REVIEW

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Visualizing presynaptic function

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Synaptic communication in the nervous system is initiated by the fusion of synaptic vesicles with the presynaptic plasma membrane and subsequent neurotransmitter release. In the 1980s, this process was characterized by electron microscopy, albeit without the ability to follow processes in living cells. In the last two decades, fluorescence imaging methods have been developed that report synaptic vesicle fusion, endocytosis and recycling. These probes have provided unprecedented insight into synaptic vesicle trafficking in individual synaptic terminals and revealed heterogeneity in recycling pathways as well as synaptic vesicle populations. These methods either take advantage of uptake of fluorescent probes into recycling vesicles or exogenous expression of synaptic vesicle proteins tagged with a pH-sensitive fluorescent marker at regions facing the vesicle lumen. We provide an overview of these methods, with particular emphasis on the challenges associated with their use and the opportunities for future investigations.

Synaptic vesicles fuse with the synaptic membrane to release neurotransmitter at prodigious speeds. Such high rates of consumption of synaptic vesicles necessitate specialized mechanisms for the recovery of vesicles. Observing this fast retrieval process requires tools beyond those that enable detection of neurotransmitter release. Development of fluorescent probes that label recycling synaptic vesicles has allowed us to visualize processes that were previously inaccessible to direct observation. These studies revealed dynamic recycling of synaptic vesicles during activity or at rest and provided insight into mechanisms that govern fusion, retrieval and recycling of synaptic vesicles. However, these studies also revealed shortcomings of fluorescent probes that limit our ability to answer the most vexing questions concerning synaptic vesicle recycling. For instance, how fast is membrane recovered under different stimulation conditions? Are synaptic vesicle proteins recovered with the membrane? Are the proteins recovered together as a unit or are they retrieved piecemeal? Do synaptic vesicles retain their structure and identity during fusion, endocytosis and recycling? Are there specialized pools of vesicles at synapses that mediate spontaneous versus evoked neurotransmitter release? In this Review, we will discuss commonly employed fluorescence probes with an emphasis on their technical strengths and weaknesses. Because of space limitations, we will not cover molecular and cell biological aspects of synaptic vesicle trafficking in depth. Recent articles provide excellent resources on the cell biology of presynaptic terminals (for example, see refs. 1,2).

Early attempts to visualize synaptic vesicle trafficking

In 1950, Bernard Katz began using newly developed fine-tipped glass microelectrodes to measure membrane potential in single frog muscle fibers. Intracellular recordings from just below the endplate revealed small spontaneous depolarizations of fixed amplitude[3,4]. From these observations, Katz proposed the quantal hypothesis, which states that neurotransmitter is released at synapses in fixed amounts[5,6]. However, it took seeing synaptic vesicles in electron micrographs[7] for Katz to refine the quantal hypothesis into the vesicle hypothesis[8]. In short, the vesicle hypothesis proposed that neurotransmission was mediated by the fusion of neurotransmitter-filled vesicles with the plasma membrane. Although the recording of postsynaptic depolarizations provided a rapid readout for neurotransmitter release, the methods were, by nature, indirect, as they relied on responses from ligand-gated ion channels. In a typical electrophysiological experiment, the properties of individual synaptic vesicle fusions become buried in the noise or are overwhelmed by an aggregate postsynaptic current. Postsynaptic responses are dynamic and are affected by diffusion and clearance of the neurotransmitter, receptor desensitization and saturation of the postsynaptic receptor field. Deriving presynaptic behavior from postsynaptic responses requires sophisticated deconvolution algorithms that rely on assumptions about the experimental conditions[9]. Importantly, electrophysiological recordings are blind to the behavior of synaptic vesicles before, during or after fusion. Thus, efforts were made to visualize vesicle dynamics in the presynaptic cell using the electron microscope.

Proving the vesicle hypothesis using electron microscopy turned out to be difficult, and 24 years passed before synapses could be stimulated and synaptic vesicles captured at the moment of fusion[10]. Nevertheless, it had been recognized that vesicles are consumed during synaptic transmission simply by observing their depletion under intense stimulation. Thus, it was assumed that mechanisms must exist to rapidly regenerate synaptic vesicles. The presence of invaginations on the plasma membrane and the uptake of fluid phase markers indicated that vesicles were being regenerated by the local recycling of membrane rather than by *de novo* synthesis from the organelles of the cell[11–13]. It was not clear at that time (nor is it today) whether the primary mechanism of recycling is the recovery of an incompletely fused synaptic vesicle or the budding of a new vesicle from the plasma membrane. Stimulation followed by rapid freezing after fixed time points demonstrated that recycling had a slow mechanism that

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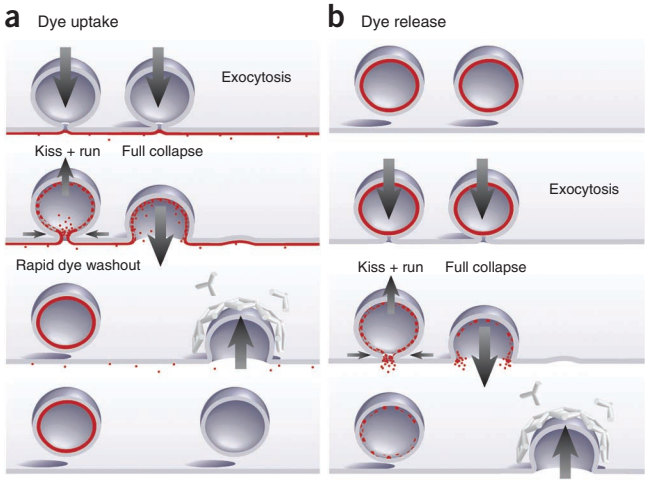

Figure 1 Optical monitoring of synaptic vesicle recycling using FM dyes. FM1-43 and its analogs are partially lipophilic dyes in which fluorescence increases almost 100-fold when they partition into membranes. Thus, they are virtually invisible in aqueous solution. The charged head group of FM dyes makes it difficult for them to cross lipid membranes, thereby limiting the fluorescence labeling to outer membranes and recycling vesicles. (**a**) Dye uptake. Stimulation in the presence of dye leads to exocytosis and the uptake of dye molecules into vesicles during endocytosis. If the dye is washed out before clathrin endocytosis occurs, only vesicles recovered via fast processes will be loaded. Although partitioning into membrane is fast, kiss-and-run vesicles may not be fully loaded by dye before they detach from the membrane. Washout of the dye at different intervals will subdivide endocytosis into rapid and slow components. (**b**) Dye release. Stimulation of loaded synapses releases the dye from synaptic vesicles and results in a decrease of fluorescence and synaptic boutons. This fluorescence loss is a result of departitioning of the dye into aqueous solution or lateral diffusion of dye in the neuronal membrane.

required about 20 s[14], and possibly employed clathrin[13]. On the other hand, it seemed that slow endocytosis of membrane could not account for all vesicle endocytosis, and it was possible that the vesicle fusion pore could reverse itself[15,16]. These models for the exocytosis and endocytosis of vesicles have become known as 'full-collapse fusion' and 'kiss-and-run' vesicle cycling (for reviews, see refs. 2,17).

Electron microscopy provided exceptional spatial resolution and enabled reconstruction of the synaptic vesicle cycle from electron micrographic images. However, because multiple fixations had to be analyzed for each time point, obtaining dynamic information was extraordinarily tedious[14]. The electron microscope was not capable of imaging the dynamics of membranes and proteins in neurons. Visualizing synaptic vesicle dynamics in live specimens became a priority and electron microscopy was sidelined.

Live imaging methods: membranes and proteins

Membrane recycling can be visualized in living specimens by using fluid phase markers such as dyes. Initially, fluorescent dyes, such as sulforhodamine, were used to label active synapses to document the elimination of synapses of polyinnervated muscles during development[18]. Starting in the early 1990s, several versatile fluorescence-imaging probes were introduced to report vesicle dynamics. Styryl dyes, such as FM1-43, are amphipathic compounds that label recycling synaptic vesicle membranes[19,20] (**Fig. 1**). There are basically two formats for these experiments: dye uptake and dye release. To measure the speed of endocytosis using dye uptake, the dye is washed away at different times after stimulation to reconstruct the kinetics of dye accumulation in terminals (**Fig. 1a**)[21,22]. However, these experiments are limited by the slow wash-off kinetics of dyes and the noise introduced by the need for multiple assays on distinct populations of synapses. As an alternative, dye release experiments can estimate the dwell time of a fusing vesicle during exocytosis (**Fig. 1b**). This experiment was further improved by using multiple dyes with increasing rates of departitioning from the vesicular membrane, which bracketed the rate of rapid synaptic vesicle retrieval (**Fig. 1b**)[22–26]. These fluid phase vesicle loading techniques enabled visualization of synaptic vesicle membrane dynamics and generated several currently debated hypotheses on synaptic vesicle recycling.

An alternative approach, genetic tagging with fluorescent proteins, allowed the analysis of the dynamics of individual synaptic vesicle proteins[27]. GFP-tagged proteins can identify mutants with severe endocytosis defects in cases where the proteins were redistributed onto axonal membranes[28–30]. However, GFP tagging was not effective at analyzing the dynamics of endocytosis because GFP remained fluorescent if it was on the surface of the axon or internalized on synaptic vesicles. A genetic-tagging approach to study vesicle dynamics emerged with the development of a pH-sensitive variant of GFP[31] (**Fig. 2**). Thus, fluorescence was quenched when the tag faced the acidic lumen of the synaptic vesicle.

Taken together, these optical tools have been instrumental in shaping our current view of presynaptic function. As described below, they have revealed that synaptic vesicle exocytosis is coupled to a highly efficient local recycling machinery that maintains synaptic transmission during sustained activity[1,2]. However, these experiments have also opened up the field to new controversies.

Analysis of membrane recycling: fluorescent dyes

The key structural feature of styryl dyes is their amphipathic nature: they are both soluble in water, but bind avidly to membranes. Moreover, they fluoresce brightly when inserted into membranes, but their fluorescence decreases 100-fold when they partition into aqueous solution. The charged head group of the dyes prevents them from crossing membranes, thereby limiting the fluorescence labeling to outer membranes and recycling vesicles. These properties made it possible to measure uptake of the dye by endocytosis after stimulation and the release of the dye by exocytosis. These experiments

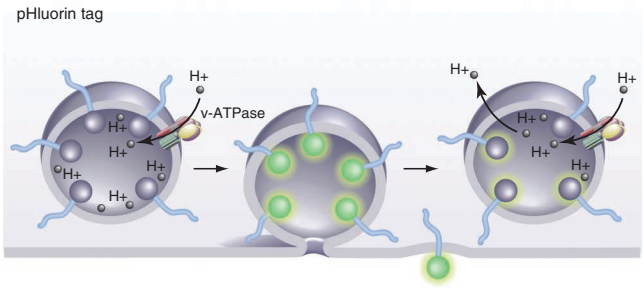

Figure 2 pHluorin protein tags. pHluorin is a pH-sensitive GFP. This fluorescent protein can be attached to the luminal domain of synaptic proteins such as synaptobrevin (shown), synaptophysin or the vesicular glutamate transporter. The lumen of a synaptic vesicle normally has an acidic pH of approximately 5.5, at which point pHluorin fluorescence is quenched. When vesicles fuse to the plasma membrane, the lumenal tag is exposed to the extracellular pH, which results in a marked increase in its fluorescence. After fusion, the pHluorin-tagged protein can diffuse laterally in the plasma membrane. After endocytosis, pHluorin fluorescence is re-quenched as the vesicle lumen is acidified by the vacuolar ATPase (v-ATPase).

indicated that the lifecycle of a vesicle, from fusion to rebirth via endocytosis and to fusion again, required about a minute[19,21]. As these dyes were applied to more preparations and under differing stimulus conditions, the full potential of these dyes, as well as their drawbacks, became clear.

Dye uptake is technically difficult because the dyes must be rapidly applied and washed out to identify distinctive uptake pathways (**Fig. 1a**). In early experiments, dye uptake following spontaneous fusion resulted in reluctant dye release, whereas dye uptake during stimulation resulted in dye easily released by further stimulation, suggesting different trafficking pathways for spontaneous versus evoked vesicle fusion[32]. However, in experiments based on styryl dye uptake, the inability to stimulate the release of the dye makes it difficult to assign fluorescence to the endocytosis of synaptic vesicles[33,34]. Nonspecific background staining arises during dye incubation; thus, dye re-release is an essential criterion for the selection of a region of interest.

Interestingly, dye uptake is also affected by the structure of the dye; different dyes are targeted to different endocytic pools[33,35,36]. In these experiments, more lipophilic dyes (such as FM1-43, FM1-84) label larger endocytic structures, such as surface membrane infoldings called cisternae, in addition to vesicles, whereas less hydrophobic dyes (FM2-10) selectively label unitary vesicles. These observations suggest caution when comparing results from different dye species[25,26]. On the other hand, they may also imply lipid diversity among distinct endocytic pathways, although the exact biophysical basis of this remains unclear[37].

Dye release presents additional challenges. To measure exocytosis, dye release should be rapid (**Fig. 1b**), but it soon became clear that dye unbinding in biological preparations is complex. The amphipathic nature of the dyes should allow the dye to freely exchange with the media. FM dyes insert into cell membranes within a millisecond and departition within a few milliseconds (~200 s^{-1})[37,38]. Rapid release from fusing synaptic vesicles can also be observed. Isolated goldfish bipolar cells can orient the presynaptic membrane toward a coverslip and dye release can then be observed using total internal reflection fluorescence microscopy. After stimulation, FM dye in fused vesicles diffused laterally into the plasma membrane and directly into the aqueous media, and fluorescence declined rapidly[38]. However, in several intact synaptic preparations, a slow destaining rate, which proceeds in seconds, has been consistently reported[25,26,35,36]. A combination of multiple factors may contribute to this discrepancy. First, destaining in the goldfish bipolar cells was measured in an isolated cell, that is, in the absence of a postsynaptic cell. Rapid dye release at synapses is impeded by the narrow cytoplasmic space in the synaptic cleft, as well as by nonspecific binding to other membranes. Second, dye release from multiple fusing synaptic vesicles in succession results in an overall slow kinetics of fluorescence loss, whereas detection of dye release from individual fusion events yields fast kinetics[23,37]. Third, the length of the lipophilic tail of particular dyes can slow the departitioning rate[22,26]. Fourth, the length of the tail also prejudices the uptake of particular dyes into distinct synaptic vesicle pools with different release properties (see above). Finally, formation of a nanometer fusion pore between the vesicle membrane and the plasma membrane constitutes a barrier against dye release and limits its dye departitioning following the formation of the vesicle-plasma membrane interface[23,25,26,39]. In contrast, a fusion pore would not present a major barrier to protons, and their loss can be measured using the pH-dependent fluorescence of quantum dots. Quantum dots can be loaded into synaptic vesicles. As a result of their large diameters, they remain trapped in the vesicle lumen. Fluorescence increases as the vesicle is alkalinized and then decreases as the vesicle is acidified, suggesting that a fusion pore could be a normal mode for neurotransmitter release[40].

Despite these caveats, dye experiments have identified a rich functional diversity among vesicles, which all appeared to be identical in electron micrographs. These pools are the readily releasable pool, which are vesicles that fuse immediately after a stimulus. The reserve pool consists of vesicles used to refill the readily releasable pool. The total recycling pool includes both the readily releasable and the reserve pools of vesicles. The resting pool, on the other hand, is normally inactive.

Studies using FM dyes revealed that fluorescence loading of few or even single vesicles was matched by exocytosis of equal amounts of dye. These data suggest that synaptic vesicles recycle without intermixing with endosomal compartments; the fluorescence probes do not get diluted into multiple vesicles[41]. By combining dye-loading experiments with electrophysiological techniques and electron microscopy, it became evident that there are active and inactive pools of synaptic vesicles[19,42]. In these configurations, dye release reports exocytosis of previously endocytosed vesicles, whereas electrical signals originate from the release of all vesicles, irrespective of their source. Differences between the two measures indicate the contribution of recycled vesicles to neurotransmitter release[43]. Dye photoconversion into electron dense signals, on the other hand, enables coupling of dye uptake with localization of recycling vesicles in electron micrographs[44,45]. In contrast with studies that solely rely on electrophysiological readout of neurotransmitter release[46], these dye photoconversion studies consistently found that only limited numbers of synaptic vesicles recycle at any given time, even during strong stimulation, suggesting that there is a large resting pool resistant to activity-dependent trafficking[47–50]. Under most experimental conditions, the resting pool seems to be inactive except during intense stimulations that fatigue the synapse. Under physiological conditions, the resting pool probably represents a specialized pool that is accessed only for spontaneous release or after mobilization by signaling pathways[51–54].

Dye studies have also led to a more nuanced view of use and reuse in the recycling pool[19,21,42]. Stimulation procedures include Ca^{2+}-dependent and Ca^{2+}-independent stimulations, such as hypertonic sucrose application (typically +500 mOsm). The readily releasable pool can be determined using hypertonic stimulation or brief bursts of action potentials (for example, 20 Hz for 2 s). The total recycling pool can be determined by the uptake and release of styryl dyes induced by depolarizations induced by elevated potassium solution or sustained activity. These strong stimuli can induce mobilization of virtually all vesicles capable of recycling in individual synapses[48]. The comparison of the two fluorescence measurements (readily releasable pool versus the total recycling pool) can reveal the allocation of vesicles to a particular pool in a given condition[55].

Styryl dye–based methods have also been used to study the behavior of single synaptic vesicles during exocytosis, endocytosis and transport[23]. These experiments corroborated the early observations that synaptic vesicles retain their identity and membrane integrity after endocytosis[23]. In a recent study, Kylachko and colleagues took advantage of the high fluorescent intensity styryl dye SGC5, examined the trajectories of single synaptic vesicles after spontaneous or action potential–evoked dye uptake[56] and found that the mobility of vesicles that are endocytosed after spontaneous fusion is more restricted than that of vesicles that endocytose during activity. As indicated above, monitoring uptake and release of single quantum dot nanoparticles from recycling synaptic vesicles provides key advantages over styryl dye–based methods for single-vesicle tracking. These nanoparticles

fluoresce at very high intensity and are resilient to photobleaching. Using quantum dots, it has been possible to show that the trajectory of vesicles before fusion depends on their use history, suggesting a link between the pathway of endocytosis and properties of subsequent fusion[57].

Despite their extensive versatility, experiments that rely on styryl dyes also suffer from substantial shortcomings. As stated above, the major caveat is the accumulation of background fluorescence after dye staining, which makes fast imaging soon after dye uptake to monitor dye re-release nearly impossible. Moreover, it is difficult to ascertain whether the background fluorescence originates from dye uptake into vesicles that are reluctant for re-release or other membranous structures or nonspecific labeling of non-recycling membranes. As a result of these problems, the vesicle reuse kinetics is typically reconstructed from a population of synapses rather than from repeated measurements at individual presynaptic terminals. These shortcomings also led to varied success of styryl dye–based approaches in brain slices[58–60], as issues associated with excessive background fluorescence, which primarily originates from unhealthy tissue found at the surface of slice preparations, curtail routine use[61,62]. The difficulty in unequivocal identification of dye fluorescence as a component in synaptic vesicle recycling has encouraged the development of alternative approaches; specifically, the monitoring of fluorescence-tagged synaptic vesicle proteins, which can be confidently assigned as components of synaptic vesicles in trafficking studies[51,63–65].

Analysis of vesicle protein recycling: fluorescent tags

SynaptopHluorin (synaptobrevin-pHluorin) is a fluorescent probe that is commonly used to monitor exocytosis and endocytosis of synaptic vesicles. SynaptopHluorin was originally engineered by Gero Miesenböck and colleagues as a fusion construct of the synaptic vesicle protein synaptobrevin (also called vesicle associated membrane protein, VAMP) with a pH-sensitive GFP at its C terminus (located in the synaptic vesicle lumen). The lumen of synaptic vesicles normally has an acidic pH of approximately 5.5, at which synaptopHluorin fluorescence is quenched. When vesicles fuse, lumenal GFP is exposed to the extracellular pH, which results in a marked increase in its fluorescence. Studies using synaptopHluorin have provided valuable information on the tight coupling between exocytosis and endocytosis[66,67]. One advantage of the synaptopHluorin probe is the molecular specificity of the fluorescence signal, as the signal originates from a synaptic vesicle protein as opposed to nonspecific lipid–based fluorescence of FM dyes. Proton diffusion from a synaptic vesicle during exocytosis is not expected to be limited; however, the time course of vesicle re-acidification may impose a delay in the fluorescence signal's return to baseline during synaptopHluorin endocytosis[68,69].

Initial studies used synaptobrevin as a carrier for the pHluorin tag; however, later work expanded the repertoire to other synaptic vesicle proteins. Surprisingly, in these experiments, different synaptic vesicle proteins tagged with pHluorin gave different results with respect to their background fluorescence (that is, surface exposure) and retrieval[69–71]. For instance, both synaptophysin (a four transmembrane domain synaptic vesicle protein) and the vesicular glutamate transporter reveal very little surface exposure (fluorescence) in the absence of stimulation[71–73]. Once stimulated, they showed a swift increase in fluorescence without substantial lateral diffusion following fusion, which was a salient feature of synaptopHluorin-based measurements[69]. The dependence of the properties of pHluorin-based measurements on the identity of the tagged protein complicates a straightforward interpretation of these experiments in terms of synaptic vesicle recycling. Despite this caveat, these measurements reveal critical information on specific cell biological attributes of synaptic vesicle proteins.

Inhibition of vesicle re-acidification has been used to measure endocytosis during stimulation. In response to brief field stimulation, synaptopHluorin fluorescence shows a swift increase and a gradual slow return to baseline, presumably as a result of slow compensatory endocytosis. However, when the same experiment is repeated in the presence of a vacuolar ATPase blocker (such as bafilomycin and folimycin), synaptophysin-pHluorin fluorescence shows a larger increase that is detectable within seconds[66,74]. The difference between these two signals can be taken as a readout of endocytosis with the assumption that re-acidification of vesicles following endocytosis proceeds rapidly and re-quenches internalized pHluorin. This premise is further supported by the selective loss of this rapid endocytotic signal in dynamin-1 knockout mice[75].

Phluorin-based imaging approaches are also amenable to detection of single synaptic vesicle exocytosis and endocytosis events. In the case of synaptopHluorin, once the surface fluorescence is quenched, it is possible to monitor single vesicle fusion events. Using this setting, Gandhi and Stevens detected single vesicle fusion events with square pulse–like rapid alkalinization and re-acidification behavior akin to single ion channel openings[68]. These findings suggest that the number of synaptopHluorin molecules per vesicle is preserved during the exocytosis and endocytosis cycle in a manner consistent with the kiss-and-run hypothesis. However, the same findings were not reproducible with a pHluorin fusion construct of synaptophysin, suggesting that rapid reversal of pHluorin signal may be a result of lateral diffusion of synaptobrevin, as synaptophysin-pHluorin does not laterally diffuse following vesicle fusion[69]. The improved optical signal-to-noise characteristics of pHluorin-tagged vesicular glutamate transporter (Vglut1-pHluorin) circumvented the need for pre-photobleaching of surface membrane fluorescence to detect single synaptic vesicle exo-endocytosis[70,72,73]. These experiments revealed that single synaptic vesicle exocytosis is coupled to endocytosis in a manner that is tightly regulated by presynaptic Ca^{2+}. The same setting can be used to extract several key properties of single release sites, such as vesicular release probability or the number of readily releasable vesicles[76].

Recent work also took advantage of red-shifted pH-sensitive fluorescence proteins to compare trafficking behaviors of distinct synaptic vesicle–associated SNARE molecules (for example, synaptobrevin2, VAMP7, VAMP4, vti1a) expressed in individual synaptic terminals[64,65]. Using pH-sensitive variants of dsRed, mOrange[77] and pHTomato[78] in combination with classical green emission pHluorin, one can monitor the simultaneous trafficking of synaptobrevin2 with other SNAREs in the same presynaptic terminal. This work uncovered substantial heterogeneity among trafficking properties of different SNAREs[64,65], which may also apply to other trafficking proteins such as synaptotagmins[79]. A combination of red-shifted fluorescence proteins and genetically encoded Ca^{2+} indicator GCaMP[80] enables simultaneous detection of presynaptic Ca^{2+} and vesicle trafficking in a given synapse[78,81]. The same probes can also be used to monitor presynaptic vesicle trafficking and postsynaptic Ca^{2+} simultaneously (**Fig. 3**). Red pHluorin tags also allow imaging of synaptic vesicle protein trafficking during light-activated stimulation of neurons by channelrhodopsin[78] (**Fig. 3**).

Comparison of current methodologies

Although FM dyes and phluorin-tagged vesicle proteins are commonly used to measure endocytosis and exocytosis, these two methods do not necessarily provide the same type of information. FM dye measurements trace membrane cycling in the synapse.

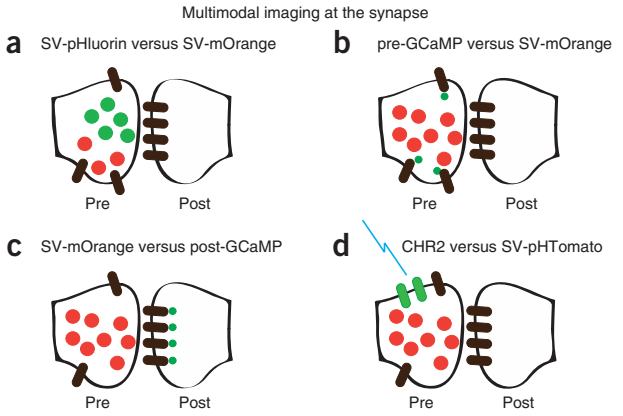

Figure 3 Red-shifted pH sensitive fluorescence proteins enable simultaneous monitoring of multiple functional modalities from individual synaptic terminals. (**a**) Using pH-sensitive variants of dsRed and mOrange in combination with the classical green emission of pHluorin, one can monitor the simultaneous trafficking of multiple synaptic proteins in the same presynaptic terminal[64,65]. (**b**,**c**) Use of red-shifted fluorescence proteins enables simultaneous detection of presynaptic Ca^{2+} (refs. 78,81) or postsynaptic Ca^{2+} together with synaptic vesicle trafficking in a given synapse. (**d**) Red-shifted pH-sensitive fluorescence proteins allow simultaneous optogenetic stimulation and presynaptic imaging[78].

Using uptake and release of FM dyes, one could study vesicle mixing with endosomal compartments or repeated use of the same vesicles during activity, which would be difficult to attain using probes such as synaptopHluorin. pHluorin-based probes report the fate of synaptic vesicle proteins (synaptobrevin or others) after fusion and endocytosis. pHluorins are genetically expressed probes that facilitate more reproducible repeated measurements on the same set of synapses (as they do not require dye uptake). Moreover, pHluorin-based probes can also be transgenically expressed in model organisms, such as mice or flies, which enables *in vivo* measurements of synaptic vesicle trafficking[82–84]. In a landmark study by Miesenböck and colleagues, expression of synaptopHluorin in the *Drosophila* olfactory system allowed live visualization of behaviorally relevant synaptic circuit activation in response to odorants[83].

Despite its numerous advantages, measurement of endocytosis with synaptopHluorin is confounded by three factors. First, commonly used transfection methods, such as electroporation or Ca^{2+} phosphate–based procedures, may yield toxicity. Second, lateral diffusion of vesicular pHluorin-based probes following fusion is commonly observed, especially when pHluorin tag is attached to synaptobrevin[69]. Thus, it is unclear whether reuptake of synaptopHluorin truly reflects endocytosis of vesicles or re-clustering and re-internationalization of synaptobrevin. It is also unknown whether endocytosis of lipid and protein components of synaptic vesicles are distinct processes or whether they are coupled[85]. Finally, the dependence of endocytosis measurements on vesicle re-acidification may delay the fluorescence signal after synaptopHluorin internalization[69,86].

There are also concerns with using FM dyes. Typically, FM dye measurements suffer from nonspecificity. FM dyes stain the surface membrane indiscriminately and are therefore less specific to synaptic vesicles. Moreover, changes in FM dye fluorescence typically occur slowly, on the order of seconds, making it difficult to detect events on the millisecond timescale, which more closely match the time course of neurotransmission. In addition, after FM dye uptake, preparations need to be washed with dye-free solution for a substantial period to reduce background fluorescence, which limits the rapid detection of synaptic vesicle re-availability after endocytosis. These issues can be addressed using extracellular fluorescence quenchers that can rapidly suppress dye fluorescence after fusion and uncover rapid kinetics of dye release[24] or help visualization of dye fluorescence after uptake by masking nonspecific surface fluorescence[62].

In summary, synaptopHluorin provides a molecularly specific probe for monitoring the coupling between exocytosis and endocytosis of individual synaptic vesicle proteins, whereas FM dyes are better at tagging vesicles during their cycle in presynaptic terminals. In the case of either probe, however, the health of neuronal preparations is a critical issue that requires substantial attention to ensure robust fluorescent imaging of synaptic vesicle trafficking. Overall, these two optical probes complement each other in monitoring distinct aspects of the synaptic vesicle cycle. Future studies will need to address the proteins that mediate the trafficking of synaptic vesicles and vesicle proteins as they move between the plasma membrane and compartments in the bouton.

Recent introduction of new probes that target endogenous synaptic vesicle proteins hold promise for alleviating the shortcomings associated with pHluorin-tagged synaptic vesicle proteins and FM dyes. For instance, cypHer5E is a red-excitable pH-sensitive cyanine dye that shows increased fluorescence with a decrease in pH from basic to acidic[87,88]. Thus, unlike pHluorin signals, vesicular uptake of cypHer5E results in fluorescence emission and enables tracking synaptic vesicles after endocytosis[87,88]. CypHer5E can be attached to antibodies that bind the synaptic vesicle lumenal region of synaptotagmin, enabling molecularly specific labeling without transfection of an exogenous probe. Along the same lines, pH-dependent quantum dots can also be attached to synaptic vesicle protein antibodies and targeted to synaptic vesicles in a molecularly specific manner. However, it is important to note that the large size of these probes may hinder their access to all recycling vesicles, especially those that are connected to the extracellular medium via narrow transient fusion pores. Thus, recent developments in the design of fluorescent false neurotransmitters and optical neurotransmitter sensors also hold substantial promise for optical detection of neurotransmitter release[89].

The molecular topography of the synapse

What is missing from our understanding of the synapse is a detailed molecular map. We need to know where particular proteins and membranes are located during the process of exocytosis and endocytosis, a molecular topography of the synapse. Robust methodologies for imaging membranes and proteins at a nanometer resolution are required. Drawing this molecular map will be aided by a return to electron microscopy.

The cast of presynaptic proteins is being ever more closely defined by biochemical approaches. We now have detailed identifications of the protein components of synaptic vesicles[90] and the active zone[91]. One promising approach to place these proteins onto synaptic ultrastructure is array tomography[92]. This method allows one to probe a ribbon of serial sections with fluorescently labeled antibodies up to nine times with antibodies to different proteins. In this setting, the ribbon is then imaged by collecting backscatter electrons on a scanning electron microscope. The localization of the fluorescently tagged antibodies is limited by the diffraction of light such that the fluorescence image is a blurred spot as large as the synapse. A way to improve resolution of the protein is to genetically tag it. The miniSOG tag generates singlet oxygens that can convert a substrate to an electron-dense product that is visible on electron micrographs[93]. Another alternative is to tag the protein with a fluorescent tag and combine super-resolution microscopy with electron microscopy (called nano-fEM). In this case, the proteins are labeled with a switchable fluorescent

protein and fixed and embedded in plastic[94]. If treated gently, the fluorescent protein retains its fluorescence and can be imaged using STED or PALM super-resolution microscopy. Under these conditions, similar to array tomography, the ultrastructure can be imaged by collecting electrons on a scanning electron microscope using a backscatter detector. On the other hand, super-resolution microscopy even in the absence of ultrastructural information can localize and orient proteins at the synapse. For example the arrangement of calcium channels and dense projection proteins such as Bruchpilot has been determined using STED microscopy[95]. Even the orientation of the dense projection protein Bruchpilot has been resolved by differential labeling of N and C termini[96].

Challenges and opportunities for the future

Looking forward, understanding the synapse will require a combination of high spatial and high temporal resolution. Eventually the molecular map must be correlated with synaptic structure in three dimensions. The advantage of electron microscopy lies in its extraordinary resolution: proteins can be observed *in situ*. However, achieving such resolution also introduces limitations, as it is extraordinarily near-sighted. One must interpret the vastness of the world with their nose to the ground and a depth of field of 50 nm. However, high-voltage electron microscopes can now reconstruct structures in synapses in 200–400-nm-thick sections using electron tomography. These reconstructed slabs can then be analyzed in 2-nm virtual sections[97,98]. These tomograms have revealed stunning details in synapses, especially when turned to the analysis of mutant synapses, such as dynamin mutants[99]. We anticipate that coupling electron tomography with super-resolution microscopy in the future will provide the detailed molecular maps that are so badly needed.

Another required advance will be to enable routine high signal-to-noise visualization of synaptic function in intact synaptic networks *in situ* and *in vivo*. Properties of available probes restrict the ability to image single synaptic vesicles during physiologically relevant sparse activity in intact synaptic networks. Nevertheless, recent attempts at imaging presynaptic function in an intact neuronal circuit have been extremely informative[82,100]. However, currently available methodologies rely on overexpression of exogenous pHluorin-tagged synaptic vesicle proteins, which leads to substantial surface fluorescence and other potential trafficking abnormalities that limit fidelity and accuracy of visualization in thick tissue. Expression of fluorescently tagged synaptic vesicle proteins from their endogenous genetic loci will provide substantial advance, as nearly all studies to date have relied on overexpression of pHluorin-tagged synaptic vesicle markers.

In summary, the last two decades have witnessed development of a large number of imaging modalities that enable live visualization of synaptic vesicle dynamics. These approaches have provided a window into processes that could only be indirectly inferred from electrophysiological recordings or electron microscopy. Although these techniques have delivered unprecedented insight into the function of individual synapses, their shortcomings, in terms of temporal and spatial resolution, require complementary measurements with electrophysiological and electron microscopic methods to arrive at dependable cell biological conclusions. Nevertheless, overcoming the current limitations of live synaptic imaging methodologies remains the only option to achieve high-resolution visualization of circuits within the functioning mammalian brain.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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