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 microsocope 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):

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \qquad \text{(S1)}$$

and

$$G_{RG}(\tau) = \frac{\langle \delta F_G(t) \cdot \delta F_R(t+\tau) \rangle}{\langle F_G \rangle \cdot \langle F_R \rangle} \qquad \text{(S2).}$$

Here, $G(\tau)$ is the autocorrelation amplitude for the correlation time τ, $F(t)$ 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:

$$\delta F(t) = F(t) - \langle F(t) \rangle \qquad \text{(S3).}$$

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):

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

Here, G_0 is the amplitude of the correlation curve at zero correlation times, $\tau = 0$. The diffusion time τ_{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]:

$$N_{\text{G/R}} = \frac{1}{G_{\text{G/R},0}} \qquad \text{(S5).}$$

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 admissibile 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 lipsosomes 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 auto-correlation curves.

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

$$G_{RG,0} = \frac{\langle \partial F_G(t) \cdot \partial F_R(t) \rangle}{\langle F_G \rangle \cdot \langle F_R \rangle} = \frac{N_{RG}}{(N_G + N_{RG}) \cdot (N_R \cdot N_{RG})}. \qquad \text{(S6).}$$

($N_G + N_{RG}$) 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:

$$G_{RG,0} = N_{RG} \cdot G_{G,0} \cdot G_{R,0} \qquad \text{(S7).}$$

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

$$N_{RG} = \frac{G_{RG,0}}{G_{G,0} \cdot G_{R,0}} \qquad \text{(S8).}$$

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_{doc}, the proportion of double- labeled liposomes needs to be compared to the proportion of liposomes, N_{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 monoexponential decay function was fitted to the histogram as described in the methods section.

N_{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, $1/\tau_{Fl}$, is a linear function of the rate for energy transfer:

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

Here, k_{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 τ_D in absence of any acceptor molecules, meaning pure Oregon Green-labeled liposomes:

$$k_{S1,0} = 1/\tau_D \qquad \text{(S10)}.$$

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

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

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:

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

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 :

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

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:

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

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. S1 b). 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):

$$R + G \rightarrow D \qquad \text{(S15)}$$

$$G + D \rightarrow GD \qquad \text{(S16)}$$

$$R + D \rightarrow RD \qquad \text{(S17) and}$$

$$D + D \rightarrow DD \qquad \text{(S18).}$$

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):

$$\frac{\mathrm{d}[D]}{\mathrm{d}t} = k_1[R][G] - k_1[R][D] - k_1[G][D] - k_1[D]^2 - k_2[D] \qquad \text{(S19)}$$

$$\frac{\mathrm{d}[RD]}{\mathrm{d}t} = k_1[R][D] - k_2[RD] \qquad \text{(S20)}$$

$$\frac{\mathrm{d}[GD]}{\mathrm{d}t} = k_1[G][D] - k_2[GD] \qquad \text{(S21) and}$$

$$\frac{\mathrm{d}[DD]}{\mathrm{d}t} = k_1[D]^2 - k_2[D] \qquad \text{(S22).}$$

If we now take into account that the intermediate described by the kinetic model includes all multiple docked species

$D_n = D+RD+GD+DD$ (S23),

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

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

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

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

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

[1] Duzgunes, N., *Preparation and quantitation of small unilamellar liposomes and large unilamellar reverse-phase evaporation liposomes.* Methods Enymol, 2003. **367**: p. 23-27.

[2] Rigaud, J.L. and D. Levy, *Reconstitution of membrane proteins into liposomes.* Methods Enzymol, 2003. **372**: p. 65-86.

[3] Zwilling, D., et al., *Early endosomal SNAREs form a structurally conserved SNARE complex and fuse liposomes with multiple topologies.* EMBO J, 2007. **26**(1): p. 9-18.

[4] Aragón, S.R. and R. Pecora, *Fluorescence correlation spectroscopy and Brownian rotational diffusion.* Biopolymers, 1974. **14**(1): p. 119-137.

[5] Elson, E.L., Madge, D, *Fluorescence correlation spectroscopy. I. Conceptual basis and theory.* Biopolymers, 1974. **Volume 13**(Issue 1): p. 1-27.

[6] Schwille, P., Meyer-Almes, Franz-Josef, Rigler, Rudolf, *Dual-Color Fluorescence Cross-Correlation Spectroscopy for Multicomponent Diffusional Analysis in Solution.* Biophys. J., 1997. **72**: p. 1878-1886.

[7] Schwille, P., Heinze, K G, *Two-Photon Fluorescence Cross-Correlation Spectroscopy.* ChemPhysChem, 2001. **2**(5): p. 269-272.

[8] Stein, A., et al., *Synaptotagmin activates membrane fusion through a Ca(2+)-dependent trans interaction with phospholipids.* Nat Struct Mol Biol, 2007. **14**(10): p. 904-11.

[9] Rigler, R., Földes-Papp, Z, Meyer-Almes, F-J, Sammet C, Völcker, M, Schnetz A, *Fluorescence cross-correlation: A new concept for polymerase chain reaction.* J Biotech, 1998. **63**: p. 97-109.