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Genetically Encoded Spy Peptide Fusion System to Detect Plasma Membrane-Localized Proteins *in vivo*

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Summary

Membrane proteins are the main gatekeepers of cellular state especially in neurons, serving either to maintain homeostasis or to instruct response to synaptic input or other external signals. Visualization of membrane protein localization and trafficking in live cells facilitates understanding the molecular basis of cellular dynamics. We describe here a method for specifically labeling the plasma membrane-localized fraction of heterologous membrane protein expression using channelrhodopsins as a case study. We show that the genetically encoded, covalent binding SpyTag and SpyCatcher pair from the *Streptococcus pyogenes* fibronectin-binding protein FbaB can selectively label membrane-localized proteins in living cells in culture and *in vivo* in *Caenorhabditis elegans*. The SpyTag/SpyCatcher covalent labeling method is highly specific, modular, and stable in living cells. We have used the binding pair to develop a channelrhodopsin membrane localization assay that is amenable to high-throughput screening for opsin discovery and engineering.

Graphical abstract

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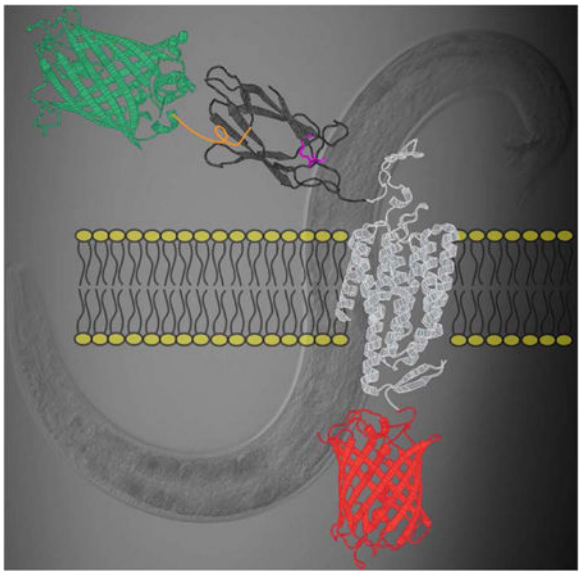

Keywords

Membrane localization; channelrhodopsin; SpyTag; SpyCatcher; Covalent labeling *in vivo*

Introduction

Real-time visualization of biochemical processes in living cells is aided by methods for specific protein labeling, including genetically encoded fluorescent proteins and synthetic probes. Since their first application as markers for transgenic protein expression and localization in live cells (Chalfie et al., 1994), genetically encoded fluorescent proteins (FP) have been engineered (Tsien, 1998) to offer a palette of colors with enhanced brightness (Goedhart et al., 2012; Tsien, 1998) and various useful properties such as reversible or irreversible photoswitching (Nienhaus and Nienhaus, 2014; Patterson and Lippincott-Schwartz, 2002; Piatkevich et al., 2013; Zhou and Lin, 2013) to aid in tracking protein dynamics (Dean and Palmer, 2014). Synthetic fluorescent probes that covalently label proteins have facilitated live cell imaging (Gautier et al., 2008; Juillerat et al., 2003; Keppler et al., 2003; Los et al., 2008; Uttamapinant et al., 2010) due to their irreversible, highly specific binding. These bright, cell permeable, spectrally diverse, fluorescent probes are ideal for microscopy of cells in culture (Lukinavicius et al., 2013). However synthetic probes must be applied exogenously, making real-time *in vivo* protein tracking difficult. Methods for specific covalent labeling using synthetic fluorescent probes also requires protein tag fusions to the protein of interest: SNAP-tag, 181 amino acids (Gronemeyer et al., 2006; Juillerat et al., 2003; Keppler et al., 2003); CLIP-tag, 181 amino acids (Gautier et al., 2008); or Halo tag, 295 amino acids (Los et al., 2008). The large size of these tags presents the risk that the assay system itself disturbs the natural compartmentalization and localization of the targeted protein.

Here we report a general method for post-translational, covalent labeling of cell surface exposed transgenic proteins using all genetically encoded components. This method

specifically and quantitatively labels membrane proteins in living cells without impacting cell viability and therefore enables further experimentation with the labeled cells (e.g. electrophysiology or imaging of protein dynamics). The method uses the covalent SpyTag-SpyCatcher peptide-protein system first described by Zakeri *et al.* (Zakeri et al., 2012) which was structurally characterized and optimized by Li *et al.* (Li et al., 2014). We show that the short peptide tag (SpyTag, 13 amino acids) fused to a membrane protein of interest can form a covalent bond with an exogenously added or expressed SpyCatcher-XFP labeling protein (SpyCatcher, 139 amino acids). This short tag system is ideal for visualizing membrane protein localization since its small size will likely minimize the effect on protein folding and membrane localization relative to the larger tag methods previously described. Here we demonstrate that the inexpensive and scalable SpyTag/SpyCatcher system can be used to 1) label membrane-localized proteins used for optogenetics (channelrhodopsins C1C2 (Kato et al., 2012) and ReaChR (Lin et al., 2013)) and receptors (TrkB) transfected in HEK cells and primary neuronal cultures; 2) aid in membrane protein engineering via an assay for membrane localization in a 96-well plate format platform; and 3) identify membrane protein localization in whole living organisms in an all-genetically encoded fashion.

Results

The SpyTag/SpyCatcher pair labels membrane-localized channelrhodopsins in live cultures

We used the SpyTag/SpyCatcher system to label membrane-localized, light-activated ion channels, channelrhodopsins (ChRs), in live cells. Since the SpyCatcher-XFP is too large to passively cross the membrane, specific labeling of membrane-localized protein requires the SpyTag be fused to a portion of the protein displayed on the extracellular surface. To limit potential disruption to the three-dimensional membrane protein structure we chose to target the SpyTag to the N-terminal region of the channelrhodopsin C1C2, a variant with a known crystal structure (Kato et al., 2012) (Figure 1A), immediately C terminal to the proposed post-translationally cleaved, signal peptide sequence (residues 1-23) (Kato et al., 2012) (Figure 1A). Though previous work on the SpyTag/SpyCatcher system has shown that it is not limited to N- or C-terminal application (Zhang et al., 2013), for our application N-terminal application was optimal. The fluorescent protein mCherry was fused to the C-terminus of the opsin as a marker of total protein expression (Tag-C1C2-mCherry) (Figure 1A). The SpyCatcher binding partner was produced separately for exogenous labeling by expression in *E. coli* with an elastin-like protein (ELP) inserted between SpyCatcher and its GFP fluorescent label (Catcher-GFP), in an attempt to minimize steric interference between the fluorescent protein and the cell membrane. A 6×His tag was inserted at the N-terminus of the SpyCatcher for purification purposes (Figure 1A). Catcher-GFP was expressed in bulk, purified and buffer exchanged to ready it for extracellular application.

The SpyTag-mCherry-labeled C1C2 channelrhodopsin was expressed in human embryonic kidney (HEK) cells, incubated with 25 μM Catcher-GFP protein for 45 min, washed and imaged. Maximum-intensity projections and single plane confocal images show that the SpyCatcher-GFP binds to the membrane-localized fraction of the Tag-C1C2-mCherry

expressed in live cells, with minimal background (Figure S1A). Intracellular Tag-C1C2-mCherry protein was not labeled by Catcher-GFP (Figure S1A). Full field, single plane confocal images show that only cells expressing Tag-C1C2-mCherry are labeled with Catcher-GFP (Figure S1A). Intracellular puncta or aggregates of Tag-C1C2-mCherry (Figure S1A) could be due to oligomerization of mCherry (Shemiakina et al., 2012). We chose mCherry because it is the most commonly used red marker for opsins used in optogenetics (Mattis et al., 2012). Because the SpyTag/SpyCatcher system is modular, any FPs can be substituted for mCherry and GFP, as long as they are spectrally distinguishable.

Labeling in live cells requires SpyTag display on the cellular surface and covalent binding to SpyCatcher

The placement of the SpyTag dictates its accessibility for labeling with SpyCatcher. In addition to the constructs discussed above that mediated stable and robust labeling with Catcher-GFP, a number of alternative constructs were built to test the requirements of the SpyTag/SpyCatcher system in live and fixed cells. As expected, Catcher-GFP applied to cultured cells expressing a C-terminal fusion of SpyTag to ChR2-mCherry does not label the inaccessible, intracellular SpyTag (Figure S2B). However, when cells were permeabilized with paraformaldehyde (PFA), SpyCatcher-GFP could label the C-terminal SpyTag (Figure S2B). Mutation of the reactive aspartic acid (D) residue in SpyTag to a non-reactive alanine (A) (Tag(DA)-C1C2-mCherry) leads to no observable labeling with Catcher-GFP when the SpyTag is expressed in HEK cells (Figure 2A), indicating that the covalent bond is required for stable labeling of the membrane-localized Tag-C1C2-mCherry. Placement of the SpyTag N-terminal to the signal peptide cleavage site (Tag^0-C1C2-mCherry) also leads to no observable labeling with Catcher-GFP when the SpyTagged construct is expressed in HEK cells (Figure 2A).

Labeling of cell surface displayed Tag with Catcher-GFP in complex media and at temperatures suitable for live cell applications

Catcher-GFP (2-50 μM) added directly to the medium of live cells expressing Tag-C1C2-mCherry shows significant labeling of the membrane-localized opsin (Figure 1A and 1B and S1B-D). SpyTag/SpyCatcher covalent binding on the surface of live cells is robust to different temperatures in the range 16-37°C (Figure S1D), consistent with reported binding results using purified SpyTag/SpyCatcher protein (Zakeri et al., 2012). Robust binding in live cells at different temperatures is particularly useful for temperature-dependent protocol such as heat-shock experiments in flies, zebra fish and nematodes i.e. (Glauser et al., 2011; Prober et al., 2008; Schwabe et al., 2013).

In Figure S1B-D the efficiency of the Catcher-GFP binding to the Tag-C1C2-mCherry is reported as the ratio of GFP fluorescence to mCherry fluorescence using measurements of individually selected cells. This binding efficiency metric is internally normalized for the total protein expression level. The results in Figure S1B show Catcher-GFP binding is saturated at 25 μM, and therefore 25 μM Catcher was used for all subsequent experiments in cultured cells. A time course for Catcher-GFP labeling of Tag-C1C2-mCherry expressing cells in culture medium indicates that binding improves with increased incubation time up to one hour (Figure S1C).

Addition of the N-terminal Tag and covalent labeling with the Catcher-GFP does not affect channelrhodopsin expression or *in vitro* function in neurons

Since the SpyTag/SpyCatcher system gave efficient labeling under optimal live cell conditions, we tested its impact on neuronal function in primary neuronal cultures commonly used for microbial opsin characterization and refinement (Mattis et al., 2012). Application of the Catcher-GFP directly to neuronal medium at 37°C for 1 hour followed by washing with MEM shows efficient membrane labeling and sustained cell health (Figure 1B). This labeling method provided efficient Catcher-GFP binding to membrane-localized Tag-C1C2-mCherry expression in neurons (Figure 1B). These data show distinct membrane labeling at the cell body as well as throughout the axon, dendrites and axon terminals (Figure 1B). Whole-cell patch-clamp recordings of neurons expressing C1C2-mCherry, Tag-C1C2-mCherry and the labeled GFP-Catcher-Tag-C1C2-mCherry complex show no significant differences in photocurrent magnitude or wavelength sensitivity (Figure 1D and 1E) to that of cells expressing similar unlabeled opsin levels (Figure 1C), indicating that the N-terminal SpyTag has no significant effect on opsin properties. Thus Spy-tagged opsin constructs can be used for optogenetic applications and then labeled for follow-up analysis.

To verify that SpyTag can be applied to other channelrhodopsins we inserted SpyTag C-terminal to residue 24 of ReaChR and observed efficient expression and labeling with Catcher-GFP in primary cultured neurons (Figure 2B). Patch-clamp electrophysiological recordings indicate that tagging ReaChR-mCherry does not effect photocurrent magnitude or spectral properties (Figure 2C), similar to the measurements for the tagged C1C2-mCherry in Figure 1E. To test the applicability of the system beyond microbial opsins, we added the SpyTag to the N-terminus of the tropomyosin-related kinase B (TrkB) receptor (Gupta et al., 2013). We observed efficient labeling of the membrane-localized protein with Catcher-GFP in HEK cells and in primary cultured neurons (Figure S3).

SpyTag/SpyCatcher can be used to screen libraries for membrane-localized ChRs

Because opsin membrane localization is a prerequisite for activity in most optogenetic applications, we have used the SpyTag/SpyCatcher system in 96-well plate format for pre-screening libraries of opsin variants for membrane localization. As shown in Figure 2B, the N-terminal Tag-ReaChR-mCherry construct shows good expression and efficient membrane localization. We used Tag-ReaChR-mCherry as a parent for preparing a library of opsin variants and tested the ability of the SpyTag/SpyCatcher membrane localization assay to eliminate mutants with lesser membrane localization. Two residue positions, E130 and N289, identified as being part of the putative channel gate (Kato et al., 2012), were targeted for saturation mutagenesis.

Site-saturation mutagenesis libraries were generated at the E130 and N289 positions. Plasmid DNA from 30 clones was purified for each library (74% coverage) and used to transfect cultured HEK cells in a 96-well format (Figure 3A). Forty-eight hours post transfection, Catcher-GFP was added to the media of expressing HEK cells to label the membrane-localized opsin (Figure 3A). Soluble Catcher-GFP was removed, the cells were washed with maintenance medium, and full field, low magnification (10×) images containing hundreds of transfected cells were analyzed for mCherry and GFP fluorescence

(Figure 3A, 3D; Figure S4A). The ratio of GFP/mCherry fluorescence (reflecting the fraction of protein that is membrane localized) for each screened variant was plotted vs. the mCherry fluorescence (total opsin expression) for the two libraries (Figure 3B). Variants from the N298 library generally showed much lower membrane localization compared to the parent (Tag-ReaChR-mCherry) and compared with the E130 library (Figure 3B and 3C).

Four variants showing membrane localization and expression equal to or above the parent Tag-ReaChR-mCherry (‘hits’) and two variants showing membrane localization significantly worse than the parent (‘poor localizers’) were selected from the E130 library (Figure 3B) and further characterized. Three ‘poor localizer’ variants from the N289 library were also selected. No variants from the N289 library gave membrane localization and expression equal to or above the parent so none were selected as ‘hits’ (Figure 3B). Selected variants were sequenced, re-streaked to obtain high purity DNA for each variant, and used to transfect HEK cells. Catcher-GFP labeling was carried out 48 hours post-transfection. Single-plane, confocal images of expressing, labeled cells of each variant show that each of the ‘hits’ have predominantly membrane-localized opsin (Tag-ReaChR (E130T, E130G, E130Q and E130L)) while all of the ‘poor localizers’ show the opsin protein split between intracellular and membrane localization (Tag-ReaChR E130Y and E130D) (Figure 3D; Figure S4B). Quantification of GFP/mCherry fluorescence measurements of individual cells within a population confirms that the variants identified as ‘hits’ have membrane localization similar to the parent while variants identified as ‘poor localizers’ have significantly lower GFP/mCherry compared to the parent (Figure 3F; Figure S4E). The mCherry fluorescence quantification shows that only one variant Tag-ReaChR (E130D) had significantly lower overall expression compared to Tag-ReaChR (Figure 3F; Figure S4E).

Electrophysiology was used to compare photocurrents of the ‘hits’ and the ‘poor localizers’ of the E130 library (Figure 3G). ‘Poor localizers’ E130Y and E130D show weak currents, both peak and steady state, compared to the Tag-ReaChR parent under green light (590 nm) activation. This decrease in current is not due to a shift in spectral sensitivity. The maximum excitation wavelength for all variants is closest to 590 nm within the wavelengths tested ranging from 390-650 nm (Figure 3H). Further the decrease in current is not due to an altered reversal potential since the currents at all holding potentials are much lower for the ‘poor localizers’ when compared with the Tag-ReaChR. The ‘hits’, on the other hand, show both high and low currents (Figure 3G). This variability is to be expected since total photocurrents are a result of both membrane localization and channel conductance. These data suggest that variants Tag-ReaChR E130T and E130L may have decreased single channel conductance resulting in low currents while variants Tag-ReaChR E130G and E130Q appear to have single channel conductance similar to the parent (Tag-ReaChR). Of particular interest is the variant Tag-ReaChR E130G which has no side chain at residue 130 while the parent has a large, negatively charged side chain, but both variant and parent appear to have similar ion conductance, while introduction of a polar, uncharged side chain (E130T) or a hydrophobic side chain (E130L) both results in what appears to be a strong decrease in the conductance of the channel.

These results indicate that the SpyTag/SpyCatcher system is a useful tool for screening libraries of opsin mutants for membrane localization. Opsin membrane localization is

sensitive to mutations in the protein, and mutations at some residue positions have more drastic effects on expression and localization than others. This assay can facilitate pre-screening of ChRs libraries to eliminate variants with poor localization and enrich for functional ChRs for further analysis using low-throughput but precise methods such as patch-clamp electrophysiology. If hits are identified as having high expression and good membrane localization then using electrophysiology to characterize the hits enables identification of single amino acid substitutions that have significant affect on the channel's electrical properties (i.e. conductance) without the confounding variable of expression and membrane localization.

Stability of SpyTag/Catcher labeling enables monitoring of protein dynamics in living cells

We hypothesized that the Spy system would be sufficiently stable in live cells to enable observation of protein dynamics. Catcher-GFP was added directly to the medium of Tag-C1C2-mCherry-expressing cells for 1 hour, at which point the cells were washed and imaged for both mCherry fluorescence and GFP fluorescence (Day 1). Labeled cells were then incubated at 37°C for an additional 24 hours and reimaged (Day 2) (Figure S5). The SpyTag/Catcher labeling was strongest on Day 1, but significant labeling was visible after 24 hours (Day 2) (Figure S5), and Catcher-GFP labeling was visible up to 3 days after the initial treatment (Figure S5). These observations indicate that even in a rapidly dividing mammalian cell line the SpyTag/SpyCatcher interaction is maintained at the cell surface over several days though there is a decrease in the observed level of Catcher-GFP.

Comparison of SpyTag/Catcher and SNAP-tag labeling methods

To test our hypothesis that N-terminal insertion of larger tags, i.e. SNAP-tag, can disturb the natural compartmentalization and localization of a membrane protein we compared the expression, membrane localization and photocurrents of the Tag-C1C2-mCherry construct with a SNAP-tag-C1C2-mCherry construct in HEK cells. The SNAP-tag-C1C2-mCherry was constructed with the SNAP-tag sequence inserted after the signal peptide sequence (residues 1-23) in the same N-terminal position as the SpyTag and the Tag-C1C-mCherry construct. The Tag-C1C2-mCherry construct is able to express and traffic to the plasma membrane more efficiently than the N-terminal SNAP-tag opsin fusion construct (SNAP-tag-C1C2-mCherry) in mammalian cell culture when imaged under the same imaging conditions (Figure 4A and Figure 4B). Due to the decrease in localization the SNAP-tag-opsin has decreased currents upon activation with 480 nm light (Figure 4C) in cells with similar levels of overall mCherry expression (Figure 4D). Though the SNAP-tag system has enabled post-translational labeling of a number of protein targets (Kohl et al., 2014; McMurray and Thorner, 2008) these results indicate that for tagging channel proteins such as opsin the SpyTag/SpyCatcher system has less effect on native protein trafficking though it should be noted that the performance of one labeling strategy over another is protein specific.

Use of SpyTag/SpyCatcher to label membrane proteins *in vivo*

Since all the components of the SpyTag/SpyCatcher labeling method are genetically encoded, it can be applied to living organisms. As proof-of-concept, we specifically

expressed Tag-C1C2-mCherry in the gonad of the nematode *C. elegans* and demonstrated that Catcher-GFP labels cells within the organ (Figure 5A). The *C. elegans* gonad arms are shaped through the migration of distal tip cells (DTCs), two cells that cap each end of the tube-like structure (Kimble and Hirsh, 1979). We generated transgenic nematodes that specifically expressed Tag-C1C2-mCherry in the DTCs using an endogenous *hlh-12* promoter and observed mCherry fluorescence both at the plasma membrane and in internal compartments (Figure 5A). Because the outer cuticle of the animal is not permeable to Catcher-GFP, the gonad was dissected out, fixed and exposed to a solution of purified Catcher-GFP. Tag-C1C2-mCherry expressing DTCs were the only cells in the gonad that were labeled by Catcher-GFP, and its localization was specific to the plasma membrane (N = 5, Figure 5A). In the control experiment, DTCs that did not express Tag-C1C2-mCherry were not labeled by Catcher-GFP (N = 7).

Since both SpyTag and SpyCatcher can be produced endogenously within the organism where the labeling reaction occurs, we then produced transgenic nematodes expressing Tag-C1C2-mCherry in the DTCs under the *hlh-12* promoter and Catcher-GFP under a heat-shock (HS) promoter. The *HS::SpyCatcher-GFP* construct was designed to be expressed in many tissues upon HS treatment and, due to its signal sequence, secreted extracellularly into the body cavity. At room temperature the DTCs expressed only Tag-C1C2-mCherry and no Catcher-GFP (N = 15, Figure 5B), three hours after a 33°C HS treatment, we observ ed specific Catcher-GFP labeling at the DTC plasma membrane (N = 6, Figure 5B). Initially we observed background cytoplasmic fluorescence from Catcher-GFP expression in the cells responsive to HS, however twenty-four hours after HS treatment, the DTC plasma membrane continues to be stably labeled by SpyCatcher-GFP (N = 13), and the background Catcher-GFP fluorescence was absent (Figure 5B). To demonstrate specificity of labeling, we HS-treated control animals expressing *HS::Catcher-GFP* but not *hlh-12::Tag-C1C2-mCherry* and observed no Catcher-GFP labeling of DTCs three hours (N = 6) or 24 hours (N = 11) after HS (Figure 5B).

Given that the SpyTagged opsin constructs described here are most useful for neuronal applications we investigated SpyTag/SpyCatcher labeling and function of Tag-ReaChR constructs in *C. elegans* neurons. *C. elegans* has 26 GABA-producing neurons, including 19 D-type neurons that reside in the ventral nerve cord and innervate dorsal and ventral body muscle (Figure 6A and 6C). Activation of these GABA neurons inhibits body muscle contractions and paralyzes the worm (Jorgensen) (Figure S6A, Movie S1). We made transgenic animals expressing Catcher-GFP under heat-shock control and also specifically expressing either Tag-ReaChR-mCherry or the mutant Tag-ReaChR(E130D)-mCherry in GABA neurons. The Tag-ReaChR(E130D)-mCherry mutant was identified in the expression/membrane-localization screen to have poor expression and membrane localization. We used this low expressing mutant both to test the sensitivity of the SpyTag/SpyCatcher screen *in vivo* and to further validate the screening method's potential to identify high and low expressers. Although the same concentration of transgenes was delivered for both Tag-ReaChR constructs, we found that Tag-ReaChR-mCherry expression is brighter than Tag-ReaChR(E130D)-mCherry (Figure 6A). The mCherry expression in neuronal cell bodies and processes was visible at 200× magnification in 47% (N=36) of animals carrying

the wild-type Tag-ReaChR-mCherry construct, but only in 4% (N=47) of animals carrying the Tag-ReaChR(E130D)-mCherry construct (Figure 6A and 6B). Expression of Tag-ReaChR(E130D)-mCherry was visible at 1000× magnification in 28% (N=47) of animals implying that the worms are transgenic but expressing the opsin mutant at very low levels. In *C. elegans* Tag-ReaChR(E130D)-mCherry appears to be expressed at lower levels than the parent molecule with the bulk of the protein localizing to the cell body rather than the cell processes (Figure 6A and 6D). These data are consistent with the mammalian cell culture results. To test labeling of the Tag-ReaChR constructs we heat-shock treated both transgenic animals, and examined labeling of Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry by Catcher-GFP 24 hours after heat-shock. We observed specific Catcher-GFP labeling of the Tag-ReaChR expressing GABA neurons and processes for both constructs, but consistent with their expression levels, the Catcher-GFP labeling was brighter in Tag-ReaChR-mCherry over Tag-ReaChR(E130D)-mCherry (Figure 6D). These results indicate that the SpyTag/SpyCatcher assay can be used *in vivo* to measure varying levels of expression and to differentiate between high and low membrane localization.

We tested whether the tagged opsin construct described in this study, could be used *in vivo* to induce light activated behaviors. We measured the impact of the Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry expression on the locomotion behavior of the animal upon light activation. We selected animals expressing high levels of Tag-ReaChR-mCherry based on mCherry visibility at 200× magnification, and of mutant Tag-ReaChR(E130D)-mCherry based on visibility at 1000× magnification. By individually assaying the animal's locomotion behavior in response to green light, we found that 100% of animals expressing wild-type (N=11) or mutant (N=10) Tag-ReaChR-mCherry immediately became paralyzed upon green light activation and recovered movement when the light was turned off (Figure S6, Movie S1). Low expressing animals tested showed no effective paralysis upon light activation. Animals expressing high levels of wild-type Tag-ReaChR-mCherry but grown without all trans-retinal (ATR) did not become paralyzed in response to green light (N=3). Catcher-GFP labeling of Tag-ReaChR-mCherry did not affect the ReaChR function as shown by the results that 100% of animals (N=6) exhibited paralysis in response to green light exposure 4 hours after heat-shock treatment.

Discussion

This work demonstrates the SpyTag/SpyCatcher as a versatile system for the characterization of membrane localization of channels and receptors in live cells and organisms. The irreversible covalent interaction between the surface-displayed SpyTag, fused to a membrane protein, and the extracellular, SpyCatcher-GFP is not affected by competing proteins in complex culture media or in cells *in vivo* and permits efficient long-term labeling without disturbing cell viability. N-terminal insertion of the SpyTag into the ReaChR (Lin et al., 2013) and C1C2 (Kato et al., 2012) ChRs had no significant effect on their expression levels, membrane localization, or photocurrents which is not the case for the SNAP-tag cell-surface labeling method tested.

An application of the SpyTag/SpyCatcher system validated here is screening membrane localization of opsins in mammalian cells in high throughput to support directed evolution

experiments for the discovery of improved opsins (Berndt et al., 2014; Hochbaum et al., 2014; Klapoetke et al., 2014; Wietek et al., 2014). Membrane localization of ChRs is crucial to their ability to mediate efficient neuronal modulation (Hausser, 2014). We demonstrate that the SpyTag/SpyCatcher system can be used in a 96-well format to enrich mutant libraries for membrane localizing variants that are therefore worthy of detailed, but time-involved, electrophysiological characterization. This method enables screening libraries to identify a reduced number of candidates for detailed characterization. This is important because the number and complexity of characteristics of a useful opsin (speed, wavelength sensitivity, photocurrent strength, ion selectivity, and reversal potential) require extensive variant-by-variant analysis (Mattis et al., 2012).

We shows that the SpyTag/SpyCatcher system can be used in live cells to label membrane-localized receptors (TrkB). The long-term stability of labeling and the neutral impact on cellular viability make the SpyTag/SpyCatcher useful for monitoring endocytosis of receptors. This is especially relevant in receptor systems in which insertion and endocytosis are critical to altering neuronal excitability, e.g. AMPA or NMDA receptors (Malenka and Bear, 2004). We have successfully applied this method for *in vivo* labeling of proteins in live *C. elegans*, while retaining protein function for subsequent behavioral assays. Even *in vivo* the SpyCatcher is able to label low levels of expression of the SpyTagged molecule. Given this work the SpyTag/SpyCatcher could be used between cells on the extracellular matrix, to track transient interactions during development, or in response to physiological changes in live animals (i.e. *C. elegans*). Our work described here is dedicated to labeling tagged heterologous membrane proteins, however, with recent advances in genome editing via, e.g. CRISPR/Cas9 (Cong et al., 2013) the SpyTag/SpyCatcher system could also be expanded to label endogenous proteins.

The SpyTag/SpyCatcher genetically encoded post-translational fusion system can be used as an affordable, highly specific, binding assay for live and fixed cells in culture and *in vivo*. The SpyTag/SpyCatcher system is between 20-50× less expensive than using SNAP-tag labeling probes (New England BioLabs, S9124S) and between 14-35× less expensive than using FLAG-tag/secondary antibodies (Sigma-Aldrich, F3165/Life Technologies, A27022). This cost advantage enables high-throughput screening and large tissue volume labeling for which the cost of the labeling molecule can be prohibitive. The SpyTag and SpyCatcher have a covalent, irreversible interaction which is advantages for experiments that require long experimental times, *in vivo* labeling, and to reduce the level of labeling variability from well-to-well for high-throughput screening. The labeling protein can be fused to any fluorescent protein or enzyme for detection and can be bulk-produced, making it a preferred option when large amounts of antibodies are required, for example staining of whole cleared organs or thick tissue slices (Chung et al., 2013; Yang et al., 2014). The SpyTag and SpyCatcher are both genetically encoded which allows for *in vivo* post-translational labeling something that is not possible with antibodies, SNAP-tag/CLIP-tag/Halo-tag or other labeling methods that rely on synthetic probes. Finally, we present the generation and validation of two SpyTagged, spectrally separate, channelrhodopsin molecules (SpyTag-C1C2 and SpyTag-ReaChR) which can be used for optogenetic experiments.

Significance

We report a stable, genetically encoded protein labeling system for the visualization of membrane protein localization in live cells. Taking advantage of the high specificity and modularity of this membrane protein labeling method we have used it to develop a channelrhodopsin membrane localization assay that is amenable to high-throughput screening for opsin discovery and engineering. We have validated the labeling method for monitoring real time protein dynamics in living organisms. We hope this work will encourage the application of the SpyTag/SpyCatcher system to living animals.

Experimental Procedures

Ethics statement

All experiments using animals in this study were approved by Institutional Animal Care and Use Committee (IACUC) at the California Institute of Technology.

Generating constructs and site-saturation library

SpyTag/Catcher & SNAP-tag fusion constructs were generated through standard molecular biology cloning techniques. All constructs were verified by sequencing and reported in Table S2. Site-saturation libraries of the *SpyTag-ReaChR-mCherry* parent were built using the 22c-trick method reported in (Kille et al., 2013) at position E130 and N298. Ten clones from each library we sequenced to test for library quality. DNA from individual clones was isolated and used to transfect HEK cells for further testing. For detailed methods see Supplemental Methods.

SpyCatcher production and labeling of HEK cells and primary neuronal cultures

Recombinant SpyCatcher for exogenous application was expressed and purified in bulk from *E. coli* strain *BL21(DE3)* harboring the *pQE80l-T5:.6×his-SpyCatcher-Elp-GFP* plasmid. Cells were grown at 37 °C in TB, expression was induced with 1 mM IPTG at 30 °C, and after 4 hours, cells were harvested. Protein purifiation was done on HiTrap columns (GE Healthcare, Inc.) following column manufactur's recommendations.

HEK cells and primary neuronal cultures were maintained and transfected using standard methods. For detailed methods see Supplemental Methods. Both HEK cells and neurons went through SpyCatcher labeling 48 hours post-transfection. Unless otherwise noted the SpyCatcher-GFP was added to the media of HEK cells at a final concentration of 25 μM and the cells were then incubated for 45 minutes – 1 hour at 25 °C. After labeling HEK cells were washed with D10 three to four times. Cells were then returned to incubate at 37 °C for 10 minutes to 1 hour before imaging. For more details on SpyCatcher labeling protocol for 96-well plate see Supplemental Methods. SpyCatcher labeling of neurons was carried out in 500 μl of the neuronal maintenance media in a 24-well plate. SpyCatcher was then added to each well of neurons for a final concentration of 25 μM. The neurons were then incubated with the SpyCatcher for 45 minutes – 1 hour at 37 °C for labeling. After labeling cells were washed in Minimal Essential Media (MEM) three to four times. After washing the neurons

were placed back into the stored neuronal maintenance media without SpyCatcher and incubated at 37 °C for 10 minutes to 1 hour before imaging.

C. elegans experiments

Transgenic *C. elegans* expressing each Tag-opsin construct were generated by DNA injection into *unc-119* mutant animals. A transgenic *C. elegans* line expressing heat-shock activated Catcher-GFP and cell-type specific expression of the tagged opsin was generated by co-injecting plasmid DNA of both constructs into *unc-119* mutant animals. To induce expression of Catcher-GFP *C. elegans* were heat-shock treated at 33°C for 15 minutes in a water bat h. Following heat-shock, animals were allowed to recover at room temperature. At specific time points they were placed on an agar pad in 3 mM levamisole and imaged. For behavioral experiments transgenic animals expressing Tag-opsin constructs were grown on NGM plates with OP50 bacteria and all-trans retinal. L4-stage transgenic animals were placed on plates and grown in the dark for approximately 16 hours. To assay paralysis, animals were transferred individually onto plain NGM plates and their movement was monitored on a dissecting microscope (Leica) at 2.5× magnification for 10 s without green light, 5 s with green light illumination, and 10 s without green light. More details on generation and maintenance of SpyTag-C1C2-mCherry, SpyTag-ReaChR-mCherry, SpyTag-ReaChR(E130D)-mCherry, and SpyCatcher-GFP transgenic *C. elegans* strains, SpyCatcher-GFP staining of dissected *C. elegans* gonad, heat-shock treatment to induce SpyCatcher-GFP expression and locomotion assay evoked by green light can all be found in Supplemental Methods.

Electrophysiology

Conventional whole-cell patch-clamp recordings were done in cultured HEK cells and cultured rat hippocampal neurons at 2 days post transfection. For detailed methods see Supplemental Methods.

Fluorescence imaging and data analysis

Fluorescence analysis of single cells was done by manually selecting regions around each cell in ImageJ and fluorescence measurements were recorded for each region of interest (ROI). The same ROI was used for both the mCherry and GFP fluorescence measurements in co-labeled cells. Fluorescence analysis and comparison between populations of cells expressing different opsin variants was done using a custom MATLAB script. For detailed methods see Supplemental Methods. Statistical methods- One-way ANOVA, unpaired student's *t*-tests and Dunnett's multiple comparison tests were performed using GraphPad Prism (version 6.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- SpyTag/SpyCatcher tracks membrane localization of proteins in live cells
- Post-translational covalent labeling of membrane-localized ChRs
- SpyTag/SpyCatcher system tracks membrane localization of ChRs in living *C. elegans*
- Screening membrane localization of opsins in a 96-well format.

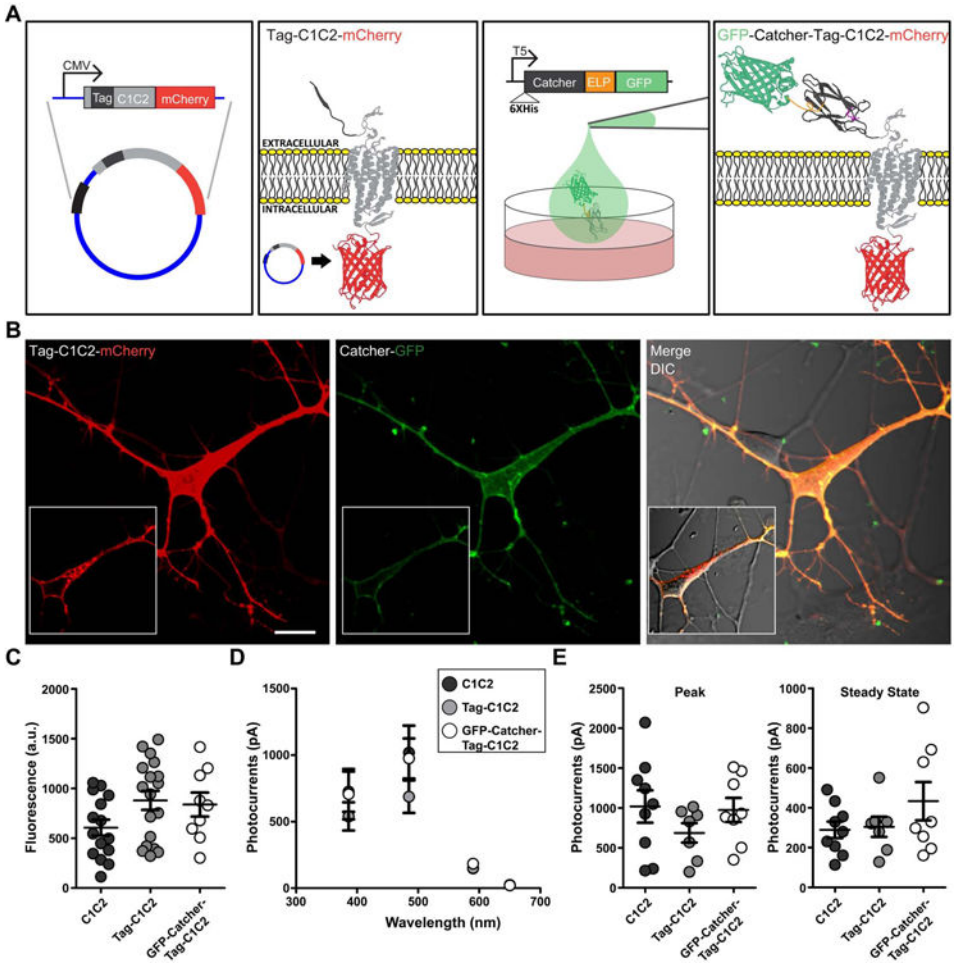

Figure 1. SpyTag fused to the N-terminus of C1C2 enables covalent binding of Catcher-GFP for membrane-localized Tag-C1C2 detection in live neurons without affecting light-induced currents

(**A**) Construct design and labeling assay workflow. (left) Schematic of SpyTag fused to the N-terminus of C1C2-mCherry (Tag-C1C2-mCherry) under a CMV promoter for expression in mammalian cells. (middle left) Correctly folded Tag-C1C2-mCherry displays the SpyTag extracellularly. (middle right) His-tagged SpyCatcher fused to a small elastin-like protein (ELP) and GFP (Catcher-GFP) with a T5 promoter for expression in *E. coli*. (right) Extracellular application of Catcher-GFP converts the membrane localized Tag-C1C2-mCherry to GFP-Catcher-Tag-C1C2-mCherry through formation of a covalent bond between the reactive lysine residue in SpyCatcher and the reactive aspartic acid residue in the surface-displayed SpyTag. (**B**) Maximum intensity projection of Tag-C1C2-mCherry expressing neurons (red), Catcher-GFP membrane-localized protein binding (green), and merge of red and green channels with DIC image of neuronal cells (inset: single plane confocal images of each) showing specific labeling of membrane-localized Tag-C1C2-mCherry. Only the cells expressing the Tag-C1C2-mCherry show binding of the Catcher-GFP. (**C**) Fluorescence measurements of mCherry in cultured neurons for C1C2-mCherry (N = 15), Tag-C1C2-mCherry (N = 18) and GFP-Catcher-Tag-C1C2-mCherry (N = 9) showing no significant difference. One-way ANOVA, P = 0.095. (**D**) Whole-cell recordings of peak photocurrents induced by different wavelengths in cultured neurons under voltage

clamp. Neurons expressing C1C2-mCherry (N = 9), Tag-C1C2-mCherry (n = 7) and GFP-Catcher-Tag-C1C2-mCherry (N = 8) show similar spectral properties. (**E**) Peak and steady-state photocurrents induced by 480 nm light in cultured neurons under voltage clamp. Cells expressing C1C2-mCherry (N = 9), Tag-C1C2-mCherry (N = 7) and GFP-Catcher-Tag-C1C2-mCherry (N = 8) show no significant difference in peak or steady state currents. One-way ANOVA, peak currents: P = 0.4 and steady state currents: P = 0.3. All population data are plotted as mean ± SEM. Not significant (ns), P > 0.05. Scale bar, 10 μm.

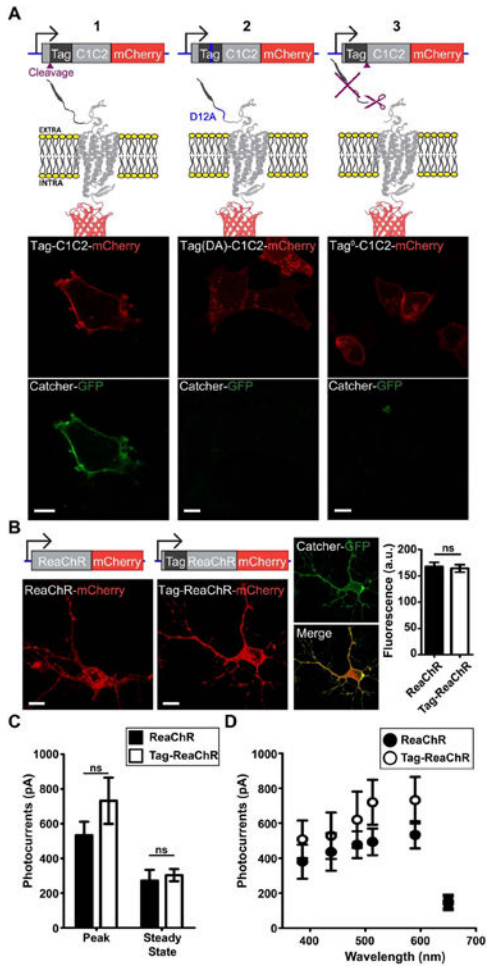

Figure 2. Opsin SpyTag fusion construct requirements for successful binding of SpyCatcher and application of the SpyTag/SpyCatcher to ReaChR

(**A**) (top) Schematic of 3 different C1C2/SpyTag designs with corresponding labeling patterns (bottom). (1) SpyTag fused to the N-terminus of C1C2-mCherry after the signal peptide cleavage site results in expression of Tag-C1C2-mCherry with the SpyTag displayed on the extracellular surface of the cell which successfully binds extracellularly applied Catcher-GFP. (2) SpyTag with the reactive aspartic acid (D12) residue mutated to alanine (A12) fused to the N-terminus of C1C2-mCherry after the signal peptide cleavage site results in expression of Tag(DA)-C1C2-mCherry. The mutated SpyTag does not bind to extracellular Catcher-GFP. (3) SpyTag fused to the N-terminus of C1C2-mCherry before the signal peptide cleavage site results in expression of C1C2-mCherry but no binding to extracellular Catcher-GFP. Single plane confocal images shown. (**B**) Maximum intensity projection of ReaChR-mCherry and Tag-ReaChR-mCherry expression in primary neuronal cultures under a CMV promoter. Application of Catcher-GFP to Tag-ReaChR-mCherry expressing neuron shows labeling. Fluorescence comparison of neurons expressing ReaChR-mCherry (N = 6) compared with neurons expressing Tag-ReaChR-mCherry (N = 5) shows no significant difference between the two opsin constructs (unpaired t-test, P = 0.7). (**C**) Whole-cell recordings of peak and steady-state photocurrents induced by 590 nm light under voltage clamp in neurons expressing ReaChR-mCherry (N = 3) and Tag-ReaChR-

mCherry (N = 5) shows no significant difference (unpaired students t-test, peak: P = 0.3 and steady state: P = 0.6). (**D**) Peak photocurrents induced by different wavelengths of light under voltage clamp in neurons expressing ReaChR-mCherry (N = 3) and Tag-ReaChR-mCherry (N = 5). ReaChR-mCherry and Tag-ReaChR-mCherry show similar spectral properties. All population data are plotted as mean ± SEM. Not significant (ns), $P > 0.05$. Scale bar, 10 μm.

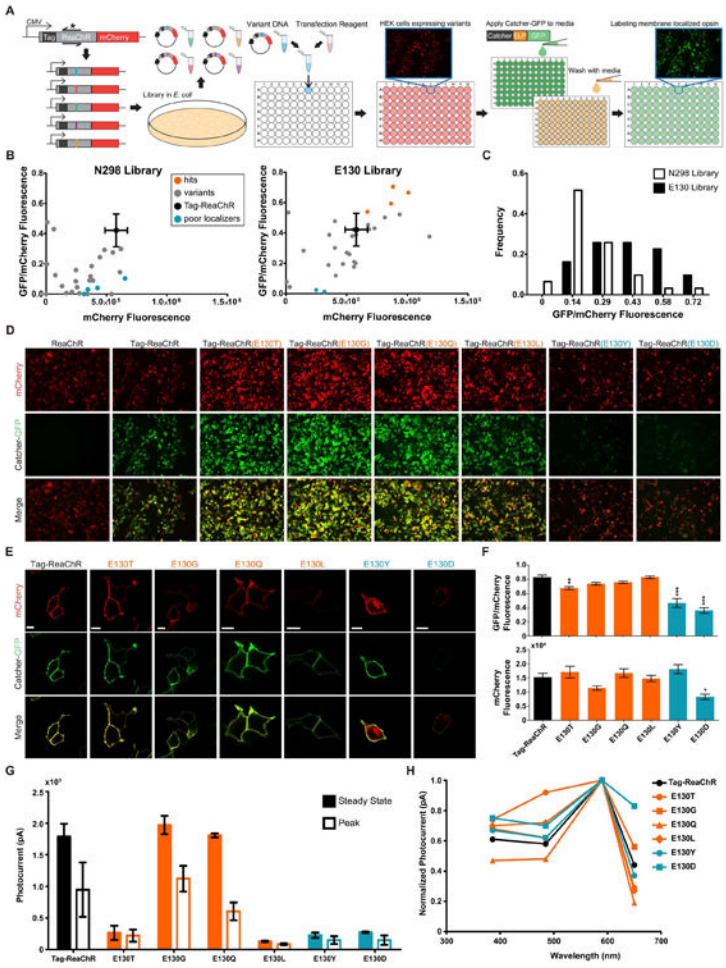

Figure 3. A screen for membrane localization based on SpyTag/SpyCatcher for optogenetics

(**A**) Screening assay workflow. From left to right: Schematic of the SpyTag/SpyCatcher opsin membrane localization assay for screening in a 96-well format. Site-saturation mutagenesis of the *CMV: :Tag-ReaChR-mCherry* backbone targeting specific amino acid locations. Transformation of the library into *E. coli*. Selection and isolation of plasmid DNA of individual clones. Transfection of HEK cells plated in a 96-well plate with each clone in a different well. Catcher-GFP is then added to each well, incubated for 1 hour and washed. Cells in each well are imaged for both mCherry fluorescence and GFP fluorescence. (**B**) GFP/mCherry fluorescence vs mCherry fluorescence for the two site-saturation libraries at amino acids N298 and E130 in ReaChR. Library ‘variants’ are shown in gray, ‘hits’ in orange and ‘poor localizers’ in blue. The mean fluorescence with SEM of the Tag-ReaChR parent is shown in black ($N = 4$). (**C**) Distribution of GFP/mCherry fluorescence ratio for each of the two site-saturation libraries. (**D**) Example images from the screening process for non-tagged control (ReaChR), parent (Tag-ReaChR), Tag-ReaChR mutant ‘hits’ and Tag-ReaChR mutant ‘poor localizers’ from the E130 library. Full field, population images were taken for each tested variant and used to measure the GFP and mCherry fluorescence. Amino acid mutations at residue 130 are highlighted in orange for the ‘hits’ and in blue for the ‘poor localizers’ in the variants label. (**E**) Single plane confocal images of parent (Tag-

ReaChR-mCherry) compared with the ‘hits’ and ‘poor localizers’ of mCherry (red), Catcher-GFP (green) and merge. (**F**) (top) GFP/mCherry fluorescence ratio or (bottom) mCherry fluorescence of Tag-ReaChR (N = 24) compared with ReaChR variants (E130T: N = 27, E130T: N = 72, E130Q: N = 43, E130L: N = 64, E130Y: N = 14, and E130D: N = 33) from single plane confocal images of HEK cells expressing the tagged opsins with intensity measurements made by selection of a region of interest around each cell and measurement of mean GFP and mCherry fluorescence across the region. Comparisons between Tag-ReaChR with each variants was done by Dunnett's Multiple Comparison Test. (**G**) Recordings of peak and steady-state photocurrents induced by 590 nm light under voltage clamp in HEK cells expressing Tag-ReaChR-mCherry (N = 6), each of the ‘hits’ (each variant, N = 3) and the ‘poor localizers’ (each variant, N = 3) from the E130 library. (**H**) Peak photocurrents induced by different wavelengths of light under voltage clamp in HEK expressing Tag-ReaChR-mCherry, each of the ‘hits’ and the ‘poor localizers’ from the E130 library. Photocurrents are normalized to show spectral sensitivity. All population data are plotted as mean ± SEM. *P < 0.05, **P < 0.01, *** P < 0.001. Scale bar, 10 μm.

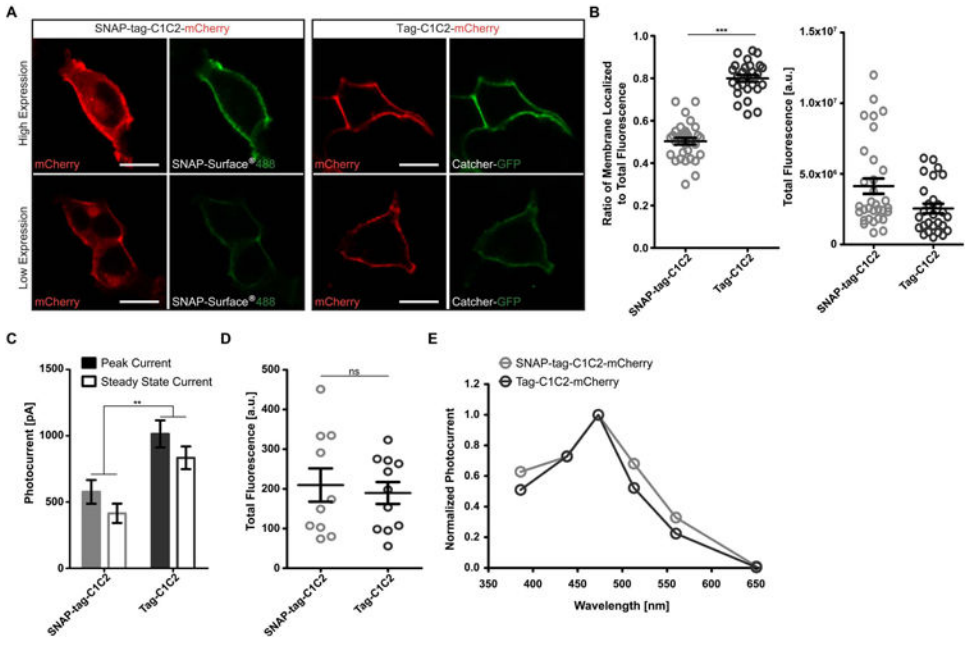

Figure 4. The N-terminal SpyTag opsin fusion construct (Tag-C1C2-mCherry) is able to express and traffic to the plasma membrane more efficiently than the N-terminal SNAP-tag opsin fusion construct (SNAP-tag-C1C2-mCherry) in mammalian cell culture

(**A**) Fluorescence images of total opsin-mCherry expression (red) and successful labeling of membrane localized expression (green). Example cell with high expression (top) and low expression (bottom) comparing two different construct/labeling sets: SNAP-tag-C1C2-mCherry/SNAP-Surface®488 (left) and Tag-C1C2-mCherry/Catcher-GFP (right). (**B**) (left) Plot of the ratio of membrane localized fluorescence to total fluorescence of the SNAP-tag-C1C2-mCherry (N = 32 cells) vs Tag-C1C2-mCherry (N = 27 cells) expressing cells. The Tag-C1C2-mCherry construct shows a larger fraction of total expression localized to the plasma membrane while the SNAP-tag-C1C2-mCherry construct has a larger fraction of its total expression internally localized. There is a significant difference in the ratio of membrane-localized opsin between the two constructs. Unpaired t-test, $p < 0.0001$. (right) Plot of the total level of fluorescence of the SNAP-tag-C1C2-mCherry (N = 32 cells) vs Tag-C1C2-mCherry (N = 27 cells) expressing cells. (**C**) Peak (filled bar) and steady-state (empty bar) photocurrents induced by 480 nm light in HEK cells under voltage clamp. Cells expressing SNAP-tag-C1C2-mCherry (N = 9), and Tag-C1C2-mCherry (N = 10) show a significant difference in peak and steady-state currents. Unpaired t-test, peak currents: p = 0.0053 and steady-state currents: p = 0.0019. (**D**) Total fluorescence measurements of mCherry in cultured HEK cells expressing either SNAP-tag-C1C2-mCherry (N = 10) or Tag-C1C2-mCherry (N = 11) used for whole-cell recordings show no significant difference. Unpaired t-test, p = 0.688. (**E**) Whole-cell recordings of peak photocurrents induced by different wavelengths in HEK cells under voltage clamp. HEK cells expressing SNAP-tag-C1C2-mCherry and Tag-C1C2-mCherry show similar spectral properties. All population data are plotted as mean ± SEM. Not significant (ns), $p > 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Scale bar, 10 μm.

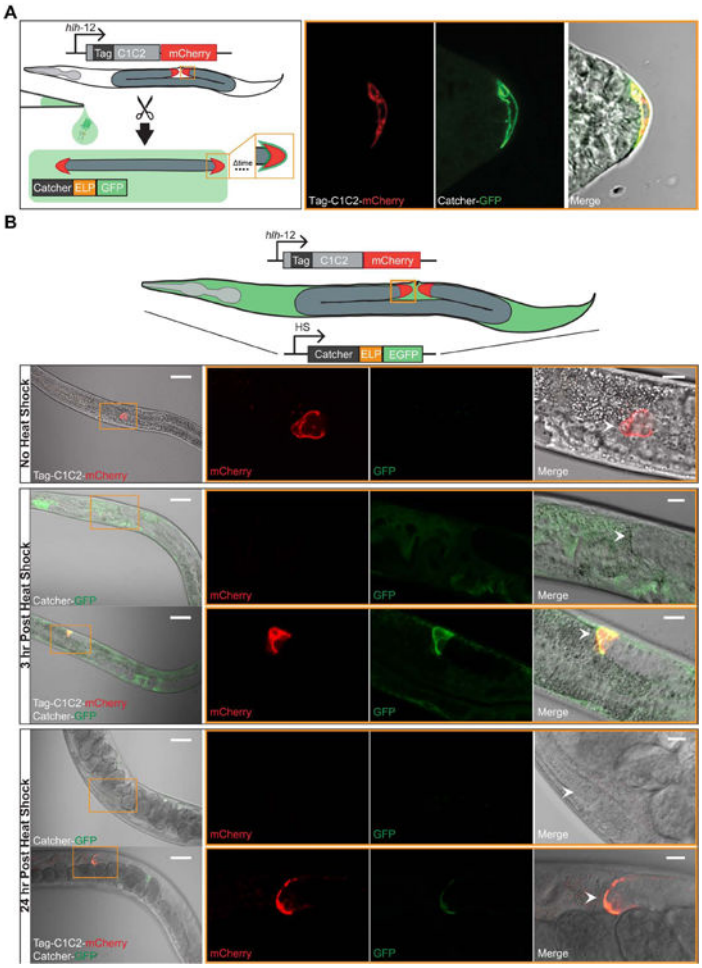

Figure 5. SpyTag fusion constructs shows efficient single-cell labeling with SpyCatcher in fixed and live *C. elegans*

(**A**) (left) Schematic of Tag-C1C2-mCherry expression in the distal tip cells (DTCs) under the *hlh-12* promoter, dissection of the expressing *C. elegans* gonad and labeling of the dissected, fixed tissue with the Catcher-GFP. (right) Single plane confocal images of Tag-C1C2-mCherry expression in one DTC (red) with efficient labeling of Catcher-GFP (green) specific to the Tag-C1C2-mCherry expressing DTC. (**B**) (top) Schematic of transgenic *C. elegans* expressing Tag-C1C2-mCherry in the DTCs under the *hlh-12* promoter and Catcher-GFP under a heat-shock (HS) promoter. The *HS::Catcher-GFP* construct expresses Catcher-GFP in many tissue types upon HS treatment. Catcher-GFP is then secreted from cells into the body cavity. Single plane confocal images of a *C. elegans* expressing Tag-C1C2-mCherry in the DTC: without HS treatment show mCherry expression in the DTC without any Catcher-GFP expression and labeling; 3 hours post HS treatment shows mCherry expression in the DTC and significant Catcher-GFP expression throughout the body cavity with specific labeling of the Tag-C1C2-mCherry. While single plane confocal images of a *C. elegans* without Tag-C1C2-mCherry expression in the DTC 3 hours post HS treatment shows significant Catcher-GFP expression throughout the body cavity without specific labeling of the DTC, imaging 24 hours after HS shows decreased levels of GFP

throughout the *C. elegans* while specific labeling of the DTC is achieved with Tag-C1C2-mCherry expression in the DTC. Scale bar, 20 μm.

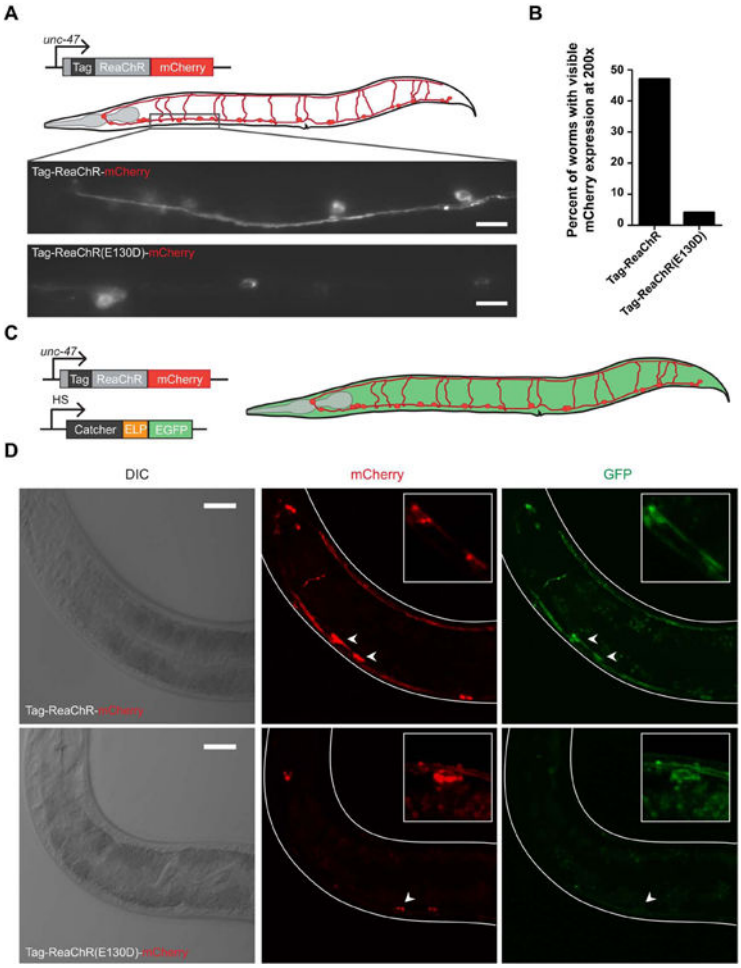

Figure 6. SpyTag opsin constructs expressed in GABA-producing neurons show efficient labeling with SpyCatcher in live *C. elegans* for both high expressing a low expressing SpyTag opsin constructs

(**A**) (top) Schematic showing Tag-ReaChR-mCherry constructs expressed in the *C. elegans* 19 D-type GABA-producing neurons that reside in the ventral nerve cord and innervate dorsal and ventral body muscle. (bottom) Expression of both Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry in cell bodies and fine processes of GABA-producing neurons in the ventral nerve cord. Scale bar, 10 μm. (**B**) Comparison of the expression levels of the Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry constructs in *C. elegans* GABA-producing neurons characterized by mCherry visibility at 200× magnification. (**C**) Schematic showing both Tag-ReaChR-mCherry constructs expressed in the *C. elegans* 19 D-type GABA-producing neurons and Catcher-GFP expression and secretion from many tissue types post heat-shock. (**D**) Confocal images of (left) DIC, (middle) mCherry and (right) GFP for both Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry constructs in *C. elegans* GABA-producing neurons 24 hr post heat-shock. Large images are maximum intensity projections of images that are power/gain matched for both constructs. Inset images show single plane confocal image of individual cell(s) (indicated with arrow in large image). For inset alone we increased the gain in low expresser for visibility. Scale bar, 20 μm.