Discrimination between docking and fusion of liposomes reconstituted with neuronal SNARE-proteins using FCS

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Neuronal exocytosis is mediated by the SNARE proteins synaptobrevin 2/VAMP, syntaxin 1A, and SNAP-25A. While it is well-established that these proteins mediate membrane fusion after reconstitution in artificial membranes, it has so far been difficult to monitor intermediate stages of the reaction. Using a confocal two-photon setup, we applied fluorescence cross-correlation spectroscopy (FCCS) and fluorescence lifetime analysis to discriminate between docking and fusion of liposomes. We show that liposome populations that are either non-interacting, or are undergoing docking and fusion, as well as multiple interactions can be quantitatively discriminated without the need for immobilizing the lipid bilayers. When liposomes containing a stabilized syntaxin 1A/SNAP-25A complex were mixed with liposomes containing synaptobrevin 2, we observed that rapid docking precedes fusion. Accordingly, docked intermediates accumulated in the initial phase of the reaction. Furthermore, rapid formation of multiple docked states was observed with an average of four liposomes interacting with each other. When liposomes of different sizes were compared, only the rate of lipid mixing depended on the liposome size but not the rate of docking. Our results show that under appropriate conditions a docked state, mediated by trans-SNARE interactions, can be isolated that constitutes an intermediate in the fusion pathway.

Intracellular membrane fusion is a hallmark of eukaryotic life. Most intracellular fusion processes are mediated by SNARE proteins that represent an evolutionarily conserved family of small membrane proteins (1). All SNAREs share a homologous sequence of 60–70 amino acids arranged in heptad repeats, referred to as SNARE motifs, which can be classified in four subfamilies (2). Appropriate sets of SNARE motifs assemble into stable bundles of four α-helixes, termed SNARE complexes, with each subfamily contributing one helix to the complex. For fusion to occur, SNARE complexes form between complementary sets of SNAREs that are localized in the two membranes destined to fuse. Accordingly, current concepts, complex formation is initiated at the N-terminal ends of the SNARE motifs, leading to a trans complex that connects the membranes. Complex formation then proceeds from the N-terminal end toward the C-terminal membrane anchors (“zippering”) which initiates fusion [see (3–5) for recent reviews].

Fusion of artificial phospholipid vesicles (liposomes) reconstituted with SNARE proteins has been an important tool for studying the mechanisms of SNARE-mediated membrane fusion (6, 7). In these studies, lipid mixing is routinely measured in solution with one of the vesicle populations containing lipids labeled with two different fluorophores at quenching concentrations. Fusion with unlabeled vesicles results in a dilution of the labeled lipids in the plane of the membrane and fluorescence dequenching (8). Using this assay, it was shown that SNARE-mediated membrane fusion shares important characteristics with biological membrane fusion reaction [e.g., inhibition by clostridial neurotoxins and lysophospholipids, the requirement for anchorage of SNAREs via transmembrane regions (9)], substantiating the view of SNAREs acting as a minimal machinery for membrane fusion (10, 11). However, it has been difficult to unravel the mechanisms of regulatory proteins such as synaptotagmins, complexins, and SM-proteins, leading to conflicting results (12–17).

In a first approximation, the fusion pathway can be divided into two steps. First, freely diffusing liposomes collide and form a docked but yet unfused intermediate, in which the two membranes are bridged by trans-SNARE complexes. In a second step, docked liposomes fuse resulting in cis-SNARE complexes. Moreover, fused liposomes may undergo additional rounds of fusion. The fluorescence dequenching assay used by most laboratories does not allow for assessing the relative contribution of these steps on the overall fusion kinetics, and it is thus not possible to discriminate the effect of regulatory proteins on docking and fusion.

To overcome this limitation, techniques were developed involving either planar membranes or immobilized vesicles which allow for discriminating between vesicle binding and membrane merger (18–22). While these approaches constitute considerable progress, the need for immobilizing membranes limits experimental throughput, and it cannot be excluded that surface attachment alters the biophysical properties of the membrane. Here we describe a procedure that is based on detection and analysis of low numbers of freely diffusing liposomes in a confocal microscope. A combination of fluorescence resonance energy transfer (FRET) and fluorescence cross-correlation spectroscopy (FCCS) allows for a distinction and concurrent quantification of different states of liposome fusion: non-interacting liposomes, tightly interacting liposomes where lipids of the two membranes are not mixed (in the following called “docked”) and liposomes where lipid mixing has occurred (“fused” liposomes). It is also possible to detect multiple interacting liposomes. The liposomes diffuse freely in an aqueous environment without any interference by supporting or immo-

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results

To measure SNARE-mediated vesicle docking and fusion, we used liposomes that were either reconstituted with synaptobrevin 2 or with a preformed complex of SNAP-25A and syntaxin 1A that was stabilized by a fragment of synaptobrevin 2 corresponding to the C-terminal part of the SNARE-motif (residues 49–96). It was shown previously that this complex contains a free N-terminal binding site for synaptobrevin 2, allowing for fast binding. When these liposomes are mixed, fusion is rapid, with the stabilizing R-SNARE fragment being displaced during the reaction (23, 24). Liposomes were labeled with Oregon Green (usually containing a stabilized acceptor complex) or with Texas Red, resulting in robust FRET upon fusion (25).

In a first step, we characterized signals observed from individual liposomes or liposome pairs diffusing through the detection volume of the confocal setup. For this, we diluted samples to a concentration of about 0.1 liposomes per focal volume to assure that the observed fluorescence bursts were produced by single freely diffusing particles, and analyzed if the bursts on the two detectors were temporally correlated. Simultaneous signals on both detectors represent either docked or fused liposomes while individual liposomes generate temporally independent signals (Fig. 1A). As expected, no correlation between photon bursts in the red and green channel was observed when red and green liposomes both contained synaptobrevin 2 (Fig. 1B). In contrast, a fluorescence burst from a fusing sample 2 min after mixing the donor and acceptor liposomes shows correlated intensity fluctuations in both channels because of simultaneous diffusional movements through the detection volume (Fig. 1C). In the case shown in Fig. 1C, the intensities of the fluorescence signals in both channels are comparable as typically seen in the onset of the fusion reaction, which indicates a liposome pair that is docked but not yet fused. Finally, Fig. 1D shows an example of a burst obtained from the same reaction as in Fig. 1C, but 2 h after mixing, that is, when fusion is largely completed. Again, the fluctuations of the fluorescence signals in both channels are correlated. However, the intensity of the red acceptor fluorescence is larger than that of the green donor fluorescence, indicating FRET between fluorophores after membrane merger.

For single burst analysis the sample needs to be very dilute, and thus fluorescence events are rare. Consequently, long recording times are needed to obtain statistically significant results, which is not compatible with measuring fusion reactions exhibiting fast kinetics. To measure the proportion of double-versus single-labeled vesicles at higher concentrations of liposomes we used FCCS, which allows for the detection of up to 100 particles per focal volume instead of 0.1 for the burst analysis. In this concentration range, even small changes in the number of liposomes diffusing in and out the focal volume still cause significant fluctuations in the fluorescence signal. Correlation or cross-correlation of fluorescence signal therefore gives accurate information about the number of liposomes present in the focal volume. The amplitude of a typical cross-correlation curve $G_{DD}$ obtained by FCCS for small correlation times $\tau$ is proportional to the number of double-labeled particles in a sample (SI Appendix and Fig. S1). When SNAP-25A/syntaxin1A liposomes were mixed with liposomes reconstituted with full-length synaptobrevin 2 the cross-correlation amplitude $G_{DD}$ as a measure of the number docked or fused liposomes increased (Fig. 2A).}

![Fig. 1. Distinction of different states of liposome fusion by single burst analysis. (A) Liposome populations expected in a typical fusion reaction and the hypothetical fluorescence bursts of photons on the two detectors expected for the various liposome populations: uncorrelated (Left), and temporally correlated for docked (Center), and lipid mixed liposomes (Right). Liposomes containing the two different classes of SNARE proteins are labeled with Oregon Green and Texas Red. Using a confocal two-photon setup, the dyes are excited simultaneously and are spectrally separately detected. Docked but unfused liposomes show no FRET, and thus the burst intensities in each channel equal to the ones of non-interacting liposomes. In liposomes that underwent lipid mixing FRET is observed, which results in an increase of the red fluorescence and a decrease of the green fluorescence. (B–D) Measured fluorescence bursts of (B) non-interacting liposomes (containing only synaptobrevin 2), (C) fusing liposomes after 2-min fusion time, and (D) fusing liposomes after 2-h fusion time.](https://www.pnas.org/doi/10.1073/pnas.0906677106)

![Fig. 2. FCCS and fluorescence lifetime analysis of a fusion reaction. (A) Open circles: FCCS curves at 30 s (black), 3 min (green), and 60 min (red) of a fusion reaction of liposomes containing a stabilized acceptor complex of SNAP-25A, syntaxin 1A and synaptobrevin 2. Solid lines: corresponding theoretical fitting curves according to equation 54. (B) Corresponding normalized fluorescence decay curves of the donor dye. Inset of (B) Average values for nine 10-s measurements of the fluorescence lifetime at 0 min (black) and 60 min (red) fusion time are shown. Error bars correspond to the standard deviations.](https://www.pnas.org/doi/10.1073/pnas.0906677106)
changes in the fluorescence lifetime were observed immediately after mixing (black and green curve in Fig. 2B). However, after 60 min, a significant decrease in the fluorescence lifetime was detected (red curve in Fig. 2B). As expected, no increase in the cross-correlation or decrease in the donor fluorescence lifetime was observed in samples where the interaction between the Q- and R-SNAREs was prevented by incubation with a soluble fragment of synaptobrevin 2 showing that the signal changes require trans-SNARE pairing.

Analysis of FCSs and fluorescence lifetime curves at various time points during a fusion reaction yielded the proportion of double-labeled (i.e., docked and lipid mixed) particles and lipid mixed liposomes (\(N_X\) and \(N_{fus}\), respectively) over the course of the reaction (Fig. 3A; Fig. 2B shows the first 10 min of the reaction; see also Fig. S3 and SI Appendix for a detailed description of the analysis). As shown in Fig. 3, the comparison of the time courses of \(N_{fus}\) and \(N_X\) revealed significant differences. In the initial phase, that is, immediately after mixing of the liposomes, the proportion of double-labeled species \(N_X\) increases much faster than the proportion of fused liposomes \(N_{fus}\). The difference between \(N_X\) and \(N_{fus}\) corresponds to the relative number of docked but non-lipid mixed vesicles:

\[
N_{doc} = N_X - N_{fus}
\]  

[1]

As shown in Fig. 3 C and D, \(N_{doc}\) reaches a maximum of about 50% of the overall lipidosome population about 2 min after mixing and then declines, showing that under our reaction conditions a relatively stable intermediate docked state exists.

To gain insights into the influence of size on the fusion kinetics we compared liposomes of a diameter of 30 nm as shown in Fig. 3 to liposomes with a 100-nm diameter (Fig. 4). In these experiments lower protein concentrations were used since the reconstitution of the stabilized acceptor complex into large liposomes at high protein density showed considerable variability in size (see SI Appendix). While the speed of docking was comparable, a clear difference between the fusion speed for large and small liposomes was observable. The larger liposomes display a lag phase in lipid mixing that is prolonged about 2- to 3-fold relative to the smaller liposomes. The data suggests that the stability of the docked intermediate is influenced by liposome size which in turn may reflect curvature and membrane elasticity effects.

It has been suggested previously that in SNARE-mediated fusion of liposomes multiple rounds of fusion can take place (27). However, the extent to which multiple fusions occur was only determined indirectly using the standard fluorescence de-quenching assay. With fluorescence correlation spectroscopy the extent of multiple interacting liposomes can be measured easily because FCS allows for the independent determination of red- and green-labeled liposomes, \(N_R\) and \(N_G\), respectively, regardless of whether the labeled particle contains exclusively red or green or both types of fluorescence labels (Fig. S4). Therefore, when liposomes dock or fuse only once, the counted number of red and green liposomes does not change because docked and fused liposomes are counted independently in each channel. However, if a docked or fused lipidosome interacts again with one or more
liposomes, the number of the corresponding species decreases. The reduction in the number of liposomes during the fusion thus allows determining how many red or green liposomes have been combined on average to form the current population of docked or fused liposomes. Fig. 5 shows that the number of liposomes drops quickly by about 40–50%. This corresponds to an interaction of 3–4 liposomes on average, with green and red liposomes participating roughly equally. Furthermore, comparison of Figs. 3A and 5 indicates, that multiple docking is completed about 6-fold faster than fusion. A comparison of single liposome fluorescence burst photon counts of a population of liposomes carrying both, green and red labels, with a population of fusing liposomes after 60 min (Fig. S5) suggests that the majority of the docked aggregates proceeds to lipid mixing. The decrease in particle number thus indicates that approximately 1.8–2 rounds of docking/lipid mixing take place during the reaction (ignoring the small population that does not proceed to lipid mixing).

For a more quantitative evaluation of the docking and fusion reactions, we fitted bi-exponential functions to the fusion curves. The resulting time constants, $\tau_1$ and $\tau_2$, are characterizing the timescales for docking and fusion for the given liposome concentrations, SNARE density and protein-lipid composition. For the data shown in Fig. 3C, the time constants for docking and fusion are $\tau_1 = 70$ s and $\tau_2 = 840$ s, respectively. The data can also be analyzed assuming a two-step kinetic model and applying the differential equations given in Fig. 6 in which multiple interactions of the liposomes have been taken into account (see SI Appendix for more details). Using this kinetic model, the time constants for docking and fusion were calculated to be approximately $\tau_1 = 100$ s and $\tau_2 = 600$ s, correlating well with the estimates obtained by the bi-exponential fit and providing further evidence for a metastable intermediate. From these time constants it can be deduced by applying Smoluchowski theory for diffusion-limited reactions that on average liposomes collide approximately $10^5$ times before they form a docked state (28).

Conclusions

Membrane fusion between vesicles requires that a physical contact is established between the membranes before the bilayers merge and SNARE proteins must interact in trans before fusion. However, it has been experimentally difficult to determine the kinetics of docking and to relate it to the kinetics of lipid mixing. The data presented here, provides evidence that in the case of the neuronal SNAREs synaptobrevin 2 and a complex of syntaxin 1A, SNAP-25A, and synaptobrevin 2 (residues 49–96) a trans-SNARE-complex is formed before lipid mixing, holding the liposomes in close contact, with lipid mixing being delayed. In the particular case studied here, the stability of the docked intermediate might be increased due to the artificial nature of the acceptor complex that requires displacement of the synaptobrevin 2 fragment before complete zippering can occur and was also shown to be dependent on the size of the liposomes.
From this intermediate fused liposomes (F) are formed with a rate constant $\kappa_1$, syntaxin 1A, SNAP-25A, and synaptobrevin 2 from Methods that are likely to act on docked vesicles and to influence docking. It is a prerequisite for the investigation of the role of proteins and especially protein to lipid ratios, which were the same conceivable that it functions in a manner comparable to the SNARE proteins including the stabilized acceptor complex. (A) Reaction scheme of a two-step fusion process: individual green (G) and red (R) liposomes interact with each other forming an intermediate state (D$_n$) with a rate constant $\kappa_1$ – $1/\tau_1$. D$_n$ includes all single and multiple docked liposomes (see SI Appendix).

From this intermediate fused liposomes (F) are formed with a rate constant $\kappa_2$ – $1/\tau_2$. (B and C) Numeric solution of the differential equations (black lines, time constants set to $\tau_1$ – 100 s and $\tau_2$ – 600 s) and experimental data (red dots) for docked (B) and lipid mixed (C) liposomes. The experiment indicates that docking occurred with a rate constant of approximately $1/100 \text{s}^{-1}$ and lipid mixing was about 6× slower.

used. In neuronal exocytosis the protein complexin is thought to stabilize such intermediate trans-SNARE complexes, and it is conceivable that it functions in a manner comparable to the synaptobrevin 2 fragment (29). It was possible to determine specific time constants for docking and lipid mixing-dependent population changes, which are intrinsic to the membrane system used depending on membrane composition, membrane curvature and especially protein to lipid ratios, which were the same as typically used in liposome fusion experiments (13), but different from the ones found in synaptic vesicles (30).

Our data show that populations of non-interacting, docked, lipid mixed, and multiple interacting species of liposomes can be determined quantitatively with FCCS under conditions where the liposomes are freely diffusing in an aqueous environment. The method allows for a concurrent determination of the formation and decay kinetics of these populations, providing a major advantage compared to classical bulk fusion assays. We believe that such a quantitative description of fusion intermediates is a prerequisite for the investigation of the role of proteins that are likely to act on docked vesicles and to influence docking and fusion kinetics such as synaptotagmin and complexin.

**Methods**

**Preparation of SNARE Proteins and Proteoliposomes.** The SNARE proteins syntaxin 1A, SNAP-25A, and synaptobrevin 2 from *rattus norvegicus* were expressed and purified essentially as described: synaptobrevin 2 [full-length: residues 1–116 (11), soluble portion: residues 1–96 (31)] was expressed from a pET28a vector in *Escherichia coli* strain B21 (DE3). A complex consisting of Syntaxin 1A (coding for a fragment including its SNARE motif and the transmembrane region, residues 183–288), SNAP-25A [residues 1–206, all cysteines mutated to serines (32)], and C-terminal fragment of synaptobrevin 2 (residues 49–96) were purified after co-expression (13).

Proteoliposomes of approximately 30-nm diameter containing either full-length synaptobrevin 2, or a purified acceptor complex consisting of SNAP-25A, the syntaxin 1A fragment (residues 183–288), and the C-terminal synaptobrevin 2 fragment (residues 49–96) were prepared as described (13), using protein to lipid ratios of 1:200–300 and the following lipid composition (molar ratios): phosphatidylcholine (5), phosphatidylethanolamine (2), phosphatidylserine (1), phosphatidylinositol (1), and cholesterol (1) (all from bovine brain, Avanti Polar Lipids). Lipids in methanol/chloroform (2:1) were dried under nitrogen and resuspended in 20 mM HEPES/KOH, pH 7.4, 150 mM KCl, 1 mM DTT, and 5% (v/v) sodium cholate, yielding a lipid concentration of 13.5 mM. Proteins in 2% (v/v) CHAPS were added and liposomes were formed by size-exclusion chromatography on a SMART system (Amersham Biosciences) using a PC 3.2/10 Fast Desalting column (GE Healthcare) equilibrated in 20 mM HEPES/KOH, pH 7.4, 150 mM KCl, and 1 mM DTT.

Typically, the liposomes contained either 1.5 mol% total lipids of the fluorescent lipid analog Oregon Green-phosphatidylethanolamine as donor dye (usually liposomes containing syntaxin 1A and SNAP-25A), or 0.1 mol% total lipids Texas Red-phosphatidylethanolamine as an acceptor dye (Molecular Probes/Invitrogen).

Liposomes of a 100-nm diameter were prepared as described in the SI Appendix.

**Burst Analysis, FCCS, and Fluorescence Lifetime Measurements.** All experiments were carried out using a two-photon confocal microscope setup (33) with a detection volume of about 1 fL and two detectors, allowing for the spectrally separated detection of the Oregon Green and Texas Red fluorescence from labeled liposomes (see SI Appendix and Fig. 5E).

The fluorescence time traces were analyzed for individual fluorescence bursts or using fluorescence auto- and cross-correlation spectroscopy (FCS and FCCS) (25, 34, 35) in combination with fluorescence lifetime analysis. FCCS allows for the determination of the average number of red or green labeled docking occurred with a rate constant of approximately $1/100 \text{s}^{-1}$ and lipid mixtures in the red or green detector that are caused by the diffusion of the particles in an out the detection volume (36) (see SI Appendix). The method is sensitive for particle concentrations in the nanomolar range (~1–100 particles in the detection volume). Similarly, FCCS allows for the determination of the average number of particles in the focal detection volume that are labeled with both red and green fluorescence dyes by analyzing the signal fluctuations from both detectors simultaneously.

For full fusion the liposome solutions as obtained by size exclusion chromatography were diluted 1:50 and mixed in equal amounts, resulting in concentrations of about 5–30 liposomes per focal volume were drawn at different times of the fusion reaction and measured nine times in the red or green detector that are caused by the diffusion of the particles in that concentration of approximately 0.1 liposomes per focal volume were obtained. To improve time resolution for the FCCS/fluorescence lifetime measurements during the first 7 min of fusion, 30 μL of a 1:50 dilution of each of the two species were mixed directly on the coverslip and measured for 7 min in 10-s intervals. For inhibition of SNARE-interacting liposomes containing the S-NAREs were preincubated with a 10-fold excess of a soluble synaptobrevin 2 fragment (residues 1–96) for 30 min at room temperature.

FRET was measured as a decrease in the donor fluorescence lifetime. We determined the fluorescence lifetime of the donor dye by fitting a monoexponential function to the fluorescence decay curve of the green donor:

$$I(t) = I_0 \cdot e^{-t/\tau_I},$$

where $I(t)$ is the fluorescence intensity observed at the time $t$ after a laser excitation pulse. $I_0$ is the amplitude of the decay curve. The reciprocal of the fluorescence lifetime $1/\tau_I$ is a linear function of the rate for energy transfer $k_{ET}$ (37).

$$\frac{1}{\tau_I} = k_{ET} + \text{const}$$

$k_{ET}$ depends linearly on the acceptor dye concentration in the membranes of fused or multiple fused liposomes. As a consequence, $1/\tau_I$ is also a linearly related to the proportion of originally free liposomes that have fused, $N_\text{free}$, even when liposomes undergo multiple rounds of fusion (see also
SI Appendix (38). Lipid mixing kinetics measured by fluorescence lifetime analysis was in good agreement with data obtained by measuring Oregon Green fluorescence intensity with a standard fluorometer (Fig. 57), and corresponded well to data obtained by the standard fluorescence quenching assays (Fig. 58).


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SI Appendix

Preparation of Liposomes with a 100 nm Diameter. To study of the influence of curvature on the kinetics of docking and fusion liposomes with a diameter of 100 nm were prepared using a different protocol than described in the method section. In a first step, large unilamellar vesicles (LUVs) were prepared by reverse phase evaporation [1]. Briefly, purified lipid extracts were mixed in 2:1 chloroform:methanol (total lipids 8 mM; from Avanti Polar lipids, Alabaster, AL, USA) consisting of phosphatidylcholin, phosphatidylethanolamine. phosphatidylserin and cholesterol in the molar ratio 50:20:20:10. The mixture contained either 0.5 mol % of total lipids of the fluorescent lipid analog Oregon Green-phosphatidylethanolamine as donor dye (liposomes containing the stabilized acceptor complex), or 1 mol % of total lipids Texas Red-phosphatidylethanolamine as an acceptor dye (Molecular Probes/ Invitrogen, Eugene, OR, USA). After solvent removal by high vacuum, lipids were dissolved in diethyl ether (1.5 mL) followed by addition of reconstitution buffer (20 mM HEPES, 150 mM KCl, 1mM EDTA, 1 mM DTT, pH 7.4, 0.5 mL) and sonication on ice (3 x 45 s). Removal of diethyl ether from the inverted suspension was achieved by vacuum evaporation, gradually lowering the pressure to approximately 150 mbar over a 2 h time period. Liposomes were then extruded using polycarbonate membranes of pore size 0.4 and 0.1 μm (Avanti Polar lipids) to give uniformly distributed LUVs in the diameter range of 100 nm as confirmed by field-flow-fractionation coupled to multi angle laser light scattering (FFF-MALLS, Wyatt Technology Corporation, Santa Barbara, CA, USA).

Recombinant synaptobrevin 2 and a stabilized SNARE acceptor complex were purified as described in the method section with the difference that n-octylglucoside (50 and 80 mM, respectively) instead of CHAPS was used as the detergent in the final ion-exchange purification step. Incorporation of the proteins into liposomes was achieved by n-octylglucoside-mediated reconstitution using a modified procedure from Rigaud and co-workers [2]: LUVs were mixed with n-octylglucoside and micellar SNAREs, with the reconstitution buffer adjusted to make the final molar ratio between the excess detergent above critical micellar concentration and the total lipid concentration equal to 2 (detergent concentration in the SNARE solution was taken into account) and the protein-to-lipid ratio equal to 1:500 (total lipid concentration 5 mM). Detergent was then
removed by overnight dialysis at room temperature (2,000 MWCO, Slide-A-Lyzer dialysis cassette, Thermo Scientific, Waltham, MA, USA). The procedure resulted in SNARE-liposomes of approximately 100 nm in diameter as confirmed by FFF-MALLS. Note that a protein concentration of 1:200 (as used in the smaller liposomes) resulted in a broader size distribution, which is the reason why we preferred the lower protein concentration for our experiments.

**Confocal Two-Photon Microscope.** Using a confocal two-photon microscope set-up with two detectors allowed for a simultaneous detection of green and red fluorescently-labeled liposomes (Fig. S6). The set-up has been described before [3]: A homebuilt titanium: sapphire laser (800 nm, 200 mW, 90 MHz) provided 100 fs pulses for excitation. The expanded excitation beam was focused onto the sample via an inverted microscope objective (Uplan Apo water immersion 40x/ 1.15, Olympus, Hamburg, Germany). Two-photon excitation and overfilling illumination of the back aperture of the high diffractive objective ensured a diffraction limited excitation spot of approximately 300 nm in diameter. The excitation energy was ~ 30 mW for all measurements. A dichroic mirror (715 DCSPXR, AHF Analysentechnik, Tübingen, Germany) and a filter (E700sp-2p, AHF) separated the excitation beam and the fluorescence light emitted by the sample. A second dichroic mirror (590 DCXR, AHF) and two bandpass filters (HQ 535/ 50 and HQ 645/ 75, AHF) separated the emission from the Oregon Green and Texas Red fluorophores. The Texas Red-signal was attenuated by a glass filter (NG11, Schott, Mainz, Germany). Photons were detected by two avalanche photodiodes (AQR-13, Perkin Elmer, Dumberry, Canada) and recorded using a single photon counter (Time Harp 200, Picoquant GmbH, Berlin, Germany) coupled to a router (PRT400, Picoquant). The brightness of the liposomes was around 10-20 kHz per particle and crosstalk was less than 5 % on both sides.
**Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross-Correlation Spectroscopy (FCCS).** To evaluate docking and multiple docking the number of single- and double-labeled liposomes was determined by applying FCS (fluorescence correlation spectroscopy) [4, 5] and FCCS (fluorescence cross-correlation spectroscopy), respectively [6, 7]. In FCS the average number of liposomes in the focal detection region of the microscope set-up can be determined by analyzing fluctuations in the number of detected photons caused by the diffusion of fluorescing particles through the detection volume. In a two detector set-up it is additionally possible to analyze how many double-labeled particles are present by means of observing cross-correlation of the signal fluctuations present on both detectors. In FCS auto- and in FCCS cross-correlation functions are calculated from the fluctuating photon numbers by using equations (S1) and (S2):

\[
G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \tag{S1}
\]

and

\[
G_{RG}(\tau) = \frac{\langle \delta F_G(t) \cdot \delta F_R(t + \tau) \rangle}{\langle F_G \rangle \cdot \langle F_R \rangle} \tag{S2}
\]

Here, \(G(\tau)\) is the autocorrelation amplitude for the correlation time \(\tau\), \(F(t)\) the photon counts at time \(t\) detected with either one detector, \(G_{RG}(\tau)\) the cross-correlation amplitude, \(F_G(t)\) and \(F_R(t)\) the photon counts on the green and red detector, brackets indicate averaged values for all times \(t\) of an entire measurement, and \(\delta F(t)\) is the deviation of the fluorescence signal from the temporal average of the signal:

\[
\delta F(t) = F(t) - \langle F(t) \rangle \tag{S3}
\]

Typical correlation curves are shown in figure 2 a.
In the case of free three-dimensional Brownian motion of the liposomes the following equation (S4) can be fitted to the correlation curves obtained using equations (S1) and (S2):

\[
G(\tau) = G_0 \cdot \frac{1}{(1 + \frac{\tau}{\tau_{\text{Diff}}})} \cdot \frac{1}{\sqrt{1 + z^2 \cdot \frac{\tau}{\tau_{\text{Diff}}}}} \quad (S4).
\]

Here, \(G_0\) is the amplitude of the correlation curve at zero correlation times, \(\tau = 0\). The diffusion time \(\tau_{\text{Diff}}\) characterizes the average time a liposome needs to diffuse through the focal volume and \(z\) is a size parameter describing the geometry of the focal area, which was set to a value of 0.25 for the confocal apparatus used in this work.

For two-photon excitation the influence of triplet state blinking on the correlation curves, as observed for one-photon excitation, can be neglected.

For decreasing particle numbers the amplitude of the correlation curve \(G_0\) increases since the relative magnitude of the observed fluorescence fluctuations becomes larger compared to the average fluorescence intensity. As a consequence, the reciprocal of the autocorrelation amplitude corresponds directly to the average number of particles in the detection volume for identical particles [6]:

\[
N_{G/R} = \frac{1}{G_{G/R,0}} \quad (S5).
\]

Liposomes used in our experiments have a size distribution of about 20-40 with an average diameter of about 30 nm [8] in diameter and are expected to show a distribution in dye content. We verified that in this case it is still admissible to approximate the average number of green and red liposomes in the detection volume, \(N_G\) and \(N_R\), as the reciprocal of the green and red autocorrelation amplitude \(G_0\), respectively, for various dilutions of red and green liposomes. For solutions containing either just one type of
liposomes as well as for a mixture of red and green liposomes the calculated number of liposomes was proportional to the dilution (Fig. S4).

The decrease in \(N_G\) and \(N_R\) over time has been used to determine the number of rounds of lipid mixing (Fig. 5).

To determine the number of all double-labeled (docked and fused) liposomes, \(N_{RG}\), equation S4 was fitted to the cross-correlation curves and the two corresponding autocorrelation curves.

The amplitude of the cross-correlation can be calculated using equation S2 [9]:

\[
G_{RG,0} = \frac{\left\langle \partial F_G(t) \cdot \partial F_R(t) \right\rangle}{\left\langle F_G \right\rangle \cdot \left\langle F_R \right\rangle} = \frac{N_{RG}}{(N_G + N_{RG}) \cdot (N_R \cdot N_{RG})}. \tag{S6}
\]

\((N_G + N_{RG})\) and \((N_R + N_{RG})\) are the total numbers of particles carrying a green or red label, respectively, including single- and double-labeled species. These numbers can be derived from the autocorrelation amplitudes according to equation S5. The cross-correlation amplitude can then be described as:

\[
G_{RG,0} = N_{RG} \cdot G_{G,0} \cdot G_{R,0} \tag{S7}.
\]

\(N_{RG}\) can then be determined from the amplitudes of all three correlation curves according to

\[
N_{RG} = \frac{G_{RG,0}}{G_{G,0} \cdot G_{R,0}} \tag{S8}.
\]

**Normalization of Data for the Direct Comparison of the Kinetics of Docking and Lipid Mixing.** In order to determine the proportion of docked liposomes, \(N_{\text{doc}}\), the proportion of double-labeled liposomes needs to be compared to the proportion of liposomes, \(N_{\text{fus}}\). For the comparison first the photon counts of a 10 s measurement were time-correlated and analyzed by FCCS as described above. Then photons of the same
fluorescence trace were summed up in a fluorescence lifetime histogram (Fig. 2b) and a
monoeponential decay function was fitted to the histogram as described in the methods
section.

$N_{\text{ fus}}$ was then derived from the change of the fluorescence lifetime of the Oregon Green-
labeled liposomes due to FRET (Förster resonance energy transfer) that is observed upon
lipid mixing (Fig. S3 c).

As the donor fluorescence intensity as well as the fluorescence lifetime are both a
measure for FRET, very similar results for the kinetics of lipid mixing were obtained
using both fluorescence lifetime analysis and the fluorescence intensity in the
conventional dequenching assay or in a Oregon Green/ Texas Red-labeled liposome
system (Figs. S7 and S8).

The reciprocal of the fluorescence lifetime of the donor dye in presence of an acceptor
dye, $1/\tau_{\text{Fl}}$, is a linear function of the rate for energy transfer:

$$1/\tau_{\text{Fl}} = k_{\text{ET}} + k_{S1,0} \quad (\text{S9}).$$

Here, $k_{\text{ET}}$ is the rate for energy transfer and $k_{S1,0}$ the rate constant for the relaxation of the
donor fluorophores excited state in the absence of any energy transfer.

$k_{S1,0}$ can easily be determined from the fluorescence lifetime of the donor dye $\tau_{\text{D}}$ in
absence of any acceptor molecules, meaning pure Oregon Green-labeled liposomes:

$$k_{S1,0} = 1/ \tau_{\text{D}} \quad (\text{S10}).$$

The rate of energy transfer in presence of an acceptor dye, $k_{\text{ET}}$, can then be calculated as
follows:

$$k_{\text{ET}} = 1/ \tau_{\text{Fl}} - 1/ \tau_{\text{D}}. \quad (\text{S11}).$$

The fluorescence energy transfer rate is linearly proportional to the average acceptor dye
concentration around any donor fluorophore in the membrane. We verified this linear
relationship for liposomes containing different ratios of donor and acceptor dye corresponding to 1 to 3 fusion rounds for each liposome species (Fig. S2).

As the energy transfer rate $k_{ET}$ for every single donor dye molecule depends linearly on the acceptor dye concentration in its environment, the average energy transfer rate of a 10 s measurement is on average proportional to the mean concentration of acceptor dye in all fused membranes. $k_{ET}$ is therefore also a linear function of the proportion of fused liposomes, $N_{fus}$. We present $N_{fus}$ as the percentage of fused liposomes relative to the value at 60 min fusion time, when fusion is largely completed:

$$N_{fus} = \frac{k_{ET}(t)}{k_{ET}(60 \text{ min})} \cdot 100 \quad \text{(S12)}.$$

To compare the fused with the docked liposomes we introduce $N_X$, defined as the proportion of double-labeled particles $N_{RG}$ relative to green particles $N_G$:

$$N_X = \frac{N_{RG}}{N_G} \quad \text{(S13)}.$$

We introduce $N_X$, because liposomes can undergo multiple rounds of docking and fusion. For example, in a case where a double-labeled liposome interacts with another green liposome this does not lead to a change in $N_{RG}$, whereas $N_X$ must increase. Again, the data presented throughout this work are the percentage of double-labeled particles (corrected for crosstalk by subtracting the values of the inhibited sample) relative to the values at 60 min fusion time:

$$N_X = \frac{N_X(t)}{N_X(60 \text{ min})} \cdot 100 \quad \text{(S14)}.$$

Similar results were obtained, when using $N_R$ instead of $N_G$ for the normalization. We verified that the percentage of double-labeled liposomes can be estimated reasonably well using equation S14 even when the liposomes are not of the same fluorescence
brightness as it would be the case for fused and docked liposomes. We prepared double-labeled liposomes containing the two dyes in such ratios that the resulting particle brightnesses corresponded to the ones of docked and fused liposomes. We then mixed single-labeled liposomes with double-labeled liposomes at different ratios and observed a linear relationship between the ratio of docked liposomes and $N_X$ (Fig. S1a).

We also mixed two populations of double-labeled liposomes with different red and green dye concentrations to simulate a situation where docked and fused liposomes are present at the same time. We measured a 100% cross-correlation for all of these mixtures (Fig. S1b). From these experiments it can be concluded that errors introduced by different brightnesses of the liposomes are small relative to the experimental errors under our experimental conditions.

Fig. S3 shows the raw data from which $N_X$ and $N_{fus}$ presented in Fig. 3 of the main manuscript were derived by the procedures described above.

**Influence of Multiple Docked Liposomes on the Kinetic Model.** A significant population of liposomes undergo multiple rounds of docking (Fig. 5). This can easily be included in the kinetic model presented in figure 6 by defining the intermediate $D_n$ as the sum of all possible multiple interacting intermediates. In the following we show this for two rounds of docking (which was also the average number of rounds of fusion observed in our experiments, Fig. 5), but the same can be easily applied to additional rounds.

The analysis shown below is based on the following assumptions:
1. Docking always occurs prior to lipid mixing, and no fused liposomes undergo docking. The latter assumption is justified because we found docking to be much faster than lipid mixing (compare Figs. 3 and 5).
2. Multiple docking occurs at the same rate as first round docking.
3. The lipid mixing speed rate $k_2$ is not dependent on the nature of docking intermediate (i.e. two or more liposomes per docking complex).
For a maximum number of two rounds of docking the following docked intermediates can be formed (Eq. S15-S18):

\[ R + G \rightarrow D \]  \hspace{1cm} (S15)

\[ G + D \rightarrow GD \]  \hspace{1cm} (S16)

\[ R + D \rightarrow RD \]  \hspace{1cm} (S17) and

\[ D + D \rightarrow DD \]  \hspace{1cm} (S18).

Here, G are green liposomes, R are red liposomes and D means a docked pair of exactly one red and one green liposome.

The formation and decay of each species can be described by the following set of differential equations with \( k_1 \) being the rate constant of docking and \( k_2 \) the rate constant of lipid mixing (Eq. S19-S22):

\[
\frac{d[D]}{dt} = k_1[R][G] - k_1[R][D] - k_1[G][D] - k_1[D]^2 - k_2[D] \hspace{1cm} (S19)
\]

\[
\frac{d[RD]}{dt} = k_1[R][D] - k_2[RD] \hspace{1cm} (S20)
\]

\[
\frac{d[GD]}{dt} = k_1[G][D] - k_2[GD] \hspace{1cm} (S21) \text{ and}
\]

\[
\frac{d[DD]}{dt} = k_1[D]^2 - k_2[D] \hspace{1cm} (S22).
\]

If we now take into account that the intermediate described by the kinetic model includes all multiple docked species
\[ \text{D}_n = \text{D} + \text{RD} + \text{GD} + \text{DD} \quad \text{(S23)}, \]

the formation and decay of this intermediate can be calculated according to

\[ \frac{d[\text{D}_n]}{dt} = \frac{d[D]}{dt} + \frac{d[GD]}{dt} + \frac{d[RD]}{dt} + \frac{d[DD]}{dt} \quad \text{(S24)}. \]

Taking into account equations S15-S18 this can be converted to

\[ \frac{d[\text{D}_n]}{dt} = k_1[R][G] - k_2[D_n] \quad \text{(S25)}. \]

Equation S25 was solved numerically to fit the kinetics of the intermediate D_n as presented in Fig. 5.


Fig. S1. Cross-correlation for mixtures of liposomes of varying brightness. To evaluate the error introduced to the cross-correlation calculated according to equation S14 when different types of single- and double-labeled particles are present in the sample (especially particles of different brightness), we prepared liposomes (at approximately equal particle concentration) carrying different label concentrations as follows: single-labeled liposomes containing 0.5% Oregon Green (G0.5), single-labeled liposomes containing 1% Texas Red (R1), double-labeled liposomes containing 0.25% Oregon Green and 0.5% Texas Red (GR0.25/0.5, “dim,” representing the dye content of a liposome after one round of lipid mixing), and double-labeled liposomes containing 1.5% Oregon Green and 1% Texas Red (GR1.5/1, “bright,” which had a brightness for each dye that was approximately equal to the brightness of the single-labeled liposomes and were therefore used to represent docked liposomes). For calibration, these liposomes were mixed in different proportions as follows: (a) Cross-correlation calculated with equation S14 for different mixtures of double-labeled liposomes (GR1.5/1) with single-labeled green (G0.5) and red (R1) liposomes. Dependency of the cross-correlation on the ratio of double-labeled to green liposomes [GR1.5/1]/[GR1.5/1 + G1] was approximately linear. In (b), we mixed the two types of double labeled liposomes representing lipid mixed (GR0.25/0.5) and docked (GR1.5/1) ones at different ratios. For all mixtures we calculated approximately 100% cross-correlation using equation S14, showing that cross-correlation was correctly determined despite variations in particle brightness.
Fig. S2. Linear dependency of the energy transfer rate $k_{ET}$ from the concentration of the acceptor dye. The rate constant $k_{ET}$ was not dependent on the donor dye concentration (which varied between 0.13 and 0.50% for the data shown) and excitation powers used in our experiment and linear proportional to the acceptor dye concentration. Data were obtained for two independent liposome preparations (red and black dots) and for excitation intensities of approximately 10 mW (open dots) and approximately 25 mW (filled dots).
Fig. S3. FCCS and fluorescence lifetime data. Fusion reaction of liposomes labeled with Oregon Green and Texas Red and containing a stabilized SNAP-25A/syntaxin1 acceptor complex and synaptobrevin 2, respectively. Red and black symbols represent two independent liposome preparations. (a) Amount of cross-correlation as calculated by dividing the number of double-labeled liposomes by the overall number of green liposomes \( \frac{N_{\text{RG}}}{N_{\text{G}}} \). Background was taken into account by subtracting the average cross-correlation measured for an inhibited sample (acceptor complex liposomes preincubated with a soluble synaptobrevin 2 fragment as a competitive inhibitor). (b) Normalized docking curve as presented in Fig. 4A, calculated from the data set in a. (c) Change in donor fluorescence lifetime \( \Delta \tau_n \). As the fluorescence lifetime of the Oregon Green-labeled liposomes varies slightly between preparations, the fluorescence lifetime of pure green liposomes has been subtracted from all values to directly compare the two preparations. (d) Normalized lipid mixing curve as presented in Fig. 4A, calculated from the data set in b.
Particle number as a function of the dilutions of liposomes. To determine the number of multiple interacting liposomes in our experiments we calculated the relative number of single-labeled liposomes from the reciprocal of the correlation amplitude $G_0$ (Fig. 5). To prove that $G_0$ is linearly dependent on the concentration, we prepared different dilutions of green (green dots) and red (red dots) liposomes (a) as well as of a mixture of red and green liposomes (b) ranging from 1:50 to 1:400. These dilutions cover the range of concentrations we expect for our experiments, where the initial concentration of the liposomes was 1:100. Liposomes were prepared as described in the Methods section, containing 1.5% Oregon Green, 1% Texas Red, and no proteins.
Fig. S5. Distributions of count ratios calculated for bursts from different liposome populations. Histograms of the ratios of the photon counts detected in the green detector divided by photon counts of the red detector during transits of red particles through the confocal detection area. Ratios are very small for free red liposomes since no green fluorescence dye is present (black curve, average ratio \(0.1\text{--}0.2\)). For a liposome population containing both, red and green labeled lipids, the ratios observed are smaller than 1, since the red acceptor fluorescence is enhanced and the donor fluorescence is decreased due to FRET (maximum at \(0.4\), red curve, compare also with Fig. 1D). The histogram for fusing liposomes measured after 60-min fusion time (green curve) is somewhat broader than the histogram of the liposomes labeled with both, Oregon Green and Texas Red (red curve). This is probably due to additional populations of free and docked liposomes. As can be seen from Fig. 1C, for docked liposomes ratios around or larger than 1 are expected. The difference of the red (100% artificially red/green-labeled liposomes) and green (fusing sample after 60 min) histograms at the right edge might therefore correspond to a small population of docked liposomes that have still not mixed lipids after 60 min.
Confocal microscope with a two-detector setup. Light emitted from single red, green or double-labeled fluorescent liposomes diffusing through the detection volume (yellow area in inset, $\Omega \approx 200–500$ nm) is collected by a microscope objective (O) and is detected by two avalanche photo diodes. BE: beam expansion, DC: dichroic mirror, M: mirror, L: lens, IF: interference filter.
Fig. S7. Comparison between fluorescence intensity and FCCS/lifetime data for Oregon Green- and Texas Red-labeled liposomes. (a) The change of fluorescence intensity of Oregon Green measured with a standard fluorometer (blue line) corresponds well with the change in the proportion of lipid mixed liposomes as calculated from lifetime analysis \(N_{\text{fus}}\) (black dots), but is significantly slower than the change in the proportion of double-labeled liposomes \(N_X\) (red dots). Liposomes contained a stabilized SNAP-25A/Syntaxin 1A-acceptor complex (labeled with 1.5% Oregon Green) and synaptobrevin 2 (labeled with 1% Texas Red). \(N_{\text{fus}}\) and \(N_X\) were calculated as described in the SI Appendix. The reciprocal of the fluorescence intensity \((F/F_0)^{-1}\) was normalized to 1 for 60-min fusion time. (b) shows the first 10 min of (A) in detail.
Fig. S8. Comparison of donor fluorescence intensity in the dequenching assay (NBD/Rhodamin Lissamine) or using Oregon Green/Texas Red-labeling. (a) Normalized fluorescence intensity ($F/F_0$) was measured for a fusion reaction of NBD (1.5%)/Rhodamine Lissamine (1.5%)-labeled with unlabeled liposomes (black line) and liposomes containing Oregon Green (0.5%) and Texas Red (1%) in different liposomes (red line). Temperature (22 °C), dilution of liposomes (1:100), and protein concentrations (1:200 protein-to-lipid) were comparable to the conditions used for FCCS/fluorescence lifetime-experiments. (b) shows the first 10 min of (a) in detail.

Other Supporting Information Files

SI Appendix