CHAPTER THREE

Fluorescence Fluctuation Spectroscopy Approaches to the Study of Receptors in Live Cells

David M. Jameson[*,1], **Nicholas G. James**[*], **Joseph P. Albanesi**[†]
[*]Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii, USA
[†]Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas, USA
[1]Corresponding author: e-mail address: djameson@hawaii.edu

Contents

Abstract

Communication between cells and their environment, including other cells, is often mediated by cell surface receptors. Fluorescence methodologies are among the most important techniques used to study receptors and their interactions, and in the past decade, fluorescence fluctuation spectroscopy (FFS) approaches have been increasingly utilized. In this overview, we illustrate how diverse FFS approaches have been used to elucidate important aspects of receptor systems, including interactions of receptors

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with their ligands and receptor oligomerization and clustering. We also describe the most popular methods used to introduce fluorescent moieties into the biological systems. Finally, specific attention will be given to cell maintenance and transfection strategies especially as related to microscopy studies.

1. INTRODUCTION

In order for multicellular organisms to exist and to function, they must be able to communicate with their surroundings, that is, to exchange chemicals and information. Communication between cells and their environment, including other cells, is often mediated by cell surface receptors, which are integral plasma membrane proteins. Signal transduction pathways are usually initiated by binding of extracellular ligands (such as growth factors, cytokines, hormones, neurotransmitters, etc.) to these plasma membrane receptors. Hundreds of different types of cell surface receptors have been identified to date—by far the largest category of these are the seven-transmembrane-spanning type (7TM) receptors.

Interestingly, nearly all of the 7TM receptors found to date are G-protein-coupled receptors (GPCRs). In fact, it is estimated that 3–4% of the human genome encodes for more than 800 different putative GPCRs and about half of currently used drugs target GPCRs (Lundstrom, 2009). Different GPCRs have selectivity for different G proteins, which in turn will regulate different signaling pathways. G proteins are heterotrimers consisting of α, β, and γ subunits. Mammals express approximately 20 αs, 5 βs, and 12 γs. In the basal state, α(GDP)-$\beta\gamma$ is inactive. The GPCR catalyzes the exchange of GTP for GDP on the α subunit and the dissociation of α(GTP) from the $\beta\gamma$ dimer (Fig. 3.1). Both α(GTP) and $\beta\gamma$ regulate downstream effectors. In addition to the GPCRs, other plasma membrane receptors include the receptor tyrosine kinases (such as the insulin receptor), nutrient receptors (such as the transferrin receptor), and ligand-gated ion channels (such as the nicotinic acetylcholine receptor). Of course, only a small subset of receptor genes are turned on in any given cell type, and hence, a typical cell may express only 50–100 different types of receptors. A smaller subset of receptors, including the nuclear hormone receptors, are soluble cytoplasmic proteins that translocate to the nucleus upon binding ligands, such as steroids or vitamins. Activated nuclear receptors (NRs) may also be considered as ligands of their own receptor, DNA, as they often serve as transcription factors or regulators.

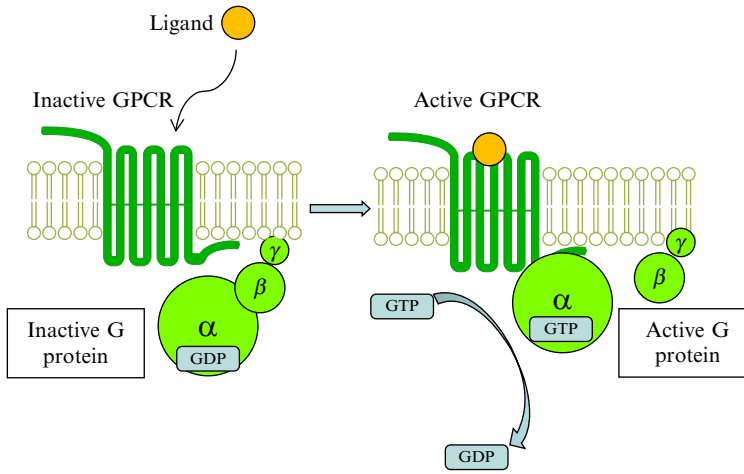


Figure 3.1 Schematic illustrating the activation of a GPCR by a ligand and the subsequent activation of a G-protein via GDP/GTP exchange.

Most nutrient uptake and signaling receptors bind to their ligands on the plasma membrane where they undergo lateral movement to promote their oligomerization and their association with signaling and trafficking elements; the insulin receptor and EGF receptor (EGFR) are among the most studied systems. Most, though not all, receptors are rapidly internalized for downregulation, for subsequent recycling to the plasma membrane, and/or for assembly into intracellular signaling complexes (e.g., Bethani, Skånland, Dikic, & Acker-Palmer, 2010). In contrast, as already mentioned, NRs often reside in the cytoplasm under resting conditions and traffic to the nucleus upon ligand binding. Hence, an understanding of receptor function must include knowledge of its oligomerization state and a dynamic picture of its life cycle, which means studying their movement both in the cell membrane and in the cell interior.

Fluorescence methodologies have been successfully used to study properties of membrane proteins for several decades. One of the first such applications to elucidate the movement of membrane proteins was the seminal study by Frye and Edidin (1970) who used fluorescent antibodies to visualize the mobility of the major histocompatibility complex in the cell membrane. Soon after it was developed, the technique of fluorescence recovery after photobleaching (FRAP) was applied to study the movement of receptors in cell membranes. For example, wheat germ agglutinin receptor complexes

on the surface of human embryo fibroblasts were studied using FRAP in 1976 (Jacobson, Derzko, Wu, Hou, & Poste, 1976), and these studies revealed that most of these receptors (>75%) were mobile with diffusion coefficients in the range of 2×10^{-11} to 2×10^{-10} cm^2/s. On the other hand, concanavalin A receptors were largely immobile on the surface of 3T3 fibroblasts. For several decades, FRAP remained one of the principal fluorescence methods to study receptor movements in the plasma membranes of cells, and it is still popular today. In the early 1980s, Axelrod developed the total internal reflection fluorescence (TIRF) approach, which became an important tool in receptor studies (Axelrod, 1981). With TIRF optics, the evanescent field associated with the exciting light only penetrates a short distance into the cell (~100–200 nm) and, therefore, is ideal for illuminating fluorophores associated with the cell membrane. In the early 1970s, Magde, Elson, and Webb published seminal papers on the theory and application of fluorescence fluctuation analysis (FFS) (Elson & Magde, 1974; Magde, Elson, & Webb, 1972, 1974). Widespread applications of FFS had to wait though until the 1990s when the advent of confocal and two-photon microscopy greatly reduced the observation volume and thus significantly improved the sensitivity of the method, even extending it to single-molecule levels (Denk, Strickler, & Webb, 1990; Eigen & Rigler, 1994; Maiti, Haupts, & Webb, 1997). In recent years, the coupling of FFS with TIRF has significantly enhanced the scope of both methods.

In the past decade, FFS approaches have been increasingly used to study receptors. Since there are many articles in this, and the previous, volume describing in detail the basics of FFS as well as the latest developments in instrumentation and analysis, we shall not dwell on the methodology. Rather, in the next section, we shall point out the main FFS methodologies that have been used to date in receptor studies. These include traditional FCS studies of diffusional rates of receptors and their ligands, photon counting histogram (PCH) and number and brightness (N&B) approaches toward measuring receptor stoichiometry, cross-correlation and two-color approaches to characterize receptor complexes, and image correlation spectroscopy (ICS) approaches to monitor receptor mobility (see Jameson, Ross, & Albanesi, 2009 for an introduction to these methods).

2. SELECTED FFS STUDIES

In this section, we give examples of studies of receptor systems that utilize FFS. We note that this area was also reviewed in 2007 by Briddon and Hill (2007). This section is organized from the perspective of the cell

biologist, namely, we have delineated specific aspects of receptor interactions and illustrated how FFS methods have been applied to address these issues. Although this report focuses on proteins, we should note that FFS approaches are also highly useful for investigations of lipid bilayers, as reviewed by Chiantia, Ries, and Schwille (2009).

2.1. Determination of receptor densities

For many years, radioligand binding was the method of choice for measuring the number and affinity of a particular receptor on a cell surface. This approach, often carried out at 4 °C to inhibit internalization of the ligand–receptor complex, typically involves separation of free and bound ligand by centrifugation or filtration. The time required to carry out these procedures may compromise the binding analysis, particularly if interactions are weak and the half-lives of ligand–receptor complexes are short. The development of fluorescently labeled ligands and antibodies encouraged the use of spectrofluorimetric methods, for example, fluorescence flow cytometry, to quantify cell surface receptor number without the need to separate bound from free ligand (Sklar, 1987). More recently, receptor number densities have been measured in live cells using a variety of image correlation methods (Kolin & Wiseman, 2007). Although FFS analyses of receptors have focused on their mobility and oligomerization (see below), several groups have used the FCS approach to measure expression levels of endogenous receptors on the cell surface. A critical step in any fluorescence-based characterization of endogenous receptors is the development of fluorescent ligand analogues with pharmacological properties reasonably similar to those of the parent molecules. For example, Hegener et al. (2004) generated an Alexa 532-labeled form of norepinephrine to characterize β2-adrenergic receptors on the surface of primary hippocampal neurons. Measurements carried out at 20 °C indicated that these neurons had a receptor density of $\sim 4.5\ \mu m^{-2}$, equivalent to ~2700 receptors/cell. Moreover, the autocorrelation analyses obtained from these binding experiments revealed the presence of two distinctly migrating populations of ligand–receptor complexes, having diffusion constants of $\sim 5 \times 10^{-8}$ and $\sim 6 \times 10^{-10}\ cm^2/s$. More recently, Chen et al. (2009) used an FITC-labeled aptamer to determine that HeLa and leukemia CCRF-CEM cells express ~550 and ~1300 copies per μm^2 of the membrane receptor PTK7 (protein tyrosine kinase 7), a regulator of cell polarity. These authors suggest that fluorophore-labeled aptamers, which can provide high-binding affinity and specificity, are potentially important tools in future FFS analyses of ligand–receptor interactions.

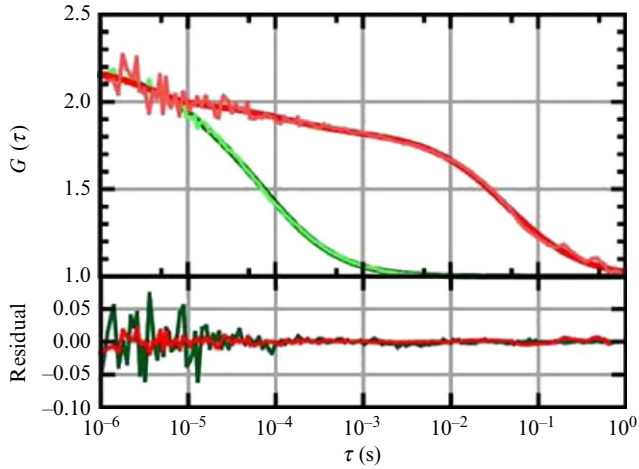


Figure 3.2 FCS results of Miagi and Maruyama (2010). Autocorrelation curves of bound Rh-EGF to CHO cells expressing EGFR–EGFP (red, right curve), and free Rh-EGF (green, left curve) are shown with their fits.

FCS has also been used to measure the number of expressed, fluorescently labeled receptors on the plasma membrane. Although of less physiological significance than the estimation of endogenous receptor number, quantification of expressed receptors is an important step in the rigorous characterization of any ligand–receptor system. As an example of such an analysis, Miagi and Maruyama (2010) used rhodamine-labeled EGF (Rh-EGF) and an EGFP-receptor construct (EGFR–EGFP) to study EGFRs in Chinese hamster ovary (CHO) cells, which do not express endogenous EGFR. The results are shown in Fig. 3.2. The green line corresponds to the autocorrelation curve for Rh-EGF free in solution, which had a diffusion coefficient of $94.3 \pm 4.7\ \mu m^2/s$, while the red line corresponds to Rh-EGF bound to the cell surface with a diffusion coefficient of $0.13 \pm 0.03\ \mu m^2/s$. The FCS data allowed them to estimate the number of expressed EGFR–EGFP receptors on the CHO cell surface to be $1.01 \pm 0.76 \times 10^5$ (number of cells = 15). By way of comparison, Scatchard analysis of the binding of Rh-EGF to these cells gave $1.70 \pm 0.11 \times 10^5$ EGFR–EFGP receptors.

2.2. Measurement of binding affinities

As indicated in the previous chapters, FCS is able to determine the concentration and the diffusional properties of fluorescent molecules in solutions and in cells. Perhaps the first use of FCS to study a receptor–ligand

interaction was the report from Rigler's laboratory (Rauer, Neumann, Widengren, & Rigler, 1996) wherein the detergent-solubilized acetylcholine receptor of *Torpedo californica* was isolated as mixed micelle complexes and the ligand α-bungarotoxin was labeled with tetramethylrhodamine. The main intent of this study was to elucidate the association and dissociation rate constants for the ligand binding. A more recent use of FCS to study ligand–receptor interactions, wherein ligands were labeled with fluorophores, was the study by Winter, McPhee, Van Orden, Roess, and Barisas (2011). These researchers utilized insulin labeled at the N-terminus with FITC and found two classes of insulin-binding sites with dissociation constants (K_d) of 0.11 and 75 n*M*. Swift, Burger, Massotte, Dahms, and Cramb (2007) used two-photon excitation fluorescence cross-correlation spectroscopy (TPE-FCCS) to study the interaction of both agonists and antagonists with the human μ-opioid receptor. Recombinant receptors were expressed containing a C-terminal hexahistidine tag that could then be targeted with fluorescently labeled antibodies (Alexa 488 was used to label the antibodies). In one of their studies, biotinylated enkephalin peptides were labeled with streptavidin-conjugated quantum dots (QDs) and equilibrium-binding constants of the ligand–receptor complexes were determined. TPE-FCCS was also used by Savatier, Jalaguier, Ferguson, Cavaillès, and Royer (2010) to measure the K_d for human estrogen receptor (ER), α and β, with the coactivator partner TIF2 in COS-7 cells. ERα and ERβ were fused with the blue fluorescent protein (FP) cerulean, while TIF2 was fused to the mCherry FP such that cross talk among the FPs was minimized. K_d values were determined for receptor–TIF2 complexes in the presence of agonist or antagonist, as well as for the unliganded system.

2.3. Receptor oligomerization state and clustering

It is known that many membrane-bound receptors are active as dimers, but not as monomers, and that ligand binding often induces dimerization, as in the case of the EGFR (Fig. 3.3). Hence, determination of the oligomerization state of a receptor, in the presence and absence of ligand, is an active research topic. Several FFS methods have been developed in the past decade to elucidate the stoichiometry of protein complexes, that is, the number of protein subunits in an oligomeric complex. These methods, described in impressive detail in other chapters in this, and the previous, volume, include the PCH method (Chen, Müller, So, & Gratton, 1999), the related fluorescence intensity distribution analysis (FIDA) method (Kask, Palo,

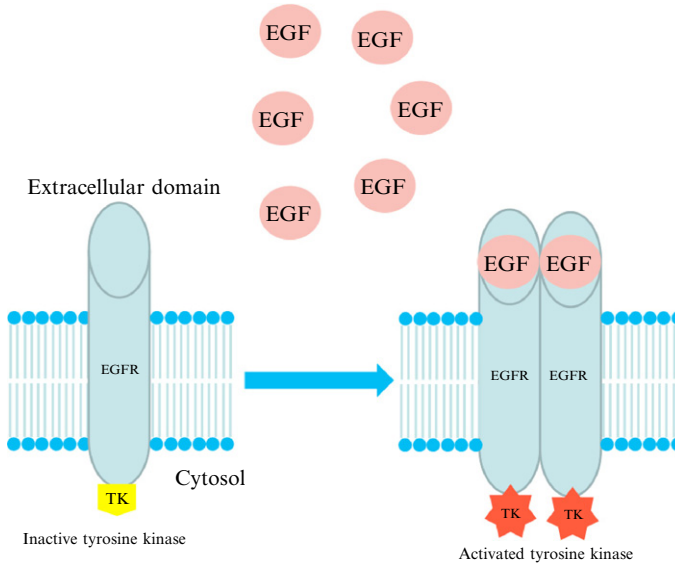


Figure 3.3 Schematic illustrating dimerization and activation of the EGFR in response to binding of the EGF ligand.

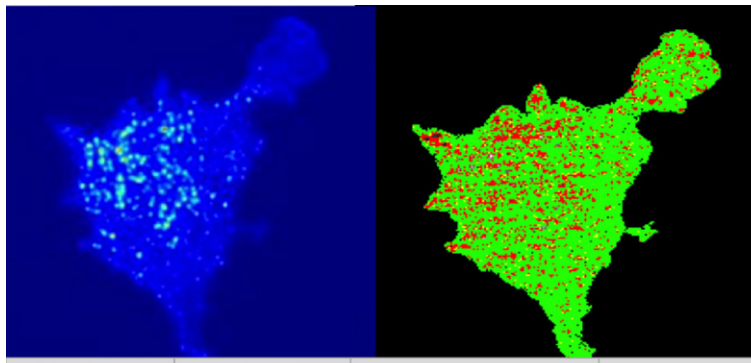
Ullmann, & Gall, 1999), and the time-integrated fluorescence cumulant analysis approach (Wu & Müller, 2005). The basic idea underlying these methods is that, in addition to providing information on fluorophore diffusion, one can determine the inherent "brightness" of the molecule under observation. True molecular brightness, for example, counts per second per molecule, is a function of photophysical parameters such as the molecule's extinction coefficient, or two-photon cross section, its quantum yield, as well as instrument parameters such as laser power, detector efficiencies and optical settings. If one has access to a molecule of standard "brightness," for example, a monomeric EGFP, then one can use that standard to calibrate the system and ascertain the "brightness" of the target molecule. For example, Chen, Wei, and Müller (2003) used monomeric EGFP and a dimeric tandem EGFP as standards to study the oligomerization of NRs, specifically the testicular receptor 4, the retinoic acid receptor, and the retinoid X receptor. Homocomplexes of receptors were observed as a function of either protein concentration or ligand binding. One interesting feature of the analysis outlined in this work is that it can be applied up to relatively high protein concentrations (for FFS), for example, $\sim$10 μM. Saffarian, Li, Elson, and Pike (2007) used FFS and the FIDA analysis to investigate clustering of the

EGFRs in response to cholesterol concentrations. In this chapter, EGFP was fused to the C-terminus of the EGFR. The data were analyzed using a modification of the FIDA method, specifically for use in a two-dimensional system using "quantal brightness," an analytical approach that provides explicit information on the distribution of the fluorescent molecules among clusters of increasing size. The results indicated that the EGFR existed as an equilibrium between single receptors and clusters of receptors and that depletion and augmentation of cholesterol led, respectively, to increased and decreased clustering of the receptor. Very recently, Herrick-Davis, Grinde, Lindsley, Cowan, and Marzurkiewicz (2012) used FCS and the PCH method to demonstrate that the serotonin 5-HT_{2C} receptors within the plasma membranes of HEK293 and rat hippocampal neurons were dimeric.

Another FFS approach to elucidate protein oligomerization is the N&B analysis method developed in Enrico Gratton's laboratory (see, e.g., Digman, Dalal, Horwitz, & Gratton, 2008). This approach allows a pixel by pixel analysis of the "brightness" of a signal, which in turn can provide information on the oligomerization state of the labeled proteins. This approach was utilized by Ross, Digman, Gratton, Albanesi, and Jameson (2011) who used TIRF and FFS to study the oligomerization state of the large GTPase dynamin in the cell membrane. As shown in Fig. 3.4, dynamin-EGFP is predominantly tetrameric on the plasma membrane although some larger aggregates are also present. A comprehensive and elegant FFS study using N&B analysis was recently carried out by Hellriegel, Caiolfa, Corti, Sidenius, and Zamai (2011) on an EGFP construct of GPI-anchored urokinase plasminogen activator receptor, which demonstrated that binding of the amino-terminal fragment of urokinase plasminogen activator is sufficient to induce dimerization of the receptor. That work discusses many of the technical issues involved in this type of study and the N&B analysis.

2.4. Analysis of nuclear receptors

FFS approaches, often in conjunction with FRAP and FRET (Förster resonance energy transfer), are being increasingly employed to define the molecular mechanisms of gene regulation by NRs and other transcriptional regulators. Properties of NRs examined by FFS include homo- and heterodimerization, transport into and out of the nucleus, and association with DNA and the transcription machinery. As with cell surface receptors, the majority of FFS investigations of NRs have utilized FCS analysis to



Region	Brightness (B)	True molecular brightness (ε)	Ratio to mEGFP
Calibration EGFP	1.068	1.36 cpsm	1×
Small aggregates	1.262	5.24 cpsm	4×
Large aggregates	1.690	13.8 cpsm	10×

Figure 3.4 Results of Ross et al. (2011) illustrating N&B analysis of TIRF data on dynamin2-EGFP oligomerization state in the plasma membrane of a mouse embryo fibroblast. Specifically, the analysis indicates that the majority of the dynamin2-EGFP is present as a tetramer. The image in the upper left corresponds to the TIRF intensity, while the image in the upper right corresponds to the N&B analysis, which shows pixels (green) with brightness levels four times those of the monomer standard.

monitor ligand-dependent changes in diffusion constants (Mazza, Stasevich, Karpova, & McNally, 2012). Once in the nucleus, NRs face a daunting variety of potential diffusional barriers, including subnuclear compartments (e.g., PML bodies, Cajal bodies, and nuclear speckles), nonspecific DNA-binding sites, and perhaps even a nuclear cytoskeleton. However, both FCS and FRAP analyses have indicated that NRs are highly mobile when unligated (Lionnet, Wu, Grünwald, Singer, & Larson, 2010). Wu, Corbett, and Berland (2009) used TPE-FCS to study the intracellular mobility of the nuclear import receptors karyopherin-α and karyopherin-β, both free and

associated with their cargoes, namely, EGFP linked to nuclear localization signals. Based on FRAP measurements, ligand binding has negligible effects on the mobility of some NRs (e.g., peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor, and thyroid hormone receptor) but significantly slows or immobilizes others (e.g., glucocorticoid receptor, ER). However, Gelman et al. (2006) suggested that these differences merely reflected the relatively low temporal and spatial resolution afforded by FRAP and showed using FCS that in fact, the mobility of PPAR is significantly reduced upon ligand binding. A compilation and analysis of FCS data on the mobility of NRs is presented by Lionnet et al. (2010).

The majority of NRs function as homo- or heterodimers, and the nature of their dimerization partners can determine whether they act as transcriptional activators or repressors. FCS analysis is generally not useful to monitor these dimerization events, as the diffusion constant for a globular protein is inversely proportional to the cube root of its mass and, hence, would only decrease by ~20% upon dimerization. In fact, as shown by Meseth, Wohland, Rigler, and Vogel (1999), to resolve two components by FCS, their diffusion times must differ by at least a factor of 1.6. To overcome this limitation, PCH analysis has been used to measure NR homodimerization (Chen et al., 2003) and dual-color FFS to measure heterodimerization (Chen & Müller, 2007).

3. CHOICE OF FLUOROPHORES: GENERAL CONSIDERATIONS

Obviously, before one can contemplate carrying out FFS, one must introduce a fluorophore into the system. Various approaches are currently used and several of the most popular shall be discussed. It is important to note that the fluorophore utilized must have a high intrinsic brightness, by which we mean a high extinction coefficient and a good quantum yield. The reason for this requisite sensitivity is that the FFS method, inherently, can usually be implemented only at low fluorophore concentrations, typically less than 0.01 μM (although exceptions exist). This concentration restriction is seldom a problem since receptors are usually present in low numbers, although these numbers vary from system to system.

3.1. Fluorescent antibodies

One of the oldest methods utilized for introducing a fluorophore into a biological system is to label an antibody with a covalently attached probe. In this approach, a fluorescent moiety is covalently attached to one of the

antibody's amino acid residues, most commonly lysine or cysteine. Perhaps the most comprehensive and thorough source for information on the chemistries of probe-protein labeling is "The Molecular Probes® Handbook—A Guide to Fluorescent Probes and Labeling Technologies," specifically the 11th edition. Texts which discuss the chemistry of protein reactive groups and diverse labeling reagents used for protein conjugation include Hermanson (1996) and Wong and Jameson (2012). Most researchers buy antibodies already labeled with a probe, often fluorescein- or rhodamine-based, though now antibodies labeled with the Alexa series of probes are also available. Regardless of whether the labeled antibody is obtained commercially or prepared "in-house," characterization of the functional properties of the labeled antibody is important. Usually, though not always, the fluorescent labels are on secondary antibodies (i.e., an antibody that recognizes the primary antibody).

Assuming suitable antibodies are available, immunofluorescence (IF) analysis of endogenous, untagged receptors should be performed to confirm that the tagged proteins localize correctly in the cell. To ascertain the specificity of the antibodies, it is desirable to carry out both IF and Western blotting in untreated cells and in cells depleted of the target proteins by RNAi. The specificity of the labeled antibody to its target should also be determined. This specificity is usually accomplished using Western blotting. We have noted a tendency of many researchers to use antibody labeled with FITC, a probe with notoriously poor photostability properties in the microscope. Although most (though not all) studies we have seen explicitly monitor and discuss the photostability issue, we note that use of more photostable probes, for example, from the Alexa (Life Technologies, Grand Isle, NY, USA), Cy (GE Healthcare, Piscataway, NJ, USA), DyLight (Thermo Fisher Scientific, Pittsburgh, PA, USA), or ATTO (ATTO-TEC GmbH, Siegen, Germany) series, would mitigate this problem significantly.

3.2. Fluorescent proteins

The use of FPs, that is, GFP-like proteins, to label cellular target proteins such as receptors has increased dramatically in recent years. Needless to say, there are now a great many members of the FP family with diverse excitation and emission properties. New FPs are being reported almost every month; a very useful review of this topic has been written by Stepanenko et al. (2011). A very recent article by Jones, Ehrhardt, and Frommer (2012) has a title which neatly summarizes the field, namely, "A never ending race for new and improved fluorescent proteins." It is manifestly impractical for us to try to

summarize this huge field, but we can point out some of the most salient aspects of choosing FPs for use with FFS, which may give those new to the field some guidelines to consider. A couple of considerations arise immediately from the biological point of view. For example, one may ask how long it takes for the FP to generate the fluorophore in the cell; this issue is usually only important when time-critical events are being monitored. A recent review on novel FPs by Wu, Piatkovich, Lionnet, Singer, and Verkhusha (2011) discusses, among other aspects, some of the so-called "fluorescent timers," which are FPs with variable maturation times. Another concern, which in fact is rarely addressed, is whether the target protein linked to the FP still possesses its inherent biological activity. It is often difficult to isolate the target-FP/fusion protein complex from eukaryotic cells, and one is usually relegated to showing that the cell appears normal or that it still can perform some particular function, such as endocytosis. In some cases, the target-FP can be isolated. For example, in our own studies on dynamin2-EGFP constructs, we isolated the recombinant protein from Sf9 cells and were gratified to determine that its GTPase activity matched that of wild-type dynamin (Fig. 3.5).

Another consideration when choosing an FP is the instrumentation available, for example, one or two-photon sources, since the excitation wavelengths available will certainly limit which FPs can be utilized. One of the most important properties of FPs, for FFS studies, is their inherent brightness, that is, how many counts per second per molecule can they deliver? This consideration is clearly important since it will determine the number of FPs that can be detected in the context of the inherent autofluorescence of the cell. An excellent review of the two-photon properties of various orange and red FPs, including two-photon absorption cross sections and two-photon brightness, is given by Drobizhev, Tillo, Makarov, Hughes, and Rebane (2009). Photostability of the FP is also a concern as is the type of "blinking" exhibited by the FP. This "blinking" is caused by dissociation and binding of protons to various amino acid residues, for example, Tyr-66 (Haupts, Maiti, Schwille, & Webb, 1998). Clearly, blinking on the right timescale may cause a fluctuation in the fluorescence signal, which can be measured by FCS and which should not be confused with other sources of signal fluctuation. We shall not go into more detail on these points since numerous articles in the primary literature address these issues (see, e.g., Liu, Kim, & Heikal, 2006; Ringemann et al., 2008; Ward, 2006; Wong, Banks, Abu-Arish, & Fradin, 2007). As stated, new and interesting FPs appear almost every month, for example, Shcherbakova, Hink, Joosen, Gadella, and Verkhusha (2012) very recently reported a new orange FP called

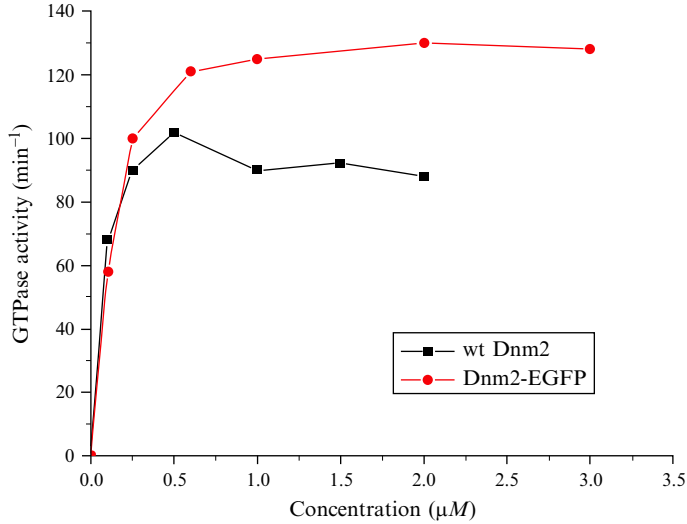


Figure 3.5 GTPase activities of C-terminally His_6-tagged (black) and EGFP-tagged (red) dynamin 2 as a function of dynamin concentration. We had previously shown that His_6-tagged dynamin behaves identically to endogenous, untagged dynamin purified from bovine brain. The specific activities of dynamins are strongly dependent on their states of self-association; hence, these activities increase as dynamins polymerize in a concentration-dependent manner. As evident from this figure, the GTPase activities of EGFP-tagged and untagged dynamins are essentially identical.

LSSmOrange (LSS stands for large-Stokes shift), which has very favorable optical properties for use with FFS or FRET measurements. Their report describes the spectroscopic properties of this FP along with several other new FPs and nicely illustrates the type of spectroscopic information required to evaluate a new FP. Finally, we should note that the positioning of FP tags should be chosen to minimize disruption of folding, activity, or interactions. Most plasma membrane receptors have extracellular N-termini and cytoplasmic C-termini. N-terminal tagging of these receptors may be problematic, as the tag must be placed after the signal sequence or it will interfere with or be removed during processing at the endoplasmic reticulum. In addition, an extracellular FP tag may impair ligand binding. For these reasons, FPs are most often fused to the C-termini of receptors, affording the additional advantage that maturation of the FP provides evidence that the entire receptor has been translated. However, even with C-terminal tags, it must be ascertained that the FP

does not interfere with critical cytoplasmic interactions, for example, with downstream signaling molecules, intracellular trafficking machinery, or posttranslational modifications, such as phosphorylation or ubiquitylation. These considerations do not apply to NRs, which are soluble proteins and therefore have both N- and C- termini available for tagging.

3.3. Biomolecular fluorescence complementation

A variant on genetically encoding FPs onto target proteins is the biomolecular fluorescence complementation (BiFC) method, which allows the study of protein–protein interaction (Ghosh, Hamilton, & Regan, 2000; Nagai, Sawano, Park, & Miyawaki, 2001). The BiFC method involves the attachment of nonfluorescent N- and C-termini of an FP, typically split between β-sheets 7 and 8, to the target proteins thought to interact. Upon association, if the three-dimensional properties of the complex permit, the FP halves can come together, which promotes refolding of the FP and maturation of the fluorescent moiety, producing a fluorescent marker of association (Fig. 3.6). Formation of the fluorescent signal after association is not instantaneous. The refolding of FP, in BiFC assays, has a half-time of seconds to minutes, and the maturation of the chromophore requires several minutes (Hu, Chinenov, & Kerppola, 2002). This process,

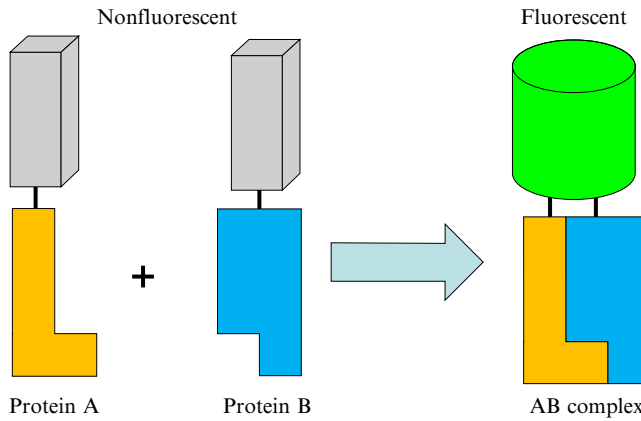


Figure 3.6 Schematic illustrating the principle underlying the biomolecular fluorescence complementation (BiFC) method. Specifically, one of the target proteins (A) is fused with part of a fluorescent protein, while the other target protein (B) is fused with the other part of the fluorescent protein. If the two target proteins interact and form a complex (AB), then it is possible for the fluorescent protein parts to fold together and form a fluorescent adduct.

formation of a mature tertiary protein attached to two target proteins, is generally considered to be irreversible, thus having the potential to capture transient interactions. However, under some conditions, BiFC has been shown to be reversible (Anderie & Schmid, 2007). This method of protein–protein detection has found great popularity among the GPCR aficionados as a means of detecting agonist-dependent protein-interacting partners, as it offers several unique advantages over FRET (Rose, Briddon, & Holliday, 2010). Although FRET measurements can be acquired in real time and are fully reversible, the analysis is often more difficult than with BiFC because appropriate controls are needed to determine energy transfer efficiency in the presence of potential complicating factors such as signal bleed-through or homo-FRET. Kilpatrick, Briddon, and Holliday (2012) combined FFS and PCH with BiFC to study the effect of β-arrestin adaptors and endocytic mechanisms on the diffusion and particle brightness of GFP-tagged neuropeptide Y receptors in the plasma membrane of HEK293 cells. In this report, Kilpatrick et al. noted that the complex photophysics of the yellow fluorescent protein (YFP), typically used in the BiFC approach, creates some difficulties with the use of FFS with BiFC. To overcome this problem, they developed a novel BiFC system based on a version of the superfolder (sf) GFP. As mentioned earlier, Herrick-Davis et al. (2012) used FFS combined with BiFC to provide evidence for the dimeric state of the 5-HT_{2C} receptor.

3.4. HaloTags/SNAP/FlAsH

HaloTags: In the HaloTag approach, one's protein of interest is fused recombinantly to a mutated form of bacterial haloalkane dehalogenase. The dehalogenase normally catalyzes the removal of halides from halogenated aliphatic hydrocarbons through nucleophilic displacement mechanisms; however, the mutant form loses the ability to release the covalent ester bond formed with an aspartic acid residue during catalysis (Los & Wood, 2006; Los et al., 2005, 2008). Hence, reaction of this mutant dehalogenase with an appropriate halogenated aliphatic substrate, covalently attached to a fluorophore, will result in the covalent linkage of the fluorescent probe to the halogenase and thus the protein of interest (Fig. 3.7). This method allows for the introduction of different fluorophores onto the target protein without having to modify the construct. Since the dehalogenase is a bacterial enzyme, the labeling is specific and cross-reaction with mammalian proteins is eliminated. This method is suitable for cell surface

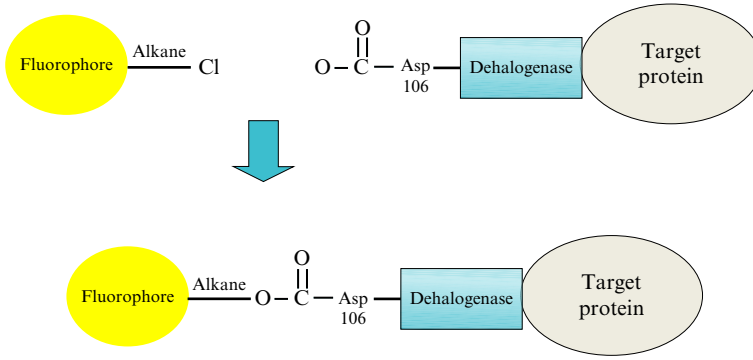


Figure 3.7 Schematic illustrating the principle underlying the HaloTag method. A fluorescent probe is linked to a haloalkane, and the bacterial dehalogenase enzyme is recombinantly fused to the target protein. The fluorescent haloalkane can form a stable covalent linkage with the dehalogenase.

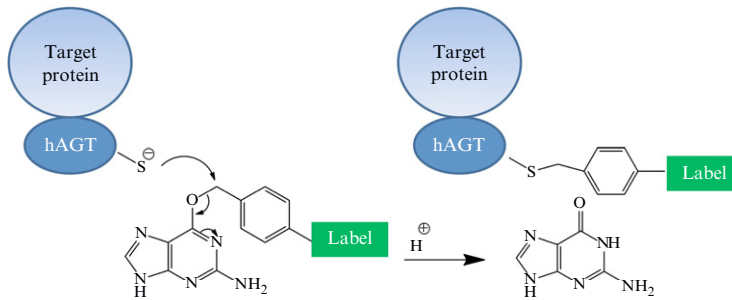


Figure 3.8 Mechanism of covalent labeling of hAGT, which is fused to a target protein, with a fluorescent label linked onto an O^6-benzylguanine derivative. *Figure modified from Keppler et al. (2003).*

proteins, such as receptors, which are accessible to the fluorescent substrate. Of course, the same considerations regarding the fusion of the dehalogenase and target protein genes mentioned in the section on FP constructs, also apply for the HaloTag approach.

SNAP Tags: Another approach that allows for labeling *in vivo* is the SNAP-tag method (Keppler et al., 2003). Covalent labeling of a target protein is accomplished by construction of a fusion protein with O^6-alkylguanine-DNA alkyltransferase (hAGT). hAGT irreversibly transfers the alkyl group from its substrate to a cysteine residue (Fig. 3.8). Substrate specificity is relatively low as hAGT can use O^6-benzylguanine (BG) or O^6-benzylguanosine with substituted benzyl rings as substrates

(Damoiseaux, Keppler, & Johnsson, 2001). Optimization of the specificity was shown by Xu-Welliver, Leitao, Kanugula, Meehan, and Pegg (1999) who used a mutated form of hGAT, namely, G160W hGAT, which has approximately five times the specific activity for BG *in vitro* compared to hGAT. The high activity of hGAT to a substrate that is typically inert makes this system ideal for targeted labeling of a protein of interest.

FlAsH: The Roger Tsien lab developed a uniquely specific probe, termed FlAsH, by modifying fluorescein to contain two arsenoxides (Griffin, Adams, & Tsien, 1998). Arsenoxides have a high affinity for closely paired cysteines and the two on FlAsH confer a strong affinity for a CCXGCC sequence, which is uncommon in naturally occurring proteins (Fig. 3.9). To implement this method, the target sequence is introduced genetically into the protein of interest and the FlAsH reagent is added to the cell's medium, whereupon it can be taken up by the cell (the 1,2-ethanedithiol (EDT) moieties on the fluorophore render it sufficiently hydrophobic to pass through the plasma membrane) and react with the cysteine sequence. Toxicity of the arsenoxides is minimized by labeling the cells in the presence of EDT. Testing of the specificity of FlAsH for different sequences led to the discovery that FlAsH has a preference for the CCPGCC motif, which adopts a hairpin rather than helical structure (Adams et al., 2002). At the same time, experiments to adjust the emission, by using rhodamine or 3,6-dihydroxyxanthone instead of fluorescein, were conducted, thus making the probe more attractive for microscopy. These experiments led to the creation of the red-emitting ReAsH as well as a blue-emitting CHOxAsH, expanding the spectroscopic diversity. A major advantage of the FlAsH tag is its small size combined with its selectivity for the CCPGCC motif. Hoffmann et al. (2005), for example, demonstrated that a GPCR labeled

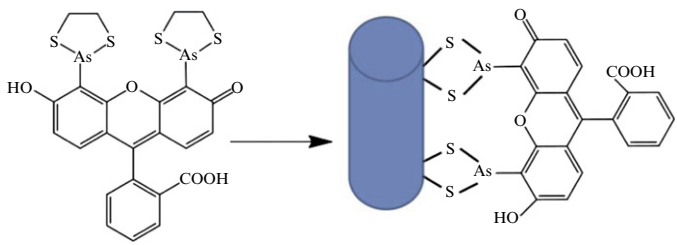


Figure 3.9 FlAsH reagent forming a fluorescent complex with an α-helix of a target protein that contains the CCXGCC FlAsH motif.

with a CFP/YFP FRET pair could not support coupling to adenylyl cyclase, whereas a CFP/FlAsH pair maintained this biological function. On the other hand, a potential limitation of the FlAsH tag approach is the possibility of significant fluorescent backgrounds.

3.5. Quantum dots

QDs are semiconductor nanocrystals, typically a few hundred to a few thousand atoms of cadmium sulfide, cadmium selenide, or cadmium telluride, which have a characteristic emission, due to exciton confinement, based on the size of the particle (first reported by Rossetti, Nakahara, & Brus, 1983). The core emission is typically weak and always unstable and so a "shell" is added to insulate the core. The shell material, for example, zinc sulfide, is typically almost entirely unreactive and completely insulates the core. A layer of organic ligands can be covalently attached to the surface of the shell, which provides a surface for conjugation to biological (antibodies, streptavidin, lectins, and nucleic acids) and nonbiological species and which makes the QDs water-soluble. QDs exhibit absorption over a wide range, extending into the visible region. Emissions can be tuned from the visible to the infrared by varying the size of the QD. In recent years, QDs have found applications in biological imaging, due to their inherent brightness and photostability. This popularity has been driven, in part, by innovations in QD surface modifications, which has facilitated the process of linking them to biological targets; a recent review of this topic has appeared (Bruchez, 2011). Heuff, Swift, and Cramb (2007) explicitly discussed the challenges involved in the use of QDs with FCS owing to their blinking characteristics. Several groups have used QDs and FFS to study receptors. For example, QDs were used by Boyle et al. (2011) to study the clustering of the T-cell receptor (TCR) with *k*-space ICS. Interestingly, the blinking statistics differed between small clusters of QDs compared to single QDs or large clusters, which allowed this function to serve as a reporter of nanometer-scale changes in QD-labeled TCR organization after T-cell activation by antigen.

4. CELLS: GENERAL CONSIDERATIONS

An important initial consideration for those embarking on FFS analysis of receptors is the choice of cell type. For example, to study the properties of recombinant, GFP-tagged EGFRs with minimal interfering contributions from endogenous unlabeled receptors, one may wish to utilize

NIH 3T3 cells, which express ~650,000 PDGF receptors (Liapi, Raynaud, Anderson, & Evain-Brion, 1990) but fewer than 10,000 EGFRs per cell (Velu et al., 1987). On the other hand, A431 cells, which express 2–4 million EGFRs per cell, may be chosen to study the trafficking of the fluorescently tagged ligand, EGF, bound to endogenous unlabeled receptors. HeLa cells, with approximately 50,000 EGFRs per cell (Berkers, van Bergen en Henegouwen, & Boonstra, 1991), could serve as a compromise. Once suitable cells for a given experiment are chosen, the success of any live-cell imaging analysis depends on the ability to maintain these cells in a healthy, functional state throughout their visualization. Critical factors that must be generally controlled, including nutrient supply, pH, and phototoxicity have been defined in numerous reviews (e.g., Frigault, Lacoste, Swift, & Brown, 2009) and are discussed only briefly here.

4.1. Cell growth and transfection

To increase the surface density of receptors and ensure that they are poised to respond to their specific ligands, cells are grown for various times, often overnight, in serum-free or serum-depleted (e.g., 0.05–0.5%) medium. For live-cell imaging experiments, cells are typically plated in dishes with coverslip bottoms, such as MatTek 35 mm #1.5 dishes having an average coverslip thickness of 0.17 mm. Slides may be uncoated or, if necessary for cell attachment, coated with collagen, polylysine, or fibronectin, for example.

Transient transfection of fluorescently tagged constructs is routinely performed using a commercially available gene transfer reagent, selected by experience for each cell type. A comparison of the efficiency and cytotoxicity of six transfection reagents in nine commonly used cell lines was recently reported (Yamato, Dai, & Moursi, 2010). It is important to establish the suitability of a particular transfection system for each cell type prior to functional experiments, although FuGENE HD (Promega) was found to be the preferred reagent for many cell types in the Yamato et al. (2010) study.

4.2. Maintenance of cell viability

Mammalian cells are most often grown in medium containing 5–10% fetal bovine serum buffered with a CO_2/bicarbonate system to maintain pH within appropriate physiological range, typically 7.2–7.4. Phenol red, a pH indicator that turns orange at pH 7.0, then yellow below pH 6.8, is routinely included in culture medium at a concentration of ~15 mg/L.

Acidification of the medium may be reflective of bacterial contamination or to the simple release of metabolic and other waste products from the cultured cells themselves. An orange color serves as a warning that the medium should be changed. Because Phenol red interferes with fluorescence imaging due to its high extinction coefficient, most conventional culture media are currently available in a Phenol red-free form. When cells are transferred from the CO_2 incubator to the often uncontrolled atmospheric conditions of the microscope stage for analysis, reduction of CO_2 levels can lead to alkalinization of the medium in less than an hour, accompanied by a change in Phenol red color to pink and then purple. To reduce the need for CO_2, bicarbonate buffer is often supplemented with or replaced by 10–25 m*M* HEPES buffer, which maintains pH even without CO_2 equilibration, and allows both survival and proliferation of cells for $\sim$10 h provided they are kept in complete medium (Frigault et al., 2009). However, even when using HEPES, the medium can alkalinize to unhealthy levels with time and should be monitored. Moreover, HEPES has been reported to form cytotoxic products, such as hydrogen peroxide, upon exposure to illumination (Zigler, Lepe-Zuniga, Vistica, & Gery, 1985). The Leibovitz L-15 culture medium has been developed to circumvent problems arising from the use of HEPES buffer or the absence of bicarbonate/CO_2. A common cause of cell stress is expression of abnormally high levels of recombinant proteins, which may also result in the generation of physiologically irrelevant data. Whenever possible, fluorescently tagged proteins should be expressed in cells depleted of their endogenous counterparts (e.g., by RNAi), using inducible promoters to control expression levels. Characteristics of dead or dying cells include detachment and rounding, formation of surface blebs and large vacuoles, and deformation of mitochondria. Numerous indicators of cell viability are available. For example, alamarBlue (Life Technologies) can be used to monitor cell metabolism and MitoTracker stain (Life Technologies) to visualize changes in mitochondrial morphology.

4.3. Photobleaching and phototoxicity

A critical consideration in live-cell imaging is to achieve a balance between maximal signal and minimal photobleaching and phototoxicity. Although photobleaching is embraced in FRAP approaches, it should be avoided or certainly minimized in most other fluorescence measurements (we note that photobleaching of the acceptor in FRET pairs is sometimes utilized). Considerable effort has been expended during the past few decades to

develop fluorescent probes with enhanced photostability. Fluorescein is still probably one of the most popular probes in use, but it is notorious for its tendency to photobleach in the microscope. For these reasons, researchers turned early on to rhodamine, a much more photostable probe, and then to newer families of probes such as the Alexa or ATTO series. We should note that not all photobleaching processes are irreversible, for example, an excited fluorophore may be transiently trapped in a triplet state, which can eventually decay to the ground state and hence resurrect the functional fluorophore (Periasamy, Bicknese, & Verkman, 1996). Phototoxicity may be coupled to fluorescence excitation, which can result in the production of toxic products, often reactive oxygen species, or to fluorescent-independent processes such as light-induced cell heating (Tinevez et al., 2012). Photodamage can be minimized by reducing illumination to a level that maintains a tolerable signal-to-noise ratio. An important contribution to photobleaching of GFPs is their ability, in the excited state, to donate electrons to cellular components such as FAD, FMN, and NAD^+, a process which has been termed "oxidative reddening" (Bogdanov et al., 2009). Oxidative reddening is influenced by the culture medium, which in turn controls the levels of biological electron acceptors. For example, the photostability of EGFP (but not the red FPs) is increased by approximately ninefold by growing cells in vitamin-depleted DMEM (Bogdanov et al., 2009). Phototoxicity can also be reduced by adding antioxidants, such as ascorbic acid or Trolox, to the culture medium.

4.4. Autofluorescence

Background fluorescence originates both from cellular components and from the cell culture medium. As stated, Phenol red is a major source of autofluorescence and should be removed from imaging medium. However, even in medium without Phenol red, background intensities from cell-free regions should be measured. Cellular sources of autofluorescence include, but are not limited to, flavins and flavoproteins (at 500–600 nm), reduced pyridine nucleotides (at 400–500 nm), and aromatic amino acids. Dead, damaged, crowded, or otherwise stressed cells increase autofluorescence, again highlighting the importance of maintaining a healthy culture environment. An interesting study of the lifetimes associated with the autofluorescence of stem cells in different metabolic stages has recently appeared (Stringari, Sierra, Donovan, & Gratton, 2012).

5. SUMMARY

In this overview, we have endeavored to elucidate the nature of cell surface receptors and the type of information that FFS methods can provide. We have also noted the principal methodologies presently used to introduce fluorophores into biological systems. Finally, we discussed key issues regarding cell maintenance and health, since no matter how sophisticated the spectroscopic technique may be, if the target cell is not functioning properly, the information attained may be suspect. With appropriate regard to all of the caveats that accompany instrumentation, choice of fluorophore, data analysis, cell transfection, and cell maintenance, however, FFS methodologies truly do open a window in the life of the cell that promises to illuminate an important undiscovered biological landscape.

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