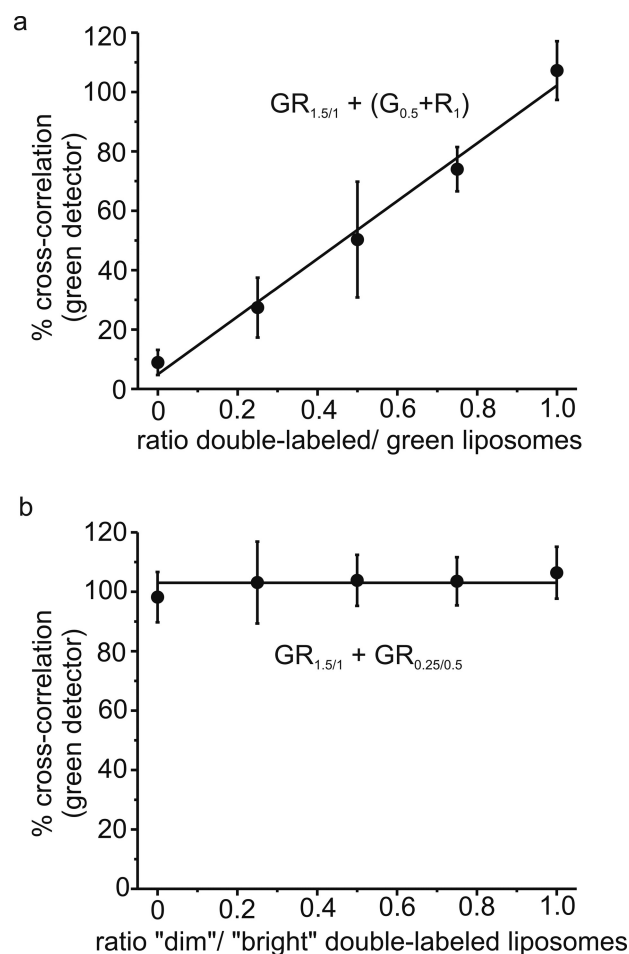
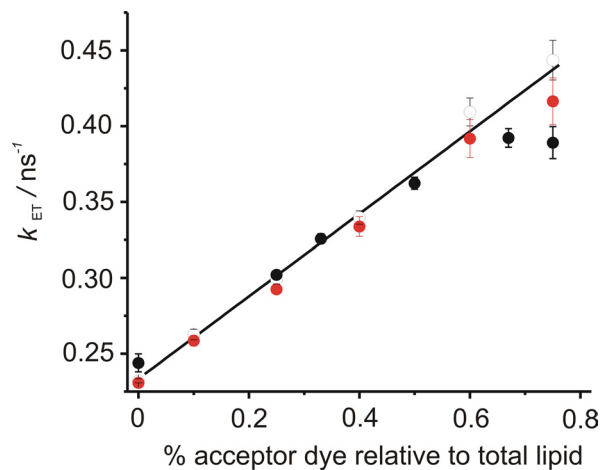


# Supporting Information

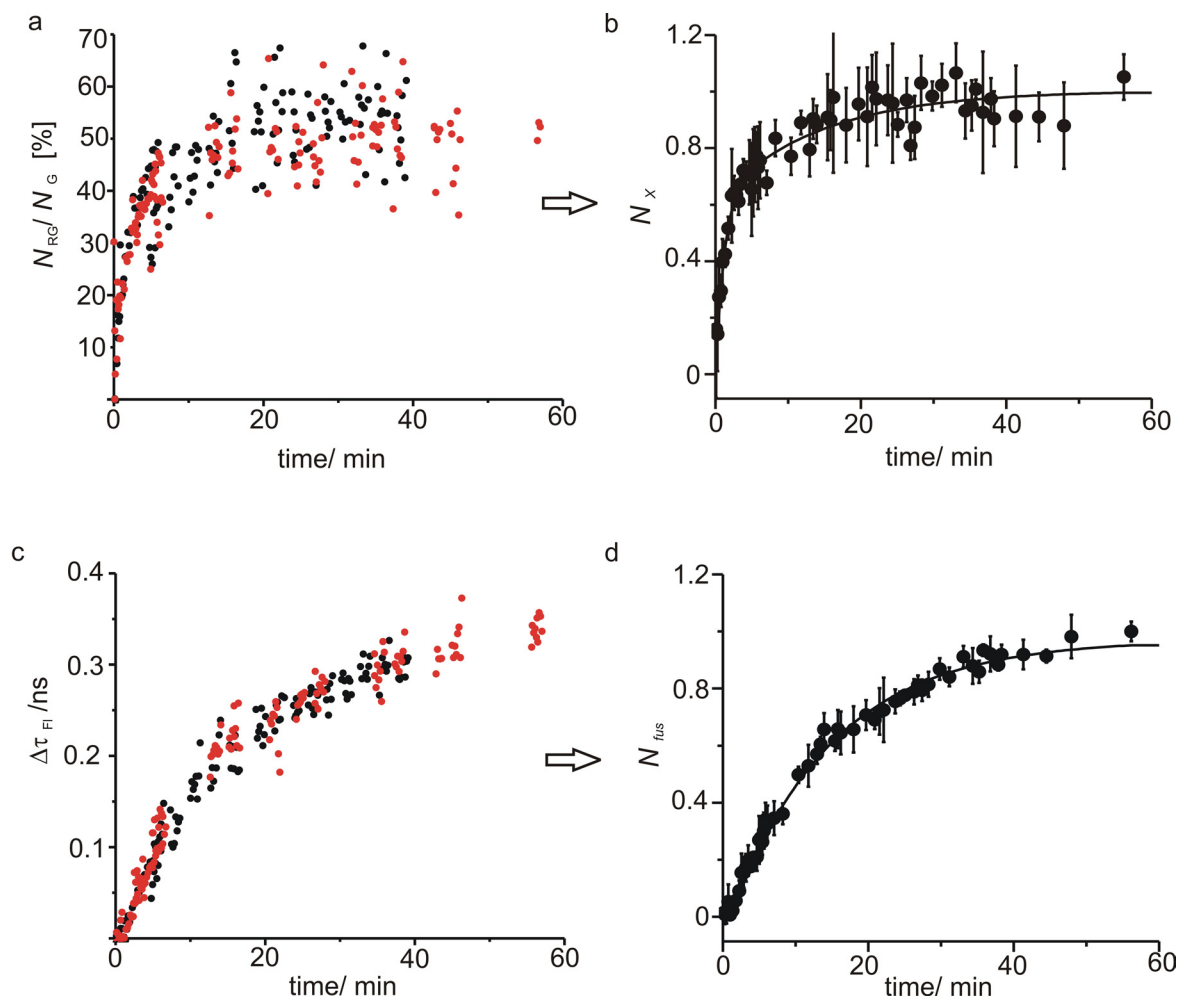
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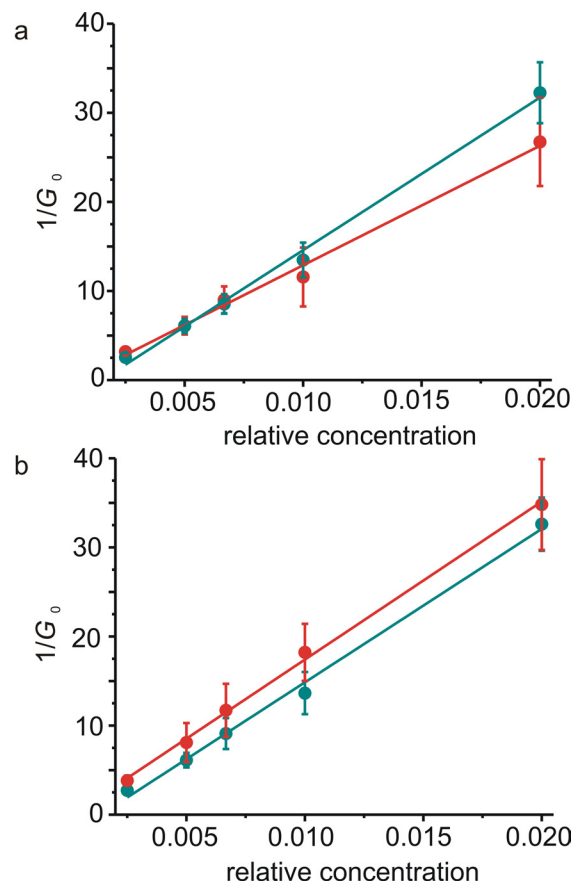
**Fig. S1.** Cross-correlation for mixtures of liposomes of varying brightness. To evaluate the error introduced to the cross-correlation calculated according to equation S14 when different types of single- and double-labeled particles are present in the sample (especially particles of different brightness), we prepared liposomes (at approximately equal particle concentration) carrying different label concentrations as follows: single-labeled liposomes containing 0.5% Oregon Green (G<sub>0.5</sub>), single-labeled liposomes containing 1% Texas Red (R<sub>1</sub>), double-labeled liposomes containing 0.25% Oregon Green and 0.5% Texas Red (GR<sub>0.25/0.5</sub>, "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 (GR<sub>1.5/1</sub>, "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 (GR<sub>1.5/1</sub>) with single-labeled green (G<sub>0.5</sub>) and red (R<sub>1</sub>) liposomes. Dependency of the cross-correlation on the ratio of double-labeled to green liposomes  $[GR_{1.5/1}]/([GR_{1.5/1}] + [G_1])$  was approximately linear. In (b), we mixed the two types of double labeled liposomes representing lipid mixed (GR<sub>0.25/0.5</sub>) and docked (GR<sub>1.5/1</sub>) 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.



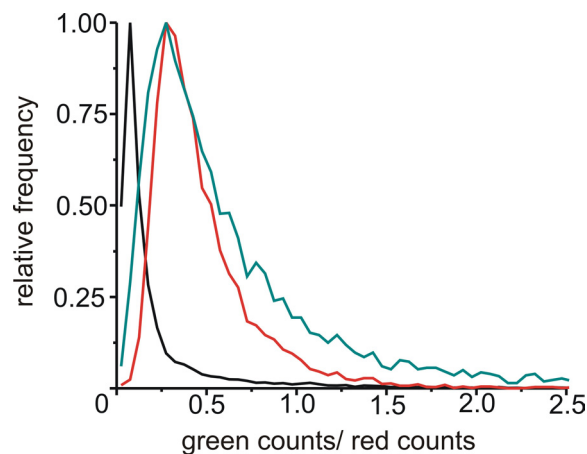
**Fig. S2.** Linear dependency of the energy transfer rate  $k_{ET}$  from the concentration of the acceptor dye. The rate constant  $k_{ET}$  was not dependent on the donor dye concentration (which varied between 0.13 and 0.50% for the data shown) and excitation powers used in our experiment and linear 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).



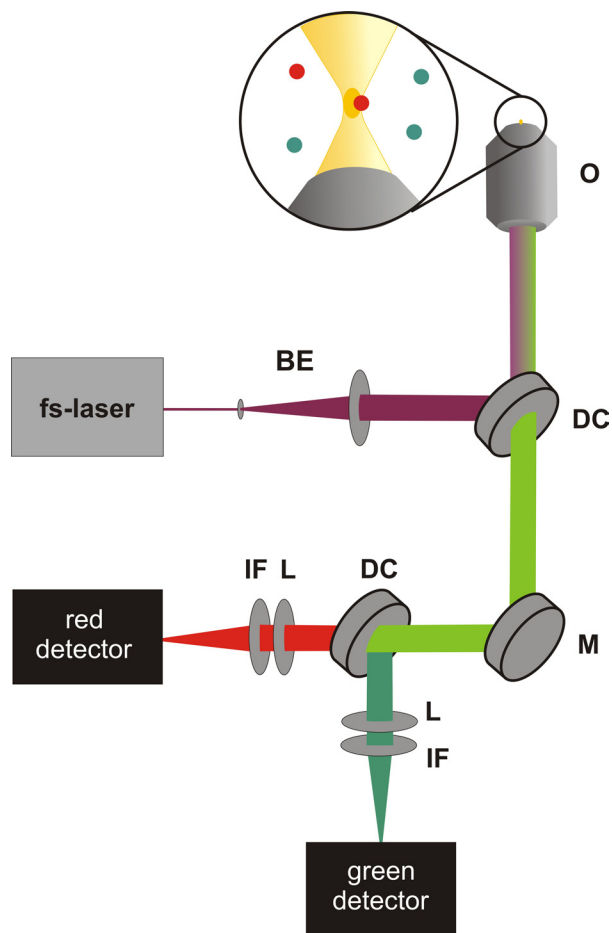
**Fig. S3.** FCCS and fluorescence lifetime data. Fusion reaction of liposomes labeled with Oregon Green and Texas Red and containing a stabilized SNAP-25A/syntaxin1 acceptor complex and synaptobrevin 2, respectively. Red and black symbols represent two independent liposome preparations. (a) Amount of cross-correlation as calculated by dividing the number of double-labeled liposomes by the overall number of green liposomes ( $N_{RG}/N_G \cdot 100$ ). 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  $\Delta\tau_{FI}$ . 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.



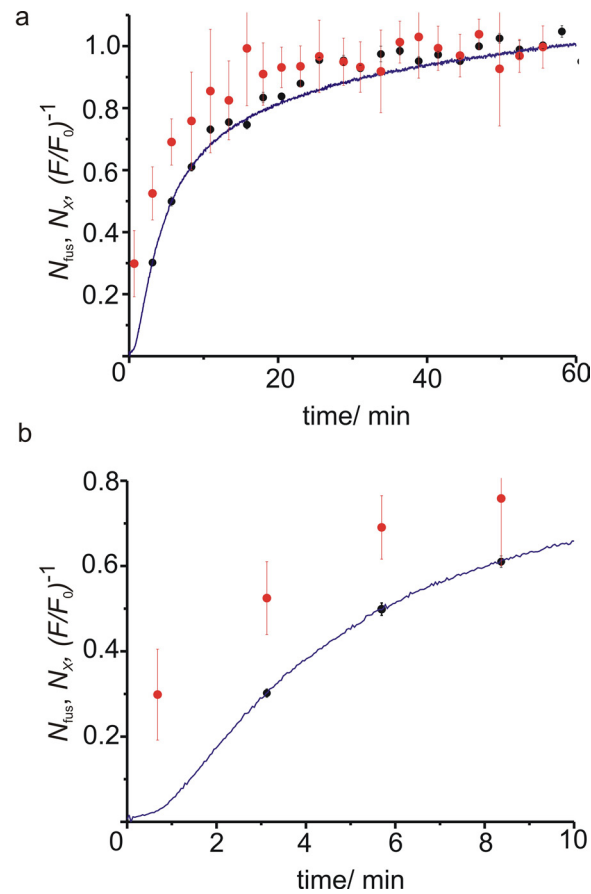
**Fig. S4.** Particle number as a function of the dilutions of liposomes. To determine the number of multiple interacting liposomes in our experiments we calculated the relative number of single-labeled liposomes from the reciprocal of the correlation amplitude  $G_0$  (Fig. 5). To prove that  $G_0$  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.



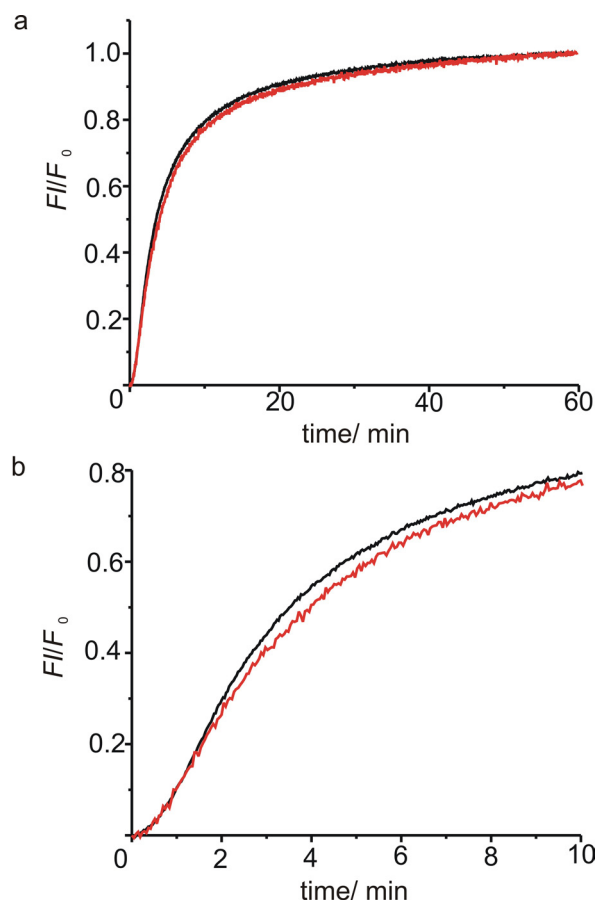
**Fig. S5.** Distributions of count ratios calculated for bursts from different liposome populations. Histograms of the ratios of the photon counts detected in the green detector divided by photon counts of the red detector during transits of red particles through the confocal detection area. Ratios are very small for free red liposomes since no green fluorescence dye is present (black curve, average ratio  $\approx 0.1$ – $0.2$ ). 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  $\approx 0.4$ , 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.



**Fig. S6.** 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,  $\varnothing \approx 200\text{--}500\text{ nm}$ ) 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 ( $N_{fus}$ , black dots), but is significant 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).  $N_{fus}$  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/Rhodamine 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](#)