 × 60 mm (Thinwall, Ultra-Clear; Beckman, cat. no. 344062)
- Carbon-coated copper grids (SPI Supplies, cat. no. 2040C-XA)
- Novex 12% (wt/vol) tris-glycine mini gels (Life Technologies, cat. no. EC6005BOX)
- Amicon Ultra-0.5, 30-kDa membrane (Millipore, cat. no. UFC503096)
- PVDF membrane, 0.2 µm (Macherey-Nagel, cat. no. 740401.50)
- FluoroNunc Maxisorp 96-well white plate (Thermo, cat. no. 437591)
- Poly-Prep chromatography columns (Bio-Rad, cat. no. 731-1550)
- Glass tube, 12 × 75 mm (Fisher, cat. no. 14-961-26)
- Vacuum desiccator
- Vacuum pump (Alcatel 2005 C1, ultimate vacuum $<10^{-4}$ mbar)
- Ultracentrifuge (optima L-90k ultracentrifuge, SW60 Ti rotor; Beckman)
- Balance
- Gas-tight glass syringe (Hamilton, cat. no. 1701RN, 1710 RN, 1750 RN)
- SpectraMax M5 multimode microplate reader
- Digital vortex mixer (VWR, cat. no. 14005-824)
- Liquid chromatography system (ÄKTA Explorer; GE Healthcare)
- Superdex 200 10/300 GL prepacked column
- Orbital shaker (Barnstead Lab, multipurpose rotator (2314Q))
- Glow discharge cleaning system (PELCO easiGlow; Ted Pella, no. 91000)
- Electron microscope (Tecnai G2 Spirit BioTWIN; FEI)
- CCD (Gatan UltraScan 4000; Gatan)
- XCell SureLock mini-cell (Life Technologies, cat. no. EI0001)
- Nitrogen tank and gauge
- Automatic high-sensitivity osmometer (5004 Micro-Osmette; Precision Systems)

REAGENT SETUP

MSP storage buffer Prepare 1 liter of a solution containing 5.85 g of NaCl (100 mM), 2.42 g of Tris/HCl (20 mM), 0.15 g of EDTA (0.5 mM); adjust the pH with HCl to 7.4. This buffer can be stored at 4 °C for months.

t-SNARE protein purification Coexpress and purify rat syntaxin 1A and mouse 6×-His-SNAP-25 with expression vector pTW34, and then store the protein mixture in 400 mM KCl, 25 mM HEPES, 1% (wt/vol) OG, 1 mM DTT and 10% (vol/vol) glycerol; adjust the pH to 7.4 as described previously[35]. t-SNARE is still active after 6 months when stored at −80 °C in the above-described solution.

6×-His-SUMO-VAMP2 Express and purify full-length, wild-type mouse VAMP2 (6×-His-SUMO-VAMP2) using expression vector pET-SUMO-VAMP2. Store the protein in 400 mM KCl, 25 mM HEPES, 1% (wt/vol) OG, 1 mM DTT and 10% (vol/vol) glycerol; adjust the pH to 7.4 as previously described[15]. VAMP2 is still active after 6 months when stored at −80 °C.

MSP1E3D1 purification Express and purify MSP1E3D1 with expression vector MSP1E3D1-pet28 (Addgene) as previously described[21]. After eluting with 400 mM imidazole, cleave off the 6×-His-tag via overnight incubation at 4 °C with TEV protease according to the enzyme manufacturer's instructions. After purification, store MSP1E3D1 in MSP storage buffer. We have successfully stored MSP1E3D1 for 6 months at −80 °C.

t-liposome reconstitution buffer Prepare 10 ml of a buffer (pH 7.4) containing 400 mM KCl, 25 mM HEPES, 1% (wt/vol) OG and 1 mM DTT. This buffer should be freshly prepared.

Dialysis buffer For liposome dialysis, prepare 4 liters of dialysis buffer (pH 7.4) containing 29.8 g of KCl (100 mM), 23.83 g of HEPES (25 mM) and 0.61 g of DTT (1 mM). This buffer should be freshly prepared.

Calcium reconstitution buffer Prepare 10 ml of a buffer (pH 7.4) containing 350 mM KCl, 25 mM HEPES, 50 mM $CaCl_2$, 1% (wt/vol) OG and 1 mM DTT. This buffer should be freshly prepared.

Calcium dialysis buffer For liposome dialysis, prepare 4 liters of calcium dialysis buffer (pH 7.4), which contains 52.2 g of KCl (175 mM), 23.83 g of HEPES (25 mM) and 0.62 g of DTT (1 mM). This buffer should be freshly prepared.

Nanodisc reconstitution buffer Prepare 1 liter of a buffer (pH 7.4) containing 5.84 g of NaCl (100 mM), 2.42 g of Tris/HCl (20 mM) and 0.15 g of DTT (1 mM). This buffer should be freshly prepared.

Dithionite buffer Prepare 100 ml of a Tris buffer (0.61 g, 50 mM, pH 10) containing 1.74 g of sodium dithionite (100 mM) as previously described[34]. To guarantee dithionite activity, immediately after preparation, quickly freeze 100-µl aliquots with liquid nitrogen. Store these aliquots at −80 °C for up to 3 months. During each experiment, thaw a dithionite buffer sample right before use. Always set a control sample in the assay and use the control sample to assess whether dithionite is still active.

Nycodenz buffer, 100% (wt/vol) Weigh 30 g of Nycodenz powder and transfer it into a 50-ml Greiner centrifuge tube; add about 10 ml of ddH_2O and rotate the mixture on a rotator overnight at room temperature (25 °C). The next day, adjust the total volume to 30 ml with ddH_2O. Prepare 5× salt buffer containing 500 mM KCl, 125 mM HEPES and 5 mM DTT. This buffer should be freshly prepared.

Nycodenz buffer, 80% (wt/vol) Pool together 12 ml of 100% (wt/vol) Nycodenz buffer and 3 ml of 5× salt buffer. This buffer should be freshly prepared.

Nycodenz buffer, 30% (wt/vol) Pool together 3 ml of 100% (wt/vol) Nycodenz buffer, 2 ml of 5× salt buffer and 5 ml of ddH_2O. This buffer should be freshly prepared.

Nycodenz buffer, 20% (wt/vol) Pool together 2 ml of 100% (wt/vol) Nycodenz buffer, 2 ml of 5× salt buffer and 6 ml of ddH_2O. This buffer should be freshly prepared.

Bio-Beads SM-2 beads Prepare the beads as indicated by the bead manufacturer. In brief, soak 5 g of beads in 50 ml of ddH_2O overnight at 4 °C. Remove the water by pipetting. Store the beads in 25 ml of methanol:water (20:80, vol/vol). Before use, wash the SM-2 beads five times with ddH_2O to remove organic solvent. Discard the ddH_2O. The humid beads are ready to use. The amount of beads used in nanodisc preparation is 0.5–0.8 g of the damp beads per every ml of the reconstitution mixture.

Uranyl acetate solution, 2% (wt/vol) Boil ddH_2O for 1 min and let it cool down at room temperature. Add 0.1 g of uranyl acetate to 5 ml of water and put it on a vortexer for 10 min. Spin the mixture at room temperature in a desktop centrifuge at 20,000*g* for 10 min and filter the supernatant through a 0.2-µm PVDF membrane. This solution can be stored at room temperature for 1 month.

Mag-Fluo-4 solution Add 73.4 µl of Milli-Q water into the tube containing 500 µg of Mag-Fluo-4 to get a 10 mM concentration. Dilute 100 times with ddH_2O to obtain the working solution, which contains 0.1 mM Mag-Fluo-4. This solution can be stored at −20 °C for 1 month.

EQUIPMENT SETUP

Plate reader setup for lipid mixing Set the incubation temperature of plate reader at 37 °C. Set the excitation/emission wavelengths at 460 nm/538 nm,

auto cutoff at 530 nm. The photomultiplier tube (PMT) is set at low. The time interval between each reading is 1 min, and the total time of acquisition is about 60 min.

Plate reader setup for the content-release assay Set the excitation/emission wavelengths at 480 nm/520 nm and the auto cutoff at 515 nm. The PMT is set at low. The time interval between each reading is 1 min, and the total time of acquisition is about 60 min.

Electron microscope Operate the FEI Tecnai electron microscope at 120 kV accelerating voltage. Take micrographs of the specimen on a Gatan Ultrascan4000 CCD camera at ×52,000 magnification.

PROCEDURE

Preparation of nanodiscs ● TIMING 32 h

1| Transfer 300 µl of v-lipid blend with a Hamilton glass syringe into a chloroform-cleaned 12 × 75 mm glass tube. Dry the lipid under gentle nitrogen flow (**Fig. 2a**). Aluminum foil should be used to cover the glass tubes containing fluorescent lipids throughout the preparation.

2| Remove traces of chloroform by leaving the glass tube in a desiccator under vacuum for at least 1 h.
▲ CRITICAL STEP Because chloroform dissolves the lipids, the preparation and experiments must be performed in a chloroform-free environment to make sure that the lipids form bilayers. Hence, it is important to completely remove chloroform from the lipid.

3| Place 15 nmol of MSP1E3D1 (59 µl of a 260 µM solution in MSP storage buffer) into a glass tube.

4| Add 6×-His-SUMO-VAMP2 (usual initial concentration of 6×-His-SUMO-VAMP2 is about 200 µM), 0.3 µl of DTT (1 M in water) and make up the required volume in the glass tube from a 20% (wt/vol) OG solution (in 400 mM KCl, 25 mM HEPES) to a final OG concentration of 1% (wt/vol). The exact volume of 20% (wt/vol) OG depends on the amount of VAMP2 added into the tube because VAMP2 storage buffer already contains 1% (wt/vol) OG. Thereafter, add nanodisc reconstitution buffer to the glass tube so that the total volume is 300 µl (**Fig. 2a**). The final copy number of VAMP2 per nanodisc is determined by the MSP1E3D1:VAMP2 ratio (**Table 1**). For example, to obtain nanodiscs containing an average of four VAMP2 per nanodisc (initial MSP:6× His-SUMO-VAMP2 = 2:2), add 75 µl of 6×-His-SUMO-VAMP2, 11.25 µl of 20% (wt/vol) OG, (stock concentration of 200 µM, i.e., 15 nmol) to the 15 nmol of MSP from Step 3 and add nanodisc reconstitution buffer to a final volume of 300 µl. Transfer this mixture to the glass tube containing the dried lipids from Step 2 (**Fig. 2a**). To prepare protein-free nanodiscs as a negative control, prepare a solution in which 6×-His-SUMO-VAMP2 is replaced by 1% (wt/vol) OG solution.
▲ CRITICAL STEP The ratio of MSP:VAMP2:lipid is crucial for the final productivity of nanodisc and to determine the copy number of VAMP2 per disc.
? TROUBLESHOOTING

5| Vortex the tube at room temperature for 15–30 min.

6| Gently shake the tube at 4 °C for at least 3 h on an orbital shaker.
▲ CRITICAL STEP A long time is required to thoroughly mix the protein and the lipids.

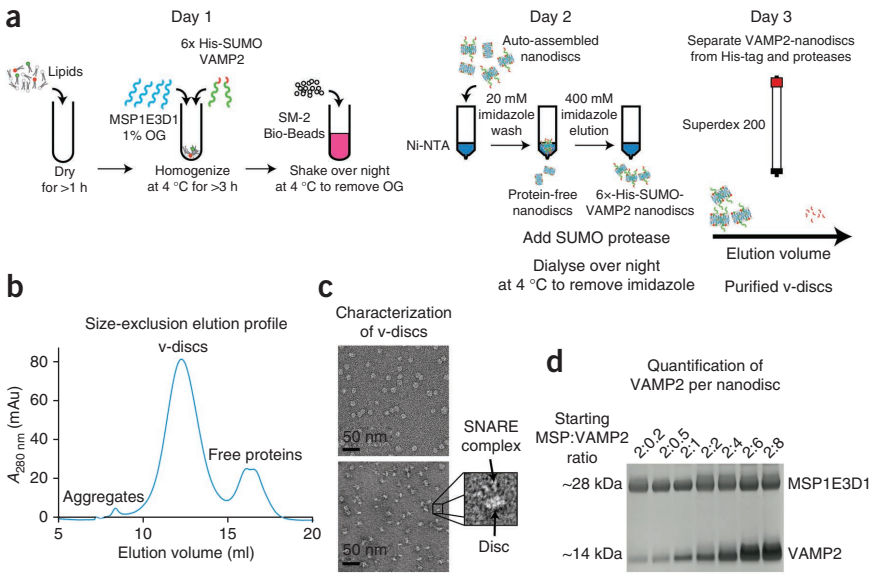

Figure 2 | Nanodisc preparation and characterization. (**a**) Overview of the procedure of preparation and purification of nanodiscs containing VAMP2. (**b**) Typical elution profile of the Superdex 200/10-300 GL column (in Absorbance units). (**c**) Typical transmission electron microscope image of v-discs after purification (top). The v-discs present a monodisperse ring shape. The v-SNAREs cannot be seen because they are small and unstructured. In order to observe them, a complex with the cytosolic domain of t-SNARE has to be formed[20] (bottom, with an ×6 magnification at right). (**d**) SDS-PAGE stained with Coomassie Brilliant Blue reveals the final protein content of the v-disc after purification. The amount of VAMP2 proteins per nanodisc can directly be estimated from the gel (**Table 1**).

7| Add ~0.3 g (equivalent to ~1.5 ml of beads in the original methanol solution) of ddH_2O-prewashed humid SM-2 Bio-Beads into the mixture, then gently shake the mixture overnight at 4 °C on an orbital shaker to ensure constant mixing of the beads and the solution. This procedure completely removes the OG. Tips can be cut to facilitate pipetting the Bio-Beads.
▲ **CRITICAL STEP** A long shaking time is required to completely remove the OG from the solution by the SM-2 beads. Remaining OG molecules would insert in the membrane, change the fusogenic properties and therefore interfere with the experiment.
? TROUBLESHOOTING

8| The next day, collect the supernatant containing the self-assembled nanodiscs and discard the SM-2 Bio-Beads.

9| Pipette 300 µl of Ni-NTA agarose slurry, as provided by the supplier (i.e., 150-µl bed volume), into a 1.5-ml tube. Briefly centrifuge the slurry and discard the supernatant.

10| Wash the agarose four times, each time by adding five bed-volumes of nanodisc reconstitution buffer to the tube from Step 9, and then centrifuging and discarding the supernatant to completely remove the ethanol in the agarose.

11| Incubate the supernatant from Step 8 with Ni-NTA agarose for 2 h at 4 °C.

12| Load Ni-NTA agarose-nanodiscs mixture into a Poly-Prep chromatography column and discard the flow-through.

13| Wash Ni-NTA agarose beads twice with ten bed-volumes of nanodisc reconstitution buffer containing 20 mM imidazole.
▲ **CRITICAL STEP** This wash step is meant to remove VAMP2-free nanodiscs from the 6×-His-SUMO-VAMP2 nanodiscs bound to the Ni-NTA agarose beads. Beads should be washed with no fewer than ten bed-volumes, and should preferably be washed with 20.
? TROUBLESHOOTING

14| Elute the nanodiscs that contain 6×-His-SUMO-VAMP-2 (v-discs) with three to five bed-volumes of nanodisc reconstitution buffer containing 400 mM imidazole (**Fig. 2a**).

15| Add SUMO protease to the eluted solution from Step 14 and incubate the solution at 4 °C overnight to cleave the 6×-His-SUMO tag. The molar ratio of the SUMO protease to VAMP2 is ~1:10. The cleavage of 6×-His-SUMO tag by SUMO protease can be performed during the dialysis process described in Step 16, as dialysis will not affect SUMO protease activity.

16| Dialyze the mixture in a D-tube dialyzer overnight at 4 °C against nanodisc reconstitution buffer (1 liter) to remove imidazole (**Fig. 2a**).

17| Perform gel filtration with the liquid chromatography system via a Superdex 200 prepacked column to separate the v-disc from the 6×-His-SUMO tag and the SUMO protease. The flow rate is set to 0.5 ml min^{-1}. The fraction is 0.5 ml (**Fig. 2b**).

18| Collect the fractions containing v-discs and concentrate them with Amicon Ultra-0.5 (30 kDa cutoff) centrifugal filter units according to the manufacturer's instructions.
■ **PAUSE POINT** After concentration, nanodiscs can be stored at 4 °C for up to 1 week or, after the addition of 10% (vol/vol) glycerol, for several months at −80 °C.

Electron microscopy ● TIMING 4 h

19| Discharge carbon-coated copper grids for 30 s with 25-mA current using a glow discharge cleaning system.

20| Put 3–4 µl of diluted sample from Step 18 on the glow-discharged carbon grid, and let the sample stand for 1 min.
▲ **CRITICAL STEP** Nanodisc samples should be diluted to reach a final concentration of 50 nM MSP, which usually provides the appropriate density on the grid to have enough nanodiscs on each image, while keeping the field unsaturated.
? TROUBLESHOOTING

21| Cut a clean piece of Parafilm and lay it on the bench; put three 40-µl droplets of 2% (wt/vol) uranyl acetate in water on the Parafilm.
? TROUBLESHOOTING

22| Blot the sample from Step 20 away with a piece of filter paper. Flip the grid by holding it with tweezers, and sequentially dab it on the top of each droplet of uranyl acetate stain for 10 s.

23| Wait for 1 min and blot the stain away with a piece of filter paper.

24| Allow the grid to dry in air for more than 1 h.

25| Put the grid into the grid box for storage and later electron microscopy.
■ **PAUSE POINT** Grids can be stored in a vacuum desiccator at room temperature for more than 6 months.

26| Observe the grid under the Tecnai electron microscope (**Fig. 2c**).
? TROUBLESHOOTING

Quantification of VAMP2 molecules per nanodisc ● TIMING ~2 h
27| According to the NuPAGE manufacturer's instructions, prepare a 10-µl of v-disc sample from Step 18 in 1× NuPAGE LDS sample buffer and 1× NuPAGE reducing agent.

28| Set up the XCell SureLock mini-cell and fill it with 1× SDS running buffer.

29| Load the 10-µl sample from Step 27 on the Novex 12% (wt/vol) Tris-glycine mini gel. Load a molecular weight ladder (for instance, Fermentas PageRuler) in another well for analysis.

30| Run the gel for 35 min at 200 V.

31| Stain the gel with Bio-Safe Coomassie stain for 1 h or overnight.

32| Destain the gel with ddH_2O by gentle shaking it at room temperature for 2–5 h. ddH_2O can be changed a few times during the shaking.

33| Quantify each band using appropriate software (ImageJ software, for instance) to determine the ratio of MSP to VAMP2, and thereby obtain the number of VAMP2 molecules per nanodisc (**Fig. 2d**).

Preparation of liposomes without cargo ● TIMING 24 h
34| Transfer 100 µl of DOPC lipid into a chloroform-cleaned glass tube. Dry the resulting mixture under nitrogen flow.

35| Remove the remaining traces of chloroform by leaving the glass tube in a vacuum for at least 1 h.

36| Add t-SNARE proteins and t-liposome reconstitution buffer to make up a total volume of 400 µl with 1:1,000 protein: lipid ratio. t-SNARE protein concentration is measured using the Bio-Rad protein assay kit.

37| Mix by vortexing for 15 min at room temperature.

38| Add 1 ml of dialysis buffer to dilute the OG concentration under its critical micelle concentration (CMC). Depending on the conditions, the CMC of OG is between 0.67% and 0.73% (wt/vol). The initial reconstitution sample is 400 µl with 1% (wt/vol) OG. After dilution with 1 ml buffer, the OG concentration decreases to 0.29% (wt/vol), which is below the CMC and enables the formation of liposomes.
▲ **CRITICAL STEP** The buffer should be added while vortexing the tube to ensure homogeneous mixing.

39| Dialyze the mixture against 4 liters of dialysis buffer overnight at 4 °C.

40| The next day, collect the liposomes with a pipette and mix with 1.4 ml of 80% (wt/vol) Nycodenz buffer. Note that, after mixing, Nycodenz concentration in the sample becomes 40% (wt/vol).

41| Prepare the Nycodenz gradient by loading the liposome sample from Step 40 into a Beckman Ultra-Clear centrifuge tube. Thereafter, successively and carefully overlay the gradient with 500 µl of 30% (wt/vol) Nycodenz buffer, 500 µl of 20% (wt/vol) Nycodenz buffer and 250 µl of dialysis buffer.
▲ **CRITICAL STEP** The different layers should be overlaid very slowly and carefully to avoid disrupting the interface between the layers.

42| Centrifuge the tube for 4 h at 350,000*g* at 4 °C.

43| Carefully collect two liposome samples (200 µl each in volume) at the interface between the layer not containing Nycodenz and the layer containing 20% (wt/vol) Nycodenz. The liposomes thus collected are ready for the fusion assay: their diameter is 50–60 nm^2 and the lipid concentration is about 3 mM.
▲ **CRITICAL STEP** Pipette slowly and carefully to collect the liposome sample, so as not to disrupt the Nycodenz gradient. Disruption of the gradient will result in the mixing of liposomes in the whole buffer and in a reduced liposome concentration.

Preparation of liposomes containing calcium ions ● TIMING 24 h
44| Repeat Steps 34 and 35.

45| Add t-SNARE and calcium reconstitution buffer to make up a total volume of 400 µl and a 1:1,000 protein:lipid ratio.

46| Mix by vortexing for 15 min at room temperature.

47| Add 1 ml of buffer (pH 7.4) containing 50 mM $CaCl_2$, 25 mM HEPES and 1 mM DTT to dilute the OG concentration under its CMC.

48| Dialyze the mixture against 4 liters of calcium dialysis buffer overnight at 4 °C. When setting up the dialysis mixture, measure the osmotic pressures of dialysis buffer and of liposome reconstitution buffer with an automatic osmometer. If the two values are different, make sure to equalize them by adjusting the osmotic pressure of the dialysis buffer by changing its KCl concentration.
▲ **CRITICAL STEP** To prevent the breaking of liposomes during the dialysis, which may cause the release of calcium, the dialysis buffer should have the same osmotic pressure as the liposome reconstitution buffer.

49| Repeat Steps 40–43.

50| (Optional) Before proceeding further, it is advisable to test for liposome leakage: record the fluorescence of the liposome sample on the plate reader with 2 µM Mag-Fluo-4 for 30 min. If the signal remains stable, that means there is no calcium ion leakage from the liposomes. Then add 10 µl of dodecyl maltoside to the sample; a marked signal jump should be observed immediately because calcium ions are released after disruption of the liposomes by detergent.
? TROUBLESHOOTING

Liposome-nanodisc lipid fusion assay ● TIMING 2 h
51| Prewarm the FluoroNunc Maxisorp 96-well plate at 37 °C for 5 min in the microplate reader. Transfer 50 µl of t-liposome from Step 43 into the prewarmed plate. Per experiment, it is better to prepare three wells, the fluorescence of which can be averaged for better accuracy.

52| Add the 2.5-µl v-discs from Step 18 into prewarmed wells containing the t-liposome mixture and gently mix by pipetting.

53| Put the plate back into the microplate reader and start the setup program of the plate reader for lipid mixing (Equipment Setup).

54| After at least 40 min, take out the plate and add 10 µl of 5% (wt/vol) dodecyl maltoside in water to each well; put the plate back into the microplate reader and read it for another 20 min to get the stable maximum signal for NBD fluorescence (**Fig. 3**).
? TROUBLESHOOTING

Dithionite assay ● TIMING 2 h
55| Prepare two samples according to Steps 51–53.

56| After at least 40 min, take out the plate and add 2.5 µl of dithionite buffer into one of the samples and 10 µl of 5% (wt/vol) dodecyl maltoside in water to the other; put the plate back into the plate reader and resume reading the NBD emission signal.

57| After 20 min, take out the plate and add 2.5 µl of dithionite buffer into the samples with dodecyl maltoside; put the

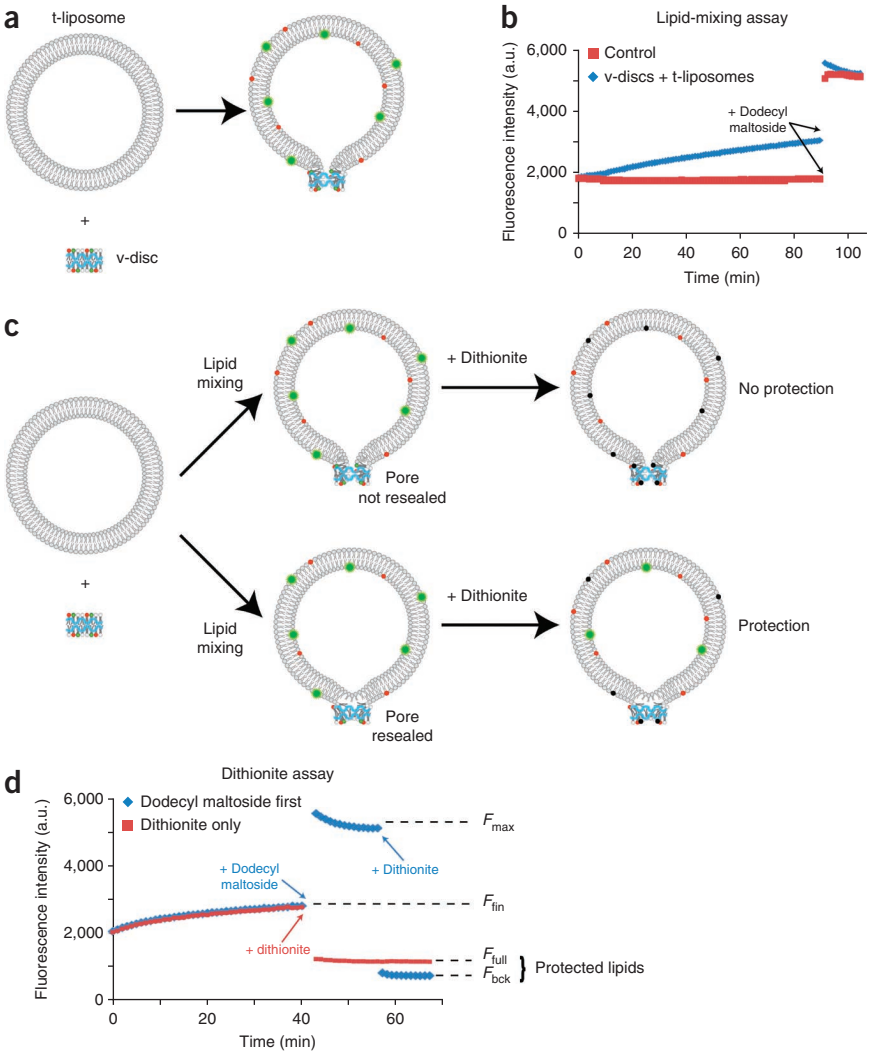

Figure 3 | Lipid-mixing and dithionite assays. (**a**) The lipid-mixing assay. If fusion between the v-disc and the t-liposomes occurs, fluorescent lipids are diluted in the resulting membrane and the NBD fluorescence can be detected. (**b**) Typical curve showing the fluorescence intensity of NBD in function of time during a lipid-mixing assay. Data were recorded on a microplate reader with excitation/emission wavelengths set at 460 nm/538 nm to monitor the specific emission signal of NBD. SNARE-mediated fusion can be specifically inhibited by addition of the cytoplasmic domain of VAMP2 (CDV) to the liposome solution before adding the v-nanodisc (negative control; red curve). Detergent (dodecyl maltoside) is then added to disrupt the liposomes and the nanodiscs to get the maximum NBD signal used for normalization. (**c**) The dithionite assay. After fusion between v-discs and liposomes, if the fusion pores are resealed, the NBD signal of the lipids localized in the inner layer of the liposomes is protected from dithionite. If the pores do not reseal, dithionite can enter the vesicles and all the NBD are quenched by addition of dithionite. (**d**) Typical curve showing the variation of fluorescence intensity of NBD with time during a dithionite assay. Data were recorded on a microplate reader (same settings as above) to monitor the specific emission signal of NBD. Detergent (dodecyl maltoside) is added to disrupt the liposomes and the nanodiscs so that all NBD signal is quenched by addition of dithionite (blue curve). The difference between the two curves shows the proportion of NBD signal protected from dithionite and that the fusion pores eventually reseals. a.u., arbitrary units.

plate back into the microplate reader and read the emission signal of NBD for another 20 min to get the stable final signal after all accessible dyes are quenched (**Fig. 3c**,**d**). Data from the set of wells in which dithionite was added after the detergent will indicate the background fluorescence when all the NBD donors are quenched, and those from the second set of wells in which dithionite was added without previous addition of detergent (see Step 56) will be used to determine the fraction of dyes actually quenched after the fusion experiment.

? TROUBLESHOOTING

Liposome content-release assay ● TIMING 2 h

58| Prewarm the FluoroNunc Maxisorp 96-well plate at 37 °C for 5 min in the microplate reader. Transfer 50 µl of t-liposome from Step 49 and 1 µl of Mag-Fluo-4 solution to the prewarmed plate. It is better to prepare three wells per experiment; the fluorescence of which can be averaged for better accuracy.

59| Add the 2.5-µl v-discs into the prewarmed wells containing t-liposomes and gently mix by pipetting.

60| Put the plate back into the microplate reader and start the program for the content-release assay (Equipment Setup).

61| After at least 40 min, take out the plate and add 10 µl of the detergent dodecyl maltoside (5% wt/vol in water) into each well to disrupt membranes and break up liposomes and nanodiscs; put the plate back into the microplate reader and read it for another 20 min to get the stable maximum Mag-Fluo-4 signal (**Fig. 4**).

Analysis of lipid-mixing assay data ● TIMING 1 h

62| Average the last data points obtained after addition of the detergent (typically three to eight data points are averaged) in Step 54. This value, FL_{max}, represents the total amount of fluorescence when all donor dyes are dequenched.

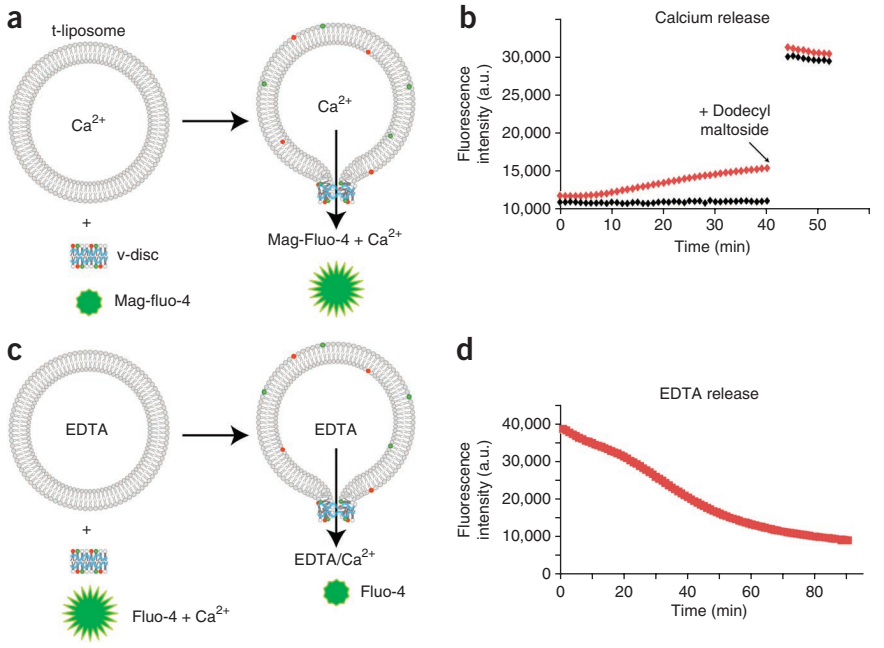

Figure 4 | Content-release assays. (**a**) The calcium release assay. Liposomes containing calcium ion (Ca^{2+}) are mixed with v-discs and Mag-Fluo-4. When the fusion pore is formed, Ca^{2+} is released and is detected by monitoring the fluorescence signal of Mag-Fluo-4. (**b**) Typical curve showing the fluorescence intensity in function of time during a calcium release assay. Data were recorded on a microplate reader with excitation/emission wavelengths set at 480 nm/520 nm to monitor the specific emission signal of Mag-Fluo-4. If no v-disc is mixed with the calcium-containing liposomes (control, black dots), no fusion pore is formed and no calcium ion release is detected. If v-discs are added, the fluorescence signal increases showing that a fusion pore is formed allowing the release of calcium ions (red diamonds). (**c**) The EDTA release assay. Liposomes containing EDTA are mixed with v-discs in the presence of Fluo-4 and calcium ion. When the fusion pore is formed, EDTA is released, and it chelates the calcium ions present in the reaction medium. Chelation results in the decrease of the initial Fluo-4 fluorescence signal. (**d**) Typical curve showing the variation of fluorescence with time during an EDTA release assay (30 mM EDTA, 2 μM Fluo-4; the calcium ion concentration was adjusted to have a start signal at ~40,000). Data were recorded on the microplate reader with excitation/emission wavelengths set at 480 nm/520 nm to monitor the specific emission signal of Fluo-4. The fluorescence intensity of Fluo-4 decreases, indicating that a fusion pore is formed that allows EDTA to be released. The same results are obtained by changing the cargo during the preparation of liposomes. This allows a refined characterization of the size of the fusion pore[20]. a.u., arbitrary units.

63| Normalize FL raw data using FL_{max} to obtain the percentage of nanodisc that have fused with a liposome:

$FL_{fused} = 100\dfrac{FL - FL_{init}}{FL_{max} - FL_{init}}$; where FL_{init} is the initial fluorescence value. Please note that, in some rare instances, upon initiation of the fusion experiment the fluorescence initially decreases before increasing. This decrease, which can last up to 5 min, is due to temperature equilibration. Qualitatively, this decrease will not change the observations and will not be a problem. However, if necessary, for a better interpretation, a correction similar to that presented for the content release (see below) can be applied.

Analysis of content-release assay data ● TIMING 1 h

64| To analyze the content-release data, first calibrate calcium ion concentration in the release experiments: on the plate reader, make measurements at various calcium concentrations with or without liposomes (**Fig. 5a**).

65| Convert all the raw fluorescence data to calcium ion concentrations, from which is subsequently subtracted the initial calcium ion concentration corresponding to the first fluorescence read at Step 60; the result will be the variation in calcium ion concentration in solution. The results can be converted to percentages of the total encapsulated calcium that was released (**Fig. 5b**).

Analysis of dithionite assay data ● TIMING 1 h

66| To determine the fraction of full-fusion events in the dithionite assay, compute the maximum fluorescence F_{max} by averaging the last data points after the addition of detergent. Compute the background fluorescence F_{bck} by averaging the last data points after the subsequent addition of the dithionite to the detergent. The intensity due to one of the liposome leaflet (the outer or the inner leaflet), if all the nanodiscs had fused, would be: $F_{lipo} = (F_{max} - F_{bck})/2$. Similarly, the initial intensity coming from one side of the nanodiscs is: $F_{ND} = (F_{init} - F_{bck})/2$; F_{init} being the initial value of the intensity.

67| Compute the fluorescence due to full-fusion events, F_{full}, by averaging the last data points after the addition of dithionite in the wells instead of the detergent (Steps 56 and 57). The fraction of full-fusion events is then equal to $f_{full} = (F_{full} - F_{bck})/F_{lipo}$. The fraction of fusion events corresponding to hemifusion-like events (in which only the outer leaflets of the liposome bilayer has fused with one leaflet of the nanodisc) is given by: $f_{hemi} = (F_{fin} - F_{bck} - 2(1 - f_{full})$ $F_{ND} - 2\,F_{lipo}\,f_{full})/(F_{lipo} - F_{ND})$; where F_{fin} is the fluorescence value at the endpoint of lipid-mixing.

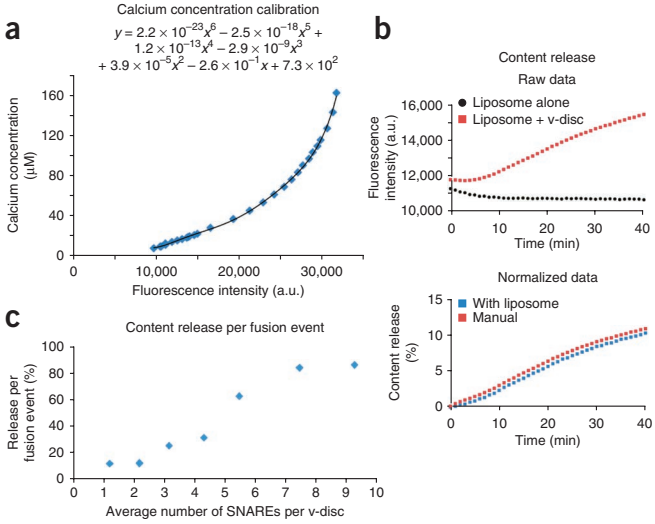

Figure 5 | Analysis of content release. In this analysis, we consider that nanodiscs can only remain intact, undergo full fusion or hemifuse (intact, fused, hemifused). We neglect other alternate states, such as a nanodisc that hemifused with two distinct liposomes (one for each face). (**a**) Calibration of the Mag-Fluo-4 fluorescence with the calcium ion concentration as indicated in PROCEDURE Step 64. A simple polynomial fit is sufficient to convert any data to obtain accurate calcium concentration. In our experiments, we used a 6th degree polynomial (R^2 = 0.9992) (**Fig. 5a**). The type of fit must be optimized for each specific case. (**b**) Example of calcium ion release monitoring (average of seven VAMP2 molecules per disc). Usually, the release curve *C(t)* shows a decrease in fluorescence intensity before an increase. This effect can easily be corrected if a buffer alone or a buffer and liposome measurement is simultaneously performed (controls). Subtracting the fluorescence due to the control calcium ion concentration from *C(t)* corrects for this temperature effect. If controls are not performed simultaneously, it is possible to correct for the initial decrease by subtracting the fastest fusion rate in *C(t)*, *r*, from the initial data points: $C(t) - rt$. Assuming that the initial rate does not vary substantially over several minutes, the result corresponds exactly to the decrease in a control experiment. Top, raw data of the experiment (red) and control (black, no v-disc). Bottom, percentage of content release using the polynomial approximation obtained in **Figure 5a** (red) and the correction using the fastest fusion rate (blue); both normalizations give the same result. (**c**) The content release per fusion event is presented for various VAMP2:disc ratios according to PROCEDURE Steps 68 and 69. The fact that the percentage of calcium ion release per fusion event plateaus close to 100% suggests that the assumption that the nanodisc can only be in three major states (intact, fused, hemifused) is reasonable. a.u., arbitrary units.

Estimation of the amount of content released during one fusion event ● TIMING 1 h

68| The fluorescence increase during lipid mixing that is due to full-fusion events, FL_{full}, can be obtained from the lipid mixing and dithionite data: $FL_{full} = 2 f_{full} FL_{max}/(1 + f_{full})$.

69| Hence, with a 1:1 nanodisc:liposome ratio, an estimate of the fraction of cargo released during a single fusion event is: FC_{fin}/FL_{full}; where FC_{fin} is the fluorescence value at the endpoint of content release (**Fig. 5c**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4	VAMP2 is not concentrated enough	Low yield in the purification of VAMP2 may lead to protein batches of low concentration	Amicon Ultra filter units can be used to bring the protein solution to the desired concentration
7	No or few self-assembled nanodiscs	OG concentration remained above the CMC	OG should be removed properly from the mixture by extensive mixing with SM-2 Bio-Beads
13	Contaminant can be observed in the preparation after Ni-NTA column	Nonspecific binding can occur on Ni-NTA column during long incubation times and/or in the absence of imidazole in the incubation buffer	Implement extensive washes and/or increase the concentration of imidazole to 50 mM during the washing step
	Nanodiscs containing VAMP2 do not bind to the Ni-NTA agarose beads	Presence of imidazole in the wash buffer can decrease the binding of His-tag on the Ni-NTA column	Decrease the concentration of imidazole in the wash buffer
		Degradation of VAMP2 can lead to the loss of the His-tag and prevent the binding of the VAMP2-containing nanodiscs on the Ni-NTA column	Check for the integrity of the protein (i.e., presence of His-tag) and re-purify 6×-His-SUMO-VAMP2, if necessary

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
20	The EM grid is saturated with nanodiscs or no nanodisc can be seen on the grid	Nanodisc concentration is too high (saturation) or too low (no nanodisc)	Different sample dilutions can be used. The time the sample is left on the grid can also be tuned but longer time will result in the saturation of the grid
21	Staining fails	Long-term storage can lead to precipitation of uranyl acetate	Prepare fresh uranyl acetate staining solution Protect the staining solution from light, as it can precipitate easily
26	The EM sample is dirty	Contamination can occur during nanodisc preparation	Centrifuge the sample at the maximum speed for 2 min to remove the contaminant
50	Calcium-loaded liposomes leak during preparation	Because liposomes are very sensitive to osmotic pressure and shear force, they could be broken during the dialysis and centrifugation, which results in the calcium release before the fusion assay	Adjust the osmotic pressure of the calcium dialysis buffer so that is similar to that of the calcium reconstitution buffer When the liposomes are collected after centrifugation, use a cut tip for aspiration and transfer to avoid too much shear
54	Fluorescence intensity is low	Fluorescent dyes on nanodiscs may get photobleached, if exposed to ambient light for too long	Take care while handling the fluorescent lipids. Aluminum foil should be used to cover glass tube all along the preparation
57	NBD is not quenched by dithionite	Sodium dithionite is stable when dry, but it is slowly oxidized by air when in solution	Use freshly prepared dithionite solutions or aliquot and freeze immediately after preparation. It can be stored at −80 °C for the short term (Reagent Setup)

● TIMING

Nanodisc preparation and purification

The preparation and purification of nanodiscs require 3 d (days 1 and 2 for auto-assembly of nanodiscs; day 3 for purification with gel filtration).

Steps 1 and 2: 1 h 30 min

Steps 3–6: 4 h

Step 7: >8 h (overnight)

Steps 8–15: 3 h

Step 16: >8 h (overnight)

Steps 17 and 18: 1 h

Electron microscopy

Steps 19–24: 2 h

Steps 25 and 26: 1 to 2 h

Quantification of VAMP2 per nanodisc

Steps 27 and 28: 15 min

Steps 29 and 30: 40 min

Steps 31–33: 1 h 30 min

Preparation of liposomes

Steps 34–50: ~1 d

Assays using nanodiscs and liposomes

Steps 51–54: 2 h

Steps 55–57: 2 h

Steps 58–61: 2 h

Data analysis

Steps 62 and 63: 1 h

Steps 64 and 65: 1 h

Steps 66 and 67: 1 h

Steps 68 and 69: 1 h

ANTICIPATED RESULTS

By using the nanodisc/liposome assay and the classical intermediate fusion states, we estimated the amount of calcium released per fusion event. We correlated this amount with the number of SNAREs in the nanodisc (**Fig. 5c**). We also showed that, as expected, cargos of increased size are released at a decreased rate, as seen in the example of EDTA and EGTA; EDTA, which is the smaller of the two molecules, is released 2.3 times faster than EGTA[20]. Both EDTA and EGTA are chelating agents with high affinity for Ca^{2+}. When they are released through the fusion pore, EDTA and EGTA chelate the calcium ions present in the reaction medium away from Fluo-4, which leads to an observable decrease of the initial Fluo-4 fluorescence signal. The observed change in the amount of cargo released is due to the nanometric size of the pore, comparable to that of the cargo, which limits the amount of molecules that can diffuse through it. Obviously, a cargo that is larger than the pore will not be released. Hence, we expect that establishing the fraction released during a single fusion event for several cargo sizes may provide direct information on the kinetics of pore formation and reseal. A similar result may be obtained by varying the size of the liposome. A bigger liposome will take more time to fully release its content. If the liposome is too large, the fusion pore will reseal before the content is fully released. Hence, the amount of content released will be correlated to the kinetics of the fusion pore.

If the kinetics of pore formation and reseal is established, the effect on it of several components could be systematically studied by mutation of the proteins involved, addition of regulatory factors or change of the membrane composition.

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AUTHOR CONTRIBUTIONS L.S., J.E.R. and F.P. designed the research. L.S., Y.J.W. and K.H. optimized the detailed protocol. Q.-T.S. performed the electron microscopy. L.S., K.H. and F.P. analyzed the experiments. L.S., K.H., Y.J.W. and F.P. wrote the paper.

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