Methods To Locate and Track Ion Channels and Receptors Expressed in Live Neurons

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ABSTRACT: Brain cells use electrical and chemical signaling to communicate with each other and to send and receive information from the body. These neurons also encode information such as memories and are constantly adapting to changes as a result of positive alterations, such as learning, or negative events, such as neurological insults or neurodegeneration. In the last two decades, it has become clear that the placement of minute branches of neurons and, more importantly for the topic of this review, the placement of individual protein molecules, are the key events that enable neuronal network building and pruning. Advances in both electrophysiology and light-based imaging have allowed neuroscientists to answer fundamental questions about the key proteins involved in memory formation, maintenance, and loss. These findings have been enabled often through the clever use of chemical biology, biotechnology, and genetic engineering. In this review, we highlight numerous examples where chemical biology was used to provide new tools to answer difficult and near impossible questions in neurobiology.

KEYWORDS: *Neuron, fluorophore, ion channel, receptor, trafficking, fluorescence microscopy*
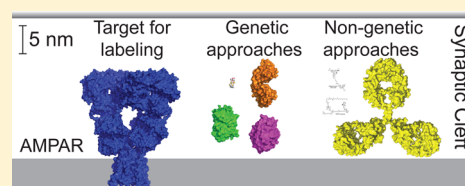
Neuronal ion channels and receptors are key molecules that underlie the adaptable nature of the brain.[1,2] These proteins can move from internal stores to surface-exposed locations and can traffic intracellularly or on the surface diffusively along dendrites, axons, and possibly at the synapse (Figure 1).[3] To visualize neuronal receptors, ion channels, and other targets on live neurons, one must be able to modify the target with a contrast agent. This generally involves the use of either small molecule fluorophores or proteinaceous fluorophores. The main methods for imaging neuronal receptors can be broadly divided into two categories, genetic methods that involve delivery or manipulation of DNA and nongenetic methods that involve biologics or small molecules. Here, we provide a review of some of the highlights of both of these methods, providing detail on certain seminal examples, and then a prospectus on potential directions for this field. It should be noted here that no perfect method presently exists, but we have attempted to offer a balanced view of the pros and cons of each example we discuss (Figure 2).

GENETIC TECHNIQUES TO PROVIDE OPTICAL CONTRAST

Intrinsically Fluorescent Proteins. Fluorescent proteins (FPs) have ushered in a new age in our understanding of important cellular functions and interactions. Our understanding of the biology and morphology of neurons have benefited greatly from FP advances as well as major improvements in imaging technology and instrumentation. One of the most striking applications of FPs and the brain has been the development of Brainbow,[4] a method that has allowed for the visualization of neurons and glia in 90 colors in model organisms such as zebrafish, *Drosophila*, and mice. Brainbow

involves the exogenous expression of cytosolic FPs to provide filled cells for serial reconstructions of connectivity. The stochastic nature of Cre/Lox recombination events determines the expression pattern of different FPs and these mixtures are perceived as different colors. By tracing individual colors through serial slices of brain tissue, one can begin to piece together a systems-level connectivity map of complex networks. However, a more commonplace use of FPs has been in the development of fluorescent fusion proteins. The cDNA of myriad neurotransmitter receptors, ion channels, and other important neuronal proteins have been genetically fused with FPs and transfected into neurons (Figure 3), thus allowing for the visualization of the location and, many times, highly dynamic alterations of protein locations in neurons.[5] FP fusions with neuronal proteins of interest are exquisitely specific because wherever the fluorophore is found, it is sure to be covalently attached to the protein of interest. This method has been used countless times to determine surface expression, receptor endo- and exocytosis mechanisms, and as a general reporter when using transient transfections to study, for instance, the biophysical properties of an ion channel in a neuronal environment. While some of these fusion proteins can be quite small (see section below on peptide tags), the vast majority of them are monomeric protein structures that are based on the GFP template originally made popular by Tsien and co-workers.[6]

Fusion proteins containing GFP variants require the attachment of a 28 kDa protein onto the receptor, which is
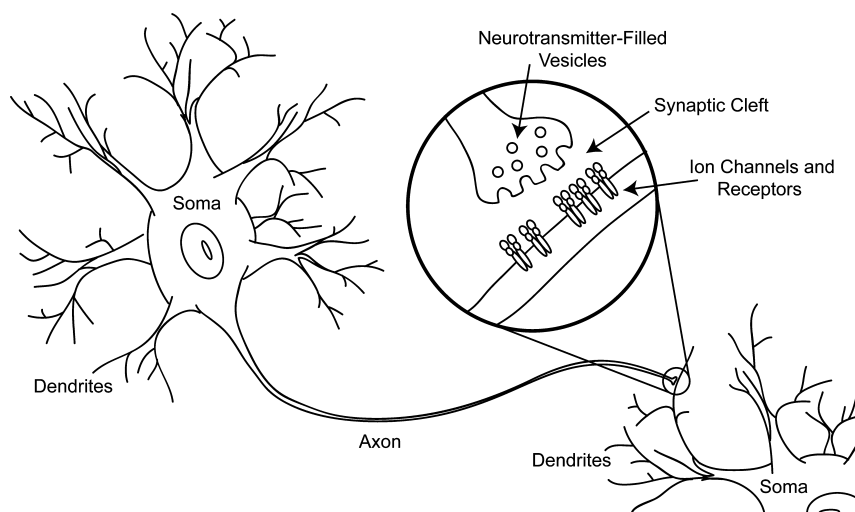
Figure 1. Cartoon depiction of two connected neurons demonstrating the connectivity between the axon of an upstream neuron and the dendrites of a downstream neuron. Magnified area shows the synaptic cleft with neurotransmitter-filled vesicles in the axon terminal and ion channels and receptors on the postsynaptic dendrite.
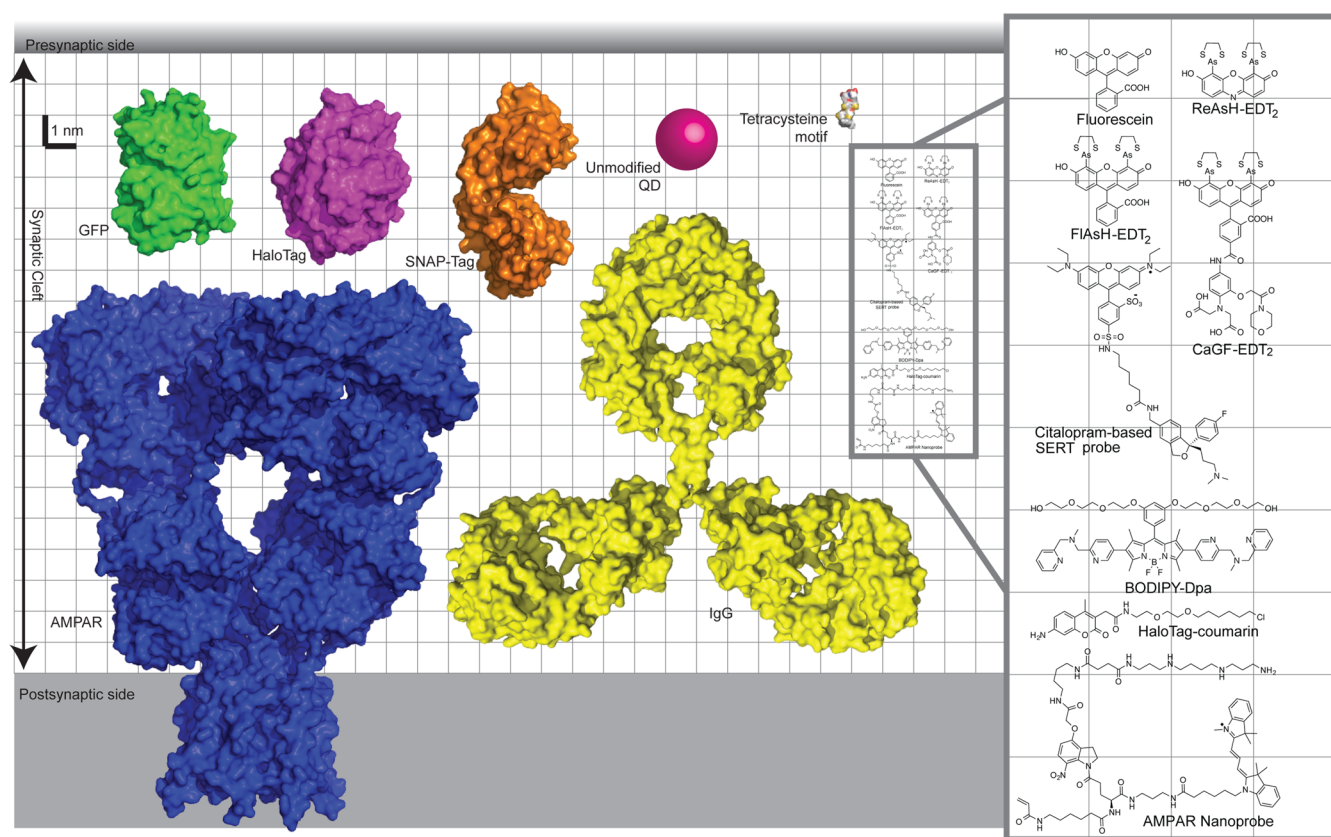

Figure 2. A depiction of a typical synaptic cleft and molecules of interest to scale. The synaptic cleft is represented by the gridded background and ranges from the presynaptic side (top) to the postsynaptic side (bottom) and has been found to be about 20 nm in width.[42] Represented as space filling models generated in Pymol[70] are the tetrameric GluA2 AMPAR assembly in blue,[71] GFP in green,[72] HaloTag in purple,[73] SNAP-tag in orange,[74] an IgG antibody in yellow,[75] a 2 nm unmodified QD in pink, and a tetracysteine motif model. On the right side of the figure are the chemical structures of various ligands, probes, and substrates that are discussed in this review. These are depicted on the same scale as the macromolecules and then larger to clearly provide the chemical structure. Background grid represents 1 nm per division for both the left and right side of figure.

itself sometimes a challenging process. When designing the fusion cDNA construct, the experimenter must consider N- or C-terminus fusion or insertion into a loop of the protein. In all cases, due diligence must be performed in comparing the

activity, location, and biophysics of the fusion protein to the native phenotype. Sometimes, these fusion proteins have been found to disrupt the natural movement of receptors as well as cause non-natural heteromultimers or homomultimers of the
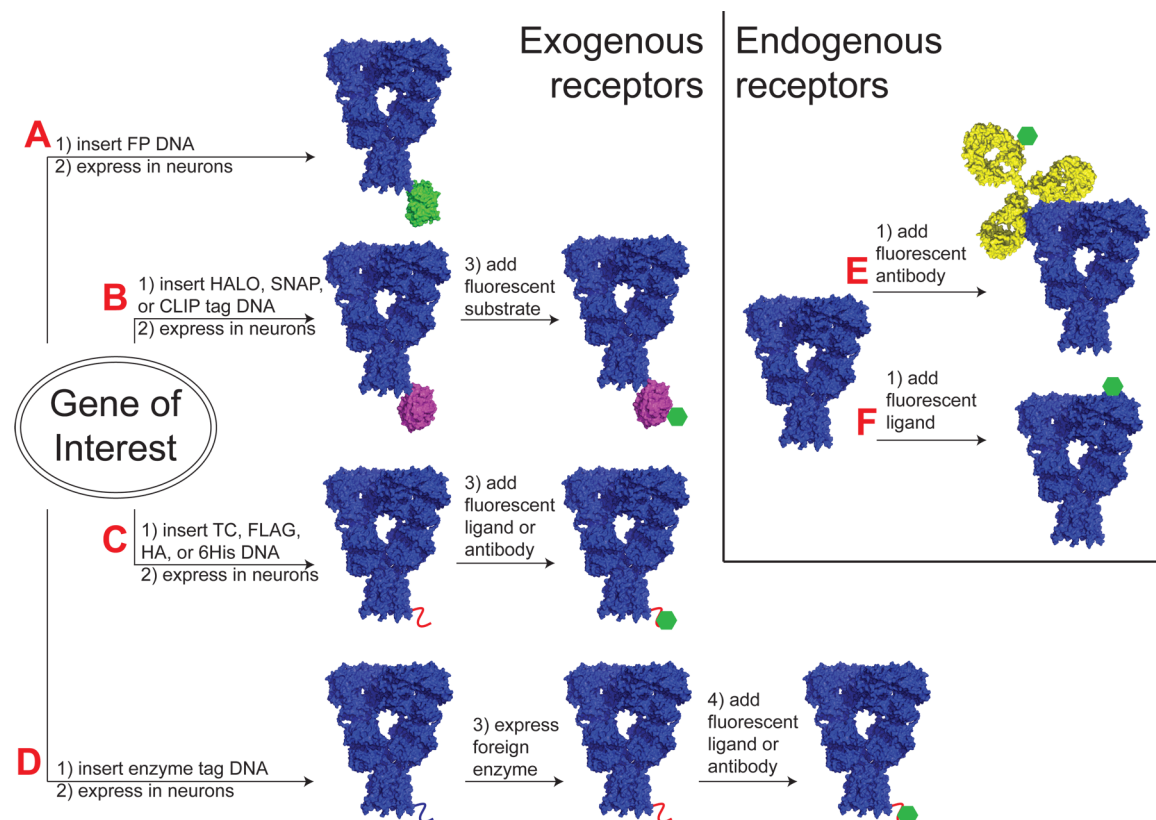
Figure 3. A schematic representation of the six main types of labeling discussed in this review. This figure provides a general overview of the steps involved in producing a receptor or ion channel that is fluorescent. Types A−D all require genetic manipulation of genomic DNA or transfection of neurons with foreign DNA. Types E and F work on endogenously expressed receptors and ion channels.

fusion protein to form.[7] To overcome the need for inserting such a large protein into the protein target, other genetic techniques have been developed that combine genetics and chemistry and are poised to preserve the native function of receptors by incorporating much smaller exogenous inserts.

Enzymatically Active Tags. *SNAP-Tag and CLIP-Tag.* A genetic technique that allows for the delivery of synthetic prostheses is the incorporation of a derivative of the DNA repair protein O^6-alkyl guanine-DNA alkyltransferase (AGT). This self-labeling enzyme, like FPs discussed earlier, can be inserted into a protein of interest. The intrinsic activity of the SNAP-tag then allows modification of the protein on which it is incorporated with synthetic derivatives of O^6-benzylguanine (BG) that contain cargo such as a fluorophore.[8] The BG probe acts as a substrate of the tag, which then delivers the cargo to a reactive cysteine in the AGT active site.[9] This system has enjoyed popularity and has been branded as SNAP-tag. Some of the key advantages of SNAP-tag are that it allows for specific labeling of intracellular proteins, has high native substrate specificity, and has low off-target reactivity. Additionally, the technology has been made commercially available and can be found in numerous expression vectors. SNAP can be used in conjunction with CLIP for use in two-color imaging experiments. CLIP, a close relative of SNAP, contains mutations of AGT so that it will react with O^2-benzylcytosine derivatives.[10]

The SNAP and CLIP tag technology has been used in a number of studies and has demonstrated great utility in deciphering multisubunit receptor assembly mechanisms and stoichiometry. The Pin group was able to use SNAP-tag technology in conjunction with time-resolved-FRET (TR-

FRET) to investigate the assembly of metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid type B (GABA_B) receptors. In the study, they labeled the N-terminus of both the GABA_{B1} and GABA_{B2} subunits with SNAP-tag and the FRET pair of probes consisting of functionalized BG probe with either europium cryptate or d2 (a propriety fluorophore from Cisbio designed to be an optimal acceptor in TR-FRET). They found that mGluRs assemble as strict dimers, but that GABA_B receptors assemble as dimers of heterodimers.[11] In more recent studies, SNAP and CLIP were used simultaneously to examine the coexpression of dopamine receptor 2 (DRD2) and ghrelin receptor (GHSR1a) in hypothalamic neurons. TR-FRET of CLIP-tagged DRD2 and both SNAP- and CLIP-tagged wtGHSR1a and GHSR1 point mutants were used, and it was found that DRD2 and GHSR1a form heterotetramers at equimolar concentration in HEK293 cells. The assembly was also found to be reliant on GHSR1a. Further experiments using TR-FRET in hypothalamic neurons from *grsh*^{+/+} mice showed the same effect demonstrating the presence of GHSR1a/DRD2 heteromers in native hypothalamic neurons.[12]

While these tagging strategies offer exquisite specificity, the one point of concern of the SNAP- or CLIP-tag technology is the sheer size of the insert that is required. The size of the O^6-alkyl guanine-DNA alkyltransferase is around 20 kDa, which is roughly 3/4th the size of FPs; therefore to employ this method, one must introduce a large protein onto a neuronal receptor. As mentioned above, interference from this increased steric burden as well as complications with natural assembly and dynamics of the protein of interest must be controlled for when conducting experiments in live cells.

HaloTag. Another enzymatic method to self-label proteins of interest is the HaloTag. This technology consists of a modified bacterial haloalkane dehalogenase, which allows for alkylation of the protein of interest with a synthetic ligand containing a primary haloalkane. The ester that is formed is on the carboxylate residue of the catalytic aspartate. The enzyme was engineered in such a way that mutation of a crucial histidine near the catalytic aspartate leaves the typically transient covalent ester intact.[13] HaloTag is itself around 34 kDa and can be genetically fused to the protein of interest via a standardized 16 amino acid linker derived from tobacco mosaic virus.[14] For optimal HaloTag activity, extensive studies revealed that the length of the alkyl linker to the fluorophore or other cargo is crucial. Luckily, the chemistry to attach different linkers to cargo is fairly trivial. The optimal ligand for HaloTag labeling has been found to be fluorescein functionalized with a chloroalkane generally six carbons in length.[13] Of course, alternative fluorophores have been employed including Alexa 488, Oregon green, tetramethylrhodamine, Dy633, and IR800.[13,15]

HaloTag has been used in sympathetic neurons grown in compartmental culture. This neuronal culture method allows for growth of distal axons, which then enables long distance effects including protein transport to be measured more efficiently and reliably than in simple dissociated cultures.[16] HaloTag was used to measure retrograde axonal transport in these cultured neurons by fusing HaloTag to the N-terminus of tublin α-1B and the N- or C-terminus of p75 neurotrophic factor (p75nTR), a protein that plays a role in the survival of sympathetic neurons because it binds both neurotrophins and preneurotrophins. The results of this work offer a method to analyze the transport of these factors both in imaging experiments and in other biochemical techniques such as SDS-PAGE and immunoblot assays.[17] Another example of HaloTag use in neurons is a HaloTag–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fusion. This system was employed to monitor the translocation of GAPDH from the cytosol into the lysozome to investigate the mechanisms of cell mediated autophagy in Purkinje cells, and this work demonstrated a new method for studying this important cell mediated process.[18]

Much like the FPs and the SNAP- and CLIP-tag systems, the main potential disadvantage of the HaloTag technique is the size of the required insert. The size of the protein is around 30 kDa; however, it should be noted that reports have indicated that the large size does not disrupt protein function.[19] Of course, individual instances will vary and caution should thus be used whenever employing a genetically modified protein of interest as discussed above.

Small, Peptide-Based Tags. *Tetracysteine Motifs.* The most widely used method to get around the concern of insert size required to use FPs is the incorporation of a tetracysteine motif into the neurotransmitter receptor of interest and to then target this motif via a biarsenical ligand.[20] This system works by making use of the relatively high affinity between sulfur and arsenic, the lack of endogenous arsenic, and the multivalency supplied by four bonds between the tetracysteine motif and the single small molecule. A variety of biarsenical ligands have been synthesized that provide a wide range of colors available for experiments. These include green fluorescein arsenical hairpin binder (FlAsH),[21] red resorufin arsenical hairpin binder (ReAsH), blue xanthene arsenical hairpin binders HoXAsH and CHoXAsH,[20] and modular approaches such as SplAsH,

which can attach a fluorophore of any color.[22] In all of these approaches, the tetracysteine motif (C-C-X-X-C-C) is genetically inserted into a protein of interest with the hope of not perturbing native function. As stated earlier, experimenters must carefully examine the function of the genetically modified protein to be certain no untoward effects are introduced that may confound data collection or analysis. The arsenic-functionalized fluorophore is typically delivered to the tetracysteine motif in complex with ethanedithiol and becomes up to 10-fold brighter upon binding and coordination to the tetracysteine motif.[20] For FlAsH and ReAsH, the optimized tetracysteine motif contains flanking residues that were engineered to provide brighter fluorescence and resistance to dithiol reduction, which allows for a relatively harsh treatment step to remove nonspecific interactions with other proteins. These sequences are HRWCCPGCCKTF and FLNCCPGCCMEP, respectively,[23] with the structure of the latter motif in conjunction with ReAsH having been solved by NMR to determine the hairpin structure.[24]

Once a fluorophore is delivered to a tetracysteine motif, imaging of protein location and dynamics is enabled. Orthogonal experiments have also been performed on fluorophore-loaded tetracysteine motifs including the use of FlAsH in fluorophore-assisted laser inactivation (FALI) to perturb systems and allow observations from a set time point.[25] Marek and Davis combined FlAsH-labeled proteins in neurons to photoinactivate synaptotagmin I at *Drosophila* neuromuscular junctions via FALI, a feat that allowed them to showcase FlAsH–FALI as a powerful method. These experiments gave comparable results as antibody-based techniques. They were also able to show that in the neuromuscular junction of *Drosophila*, synaptotagmin I is involved in a postdocking step of vesicle fusion and that it is required for calcium dependent transmitter release.[26] Soon after, Robert Malenka's group used a combination of FlAsH and ReAsH to examine the intracellular trafficking of GluA1 and GluA2 subunits by inserting the tetracysteine motif on the C-terminus.[27] They observed that the dyes were specific toward the receptors of interest, could report on protein synthesis, and were nontoxic. They demonstrated that GluA1 and GluA2 subunits were locally synthesized in dendrites and not just in the soma by first applying a pulse of FlAsH and then applying a ReAsH chase 4–8 h later.

The tetracysteine motif has also been employed in live cell calcium imaging because it can bind myriad differently functionalized biarsenical-containing fluorophores. Using a calcium-sensitive chromophore, Roger Tsien and colleagues used the tetracysteine tag to detect and report on the fast kinetics of calcium release. Tsien then improved on his FlAsH system by synthesizing Calcium Green-FlAsH (CaGF). The core of CaGF provides improved fluorescence, offering a larger dynamic response upon calcium binding. CaGF was developed further as a cell-permeable AM-ester, because the multiple carboxylates on CaGF make it less membrane permeable than either FlAsH or ReAsH. CaGF was then showcased by monitoring L-type calcium channels at gap junctions.[28]

Advantages of the tetracysteine technology are that the binding tag itself is very small (ca. 0.7 kDa) and thus can more easily be developed into a silent genetic alteration. In addition to the small tag size and variety of compatible fluorophore colors, the coordination between the tetracysteine tag and biarsenical fluorophore can be quickly reversed by treatment with 2,3-dimercapto-1-propanol (BAL) or 1,2-ethanediol

(EDT) allowing for dynamic studies. Finally, the tetracysteine motif has been employed for both intracellular domain labeling and protein domains that are found in oxidizing environments such as extracellular space.[25]

Introduction of a polyhistidine tag (His-tag) onto a protein of interest has been an invaluable method to make protein purification routine. The method is made possible by exploiting the affinity and multivalency of polyhistidine for nickel nitrilotriacetic acid (NTA) resin. More recently, the His-tag has been used for imaging experiments by employing fluorescent probes that have affinity for the exogenous polyhistidine motif.[29] Others were able to improve the selectivity of the interaction between polyhistidine sequence and Ni-NTA probe by nearly 1000-fold with the introduction of multiple multivalent chelators onto one ligand.[30,31] Further, the addition of polyproline II helices as rigid spacers in the ligand design improved the quantum yield and made the ligand amenable for use in single-molecule experiments. These were termed *tris*NTA-PPII-fluorophore probes, and this method was employed on a His-tagged GFP-fused GluA1 receptor subunit where colocalization of *tris*NTA-P_8-ATTO647N fluorophore was observed, thus demonstrating the applicability of the probe for use with super-resolution molecular imaging techniques. A dual advantage to this application is that the *tris*NTA-fluorophores are nontoxic and can be refreshed into the bath to combat photobleaching.[32] A similar concept was used in the introduction of oligo-aspartate tags, which demonstrate affinity for multinuclear zinc complexes. The design is such that an oligo-aspartate sequence (D4 tag) is encoded on the protein of interest and is targeted by binuclear Zn(II)-2,2′-dipicolylamine presented on a tyrosine scaffold (Zn(II)-DpaTyr). The choice of Zn(II)-DpaTyr allows for a multivalent metal−ligand interaction between zinc and aspartates on a scaffold that can be easily modified. The advantage of this tag design is that it allows for fluorescent molecules to be noncovalently incorporated on the protein of interest yet retain an interaction that is stable enough to be used in further postlabeling analysis such as Western blots.[33,34] This approach was used in imaging neurofibrillary tangles from the brains of Alzheimer's disease patients. By functionalization of the binuclear Zn(II) complex with a BODIPY fluorophore, visualization of binding to neurofibrillary tangles could be achieved in brain slices from patients.[35]

Another method that employs short, molecularly recognizable tags is to target fluorescent antibodies to engineered sequences. Antibodies have been extremely useful as a diagnostic tool for identification of protein interactions. Antibodies bridge the gap between genetic and nongenetic techniques because they may make use of aspects from each. Antibodies are genetic in the sense that they are proteins raised to label a specific epitope or target sequence on the protein of interest. In fact, there are numerous examples of researchers expressing a specific epitope tag into a protein of interest to allow for a single antibody to be used. FLAG-tag (DYKDDDDK)[36] and HA-tag (YPYDVPDYA)[37] are two of the most popular methods that have been used by the neuroscience community to impart orthogonal labeling of proteins.

Enzymatic Substrate Tags. A creative alternative was developed recently by the Ting lab, which involves attachment of a 15 amino acid acceptor peptide to the protein of interest and then targeting this substrate sequence with exogenously expressed biotin ligase.[38] This results in bioconjugation of the peptide with biotin that can then be targeted with a streptavidin labeled moiety. The biotin ligase strategy has been adapted for single molecule imaging in live cells in conjunction with HaloTag and quantum dots.[39] The biotin ligase system is analogous to the powerful formylglycine strategy by Carolyn Bertozzi in that a separate enzyme is used to provide recognition of the substrate sequence and results in post-translational chemistry amenable for downstream bioconjugation.

For all of the findings that experiments using short tags along with accompanying small molecules have brought about, there are some caveats that must be considered. One point of concern when employing tetracysteine motifs comes from the relatively low specificity when using the system in an environment containing cysteine-rich proteins. The biarsenical ligand does not discriminate between the exogenously inserted tetracysteine motif and areas on the protein that are rich with cysteine. This can lead to misdirected targeting by the fluorescent ligand, confounding the desired outcome, since imaging may be of a mixed population of target protein as well as off-target, cysteine-rich proteins.

■ NONGENETIC TECHNIQUES TO PROVIDE OPTICAL CONTRAST

Nongenetic techniques for targeting neurotransmitter receptors include the use of antibodies and ligand-directed chemistry to deliver fluorophores. Due to the partial or full synthetic nature of these techniques, a broad range of colors and fluorophore properties are available for imaging after being delivered to a protein of interest. When designing a nongenetic tag for detection of neuronal proteins, there are many factors that should be considered. An ideal probe should be selective, water-soluble, nontoxic, and able to tolerate the environment inside the cell if it is targeted intracellularly. The fluorophore attached to either an antibody or small molecule probe should retain these properties, maintain brightness when bound to the system of interest, and be amendable for current imaging techniques. An excellent review of reactive and responsive fluorophores that have been used in bioimaging was done in 2012.[40]

Fluorescent Primary Antibodies. Antibodies can many times involve no genetic manipulation when the antibody is raised against a native epitope sequence that is part of a target protein. Antibodies have been used in both live cell and fixed cell imaging of neuronal targets where they have enabled location, quantification, and observation of the dynamic movements of neurotransmitter receptors. Antibodies are exquisitely specific toward their intended epitope, but they are large proteins, typically measuring around 10−15 nm along their longest dimension.[41] The synaptic cleft is approximately 20 nm,[42] so after conjugation with a primary antibody alone (not to mention that many times a fluorescent secondary antibody is used for visualization), the size of the entire complex is larger than the synaptic cleft, limiting their value for observing protein dynamics that occur at the synapse.

Quantum Dots. Quantum dots, QDs, are nanosized colloidal semiconductors that have properties that can be tuned during their synthesis. They can also be targeted to a biomolecule of interest by graphing ligands onto the surface of the QD. Targeting of QDs to a protein of interest often relies on the interaction of streptavidin and biotin[43] but also can be accomplished through acid−base interactions[44] and covalent ligand attachment to a polymer on the surface of the QD.[45]

QDs have the advantage of being brighter and much more photostable than organic fluorophores; however blinking of individual dots can lead to complications.[46,47] Another consideration is the size of QDs. The smallest useful QDs are around 2 nm, but after decoration with passive shells and other functionalities including targeting ligands or proteins, on average they are 20 nm in diameter, which is about the same size as the bank to bank distance of the synaptic cleft.[48]

QDs have been used in conjunction with antibodies for long-term tracking of proteins involved in synaptic transmission. An early and exciting use of QDs on live neurons was to track individual glycine receptors. To do so, mouse primary antibodies were raised against glycine receptors, which were then incubated with a secondary biotinylated anti-mouse Fab antibody and then streptavidin-coated QDs for visualization. Glycine receptors were found to localize in both the soma and dendrites where they were found in synaptic and extrasynaptic domains.[46] More recently, single particle tracking has been used to observe changes in populations of NMDA receptor subtypes NR2A and NR2B by the Choquet group. Primary antibodies were raised against these receptor subunits and the secondary antibody was decorated with either a QD or a single organic fluorophore. They found that synaptic subtype concentration changed based upon maturation of the cell, with the concentration of NR2A increasing and the concentration of NR2B decreasing over time. The use of QDs enabled them to track movements in specific synaptic compartments for up to 30 min. However, the authors of this study have pointed out that the use of QDs could result in a bias for some cellular compartments within a confined space, but a single molecule approach should reduce this bias.[49] Antibodies have also been used in live cell imaging for the detection of AMPARs, the main excitatory receptor family expressed in the CNS. Antibodies were raised against the extracellular N-terminus of GluA2 and receptor mobility was determined by performing fluorescence-recovery after photoinactivation (FRAP) studies.[50] They observed that GluA2-containing AMPARs were located in extrasynaptic areas in culture. They also measured the lateral diffusion of AMPARs and found that they can move at speeds above 0.25 $\mu m^2/s$, although this may underestimate the true mobility due to the steric burden imposed by the QD.

Ligand-Based Techniques. Another method for visualizing neuronal receptors involves the use of small molecules alone. Ligands, in conjunction with other chemical entities, have been used extensively to deliver cargo to receptors of interest. A tethered ligand that has been designed to bind to the receptor of interest is a tried and true way to bioconjugate the target receptor with a fluorophore or other cargo. These modifications can be covalent or noncovalent in nature, depending on the specific design of the probe. Our group has recently synthesized a polyamine based probe for the detection of calcium permeable AMPA receptors.[51] Borrowing structure–activity relationship data from the known polyamine toxins that act as use-dependent antagonists of calcium-permeable AMPA receptors, we incorporated an electrophile for bioconjugation and a fluorophore for tracking. In addition, part of our molecule was engineered to be photoreleasable where upon UV light can be used to excise the ligand via a nitroindoline-containing core, leaving the fluorophore behind attached to a nucleophile on the receptor. This strategy allows for tracking of receptor motion in a nonperturbed fashion.

Another ligand-directed method to deliver cargo to receptors of interest is to modify existing drug molecules so that they can be decorated with a fluorophore via a linker that does not interfere with target binding. Newman and co-workers have developed ligand-directed probes for the dopamine transporter (DAT), which include a synthetic ligand tethered to a fluorophore.[52] The result is a probe that can target the natively expressed DAT protein in neuronal culture. This is an important feature of the ligand-based approach to labeling. Since some neuronal receptors and ion channels are expressed at extremely low levels, the ligand-based approach does not require the overexpression of the target for visualization. Prior methods to visualize the DAT have relied on using heterologously transfected non-neuronal systems to avoid the low expression problem. They were able to label the DAT in rat midbrain neurons and to demonstrate that DAT was present in the soma, dendrites, and varicosities.[53] They were also able to show that the receptor is mobile and able to undergo internalization via endocytosis. Recently, an analogous probe was synthesized for the serotonin transporter (SERT) using similar methodology based on an analogue of the selective serotonin reuptake inhibitor citalopram. This probe was tethered to rhodamine and used in HEK293T cells expressing the SERT as a proof of concept.[54,55] A similar technique was also used in the development of a NMDA receptor probe by the Strømgaard group.[56] By affixing a fluorophore to the end of a naturally occurring antagonist, they were able to visualize the natively expressed NMDA receptors in live neurons, thus demonstrating the utility of the probe for visualization of active ionotropic glutamate receptors in culture.

QDs have also been conjugated with ligands to allow their direction to receptors of interest. In one example, GABA-$\alpha 1\beta 2\gamma 2$ heteropentameric receptors were labeled with a QD that had been functionalized with either muscimol or GABA itself, both GABA receptor agonists. The small molecules were appended to the QD via PEG 3400 chains that were in turn attached to an aminohexanoyl spacer. These ligand-decorated QDs were tested using *Xenopus* oocytes injected with mRNA encoding the GABA receptor subunits of interest and showed promising receptor specific binding.[57,58]

Peptide-Based Ligand Delivery. Another recent and fruitful method that is relevant to this review is the use of peptide-based ligands for targeting native proteins. Peptide synthesis is, today, trivial, and thus a plethora of peptide-based ligands can be optimized rapidly. Choquet and Imperiali have devised a synthetic biomimetic probe that is targeted to PDZ domains at synapses. PDZ domains are believed to govern the general dynamics of receptors and ion channels at the postsynaptic side of a synapse. In their strategy, a library of peptides derived from the C-terminus of stargazin, a transmembrane AMPAR regulatory protein (TARP), and the PDZ domains of PSD-95 were synthesized using solid phase peptide synthesis. They then selected the peptide with the highest affinity and appended chromophores such as 4-dimethylaminophthalimide (4-DMAP) or 6-dimethylaminonaphthalimide (6-DMN). These peptide-fluorophore constructs were tested in experiments involving AMPA and NMDA receptor dynamics in neuronal cultures. Initial testing of these probes revealed the need for newer probes to include divalent ligand attachment points in order to effectively disrupt the interaction between native receptors and their anchoring proteins. This is likely a result of the fact that AMPA receptors contain a stoichiometry of 2–4 anchoring proteins per functional AMPAR, as indicated by a block of synaptic AMPAR expression, but an uptick in endocytosis and lateral motion of those on the surface.[59] For

NMDA receptors, the dynamics of the 2A and 2B subunits were studied, and the PDZ biomimetic probe was able to block the interaction of the NMDAR-2A subunit and native PDZ proteins but not block the interaction of NMDAR-2B subunits. This suggests that the anchoring mechanism of the two is different.[60] In both of these cases, visualization of the proteins was enabled using QDs, and based on these results, a caged version of this probe is being developed to allow for high spatial and temporal control, which will in turn allow for precise targeting to areas of interest.[61]

A different peptide-fluorophore hybrid tool was devised by the Zenisek group to investigate the role of calcium at the ribbon synapse. The ribbon synapse is an interesting biological target because, unlike traditional synapses, the ribbon synapse undergoes essentially constant multivesicular release and has a very precise dependence on calcium signaling. The probe that they designed is a peptide dimer with the two targeting sites separated by a linker and is decorated with either Texas-Red or Fluo-4 for visualization. Modifications to the peptide have allowed for binding with higher affinity and, when used with Fluo-4, can report on local changes to the Ca^{2+} influx at the ribbon.[62]

Finally, the Desai group conjugated βNGF, a small protein that is crucial for autonomic, motor, and sensory neuron development and survival, to QDs. This complex retains bioactivity when tested in PC12 cells, and it was able to activate TrkA receptors to initiate neuronal differentiation. This technique could be used as a method to target ligand−receptor activity.[63]

The advantage of ligand-directed probes is that the specificity provided by the ligand drives the specificity of labeling the target. However, one of the main limitations for this method is that many times ligands are not completely specific for the target and have known interactions with alternate subunits of the same protein as well as interactions with off-target receptors. Another consideration with this method is cell permeability and target location. Some probes may be difficult to deliver inside of cells, and still others may be less than optimal for visualization of receptor recycling if they are sensitive to acidic or reducing environments. Issues do also arise from the use of heterologous systems for initial testing of probes. Since heterologous systems lack the same complex and nuanced downstream signaling pathways that occur in neurons, this can lead to false impressions of probe success and may not reflect how the probe will interact with the target receptor when used in native neurons.

Fluorescent False Neurotransmitters. Fluorescent false neurotransmitters (FFNs) are small molecules that structurally or chemically resemble naturally occurring neurotransmitters such that the neurons treat them as such. Yet these molecules can be optically tracked using fluorescence microscopy. The Sames and Sulzer groups used a fluorescent version of their neurotransmitter of interest for tracing dopaminergic signaling. To study presynaptic vesicular uptake and release of mono-amines including dopamine, they developed a small, fluorescent monoamine probe, which acts as an optical tracer for dopamine release. Current versions of the probe work with both the neuronal vesicular monoamine transporter-2 (VMAT2) and DAT.[64] FFNs were able to label presynaptic terminals in the striatum, showcasing the utility of this method as an optical tracer for dopamine signaling. Current versions of the probe have incorporated a pH sensitive fluorophore and are coumarin-based dyes. They act as VMAT substrates and are being used to investigate neurotransmitter accumulation and release during vesicular fusion regulated by synaptic plasticity. The environment-sensitive fluorophores also aid in detecting the pH gradient between the cytosol and vesicular lumen.[65]

■ SUMMARY AND PROSPECTIVE

As a whole, the techniques presented in this review have significantly expanded our collective knowledge about neurons and neuronal proteins. The creative scientists who have dreamed up genetic and nongenetic techniques to visualize proteins expressed in neurons have expanded the number of available tools for carrying out detailed studies of receptor dynamics, trafficking, localization, and organization. While all of the techniques described here have added significant knowledge to our understanding of neurons, the "holy grail" of this field is to be able to use fluorescence imaging for visualization of processes occurring in an active and behaving brain. The challenges surrounding this goal are numerous since deep-brain imaging at an almost subcellular magnification in a potentially moving model organism is required.

Near-IR fluorophores will have a large impact on our knowledge of fundamental neuroscience. These dyes allow for longer wavelengths of light to be used for excitation and emission, making use of the relative transparency of brain tissue in this optical region. Thus, light can penetrate deeper into tissue allowing for use in live and behaving animals via windows installed into the skull. These dyes are also spectrally far removed from much of the autofluorescence that the tissue produces, allowing for clearer imaging results. The use of these dyes could shed information on how complex neural pathways and systems as a whole work, rather than what has been seen in neuronal culture and slice preparations. Additionally, methodology such as CLARITY, a technique used to chemically fix and then clear endogenous chromophores from the brain, has already opened new avenues of research to help map the connectome.[66]

The recent development of precise genome targeting and editing via manipulations of the bacterial and archaea host defense system may revolutionize neuronal imaging. Using RNA guided Cas9 nuclease from a type II prokaryote (*Streptococcus pyogenes* SF370) clustered regularly interspaced short palindromic repeats (CRISPR), researchers have been able to heterologously introduce the CRISPR machinery (Cas9 nuclease, CRISPR RNAs (crRNAS), trans-activating crRNAs, and precursor crRNA) into mammalian cells for RNA guided genome editing.[67,68] CRISPR technology opens the door for experiments in model systems and will be of great value in the field of fluorescence imaging. One can imagine that soon directed genome editing will enable the introduction of fluorescent moieties (i.e., entire FPs or some of the enzymatic tags discussed in this review) or even a single directed codon mutation that would alter, for instance, a serine codon to cysteine (e.g., AGC to TGC). A single cysteine that is engineered into an extracellular loop of a protein is often chemically addressable and allows for covalent modification with functionalized maleimides thereby enabling bioorthoginal chemistry.[69] The future of genome editing will allow, for instance, the investigation of fluorescently tagged neuro-transmitter receptors that are expressed under endogenous control, thereby allowing visualization of receptor movement and production in as silent a way as possible. Of course, the addition of relatively large genetic fluorophores or tags may suffer from the steric problems discussed previously. However,

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small tags may result in receptors with no perturbations. Genome editing, in conjunction with deep-brain imaging with near-IR dyes, will soon open up even more experiments that today would be impossible.

■ AUTHOR INFORMATION

Notes
The authors declare no competing financial interest.

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■ DEFINITIONS OF TERMS

neuron, a cell in the brain that communicates with either other neurons or peripheral tissue; fluorophore, a molecule that can be excited by a photon of one wavelength of light and relaxes by emitting a photon of a lower energy; ion channel, an integral membrane protein that passes ions between the cytoplasm and extracellular space; receptor, a protein (many times an integral membrane protein) that binds to a ligand and modulates a downstream signaling event; trafficking, the movement of ion channels and/or receptors either on the surface of a neuron or from or to internal stores; fluorescence microscopy, the use of filtered light microscopy to excite a fluorophore with a higher energy light and then observing the lower energy light that is emitted. traditionally this is performed by using a series of optical filters that allow the user to select the wavelength of light allowed to pass while rejecting other wavelengths

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