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Optical highlighter molecules in neurobiology

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The development of advanced optical methods has played a key role in propelling progress in neurobiology. Genetically-encoded fluorescent molecules found in nature have enabled labeling of individual neurons to study their physiology and anatomy. Here we discuss the recent use of both native and synthetic optical highlighter proteins to address key problems in neurobiology, including questions relevant to synaptic function, neuroanatomy, and the organization of neural circuits.

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Introduction

GFP and other conventional fluorescent proteins have had a tremendous impact on biology, and their subsequent development into optical highlighters (photoactivatable, photoconvertible, and photoswitchable fluorescent proteins) has opened even more avenues of experimentation. Respectively, these molecules are initially synthesized as molecules lacking or having low fluorescence, molecules initially fluorescing at wavelengths other than their 'activated' wavelength, and molecules exhibiting fluorescence which can be switched 'on' and 'off' repeatedly. These features enable experiments not possible through conventional photobleaching of constitutively fluorescent proteins: by definition, photobleaching experiments track the properties of those molecules not subject to the photobleaching itself, whereas optical highlighting enables researchers to track precisely those molecules that have been photostimulated. Recounting the >20 variations of these molecules is outside the purview of this review, but interested readers are directed to reviews detailing their characteristics [1,2].

Optical highlighters at the synapse

Chemical and electrical stimulation protocols induce defined forms of plasticity *in vitro* whose *in vivo* correlates are thought to instantiate network-level processes such as learning and memory. These experimental manipulations trigger both local alterations in protein translation, localization and biochemical activity and neuron-wide changes in transcription; together these events respecify the molecular landscape within individual synapses, culminating in altered synapse anatomy and function [3–5].

The use of live imaging techniques has promised to enable visualization of the anatomic and molecular changes coincident with the induction of synaptic plasticity in real-time. However, many of the techniques traditionally deployed by researchers — such as the use of GFP-fusion proteins to track single molecules — do not afford sufficient temporal or spatial resolution to effectively address questions about molecular dynamics at single synapses on the timeframes relevant to plasticity. By conferring tight control over molecular labeling both in space and in time, optical highlighters have circumvented many of the technical limitations of conventional approaches, enabling key experiments to characterize synapses and the individual molecules within them.

For example, the observation that isolated synapses on a single neuron exhibit distinct functional properties suggests that the anatomic structure of dendritic spines — whose bulbuous heads are connected to the dendritic shaft by narrow necks only a few hundred nanometers across — may act as electrical or chemical compartments, enabling the spine to act as an isolated computational unit [6]. By using multiphoton techniques to photoactivate PA-GFP within individual spines in hippocampal slice culture and then monitoring the rate of PA-GFP exit into the dendrite, Bloodgood and Sabatini [7] could establish that spines are diffusionally coupled to the dendritic shaft (Figure 1). Further, by manipulating levels of electrical activity in the slice and then photoactivating PA-GFP within spines they showed that degree of effective continuity between spines and dendrites could be dynamically altered [7].

The observation of regulated diffusional coupling between the spine head and the dendrite suggests that molecular mediators of plasticity may be differentially trapped within or released from spines in response to trans-synaptic cues [8]. As PA-GFP diffusion may not reflect the biophysics of native spine-localized molecules, researchers have measured the diffusion constants of various synaptic molecules fused with optical

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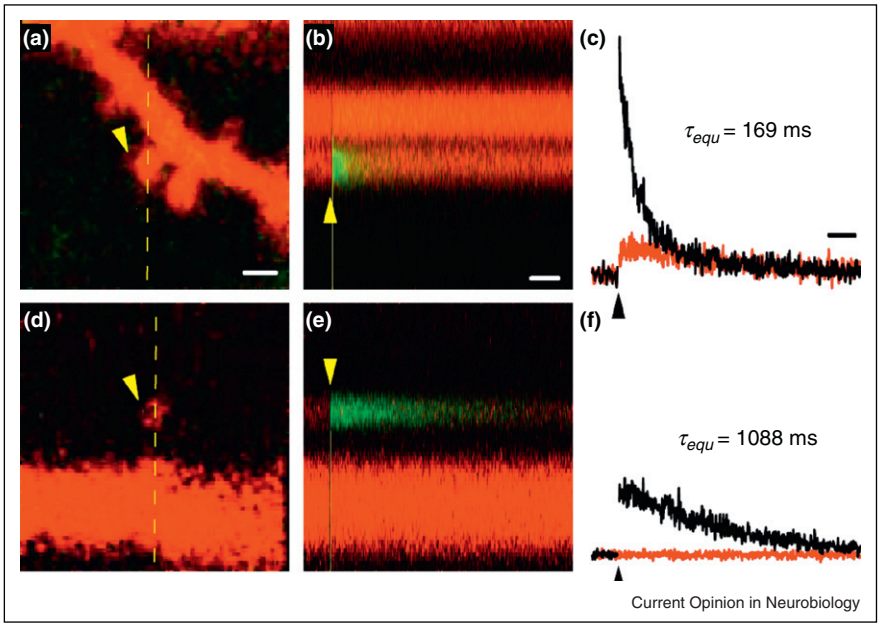
Figure 1



Using PA-GFP to measure diffusional coupling between spines and dendrites. **(a)** Constructs expressing DsRed and PA-GFP were biolistically delivered to pyramidal neurons in hippocampal slice culture. Multiphoton imaging reveals dendrites and individual dendritic spines (yellow arrow). Localized photoactivation of PA-GFP through targeted illumination of the spine with 710 nm light effectively photoconverts PA-GFP as revealed by a linescan through the spine and the adjacent dendrite (**b**, see dashed line in (b) for region of linescan, yellow arrow in (b) is time of photoconversion). By quantitating the fluorescence transient and fitting the decay to a single exponential **(c)** one can determine the tau_{equ}, the time constant of diffusional equilibration across the spine neck. By using PA-GFP as a diffusional probe in this manner on many spines (e.g. **d**–**f**), Bloodgood *et al.* could determine that most dendritic spines are diffusionally coupled to the parent dendrite, but their individual diffusion constants vary widely (compare **c** and **d**) and are regulated by activity.
Images courtesy of Brenda Bloodgood and Bernardo Sabatini (Harvard University).

highlighters. Although the molecular weight of fusion proteins significantly differs from that of the native proteins, diffusion coefficients are only weakly related to total molecular weight (approx. $MW^{1/3}$) [9], enabling fusion proteins to provide an adequate estimate. Several groups have fused PA-GFP to PSD-95, a core protein within the postsynaptic density (PSD) whose local concentration may scale with synapse size and strength [9,10•,11•,12•,13••]. Photoactivation of PSD-95-PA-GFP in spines *in vivo* has revealed that PSD-95 leaves unstimulated synpases on timescales of 10 s of minutes to hours (instead of seconds as measured for free PA-GFP) and that this rate increases in response to activity. Highlighter proteins have also been fused with other adapter proteins, enzymes and ion channels resident in synaptic spines, including Shank2, Shank3, CaMKIIα, CaMKIIβ, GluR2, stargazin, Ras, Rho, and Cdc42 [10•,14•,15•,16,17] revealing a diversity of diffusion rates that constrain molecular models of plasticity. These experiments have also shown that the spine concentration of certain molecules (PSD-95, CaMKII) scales with spine size. Importantly, while protein diffusion may facilitate the spread of signals from activated spines to adjacent spines (as is the case for PSD-95), in at least some cases diffusion may also promote enzyme inactivation. For example, PA-GFP-tagged cdc42 diffuses rapidly out of activated spines, but (as revealed by 2P-FLIM) its enzymatic activity is disabled upon entry into the dendrite [15•].

Optical highlighters have also been used to probe actin dynamics within spines, where actin plays a critical role in synaptic function by acting both as a tether (through interactions with signaling molecules) and as a strut [18]. Expression and photoactivation within spines of PA-GFP-β-actin fusion proteins in hippocampal neurons revealed two populations of filamentous F-actin: a treadmilling pool of actin that flows from the spine periphery toward the center, and a stable pool of actin at the spine base whose size is proportional to the size of the spine itself [12•,13••]. Stimulation via single spine uncaging of MNI-glutamate causes formation of a third unlocalized pool of actin that may play a role in structural plasticity [12•]. Photoconvertible protein actin chimeras have also been used in a superresolution microscopy technique, Photoactivatable Localization Microscopy (PALM) [19]. PALM and other molecular localization microscopy techniques, such as fluorescence-PALM [20], Stochastic

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Optical Reconstruction Microscopy (STORM) [21], PALM with independent running acquisition (PALMIRA) [22] and many others [23], rely on the precise localization of single molecules by imaging and fitting their fluorescence signals to two-dimensional Gaussian distributions. These experiments revealed that most actin fibers are short (<300 nm) and that the most dynamic fibers are irregularly distributed within the spine in numbers proportional to spine size [13••,24•]. As an alternative to direct actin tagging, Izeddin *et al.* [25•] developed an actin binding protein (ABP)-tdEosFP fusion for PALM with which they have visualized actin redistribution after exposure to AMPA agonists in hippocampal neurons [25•].

Like their postsynaptic counterparts, presynaptic boutons have been proposed to serve as compartments that differentially isolate second messengers and specific pools of synaptic vesicles. By expressing and locally photoactivating PA-GFP fused to presynaptic active zone components, an active exchange of presynaptic material between adjacent boutons was observed; the exchange of certain molecules (such as synapsin) was promoted by activity, whereas the exchange of other molecules (such as bassoon) was not [17,26]. Similar synaptophysin-Dendra2 experiments demonstrated that presynaptic vesicles decorated with synaptophysin are also shared in 'packets' between adjacent presynaptic boutons [27]. These results indicate that local exchange of multiple components occurs within presynaptic structures, and that optical highlighters afford sufficient temporal and spatial resolution to track this process.

Tracking translation and transcription with highlighters

In addition to enabling molecule tracking at the synapse, optical highlighters can enable real-time tracking of *de novo* protein synthesis. Certain forms of synaptic plasticity likely require the local translation of specific mRNAs into protein via synthetic machinery located within the dendrite [28,29]. Although pharmacological and molecular studies (often utilizing pulse-chase approaches) have strongly suggested that protein translation both occurs locally and may be causally involved in synaptic plasticity, visualization of this process has been difficult. The generation of translational reporters consisting of the cDNAs for two-color photoconvertible proteins fused to the UTRs of translationally regulated messages has facilitated the identification of both the signaling pathways that impinge upon the local translational machinery and the cis-acting elements that confer translational regulation upon specific messages. In these experiments typically a photoconvertible fluor such as Kaede is converted to its active red color, and then the stimulus-induced rate of translation of the reporter is revealed as *de novo* green fluorescence. This strategy has been used to examine the dendrite-specific translation of Kv1.1, CaMKII and Lypla1, and the axon-specific translation of β-actin [30–37]. Recently Martin and colleagues used a Sensorin-Dendra2 reporter to test synapse-specific mRNA translation in a culture system in which single, defined synapses between Aplysia sensory and motor neurons can be both manipulated and imaged [38••]. These experiments demonstrated that stimuli known to initiate facilitation (such as five pulses of serotonin) trigger synapse-specific translation, whereas stimuli that are ineffective (such as a single pulse of serotonin, or a pulse of FMRFamide) fail to generate new protein. It has also recently been observed that certain forms of synaptic plasticity (such as homeostatic plasticity [39]) may require targeted protein degradation; because photoconversion allows photoconvertible proteins to be used as optical pulse-chase reagents, these molecules can track stimulus-induced protein destruction. For example, degradation reporters have been generated in which PA-GFP is fused to a variety of protein motifs that can target peptides to the proteasome, and used to show that the chronic manipulation of activity can alter dendritic protein turnover [34,35].

Optical highlighters have also been used to reveal mechanisms of new gene expression, which is thought to be essential to the induction of synaptic plasticity over long time scales [36,37]. This process canonically involves recruitment of cellular signal transduction molecules that culminate in the regulation of transcription factors in the nucleus. However, it has recently been suggested that some transcription factors are held in abeyance in the dendrite, but that upon stimulation these factors are released and imported into the nucleus where they alter gene expression to promote synaptic modification [40]. For example, in the Aplysia system the transcription factor CREB2 has been suggested to translocate from dendrites to the nucleus, where in response to specific types of synaptic stimulation it initiates new gene expression required for long term depression. By tagging CREB2 with Dendra2, Lai *et al.* [41] demonstrated that CREB2 translocates to the nucleus in response to FMRFamide in an importin-dependent manner [41]. Similarly, tagging of the p65 subunit of NF-kB with PA-GFP has been used to demonstrate that dendritically localized NF-kB is actively transported from the synapse to the nucleus [42].

Axonal transport and long range protein movement

The long distances between the soma and the tips of an axon present challenges to the cell in moving proteins to and from these distal points. In addition to translating mRNAs at peripheral sites (as discussed earlier), neurons deliver proteins to axons via least three other mechanisms, each of which has been clarified through the use of optical highlighter-cargo fusion proteins. Neurofilament fusion proteins, for instance, have been shown to move along microtubules [43] and rely on myosin Va to keep them on microtubule tracks [44]. Fusions of at least two

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soluble cytosolic proteins, synapsin and CamKII, have been found to be transported via a microtubule-based mechanism in which large multimeric complexes interact with motor proteins [45•]. Diffusion can also be a primary mode of transport. For instance, highlightable fusions of the microtubule binding protein tau undergo rapid binding to and unbinding from microtubules during transit to the end of an axon, with transport kinetics indicative of free diffusion to the tip of the neurite [46,47]. Fusions of even slowly diffusing transmembrane proteins, such as the beta-adrenoceptor [48] and voltage-gated potassium channel isoforms 1.3, 1.4, and 2.1 [49], have been shown to move by this mode. These studies of diffusive mechanisms have particularly benefited from using highlighting methods since measurements can be made at many distal points in the cell in addition to the irradiation spot. Consequently, mobility fraction and diffusion coefficient maps over large areas of the membrane and various axon segments can be easily established.

Finally, optical highlighters are also well suited for studying trafficking in the secretory pathway. Since new proteins are often continuously produced over the course of an experiment, photoactivated or photoconverted pools effectively provide a time stamp for a given population of molecules. Transport studies of UNC-2, the *C. elegans* voltage-gated calcium channel, CaV2, found that a newly discovered endoplasmic reticulum (ER) localized chaperone, CALF-1, is required in conjunction with a calcium channel subunit, alpha2 delta, for proper exit of UNC-2 from the ER [50]. Photoconverted (and thus time stamped) Dendra2-tagged UNC-2 exhibited transport from the ER to synapses, but only in the presence of calf-1, indicating that preexisting protein within the ER exits through interactions with this newly described chaperone.

Cell fate mapping

Mapping the fates of individual cells or small numbers of cells during development requires the ability to introduce a label into a sparse number of cells at a specific point in time. Traditionally this has been achieved through laborious single cell methods like DNA electroporation, through the use of genetic chimeras, or through clever genetic approaches such as MARCM and MADM [51]. However, these physical or genetic methods are often impractical or impossible (i.e. because of physical access issues or the absence of effective mitotic recombination in the chosen model organism). The ability to generate focal signals with high contrast ratios through precision photoactivation — particularly via multiphoton methods — potentially circumvents these limitations, and has led to the widespread use of optical highlighters to track cell fates during development. This approach was first used to track the fate of neural crest precursors in chick embryos; chicks were electroporated *in vivo* with plasmids expressing PA-GFP under the control of a ubiquitous promoter, and then single neural crest cells (NCCs, located on the dorsal surface of the neural plate) were photoactivated and imaged [52].

Since this initial demonstration, a number of different highlighters have been successfully deployed in the chick [52–57], zebrafish [58,59,60••,61•,62], xenopus [63], ciona intestinalis [64••] and in mammals [52,54,57,61•,63,65,66] to track the fates of neural precursors during development. These experiments have correlated the initial positions of neural precursors with the final positions of differentiated neurons [52,54,57,61•], visualized interactions between migrating neurons [65], assessed proliferation during migration [57,65], followed the fate of cells during metamorphosis [64••] and tested the role of individual signaling molecules and transcription factors in cell migration processes [54,63,66]. By restricting the pattern of optical highlighter expression through the use of cell-type specific promoters researchers can precisely characterize the tracked neuron's function. For example, interneuron subtype-specific promoters driving Kaede expression in zebrafish have been used to address the relative role of CiD and MCoD interneuron types in fast and slow forms of swimming, respectively [61•]. Furthermore, a combinatorial technique called BAPTISM, which combines promoter-specific expression of a photoconvertible fluorescent protein (*huc::kaede*, for example) with EGFP expression under the control of a second cell-type specific promoter (*p2x3b::egfp* and *trpa1-b::egfp*, for examples), has enabled researchers to birthdate multiple different cell types was enabled within a specific pool of neurons (Figure 2) [60••].

Circuit mapping

Freely diffusing optical highlighters not targeted to specific subcellular compartments have been used to great advantage in measuring diffusional coupling between spines and dendrites (see above) [7]. To a first approximation, highlighted molecules within any cytoplasmic compartment of the neuron have free access to the rest of the neuron. Because the anatomy of the neuron is regionally specialized based upon connectivity — with input-receiving dendrites spatially segregated from output-transmitting axons — researchers have used photoactivation methods to focally activate a protein within the dendrite or axon and then utilize its de novo fluorescence to visualize (via diffusion) the contiguous cell bodies, axonal arbors, and dendrites. While labeling is restricted to contiguous structures within a given neuron, the complete morphology of targeted neurons and potentially the direction and nature of information flow (at least as constrained by anatomy) within a neural circuit can be revealed with this approach.

This strategy was first deployed in a paper in which Kaede was expressed under the control of the neural HuC promoter in zebrafish followed by focal photoconversion

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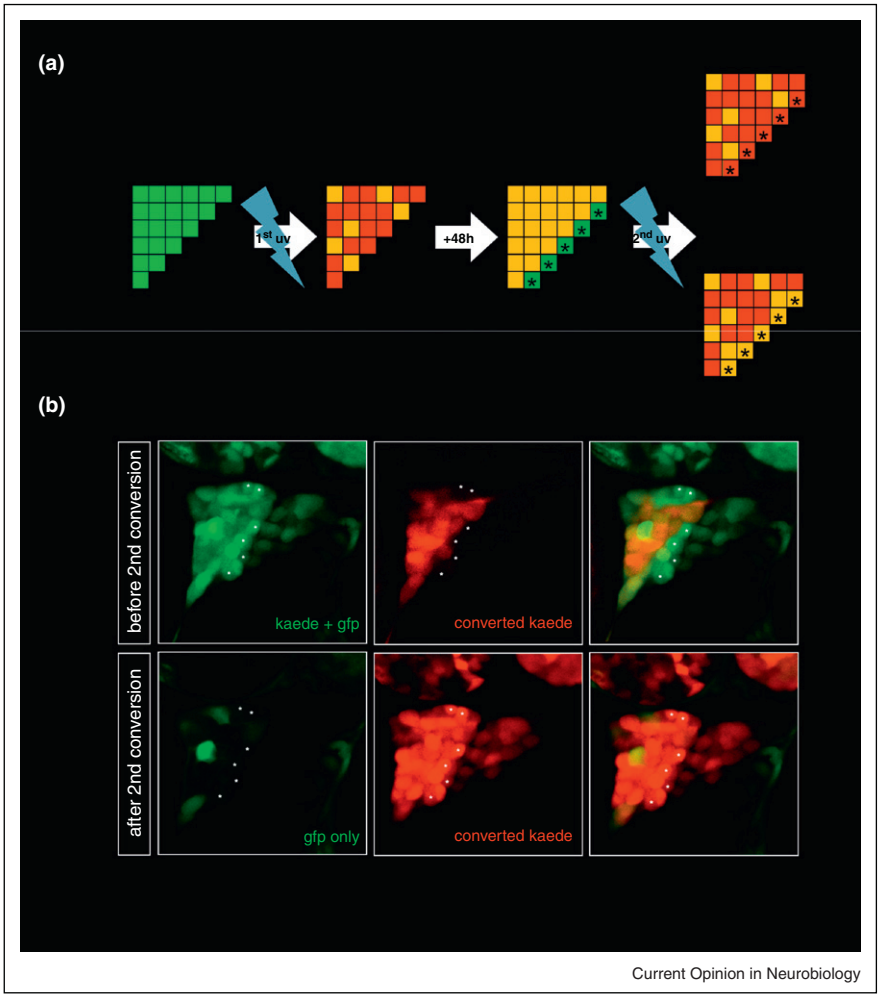
Figure 2



BAPTISM allows birthdate analysis of genetically marked subpopulations of neurons. **(a)** Expression (using different drivers) of Kaede (green) and a GFP marker to label specific populations of neurons (green) has all cells initially in a green state; after photoconversion, those cells that co-expressed Kaede and the marker are yellow, while those that expressed Kaede alone are red. Newly born cells are green. After a second round of photoconversion, cells that are in the GFP-expressing genetically marked population are yellow, whereas those that are GFP-negative are red. **(b)** Example use of BAPTISM in zebrafish sensory neurons. Kaede is expressed under control of a general neural promoter, and GFP under the control of a promoter for the gene trpa1b. After a single round of photoconversion and a delay period, newly developed cells can be identified based upon green fluorescence (upper right panel, see white dots). To determine whether these cells express trpa1b, a second round of photoconversion was performed; because the resultant cells are red (and not yellow), these cells do not express trpa1b (lower right).
Images and cartoon courtesy of Sophie Caron and Alex Schier (Harvard University).

to reveal the complete anatomy of targeted trigeminal neurons and Rohan-Beard cells in the ventral spinal cord [67]. Aramaki *et al.* [59] performed a similar experiment in zebrafish, but with a twist: by using the 'rewritable' photoswitchable fluor Dronpa (expressed in neurons using the Gal4–UAS system), these researchers were able to interrogate a targeted neuron's anatomy, erase the fluorescence, and then target and image a second neuron (and so on with multiple neurons in series) [59]. In this iterative manner, structural features of a number of different neurons presumed to be within a single neural network were identified.

While these two proof-of-principle papers demonstrated that optical highlighters could be used as long-range neural tracers, subsequent papers in the fruit fly Drosophila melanogaster illustrate the power of this approach to dissect the structure and function of behaviorally relevant neural circuits. One such circuit is responsible for sexually dimorphic behaviors in response to the pheromone cis-vaccenyl acetate (cVA), which (in particular contexts) elicits aversive behaviors in males but is attractive to females [68]. To address whether differences in downstream circuits are responsible for sexually dimorphic responses to cVA, Datta *et al.* [69••] expressed PA-GFP

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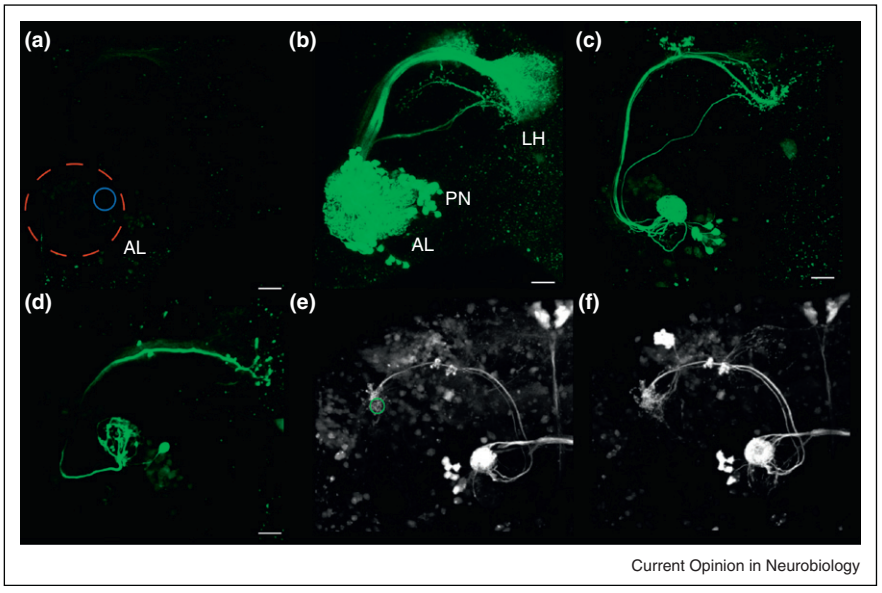
in most projection neurons in the antennal lobe, and then took advantage of the spatial specificity afforded by multiphoton techniques to photoactivate the PA-GFP specifically within the DA1 glomerulus; the PA-GFP then diffused, labeling the connected cell bodies and revealing sexual dimorphism in the DA1 projection neuron axonal arbors (Figure 3) [69••]. Furthermore, the induced fluorescence enabled electrophysiological characterization of DA1 projection neurons via cell-attached recordings.

Because this approach depends upon the inherent diffusional characteristics of PA-GFP, the total neurite length that can be traced is limited, and effective tracing requires photoactivation of a significant amount of PA-GFP (through repeated cycles of activation over relatively large target regions). These constraints have been partially circumvented through the development of two variants of PA-GFP (C3PA and SPA) that exhibit improved apparent rates of diffusion *in vivo* [70••]. By expressing C3PA and SPA in neurons potentially connected in a circuit, Ruta *et al.* [70••] traced a single neuron and identified its axonal arbor, and then irradiated the newly labeled axonal arbor, thereby taking advantage of the spatial overlap with the connected dendritic spines to highlight the downstream neuron (Figure. 3). By iteratively repeating this process, a behaviorally relevant neural circuit was traced across multiple synapses and through multiple neurons [70••].

As work in neural circuits becomes increasingly focused on interrogating the role of individual neurons in driving behaviors, researchers are finding new roles for optical highlighter proteins in disambiguating functionally relevant neurons from bystander cells. For example, a common difficulty is that the genetic drivers that are used to functionally manipulate the activity of neurons promote gene expression within large numbers of cells, making it difficult to identify the particular neurons responsible for a specific behavior. Claridge-Chang *et al.* used optical highlighters to identify which subset of cells labeled by their driver was relevant to behavior on the basis of connectivity revealed by targeted photoactivation of specific neurons [71••]. Similarly, a number of researchers have begun co-expressing various photoconvertible proteins along with optogenetic reagents; optical stimuli then both trigger changes in electrical activity via the light-gated channel, and photoconvert and label the

Figure 3



Neural tracing using PA-GFP. **(a)** Image of a fly brain (obtained with multiphoton imaging) in which olfactory projection neurons are labeled with PA-GFP. Note that only background fluorescence is apparent, but this background is sufficient to identify the antennal lobe (AL, red circle), as well as specific glomeruli within the AL such as the DA1 glomerulus (blue circle). **(b)** Photoactivation of the AL (red circle from (a)) causes the PA-GFP to photoconvert, labeling all of the connected projection neuron cell bodies (PN) and axonal projections to the lateral horn (LH). **(c)** By photoactivating only the DA1 glomerulus (blue circle from (a)) one can label and identify those specific projection neurons that innervate the DA1 glomerulus and trace their projections to the LH. Note that the induced fluorescence in the cell bodies is sufficient to guide electrodes for physiological characterization of these neurons. **(d)** To label single DA1 PNs using this technique, one can very briefly photoconvert the DA1 glomerulus, and then identify those PN cell bodies that are connected to the glomerulus through their transient increase in fluorescence. By using this transient signal one can then target the cell body itself for strong photoconversion, enabling tracing of single neurons. **(e)** Pseudo-trans-synaptic tracing using SPA-GFP to identify neurons connected to the DA1 PNs. Flies were generated in which many neurons relevant to sexual behaviors and putatively connected into functional circuits express SPA-GFP (Fru::SPA-GFP), the DA1 glomerulus was photoconverted, and the axonal arbor within the LH identified. The ventral male-specific region of the arbor was then targeted for photoconversion (green circle), thereby photoconverting SPA-GFP within the dendrites of connected neurons **(f)**. Diffusion of the photoconverted SPA-GFP, even from this limited region of dendrite, is sufficient to label cell bodies and axons of the connected neurons.

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stimulated cells, enabling posthoc verification of their identity [72•,73•,74•]. Photoconvertible fluors are thus proving to be useful adjuncts in optogenetic experiments aimed at testing neural function.

Cell and organelle continuities

Similar to mapping circuits, the advantages of an optical highlighter become apparent when determining organelle continuities as well as both intracellular and intercellular continuities. Axonal regeneration [75] has been confirmed by photoconverting freely diffusing protein either in the cell body or distal axon and monitoring the presence of red and green signal mixtures. Continuity between cells has been established by observing photoconverted KikGR moving bidirectionally into neighboring cells within the neural crest of chick embryos via cytoplasmic bridges [76]. Finally, axon degeneration has been confirmed by highlighting fragmented axon segments to ensure that subsequent re-fusion of fragments did not take place [77].

Summary and outlook

Some of the experiments discussed here represent approaches that are difficult or impossible with conventional fluorescent protein imaging, demonstrating that optical highlighters have moved from being neat little fluorescent protein tricks to bona fide neurobiology tools. Yet, the challenges of imaging in the nervous system will likely require further optimization of these molecules, with emphasis on brightness, contrast, photostability, monomeric behavior, red-shifted wavelengths, improved folding efficiency, faster folding kinetics, and higher photoactivation quantum efficiency. Improvements in any and all of these parameters will assist in neurobiology experiments that seem to be delving deeper into tissue, imaging with faster kinetics, and observing over longer timescales. If history provides any reasonable forecast, the rapid development of highlighter molecules over the past 10 years is likely to continue and provide tools to facilitate new and innovative approaches.

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- •• of outstanding interest

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PSD-95, CaMKII alpha and beta, GluR2, and stargazin were tagged with PA-GFP to determine their relative diffusion kinetics. Each is found to have different kinetics of turnover in individual spines in hippocampal slice CA1 neurons, with PSD-95 being the slowest and retaining ~90% fluorescence after 30 min. Palmitoylation of PSD-95 was found to be responsible for the slow kinetics and a structure/function analysis showed that only the two N-terminal PDZ domains, which interact with NMDA and shaker channels, are required for stabilization of photoactivated PSD95 within the spine. On the other hand, a third PDZ domain and the C-terminal portion of the protein were implicated in establishing the PSD lattice within the spine.

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Photobleaching of PSD-95-GFP and photoactivation of PSD-95-PA-GFP was used to track PSD-95 dynamics within single spines. Optically tracking PSD-95 revealed that each spine had structural dynamics that were independent from those observed in adjacent spines, and that the post-synaptic density followed those dynamics. However, little exchange of PSD-95 was observed within the PSD (on timescales ranging from 60 s to 30 min), leading the authors to suggest a model in which the PSD is largely stable but capable of flexible conformational changes to accommodate alterations in spine shape.

12. • Honkura N, Matsuzaki M, Noguchi J, Ellis-Davies GC, Kasai H: **The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines**. *Neuron* 2008, **57**:719-729.
Photoactivation of actin-PA-GFP was used to observe treadmilling and revealed that two pools of actin are normally present in spines. The actin toward the spine tip shows high turnover (tau = 40 s) while actin toward the base shows low turnover (tau = 17 min) and represents ~15% of actin in spine. Activation with caged glutamate resulted, in a third stable pool that is confined to the spine and is necessary for spine enlargement. Photoactivation at spine tips showed a retrograde stream of actin-PA-GFP, which does not join the stable pool at the base.

13. •• Frost NA, Shroff H, Kong H, Betzig E, Blanpied TA: **Single-molecule discrimination of discrete perisynaptic and distributed sites of actin filament assembly within dendritic spines**. *Neuron* 2010, **67**:86-99.
This study uses two-photon excitation microscopy to photoconvert and image single actin molecules in spines. Diffusion was found to be slow, consistent with treadmilling in the spine. By differentially targeting regions of spine, treadmilling was noted to be faster in the tip than in the core. PALM using one-photon photoconversion of actin-mEos2 revealed numerous single molecule trajectories whose measured velocities were faster than those measured by bulk flow; an inverse relationship between velocity and total distance traveled was noted. Blocking polymerization with jasplakinlide arrested most movement. Two color PALM on fixed samples with Dronpa-GKAP to mark the PSD showed that actin abuts the PSD. Single color PALM on actin plus regular diffraction-limited imaging with PSD95-cerulean showed that actin density is maximal at edges of the PSD.

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required combining de novo labeling of these neurons with PA-GFP with image registration techniques. In addition, the fluorescence induced by photoactivation of dendrites of these neurons revealed cell bodies that could be targeted for cell-attached electrophysiological recordings. These experiments showed that axonal projections to higher brain of this class of projection neuron are sexually dimorphic and under control of the genetic sex determination hierarchy, with male neurons elaborating a male-specific process within the lateral horn.

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