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REVIEW

Real-time imaging of single synaptic vesicles in live neurons

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Abstract **Recent advances in fluorescence microscopy have provided researchers with powerful new tools to visualize cellular processes occurring in real time, giving researchers an unprecedented opportunity to address many biological questions that were previously inaccessible. With respect to neurobiology, these real-time imaging techniques have deepened our understanding of molecular and cellular processes, including the movement and dynamics of single proteins and organelles in living cells. In this review, we summarize recent advances in the field of real-time imaging of single synaptic vesicles in live neurons.**

Keywords single synaptic vesicle, real-time imaging, exocytosis, tracking

Introduction

The past two decades have seen considerable advances in fluorescence imaging techniques (Liuet al., 2015; Manzo and Garcia-Parajo, 2015), including extremely sensitive detectors such as electron-multiplying charge-coupled device (EMCCD) cameras (Kwakowsky et al., 2013; Michaletet al., 2013; Gustet al., 2014), high numerical aperture (NA) objectives (Joo et al., 2008; Kwakowsky et al., 2013; Gust et al., 2014), and extremely bright photostable fluorescent probes (Giepmans et al., 2006; Fernández-Suárez and Ting, 2008; Xia et al., 2013; Lavis and Raines, 2014). These advances have enabled researchers to measure single molecules at room temperature (Xie and Trautman, 1998; Blum et al., 2004) and to observe the *in vitro* dynamics of single molecules in real time (Deniz et al., 2008; Joo et al., 2008; Duzdevich and Greene, 2013; Gust et al., 2014). Importantly, these advances have also made it possible for researchers to observe the real-time movement and dynamics of single molecules and organelles in live cells (Coelho et al., 2013; Monico et al., 2013; Kusumi et al., 2014). Despite these technological breakthroughs, however, imaging single molecules and organelles in live cells remains challenging, due in part to several complicating factors such as high cellular background caused by autofluorescence (Joo et al., 2008; Xia et al., 2013; Haas et al., 2014).

Nevertheless, real-time imaging of single molecules and organelles in live cells has yielded in-depth knowledge regarding the molecular and cellular mechanisms that underlie biological processes (Levi and Gratton, 2007; Zhou and Wang, 2010; Manzo and Garcia-Parajo, 2015). In particular, these imaging techniques have provided insight into many key neurobiological processes (Triller and Choquet, 2008; Tatavarty et al., 2012; Choquet and Triller, 2013; Buxbaum et al., 2015; Ifrim et al., 2015). For example, real-time imaging of single glutamatergic AMPA receptors (AMPARs) in synapses has revealed that AMPARs are extremely mobile in the synapse (Tardin et al., 2003; Opazo et al., 2012); moreover, AMPAR trafficking is tightly regulated during synaptic plasticity (Chater and Goda, 2014), for example during the formation of long-term potentiation (Makino and Malinow, 2009). Moreover, real-time imaging of single synaptic vesicles in live neurons has provided valuable information regarding the dynamics of synaptic vesicles (Kavalali and Jorgensen, 2014; Maschi and Klyachko, 2015). Here, we review the recent advances on the use of real-time imaging for monitoring exocytosis and tracking single synaptic vesicles in synapses.

Visualizing the exocytosis of single synaptic vesicles

Neurons communicate with other neurons and other cellular

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targets by releasing the contents of presynaptic vesicles through exocytosis at the presynaptic terminals (Murthy and De Camilli, 2003, Südhof, 2004, 2008). Extensive research has revealed key roles for synaptic vesicles in synaptic transmission (Sakaba, 2006; Alabi and Tsien, 2012) and synaptic plasticity (Liu, 2003; Fioravante and Regehr, 2011; Mochida, 2011), including both short-term plasticity (Fioravante and Regehr, 2011; Regehr, 2012) and long-term plasticity (Yang and Calakos, 2013); in addition, studies have revealed details regarding key components in synaptic vesicles (Takamori et al., 2006; Jahn and Fasshauer, 2012). Despite these advances, however, the extremely small size of synaptic vesicles (with a diameter of 40–50 nm, which is far below the diffraction limit of resolution of conventional light microscopy) has hampered our efforts to investigate the dynamics of single synaptic vesicles, particularly in live neurons. Nevertheless, important advances in real-time imaging have enabled researchers to directly observe the exocytosis of single synaptic vesicles in live neurons (Zenisek et al., 2000; Aravanis et al., 2003; Gandhi and Stevens, 2003; Zhang et al., 2009; Park et al., 2012; Midorikawa and Sakaba, 2015).

The first report of using real-time imaging to monitor the exocytosis of single synaptic vesicles in live neurons came from Wolfhard Almers' group in 2000 (Zenisek et al., 2000). In this seminal study, Zenisek *et al*. used a high numerical aperture objective (NA 1.65 objective) and a sensitive charge-coupled device (CCD) camera together with total internal reflection fluorescence microscopy (TIRFM)—which restricts the illumination depth to 100 nm using an evanescent wave (Axelrod et al., 1983; Steyer and Almers, 2001)—to image single synaptic vesicles loaded with the fluorescent lipid FM 1-43 in big ribbon synapses in live goldfish retinal bipolar neurons (Fig. 1A1). This important study showed that newly arriving vesicles move to the active zone during stimulation and release FM 1-43 by exocytosis (Fig. 1A2). Examining the fluorescence traces revealed that nearly all of the FM 1-43 was released from the vesicle (Fig. 1A2), indicating that these synaptic vesicles underwent full-collapse fusion, a fusion mode in which the fusion pore of the vesicle dilates rapidly, causing the vesicle to "flatten" into the plasma membrane (Alabi and Tsien, 2013). Recent live imaging of single synaptic vesicles at the calyx of Held using TIRFM revealed that tethered vesicles are released after the readily releasable pool (RRP) is depleted (Midorikawa and Sakaba, 2015), indicating that tethered vesicles replenish the RRP. Moreover, new vesicles that entered from outside the imaging field did not release in response to another wave of Ca^{2+} influx, suggesting that tethering takes longer than the docking and priming steps.

Three years later, the first real-time imaging of individual synaptic vesicles in the small synapses of cultured hippocampal neurons was reported simultaneously by two independent groups (Aravanis et al., 2003; Gandhi and Stevens, 2003). Both groups used epifluorescence microscopy because of technical difficulties associated with using TIRFM, which requires the presynaptic terminals to directly contact the bottom of the coverslip. Aravanis *et al*. sparsely labeled synaptic vesicles with FM 1-43 and then tracked the release of the dye in single synaptic vesicles. Some synaptic

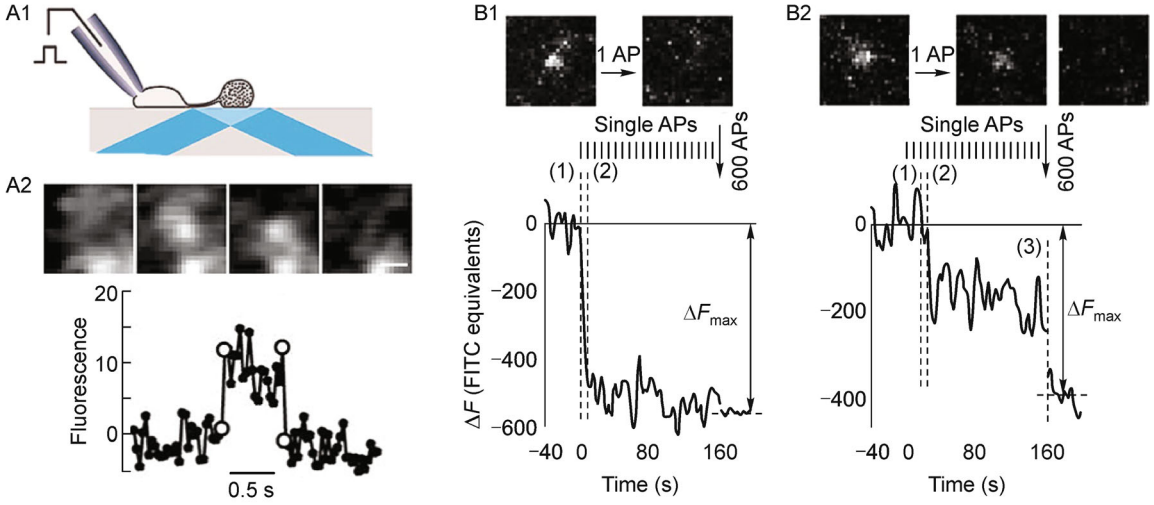

Figure 1 Early examples of monitoring exocytosis of single synaptic vesicles. (A) Exocytosis of single vesicles loaded with FM 1-43 in presynaptic ribbon terminals. (A1) The experimental scheme. Total internal reflection fluorescence microscopy (TIRFM) is used to illuminate the sample. (A2) A newly arriving vesicle enters the active zone during stimulation and nearly fully releases FM 1-43. The open symbols correspond to the images shown above. The scale bar represents 0.5 microns. Modified from (Zenisek et al., 2000). (B) Two distinct fusion modes can be triggered by stimulation with a single action potential (AP). (B1) Images (above) and a corresponding time course showing full-collapse fusion triggered by a single AP. Note that the synaptic vesicle released its entire FM 1-43 contents in one step. (B2) Images and a corresponding time course showing kiss-and-run fusion triggered by a single AP. Note that the synaptic vesicle released only a fraction of its FM 1-43 contents. Modified from (Aravanis et al., 2003).

vesicles lost their entire fluorescence signal during a train of action potentials (APs) (Fig. 1B1), which was reminiscent of full-collapse fusion observed in ribbon synapses. However, the majority of vesicles lost only a fraction of their FM 1-43 during the AP train (Fig. 1B2), suggesting that under these experimental conditions, majority of synaptic vesicles in small hippocampal synapses undergo "kiss-and-run" fusion, a fusion mode in which the vesicle fuses with the presynaptic membrane briefly and reversibly, producing a narrow pore through which a small amount of neurotransmitter is released (Alabi and Tsien, 2013). In contrast, Gandhi and Stevens monitored exocytosis and endocytosis using a pH-sensitive probe called SynaptopHluorin, which contains a pH-sensitive GFP moiety fused to the luminal domain of VAMP (vesicle-associated membrane protein). Importantly, SynaptopHluorin is non-fluorescent at the acidic pH (~5.5) inside the synaptic vesicle lumen, but becomes brightly fluorescent at neutral pH (~7), for example when exposed to extracellular solution (Miesenböck et al., 1998, Gandhi and Stevens, 2003; Dreosti and Lagnado, 2011). Using this approach, Gandhi and Stevens observed both full-collapse fusion and kiss-and-run fusion. Interestingly, they also found that kiss-and-run fusion was the predominant fusion mode in synapses with low release probability, whereas full-collapse fusion was the predominant mode in synapses with high release probability. Later, other methods for imaging single synaptic vesicles in real time using pH-sensitive GFP (in this case, fused to the vesicular glutamate transporter vGlut1) revealed that exocytosis and endocytosis are tightly coupled in a Ca^{2+}-dependent manner (Balaji and Ryan, 2007; Leitz and Kavalali, 2011; Leitz and Kavalali, 2014). Heinemann and colleagues conjugated four pH-sensitive GFP moieties to the luminal domain of Synaptophysin, thereby significantly increasing the signal-to-noise ratio (SNR) (Zhu et al., 2009). With this high SNR, they were able to distinguish between two endocytosis pathways: fast endocytosis, which occurs within approximately 3 s of fusion, and slow endocytosis, which occurs more than 10 s after fusion.

Quantum dots are extremely bright, photostable nanocraystals (Mattoussiet al., 2012; Maysinger et al., 2015) with a smaller diameter than a synaptic vesicle. Because of these unique properties, quantum dots have been used to study both exocytosis (Zhang et al., 2007; Zhang et al., 2009; Park et al., 2012; Gu et al., 2015) and the movement (Lee et al., 2012; Park et al., 2012) of single synaptic vesicles. Importantly, a single quantum dot coated with carboxyl groups can be loaded into a single synaptic vesicle without affecting the vesicle's functional properties, including the ability to produce excitatory postsynaptic currents (Zhang et al., 2007). Similar to the GFP-tagged proteins described above, the fluorescence of some quantum dots is pH-sensitive; thus, these pH sensitive quantum dots have been used to monitor kiss-and-run fusion events in real time (Zhang et al., 2009) (Fig. 2A). Vesicles loaded with a single quantum dot have been used to measure the prevalence of the kiss-and-run fusion mode in small synapses in cultured hippocampal neurons, revealing that kiss-and-run is prevalent in vesicles from the readily releasable pool (RRP) and at higher stimulation frequencies (Zhang et al., 2009). Recently, Zhang and colleagues applied this method to dopaminergic synapses derived from embryonic stem cells and found that a significant number of kiss-and-run fusion events occur in these synapses (Gu et al., 2015).

Biotinylated antibodies binding against the luminal domain of vesicular proteins have been used to load a single quantum dot into a specific subset of synaptic vesicles (Park et al.,

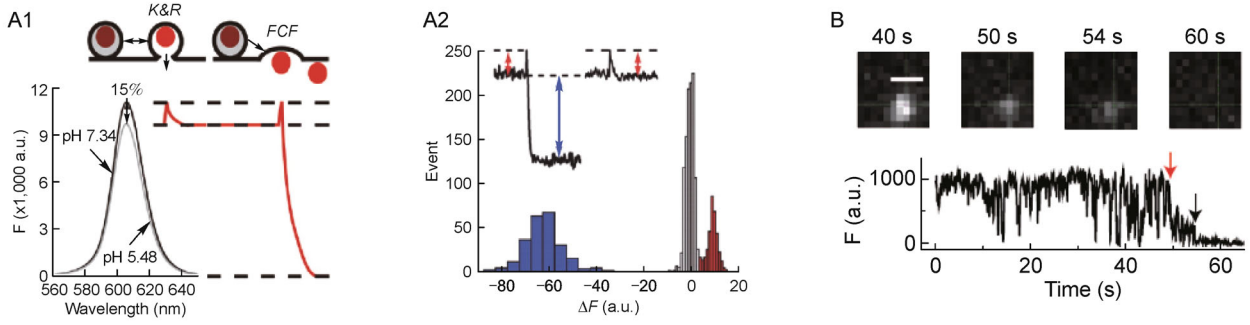

Figure 2 Kiss-and-run fusion and full-collapse fusion detected by the fluorescence change of a single quantum dot. (A) Kiss-and-run is revealed by a 15% increase in fluorescence intensity triggered by the transient opening of the fusion pore in a synaptic vesicle loaded with a single quantum dot (shown as an "uptick" in the red trace). (A1) Schematic diagram depicting the increase in fluorescence upon exposure to the extracellular solution (pH ~7.3). (A2) The change in the quantum dot's fluorescence signal during kiss-and-run events (red arrows in the trace and red bars in the histogram) and upon full-collapse fusion (the blue arrow in the trace and the blue bars in the histogram). Modified from (Zhang et al., 2009). (B) Kiss-and-run fusion was detected by the partial quenching of fluorescence of a single quantum dot inside a vesicle. The images and corresponding fluorescence trace indicate that a single quantum dot residing inside a vesicle undergoing kiss-and-run fusion was partially quenched by trypan blue in the extracellular solution. During the kiss-and-run event, the fluorescence signal dropped partially (indicated by the red arrow), then was completely quenched upon full-collapse fusion (indicated by the black arrow). The scale bar represents 0.8 microns. Modified from (Park et al., 2012).

2012). Specifically, biotinylated antibodies binding against the luminal domain of Synaptotagmin I and the vesicular GABA transporter (VGAT) were conjugated to streptavidin-coated quantum dots and loaded into general synaptic vesicles and inhibitory synaptic vesicles, respectively. By including trypan blue (which quenches quantum dots fluorescence) in the extracellular solution, the authors distinguished between kiss-and-run fusion and full-collapse fusion using different pore opening time; the brief pore opening in kiss-and-run fusion causes partial quenching, whereas full-collapse fusion causes complete quenching (Fig. 2B). By measuring the degree and kinetics of quenching, they found that the fusion mode is determined by the vesicle's location—specifically, kiss-and-run fusion occurred close to the presumed center of the active zone, whereas full-collapse fusion occurred throughout the synapse. This differential prevalence of fusion modes based on location in the presynaptic terminal was suggested to correlate with distinct distributions of NMDA receptors and AMPA receptors in the postsynaptic compartment (Kharazia and Weinberg, 1997).

Tracking single synaptic vesicles

Despite many successes described in the previous section, tracking individual small-diameter (*ca*. 40–50 nm) synaptic vesicles in small synapses in live neurons has remained a challenge. By combining brighter dyes with TIRFM, a new fluorescence imaging technique called FIONA (Fluorescence Imaging with One-Nanometer Accuracy) has allowed researchers to track a single fluorescent molecule with an accuracy of one nanometer (Yildiz et al., 2003; Yildiz and Selvin, 2005; Park et al., 2007). Although a single organelle containing many GFP molecules or quantum dots can be localized with nanometer-accuracy using TIRFM (Nan et al., 2005; Kural et al., 2005; Chang et al., 2008), the inability to use TIRFM in cultured neurons attached to coverslips has hindered our ability to accurately track single synaptic vesicles in live cells. However, new advances in imaging techniques have enabled researchers to track single vesicles in hippocampal neurons in real time (Maschi and Klyachko, 2015).

Hell and colleagues reported the first real-time tracking of individual synaptic vesicles in hippocampal neurons with an accuracy of tens of nanometers (Westphal et al., 2008). In their experiments, they used an enhanced version of stimulated emission depletion (STED) microscopy (Müller et al., 2012; Bianchini et al., 2015; Chéreau et al., 2015). By using faster STED imaging in a small area (1.8 μm × 2.5 μm), Westphal *et al.* were able to track individual synaptic vesicles at a rate of 28 frames per second. They observed that synaptic vesicles moved faster when outside the synapse and slower when located at the synapse. Interestingly, they also found that vesicles entered locations at the synapse called "hot spots," where they remained temporally. Within these hot spots, the synaptic vesicles were clustered tightly and remained stationary (Pechstein and Shupliakov, 2010). Tracking synaptic vesicles using STED imaging also revealed that recently endocytosed vesicles remain highly mobile after endocytosis and then join clusters of vesicles that are less mobile (Kaminet al., 2011). Moreover, STED imaging confirmed that synaptic vesicles are exchanged between synapses, which was previously reported in clusters of synaptic vesicles (Darcy et al., 2006; Fernandez-Alfonso and Ryan, 2008; Staras et al., 2010; Herzog et al., 2011; Ratnayaka et al., 2011). These results obtained at the single-vesicle and ensemble levels suggest that the movement of vesicles and proteins between synapses is a possible mechanism underlying changes in synaptic strength (Staras et al., 2010).

The three-dimensional position of single synaptic vesicles was measured recently using extremely bright quantum dots and dual-focus imaging optics with an epifluorescence microscope (Park et al., 2012). Using this approach, the three-dimensional movement of individual synaptic vesicles containing a single quantum dot was followed until the moment of exocytosis along with the three-dimensional centroids of a presynaptic marker (FM 4-64−labeled vesicles) and a corresponding postsynaptic marker (GFP fused to PSD-95) (Fig. 3A). Thus, the three-dimensional movement of synaptic vesicles between neighboring synapses—and their subsequent exocytosis—was observed at the single-vesicle level for the first time (Fig. 3B). These highly accurate spatiotemporal real-time measurements of single synaptic vesicles revealed that vesicles in the RRP are located close to the fusion site, suggesting a clear relationship between a vesicle's position and the pool to which it belongs (i.e., groups of synaptic vesicles which have similar properties, including release time) (Alabi and Tsien, 2012).

The relationship between synaptic vesicle mobility and synaptic plasticity was confirmed using real-time two-dimensional tracking of individual synaptic vesicles, each containing a single streptavidin-conjugated quantum dot (Lee et al., 2012). Synaptic potentiation increased the actin- and microtubule-dependent movement of vesicle at synaptic and intersynaptic area, respectively, whereas synaptic depression decreased vesicle mobility. These results suggest that the mobility of synaptic vesicles can affect synaptic activity by modulating the number of vesicles that are available for release.

Real-time tracking of single synaptic vesicles has also revealed differences in the mobility of synaptic vesicles labeled using spontaneous release versus evoked release (Peng et al., 2012). Despite a large number of extensive studies (Atasoy et al., 2008, Chung et al., 2010; Hua et al., 2010; Wilhelm et al., 2010; Ramirez and Kavalali, 2011; Sara et al., 2011; Andreae et al., 2012; Loy et al., 2014; Kavalali, 2015), whether the same pools of synaptic vesicles release during spontaneous activity and evoked activity had remained controversial. To investigate possible differences in the mobility of synaptic vesicles undergoing spontaneous versus evoked release, Klyachko and colleagues used SGC5—a

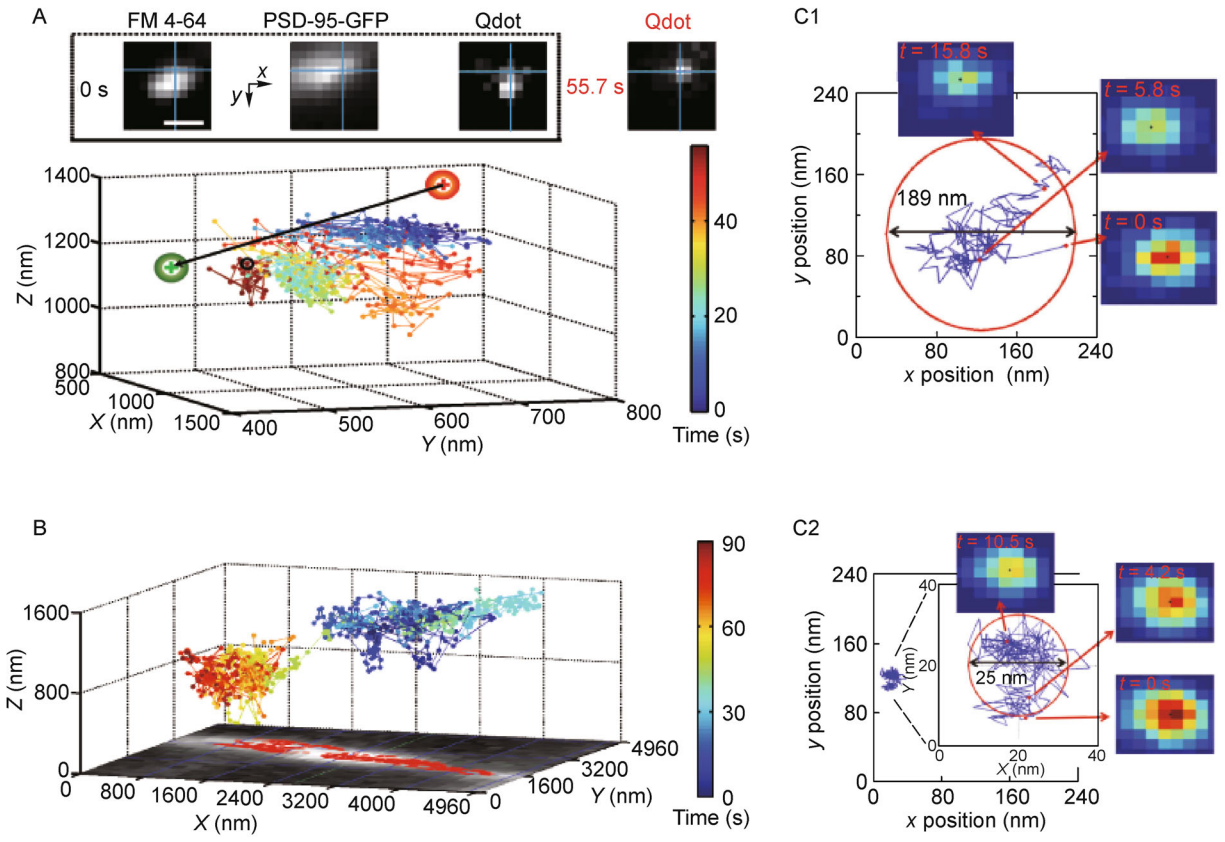

Figure 3 Tracking single synaptic vesicles. (A) Representative images of a synaptic vesicle loaded with a single quantum dot, a presynaptic terminal containing FM 4-64–labeled vesicles, and a postsynaptic compartment labeled with GFP-tagged PSD-95 (PSD-95-GFP). The graph below shows a three-dimensional trace of the quantum dot-loaded vesicle, showing that this vesicle moved from the center of the presynaptic terminal to the synaptic cleft, where it underwent exocytosis. The scale bar represents 0.8 microns. (B) Three-dimensional trace of one single synaptic vesicle that moved from one synapse to another synapse, and then underwent exocytosis. Modified from (Park et al., 2012). (C) Differential mobility of single synaptic vesicles labeled with the styryl dye SGC5 either by spontaneous activity or by evoked activity. (C1) Images and the corresponding trace of a single synaptic vesicle labeled by evoked activity. (C2) Images and the corresponding trace of a synaptic vesicle labeled by spontaneous activity. Vesicles labeled by evoked activity were more mobile than vesicles labeled by spontaneous activity. Modified from (Peng et al., 2012).

novel styryl dye with several-fold higher fluorescence intensity compared to FM dyes (Wu et al., 2009)—to label individual vesicles that were undergoing exocytosis; they then measured the two-dimensional mobility of these vesicles. Surprisingly, their analysis revealed that vesicles labeled during spontaneous exocytosis were less mobile than vesicles labeled during evoked exocytosis. Moreover, evoked release vesicles had more directed movement than spontaneously released vesicles, and their movement was dependent upon myosin II. These results suggest that myosin II also plays an important role in the directed movement of synaptic vesicles during synaptic transmission (De Pina et al., 2007).

Conclusions

The ability to image single synaptic vesicles in real time has enabled researchers to observe the behavior of single synaptic vesicles in live neurons, a breakthrough that was not previously possible using conventional imaging of clusters of vesicles. Observing the spatiotemporal behavior of single vesicles has shed light upon their dynamics and the complex mechanisms underlying exocytosis and vesicle recycling. Despite these important breakthroughs, we have only just begun to address the relationships between the behavior of single synaptic vesicles and the behavior of vesicle pools, synaptic transmission, and synaptic plasticity; in this respect, imaging single synaptic vesicles in real time will likely shed light on these fundamental yet important relationships, as well as the dynamic interactions between vesicles and other proteins such as motor proteins, synapsins, and proteins in the active zone. Although the state-of-the-art imaging techniques discussed in this review have yielded valuable insight into the detailed mechanisms underlying dynamic synaptic processes,

further refinement of existing tools and the development of new methodologies—for example, more effective loading methods, brighter dyes, and methods to image single synaptic vesicles in a slice or even *in vivo*—are clearly needed. Moreover, combining real-time imaging of single vesicles with powerful techniques such as optogenetics, FRAP (fluorescence recovery after photobleaching) and super-resolution imaging will significantly advance our knowledge regarding the cellular and molecular processes that underlie synaptic transmission and neurobiology.

Box 1. Quantum dots

A quantum dot is a light-emitting nanometers sized (1–10 nm) semiconductor nanocrystal that confines electrons or electron holes in three dimensions (Smith and Nie, 2010). When a quantum dot absorbs a photon with energy higher than its band gap energy, an electron of the quantum dot will get excited, which generates an electron-hole pair (Michalet et al., 2005). Due to the relative symmetric structure in a quantum dot, the radiative recombination events between electrons and holes produce a narrow and symmetric emission spectrum (Medintz et al., 2005; Michalet et al., 2005). The hole-electron pair can be considered as confined within a box. By controlling the size of a quantum dot, one can control the emission wavelength of the quantum dot from violet to near-infrared (Medintz et al., 2005).

Good optical properties of quantum dots, such as broad excitation spectra, narrow emission spectra, tunable emission peaks, long fluorescence lifetimes, and negligible photobleaching, provide a wide range of application in imaging biological samples (Barroso, 2011). In particular, greater quantum yield and photostability of a quantum dot compared with existing fluorescent molecules make a quantum dot a powerful probe for single particles tracking in live cells (Chang et al., 2008). Using quantum dots, researchers tracked single membrane receptors including glycine receptors (Dahan et al., 2003), NMDA and AMPA receptors (Groc and Choquet, 2006) in live neurons, and measured the mobility of individual neurotransmitter receptors in synapses. In addition, tracking single synaptic vesicles containing a quantum dot revealed dynamic regulation of exocytosis of single synaptic vesicles in synapses (Zhang et al., 2009; Park et al., 2012). However, quantum dots have a few disadvantages such as the potential toxicity (Bottrill and Green, 2011), relative large size and photoblinking (Baba and Nishida, 2012). Many efforts have been made to overcome disadvantages including decreasing the size (Howarth et al., 2008) and reducing photoblinking of quantum dots (Mahler et al., 2008)

Box 2 Fluorescence imaging with one-nanometer accuracy (FIONA)

The diffraction causes the image of a fluorescent molecule in a microscope to blur to the diffraction limit. The diffraction limit (d) was described by Ernst Abbe in 1873 as

$$d = \frac{\lambda}{2nsin\theta}$$

where λ is the wavelength, and n is the refractive index, θ is the maximal half-angle of the cone of light and $nsin\theta$ is the numerical aperture (NA) of an objective in a microscope (Park et al., 2007). The diffraction limit in visible light in a microscope with an objective with NA = 1.4 is 200 – 300 nm. However, the centroid of a fluorescent molecule can be localized with a higher precision by determining the center of the point-spread function (PSF) of a fluorescent molecule because the center of the PSF is the same as the centroid of a fluorescent molecule. Fluorescence imaging with one-nanometer accuracy (FIONA) is a method to localize the center of single molecules or organelles within an accuracy of one nanometer using two dimensional Gaussian fitting (Yildiz et al., 2003). The uncertainty of the localization (σ_μ) is given by

$$\sigma_\mu^2 = \frac{\sigma^2}{N} + \frac{a^2}{12N} + \frac{8\pi\sigma^4 b^2}{a^2 N^2}$$

where σ is the standard deviation of the point-spread function (PSF), N is the number of collected photons, a is the effective pixel size of camera, and b is the standard deviation of the background (Thompson et al., 2002; Park et al., 2007). The first term (σ^2/N) originates from photon noise, the second term ($a^2/12N$) is caused by limited pixel size of the detector, and the last term represents the background noise.

Bright fluorescent molecules, such as Cy3 and ReAsH, and nanocrystals like quantum dots, were demonstrated to be localized with an accuracy of nanometers using FIONA (Yildiz et al., 2003; Park et al., 2004, 2007; Warshaw et al., 2005; Zhang et al., 2011).

Box 3. Stimulated emission depletion (STED) microscopy

Owing to diffraction, it is difficult to separate two fluorescent molecules within Abbe's the diffraction limit. To overcome this limit, several super-resolution microscopy techniques such as stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994), photoactivated localization microscopy (PALM) (Betzig et al., 2006) and stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006) have been developed. STED microscopy uses an additional depletion laser to force the electrons of fluorescent molecules at an excited state to go down to a ground state (Müller et al., 2012). The depletion laser uses a specific wavelength which can deplete the electrons at an excited state before the fluorescent molecules emit photons by stimulated emission (Hell and Wichmann, 1994). The beam profile of this depletion laser is shaped into a donut-like pattern after phase modulation (Willig et al., 2006), and thus only depletes

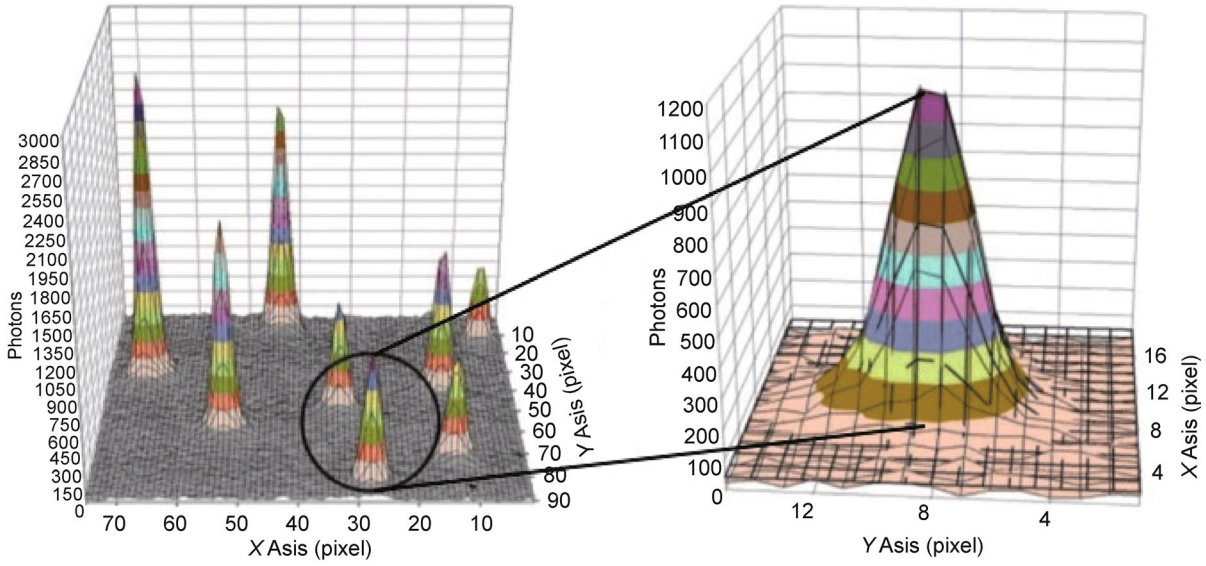

Figure box2 Point-spread functions (PSFs) of Cy3 labeled DNA. The expanded PSF had a width of 287 nm, photon counting of 14200 and signal to noise ratio of 32. The localization accuracy in the example was 1.3 nm. Modified from (Yildiz et al., 2003).

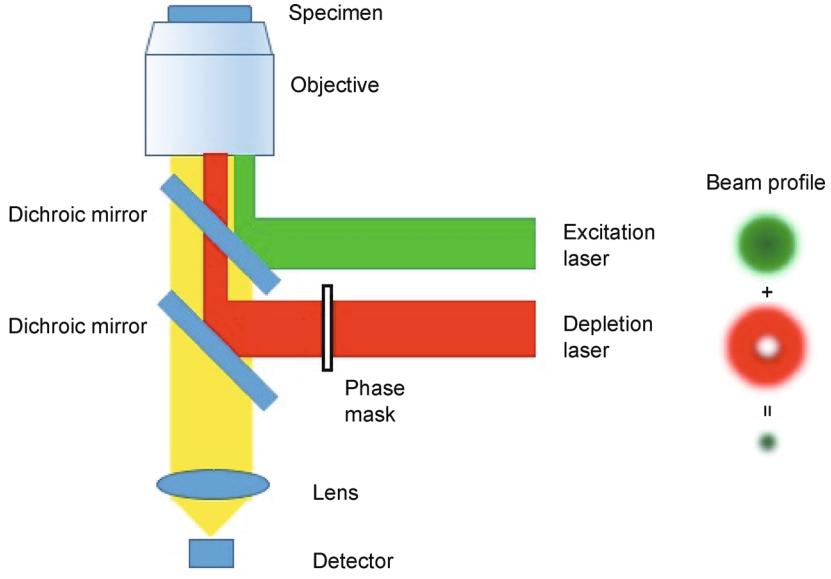

Figure box 3 Schematics of stimulated emission depletion (STED) microscopy. The depletion laser creates a donut-like beam pattern using a phase mask and selectively suppresses fluorescent molecules at the periphery. Fluorescent molecules in the tiny central region are allowed to emit photons.

fluorescent molecule at the periphery. Only fluorescent molecules in the tiny central area can emit photons, whereas fluorescent molecules in the periphery are suppressed not to emit photons by depleted laser. By scanning, STED microscopy generates super resolution images with a resolution < 25nm (Harke et al., 2008). Many fluorescent molecules, such as Atto and Alexa, fluorescent proteins including eGFP and eYFP, and nanocrystals like quantum dots, can be used with STED microscopy (Müller et al., 2012). The STED microscopy has provided dynamic information of live cells as well as detail structures about organelles, which were inaccessible with conventional microscopy (Müller et al., 2012; Neupane et al., 2014).

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Compliance with ethics guidelines

The authors declare no competing interest.

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