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Dark Dyes–Bright Complexes: Fluorogenic Protein Labeling

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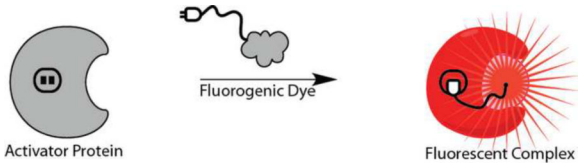
Abstract

Complexes formed between organic dyes and genetically encoded proteins combine the advantages of stable and tunable fluorescent molecules and targetable, biologically integrated labels. To overcome the challenges imposed by labeling with bright fluorescent dyes, a number of approaches now exploit chemical or environmental changes to control the properties of a bound dye, converting dyes from a weakly fluorescent state to a bright, easily detectable complex. Optimized, such approaches avoid the need for removal of unbound dyes, facilitate rapid and simple assays in cultured cells and enable hybrid labeling to function more robustly in living model organisms.

Graphical Abstract



Introduction

The emergence of new imaging approaches that interrogate cellular and organismal behavior with resolution and timescales previously inconceivable have placed new requirements on the performance of fluorescence labels used in these imaging approaches. In particular, brightness and photostability enhancements are required to provide increased resolution in methods such as stimulated emission depletion (STED) microscopy[1,2] and structured illumination microscopy (SIM) [3,4], both of which effectively oversample the specimen relative to conventional confocal and widefield microscopy to produce resolution gains. Localization microscopy methods such as photoactivation localization microscopy (PALM) [5] and stochastic optical reconstruction microscopy (STORM) [6] require high photon output molecules that can be precisely localized at a single molecule level. The use of these approaches in living cells, where molecules move (e.g. sptPALM) [7] require both sustained photon output and high brightness probes. The use of hybrid tagging approaches (i.e. a

synthetic dye that labels a specific genetically encoded reporter) has been essential in establishing superresolution imaging as a viable biological imaging approach in conditions where the fluorescence brightness, photostability and photochemical properties of intrinsically fluorescent proteins are limiting.

Initial applications for many of these reagents and imaging approaches were based in cultured cell models. As the approaches for high-resolution and high-speed imaging expand, there is a persistent drive to move into more complex milieu[8,9]. This poses challenges for many labeling approaches that work in cell culture, because the removal of unbound probe is usually required to produce specific detectable labeling. To alleviate these challenges, chemical biologists have developed a series of reagents that consist of initially dark or quenched dyes that are activated upon target binding. Ideally, these reagents can provide very specific and selective activation upon target labeling, producing a low-background, high-contrast imaging approach that powers high-speed, high-resolution imaging in living animals.

An essential benefit to the use of fluorogenic protein labeling arises from the ability to control the time of dye addition[10]. These fluorogenic tagging approaches are well-suited to pulse-chase labeling, or differential labeling of distinct compartments using chemical modifications of the fluorogenic dye that are compartment-restricted. In addition, the stability and binding-dependent activation of these probes have generated unique applications in single molecule and superresolution imaging.

Fluorogenic protein labeling

The overall goal of fluorogenic protein labeling is to develop a system that consists of a non-fluorescent dye and an apoprotein, where the binding of the dye results in a significant change in the fluorescence brightness[11,12]. An important metric for a fluorogenic labeling system is the “activation ratio” (AR), typically given as the fluorescence intensity of the fully bound dye divided by the fluorescence intensity of the unbound dye under the same excitation conditions. This definition allows three critical parameters to control the AR. Changes in the fluorescence quantum yield, spectral position, or dye extinction coefficient upon binding can be used to achieve a fluorogenic labeling effect. (Figure 1) To optimize the performance of a fluorogenic protein/peptide tag, the protein should be well folded in various cellular compartments, the dye should be freely cell permeant, and show low background within the cell in the absence of the expressed protein, and the interaction between the dye and protein should take place at a low concentration of dye, to ensure the highest activation ratio in a labeled cell. Fluorogenic labeling, because it takes place in the background of unremoved dye, will be directly affected by the ratio of free to bound dye in the specimen, so while the AR provides some useful information on the imaging contrast, the ultimate performance will depend on the AR and the excess concentration of dye required to achieve complete labeling. (Figure 2) From this perspective, it is important to have a fluorogenic system with a high AR that also functions effectively at low concentrations of added dye.

Fluorogen activation mechanisms

FRET Quenching—Because many of the earliest hybrid tagging approaches involved ligating a protein to a chemically modified substrate, it was recognized that coupling of an energy transfer acceptor at a short distance could result in efficient quenching of the donor. Coupling to the tagging motif through chemistry that releases the linked quencher results in efficient reactivation of the fluorescent dye[13,14]. For dyes that are efficiently quenched by the proximal donor, the reactivation can typically bring 10-fold or higher activation. Although the distance-dependence in the Förster relation would predict 1000-fold quenching for a dye pair held at ~1/3 the Förster radius, the orientation dependence for dyes on a short linker alters this efficiency, and only moderate fluorescence activation has been achieved using these approaches (typically ~100-fold maximum activation).

Direct—Because the electronic ground and excited states of conjugated molecules have differences in their rigidity and charge distribution, many show sensitivity to the local environment that can be exploited for fluorogen activation. Polarity sensing dyes are typically those that have differences in the charge distribution between ground and excited states, and respond to solvent stabilization of one or the other states with a spectral shift. Many polarity sensing dyes are “push-pull” or “donor-acceptor” chromphores, which have an electron donating group electronically coupled to the acceptor group. Viscosity sensing dyes, on the other hand, typically have some vibration or rotational mode in the excited state that can couple directly to the ground electronic state, resulting in internal conversion and relaxation without fluorescence. High viscosity or conformational constraints can provide significant barriers to these relaxation modes, and significantly activate the fluorescence. Finally, excited state charge transfer (electron or proton) can allow direct relaxation, and chemical modification or binding to the charge donor (or acceptor) group can activate the fluorescence directly. While this method has primarily been used for direct chemical sensing (e.g. BAPTA based Ca^{2+} indicators[15,16]), some recent approaches have shown this to be useful for direct fluorogenic labeling of proteins with suitable ligands[2,17].

While each of these methods have been used for “activity-based” labeling, where the construct is targeted to enzymes to assess activity in a living cell or animal, those applications are beyond the scope of this review, and have been reviewed elsewhere. In this work, we focus on using fusion-tags to label specific proteins for imaging applications.

Fluorogen activating proteins/peptides

Apart from activity-based probes, most fluorogens are activated by a dye-binding or dye-anchoring domain genetically fused to the target of interest. The target can be a cellular protein, which is labeled and expressed in live cells directly[18], or the protein can be fused to a targeting domain, such as an affibody[19] or a scFv[20], which is expressed recombinantly and then used to label endogenous proteins on cells or in tissues. For effective protein labeling, the fluorogenic dye should be bound and activated at the expressed protein target. Figure 2 shows the various ways in which fluorogens can be activated by their target proteins. Covalent labeling can chemically transform the dye, creating a conjugated structure or removing a quencher to activate the detectable fluorescent probe at the labeled site (e.g. [21]). Once converted, however, these dyes are always “on”.

Covalent linking of an environmentally sensitive dye to a protein of interest, allows local interrogation of the chemical environment, and selective activation of the dye in the proper environment. Targeting of pH sensors can be used to assess protein localization to acidic compartments such as endosomes[22], and targeting of lipid probes can be used to detect membrane associated proteins fluorogenically, as recently demonstrated with SNAP-tag directed Nile Red[23].

Alternative proteins directly activate the fluorogen dye upon binding (e.g. [11,12,24]). Binding of fluorescent molecules to a specific stereoelectronic environment inside a protein can activate the fluorescence directly without any chemical modification of the protein or the dye itself. An assortment of dye structures have been used as fluorogens, and these include dyes that are inherently rotationally relaxed in the excited state[12,25–27], those that are enhanced by solvent polarity[28], and those that are activated by anchoring in the protein environment, potentially through altered spirolactone formation[24,29]. Some of these fluorogens are shown in Figure 3, with the associated AR values reported for these in the optimal fluorogen activating protein. These dyes are typically weakly or completely non-fluroescent in aqueous media, and activation ratios of up to ~20,000 have been reported with MG-binding scFv based activating proteins, with equilibrium dissociation constants in the low pM range[30]. Many of these FAP-fluorogen pairs function at the cell surface, but so far, only MGe-FAP[18], aminocoumarin-PYP[28] and the SiR-SNAP[29] and SiR-halo ligands (and analogous azetidine containing JF646 ligands[24]) complexed to their target proteins function as fluorogens robustly within living cells. These challenges are three-fold: dye penetration across the plasma membrane requires a compact, low-polarity fluorogen; the protein targeting domain must fold robustly at the target site in the cell; and the dye must not activate nonspecifically within living cells, even at sites of high accumulation (e.g. mitochondria or DNA in the nucleus). Modifications to the fluorogens can potentially reduce these interactions, and improve the function within living cells[24,31].

Applications

An essential advantage of a high-affinity, high AR fluorogenic labeling approach is the ability to selectively and rapidly label protein in living, behaving cells and organisms, without any need for washing away of unbound dye for quantitative detection. Although labeling inside the cell is practical, the timescale for labeling is still on the order of minutes, since dye permeation through the plasma membrane is required[18,28]. A number of applications for rapid fluorogen activation have evolved in the past two years that exploit the rapid, no-wash labeling of surface displayed proteins for quantitative analysis of protein plasma membrane abundance and protein trafficking.

Malachite green itself is a cell-permeant, cationic dye that shows significant fluorescent activation in cells, as well as some growth suppression when incubated with growing yeast. Substitution of the base phenyl ring with a phenolic ether allowed preparation of various dye analogs with low nonspecific and tailored cell permeability properties, allowing chemical discrimination between cell surface and intracellular fractions of membrane proteins[12]. Recently, Yan et al. [31] prepared bis-sulfonated versions of this malachite green chromophore, and showed that they had improved cell exclusion, further reduced

nonspecific activation on dying cells, and rapid labeling kinetics (k_{on} ~10^5 $M^{-1}s^{-1}$) for FAP-labeled proteins in the plasma membrane. Using dye addition and flow cytometric analysis, labeling is complete within <30 sec, and results are in quantitative agreement with surface-selective antibody labeling approaches for measurement of G-protein coupled receptor (GPCR) desensitization. This approach substantially reduces the variability seen with antibody labeling approaches, and potentially opens the door to very high-throughput analysis of cell surface abundance of proteins in high throughput flow cytometry[32–34].

Fluorogens based on sulfonated thiazole orange analogs were shown to be cell excluded, and suitable for labeling FAP-tagged proteins at the plasma membrane with rapid labeling kinetics[35]. This fluorogen could be linked to a variety of dye acceptors[36], including pH sensors and far-red energy transfer acceptors, producing multicolor labeling reagents. Grover et al. prepared a pH senstitive analog of the TO1 dye, which reported in real-time on changes in GPCR endocytosis and intracellular acidification of the endosomes[37]. Fisher et al. prepared a pH insensitive analog of this dye, and used it as a two-color pulse-chase labeling reagent. By pre-labeling receptors with the low-affinity TO1-2p-Cy5 fluorogen, treating with an agonist, and then labeling with the higher-affinity TO1-2p, internalized receptors retained red-shifted fluorescence (488 nm excitation/670 nm emission) and residual surface receptors were displaced with the higher-affinity dye, (488 excitation/530 nm emission) (Figure 4). The assay was shown to function robustly for a wide variety of receptors and agonists[38].

The simplicity of a noncovalent interaction to activate a fluorogen leads to an interesting and useful feature of this labeling approach. Regardless of expression level, a sparse, optically resolvable subset of target sites can be labeled by using a dye concentration that is considerably below the saturation concentration. Over time, this population can be analyzed at a single molecule level, revealing single particle trajectories or superresolution reconstructions of intracellular structures[39]. Although currently available constructs have slow dissociation kinetics, selection and optimization of these properties may enable rapid and dynamic superresolution imaging in living cells. At the surface, stochastic labeling of FAP-tagged FcεRI was used to show that diffusion properties of these receptors did not change upon binding of cytokinergic (activating) IgE molecules, illustrating a different mode of activation than that obtained with normal IgE crosslinking for activation of the receptor[40]. The sparse labeling is compatible with continuous application of low dye concentration, so there is a persistent, low density of labeled receptors throughout the experiment. This allows for on-the-fly changes in the media, which can be used to test “before” and “after” challenges in the same cell, for example addition of a biological ligand, while examining the same population of receptors.

Conclusions

The utility of fluorogens as a chemical-genetic protein labeling method is now established as a general approach that can function at various intracellular sites and in various experimental approaches. Extension to living model organisms has lagged behind the applications in cells, but remains a very exciting future direction[41]. For these in-vivo applications, the optimal fluorogens will need to be coupled with highly active, highly robust fluorogen activating

proteins that are functional in various intracellular compartments. Future applications that integrate acute fluorogenic labeling with chemical sensing may provide powerful new measurements of living systems and their dynamic changes.

Acknowledgements

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Highlights

- Protein-dye complexes that activate fluorescence allow no-wash protein labeling.
- Quencher removal, environmental change and direct binding can activate fluorescence.
- Modifications of the chromophore and linker can influence fluorogen performance.
- Pulse-chase and order-of-addition experiments can reveal dynamic cellular changes.
- High-quality fluorogen imaging in organisms will reveal new biological processes.

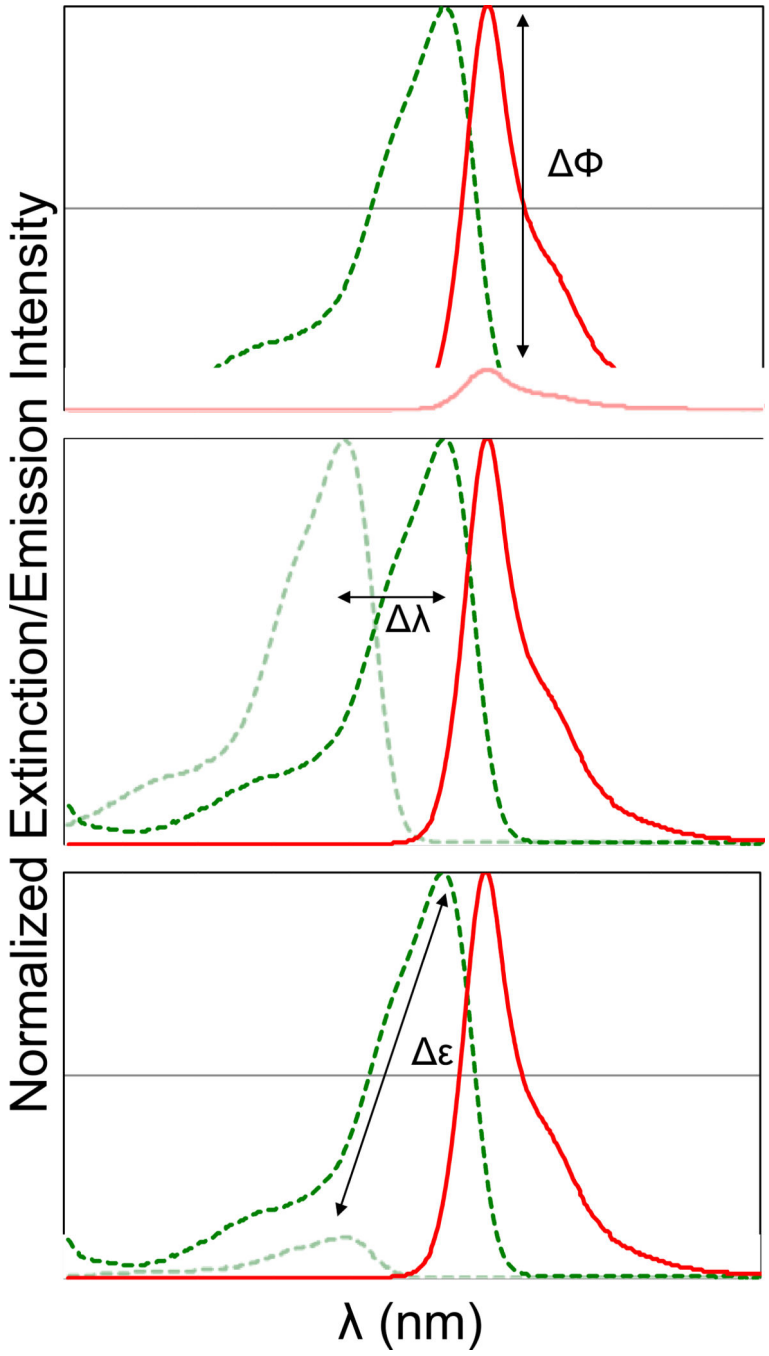

Figure 1. Spectroscopic changes utilized for fluorogen activation

Binding of a dye to a protein domain can result in changes in quantum yield (top), excitation wavelength (middle) or extinction coefficient/excitation cross-section (bottom). Combinations of these properties will result in high activation ratios.

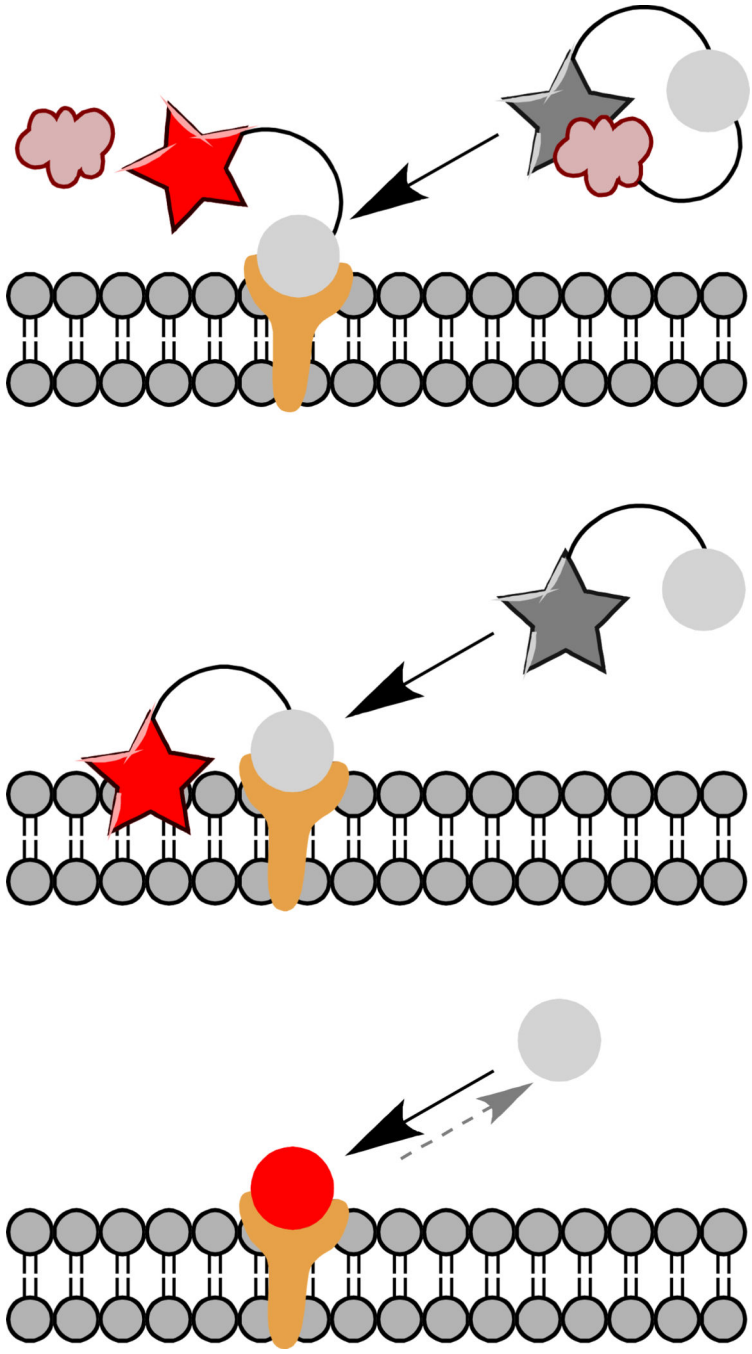

Figure 2. Fluorogen activation approaches

Binding of the fluorogen to an expressed domain can be used to release a quenching group (top), to bring a dye near to an environment that activates it, for example a membrane or protein pocket (middle), or to directly bind the fluorogen dye in a fluorescent complex (bottom). Direct binding of the fluorogen to the fluorogen activating protein is reversible, unlike cleavage or covalent linkage, which is advantageous for some applications.

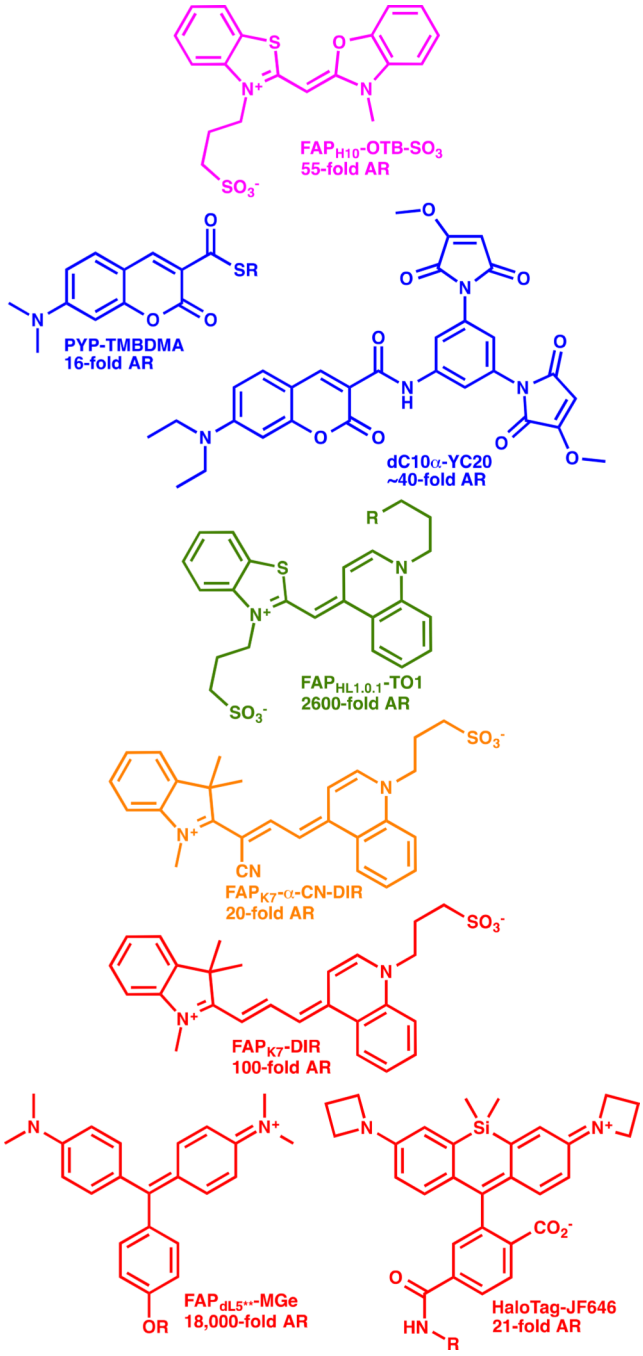

Figure 3. Fluorogen dye structures spanning the visible spectral range

Fluorogenic dyes of various structure and spectral properties have been demonstrated for labeling of living cells. The dye structure is shown, along with the activating protein (as named in the cited references), and the reported activation ratio (AR). References: OTB-SO_3 [25]; TMBDMA [28]; YC20 [21]; TO1 [12]; α-CN-DIR [27]; DIR [26]; MG [30]; JF646 [24].

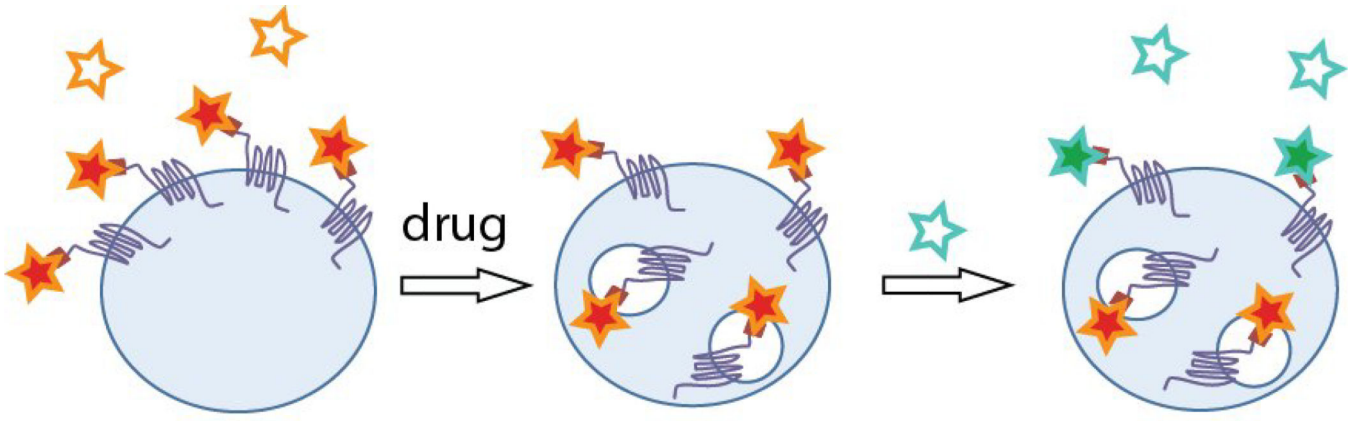

Figure 4. Fluorogen labeling for protein trafficking at the membrane

Pulse-treat-chase labeling of cell-surface receptors with exchangeable fluorogens of two resolvable colors results in differential coding of proteins protected (from exchange) in endosomes and those still exposed at the plasma membrane.