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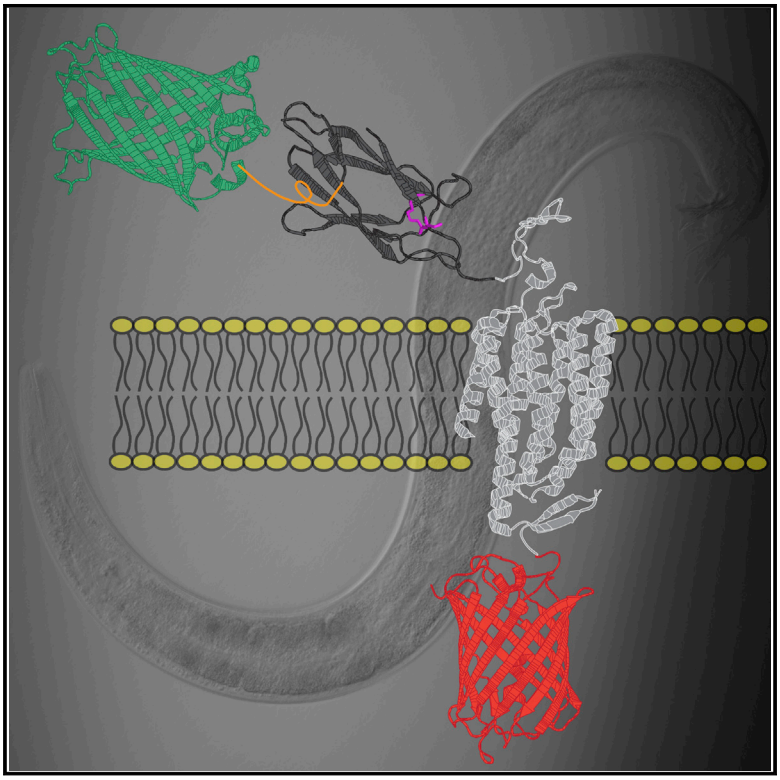
Genetically Encoded Spy Peptide Fusion System to Detect Plasma Membrane-Localized Proteins In Vivo

Graphical Abstract



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In Brief

Bedbrook et al. describe a method for specifically labeling the membrane-localized fraction of channelrhodopsin expression using the genetically encoded, covalent binding SpyTag and SpyCatcher pair in live neurons and in vivo in *Caenorhabditis elegans*.

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

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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 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.

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 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 affecting 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. (2012), which was structurally characterized and optimized by 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. We demonstrate here that the inexpensive and scalable SpyTag/SpyCatcher system can be used to: (1) label membrane-localized proteins used for optogenetics (channelrhodopsins [ChRs] C1C2 [Kato et al., 2012] and ReaChR [Lin et al., 2013]) and receptors (tropomyosin-related kinase B [TrkB]) transfected in human embryonic kidney (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.

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RESULTS

The SpyTag/SpyCatcher Pair Labels Membrane-Localized ChRs in Live Cultures

We used the SpyTag/SpyCatcher system to label membrane-localized, light-activated ion channels, 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 ChR C1C2, a variant with a known crystal structure (Kato et al., 2012), immediately C-terminal to the proposed post-translationally cleaved, signal peptide sequence (residues 1–23) (Kato et al., 2012) (Figure 1A). Although 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 *Escherichia 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 6xHis 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 ChR was expressed in 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 fluorescent proteins 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, SpyCatcher-GFP was able to 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 (Figures 1A and 1B; Figures S1A–S1D). SpyTag/SpyCatcher covalent binding on the surface of live cells is robust to different temperatures in the range 16°C–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 (HS) experiments in flies, zebrafish, and nematodes (Glauser et al., 2011; Prober et al., 2008; Schwabe et al., 2013).

In Figures S1B–S1D 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 that Catcher-GFP binding is saturated at 25 μM; 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 1 hr (Figure S1C).

Addition of the N-Terminal Tag and Covalent Labeling with the Catcher-GFP Does Not Affect ChR 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 hr followed by washing with minimal essential medium (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 difference in photocurrent magnitude or wavelength sensitivity (Figures 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, SpyTagged opsin constructs can be

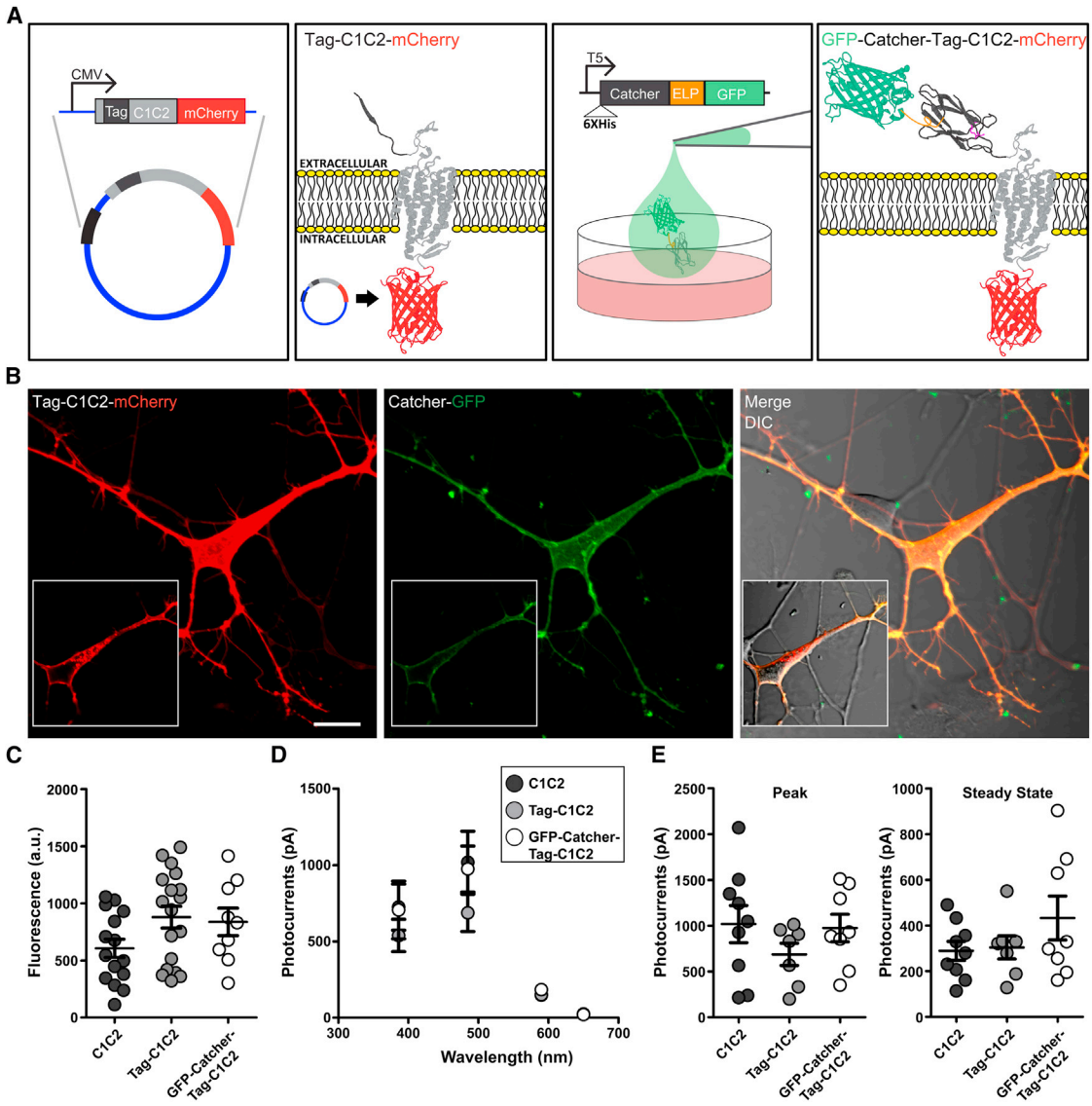


Figure 1. SpyTag Fused to the N Terminus of C1C2 Enables Covalent Binding of Catcher-GFP for Membrane-Localized Tag-C1C2 Detection in Live Neurons without Affecting Light-Induced Currents

(A) Construct design and labeling assay workflow. (Left) Schematic of SpyTag fused to the N terminus of C1C2-mCherry (Tag-C1C2-mCherry) under a cytomegalovirus (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 *Escherichia 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 differential interference contrast (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 means ± SEM (error bars). Not significant (ns), $p > 0.05$. Scale bar, 10 μm.

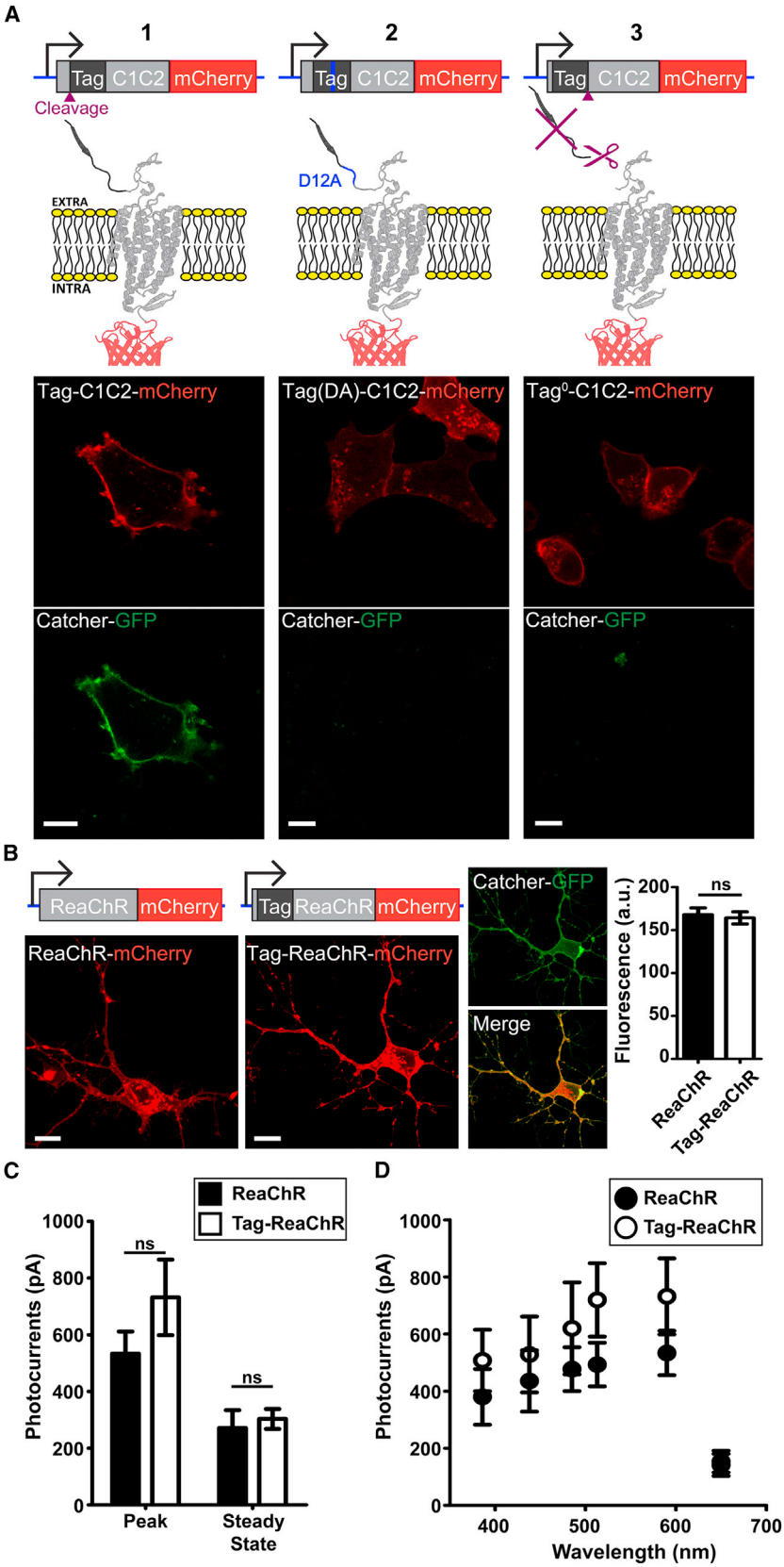


Figure 2. Opsin SpyTag Fusion Construct Requirements for Successful Binding of SpyCatcher and Application of the SpyTag/SpyCatcher to ReaChR

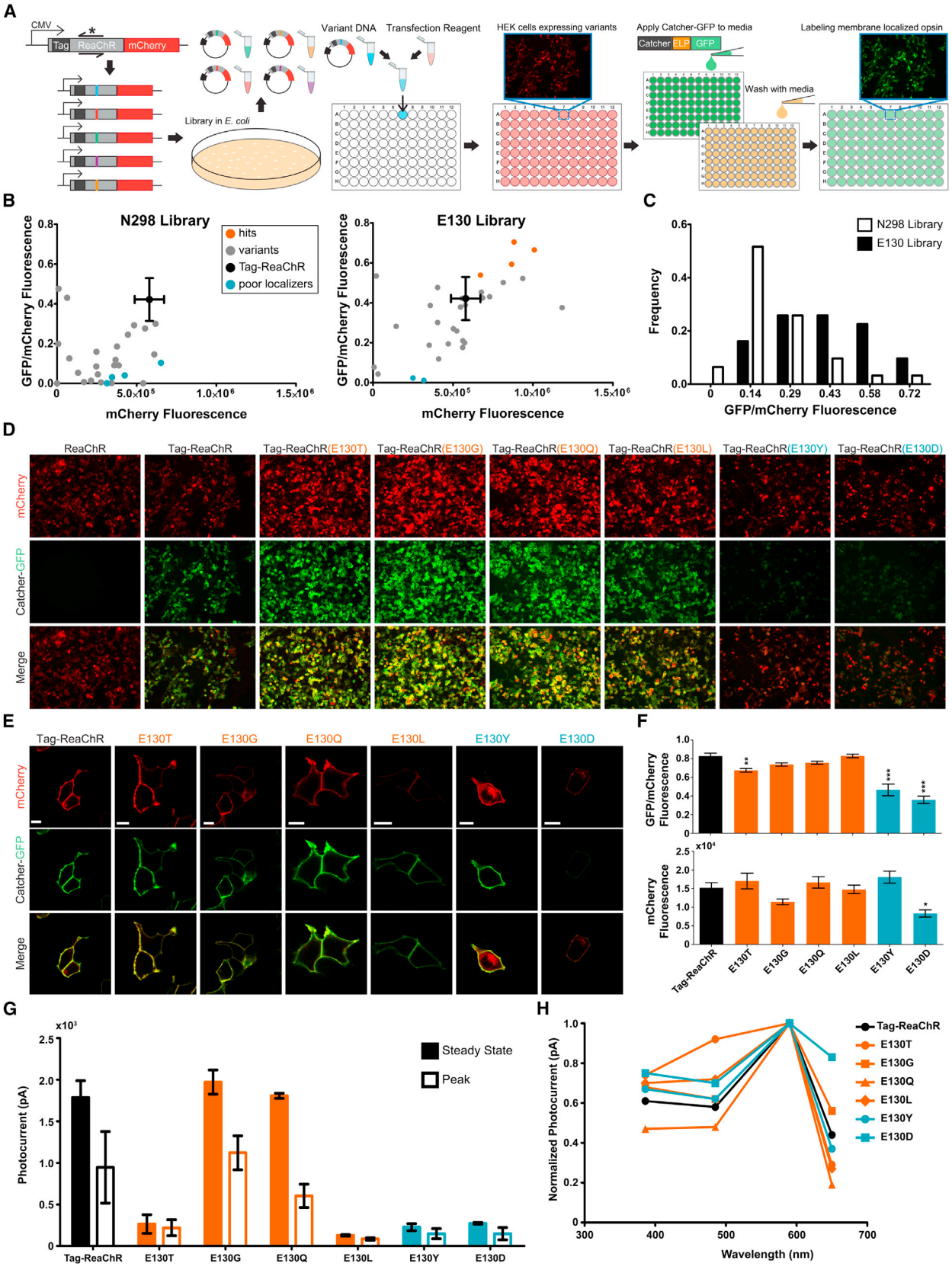
(A) Schematic of three different C1C2/SpyTag designs (top) with corresponding labeling patterns (bottom). (1) SpyTag fused to the N terminus of C1C2-mCherry after the signal peptide cleavage site results in expression of Tag-C1C2-mCherry, with the SpyTag displayed on the extracellular surface of the 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 are 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 Student's 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 means ± SEM (error bars). Not significant (ns), $p > 0.05$. Scale bars, 10 μm.



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used for optogenetic applications and then labeled for follow-up analysis.

To verify that SpyTag can be applied to other ChRs, 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 affect photocurrent magnitude or spectral properties (Figures 2C and 2D), 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 TrkB receptor (Gupta et al., 2013). We observed efficient labeling of the membrane-localized protein with Catcher-GFP in HEK cells and 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 N298, 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 N298 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 after 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 (Figures 3A and 3D; Figure S4A). The ratio of GFP/mCherry fluorescence (reflecting the fraction of protein that is membrane localized) for each screened variant was plotted versus the mCherry fluorescence (total opsin expression) for the two libraries (Figure 3B). Variants from the N298 library generally showed much lower membrane localization compared with the parent (Tag-ReaChR-mCherry) and the E130 library (Figures 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 N298 library were also selected. No variants from the N298 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 hr 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 3E; 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 with the parent (Figure 3F; Figure S4C). The mCherry fluorescence quantification shows that only one variant, Tag-ReaChR (E130D), had significantly lower overall expression compared with Tag-ReaChR (Figure 3F; Figure S4C).

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 with 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

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::SpyTag-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 hr, and washed. Cells in each well are imaged for both mCherry fluorescence and GFP fluorescence.

(B) GFP/mCherry fluorescence versus 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; 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 and each variant was done using 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), and each of the hits (each variant, n = 3) and 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, and each of the hits and poor localizers from the E130 library. Photocurrents are normalized to show spectral sensitivity.

All population data are plotted as means ± SEM (error bars). *p < 0.05, **p < 0.01, ***p < 0.001. Scale bars, 10 μm.

wavelength for all variants is closest to 590 nm within the wavelengths tested ranging from 390–650 nm (Figure 3H). Furthermore, 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 that of 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 result 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 ChR libraries to eliminate variants with poor localization, and enrich 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, using electrophysiology to characterize the hits enables identification of single amino acid substitutions that have a significant effect on the electrical properties of the channel (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 hr, 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 hr and re-imaged (day 2) (Figure S5). The SpyTag/Catcher labeling was strongest on day 1, but significant labeling was visible after 24 hr (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, although 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 (Figures 4A and 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). Although 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, although 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 select cells of the gonad of the nematode *Caenorhabditis 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 a cell-specific *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 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); 3 hr after a 33°C HS treatment, we observed 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, 24 hr after HS treatment the DTC plasma membrane continued to be stably labeled by Catcher-GFP (n = 13), and the background Catcher-GFP fluorescence was absent

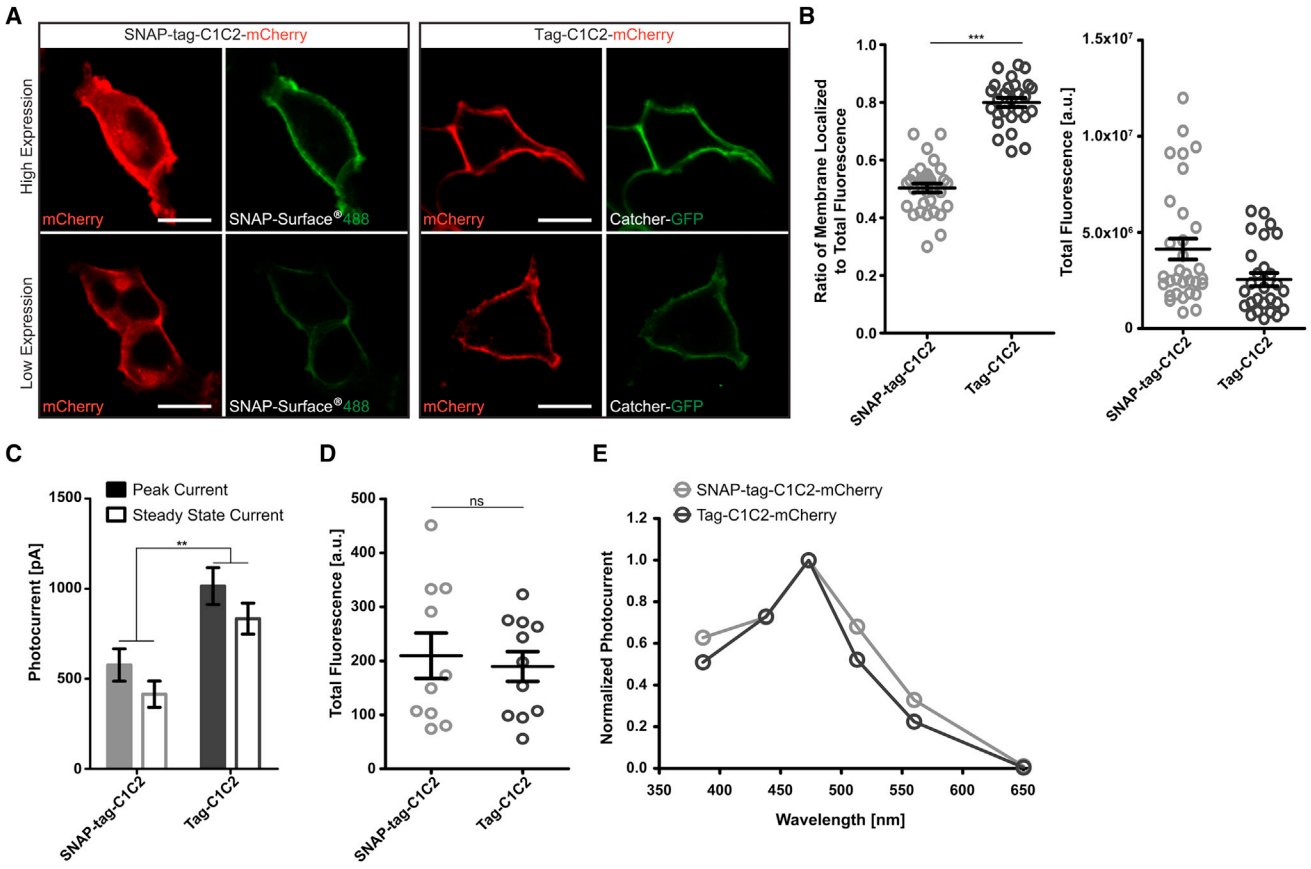

Figure 4. The N-Terminal SpyTag Opsin Fusion Construct, Tag-C1C2-mCherry, Is Able to Express and Traffic to the Plasma Membrane More Efficiently Than the N-Terminal SNAP-Tag Opsin Fusion Construct, SNAP-tag-C1C2-mCherry, in Mammalian Cell Culture

(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-Surface488 (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) versus 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) versus 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 means ± SEM (error bars). Not significant (ns), $p > 0.05$; **$p < 0.01$; ***$p < 0.001$. Scale bars, 10 μm.

(Figure 5B). To demonstrate specificity of labeling, we HS-treated control animals expressing *HS::SpyCatcher-GFP* but not *hlh-12::SpyTag-C1C2-mCherry*, and observed no Catcher-GFP labeling of DTCs 3 hr (n = 6) or 24 hr (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 γ-aminobutyric acid (GABA)-producing neurons, including 19 D-type neurons that reside in the ventral nerve cord and innervate dorsal and ventral body muscle (Figures 6A and 6C). Activation of these GABA neurons inhibits body muscle contractions and paralyzes the worm (Jorgensen, 2005) (Figure S6A; Movie S1). We made transgenic animals expressing Catcher-GFP under HS 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

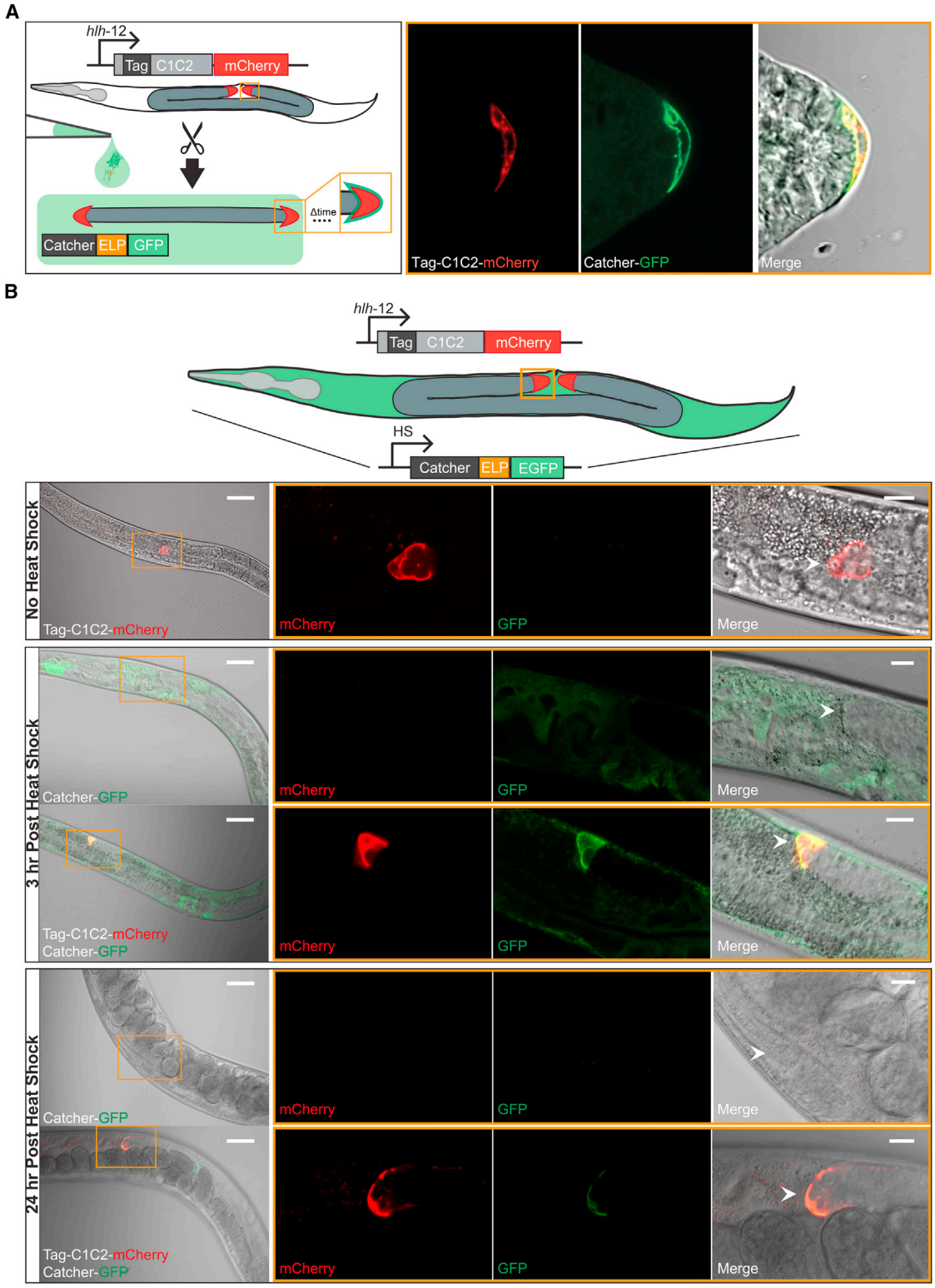

Figure 5. SpyTag Fusion Constructs Shows Efficient Single-Cell Labeling with SpyCatcher in Fixed and Live *C. elegans*

(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. ELP, elastin-like protein.

(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::SpyCatcher-GFP* construct expresses Catcher-GFP in many tissue types upon HS treatment. Catcher-GFP is then secreted from cells into the

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potential of the screening method 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 (Figures 6A and 6B). Expression of Tag-ReaChR(E130D)-mCherry was visible at 1,000× magnification in 28% (n = 47) of animals, implying that the worms are transgenic but express the opsin mutant at very low levels. In *C. elegans*, Tag-ReaChR(E130D)-mCherry appears to be expressed at lower levels than in the parent molecule, with the bulk of the protein localizing to the cell body rather than the cell processes (Figures 6A and 6D). These data are consistent with the mammalian cell culture results. To test labeling of the Tag-ReaChR constructs we HS-treated both transgenic animals, and examined labeling of Tag-ReaChR-mCherry and Tag-ReaChR(E130D)-mCherry by Catcher-GFP 24 hr after HS. 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 than in 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 1,000× magnification. By individually assaying the locomotion behavior of the animal 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 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 hr after HS 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 show 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 the endocytosis of receptors. This is especially relevant in receptor systems where insertion and endocytosis are critical to altering neuronal excitability, e.g. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate or *N*-methyl-D-aspartate 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

body cavity. (Bottom) 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; images 3 hr post HS treatment show mCherry expression in the DTC and significant Catcher-GFP expression throughout the body cavity with specific labeling of the plasma membrane-localized Tag-C1C2-mCherry. While single-plane confocal images of a *C. elegans* without Tag-C1C2-mCherry expression in the DTC 3 hr post HS treatment show significant Catcher-GFP expression throughout the body cavity without specific labeling of the DTC, imaging 24 hr 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. Orange insets (leftmost images) are magnified (right panels) to show the detail of DTC (white arrowheads in the "Merge" panels). Scale bars, 20 μm.

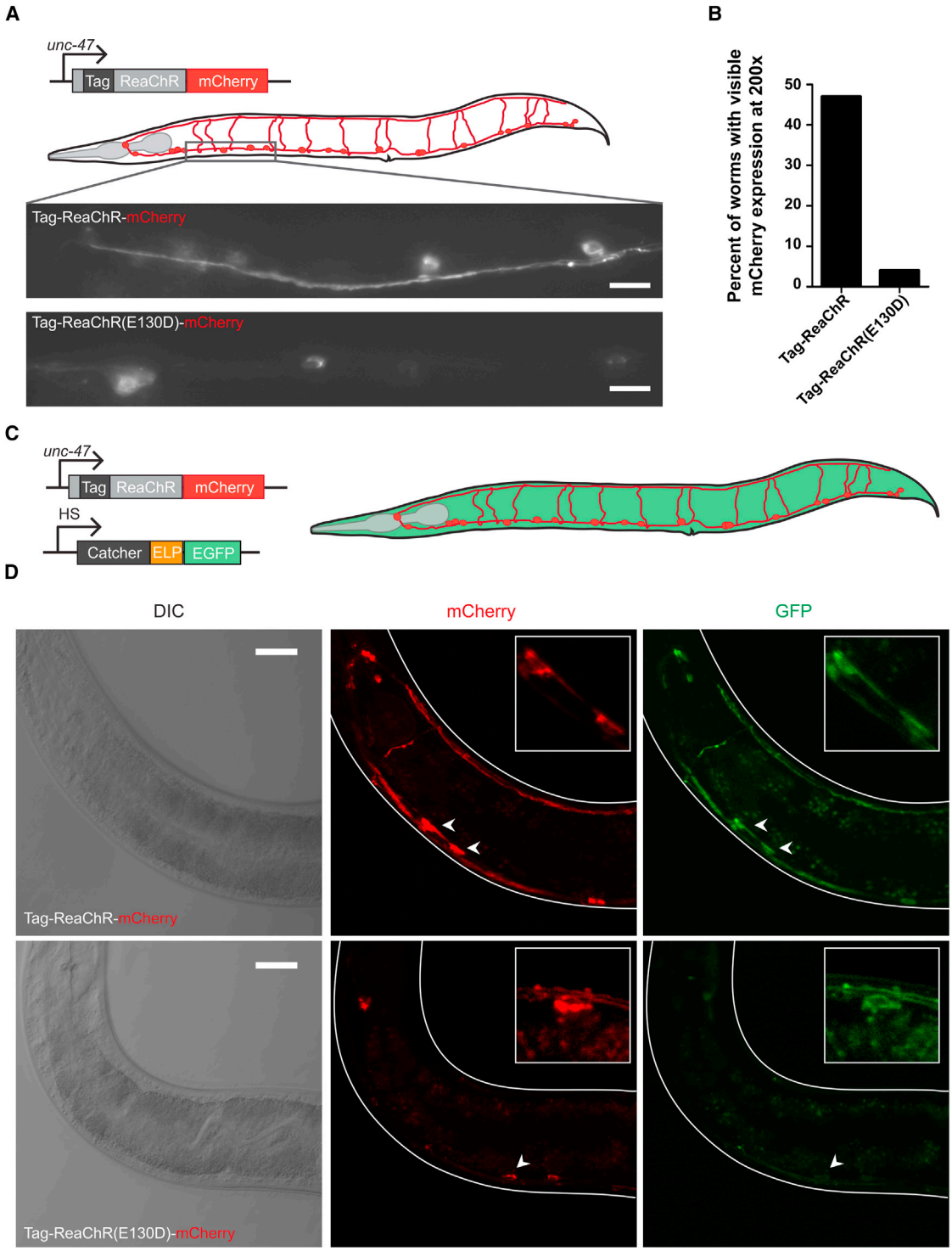

Figure 6. SpyTag Opsin Constructs Expressed in GABA-Producing Neurons Show Efficient Labeling with SpyCatcher in Live *C. elegans* for Both High-Expressing and Low-Expressing SpyTag Opsin Constructs

(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 bars, 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 after HS.

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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 and 50 times less expensive than using SNAP-tag labeling probes (New England BioLabs, S9124S) and between 14 and 35 times 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 that is advantageous for experiments that require long experimental times, in vivo labeling, and reducing 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 produced in bulk, making it a preferred option when large amounts of antibodies are required, e.g. for 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, ChR 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 ChR 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 that 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/SpyCatcher and SNAP-tag fusion constructs were generated through standard molecular biology cloning techniques. All constructs were verified by sequencing and are 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 positions E130 and N298. Ten clones from each library were 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 Experimental Procedures.

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::6xhis-SpyCatcher-Elp-GFP* plasmid. Cells were grown at 37°C in Terrific Broth, expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C, and after 4 hr cells were harvested. Protein purification was done on HiTrap columns (GE Healthcare) following the manufacturer's recommendations.

HEK cells and primary neuronal cultures were maintained and transfected using standard methods (for detailed methods, see Supplemental Experimental Procedures). Both HEK cells and neurons went through SpyCatcher labeling 48 hr 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 min to 1 hr 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 min to 1 hr before imaging. For more details on the SpyCatcher labeling protocol for a 96-well plate, see Supplemental Experimental Procedures. 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 SpyCatcher for 45 min to 1 hr at 37°C for labeling. After labeling, cells were washed in 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 min to 1 hr 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 HS-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 HS treated at 33°C for 15 min in a water bath. Following HS, 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 nematode growth media (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 hr. 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, HS treatment to induce SpyCatcher-GFP expression, and locomotion assay evoked by green light can all be found in Supplemental Experimental Procedures.

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 Experimental Procedures.

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

(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 HS. 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 by arrowheads in larger images). For the inset alone we increased the gain in low expresser for visibility. Scale bars, 20 μm.

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 Experimental Procedures). Statistical methods, namely one-way ANOVA, unpaired Student's t test, and Dunnett's multiple comparison test, were performed using GraphPad Prism (version 6.04 for Windows; GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, four tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.06.020.

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Chemistry & Biology, Volume *22*

Supplemental Information

Genetically Encoded Spy Peptide Fusion System to Detect Plasma Membrane-Localized Proteins In Vivo

Claire N. Bedbrook, Mihoko Kato, Sripriya Ravindra Kumar, Anupama Lakshmanan, Ravi D. Nath, Fei Sun, Paul W. Sternberg, Frances H. Arnold, and Viviana Gradinaru

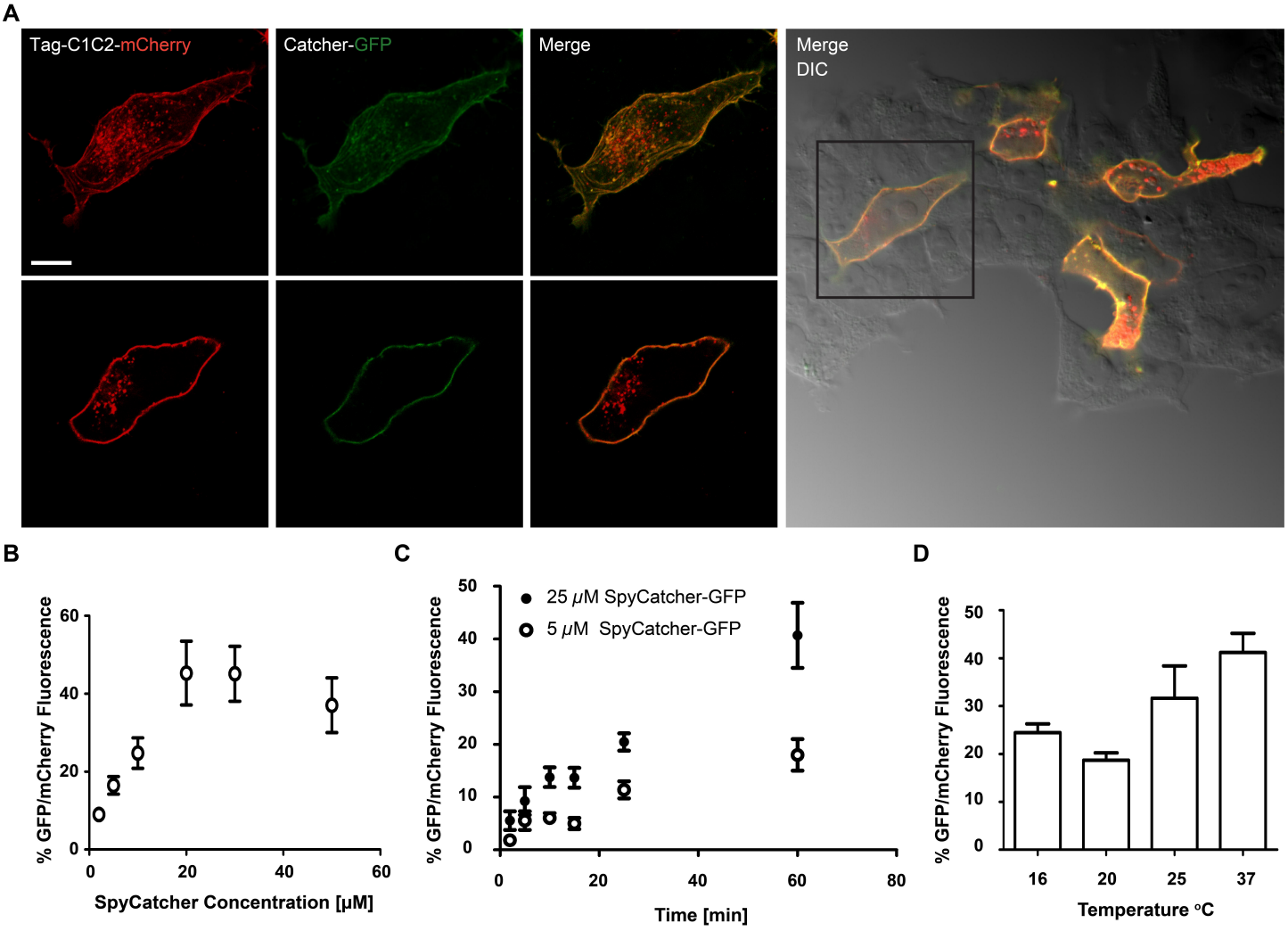

A
Tag-C1C2-mCherry
Catcher-GFP
Merge
Merge
DIC
B
% GFP/mCherry Fluorescence
SpyCatcher Concentration [μM]
C
% GFP/mCherry Fluorescence
25 μM SpyCatcher-GFP
5 μM SpyCatcher-GFP
Time [min]
D
% GFP/mCherry Fluorescence
Temperature °C

Figure S1, Related to Figure 1. Catcher-GFP labeling of membrane-localized Tag-C1C2-mCherry in live HEK cells and optimization of SpyTag/SpyCatcher binding efficiency in complex media used for mammalian cell cultures.

(**A**) Top row: (left) Maximum intensity projection of Tag-C1C2-mCherry expression in HEK cells (red), (middle left) Catcher-GFP membrane localized protein binding (green) and (middle right) a merge. Bottom row shows single plane confocal images of cell in each channel. (right) Single plane confocal image of a population of HEK cells with only a fraction of cells expressing Tag-C1C2-mCherry. Black box indicates cell shown to the left. Only the cells expressing the Tag-C1C2-mCherry show binding of the Catcher-GFP. (**B**) Effect of different concentrations of extracellular Catcher-GFP. Plot shows quantification of GFP fluorescence relative to mCherry fluorescence of individual labeled Tag-C1C2-mCherry expressing cells. Fluorescence measurements were obtained from single plane confocal images of Catcher-GFP bound to membrane-localized Tag-C1C2-mCherry after treatment of HEK cells with Catcher-GFP for 1 hour in D10 medium (*N* = 12-14 cells for each concentration). (**C**) Testing different incubation times from 2-60 min at 25^{o}C. Plot shows the percent of GFP fluorescence relative to mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells covalently bound to Catcher-GFP after incubation of Tag-C1C2-mCherry expressing cells with either 5 μM (empty circles) or 25 μM (filled circles) Catcher-GFP (*N* = 11-14 for each time point). (**D**) Effect of temperature from 16 to 37 ^{o}C. Plot of the percent of GFP fluorescence relative to mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells bound to Catcher-GFP after incubation of Tag-C1C2-mCherry expressing cells with 25 uM Catcher-GFP for 1 hour (*N* = 9-14 for each temp). All population data are plotted as mean ± SEM.

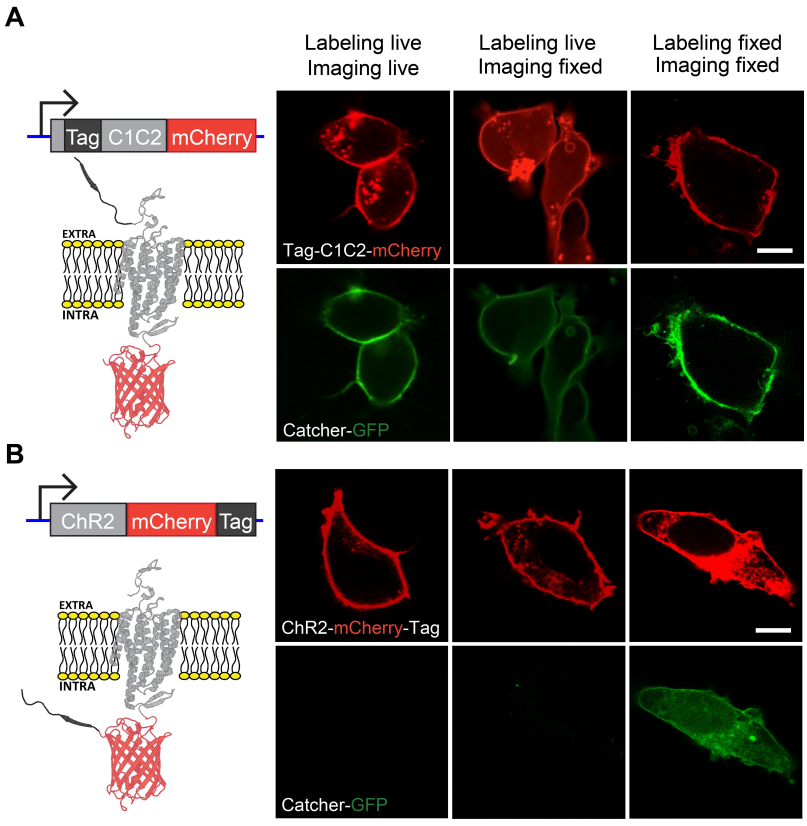

A
Labeling live
Imaging live
Labeling live
Imaging fixed
Labeling fixed
Imaging fixed
Tag
C1C2
mCherry
EXTRA
INTRA
Tag-C1C2-mCherry
Catcher-GFP
B
ChR2
mCherry
Tag
EXTRA
INTRA
ChR2-mCherry-Tag
Catcher-GFP

Figure S2, Related to Figure 2. SpyTag/SpyCatcher system works with both live and fixed cultured cells and can be used to identify the signal peptide of ChR2 and its positioning can affect ChR2 membrane localization.

(**A**) (left) Schematic of N-terminal SpyTagged opsin construct (Tag-C1C2-mCherry) in the cell membrane with the SpyTag displayed on the extracellular surface. (**B**) (left) Schematic of C-terminal SpyTagged opsin construct (ChR2-mCherry-Tag) in the cell membrane with the SpyTag displayed on the intracellular side of the cell. (**A**) & (**B**) (right) Single plane confocal images of the two opsin constructs with varying labeling and fixation methods. Column 1: both constructs show expression of the tagged ChR-mCherry. With extracellular application of Catcher-GFP only the N-terminal SpyTag shows Catcher-GFP binding since the Catcher-GFP cannot penetrate the membrane to label the C-terminal SpyTag. Column 2: fixation in paraformaldehyde (PFA) has no effect of the membrane-localized tagging after covalent binding of the Catcher-GFP. Column 3: fixation with PFA permeabilizes the cells allowing Catcher-GFP to get through the membrane and then covalently bind total ChR-mCherry for both the N-terminal and C-terminal SpyTagged constructs. Scale bar, 10 μm.

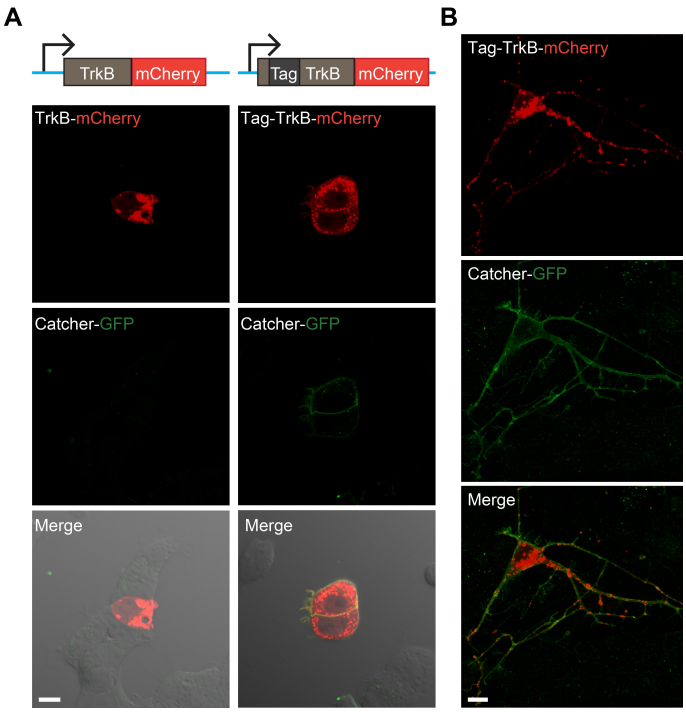

A
TrkB
mCherry
Tag
TrkB
mCherry
TrkB-mCherry
Tag-TrkB-mCherry
Catcher-GFP
Catcher-GFP
Merge
Merge
B
Tag-TrkB-mCherry
Catcher-GFP
Merge

Figure S3, Related to Figure 2. SpyTag/SpyCatcher labeling of TrkB receptor transfected in HEK cells and neurons.

(**A**) The SpyTag was placed at the N-terminus after the signal peptide cleavage site of the TrkB-mCherry fusion. Single plane confocal images of HEK cells expressing TrkB-mCherry and Tag-TrkB-mCherry (red) after 1-hour incubation with Catcher-GFP (green). Only the Tag-TrkB-mCherry expressing cells show labeling with Catcher-GFP. (**B**) Maximum intensity projection of the Tag-TrkB-mCherry expressed in primary neuronal cultures (red) labeled with Catcher-GFP (green). Scale bar, 10 μm.

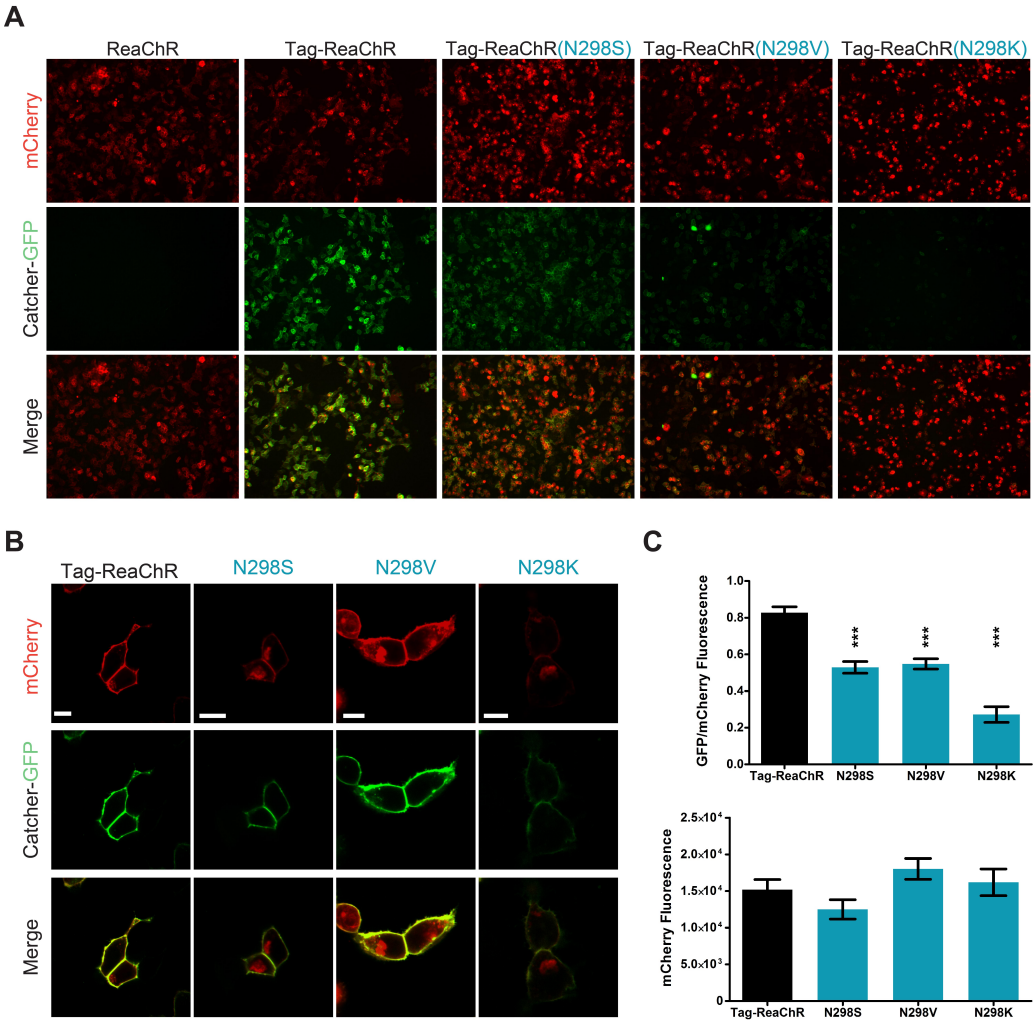
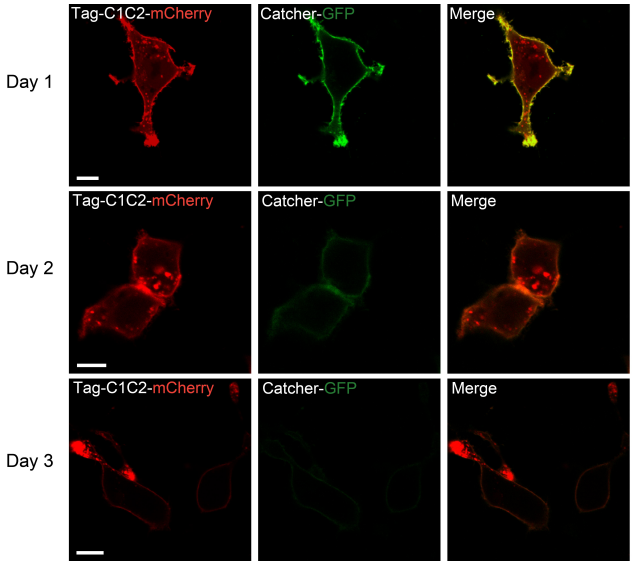

A
ReaChR
Tag-ReaChR
Tag-ReaChR(N298S)
Tag-ReaChR(N298V)
Tag-ReaChR(N298K)
mCherry
Catcher-GFP
Merge
B
Tag-ReaChR
N298S
N298V
N298K
mCherry
Catcher-GFP
Merge
C
GFP/mCherry Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
Tag-ReaChR
N298S
N298V
N298K
mCherry Fluorescence
2.5x10⁴
2.0x10⁴
1.5x10⁴
1.0x10⁴
5.0x10³
0
Tag-ReaChR
N298S
N298V
N298K

Figure S4, Related to Figure 3. Characterization of a subset of variants with poor membrane localization identified in the SpyTag/SpyCatcher screen of the ReaChR N298 library.

(**A**) Example images from the screening process for non-tagged control (ReaChR), parent (Tag-ReaChR), and Tag-ReaChR mutant 'poor localizers' from the N298 library. Full field, population images were taken for each tested variant and used to measure the GFP and mCherry fluorescence. Amino acid mutations at the 298 residue position are highlighted in blue for the 'poor localizers' in the variants labeled as in Figure 3*D*. (**B**) Single plane confocal images of parent (Tag-ReaChR-mCherry) compared with the 'poor localizers' of mCherry (red), Catcher-GFP (green) and merge. All 'poor localizers' show high levels of internal mCherry localization. (**C**) (top) GFP/mCherry fluorescence ratio or (bottom) mCherry fluorescence of Tag-ReaChR (N = 24) compared with ReaChR variants (N298S: N = 44, N298V: N = 68, and N298K: N = 26) 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 parent with each variant was done by Dunnett's Multiple Comparison Test. All population data are plotted as mean ± SEM. *P < 0.05, **P < 0.01, *** P < 0.001. Scale bar, 10 μm.


Day 1
Day 2
Day 3
Tag-C1C2-mCherry
Catcher-GFP
Merge
Tag-C1C2-mCherry
Catcher-GFP
Merge
Tag-C1C2-mCherry
Catcher-GFP
Merge

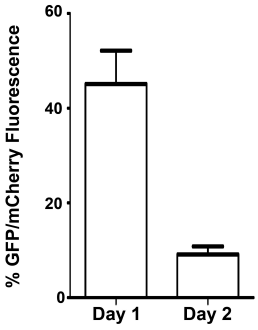
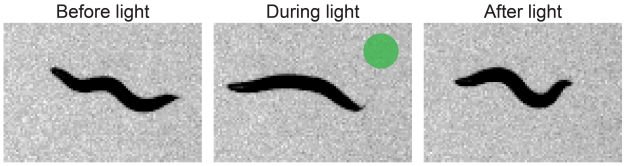

% GFP/mCherry Fluorescence
60
40
20
0
Day 1
Day 2

Figure S5, Related to Figure 5. Long-term stability of SpyTag/SpyCatcher labeling.

Single plane confocal images of Tag-C1C2-mCherry expression (red) and Catcher-GFP membrane-localized protein binding (green) and merge. Day 1 is imaged shortly after a 1-hour incubation of Catcher-GFP with HEK cells expressing Tag-C1C2-mCherry in D10 and washing with MEM. Cells were then left in D10 at 37^{o}C for 24 hours and imaged again for Tag-C1C2-mCherry expression (Day 2). Cells were then left in D10 at 37^{o}C for another 24 hours and imaged again for Tag-C1C2-mCherry expression (Day 3). (right) Plot of the percent of GFP/mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells covalently bound to Catcher-GFP on Day 1 vs Day 2 (*N* = 12 for each day). All population data are plotted as mean ± SEM. Scale bar, 10 μm.

A



B

	Catcher-GFP labeling?	ATR?	Fraction worms showing light induced paralysis
Tag-ReaChR-mCherry	NO	YES	11/11
Tag-ReaChR-mCherry	YES	YES	6/6
Tag-ReaChR-mCherry	NO	NO	0/3
Tag-ReaChR(E130D)-mCherry	NO	YES	10/10

Figure S6, Related to Figure 6. Functional characterization of Tag-opsin constructs in locomotion behavioral assay in *C. elegans*.

(**A**) Three frames of video of a *C. elegans* expressing Tag-ReaChR-mCherry specifically in GABA-producing neurons (19 D-type neurons) before (left), during (middle) and after (right) green light stimulation. Activation of these GABA neurons paralyses the worm. Activation of Tag-ReaChR-mCherry with green light shows clear induction of paralysis as shown by the posture change evident during light stimulation. (**B**) Table showing the fraction of worms with high opsin expression that have light induced paralysis under different conditions.

Movie S1, Related to Figure 6. Light activation of *C. elegans* GABA-producing neurons expressing Tag-ReaChR-mCherry to inhibit body muscle contractions and cause paralysis of the animals.

Video shows moving worm under low red light illumination then upon green light exposure (indicated by bright light in video) the worm becomes paralyzed. Once the green light is turned off the worm immediately recovers and begins moving again.

Table S1, Related to Experimental Procedures. Comparison between size of SpyTag with other covalent labeling methods.

Tag Name	Size [amino acids]	Reference
SpyTag	13	(Zakeri et al., 2012)
SpyTag optimized	10	(Li et al., 2014)
SpyCatcher	139	(Zakeri et al., 2012)
SpyCatcher optimized	84	(Li et al., 2014)
SNAP-Tag	181	(Gronemeyer et al., 2006)
CLIP-Tag	181	(Gautier et al., 2008)
Halo Tag	295	(Los et al., 2008)
GFP	238	(Tsien, 1998)

Table S2, Related to Experimental Procedures. Summary of constructs built with protein product name used in the text.

Construct	Protein
pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-EYFP*	Tag-C1C2-EYFP
pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-mCherry*	Tag-C1C2-mCherry
plenti-CMV/CaMKIIa::SNAP-tag-C1C2-TS-mCherry*	SNAP-tag-C1C2-mCherry
pLenti-CMV/CaMKIIa::C1C2-TS-mCherry*	C1C2-mCherry
pQE80l-T5::6xhis-SpyCatcher-Elp-GFP	Catcher-GFP
pLenti-CMV/CaMKIIa::SpyTag(DA)-C1C1-TS-mCherry*	Tag(DA)-C1C1-mCherry
pLenti-CMV/CaMKIIa::SpyTag(0)-C1C1-TS-mCherry*	Tag^0-C1C1-mCherry
pGP-CMV::ChR2-mCherry	ChR2-mCherry
pGP-CMV::ChR2-mCherry-SpyTag	ChR2-mCherry-Tag
pLenti-CMV/CAMKIIa::ReaChR-TS-mCherry-WPRE*	ReaChR-mCherry
pLenti-CMV/CAMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE*	Tag-ReaChR-mCherry
pLenti-CMV/hSyn1::TrkB-3xGS linker- mCherry-WPRE*	TrkB-mCherry
pLenti-CMV/hSyn1::SpyTag-TrkB-mCherry-WPRE*	Tag-TrkB-mCherry
hlh-12::SpyTag-C1C2-mCherry	Tag-C1C2-mCherry
HS::lin-3 signal sequence::SpyCatcher-GFP	Catcher-GFP
pSM*::unc-47::SpyTag-ReaChR-TS-mCherry*	Tag-ReaChR-mCherry
pSM*::unc-47::SpyTag-ReaChR(E130D)-TS-mCherry*	Tag-ReaChR(E130D)-mCherry

**Constructs denoted to have a CaMKIIa or hSyn promoter also have an upstream CMV promoter. The CMV promoter is responsible for expression in transfected cells while the CaMKIIa or hSyn promoter controls expression in virally infected cells. The work in this paper uses only transfected cells so the CMV promoter drives all reported mammalian cell expression.*

Table S3, Related to Experimental Procedures. Addgene plasmids with accession codes used for construct designs used in this paper.

Construct	Addgene #
pAAV-CaMKIIa-C1V1 (t/t)-TS-mCherry	35500
pLenti-CaMKIIa-C1C2-TS-EYFP[*]	35520
pLenti-CaMKIIa-hChR2(H134R)-mCherry-WPRE[*]	20943
pGP-CMV-GCaMP6f	40755
pLenti-hSyn-eNpHR 3.0-EYFP[*]	26775

[*]*Constructs denoted to have a CaMKII, CaMKIIa or hSyn promoter also have an upstream CMV promoter. The CMV promoter is responsible for expression in transfected cells while the CaMKIIa or hSyn promoter controls expression in virally infected cells. The work in this paper uses only transfected cells so the CMV promoter drives all reported mammalian cell expression.*

Table S4. Cloning primers used for construct design and construction.

Primer	Sequence (5' …. 3')	Used to generate
plenti-CaMKII_F	GTCAAGCCGGTTCTCCG	External primers for all genes in the pLenti vector
plenti-CaMKII_R	GTTAAGAATACCAGTCAATCTTTCAC	
SpyTag_C1C2_F	GCCCACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTCGACTGGCAGTGACG	*pLenti-CaMKII::SpyTag-C1C2-TS-mCherry/EYFP*
SpyTag_C1C2_R	TTGGTGGGCTTGTAGGCGTCCACCATCACGATGTGGGCGGCTCCTGCGCTGC	
pLenti-C1V1-3	GTTCGCATCCCCTTCTCCAAC	*pLenti-CaMKII::SNAP-tag-C1C2-TS-mCherry*
C1C2-NS-R	GCGCTTCATTTCGCAGTCTTTGTCGGCTCCTGCGCTGCCGGCCGCCAG	
C1C2-SNAP-NS-start	GAGCCGACAAAGACTGCGAAATGAAGCG	
C1C2-SNAP-NS-end	CTGCCAGTCGAACCCAGCCCAGGCTTGCCCAGTCTGTG	
SNAP-CIC2-NS-mid	GCCTGGGCTGGGTTCGACTGGCAGTGACGCGACGG	
WPRE-R	GTTAAGAATACCAGTCAATCTTTCAC	
TS_For	GAGCAGGATCACCAGCG	Primers specific to the TS sequence between opsin and marker
TS_Rev	CGCTGGTGATCCTGCTC	
C1C2_Spy_TagDA_F	GTGATGGTGGCCGCCTACAAGCCCACCAAGTCGACTGGCAGTGACG	*pLenti-CaMKII::SpyTag(DA)-C1C1-TS-mCherry*
C1C2_Spy_TagDA_R	GTAGGCGGCCACCATCACGATGTGGGCGGCTCCTGCGCTGC	
N_term_Tag_C1C2_2	CGGGGGATCCCCGGGTACCGGTAGGCCACCATGGCCCACATCGTGATGGTGGACG	*pLenti-CaMKII::SpyTag(0)-C1C1-TS-mCherry*
N_term_Tag_C1C2_1	CCCACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTCGCGGAGGCCATGGC	
pGP_Gib_ChR2_F	GCTAGCGCTACCGGACTCAGATCTCGCCACCATGGACTATGGCGGCGCT	*pGP-CMV::ChR2-mCherry*
pGP_Gib_ChR2_R	ATGGCTGATTATGATCTAGAGTCGCGGCCGCTTACTTGTACAGCTCGTCCA	
ChR2_SpyTag_F	CACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTGAGCGGCCGCGACTCTAG	*pGP-CMV::ChR2-mCherry-SpyTag*
ChR2_SpyTag_R	GGGCTTGTAGGCGTCCACCATCACGATGTGGGCCTTGTACAGCTCGTCCATG	
pGP-Gib_F	GTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGG	External primers for all genes in the pGP vector
pGP-Gib_ R	CAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTAGAG	
ReaChR_fwd	GCGGGGGATCCCCGGGTACCGGTAGGCCACCATGGTGAGCAGAAGACCCTGGCTGC	*pLenti-CAMKIIa::ReaChR-TS-mCherry-WPRE*
ReaChR_rev	CCGCGCTGCTCTCGTACTTATCTTCTTC	
pA-TS-mcherry-fwd	GATAAGTACGAGAGCAGCGCGGCCGCCAAGAGCAGGATCAC	
WPRE-rev	GTTAAGAATACCAGTCAATCTTTCAC	
CAMKIIa-fwd	GTT CGC ATC CCC TTC TCC AAC	
Spy-ReaChR-fwd	CATCGTGATGGTGGACGCCTACAAGCCCACCAAGAGCACCGGCAGCGACGCCAC	*pLenti-CAMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE*
Spy-ReaChR-rev	GTAGGCGTCCACCATCACGATGTGGGCGGCGCCGGCGCTGCCGGCGGCC	
trkB-fwd	GTCGTGCCTGAGAGCGCAGTCGAGAAACCGGTGCCACCATGAGCC	*pLenti-hSyn1::TrkB-3xGS linker- mCherry-WPRE*
3xGS-trkB-rev	CGCCCTTGCTCACGCTACCGCTGCCGCTACCGCCCAGGATGTCCAGGTACACGG	

3xGS-mcherry-fwd	CTGGACATCCTGGGCGGTAGCGGCAGCGGTAGCGTG AGCAAGGGCGAGGAGGATAAC	
mcherry-rev	GATTATCGATAAGCTTGATATCGAATTCTCATTACTTGT ACAGCTCGTCCATGCCGCC	
spy-trkB-rev	CACATCGTGATGGTGGACGCCTACAAGCCCACCAAGG GCGGTAGCGGCTGCCCCATGAGCTGCAAGTGCAG	*pLenti-hSyn1::SpyTag-TrkB-mCherry-WPRE*
spy-trkB-fwd	GATCCGGGTGGTGCTGCACTTGCAGCTCATGGGGCAG CCGCTACCGCCCTTGGTGGGCTTGTAGGCGTCCACCA TCACGATGTGGGCGGCCAGGCTGGCCCGCCAGAAGC	
hsyn-fwd	CAGGGACAGCAGAGATCCAGTTTGGTTAATTAAGTGTC TAGACTGCAGAGGGCCCTGCG	
Worm1	CCCTGCAGCAGTTTATCAGTTATCAGCAAGCAG	*hlh-12::SpyTag-C1C2-mCherry*
Worm2	CCGGATCCTTTAATAAAATTGTGTAAGATGACGC	
Worm3	GCGGTACCATGTCGCGGAGGCCATGGCTTCTT	
Worm4	GCGGTACCTCACTTGTACAGCTCGTCCATGCC	
Worm5	CGGGTACCATGCGGAAAATGCTACTTTTTTGC	
Worm6	CGGGTACCTCATGTGTGTCGAATCATTGGTTC	
Worm7	CGGGTACCATGAAAGGCAGCAGCCATCATCAT	*HS::lin-3::SpyCatcher-GFP*
Worm8	CGGGTACCTTATTTGTAGAGCTCATCGATGCC	
Worm9	GACCATGATTACGCCAAGCTTGCA	
Worm10	TGAAACTGTAAATTGAGGCATAAAGAGTAG	
Worm11	TTTATGCCTCAATTTACAGTTTCAATGAAAGGCAGCAG CCATCATCAT	
Worm12	ATCACCGAAACGCGCGAGACGAAA	
Worm13	CGGGGTACCATGGTGAGCAGAAGACCCTGGCTG	*unc-47::SpyTag-ReaChR-TS-mCherry*
Worm14	CCAGAGGTTGATTATCGATAAGC	
Worm15	CGGGCCGGCCATGTTGTCATCACTTCAAACTT	
Worm16	CGGGCGCGCCCTGTAATGAAATAAATGTGACG	
Worm17	TTTATGCCTCAATTTACAGTTTCAATGAAAGGCAGCAG CCATCATCAT	*pSM-HS::lin-3::SpyCatcher-GFP*
Worm18	CGGGTACCATGCGGAAAATGCTACTTTTTTGCATCCTT CTACTCTTTATGCCTCAATTT	
Worm19	CGGAATTCTTATTTGTAGAGCTCATCGATGCC	
Worm20	CGGGCCGGCCGACCATGATTACGCCAAGCTTGCA	
Worm21	CGGGCGCGCCGATCCGATGAGGATTTTCGAAGTT	
ReaCh_E130_NDT_F	CGCTGATTNDTATGATGAAAAGC	Primers for site-saturation at position E130
ReaCh_E130_VHG_F	CGCTGATTVHGATGATGAAAAGC	
ReaCh_E130_TGG_F	CGCTGATTTGGATGATGAAAAGC	
ReaCh_E130_AHN_R	CATCATAHNAATCAGCGCCACATA	
ReaCh_E130_CDB_R	CATCATCDBAATCAGCGCCACATA	
ReaCh_E130_CCA_R	CATCATCCAAATCAGCGCCACATA	
ReaCh_N298_NDT_F	CGAAGNDTATGTGGGGC	Primers for site-saturation at position N298
ReaCh_N298_VHG_F	CGAAGVHGATGTGGGGC	
ReaCh_N298_TGG_F	CGAAGTGGATGTGGGGC	
ReaCh_N298_AHN_R	ACATAHNCTTCGCAATCAGATC	
ReaCh_N298_CDB_R	ACATCDBCTTCGCAATCAGATC	
ReaCh_N298_CCA_R	ACATCCACTTCGCAATCAGATC	

Highlighted text is to indicate degenerate nucleotides for site-saturation library

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SpyTag/SpyCatcher & SNAP-tag fusion constructs

The mammalian codon optimized *SpyTag* was first introduced into the N-terminus of *pLenti-CaMKIIa-C1C2-TS-EYFP* (**Table S2**) after the signal peptide cleavage site (between amino acid position 23 and 24 in the C1C2 sequence) by overlap extension PCR using external primers plenti-CaMKII_F and plenti-CaMKII_R, and internal primers SpyTag_C1C2_F and SpyTag_C1C2_R (**Table S3**). To generate the *pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-EYFP* (**Table S1**) the assembly product was then inserted into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* vector (**Table S2**). The *pLenti-CMV/CaMKIIa::SpyTag-C1C2-mCherry* construct (**Table S1**) was built by first amplifying *SpyTag-C1C2-TS* from the *pLenti-CMV/CamMKIIa-SpyTag-C1C2-TS-EYFP* (**Table S1**) construct using the plenti-CaMKII_F and TS_Rev primers (**Table S3**) and amplifying the *TS-mCherry* from *pAAV-CaMKII-C1V1-TS-mCherry* (**Table S2**) using TS_For and plenti-CaMKII_R (**Table S3**). The fragments were then assembled using overlap extension PCR with plenti-CaMKII_F and plenti-CaMKII_R primers (**Table S3**). The assembly product was then inserted into *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* (**Table S2**) vector using Gibson assembly. A similar process was used to generate the *pLenti-CaMKIIa::C1C2-mCherry* construct only the initial amplification was done using the *pLenti-CamMKIIa::C1C2-TS-EYFP* backbone. Note that all vectors denoted as having a *CaMKII*, *CaMKIIa* or *hSyn1* promoter also have an upstream *CMV* promoter. For the construct built for this work we have labeled the promoter as *CMV/CaMKIIa* since both promoters are present. The *CMV* promoter drives expression in transfections while the *CamKIIa* promoter would drive expression upon viral infection. These constructs can be used for both transfection of viral production and infection.

The mammalian codon optimized *SNAP-tag* sequence was first introduced into the N-terminus of *pLenti-CaMKIIa-C1C2-TS-mCherry* (**Table S2**) after the signal peptide cleavage site (between amino acid position 23 and 24 in the C1C2 sequence). The SNAP-tag sequence was amplified from $pSNAP_f$ vector (NEB, cat N9183S) with primers: C1C2-SNAP-NS-start and C1C2-SNAP-NS-end, and fused to C1C2 and mCherry with internal primers C1C2-NS-R and SNAP-CIC2-NS-mid, and external primers plenti-C1V1-3 and WPRE-R by overlap extension PCR method (**Table S3**). To generate the *pLenti-CMV/CaMKIIa::SNAP-tag-C1C2-TS-mCherry* (**Table S1**) the assembly product was then inserted into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-mCherry* vector using Gibson assembly method (**Table S2**).

Substitution of the aspartic acid, the reactive residue in the SpyTag, to the non-reactive alanine was done through mutation of the codon from *GAC* to *GCC*. This mutation was introduced through overlap extension PCR. The *SpyTag-C1C2-mCherry* was amplified into two separate fragments with the mutation introduced at the beginning of one fragment and the end of the other fragment using the C1C2_Spy_TagDA_F/plenti-CaMKII_R and plenti-CaMKII_F/C1C2_Spy_TagDA_R primer pairs (**Table S3**). These fragments were assembled though PCR, digested with *BamHI*/*EcoRI* and then ligated into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* vector (**Table S3**) to produce the *pLenti-CMV/CaMKIIa::SpyTag(DA)-C1C1-TS-mCherry* construct.

To generate SpyTag-ChR2-mCherry variants *ChR2-mCherry* was amplified from *pLenti-CaMKIIa-hChR2(H134R)-mCherry-WPRE* (**Table S2**) using pGP_Gib_ChR2_F and pGP_Gib_ChR2_R primers (**Table S3**). Gibson assembly method was then used to insert the *ChR2-mCherry* amplification product into the *pGP-CMV-GCaMP6f* vector (**Table S2**) cut with *BglII/XbaI*. This produced the *pGP-CMV::ChR2-mCherry* construct that was then used for all ChR2, SpyTag fusions. The C-terminal fusion of *SpyTag* to *ChR2-mCherry* (*pGP-CMV::ChR2-mCherry-SpyTag*) was generated by overlap-extension PCR by first amplifying the *ChR2-mCherry-SpyTag* in two parts with ChR2_SpyTag_F/ pGP-Gib_ R and pGP-Gib_ F/ ChR2_SpyTag_R primer pairs. The two amplified fragments were then assembled using ChR2_SpyTag_F/ ChR2_SpyTag_R primers. The assembly product was inserted into the *pGP-CMV-GCaMP6f* vector (**Table S2**) cut with *BglII/XbaI*. Stepping of the SpyTag at the N-terminal end of ChR2 was done using the same method using different SpyTag insertion primers labeled as SpyTag_ChR2_# based on the position described in **Figure S4**.

ReaChR rhodopsin was fused to the mCherry reporter after a three-alanine residue linker, and a trafficking signal (TS) KSRITSEGEYIPLDQIDINV (Gradinaru et al., 2010). The *ReaChR* gene was amplified from *AAV-EFIa-ReaChR-mCitrine-FLEX* vector (**Table S2**) using ReaChR_fwd and ReaChR_rev primers (**Table S3**). The *3xA-linker-TS-mCherry* was amplified from *pLenti-CAMKIIa-C1C2-TS-mCherry* (**Table S1**) plasmid using pA_TS_mcherry_fwd and WPRE_rev primers (**Table S3**). *ReaChR-TS-mCherry* was assembled using overlapping assembly PCR and digested with *EcoRI* and *BamHI*. Digested insert was ligated into an *EcoRI*/*BamHI* digested Lentiviral vector containing the *CMV*/*CaMKIIa* promoters and WPRE to obtain the *pLenti-CMV/CaMKIIa::ReaChR-TS-mCherry-WPRE* clone (**Table S1**). *SpyTag* was inserted at the N-terminus of *ReaChR* after the signal peptide cleavage site (24 amino acids from the N-terminus) using overlap extension

PCR with primers Spy_ReaChR_fwd, Spy_ReaChR_rev, WPRE_rev and CAMKIIa_fwd (**Table S3**). Digestion and ligation of the assembled product into the template lentiviral vector produced the *pLenti-CMV/CaMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE* clone (**Table S1**).

The *pLenti-CMV/hSyn1::TrkB-mCherry-WPRE* vector (**Table S1**) was built by Gibson assembly method. A lentiviral vector containing *human synapsin I* (*hSyn1*) promoter and *WPRE*, *pLenti-hSyn-eNpHR 3.0-EYFP* (**Table S2**), was digested with *AgeI* and *EcoRI* enzymes and used as backbone for all TrkB constructs. *TrkB* was synthesized from GenScript USA Inc, fused with *EYFP* reporter and inserted into this lentiviral vector to build *pLenti-CMV/hSyn1::TrkB-EYFP-WPRE* vector. To replace the *EYFP* marker with *mCherry* the *mCherry* gene from *pLenti-CaMKIIa-ReaChR-TS-mCherry-WPRE* vector was amplified with 3xGS_mcherry_fwd and mcherry_rev primers (**Table S3**). Assembly product of the *TrkB*, *mCherry* fusion was generated using TrkB_fwd and 3xGS_TrkB_rev primers, and then inserted into the digested lentiviral vector. *pLenti-CMV/hSyn1*::SpyTag-TrkB-mCherry-WPRE* (**Table S1**) was built by inserting the *SpyTag-GGSG* linker at the N-terminus of *TrkB* after the signal peptide cleavage site (31 amino acids from the N-terminus) using the overlapping primers spy_trkB_rev and spy_trkB_fwd, and assembled with end primers hsyn_fwd and mcherry_rev primers. This was then inserted into the template lentiviral vector containing *hSyn1* promoter at sites *AgeI* and *EcoRI* using Gibson assembly method.

To generate *C. elegans* expression plasmid *hlh-12::SpyTag-C1C2-mCherry*, 1.2 kb of the *hlh-12* 5' region was PCR amplified from genomic DNA using primers Worm1 and Worm2, and cloned into pPD49.26 (Fire vector) using *PstI* and *BamHI* restriction sites. Then, the *SpyTag-C1C2-mCherry* sequence was PCR amplified from plasmid *pLenti-CaMKIIa::SpyTag-C1C2-mCherry* (**Table S1**) using primers Worm3 and Worm4, and was cloned into ppD49.26 *hlh-12* vector using the *KpnI* restriction site. Plasmid pSM*::unc-47::SpyTag-ReaChR-TS-mCherry* was generated by first PCR amplifying *SpyTag-ReaChR-TS-mCherry* from *pLenti-CaMKIIa::SpyTag-ReaChR-TS-mCherry* using primers Worm13 and Worm14 and inserting the PCR product into vector pSM::GFP (gift from Cori Bargmann) using *KpnI* and *EcoRI* restriction sites. pSM*::unc-47::SpyTag-ReaChR(E130D)-TS-mCherry* was constructed in the same way only by PCR amplifying *SpyTag-ReaChR(E130D)-TS-mCherry* from *pLenti-CaMKIIa::SpyTag-ReaChR(E130D)-TS-mCherry*. 1.5 kb of the *unc-47* 5' region was PCR amplified from genomic DNA using primers Worm15 and Worm16, and cloned into pSM::*SpyTag-ReaChR-TS-mCherry* using *FseI* and *AscI* restriction sites (**Table S1**).

PCR fusion product *HS::lin-3 signal sequence::SpyCatcher-GFP* was constructed by PCR fusion of PCR products from a *HS::lin-3* plasmid and *SpyCatcher-GFP* plasmid. To generate *HS::lin-3* plasmid, a partial *lin-3* (*C. elegans* EGF) sequence was PCR amplified from genomic DNA using primers Worm5 and Worm6, and inserted into plasmid *pPD49.83* (Fire vector containing HS promoter) using the *KpnI* restriction site. *SpyCatcher-GFP* plasmid was generated by amplifying *SpyCatcher-GFP* sequence from plasmid *pQE80l-T5::6xhis-SpyCatcher-Elp-GFP* using primers Worm7 and Worm8 and inserting into plasmid pPD49.83 using the *KpnI* restriction site. *HS::lin-3 signal sequence* was PCR amplified from the *HS::lin-3* plasmid using primers Worm9 and Worm10, and *SpyCatcher-GFP::unc-54 3' UTR* sequence was amplified from *SpyCatcher-GFP* plasmid using primers Worm11 (containing overlap to the *lin-3* signal sequence) and Worm12. *HS::lin-3 signal sequence* and *SpyCatcher-GFP::unc-54 3' UTR* PCR products were fused through a second PCR reaction using both products as templates and primers Worm9 and Worm12.

Plasmid *pSM-lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR* was generated by adding on the *lin-3 signal sequence* to *SpyCatcher-GFP::unc-54 3'UTR* through two PCR reactions, first using primers Worm17 and Worm19 and *SpyCatcher-GFP* plasmid as template. The PCR product was then amplified using primers Worm18 and Worm19. The product was cloned into *pSM-GFP* vector using the *KpnI* and *EcoRI* restriction sites. *HS* sequence was PCR amplified from *pPD49.83* using primers Worm20 and Worm21 and the product was cloned into *pSM-lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR* using *FseI* and *AscI* restriction sites to generate the final plasmid, *pSM-HS::lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR.*

To build the *pQE80l-T5::6xhis-SpyCatcher-Elp-GFP* construct new restriction sites were introduced for our system (for details see Sun *et al*.). The original restriction sites following His6 tag in pQE-80l were removed. This SpyCatcher-GFP construct was derived from SpyCatcher-Elp-SpyCatcher (pQE-BB) described in Sun *et al.* (Sun et al., 2014). The GFP gene with a TAA stop codon was inserted between *SacI* and *SpeI* sites to generate the final construct.

Generating site-saturation library from the Tag-ReaChR-mCherry parent

Primers designs are listed in **Table S3** with degenerate residues highlighted in yellow. Mutations were introduced by overlap extension PCR of the *pLenti-CaMKIIa::SpyTag-ReaChR-TS-mCherry* parent backbone

with external primers plenti-CaMKII_F/ plenti-CaMKII_R used for amplification and assembly. Assembly product was then digested with *EcoRI/BamHI* and ligated into *EcoRI/BamHI* cut *CaMKIIa::SpyTag-ReaChR-TS-mCherry* vector. Each library was then transformed into *E. coli*, single colonies were picked and 2-5 ml cultures were grown for each variant. DNA for each variant was purified and the concentration of DNA for each variant was normalized to 100 ng/ul for transfection into HEK cells.

HEK cell maintenance and transfection

HEK 293F cell were cultured at 37 ^{o}C and 5% CO2 in D10 (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 1% sodium bicarbonate, 1% sodium pyruvate, and penicillin-streptomycin). For low throughput confocal imaging constructs were transfected with Fugene6 into HEK cells according to the manufacturer's protocol plated at a density of 5,000 cells per cm^2 onto 12 mm- PolyDLysine coated coverslips at 18 hours post-transfection. The HEK cells were then left to adhere to coverslips and continue to express for another 30 hours (so total expression for 48 hours post transfection) before labeling with SpyCatcher and imaging. For the 96-well format screening HEK cells were seeded at low density in tissue culture treated 96-well plates (BD Falcon Microtest^{TM} 96). Cells were left to divide until they reached ~20-30% confluency. Library variants were transfected with Fugene6 into HEK cells according to the manufacturer's recommendations with one variant per well (with pre-normalized DNA concentration of each variant). Cells were then labeled with SpyCatcher 48 hours post transfection and imaged.

Preparation and transfection of primary neuronal cultures

Rat hippocampal cells were dissected from Wistar embryos (prenatal days E18, Charles-River Labs), and cultured at 37oC, 5% CO2 in Neurobasal media supplemented with B27, glutamine, and 2.5% FBS. 3 days after plating, glial growth was inhibited by addition of FUDR. Cells were transfected 4-5 days after plating with SpyTag-opsin variants using calcium chloride. Neurons were labeled with SpyCatcher and imaged 2-5 days after transfection.

SpyCatcher labeling of HEK cells in 96-well format

SpyCatcher labeling protocol for 96-well plate. To avoid any variability in labeling in the 96-well format screen a saturating concentration of the SpyCatcher (30 μM) was used for labeling experiments. A 75 μM SpyCatcher stock was made and 20 μl of the stock was added to 30 μl of D10 in each well for a final concentration of 30 μM SpyCatcher per well. The cells were then incubated with the labeling protein at room temperature for 45 minutes. After the labeling the cells were washed. To avoid complete removal of media from the cells 200 μl of fresh D10 was added to each well to dilute the SpyCatcher concentration and then 200 μl was removed from each well. This washing/dilution was repeated four times. After washing the 96-well plates of cells were returned to a 37 ^{o}C incubator and left for 30 minutes before imaging. For imaging of cells in each well the media was replaced with extracellular buffer (in mM: 140 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.35) to avoid the high autofluorecence of the D10.

SNAP labeling of HEK cells

SNAP-Surface® 488 was purchased from NEB (cat S9124S). Labeling of live HEK cells transfected with *pLenti-CaMKII::SNAP-tag-C1C2-TS-mCherry* was done following manufacturer's instructions for cellular labeling. In brief, the SNAP-Surface® 488 reconstituted in DMSO to make a 1 mM stock solution. The stock solution was then diluted 1:200 in D10 media to yield a labeling medium of 5 uM dye substrate. The SNAP-tag-C1C2-mCherry expressing HEK cells were then incubated in the labeling medium for 30 min at 37^{o}C. After labeling the cells were washed 3-4x with D10 media before confocal imaging.

Generating and maintaining SpyTag and SpyCatcher transgenic *C. elegans* strains

C. elegans strains were cultured at room temperature using standard protocols unless indicated otherwise (Brenner, 1974). Strains used in this study were *him-5*(*e1490*) (Hodgkin et al., 1979) and *unc-119(ed4)* (Maduro and Pilgrim, 1995). Transgenic *C. elegans* expressing Tag-C1C2-mCherry was generated by co-injecting plasmid *hlh-12::SpyTag-C1C2-mCherry* (14 ng), *unc-119* rescue plasmid (60 ng), and 1kb ladder carrier DNA (50ng) into *unc-119* mutant animals. A transgenic *C. elegans* line expressing heat-shock activated Catcher-GFP and specific expression of Tag-C1C2-mCherry in DTCs was generated by co-injecting plasmid *hlh-12::SpyTag-C1C2-mCherry* (14 ng), PCR fusion product *HS::lin-3 signal sequence::SpyCatcher-GFP* (40 ng), 1kb ladder carrier DNA (50 ng), and *unc-119* rescue plasmid (60 ng), into *unc-119* mutant

animals. Transgenic animals expressing heat-shock activated Catcher-GFP and either wild-type or mutant *SpyTag-ReaChR-TS-mCherry* in GABA neurons was generated by co-injecting plasmid *unc-47::SpyTag-ReaChR-TS-mCherry* (wild-type or mutant 90 ng), plasmid *HS::lin-3 signal sequence::SpyCatcher-GFP* (50 ng), 1kb ladder carrier DNA (50 ng), and *unc-119* rescue plasmid (60 ng), into *unc-119* mutant animals.

SpyCatcher staining of dissected *C. elegans* gonad

To extrude gonads from animals, hermaphrodites were placed in 6 mL of PBS (phosphate buffered saline) on a Superfrost plus microscope slide (Fisher Scientific) and cut below the pharynx with a razor blade as described previously (Chan and Meyer, 2006). 6 mL of 4% p-formaldehyde solution was added, sandwiched with a coverslip, and fixed for 10 minutes. The entire slide was then submersed in liquid nitrogen for a few minutes, and immediately upon removal, the coverslip was removed and the slide was washed with PBS three times. 30 mL of purified Catcher-GFP in PBS solution (20 μM) was applied to the fixed gonads on the slide and incubated for 30 minutes at room temperature. The slide was washed 3x5 minutes with PBS and imaged after mounting with Vectashield mounting media (Vector Laboratories).

Heat-shock treatment to induce SpyCatcher expression

C. elegans strain carrying transgenes *hlh-12::SpyTag-C1C2-mCherry* and *HS::lin-3 signal sequence::SpyCatcher-GFP* was heat-shock treated at 33°C for 15 minutes in a water bath. *C. elegans* strain carrying transgenes *HS::lin-3 signal sequence::SpyCatcher-GFP and either wild-type or mutant unc-47::SpyTag-ReaChR-TS-mCherry* were heat-shock treated at 33°C for 30 minutes. 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.

Locomotion assay evoked by green light

Animals expressing either wild-type or mutant *unc-47::SpyTag-ReaChR-TS-mCherry* were grown on NGM plates with OP50 bacteria and all-trans retinal. 150μL of OP50 culture alone or with 100μM all trans-retinal (0.15μL of 100mM stock in ethanol; Sigma-Aldrich) was added to NGM plates and dried for several hours in the dark. 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 2.5x magnification for 10 s without green light, 5 s with green light illumination, and 10 s without green light. Green light (650±13 nm) was generated using LED illumination using a Lumencor SPECTRAX light engine at a power of 1 mW. White light illumination, which was constant throughout the experiment, was filtered to remove blue/green light. Paralysis upon illumination was scored as a positive.

Fluorescence Imaging

For non-confocal imaging of cultured neurons expressing different opsin variants a Zeiss Axio Examiner.D1 microscope with a 20x 1.0 NA water immersion objective (Zeiss W Plan Apochromat 20x/1.0 DIC D=0.17 M27 75mm) was used. Images of neurons were taken before electrophysiological recordings and the images we analyzed for fluorescence level comparison between variants. Imaging of the mCherry fusion fluorescence was excited with 650±13 nm, and imaging of the GFP label fluorescence was excited with 485±20 nm. Both wavelengths of light were generated with LED illumination using a Lumencor SPECTRAX light engine with quad band 387/485/559/649 nm excitation filter, quad band 410/504/582/669 nm dichroic mirror and quad band 440/521/607/700 nm emission filter (all SEMROCK).

Confocal imaging was preformed on a Zeiss LSM 780 Confocal Microscope. Imaging of live cultured HEK cells and neurons was preformed with a Zeiss W Plan-APOCHROMAT 20x/1.0 DIC(UV) Vis-IR objective. Imaging of live *C. elegans* was preformed using a Zeiss LD LCI Plan-APOCHROMAT 25x/0.8 Imm Korr DIC M27 objective. GFP fluorescence was excited with a 488 nm laser and mCherry fluorescence was excited with a 561 nm laser. Fluorescence emission was imaged using the LSM 780's GaAsP detectors with a detection range of 499-606 nm for GFP and 578-695 nm mCherry. Imaging was done with excitation and emission measurements of GFP and mCherry done on separate tracks to avoid crossover. Imaging settings were matched across experiments to enable comparison.

Full population images of cells in 96-well plates were taken with a Leica DM IRB microscope and the Leica microsystems objective HC PL FL 10x/0.30 PH1. Cells were illuminated with LEJ ebq 50 ac mercury lamp. GFP fluorescence was imaged with SEMROCK Blue light filter set: SEMROCK BrightLine® single-band filter set with BrightLine® single-band bandpass excitation filter (482/18 nm), emission filter (520/28) and 495

nm edge BrightLine® single-edge dichroic beamsplitter. mCherry fluorescence was imaged with Leica's N2.1 filter cube with bandpass excitation filter (515-560 nm), longpass suppression filter (590 nm) and dichromatic mirror (580).

Electrophysiology

Conventional whole-cell patch-clamp recordings were done in cultured HEK cells and cultured rat hippocampal neurons at least 2 days post transfection. Cells were continuously perfused with extracellular solution at room temperature (in mM: 140 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.35) while mounted on the microscope stage. Patch pipettes were fabricated from borosilicate capillary glass tubing (1B150-4; World Precision Instruments, Inc., Sarasota. FL) using a model P-2000 laser puller (Sutter Instruments) to resistances of 2-5 MΩ. Pipettes were filled with intracellular solution (in mM): 134 K gluconate, 5 EGTA, 10 HEPES, 2 $MgCl_2$, 0.5 $CaCl_2$, 3 ATP, 0.2 GTP. Whole-cell patch-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), a Digidata 1440 digitizer (Molecular Devices), and a PC running pClamp (version 10.4) software (Molecular Devices) to generate current injection waveforms and to record voltage and current traces.

Patch-clamp recordings were done with short light pulses to measure photocurrents. Photocurrents induced by green light were measured using 590±25 nm LED at 1 mW mm^{-2} for ReaChR-mCherry and Tag-ReaChR-mCherry variants. While C1C2-mCherry and Tag-C1C2-mCherry variant's photocurrents were induced by cyan light were measured using 485±20 nm LED at 0.3 mW mm^{-2}. Photocurrents were recorded from cells in voltage clamp held at -50 mV with 3-10 light pulse trains (0.5 s each pulse; 2 s apart). Both wavelengths were produced using LED illumination using a Lumencor SPECTRAX light engine with quad band 387/485/559/649 nm excitation filter, quad band 410/504/582/669 nm dichroic mirror and quad band 440/521/607/700 nm emission filter (all SEMROCK).

Action spectra measurements were performed for the following wavelengths: 386±23 nm, 485±20 nm, 590±25 nm, and 650±13 nm with light intensity matched across all experiments at 0.1 mW mm^{-2}. Each light pulse was delivered for 0.6 s with 10 s breaks between light pulses. All wavelengths were produced using LED illumination from a SPECTRAX light engine (Lumencor). Cell health was monitored through holding current and input resistance.

Data analysis

A MATLAB script was written to compare area above a threshold level of fluorescence in a population of cells. This was done for both mCherry fluorescence and GFP fluorescence. The mCherry-above-threshold-area was then used to normalize the GFP-above-threshold-area so that the density of cells within the image was not a confounding factor. The ratio of GFP-above-threshold-area to mCherry-above-threshold-area was the metric used to compare across the libraries reported in **Figure 3**.

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