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Single molecule studies of the neuronal SNARE fusion machinery

Axel T. Brunger[1,*], **Keith Weninger**[2], **Mark Bowen**[3], and **Steven Chu**[4,*]

[1] The Howard Hughes Medical Institute and Departments of Molecular and Cellular Physiology, Neurology and Neurological Sciences, Structural Biology, and Photon Science, Stanford University, CA

[2] Department of Physics, North Carolina State University, Raleigh, NC 27695-8202

[3] Department of Physiology and Biophysics, Stony Brook University Medical Center, Stony Brook, NY, 11794-8661

[4] Lawrence Berkeley National Lab, Berkeley, California, and Departments of Physics and Molecular and Cell Biology, University of California, Berkeley, CA 94720

Abstract

SNAREs are essential components of the machinery for Ca^{2+}-triggered fusion of synaptic vesicles with the plasma membrane, resulting in neurotransmitter release into the synaptic cleft. While much is known about their biophysical and structural properties and their interactions with accessory proteins such as the Ca^{2+} sensor synaptotagmin, their precise role in membrane fusion remains an enigma. Ensemble studies of liposomes with reconstituted SNAREs have demonstrated that SNAREs and accessory proteins can trigger lipid mixing/fusion, but the inability to study individual fusion events has precluded molecular insights into the fusion process. Thus, this field is ripe for studies with single molecule methodology. In this review we discuss first applications of single-molecule approaches to observe reconstituted SNAREs, their complexes, associated proteins, and their effect on biological membranes. Some of the findings are provocative, such the possibility of parallel and anti-parallel SNARE complexes, or vesicle docking with only syntaxin and synaptobrevin, but have been confirmed by other experiments.

Keywords

FRET; membrane fusion; neurotransmission; synaptic vesicle

Introduction

Synaptic neurotransmitter release involves the Ca^{2+}-triggered fusion of synaptic vesicles with the plasma membrane in the presynaptic terminal, releasing neurotransmitter into the synaptic cleft. Synaptic vesicles are recruited to the active zone in the presynaptic membrane, but do not readily fuse. Instead, an average of ten vesicles are stably docked at the active zone awaiting an action potential (1–4). Exocytosis is triggered within approximately 0.2 msec of the Ca^{2+} influx that follows arrival of an action potential (5,6). Although extremely rapid, the neurotransmitter release probability has a significant heterogeneity in single synaptic release sites in hippocampal neurons (7). At most one synaptic vesicle per synapse undergoes exocytosis upon depolarization in the central nervous system (CNS) (8). Thus, regulation of neurotransmitter release occurs at the level of synaptic vesicle release probability. There is also

*Correspondence: brunger@stanford.edu, krwening@ncsu.edu, mark.bowen@sunysb.edu, schu@lbl.gov.

a background rate of fusion of about one per minute per synapse in the absence of action potentials.

Synaptic vesicle fusion involves a highly conserved family of proteins termed SNAREs (soluble N-ethyl maleimide sensitive factor attachment protein receptors) (9–11). SNAREs are directly linked to Ca^{2+} triggering of exocytosis in conjunction with a Ca^{2+} sensor, such as synaptotagmin (12–14). Genetic rescue experiments with mutants of synaptotagmin have now firmly established that synaptotagmin is the Ca^{2+} sensor for the synchronous component of synaptic exocytosis (15), but the mechanism of action of the synaptotagmin·SNARE·membrane fusion machinery remains a matter of intense research (16–21,14,22,23). Numerous other auxiliary proteins have been found to be essential for Ca^{2+} dependent neurotransmitter release, such complexin, Munc18, and Munc13. Thus, SNAREs form only one part, albeit the central part, of the complex system of synaptic neurotransmission. In this review we focus on the first single molecule studies of the neuronal SNAREs syntaxin, synaptobrevin, SNAP-25, some of their binding partners and complexes, and their role in synaptic vesicle docking and fusion.

Single molecule approaches to study membrane protein interactions and fusion

One of the key advantages of single molecule experiments is that they allow one to study the behavior of a single or countable number of molecules or molecular complexes, and their action on an individual molecule or "particle" such as a synaptic vesicle. This is especially useful for systems that show significant variability of individual molecular "trajectories" for a biological or chemical process such as protein folding, protein synthesis, or protein-assisted membrane fusion. The ability of observing individual events therefore removes the requirement of synchronizing events in ensemble experiments that monitor the average behavior of many individual molecules or particles (often in the order of Avogadro's number), and it allows one to perform statistical analysis of a population of individual trajectories that would not be possible in bulk due to ensemble averaging. There are several recent reviews that discuss applications of single molecule techniques to biological systems (24–28).

Here we briefly discuss the principles and focus primarily on single molecule fluorescence approaches such as the particular experiment shown in Figure 1a. Protein-protein interactions are monitored between membrane-bound syntaxin·SNAP-25 complex and synapobrevin that is introduced above the supported bilayer (29). Fluorescent labels are covalently attached at different positions in the individual proteins. These dyes are planar aromatic ring structures that range in size from around 0.5 to 1.2 kDa and may be charged as well. Since any covalent modification of a macromolecule with such a dye can affect the energtic and kinetic properties of the system it is important to study this effect. In our experiments we therefore tested for the potential influence of the labels by repeating them with different labeling combinations. Using available crystal structures, we selected sites that were surface exposed in the particular macromolecules that we studied. As such we usually found that the effect of the labels is small on qualitative properties such as co-localization of dyes or presence or absence of FRET efficiency. However, labeling sites near binding interfaces can affect protein folding and interactions {Li, 2007 #707}.

Quantitative interpretation of FRET efficiencies in terms of absolute distances is not straightforward. It requires careful measurement of fluorophore dynamics to correct the anisotropy and quantum yield terms in the Forster radius {Antonik, 2006 #766}. Interpreting quantitative smFRET values in terms of macromolecular structure is a developing field {Lee, 2005 #768;Margeat, 2006 #770;Schuler, 2005 #772;Watkins, 2006 #771}. An ideal fluorophore attachment site has free, isotropic rotation so there is a high degree of uncertainty

when correlating fluorophore separation to protein structure. Recently, molecular dynamics simulations of protein attached dyes show some promise in obtaining a conversion function between FRET efficiency and absolute FRET distance {Wozniak, 2008 #773}.

The sample is illuminated with laser light using total internal reflection in order to restrict illumination to the region near the surface (the electric field intensity is restricted to a surface layer with decay length around 100 nm) and thus reduce background fluorescence. Two or more lasers emitting at different wavelengths are used to study co-localization and FRET between the fluorescent dyes. A similar setup was used to study single vesicle interactions between protein-containing liposomes and deposited bilayers while simultaneously monitoring content mixing (30).

An alternative to the supported bilayer geometry is to tether “acceptor” liposomes to an inert surface and then to monitor the interaction of these liposomes with “donor” liposomes in solution. This geometry has been used for single vesicle studies (31). Both geometries have advantages and disadvantages. The supported bilayers mimic the geometry of synaptic vesicles docked to a relatively flat presynaptic membrane, but they tend to produce limited protein mobility for at least a portion of the reconstituted proteins due to interactions of the proteins with the underlying surface (32,30). On the other hand, the tethered liposome approach suffers from non-specific binding of the free liposomes with the surface, and the protein density in the tethered liposomes is more difficult to control than in supported bilayers (33).

Another very useful approach to study individual biological processes is single molecule atomic force microscopy often combined with optical trapping or fluorescence measurements (34–37). A particular important application in the context of membrane fusion is the determination of the force and formation enthalpy between individual interacting proteins (38,39).

SNAREs

For a comprehensive review of the chemical, biophysical, and structural properties of SNARE proteins see ref. (40). Both syntaxin and synaptobrevin have a C-terminal transmembrane domain and an adjacent domain that is involved in interacting with SNAREs – we will refer to these domains as SNARE core domains (Figure 2a).

Syntaxin has a folded N-terminal domain consisting of a three-helix bundle that is connected to the partially unfolded SNARE core domain by a short linker (41). Syntaxin switches between closed and open conformations. In the closed conformation the N-terminal domain interacts with part of the SNARE core domain preventing interactions with other SNAREs whereas in the open conformation the syntaxin’s SNARE core domain is free to interact with synaptobrevin and SNAP-25 (42). Fluorescence correlation spectroscopy of individual molecules indicated frequent switching between both conformations with a relaxation time of 0.8 ms (43) while an ensemble NMR study primarily showed a closed conformation (44).

Synaptobrevin has a short unstructured N-terminal region that is adjacent to the SNARE core domain. The entire cytoplasmic domain of isolated synaptobrevin is unfolded in solution (45,46).

Isolated SNAP-25 is an unfolded protein consisting of two SNARE core domains (termed SN1 and SN2, respectively) (47), and a linker that includes four palmitoylated cysteine residues. These palmitoylated cysteines are probably important for membrane association and exocytosis although mutation of all four cysteines in chromaffin cells has surprisingly mild effects on exocytosis and electrophysiological parameters (48).

The SNARE core domains exhibit a plethora of configurational, conformational, and oliogomeric states (40). These different states allow SNAREs to interact with their matching SNARE partners or auxiliary proteins, sometimes in a mutually exclusive fashion. SNAREs undergo progressive disorder to order transitions upon interactions with binding partners, culminating with the fully folded ternary SNARE complex consisting of an elongated four-helix bundle (49) (Figure 2b). These SNARE folding and assembly events are intimately coupled to their function in synaptic vesicle docking and fusion. We discuss in the following three of the assembly states that have been studied with single molecule methods.

Single molecule studies of the syntaxin·SNAP-25 binary complex

The binary interaction between syntaxin and SNAP-25 is generally considered to be the first intermediate in the path to SNARE complex formation. This complex has also been called the target (t)-SNARE or acceptor complex because it forms the binding site on the plasma membrane for synaptic-vesicle localized synaptobrevin (50). The binding of synaptobrevin to the binary syntaxin·SNAP-25 complex thus serves to stably dock synaptic vesicles near the active zone of the synapse.

Syntaxin and SNAP-25 readily form a stable “dead-end” 2:1 complex *in vitro* where a second syntaxin SNARE domain takes the usual position of the synaptobrevin helix in the SNARE complex (47,50). The 2:1 state forms a stable four-helix bundle (51,52). The prevalence of this 2:1 species during solution assembly of SNARE proteins made it impossible to study the 1:1 binary complex by ensemble methods. Using an extremely low concentration of syntaxin (about 100 molecules in an area of 50x50 μm) embedded in a supported bilayer it was possible for the first time, to study the structure and dynamics of the neuronal binary complex in its 1:1 state with single molecule methods (29) (Figure 1b).

The conformation of the 1:1 binary complex is more variable than one would expect if it formed a stable three-helix bundle. With a labeling site pair in syntaxin and SN1, and a dual labeling site pair in SNAP-25, dynamic changes in smFRET efficiency levels were observed. The discovery of fluctuating molecular structural states is a common feature of many single molecule studies (53,54,25). The dynamic changes of the binary complex included both frame-by-frame variability in smFRET efficiency as well stochastic switching between stable intermediate and high smFRET states (Figure 3a). These large changes in smFRET efficiency indicate conformational transitions within the binary complex involving large (> 5 nm) movements. These results are consistent with earlier studies using circular dichroism that indicated some induced structure upon binary complex formation, but much less than what one would expect it were to form a helix bundle (47).

An approximate model of the configurations of the 1:1 binary complex is shown in Figure 3b (lower panels). Prior to synaptobrevin binding, an equilibrium exists between a configuration consisting of the SNARE domains of syntaxin and SNAP-25 (SX-SN1-SN2), and two configurations involving the SNARE domain of syntaxin and either one of the two SNAP-25 SNARE domains with the other SNAP-25 domain dissociated (SX-SN1 and SX-SN2). This model assumes that the syntaxin N-terminal domain is in the open conformation (41) since if it were in the closed conformation, it would prevent interactions between syntaxin and SNAP-25, at least in the N-terminal portion of the SNARE domains.

Upon addition of synaptobrevin, the equilibrium shifts towards the three-helix bundle (SX-SN1-SN2) configuration (Figure 3b). This effect occurs on a fast scale, orders of magnitude faster than the rate constants for synaptobrevin binding to the dead-end 2:1 binary complex. Addition of synaptobrevin to the 1:1 binary complex also completely eliminated dynamic variability in smFRET efficiency levels and stochastic switching (29).

Single molecule studies of the syntaxin-synaptobrevin binary complex

A weak interaction exists between syntaxin and synaptobrevin as corroborated by a small increase in circular dichroism helicity and proton NMR chemical chemical shifts upon binding (45,46). The observed structural changes are much smaller than those for the formation of the syntaxin·SNAP-25 binary complex. Still, single molecule experiments showed that docking and fusion of liposomes to deposited bilayers can be accomplished with just synaptobrevin and syntaxin in different membranes, despite the absence of SNAP-25 (30,55) as was hinted previously by ensemble experiments (56). Furthermore, single molecule atomic force microscopy studies have revealed that the activation free energy for the formation of the syntaxin-synaptobrevin complex comparable to that of leucine zippers (38,39). In comparison, an ensemble study using a surface force apparatus produced an estimate for the formation enthalpy of the *trans* ternary SNARE complex between two separate membranes (35 k_BT) (57). Bulk liposome and single molecule force measurements revealed that the ternary SNARE complex is much more stable than the syntaxin-synaptobrevin complex (58,38). Furthermore, in order to obtain a high FRET efficiency signal between syntaxin and synaptbrevin labeling sites, SNAP-25 had to be added suggesting that the syntaxin-synapbrevin complex is not a helical bundle. Interestingly, studies of SNAP-25 knockout mice revealed a phenotype where vesicle docking and stimulus-independent (spontaneous) fusion persisted although Ca^{2+} triggered release was abolished (59,60). Thus, an explanation of these *in vivo* experiments could be that even in the absence of SNAP-25 and its homologues, the weak interaction between synaptobrevin and syntaxin can promote synaptic vesicle docking and fusion, although no Ca^{2+} triggering is possible.

Single molecule studies of the ternary SNARE complex: parallel or anti-parallel?

The ternary SNARE complex consisting of syntaxin, synaptobrevin, and SNAP-25 can be readily isolated from neuronal cell extracts (61). The membrane anchors are not required for the assembly of the SNARE complex, so many biophysical and structural studies were carried out with recombinant proteins in the absence of the transmembrane domains and palmitoylated cysteines. These truncated SNARE constructs readily form a variety of helical bundles of varying composition and configuration (47,45,40).

Ternary SNARE complex formation induces major disorder to order transitions in SNARE core domains (45), in addition to those observed in the binary complexes discussed above. The assembled ternary SNARE complex consists of a parallel four-helix bundle (49,62) (Figure 2b). The core of the four-helix bundle of the SNARE complex is composed of 16 primarily hydrophobic layers formed by interacting sidechains from each of the four α-helices. At the center of the core complex, a conserved ionic layer is present consisting of an arginine and three glutamine residues contributed from each of the four α-helices. This ionic layer is sealed off against solvent by adjacent hydrophobic layers, but it contains a buried water molecule (62). Structures of the neuronal, endosomal, and yeast SNAREs complexes are very similar, indicating a high degree of evolutionary conservation (49,63,62,64,65).

smFRET experiments revealed a surprising characteristic of SNARE complex assembly: a mixture of parallel as well as anti-parallel configurations was found between the SNARE core domains of syntaxin and synaptobrevin and to a lesser degree between those of syntaxin and SNAP-25; we confirmed that this result is not an artifact by the introduction of covalent dye labels by using different labeling pair combinations to probe for parallel and anti-parallel configurations (66). The subpopulation with the parallel four-helix bundle configuration could be greatly enriched by an additional purification step in the presence of denaturant, indicating that the parallel configuration is the energetically most favorable state. This explains why only

the parallel configuration was found in the crystal structures of the SNARE complex since extensive purification including the temporary use of denaturants was performed for crystallization. Inter-conversion between the parallel and anti-parallel configurations was not observed on the hour time scale. The discovery of mixtures of parallel and anti-parallel configurations now explains previously puzzling results of smaller than expected mean FRET between parallel reporting sites in ensemble studies of SNARE complexes (67), and of non-zero mean FRET in ensemble studies of syntaxin C-terminally fused to blue fluorescent protein (BFP) and synaptobrevin N-terminally fused to green fluorescent protein (GFP) in 20S complexes (68).

To investigate if such mixtures exist in the membrane environment of docked liposomes, smFRET efficiency studies were carried out to determine the configuration of the SNARE complexes involved in docking liposomes to deposited bilayers (30). The anti-parallel population was approximately 1/5 the size of the parallel population. Thus, liposome docking to a supported bilayer favors the assembly of SNAREs into the parallel configuration as seen in the crystal structure (Figure 2c).

Evidence for a *trans* state of the ternary SNARE complex?

Numerous biochemical, structural, and genetic studies have lent support to the zipper model which posits that SNARE complex assembly begins in *trans* (i.e, residing on opposite membranes), with separate SNAREs on the donor and acceptor membranes, and ends with formation of a *cis* complex (i.e., residing on the same membrane). Directional folding of SNAREs into a highly stable parallel four-helix bundle is then thought to drive membrane juxtaposition and, eventually, fusion (69–73) (Figure 2c). Single molecule atomic force microscopy has shown that the assembly of the ternary SNARE complex in principle provides sufficient energy to drive membrane juxtaposition and fusion (39). In addition to juxtaposing membranes through SNARE complex formation, the SNARE transmembrane domains may also participate in the later stages of fusion (74), e.g., by stabilizing or destabilizing fusion intermediate states (75,31).

The zipper model suggests that SNAREs exist in a partially assembled state prior to the arrival of the Ca^{2+} signal (76–79) (Figure 2c). In this state, the SNAREs would be still susceptible to cleavage by a subset of clostridial neurotoxins (80–82). Furthermore, folding-specific SNARE antibodies SNAREs affect some but not all components of the electrophysiological response (77). However, these experiments did not show a direct interaction between the membrane distal regions of the ternary SNARE complex while the transmembrane domains are in opposite membranes. An important first step in this direction is a smFRET study where SNAREs promoted liposome docking to supported bilayers and high FRET was observed between labels at the membrane distal ends of syntaxin and synaptobrevin (Table 1 in ref. (30)). It is unlikely that the observed FRET signal occurred after liposome fusion since fusion was a rare event in these experiments although this question deserves further study.

Models of membrane fusion

Models of membrane fusion have been largely restricted to a phenomenological description of elastic materials representing monolayers (for a review, see (83)). These models make the assumptions that the elastic moduli of the monolayers are uniform and that their surfaces vary smoothly. A commonly used model has emerged from these largely theoretical studies: fusion starts with a stalk state, and proceeds through one or more hemifusion diaphram intermediates states, leading to the formation of a fusion pore (Figure 4, pathway 1). A hemifusion diaphram state is characterized by outer leaflet mixing with no inner leaflet mixing (84,85). However, the only intermediate state that has actually been observed experimentally is the stalk state (86). Furthermore, this dogma of a stalk-diaphram-fusion pathway has been challenged by

computer simulations that demonstrate the possibility of direct transitions from stalk to fusion pore using Monte Carlo (87) and coarse-grain ensemble molecular dynamics algorithms (88) (Figure 4, pathway 2). The relative importance of these two pathways depends on the lipid composition; about 90% of the fusion events proceed via pathway 2 for 2:1 palmitoyloleoyl phosphatidylcholine (POPC): palmitoyloleoyl phosphatidylethanolamine (POPE) lipid mixtures whereas most fusion events proceed via pathway 1 for liposomes containing pure POPE (89). In experimental support of this alternative pathway, polyethylene glycol (PEG)-mediated liposome fusion could only be modeled with direct transitions between stalk and fusion pore in addition to the pathway via a hemifusion diaphram intermediate (85). To uncover these underlying intermediates and transitions, it was essential to simultaneously monitor lipid mixing, content mixing, and leakiness of the fusing liposomes (58).

Reconstitution of SNARE-mediated membrane fusion

Rothman and co-workers developed the first *in vitro* assay to study SNARE-mediated vesicle docking and fusion (90). Co-expressed acceptor complex (syntaxin·SNAP-25) and synaptobrevin were reconstituted into separate liposomes, and lipid mixing was observed on a minute time scale. In the literature the lipid mixing events are interpreted as “fusion” even though these experiments typically do not probe content mixing. We therefore refer to the events observed in these experiments as “lipid mixing/fusion” since the experimental data cannot resolve this ambiguity. We refer to this assay and its subsequent variants as “bulk liposome assay”.

The bulk liposome assay was an important advance since it demonstrated that SNAREs are capable of docking and lipid-mixing/fusion of liposomes *in vitro*. However, it suffers from several deficiencies: only lipid mixing is observed, the protein to lipid ratio can be too high, and no individual fusion events can be observed. It is essential to measure both lipid mixing and content mixing in order to obtain a detailed kinetic model of the fusion process (84,85), in order to distinguish fusion events from liposome instability and leaking (58), in order to account for lipid mixing arising from lipid “flipflop” without fusion (69), and in order to account for possible spontaneous lipid dye transfer between adjacent membranes (91). Historically, only lipid mixing was probed in bulk liposome experiments except in one case where duplex formation of oliognucleotides was used to assay content mixing (92).

The protein density in the early bulk liposome assays is now considered generally too high to be physiologically relevant (e.g., 750 synaptobrevins per 45 nm vesicles (90) compared to the physiological density of roughly 70 synaptobrevins per 50 nm synaptic vesicle (93)). In a more recent experiment, physiological average protein to lipid ratios were used (94). SNARE complex formation was assayed with C-terminal FRET labels and lipid mixing was observed with liposome dye dequenching. Both signals were highly correlated and application of botulinum neurotoxin (serotype E) or tetanus neurotoxin disrupted both processes. No content mixing indicator was used, so fusion was inferred indirectly by an increase of liposome size as observed by electron microscopy. However, the ensemble time scale of the observed lipid mixing/fusion events was still on the minute time scale. Furthermore, even though the average concentration of reconstituted proteins was physiological in these and some more recent studies, there is still a concern since there can be a large variation of reconstituted protein density in individual liposomes depending on the reconstitution protocol (33), resulting in a subpopulation of unstable liposomes which could give raise to false “fusion” positives and consequently results that do not agree with physiological data.

The lipid-mixing/fusion events observed in the bulk liposome experiments are often infrequent or slow, resulting in rounds of fusion that occur over a minute time scale, orders of magnitude slower than individual fusion events that occur in synaptic neurotransmission. For comparison,

in goldfish bipolar neurons, activation of Ca^{2+} current drives secretion at a rate of 10,000 synaptic vesicles per sec (95), and in calyx of Held nerve terminals three to five readily releasable synaptic vesicles fuse within < 1 msec at each active zone upon Ca^{2+} triggering (96).

Despite the deficiencies of the bulk liposome assay, a number of results have been obtained that are consistent with structural knowledge of SNAREs and their complexes. For example, an increase in the number of rounds of lipid-mixing/fusion within a time interval resulted from removal of the N-terminal domain of syntaxin (73). Lipid-mixing/fusion was sensitive to particular SNARE pairings (97–99) presumably caused by kinetic differences of the assembly of these pairings despite very similar thermal stabilities (100,101). Finally, a dramatic acceleration of the lipid-mixing/fusion rate was obtained by the addition of a fragment of synapotobrevin that reduces the prevalence of the "dead-end" 2:1 syntaxin·SNAP-25 complex (50). This work also showed that co-expression of syntaxin and SNAP-25 as opposed to formation of the binary complex by addition of SNAP-25 to membrane reconstituted syntaxin is not essential for the efficiency of the lipid-mixing/fusion rates contrary to what had been suggested in earlier works (90).

The importance of membrane anchors was studied by replacement of the SNARE transmembrane domains with covalently attached lipids (102,103). Replacing either syntaxin or synaptobrevin transmembrane domains with a covalent phospholipid anchors prevented lipid-mixing/fusion, but still allowed docking of liposomes. The membrane proximal region of synaptobrevin could be modified by helix-breaking proline residues with little effect on the assay, and insertion of a flexible linker had a moderate effect with increasing influence for longer linkers (104).

A recent bulk liposome study of SNARE-mediated vacuolar fusion showed that it requires physiological lipid compositions and protein to lipid ratios in order to obtain a reconstituted system that faithfully reproduces the critical dependence on other factors in addition to SNAREs for vacuolar fusion (105). Furthermore, careful controls were performed to rule out instability or leakiness of the liposomes. It is disappointing that such carefully designed bulk liposome experiments are rare for neuronal SNAREs.

Single molecule microscopy and spectroscopy can overcome some of the key deficiencies of the bulk liposome assay since individual events can be observed rather than an ensemble average. Using multiple dye reporters, content and lipid mixing, and protein localization can be monitored simultaneously. In principle, **smFRET** studies should also allow the study of single protein-protein interactions conditional on the occurrence of a fusion reaction although there are many challenges to overcome to perform this experiment. With synaptobrevin reconstituted in liposomes and syntaxin·SNAP-25 in deposited bilayers, efficient SNARE-dependent docking was observed (30). Infrequent fusion events were also observed using the content mixing reporter calcein. The fusion events appeared to be triggered by the start of the laser illumination. The most likely explanation of this effect is that the illumination of the calcein dye caused heating which provided the energy to initiate fusion. Another explanation might be photobleaching induced radical formation. Clearly, illumination induced heating or radical formation is not restricted to single molecule experiments, but could also occur in ensemble experiments. It is possible that the use of a supported bilayer may have affected the kinetics of the system, requiring heat to trigger fusion. Further ensemble and single molecule studies are required to investigate these effects since they might affect the observed energetics and kinetics.

The time scale of these individual fusion reactions, once initiated, was faster than the time resolution of the camera employed in these experiments, indicating that the fusion is

intrinsically faster than 100 msec (Figure 5). A surprising result was found when SNAP-25 was left out: docking and thermally induced fusion still occurred. The previously mentioned weak interaction between syntaxin and synaptobrevin is thus sufficient for constitutive docking and fusion *in vitro*. Clearly, for a fully functional Ca^{2+}-sensitive system, SNAP-25 is required since it interacts with synatotagmin and manipulations of SNAP-25 (e.g., by the action of clostridial neurotoxins (CNTs) affect neurotransmission (13).

A similar liposome/bilayer topology was used in the single vesicle study by Liu et al. (55, 106), although the bilayer preparation and lipid compositions were quite different, the lipid density lower than that of Bowen et al. (30), and only lipid mixing was monitored. SNARE-dependent docking of liposomes was observed at the start of the experiment. Lipid mixing was monitored by dequenching of fluorescence from lipid dyes incorporated into the liposomes. Fusion events were thus inferred from dequenching and subsequent decay of the lipid dyes due to diffusion within the deposited bilayer. After initiation of the experiment, 65% of the docked vesicles exhibited lipid mixing within less than 25 msec after docking. When the concentration of syntaxin·SNAP-25 was increased about 100 fold, only few events were observed. This observation was explained by the formation of large aggregates at these higher concentrations of SNAREs as revealed by atomic force microscopy (55). This presumably results in SNAREs incapable of promoting lipid mixing, which should serve as a cautionary tale for everyone working with these proteins.

The experiments by Liu et al. (30) and Bowen et al. (55) agree on three aspects: first, individual "events" (content mixing observed by (30) or lipid mixing observed by (55)) are fast (faster than 100 and 25 msec, respectively). Second, Ca^{2+} has no effect on this SNARE-only system. Third, SNAP-25 is not required for SNARE-mediated docking and fusion. There are also differences between the two experiments. Only thermally-induced fusion events were observed by (30), in contrast to the burst of lipid mixing events at the start of the experiment by (55). It should be noted however that the experimental set up by (30) prevented measurement of events at the early stage since the system was initially equilibrated to establish single molecule conditions and to avoid non-specific liposome binding. It is therefore possible, that initial "events" might have also occurred in the experiments by (30), albeit unobservable. In summary, the studies by (55) and (30) both produce individual events on the millisecond timescale, while they differ in terms what is being observed: (55) observed lipid mixing events that occurred spontaneously, while thermally observed fusion events were observed by (30).

The notion that *trans* SNARE complexes alone are insufficient for fusion but require a trigger such as the Ca^{2+} sensor synaptotagmin or thermal heating (*in vitro*) to promote efficient fusion (30) is supported by bulk liposome experiments of SNARE-containing vesicles that are brought into contact by a low concentration of PEG (58). In this experimental setup the neuronal SNARE complex alone did not trigger fusion as determined by a content mixing indicator. SNAREs did enhance PEG-triggered fusion by favoring formation of the stalk intermediate. These studies also revealed that high protein to lipid ratios for syntaxin (> 1:500), and to a lesser degree synaptobrevin (> 1:100), cause liposomes to loose integrity, calling into question bulk liposome studies carried out at high protein to lipid ratios.

Single vesicle studies revealed apparent hemifusion states induced by neuronal SNAREs in the presence of 20% DOPE (dioleoyl phosphatidylethanolamine), but not in the presence of 15% DOPS (dioleoyl phosphatidylserine) (106). Apparent intermediate hemifusion states were also induced the yeast homologues of neuronal SNAREs (31), even in the absence of phosphatidylethanolamine. However, since no protein and content mixing reporters were used, it is not possible to directly correlate the state of the membrane (e.g. hemifusion diaphram) with formation of fusion pores and protein conformational changes.

Additional insights into SNARE – membrane interactions have been reported by using the Langmuir-Blodgett trough to obtain single planar phospholipids bilayers supported on PEG cushions (107). Reconstituted synaptobrevin exchanged between the supported bilayer and vesicles in solution (i.e., unbound) (107). However, little protein transfer was observed in the single molecule experiments by (30), although this difference could be due to differences in the experimental conditions. Reconstituted syntaxin·SNAP-25 binary complexes partitioned into a mobile and fixed fraction in deposited bilayers; the mobile fraction was significantly reduced in the presence of negatively charged lipids, such as PS or PIP_2 (32). Similarly, Bowen et l. (30) observed a significant fraction of immobile reconstituted syntaxin molecule in deposited bilayers that were obtained by liposome condensation on the quartz surface; some of the reduced mobility may be related to interactions between syntaxin transmembrane domains involving up-side-down syntaxin molecules whose cytoplasmic domain interacts with the surface (30).

Number of SNARE complexes involved in synaptic vesicle fusion

It is still an open question how many SNARE complexes are involved in docking and fusion of synaptic vesicles and whether these SNARE complexes interact with each other. There are some estimates that generally suggest a low number of SNARE complexes involved in these processes. One such study involved a permeabilized PC12 cell system (108). Upon injection of the cytosolic domain of synaptobrevin exocytosis was inhibited. The increased inhibition of fusion with increasing synaptobrevin concentration was best fit to a function involving three SNARE complexes. A model based on mutagenesis studies of syntaxin transmembrane segments suggested five to eight complexes involved in formation of a putative fusion pore (74). SNARE complexes obtained from brain extracts as well as from purified components appeared as star-shaped oliogomers in negative stain electron micrographs (109). However, a different atomic force microscopy experiment revealed that as little as one SNARE complex can dock a liposome to a target membrane (38). A previous study using single molecule fluorescence microscopy came to a similar conclusion: as little as one complex per liposome is sufficient for docking to supported bilayers (30).

Synaptotagmin

Synaptotagmin is the Ca^{2+} sensor for the synchronous component of synaptic exocytosis (15). Synaptotagmin occurs in both neuronal and non-neuronal cells and there are at least 16 different isoforms in different subcellular localizations in the brain {Mittelsteadt, 2009 #774}. For example, synaptotagmins I and II are localized on synaptic vesicles (110); these isoforms are often collectively referred to as simply synaptotagmin in the following. In contrast, synaptotagmin III is primarily found in the plasma membrane. Synaptotagmins are composed of a short intravesicular (luminal) amino-terminal region, a single membrane spanning domain, a lysine and arginine-rich juxtamembrane region, and two homologous C2 domains, termed C2A and C2B. A fragment consisting of both C2 domains interacts in a Ca^{2+}-dependent manner with acidic lipids, and both in a Ca^{2+}-dependent and independent manner with SNAP-25 and syntaxin, the binary complex, and the ternary SNARE complex (111–113). The Ca^{2+}-dependent interactions with the SNARE complexes are very salt-dependent – at 200 mM salt concentration, the Ca^{2+} dependence of these interactions disappears (14).

It should be noted that resequencing of the synaptotagmin I cDNA revealed an accidental mutation (Gly374Asp) in the C2B domain of the original clone (114) resulting in misfolding, so *in vitro* studies prior to 2000 and papers that reference these early works are affected by this mutation.

The X-ray crystal structure of the C2A domain of synaptotagmin I revealed a β-sandwich fold with a cluster of three Ca^{2+}-binding loops at the apex of the fold (115). Upon Ca^{+2} binding,

few structural changes occurred in the divalent cation binding pocket of the C2A domain, apart from changes in the sidechain rotamers for the Ca^{2+} coordinating aspartate residues and a general decrease in flexibility of the domain (115,116). Crystal structures of the C2A-C2B fragments of synaptotagmins I and III without Ca^{2+} (117,118) and that of synaptotagmin III with Ca^{2+} (Vrljic et al., submitted) are also available. The relative position between the C2 domains are very different in these structures and thus indicate a high flexibility of the linker connecting the two C2 domains. Single molecule studies of the C2A-C2B fragment of synaptotagmins I and III revealed highly dynamic interactions between the two C2 domains, and indicated that interactions with SNARE complex and/or liposomes stabilize one of the conformations (Vriljic et al. submitted; Choi et al., submitted). No high-resolution structures are available at this time of complexes between synaptotagmin, SNAREs, phospholipids, or other binding partners.

A direct interaction between synaptotagmin and the syntaxin·SNAP-25 binary complex was revealed by smFRET experiments (29). Synaptotagmin stabilizes the helix bundle configuration of the binary complex to the same extent as synaptobrevin, even in the absence of Ca^{2+}. smFRET between acceptor labeled binary complex and donor labeled synaptotagmin confirmed the molecular interaction. The observed stabilization of the binary t-SNARE complex might provide an explanation for the increase in lipid-mixing/fusion in bulk liposome assays upon incubation of syntaxin·SNAP-25 liposomes with C2A-C2B fragment of synaptotagmin I (20,119). However, since synaptotagmin I/II and the binary complex are thought to primarily reside on opposite membranes, this interaction could only take place only if the synaptic vesicle is already docked to the target membrane. Furthermore, the lipid-mixing accelerating property of the C2A-C2B fragment is eliminated when the syntaxin·SNAP-25 complex is "activated" with a C-terminal fragment of synaptobrevin (22). Another report showed no Ca^{2+}-dependence of lipid-mixing/fusion using full-length synaptotagmin (minus the luminal domain) (120). Furthermore, bulk-liposome studies do not reproduce the observation that disrupting Ca^{2+} binding to the C2B domain impairs neurotransmitter release more strongly than with C2A (15,121–124).

Synaptotagmin preferentially binds to curved membranes suggesting possible interactions with highly curved fusion intermediates (125). However, the model drawn from this study does not explain the abovementioned higher sensitivity of mutations of the C2B domain compared to the C2A domain to neurotransmitter release. Furthemore, it is of interest to note that purified synaptic vesicles fuse with binary complex containing proteoliposomes in a Ca^{2+}-independent manner (23) despite the fact that the synaptic vesicles contain both synaptobrevin and synaptotagmin. Thus, the observed synaptotagmin-accelerating effect in current *in vitro* experiments is not yet representative of the mechanism of Ca^{2+} triggering in neurons.

smFRET studies revealed structural insights for the interactions between a C2A-C2B fragment of synaptotagmin I and the ternary *cis* SNARE complex although the generally noisy FRET efficiency data and the unknown fluorophore dynamics precluded quantitative interpretation in terms of absolute distances (126). Interactions were found between the C2B domain of synaptotagmin I and the membrane-proximal portion of the SNARE complex, but only in the presence of Ca^{2+}. Few high FRET efficiency interactions were observed to the C2A domain. Thus, the low number of FRET events observed between C2A and the SNARE labeling sites suggests that the C2A domain does not closely interact with the SNARE core complex.

The labeling sites on the SNARE complex showing high FRET efficiency with labeling sites on the C2B domain were near the ionic layer and the membrane proximal region, in qualitative agreement with biochemical studies that implied the membrane proximal region of the SNARE complex in synaptotagmin binding (127–131). Since the fluorescent probes were in a loop distal to the Ca^{2+} binding sites, the appearance of high FRET efficiency between the SNARE

complex and C2B is more consistent with the Ca^{2+} binding sites oriented away from the SNARE complex rather than in direct contact. This would leave the Ca^{2+} binding sites accessible for phospholipid binding, allowing concurrent binding of both SNAREs and membrane. This prediction has been confirmed by monitoring the partitioning of synaptotagmin to POPS containing membranes vs. SNARE-reconstituted DOPS membranes (132): synaptotagmin preferentially binds to the SNARE-containing membrane while its Ca^{2+}-binding loops are inserted into the membrane. Clearly, these studies need to be extented to investigate the interaction between synaptotagmin and the *trans* SNARE complex as this is probably the most relevant interaction in the context of synaptic vesicle fusion.

Complexin

Complexin is a soluble protein of molecular weight ~15 kDa that shows rapid and high-affinity binding to the SNARE complex (133,134). Solution NMR studies of complexin and its interactions with SNAREs revealed an α-helical region involved in SNARE binding and an unstructured portion (135). Complexin binds in an antiparallel α-helical conformation to the groove between the synaptobrevin and syntaxin α-helices of the ternary SNARE complex (136,137). A report of complexin-dependent oliogomerization of SNARE complexes (138) was probably an artifact caused by disulfide bond formation (134). Knockout experiments in mice showed that complexin is essential for the Ca^{2+} dependency of synaptic vesicle release with a phenotype related to that of synaptotagmin knockout mice (139). However, since complexin has no Ca^{2+} binding sites the observed phenotype must be related to some interplay between the SNAREs, complexin, and synaptotagmin (14).

Single molecule experiments indicated a novel interaction between complexin and the membrane-bound syntaxin·SNAP-25 complex (29). Previously, no interaction had been observed between complexin and the individual SNAREs or the binary complex by using isothermal titration calorimetry in solution (134). In these experiments the binary complex was likely in the 2:1 state which might have prevented complexin binding. Interestingly, the N-terminal accessory helix of complexin (residues 29–48) has an inhibitory effect on neurotransmitter release, suggesting that this region serves as a placeholder for the C-terminal portion of the synaptobrevin SNARE motif, thereby regulating assembly of the SNARE complex (140). This result suggested possible interactions between complexin and the binary complex. Single molecule experiments have now clearly established that there is a direct interaction between complexin and the 1:1 binary complex, and have shown that this interaction dramatically stabilizes the helix bundle configuration. Recently, this interaction has also been confirmed with two entirely different methods: liposome co-floatation assays (141) and electron paramagnetic resonance (EPR) spectroscopy (142).

Kinetic binding studies between complexin and the *cis* ternary SNARE complex were carried out by stopped flow fluorescence and isothermal titration calorimetry (134) and by smFRET spectroscopy (126,143). The measured dissociation constant (K_D) was in the nanomolar range with fast on and off rates. The single molecule experiments revealed a transient nature of the complexin·SNARE interaction despite its relatively high affinity. Although the dissociation constants are similar there are signficant variations for the on and off rates in the two smFRET studies (126,143) which is likely due to the different dye labeling site altering the kinetics of this interaction. FRET efficiency distributions involving a label attached to the unstructured C-terminal portion of complexin were broader than those seen for the structured SNARE-binding region of complexin (126). Again, these studies need to be extended to interactions between complexin and the *trans* SNARE complex.

Complexin-dependent Ca^{2+}-accelerated lipid mixing between SNARE containing liposomes was observed in a single vesicle studies (142) although only a fraction of the liposomes showed

this Ca^{2+} effect. The observed Ca^{2+} stimulation is puzzling since neither SNARE nor complexin contain Ca^{2+} binding sites. The distribution of reconstituted proteins in liposomes can vary significantly, thus some liposomes may have a much higher protein concentration, effectively destabilizing the vesicle. Furthermore, it should be noted that this study used a relatively high protein to lipid ratio (1:100 for syntaxin) that might have made the liposomes leaky. Upon addition of complexin, these liposomes could thus be further destabilized making them prone to non-specific Ca^{2+}-induced "events" in combination with POPS containing membranes. As the authors suggest it is possible that this type of synaptotagmin-independent Ca^{2+} triggering could play a role in asynchronous release, but it clearly does not explain the requirement for complexin for synchronous release where synaptotagmin is also required. In this context, synaptotagmin 1 knockout mice exhibited some Ca^{2+}-triggered release in the presence of an N-terminal fragment of complexin (140). A possible role of complexin function in synchronous release is provided by experiments using the bulk liposome assay involving both synaptotagmin and complexin: complexin impaired inner leaflet mixing and the addition of synaptotagmin in the presence of Ca^{2+} releaved this inhibition and lead to lipid mixing (144). Taken together, these studies indicate both activating and inhibitory activities of complexin.

Munc18

Sec1/Munc18 (SM) proteins are a small family of cytoplasmic proteins that play an important but poorly understood role in intracellular membrane fusion. Interactions between the neuronal SM protein Munc18 and syntaxin (145,42), the binary syntaxin·SNAP-25 SNARE complex (146), and the ternary SNARE complex have been found (147). Based on the available structural and biophysical information, several possible interaction interfaces and conformations have been found: a tight interaction between the closed form of syntaxin and Munc18 involving part of the syntaxin SNARE motif, and the N-terminal domain of syntaxin (42), as well as interactions between the SNARE domains of the ternary complex and the short N-terminal sequence of syntaxin (148). Now smFRET experiments have demonstrated for the first time a direct interaction between the MUN domain of Munc18 and the membrane-anchored 1:1 binary complex accompanied by stabilization of the helix bundle configuration (29). Despite increasing information about Munc18 – SNARE intearctions, the function of this protein in membrane fusion is still unclear (149,148).

Munc13

Munc13 is a ~ 200 kDa protein that is essential for priming of synaptic vesicles to the release-ready state (150), and is also involved in presynaptic plasticity (151–153). It has been suggested that Munc13 catalyzes the transition from the closed to the open state of syntaxin based on the observation that double-knockout (Munc13 and syntaxin) *C. elegans* mutants are rescued by a constitutively open syntaxin (154). The α-helical MUN domain of Munc13 is sufficient for rescuing neurotransmitter release in hippocampal neurons lacking Munc13s (155). It does not interact with syntaxin alone, but single molecule experiments for the first time reported an interaction with the membrane-bound binary syntaxin·SNAP-25 complex that has a similarly pronounced effect as complexin leading to stabilization of the helix bundle configuration (29). The Mun domain of munc13 is highly alpha-helical, so its strong effect on diminishing the SX-SN1 and SX-SN2 configurations could be explained by stabilization of the SX-SN1-SN2 complex by four-helix bundle formation. These single molecule results have been reproduced with an entirely different approach, using liposome co-floatation assays (141).

Summary and future issues

Single molecule studies have revealed a number of new and sometimes provocative features of SNAREs and their accessory proteins. Mixtures of parallel and anti-parallel SNARE complexes were found in solution, but mostly parallel complexes are involved in the docking of liposomes to supported bilayers. Transient docking of liposomes can occur with only syntaxin and synaptobrevin, i.e., without SNAP-25. Very few (1–3) SNARE complexes are sufficient for stable docking of liposomes. SNARE-induced fusion mechanism is intrinsically fast (less than 100 msec), but infrequent. The binary syntaxin·SNAP-25 complex is flexible with three different rapidly interconverting configurational states that collapse into a single state upon binding of synaptotagmin (Fig. 3b). The syntaxin·SNAP-25 complex can also be stabilized by the accessory factors (in decreasing order) synaptotagmin (with and without Ca^{2+}), complexin, Munc13, and Munc18. We conclude that in the cellular environment the binary complex will be primarily in the three-helix bundle configuration since there is a high likelihood that at least one of the accessory proteins is near a particular syntaxin·SNAP-25 complex. Because it is this configuration to which synaptobrevin can readily bind, the formation of this configuration of the syntaxin·SNAP-25 complex is likely not going to a limiting step in neurotransmitter release

SNARE-mediated fusion events are relatively rare in single molecule experiments (or “slow” in bulk liposome assays). However, single molecule experiments revealed a intrinsically fast kinetics (< 10 msec) for individual fusion events. Using the bulk liposome assay some acceleration of lipid-mixing/fusion has been observed upon addition of other factors, but it is desirable to have these accelerating effects also examined with single molecule methodologies. Thus, the single molecule experiments need to be expanded in order to mimic the pertinent properties of Ca^{2+} triggered synaptic vesicle fusion. Such a system would contribute to the understanding of the molecular machinery of vesicle fusion since the sequence of protein-protein, lipid-lipid, and protein-lipid interactions could be studied during the fusion process. Other open questions are: do anti-parallel SNARE configurations exist in the physiological context and are they regulated by chaperones? Is the fusion pore of pure lipidic character or does it involve SNAREs or other proteins? How many SNARE complexes are required for synaptic vesicle fusion? Single molecule methods will certainly play a major role in addressing these fundamentally important questions about the molecular mechanism of Ca^{2+} triggered neurotransmitter release.

The probabilistic nature of synaptic vesicle release results in one (or zero) synaptic vesicles fusing in response to an action potential out a larger readily releasable pool. Fusion probability is dynamically regulated by many factors and contributes to presynaptic plasticity. Thus each synaptic vesicle undergoes a series of sequential interactions as they mature from docking to a fusion competent state. Reconstituting this type of dynamic and heterogeneous reaction pathway is precisely where single molecule methods excel and thus these methods will be invaluable in untangling this complex system.

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Acronyms

BFP	blue fluorescent protein
CNS	central nervous system

CNT	clostridial neurotoxin
DOPE	dioleoyl phosphatidylethanolamine
DOPS	dioleoyl phosphatidylserine
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
NMR	nuclear magnetic resonance spectroscopy
NSF	soluble N-ethyl maleimide sensitive factor
PEG	polyethylene glycol
PE	phosphatidylethanolamine
POPC	palmitoyloleoyl phosphatidylcholine
POPE	palmitoyloleoyl phosphatidylethanolamine
POPS	palmitoyloleoyl phosphatidylserine
PS	palmitoyloleoyl phosphatidylserine
smFRET	single molecule fluorescence resonance energy transfer
SNARE	soluble N-ethyl maleimide sensitive factor Attachment Protein Receptors

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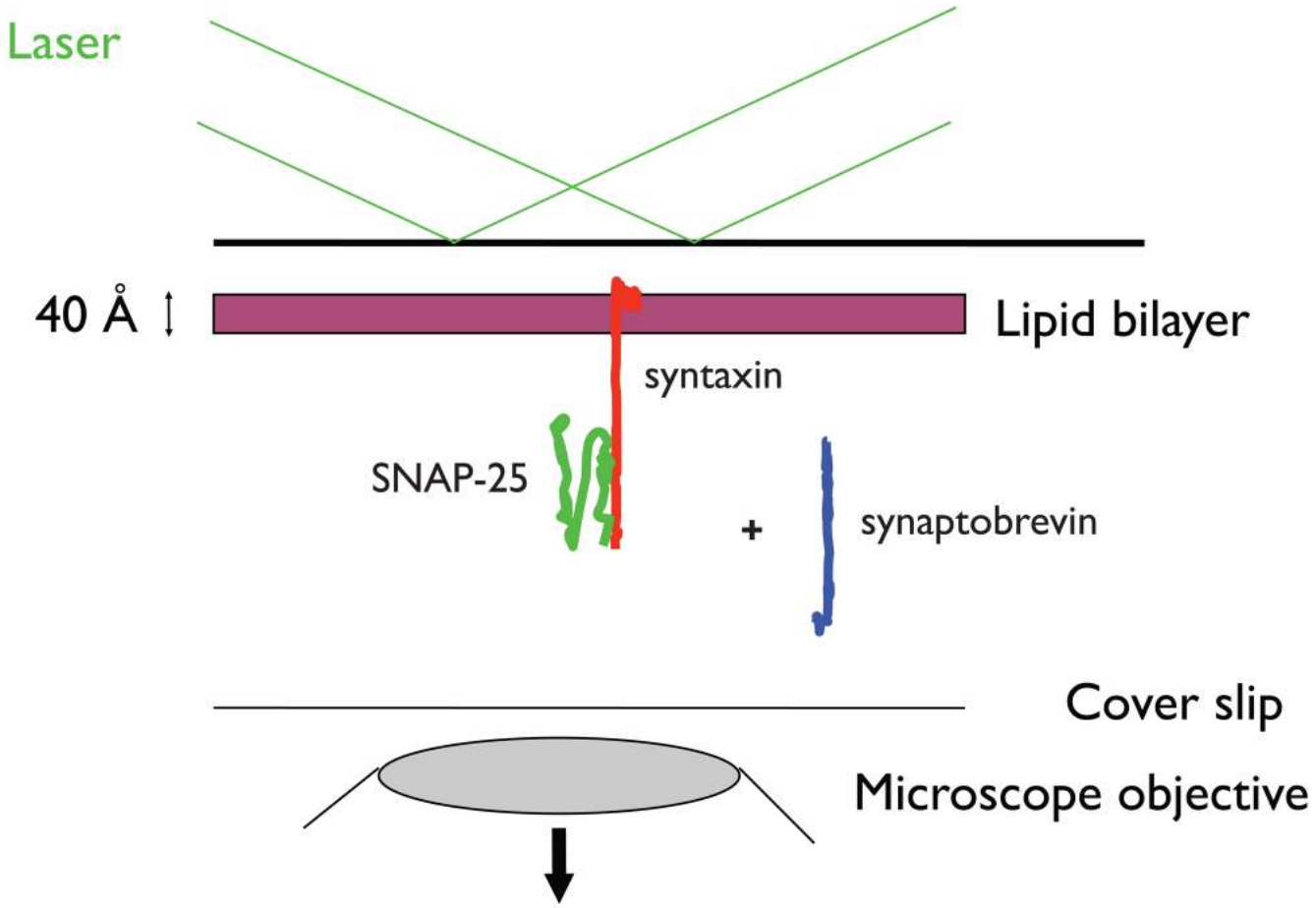

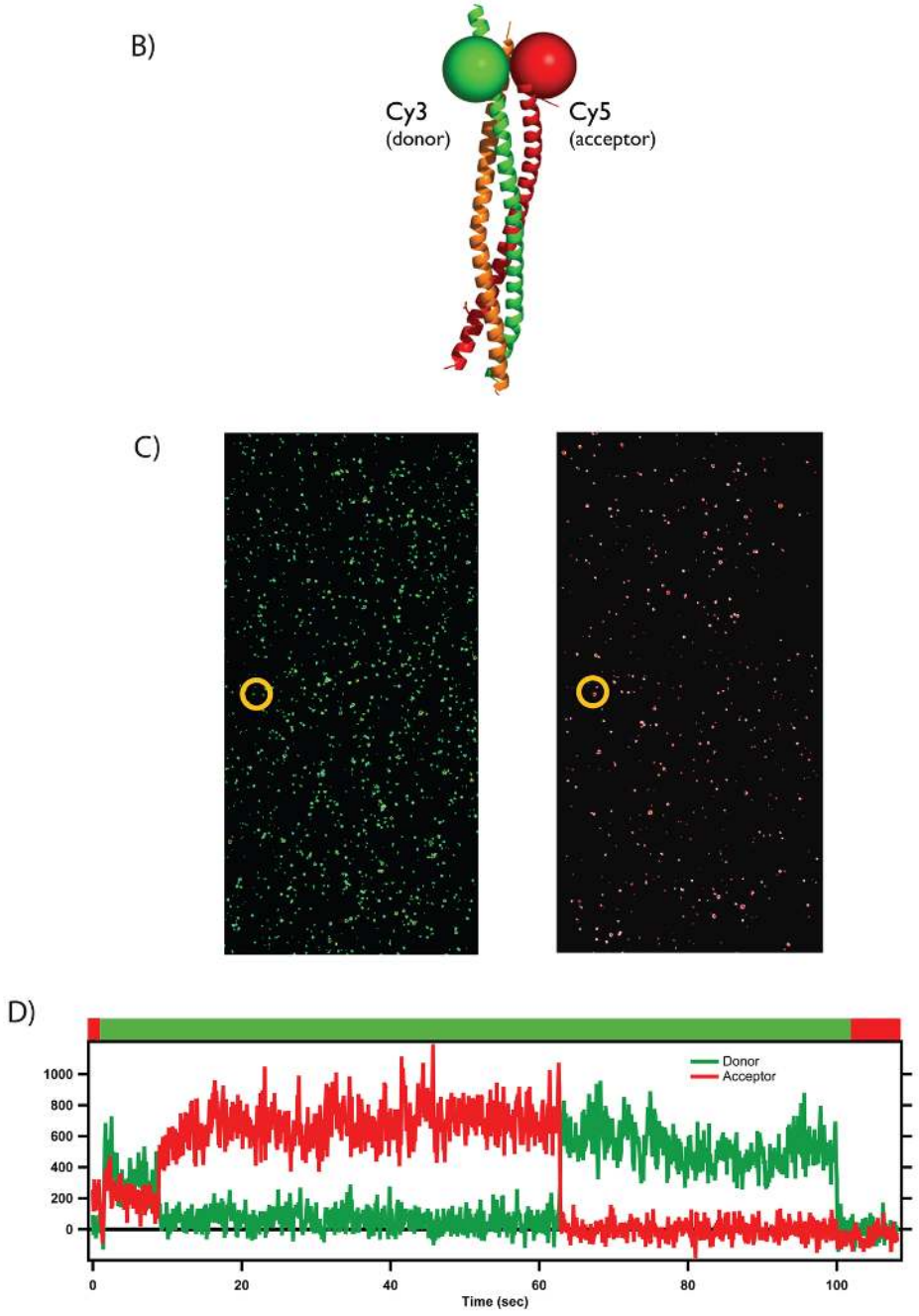

Figure 1. A typical smFRET experiment

A. Shown is the experimental setup for the smFRET experiments of the binary complex (29). Briefly, dual dye (donor/acceptor) labeled binary complex (syntaxin·SNAP-25) was reconstituted into a supported bilayer. Evanescent wave illumination was performed through total internal reflection. Laser light was chosen at two wavelengths to monitor donor and acceptor fluorescence. Synaptobrevin or other factors were injected and binding to binary complex monitored by a change in FRET from the dual labeled syntaxin·SNAP-25. A similar setup was used for docking and fusion experiments where synaptobrevin was reconstituted into liposomes that contained the soluble dye calcein that served as a content mixing indicator (30).

B. Donor (green) and acceptor (red) dye labeling positions in the dual-labeled SNAP-25 molecule that forms the binary complex with syntaxin. Shown is a model of the three-helix bundle complex consisting of the SNARE core domains of syntaxin (orange), SNAP-25 SN1 (green), and SNAP-25 SN2 (red).
C. Fields of view (50 × 100 μm) of donor (left) and acceptor (right) fluorescence arising from a dual-labeled SNAP-25 molecule in the syntaxin·SNAP-25 binary complex.
D. Selected time trace of the donor and acceptor fluorescence arising from a co-localized spot (similar to the marked one in panel C). In this case, synaptobrevin is bound to the binary complex, so high FRET is observed until the acceptor photobleaches.

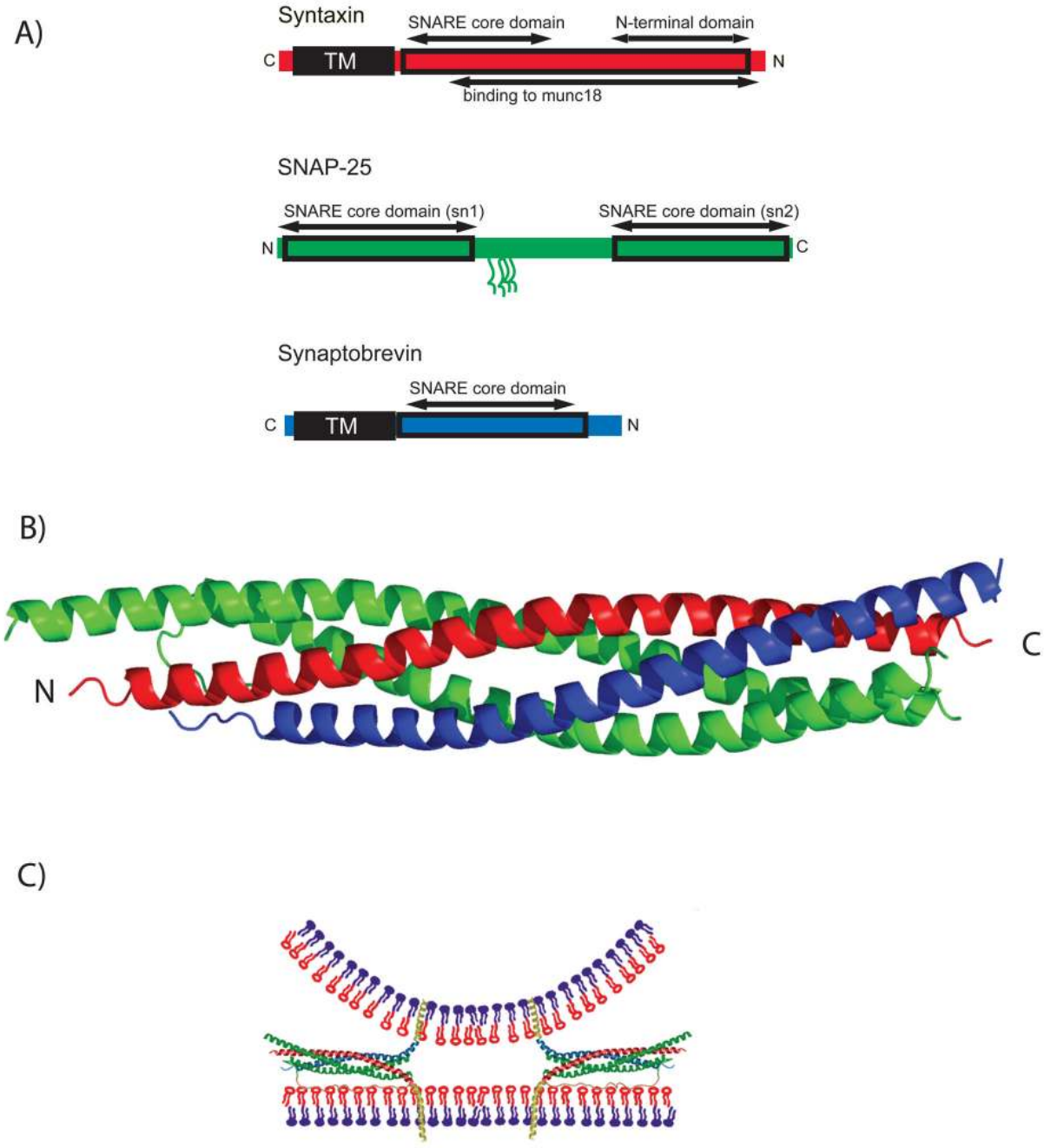

Figure 2. The neuronal SNARE complex

A. Primary structure diagram for syntaxin (red), SNAP-25 (green), and synaptobrevin (blue). The experiments referenced in this review refer to the following isoforms: syntaxin 1A, synoptobrevin II, and SNAP-25A which we simply refer to as syntaxin, synaptobrevin, and SNAP-25. TM: transmembrane domain. The SNARE core domains are defined through the 16 layers as found in the crystal structure of the neuronal SNARE complex (49). For SNAP-25, the palmitoylation sites are indicated by green lines.

B. X-ray crystal structure of the core of the neuronal SNARE complex consisting of synaptobrevin (blue), SNAP-25 (green), and syntaxin (red) (PDB ID 1SFC) (49). This structure

represents the fully folded post-fusion state of the complex, also referred to as the *cis*-state. The N- and C-terminal sides of the core complex are indicated.

C. Model of the *trans* state of two SNARE complexes that dock a liposome to a supported bilayer *in vitro*. This model was obtained by modifying the membrane proximal end of the crystal structure of the neuronal SNARE complex in order to allow the transmembrane domains to enter into the juxtaposed membranes. The transmembrane domains were assumed to be helical (156). The connecting region between the transmembrane domains and the core complex are likely flexible. Two SNARE complexes are shown; the exact number is unknown, but 1–2 SNARE complexes suffice to dock liposomes (30).

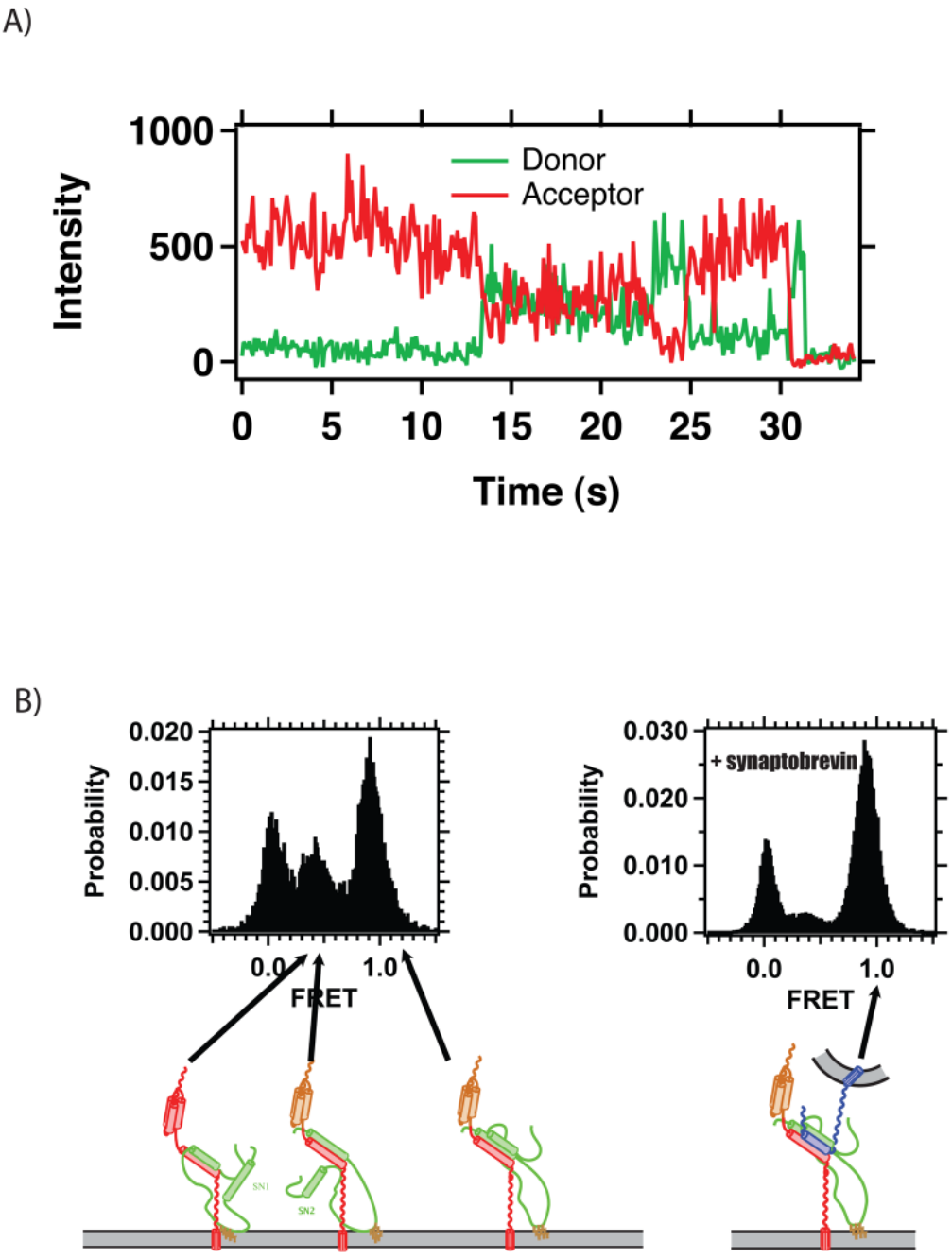

Figure 3. Single molecule FRET (smFRET) studies of the binary complex

A. Selected time trace of the donor and acceptor fluorescence of the binary complex (syntaxin·SNAP-25, see Figure 1) arising from a co-localized spot (similar to the marked one in panel C) (29). Note, the switching between two different FRET states as indicated by the correlated changes in donor and acceptor fluorescence.

B. FRET distributions of donor and acceptor dyes on the binary complex before (left panel) and after addition of synapbrevin (right panel). Note, that the intermediate FRET states have disappeared after addition of synaptobrevin (29). Below the FRET distributions, models of the binary complex conformations are shown. Before the addition of synaptobrevin, the binary complex exhibits three configurations: only the SN2 SNAP-25 domain bound to syntaxin (SX-

SN2), only the SN1 domain of SNAP-25 bound to syntaxin (SX-SN1) or both SNAP-25 SNARE domains bound to the syntaxin SNARE domain (SX-SN1-SN2). Upon addition of synaptobrevin (right) or accessory proteins, these configurations collapse into the SX-SN1-SN2 configuration.

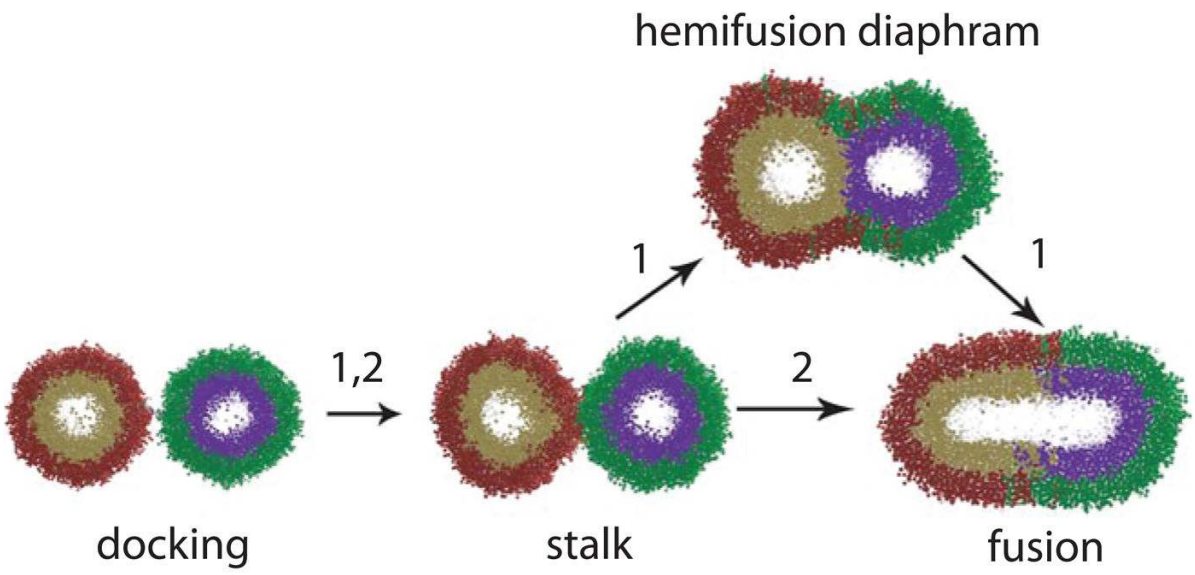

Figure 4. Alternative pathways for liposome fusion

Computer simulations of liposome-membrane fusion (88). Pathway 1 shows the canonical progression from an unfused starting state through a stalk intermediate and a hemifused diaphram intermediate to the fully fused state. Pathway 2 shows an alternative reaction pathway observed in ensemble molecular dynamics simulations (88): rapid fusion from the stalk intermediate to the fully fused state. All renderings are of snapshots from observed reaction trajectories; lipids are colored to distinguish the outer (red and green) and inner (gold and blue) lea ets of each vesicle. Explicit water was present in all simulations but not rendered.

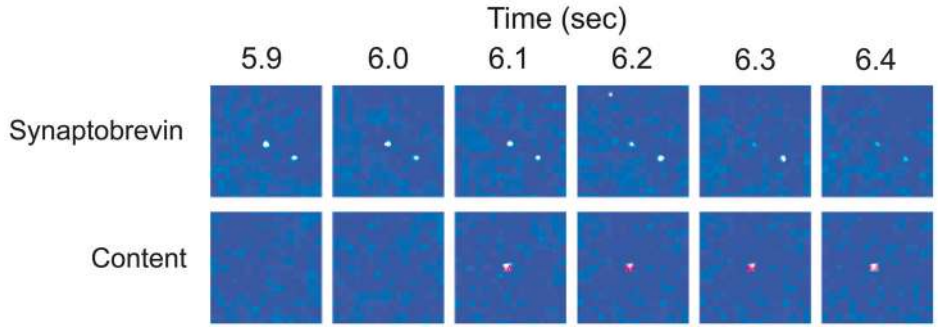

Figure 5. Individual fusion event observed by single particle studies

Liposomes containing the content dye calcein were reconstituted with dye-labeled synaptobrevin molecules and then introduced above PC/PS bilayers with reconstituted syntaxin in complex with SNAP-25 (30). The images represent a single 11 μm by 11 μm patch of membrane with docked liposomes observed in two different spectral ranges to detect the content dye and synaptobrevin dye fluorescence. Two liposomes are docked to the bilayer in the field of view as indicated by the synaptobrevin dyes. A single fusion event occurs at 6 secs as indicated by the sudden appearance of a bright content dye signal. The increase of content dye fluorescence is due to dequenching. Fusion proceeds faster than the time resolution of the camera used in this experiment; in other words the fusion reaction is faster than 100 msec.