Review

Fluorescence imaging of synapse formation and remodeling

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Abstract
Brain function is based on proper connectivity between neuronal cells. In the developing brain, neurons extend axons and form synaptic connections with appropriate postsynaptic neurons. Molecular mechanisms underlying establishment of proper synaptic connections are one of the most important topics in the field of developmental neurobiology. Dynamics of synaptic structure and local recruitment of synaptic molecules can be studied by live-cell imaging of neurons expressing fluorescent probes of synaptic molecules. In this review, examples of live-cell fluorescence imaging are presented and their contributions to our understanding about the molecular mechanisms of synapse formation and remodeling are discussed. Imaging of synaptic proteins in living neurons revealed rapid formation of individual synapses within hours and extensive remodeling of synaptic connections. Different types of neurons express unique protrusions from dendrites and axons, which play important roles in synapse formation and maturation. Rapid formation of synaptic structure is associated with continual assembly and disassembly of synaptic scaffolding proteins, which are essential building blocks of the presynaptic active zone and the postsynaptic density (PSD). Quantitative analyses of PSD scaffolding proteins further confirmed their essential roles in maintenance of the synaptic structure. These examples clearly indicate that fluorescence-based live-cell imaging is an indispensable technique in the research on synapse development and its impact will further increase in combination with development of new light microscopic techniques in the future.

Keywords
live-cell imaging, fluorescent proteins, synapse, postsynaptic density, glutamate receptors, two-photon microscopy, super-resolution microscopy

Introduction
In the central nervous system (CNS) of vertebrates, neuronal precursors are differentiated from the neuroepithelial cells, migrate within the nervous tissue and settle within specific cortical layers or nuclei. After migration, neurons extend long axonal processes and start to connect with their target neurons. The contact sites between presynaptic axons and postsynaptic dendrites start to differentiate into synapses, which are the structures important in information processing and memory storage.

Electrophysiological and structural properties of mature synapses were studied extensively in the late 20th century. However, appropriate techniques for the reliable detection and measurement of nascent synapses during development had not been available until recently. Therefore, researchers initially focused on several types of model synapses in the peripheral nervous system, such as the neuromuscular junctions (NMJs), which were larger than synapses in the CNS and easier to manipulate experimentally [1]. Nascent NMJs could be identified.
using specific fluorescent probes, and the mechanisms of synaptic competition between multiple presynaptic axons targeted to the same postsynaptic muscle could be analyzed in detail. In the CNS, however, several factors prevented direct detection and analyses of developing synapses. First, synaptic structures in the CNS are much smaller than NMJs and the resolution of light microscopy is not sufficient for the detection of their detailed morphology. Second, the mammalian brain is covered by the cranium and direct access to the brain parenchyma requires invasive surgery. Because the brain tissue is fragile and easy to develop edema, highly sophisticated surgical procedures should be invented and applied to small animals. To circumvent this difficulty, isolated preparations of the nervous system, such as dissociated neuronal cultures and slice cultures, have been developed and widely used for the analyses of synapse development within the CNS [2,3]. Imaging experiments in these reduced preparations in combination with the development of green fluorescent protein (GFP)-based fluorescent probes provided essential information about the behavior of nascent synapses and the time course of their differentiation. Another possible approach toward the understanding of synapse development in the CNS is the histological analyses of brain sections taken from different stages of brain development. Analyses of synapse development in vivo by electron microscopy revealed structural details of nascent synapses quantitatively [4]. Fluorescence imaging of living neurons and ultrastructural studies of the fixed brain tissue provide us with complementary information and their integration has facilitated our understanding about CNS synapse development.

**Structural components of synapses and molecules involved in the assembly of presynaptic and postsynaptic specializations**

Detailed morphological characteristics of synapses have been studied by using transmission electron microscopy of chemically fixed or quickly frozen samples of the nervous tissue (Fig. 1). Synapses are the sites of membrane adhesion between presynaptic axons and postsynaptic dendrites or cell bodies. Axonal boutons are the cytoplasmic swellings formed at the contact sites between the axonal plasma membrane and the target neurons along the course of axonal trajectories. Axonal boutons contain synaptic vesicles, which are the storage sites of neurotransmitters, such as glutamate and γ-Aminobutyric acid (GABA). Arrival of action potentials to the presynaptic terminal activates voltage-gated calcium channels and triggers exocytosis of synaptic vesicles. Neurotransmitters released from the presynaptic membrane diffuse across the synaptic cleft and activate neurotransmitter receptors on the postsynaptic membrane.

Most excitatory synapses in the forebrain pyramidal neurons are formed onto dendritic spines, small protrusions containing densely packed actin filaments [5]. Another morphological feature of excitatory synapses is the presence of the postsynaptic density (PSD), located at the plasma membrane of the dendritic spine and apposed to the presynaptic active zone [6]. The PSD is composed of a variety of proteins, including membrane proteins, such as glutamate receptors and cell adhesion molecules, together with PSD scaffolding proteins and signaling molecules. In addition to the PSD, the postsynaptic cytoplasm contains several other microstructures, such as spine apparatus, endosomal membranes and mitochondria.

The sizes of both presynaptic axonal boutons and dendritic spines are in the order of several micrometers. The PSDs are disk-like structures with diameters of 200–500 nm and thicknesses of 30–60 nm [7]. Synapse morphology and distribution of synaptic proteins are difficult to resolve without the aid of fluorescence microscopy. Under optimized imaging conditions, structural distinction between different types of spines, such as thin, stubby and mushroom spines, can be reliably achieved and could be confirmed by retrospective electron microscopy [8,9]. In order to identify nascent synaptic structures, it is essential to develop techniques that enable expression of fluorescent protein (FP)-tagged synaptic molecules and subsequent detection of fluorescence signals in living neurons. A number of FP-based probes for presynaptic and postsynaptic components have been developed and utilized for live-cell imaging. Formation and remodeling of presynaptic structures can be monitored...
using GFP-tagged synaptophysin or synaptobrevin2/VAMP2 [10,11]. Both of these probes show selective accumulation in axonal boutons. Synaptophysin is one of the most abundant proteins of synaptic vesicles. Synaptophysin contains four transmembrane domains with cytoplasmic N- and C-termini. Synaptophysin tagged with GFP at its C-terminus can be properly recruited to the synaptic vesicles and is utilized as a reliable marker of local synaptic vesicle accumulation [11]. Synaptobrevin2/VAMP2 is also an integral synaptic vesicle protein with a single transmembrane domain and a cytoplasmic N-terminal domain. In the process of exocytosis, the assembly of a SNARE (soluble N-ethylmaleimidesensitive factor attachment protein [SNAP] receptor) complex from SNAP-25, syntaxin and synaptobrevin2/VAMP2 is proposed to drive the formation of fusion pore between vesicles and the plasma membrane. Synaptobrevin2/VAMP2 tagged with GFP at its N-terminus is functional and does not perturb synaptic vesicle exocytosis. GFP-tagged synaptobrevin2/VAMP2 is localized at the sites of synaptic vesicle accumulation and can be utilized as a reliable presynaptic marker [10].

Detection of the postsynaptic specialization can be achieved using FP-tagged PSD molecules. 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA)-type and N-methyl-D-aspartic acid (NMDA)-type glutamate receptors are enriched in the biochemically purified PSD fraction and their postsynaptic localization was confirmed by immunoelectron microscopy. AMPA receptors and NMDA receptors are essential components of glutamate-mediated synaptic transmission and their presence at the postsynaptic sites can be detected by expression of FP-tagged receptors. In the case of AMPA receptors, their distribution on dendritic membrane is diffuse and local density of AMPA receptors on the postsynaptic membrane is not extremely high compared with extrasynaptic receptors [12]. Furthermore, it is proposed that nascent synapses may lack AMPA receptors (silent synapses) and local activation of NMDA receptors triggers recruitment of AMPA receptors to silent synapses [13]. From these considerations, FP-tagged AMPA receptor subunits, such as GFP-GluA1 and GluA2, have not been utilized frequently to detect nascent synapses. However, these GFP-tagged AMPA receptors are useful in the detection of activity-dependent modifications of postsynaptic functions [14]. Recruitment of NMDA receptors to the postsynaptic sites takes place in the early stage of synaptogenesis. NMDA receptors are tetramers composed of two GluN1 subunits and two GluN2 subunits.
subunits. NMDA receptors tagged with GFP at their extracellular N-termini have been utilized for the detection of postsynaptic sites in cultured cortical neurons [15].

PSD scaffolding molecules are highly concentrated in the postsynaptic sites and are good candidates for postsynaptic markers. PSD-95 is one of the most abundant scaffolding molecules in the PSD [16]. PSD-95 can interact directly with NMDA receptors and also indirectly with AMPA receptors through its binding to transmembrane AMPA receptor regulatory proteins. PSD-95 interaction partners are not restricted to glutamate receptors, but include cell adhesion molecules, such as neuroligins and synCAMs, scaffolding molecules, such as guanylate kinase-associated protein (GKAP), and signaling molecules, such as synaptic GTPase-activating protein for Rac (SynGAP), neuronal nitric oxide synthase (nNOS) and spine-associated Rap-Gap (SPAR) [17]. Multiple binding partners of PSD-95 indicate its essential role in PSD organization. PSD-95 with its C-terminal tagged with GFP shows postsynaptic distribution indistinguishable from that of endogenous PSD-95 and has been utilized as a reliable fluorescent marker of the postsynaptic specialization (Fig. 2a) [18,19]. In addition to PSD-95, other GFP-tagged PSD scaffolding proteins, such as GFP-Homer and GFP-Shank, have also been utilized as probes for the PSD detection (Fig. 2b) [20,21]. The basic properties of these scaffolding proteins are similar, with quantitative difference in their local turnover rate and response to neuronal activity.

Electron microscopic studies reported ultrastructural difference between asymmetrical (Gray type 1; correspond to excitatory glutamatergic synapses) and symmetrical (Gray type 2; correspond to inhibitory GABAergic synapses) synapses [22]. These structural differences are derived from the difference in thickness of the postsynaptic membrane specialization. Postsynaptic membranes of excitatory synapses develop thicker meshwork of protein assembly and are detected as prominent electron-dense structures [6]. Typical PSD proteins, such as PSD-95, Homer and Shank, are specifically

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**Fig. 2.** Clustering of PSD scaffolding proteins tagged with GFP at the postsynaptic sites and their turnover in living hippocampal neurons in culture. (a) A cultured hippocampal neuron expressing PSD-95-GFP (green) stained with a lipophilic dye Dil (red). Arrows indicate colocalization of PSD-95-GFP puncta (green) and dendritic spines (red). Arrowheads indicate a single straight axon devoid of PSD-95 puncta. Scale bar, 10 μm for the main panel and 4 μm for the lower small images. (b) Formation and elimination of the PSD detected by expression of GFP-tagged Homer1c in cultured hippocampal neurons. Imaging of the same dendritic segments with an interval of 24 h for 6 days revealed continual remodeling of the PSDs, with gradual increase in the total number of GFP-Homer1c clusters. Scale bar, 4 μm. Reprinted from Okabe et al. [19].
accumulated at excitatory asymmetrical synapses and their GFP-tagged probes can be utilized as specific markers of this type of synapses. In turn, GFP-tagged scaffolding proteins showing specific localization at inhibitory postsynaptic sites, such as gephyrin-GFP, can be utilized for the detection of symmetrical synapses in living neurons [23,24].

By using GFP-tagged presynaptic and postsynaptic molecules, time-lapse imaging of living neurons revealed the process of synapse formation and timing of recruitment of synaptic molecules. These analyses revealed basic principles of synapse formation and mechanisms of molecular assembly at the synaptic junctions [11,25–27].

**Live-cell fluorescence imaging of synaptic molecules and structure**

Before GFP technology enabled us to visualize dynamics of synaptic molecules in living neurons, discussions on the time-course of synapse development had been based on the comparison of immunocytochemical and electron microscopic data of fixed preparations. Because the density of synapses and dendritic spines increases gradually in both culture preparations and in vivo, it was widely accepted that the differentiation of individual synapses is also a slow process which may take a few weeks. However, time-lapse imaging of PSD-95 tagged with GFP in hippocampal pyramidal neurons in culture revealed rapid establishment of PSD-95-GFP fluorescent clusters on time scales of several hours [19]. These PSD-95-GFP clusters were apposed to the presynaptic boutons and were selectively localized within dendritic spines, suggesting their identity as synaptic PSD structures [11]. A similar time course of postsynaptic molecular assembly was confirmed by several independent groups and has been taken as convincing evidence for rapid establishment of synaptic specialization [28,29]. If synapse assembly is a rapid process, how is relatively slow increase of overall synaptic density achieved? A possible explanation is that synapse addition and elimination take place simultaneously and the rate of addition is maintained to be moderately higher than the rate of elimination. Indeed, this difference in addition and elimination of PSD-95-GFP clusters existed in culture preparations and could quantitatively explain the overall rate of synapse density (Fig. 2b) [18,19].

It is possible to determine the time course of molecular assembly at presynaptic and postsynaptic sites simultaneously by using multicolor imaging of FP-tagged synaptic proteins. We expressed PSD-95 tagged with yellow fluorescent protein (YFP) together with synaptoplakin tagged with cyan fluorescent protein (CFP) to determine whether presynaptic and postsynaptic molecules appear at synaptic sites with different time courses [11]. We found a strong temporal correlation between clustering of PSD-95-YFP and that of synaptoplakin-CFP at the synaptic contact sites (Fig. 3d and e).

**Fig. 3.** Simultaneous detection of multiple synaptic components by dual-color time-lapse imaging. (a–c) Imaging of the PSDs by expression of PSD-95-yellow fluorescent protein (YFP) (b) together with detection of spine structure by cyan fluorescent protein (CFP) (green) was also presented. (c) Formation of a PSD-95-YFP cluster (arrows) is coordinated with the enlargement of the spine head. Scale bar, 3 μm. (d–e) Simultaneous imaging of synaptoplakin-CFP (d) and PSD-95-YFP. (e) Accumulation of synaptic vesicles detected by synaptoplakin-CFP is synchronized with clustering of PSD-95-YFP (arrows). Scale bar, 3 μm. Reprinted from Okabe et al. [11,21].
Interestingly, appearance of synaptophysin-CFP clusters tends to precede clustering of postsynaptic PSD-95-YFP with time intervals of <1 h, indicating the existence of temporal orders in synapse differentiation.

Immature hippocampal pyramidal neurons express dendritic filopodia, which are highly motile and have lifetimes shorter than those of spines. It has been proposed that dendritic filopodia serve as sensors of the surrounding tissue environment and are transformed into spines after physical interaction with nearby axons. To determine structural changes of immature dendritic protrusions and their relationship to clustering of PSD-95, we expressed both PSD-95-YFP and CFP in dissociated hippocampal neurons [11]. CFP was utilized as a volume label for morphological identification of dendritic protrusions. Imaging of PSD-95-YFP and CFP revealed the presence of filopodia-like protrusions on pyramidal neuron dendrites and appearance of PSD-95-YFP clusters in a small subset of these protrusions (Fig. 3a–c). After establishment of PSD-95-YFP clusters, these protrusions were stabilized and increased their volume, indicating their morphological transformation into spine-like structures. Importantly, none of the PSD-95-YFP clusters within dendritic shafts induced the formation of dendritic protrusions. These observations indicate that filopodia-like protrusions from dendritic shafts play an important role in initial contact with nearby axons and subsequent differentiation of spines with synaptic contacts.

Roles of dendritic and axonal protrusions in synaptogenesis

Neuronal networks in the mammalian cortex are composed of two types of neurons, excitatory pyramidal-shaped neurons and inhibitory neurons. Although glutamatergic excitatory synapses are formed on dendrites of both pyramidal neurons and interneurons, their postsynaptic morphology and molecular composition are distinct. The most obvious difference is the absence of dendritic spines in excitatory synapses on interneuron dendrites [30,31]. Dendritic filopodia from pyramidal neurons are thought to be the precursors of spines and important in searching nearby axons. It has not yet been clarified whether interneuron dendrites have any searching systems to contact nearby axons. Without such a mechanism to enhance the chance of contacting nearby axons, the ability of interneurons to increase synaptic density should be quite limited. However, previous electron microscopic studies reported that dendritic shafts of mature interneurons are densely covered with glutamatergic synapses, suggesting the presence of interneuron-specific strategy to increase synaptic contacts [32]. To solve this problem, time-lapse imaging of synapse formation on interneuron dendrites was performed and two important observations were made [33]. First, dendritic protrusive activity of interneurons was developmentally regulated. Although dendrites of mature interneurons had few protrusions, immature interneurons expressed numerous dendritic protrusions, which were longer than typical filopodia of pyramidal neurons. Second, PSD-95 clusters were frequently observed on these dendritic protrusions and showed slow translocation toward the base of protrusions (Fig. 4). These observations indicated that dendritic protrusions of interneurons serve as conduits for retrograde translocation of synaptic structure to the parental dendrites. This translocation was dependent on microtubules present within dendritic protrusions and was driven by dynein motor system. These experimental data indicate that the behavior of synaptic structure may differ between different types of synapses even within the same brain area. In the case of pyramidal neurons, the positions of individual synapses along dendritic shafts are precisely determined by the position of initial protrusive activity of dendritic filopodia. On the other hand, the positions of excitatory synapses along interneuron dendrites may be more flexible and the synaptic connectivity can be increased effectively by using long protrusions as synaptic conduits.

Assembly of synaptic structure is a stochastic process and the behavior of a large number of synapses with different stages of maturation is difficult to be classified and characterized. If synapse maturation can be synchronized, the time course of synapse development may be determined more precisely and molecular markers and structural features for each developmental stage may be characterized more easily. In the cerebellum,
synaptogenesis between the axons of granule cells (parallel fibers) and the dendrites of Purkinje cells is regulated by a soluble factor Cbln1, a C1q family protein produced and secreted from granule cells. Cbln1 released from parallel fibers binds to both presynaptic receptor neurexin (Nrx) and postsynaptic receptor glutamate receptor delta 2 (GluD2). It is likely that the tripartite Nrx-Cbln1-GluD2 complex affects synaptogenesis of both presynaptic and postsynaptic components. We took advantage of slice cultures derived from Cbln1 knockout (KO) mice and induced synaptogenesis in slices by exogenous application of Cbln1 [34]. Without Cbln1, parallel fibers showed little morphological remodeling and subsequently generated fewer axonal boutons. However, application of Cbln1 increased both motility of synapses and their subsequent transformation into axonal boutons. On the basis of the data obtained by using this artificial synchronization system of synaptogenesis, we proposed ‘bidirectional interaction model’ of synapse maturation between parallel fibers and Purkinje cell dendrites. This model is based on our observation that a specific type of axonal protrusions with complex morphology appears during the intermediate stage of synapse formation and increase in the contact area between axons and dendritic spines enhances both Nrx-dependent presynaptic maturation and GluD2-dependent spine maturation. We learned from this study that time-lapse imaging of the synchronized formation of neural circuits is highly informative in defining precise stages of synaptogenesis.

**Turnover of postsynaptic scaffolding molecules and their structural roles**

We proposed that synapse addition and elimination take place simultaneously and the slight excess of added synapse over eliminated synapse determines the gradual increase of overall synapse density. This model indicates the importance of a regulatory mechanism that controls the rates of synapse formation and elimination through the assembly and disassembly of synaptic molecules. To determine the turnover rate of a given synaptic molecule at local synaptic sites precisely, fluorescence recovery after photobleaching (FRAP) and fluorescence activation were performed. With both techniques, the turnover rate of fluorescent molecules in a local environment can be directly measured. Interestingly, FRAP analyses of four different PSD scaffolding molecules revealed distinct kinetic properties (Fig. 5) [20]. PSD-95, a scaffolding molecule directly interacting with the plasma membrane via its S-palmitoylation, showed low mobility by FRAP analysis. In contrast, Homer1c, a scaffolding molecule containing binding sites for actin, showed a much higher turnover rate. Because actin molecules in spines show very rapid exchange between soluble and polymerized pools [35], it is likely that dynamics of PSD scaffolding proteins is heterogeneous and scaffolding proteins
enriched at the cytoplasmic surface of PSDs are dependent on actin cytoskeleton for their localization and stability.

To further clarify the roles of PSD scaffolding proteins in the molecular architecture of PSDs, information about the molecular content of individual scaffolding proteins per single PSD is essential. Even if functional studies suggest importance of a given molecule by gene knockout and knockdown approaches, its importance as a structural component can be proved only by measuring its molecular content per synapses. To this end, we developed a technique of quantitative measurement of protein content per synapses by using fluorescent microspheres as a calibration standard of GFP molecules (Fig. 6) [36]. We first determined the fluorescence intensity of single GFP molecules and compared the fluorescence of single microspheres with that of single GFP proteins. Our measurements indicated that the fluorescence microspheres are equivalent to the fluorescence intensity of about 4000 GFP proteins. By using these calibrated microspheres,
we next determined the number of GFP-tagged PSD scaffolding proteins present in single postsynaptic sites. We could successfully estimate the absolute numbers of four key scaffolding proteins, PSD-95, GKAP, Shank and Homer, per synapses. Interestingly, the synaptic contents of the four scaffolding proteins were quite similar and were in the range 100–450 molecules per synapses. Because these four scaffolding molecules were known to interact with each other, we proposed that their binding stoichiometry is relatively simple. Furthermore, the total protein mass of four scaffolding proteins was calculated to be 100 MDa, which corresponded to about 10% of the total mass of single PSD (1.1 GDa). This suggests that only four types of PSD scaffolds occupy a substantial fraction of the total PSD mass. This conclusion is consistent with the idea that a small subset of PSD scaffolding proteins are responsible for the construction of the main PSD structure, which may function for anchoring and stabilization of other functional molecules, such as neurotransmitter receptors and cell adhesion molecules [16].

Several hundreds of PSD-95 molecules in a single synapse are sufficient to anchor glutamate receptors, which are estimated to be accumulated to single synapses in the range 10–200 tetramers per synapse [37,38]. It is important to note that the number of PSD-95 per synapse and the number of glutamate receptor complexes per synapse are set to be in a similar range. This relationship may enable efficient regulation of glutamate receptor numbers through the synaptic content of PSD-95. This idea was supported by electrophysiological recordings of slices from PSD-95 knockout mice. In the absence of PSD-95 and related membrane-associated guanylate kinase proteins, synaptic transmission, which was mediated by AMPA-type glutamate receptors, could be detected, but its amplitude was significantly down-regulated, suggesting PSD-95-dependent regulation of AMPA receptor content per synapse [39].

Future directions of imaging research on synapse dynamics

Recent advancement of imaging technologies provided a wide range of opportunities for the applications of new optical principles, light sources and sample preparations techniques to the analyses of synapse formation and remodeling. Especially, two important technologies, in vivo two-photon imaging and super-resolution microscopy, may greatly accelerate our understanding of synapse dynamics.

Two-photon excitation laser scanning microscopy is a technique appropriate for high-resolution imaging of neuronal microstructure in vivo [40]. An infrared pulsed laser beam can penetrate the brain tissue more effectively than the shorter wavelength light and can be focused to the volume small enough to resolve submicron structures within the nervous tissue [41,42]. By using two-photon microscopy, activation of fluorescence molecules is restricted to a small volume without unnecessary excitation above and below the focal plane, which may induce phototoxic effects after repeated imaging of a large volume of samples. Two-photon microscopy enabled researchers to resolve dendritic spines filled with GFP in the neocortex of living mice with time intervals of several days to months (Fig. 7) [43,44]. By comparing the images of dendritic segments and attached spines at different time points, the rate of formation and elimination of spines could be successfully measured in vivo. Initial studies revealed the highly stable nature of dendritic spines in the mature neocortex: more

Fig. 7. In vivo imaging of layer 5 pyramidal neurons expressing GFP in the somatosensory cortex. (a) Two-photon imaging of the apical dendrites in the layer 1 of an adult transgenic mouse expressing GFP under the control of Thy1 promoter. (b) Dendritic spines visualized in vivo with an interval of 14 days. Arrow indicates an eliminated spine. Most of the imaged spines were stable over 14 days in vivo. Scale bar, 5 μm.
than 90% of spines imaged in the adult mouse neocortex could survive when they were re-examined several months later [43,45]. This stability is in contrast with rapid turnover of synapses in culture conditions. There are two possibilities that can explain this discrepancy. First, synapse dynamics in culture conditions may be artificially upregulated by the presence of factors specific to the culture condition or the absence of factors present in the native tissue environment. For example, culture medium and serum supplement may upregulate synapse elimination in vitro. It is also possible that mature astrocytes may protect newly formed synapses and the lack of glial support may explain the higher turnover rate of synapses in culture [41]. The second possibility is that rapid turnover of synapses in vitro reflects the situation of synapses in the early phase of synapse development in vivo. Indeed, quantitative analyses of spine density in the early postnatal period of rat visual cortex revealed that significant increase in spine density occurs between postnatal days 6 and 21 [46]. Application of in vivo two-photon imaging in the early postnatal period may clarify this point. However, this experiment has not yet been accomplished, mainly because of the technical difficulty of imaging nascent synapses in vivo. At present, most of the in vivo synapse analyses rely on dendritic spines as a structural marker of synapses. In the future [47], application of GFP-tagged postsynaptic proteins, such as PSD-95-GFP, will increase the precision of identifying nascent synapses in vivo and will reveal the degree of synapse turnover in the early postnatal period.

Another important advancement in the field of synapse imaging is application of super-resolution imaging to molecular distribution and dynamics of synaptic proteins [48]. Recently, two papers reported significant improvement in spatial resolution of postsynaptic molecular movement and PSD structure by using photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) techniques [49,50]. PALM is the imaging technique based on photoactivation of FPs and STORM is based on stochastic activation of organic fluorescent dyes attached to appropriate antibodies. Recent application of PALM to the behavior of single actin molecules revealed the presence of actin flow in spines and confirmed the results of previous photoactivation studies of actin tagged with photoactivatable GFP [50]. Furthermore, these PALM studies revealed heterogeneity in the speed of actin movement within single spines. In the cytoplasm close to the PSD, actin velocity was specifically higher than in the other area. These PALM experiments of actin mobility in spines suggested that overall organization of actin filaments in spines is based on short filaments with less aligned orientation and heterogeneous velocity. It is also possible to evaluate molecular distribution of multiple synaptic proteins at the synaptic junctions by using super-resolution imaging techniques. Dani et al. reported distribution of PSD scaffolding proteins and glutamate receptors within the postsynaptic membrane of fixed brain sections labeled with antibodies against presynaptic and postsynaptic molecules [49]. They could successfully detect differential distribution of AMPA and NMDA receptors in individual synapses by using the STORM technique. In the future, precise organization of glutamate receptors and scaffolding proteins can be determined by using the PALM/STORM techniques and their intrasynaptic redistribution associated with synaptic plasticity may be directly visualized.

Conclusions

Before the introduction of live imaging techniques of synaptic structure and function, electrophysiological recording was the only possible technique to analyze functional modification of synapses. Although electrophysiological recording is an indispensable technique to record activity of synaptic receptors and ion channels, it usually lacks the information of single synapses and is not suitable for long-term monitoring of synapse behaviors. Introduction of GFP technology and its application to the research on synapse formation led to direct monitoring of single synapse behavior for a long time with minimal perturbation. These imaging studies in the last decade provided a clear picture of synapse formation in the mammalian neocortex and hippocampus. Two-photon excitation microscopy, combined with various gene expression technologies, such as generation of transgenic mice and
infection of viral vectors, further extended the possibility of imaging synapse formation and remodeling within tissue environment. Especially, two-photon imaging of neocortical neurons through optical windows on the rodent cranium enabled researchers to monitor the lifetime and the behavior of single synapses in vivo. In the near future, using in vivo imaging technologies combined with techniques of manipulating neuronal activity, we can expect that the basic principles of neural connectivity in living animals can be extracted with firm experimental evidences.

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**References**


