Review paper

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Visualization of synaptic vesicle dynamics with fluorescence proteins

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Abstract

Synaptic vesicle (SV) will be transported to the bouton along the axon, once it is formed in a cell body. After docking in the active zone, neurotransmitters will be released upon the stimulation, and then transmission of chemical signals will be initiated. Presently, many advanced technologies and burgeoning molecular sensors are being used to explore the synaptic transmission. These studies provide a new sight into the presynaptic structure and its function. The present review summarizes the application of fluorescent proteins (FPs) for SV tracking and recycling. Some FPs and relevant imaging technologies such as fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime imaging microscopy (FLIM), are introduced here. In addition, some examples are also analyzed to visualize the dynamics of SVs in living cells with the help of some FPs.

Key words: *synaptic vesicle, fluorescent proteins, fluorescent technologies, dynamics.*

Introduction

Synaptic vesicles (SVs) play essential roles in information transferring such as visual sense, auditory sense and even emotions. The small spherical SV fuses with presynaptic membrane and releases its contents of the neurotransmitter (exocytosis). Subsequently, the neurotransmitters diffuse across the cleft between pre- and postsynaptic membranes, leading to the activation or inhibition of the postsynaptic neuron. Consequently, an intact information transfer is accomplished during this period. At the same time, the components of SVs are recovered to the presynaptic membrane for next transfer preparation (endocytosis).

Researchers began their studies on SVs by electrophysiological ways because some electrophysiological parameters such as current across the membrane would be changed during exocytosis or endocytosis [97]. Therefore, ion fluxes through neurotransmitter-gated channels can be measured electrically via recording the whole cell from soma or dendrites of postsynaptic neurons [7,20]. Similarly, SV recycling [98], neurotransmitters secretion [40], and membrane fusion [65], have also been detected via electric current, capacitance and some other electrophysiological parameters. However, these methods usually face some difficulties in reporting the SVs release from an individual release site or synapse, and they cannot show the complete spatial information about the origin and destination [42].

As the fluorescent technology develops, biological experts can employ the fluorescent probes to

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report the neurotransmitters release at the level of individual synapse, and collect more information about vesicle tracking and fusion [49]. One of these probes adopted fluid phase markers such as fluorescent dyes to visualize membrane recycling through two basic formats (i.e., dye uptake and dye release) [3,41]. Dye-based methods mainly focus on lipid cycling in an active-dependent manner. Thanks to rapid uptake and release of the dye, the same vesicle was found to be reused several times during routine neurotransmission with the help of FM1-43 [4]. These fluorescent dyes have high molar absorptivity, strong fluorescence and great photostability [23]. Interestingly, they also become brighter distinctly once their tail domains partition into the membrane [37]. For example, FM1-43 increases fluorescence 100 times brighter in the membrane than in the aqueous solution [51]. Such changes in fluorescence intensity provide possibility for analyzing SVs fusion and internalization. Although it is easy to mark SVs with dyes [26], these measurements are still limited by the non-specificity of dyes since they stain the membrane randomly [86]. Furthermore, SVs release neurotransmitters in a millisecond time scale, which makes it tough for fluorescent dyes to be detected as their fluorescence changes in seconds [42]. The worst thing is the toxicity of dyes. For example, FM1-43 behaves as a permeant blocker of mechanosensitive channels in sensory neurons [21,27].

Contrarily, some other probes take advantage of genetic expression of SV protein tagged with fluorescent proteins (FPs) to measure the kinetics of vesicles intuitively [52]. These probes focus on proteins cycling without any perturbation to the original function mostly, since they are constructed on DNA by subcloning and expressed by cells themselves after transfection. Therefore, SV-related proteins could be labeled specifically and detected in real time with the help of FPs. The kinetics of SVs with an emphasis on the application of fluorescent proteins to SVs is introduced below.

Fluorescent proteins and relevant imaging technologies

FPs technology has been confirmed to be a very useful tool for imaging living cells since its vast application in the research field in recent years. The first FP found in jellyfish Aequorea victoria is green fluorescence protein (GFP) with two different chromophores [12,74]. A mass of FPs have been developed later, including short wavelength FPs derived from ECFP and long wavelength FPs derived from DsRed [92]. They supply various colored tools on a molecular level for the visualization of single or multiple target molecules in living cells [85]. These FPs can also be used to track the movement of vesicle-associated proteins in real time. For example, it is easy to view the distribution of synapsin Ia during stimulation by using EGFP-synapsin Ia fusion protein generated through subcloning synapsin Ia into pEGFP-C1 vector [18]. Some variants of FPs are found sensitive to regional changes like pH, ionic concentration or oxidability [92]. For example, pHluorin is a widely used pH-dependent variant of GFP whose fluorescence is quenched at acidic pH while recovered after being exposed to near-neutral pH in the solution [2]. As the pH of SV lumen changes from acidic to near-neutral during exocytosis and re-acidic after endocytosis, the changes in fluorescence intensity caused by varying pH can reflect SVs' recycling and can be used for quantitative assessment [50,93]. Moreover, a set of similar FPs have been attained via different directed point mutations [10,61,89]. Such properties have provided us with efficient tools for detecting ionic fluxes or pH changes *in vivo*.

With the development of diversified FPs, advanced imaging technologies based on FPs have been put forward, including fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime imaging microscopy (FLIM). FRET is a quantum mechanical phenomenon where a donor FP transfers energy to an acceptor FP within a close distance if donor's emission spectrum overlaps the excitation spectrum of the acceptor [53]. FRET efficiency, which is calculated by the acceptor/donor emission ratio, has a pally relationship with the distance and relative angles between the donor and the acceptor. Thus, the conformational changes of a target molecule, which has been inserted into FRET pair proteins, result in a FRET signal which can be utilized to explore the interaction between two presynaptic proteins [16]. For instance, with EGFP and tdTomato fusing to presynaptic protein dNSF1 and synaptosomal-associated protein of 25kDa (SNAP-25) respectively, FRET efficiency increased once the two target proteins interacted with each other and shortened their distances [99]. FRAP is a process where the fluorescence signals are selectively photobleached and recovered in the same region over a period of

time. Usually, this technology is usually used to analyze the mobility of fluorescent molecules [9]. For example, the mobility of dNSF1 tagged with EGFP could be estimated through the speed of fluorescence recovery after photobleaching [99]. FLIM is a technique for visualizing the lifetime of the excitation state of the spatially distributed fluorescence molecules [91]. This technology can be applied to determine the interaction between the donor and the acceptor along with FRET because the fluorescence lifetime of donor changes during the occurrence of FRET [90,92].

Vesicle associated protein: fluorescent tags

SVs are released at the presynaptic active zone mainly in three steps: docking, priming and release [79]. The precursor vesicle is formed in the neural cell body and internalizes SV proteins which are synthesized in endoplasmic reticulum (ER) or Golgi apparatus. Then the vesicle is transported to the synapse along the axon with a directed motion. After being placed SV proteins in the right position and embellished with cofactors or other necessary proteins, basically the first SV comes into being [70]. Currently, the SV has no function until it is acidized by a proton pump called vATPase and filled with neurotransmitters [22,73]. Then SV is driven to the active zone with an unclear docking mechanism. One mode holds a view that soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [19], which are the core of the docking-fusion complex comprised of syntaxin [96], synaptobrevin (also called vesicle-associated membrane protein, VAMP) [82], and SNAP-25 [28], tether vesicles to the presynaptic plasma membrane via interactions of proteins (Fig. 1) [58,69,75]. In addition, Rab3-interacting molecule (RIM) helps the vesicle to connect with Ca^{2+} channel protein [39]. Complexin [59], which stabilizes SNAREs complex and RIM, promotes the process of dock and prepares the vesicle for release [80]. Under proper conditions like the sharp entry of calcium, SVs fusion and release may take place with the help of synaptotagmin [15,81].

Many other proteins have also taken part in the exocytosis, for example, RIM-binding protein (RIM-BP) [54], mammalian homologue of Caenorhabditis elegans unc-13-1 (Munc13-1) [36], liprin [6] and so on.

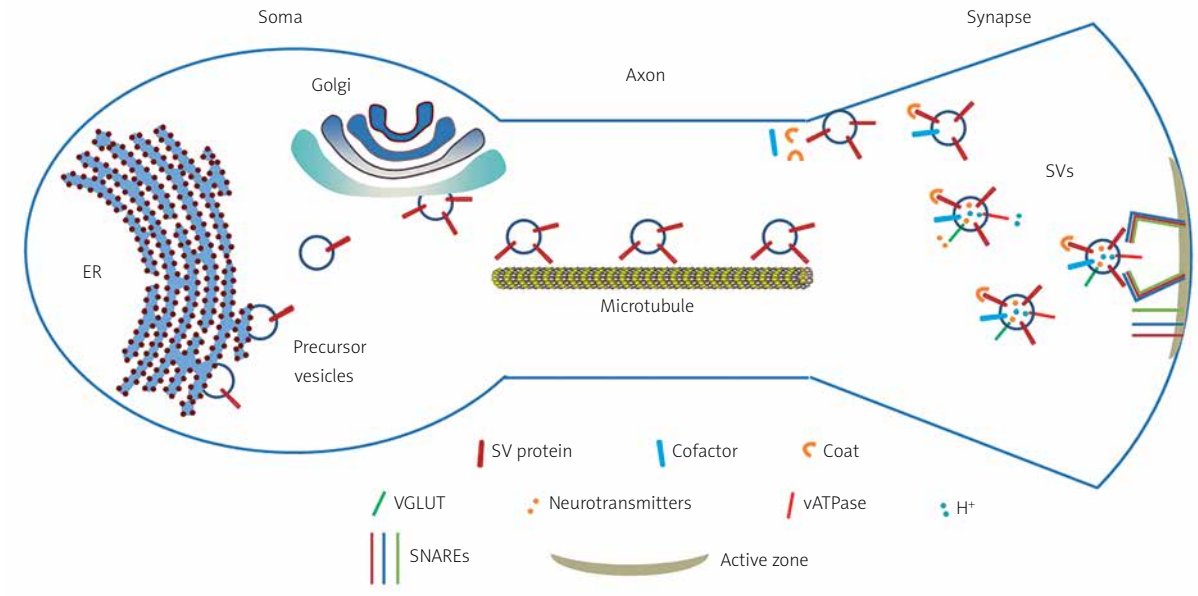

Fig. 1. SVs are transported from soma to synapse [19,22,70,73]. SVs are first formed in neural cell body (termed precursor vesicle), and modified with SV proteins in ER and Golgi. Then they are transported along with microtubule toward presynaptic terminal. Within synapse, SVs are formed basically after sorting of SV components and being embellished with necessary proteins like cofactors and coats. After that, SVs are acidized and filled with neurotransmitters before being tethered to active zone for release, with the help of SNAREs, and maybe some other proteins like RIM and Munc13-1.

These proteins play important roles in regulating SVs' cluster, transport and recycle. In this section, some FPs-dependent sensors and FPs-associated technologies will be introduced along with some main presynaptic proteins in order to give an intuitionistic description of the kinetics of SVs.

Synaptobrevin/VAMP

VAMP is identified as an important regulator of vesicular transport with seven kinds of isoforms, VAMP-1/2/3/4/5/7/8, which are expressed differently in the central nervous system [84]. Previous studies have reported that VAMP was involved in rapid and slow endocytosis together with SNAP-25 and syntaxin [96]. Additionally, it may migrate with SVs synchronously during SVs' activity because of its location on the SV membrane [38]. Thanks to this property, VAMP is usually used to explore the mobility of SVs. For example, synaptobrevin 2 tagged with EGFP (syb2-EGFP) was applied to measure the speed of moving SVs cluster by taking images of syb2-EGFP labeled material moving in intersynaptic areas [77]. Furthermore, by using DsRed2-synaptobrevin2, the formation of presynaptic vesicle clusters could be seen during synapse maturation, and its regulation by N-cadherin could be visualized with EGFP-VAMP2 through the technology of FRAP [78]. Recently, VAMP-pHluorin (also named as synaptobrevin-pHluorin, spH) was also used to study the mobility of recycling SVs in living hippocampal nerve terminal combined with Atto647N-conjugated nanobodies [19]. Some researchers even took advantage of VAMP-pHluorin to test the effects of different genes on SV cycling [30]. VAMP-pHluorin is a common fluorescent probe to monitor the exo- and/or endocytosis [13]. Its pH-sensitive pHluorin lies in the SV lumen

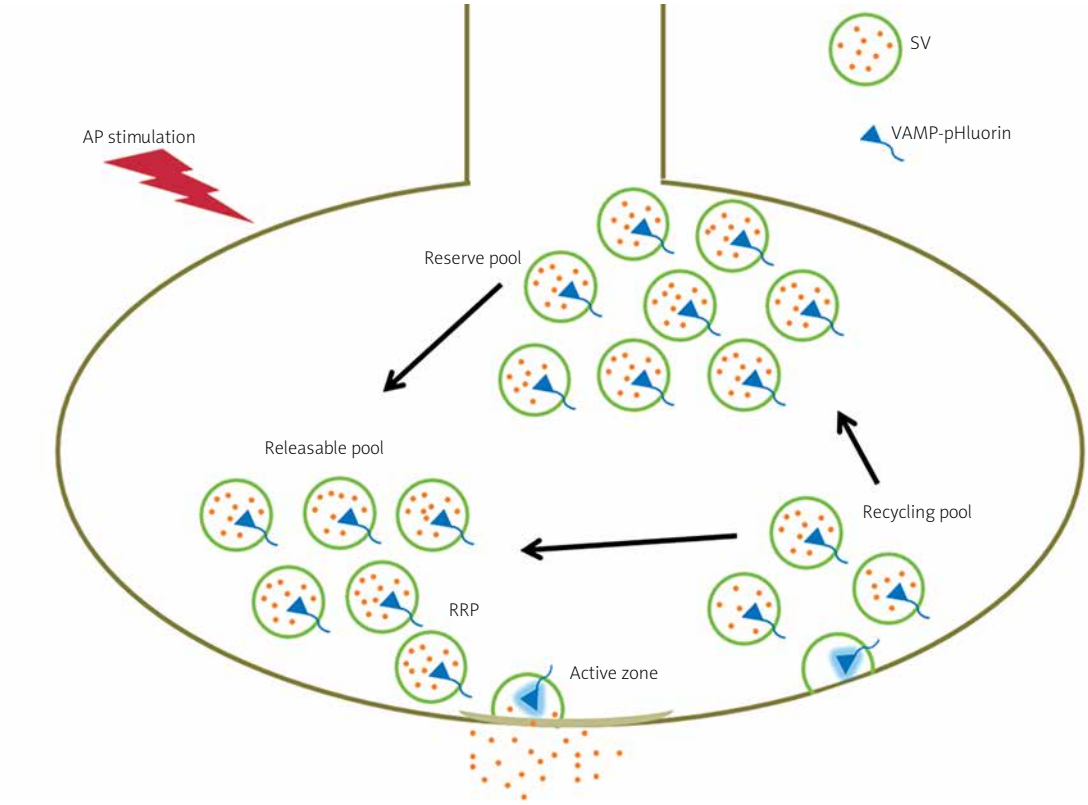

Fig. 2. Balance between exocytosis and endocytosis revealed by VAMP-pHluorin [2,24]. VAMP-pHluorin was used to monitor the accumulation of SVs on the presynaptic membrane and its fluorescence increased about 20 times when exocytosis happened. According to the changes in fluorescence intensity induced by different frequencies of stimulation at physiological temperature, it was concluded that the releasable SV pool was not depleted significantly under stimulation up to 10 Hz. This result indicated that the other two pools supplemented releasable pool with SVs to maintain the balance between exocytosis and endocytosis.

which has an acidic environment, so it has no fluorescence until it is exposed to the near-neutral pH environment after vesicles fuse to membrane. Therefore, a change in fluorescence intensity can assess the synaptic transmission. Surprisingly, fluorescence intensity changed inhomogenously throughout an individual presynaptic terminal no matter which frequency of stimulation was applied. Further experiment revealed that changes in fluorescence intensity had a significant linear correlation with the abundance of local SVs [94]. According to the three-pool model, local vesicles come from readily releasable pool (RRP), recycling pool and reserve pool. RRP has the highest probability for releasing vesicles at each bouton while the other two pools act as replenishments [2]. With the help of spH, it has been found that the releasable SV pool was not depleted significantly under action potential (AP) stimulation (less than 10 Hz) at physiological temperature, indicating a balance between the exocytosis and endocytosis (Fig. 2) [24]. It was reported that VAMP also played its role in SVs fusion. Xia *et al*. developed a FRET-based biosensor comprised of YFP-synaptobrevin and CFP-SNAP-25. They found that the FRET efficiency increased with given stimulation, suggesting the assembly of SNAREs by synaptobrevin and SNAP-25 [95]. This assembly might help SVs get closer to the presynaptic membrane and be ready for release.

Synaptophysin

Synaptophysin is the most abundant intrinsic membrane glycoprotein of SVs with four transmembrane domains and two loops on the lumen side [5,87]. It has a Ca^{2+}-binding site on the cytoplasmic side and may be involved in the Ca^{2+}-trigger mechanism that initiates the opening of a fusion pore [17]. Currently, synaptophysin is an important marker of SV which provides reliable data for the synaptic structure [43]. Moreover, it can be used to track the movement or recycling of SVs either through FRAP with synaptophysin I-EGFP or by fusing with FPs alone (Fig. 3) [29,63,102]. Amazingly, it was found that two distinct vesicles were endocytosed while only one vesicle fused with the presynaptic membrane when imaging the synaptophysin-pHluorin (SypHluorin) in an individual vesicle under low-frequency stimulation [101]. This phenomenon seemed to disrupt the balance between exocytosis and endocytosis, shown by the application of spH to the vesicle pool [24]. In general perception, there are two pathways of SV recovery: one fast pathway in which the vesicles remain or are recycled around the active zone, and the other slow one, a clathrin-involved pathway [32,71]. By using SypHluorin, it was found that the slower endocytosis was accelerated and became the predominant pathway with increased stimulation frequency [101]. As a result, a new match was rebuilt between endocytosed vesicles and exocytosed vesicles.

Besides, SypHluorin was also used to measure the release possibility of SVs which was an important component of synaptic strength [57]. Other functional properties like evoked release and total vesicle pool size, were also studied upon SypHluorin applications [67]. Recently, SypHluorin has been widely used to test drugs and explore proteins function within synapses [31]. It was found that *N*-cadherin and Neuroligin 1 could significantly promote exocytosis [88], and presenilin 1 could facilitate exocytosis directly by interacting with synaptotagmin 1 [102]. In addition, synaptophysin marked with EYFP and

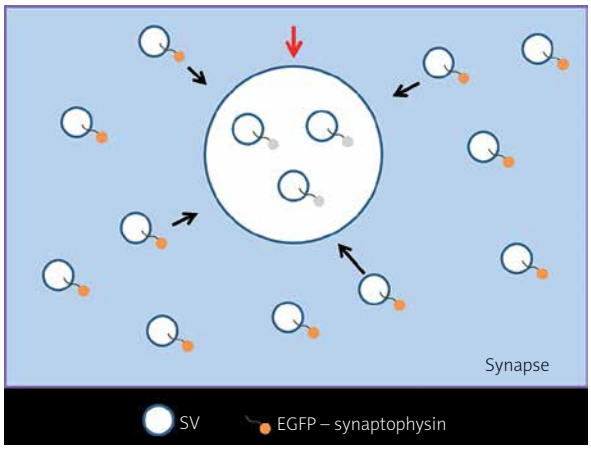

Fig. 3. Tracked SVs with EGFP-synaptophysin via the technology of FRAP [29,63,102]. The fluorescence of selected region of interest (ROI, the red arrow) decreased drastically during photobleaching experiments, and recovered over a period of time after bleaching because surrounding SVs marked with EGFP-synaptophysin migrated to ROI again. By recording the recovery time, the speed of SVs' movement could be measured easily. Besides, this biosensor could be used to explore the influences of proteins or protein interactions on the tracking of SVs through disturbing the expression of relevant proteins.

ECFP-VAMP2 were designed for a FRET-based biosensor, which could detect the interaction between synaptophysin and VAMP2 during exocytosis by analyzing the changes in FRET signals [68]. Moreover, with the development of calcium indicator GCaMP [60], more methods of combining two FPs with non-overlapping color spectra, such as SyGCaMP2 and SypHluorin, have been used to monitor simultaneously the spatial and temporal relationships of calcium influx and the dynamics of SVs [48,100], which makes it possible for the detection of signals in parallel.

Vesicular glutamate transporter (VGLUT)

VGLUT has three isoforms named VGLUT1/2/3 which are distributed in different places [34,77]. It participates in the probability of SVs release and synaptic plasticity because it acts as a carrier that loads the neurotransmitter into SVs [72]. The relationship between changes in calcium ion concentration and vesicle recycling has been analyzed by means of VGLUT1-mOrange2 and SyGCaMP3 [47]. It has also been discovered that Ca^{2+} increases the SV fusion probability but decreases the rate of SV retrieval with VGLUT-pHluorin by contrast [45]. Focusing on the single SV, Balaji and Ryan gave more details about the retrieval time. They found that the recovery occurred over a wide range of time with an average constant of 14 seconds, suggesting a stochastic process of retrieval [8]. In addition, by utilizing VGLUT-pHluorin, the increase in fluorescence was viewed after using tetrodotoxin [46], a voltage-gated Na^{+} channel blocker. It was concluded that SVs should be released spontaneously except for responding to the action potential. Besides, the fluorescence delay was very short (less than 370 ms), indicating that SVs were recycled at an extremely fast speed [46]. It was supposed that vesicles were endocytosed into the recycling pool in a faster way that SNARE-binding protein Tomosyn was involved in the regulation of dynamic partitions of SVs pool, and knockdown of Tomo1 might lead SVs to total recycling pool, including RRP and recycling pool [14].

Other proteins

FPs have also been used to explore the function of some other proteins in synaptic vesicle dynamics. Synapsin involves in managing the presynaptic reverse pool and plays an important role in synaptic short-term plasticity [62]. By using synapsin Ia-EYFP or synapsin IIa-EGFP with the application of FRAP, the extent of fluorescence recovery was found to be less than that in synapsin-triple-knockout neurons. It indicated that synapsins could immobilize SVs and control the mobility of the resting pool vesicle [63]. By employing the same probe, changes in fluorescence puncta could be seen clearly in the stimulated neuron. Thus it was concluded that exocytosis and endocytosis were necessary for synapsin to redistribute into the axon by analysis of fluorescence images [64]. Combined EGFP-synapsin I with FRAP, Huntingtin-associated protein-1 (HAP1) was found to be a new partner of synapsin I and was involved in regulating the axonal transport of synapsin I-containing vesicles [56]. Another presynaptic protein, $GABA_B$ receptor, has been reported to regulate neurotransmitter release [55]. By labeling two subunits of $GABA_B$ receptor with a FRET pair, CFP and YFP, respectively, Laviv et al. found that high FRET efficiency was associated with low SVs release probability, which suggested an important role of $GABA_B$ receptor played in mediating SVs release [44]. Furthermore, some other proteins tagged with FPs have also been used to explore the dynamics of SVs. For example, EGFP-DOC2B was used to study the prime process [25]. The GFP-vesicular monoamine transporter and GFP-vesicular acetylcholine transporter were used to view the release of relative neurotransmitters [76]. Thanks to FRAP, a faster process of endocytosis mediated by clathrin could be seen with clathrin-EGFP/mCherry application [66].

Conclusions

It is clear that SVs are indispensable for the information transfer between neurons, and a large number of proteins participate in the regulation of this process. How do SVs travel from soma to neural terminal and fuse to the presynaptic membrane or exchange among different SVs pools? What kinds of modes do there usually exist in the cycling of SVs recovery? These questions remain unclear while partly being answered with the help of FPs in this review. There is no doubt that FPs technology has widened the research field of life sciences and advanced our understanding of the presynaptic structure and function since the first GFP was discovered. FPs provide a powerful tool for direct observation and quantification of cellular processes in living cells [11]. They are usually applied to view the distribution of proteins [33], track the migration of SVs [83], and study protein activity during stimulations in presynaptic termi-

nal [35]. Applications of both target proteins labeled with FPs and relative fluorescent technologies allow us to follow the fate of an individual vesicle at synapses with high spatial and temporal resolution, varying from the formation, cluster, transport, dock, prime and release. In addition, FPs also contribute to exploring the roles which SVs-associated proteins play in the regulation of the movement or recycling of SVs. Such fluorescent probes are convenient for us to study the dynamics of SVs visually especially on a molecularly specific level [1]. However, the fidelity and accuracy are limited when the biosensor is applied to visualize thick tissues. In addition, the fluorescent decay, which will cause deviation, cannot be avoided once utilizing pH-sensitive FPs. This shortcoming has to be taken into consideration if this kind of FP is employed to explore the fast process like exo- or endocytosis. Furthermore, there are still some limitations in detecting the multiple molecular signals simultaneously, which means more colorful biosensors are needed to be developed. Hopefully, with advanced imaging devices and novel or improved biosensors, the kinetics of SVs can be studied more accurately and deeply.

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Disclosure

The authors report no conflict of interest.

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