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Fluorogen Activating Proteins in Flow Cytometry for the Study of Surface Molecules and Receptors

Matthew J. Saunders^{1,*}, Christopher Szent-Gyorgyi¹, Gregory W. Fisher¹, Jonathan W. Jarvik^{1,2}, Marcel P. Bruchez^{1,2,3}, and Alan S. Waggoner^{1,2}

¹Molecular Biosensor and Imaging Center. Carnegie Mellon University, 4400 Fifth Ave. Pittsburgh, PA 15213

²Department of Biological Sciences. Carnegie Mellon University, 4400 Fifth Ave. Pittsburgh, PA 15213

³Department of Chemistry. Carnegie Mellon University, 4400 Fifth Ave. Pittsburgh, PA 15213

Abstract

The use of fluorescent proteins, particularly when genetically fused to proteins of biological interest, have greatly advanced many flow cytometry research applications. However, there remains a major limitation to this methodology in that only total cellular fluorescence is measured. Commonly used fluorescent proteins (e.g. EGFP and its variants) are fluorescent whether the fusion protein exists on the surface or in sub-cellular compartments. A flow cytometer cannot distinguish between these separate sources of fluorescence. This can be of great concern when using flow cytometry, plate readers or microscopy to quantify cell surface receptors or other surface proteins genetically fused to fluorescent proteins. Recently developed fluorogen activating proteins (FAPs) solve many of these issues by allowing the selective visualization of only those cell surface proteins that are exposed to the extra cellular milieu. FAPs are GFP-sized single chain antibodies that specifically bind to and generate fluorescence from otherwise non-fluorescent dyes ('activate the fluorogen'). Like the fluorescent proteins, FAPs can be genetically fused to proteins of interest. When exogenously added fluorogens bind FAPs, fluorescence immediately increases by as much as 20,000 fold, rendering the FAP fusion proteins highly fluorescent. Moreover, since fluorogens can be made membrane impermeant, fluorescence can be limited to only those receptors expressed on the cell surface. Using cells expressing beta-2 adrenergic receptor ($\beta 2AR$) fused at its N-terminus to a FAP, flow cytometry based receptor internalization assays have been developed and characterized. The fluorogen/FAP system is ideally suited to the study of cell surface proteins by fluorescence and avoids drawbacks of using receptor/fluorescent protein fusions, such as internal accumulation. We also briefly comment on extending FAP-based technologies to the study of events occurring inside of the cell as well.

Keywords

Fluorogen Activating Protein (FAP); fluorescence; receptor internalization; fluorescence amplification; fluorescent proteins; dose-response

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*corresponding author, matsaun@andrew.cmu.edu (412) 268-9675. Fax (412) 268-6571.

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1 Introduction

Cell cytometry and microscopy methodologies rely heavily upon fluorescent dyes or proteins for measurements of quantities and localization. The fluorophore systems used in such studies are usually of two types: *i)* Small organic dyes that are conjugated to biological molecules, and then added to cells; and *ii)* Peptide chromophores intrinsic to proteins that are genetically fused to proteins of interest, and then expressed in cells. For detection and quantitation, many organic dyes are coupled to antibodies that specifically target a protein. In the case of antibodies that target protein molecules on the cell surface, cells can remain viable and be sorted by fluorescence activated cell sorting (FACS). Antibodies against internal cellular proteins require that cells are fixed and permeabilized, making them no longer viable for sorting and growth. Analysis and/or sorting by flow cytometry using small organic dyes coupled to antibodies also requires a suitable antibody. Studies involving surface receptor internalization or functions of surface based proteins measuring downstream events are often times hindered by the large size of antibodies, which prevents other molecules from interacting with surface proteins [1, 2] This can be of particular concern where receptor agonist or antagonist studies are being performed, as the antibodies used to visualize or label receptors can potentially hinder or affect receptor binding or internalization.

Protein based fluorophores such as enhanced green fluorescent protein (EGFP) or other related proteins are genetically encoded and expressed as fusion proteins in living cells. They become fluorescent within minutes to hours of expression and are stably fluorescent until irreversible photobleaching occurs[3]. For protein fluorophore applications, cells do not have to be permeabilized or fixed to measure intracellular localization or surface expression of the protein of interest/EGFP fusion protein. Recent advances in protein-based fluorophores have enabled a wide spectrum of available colors and provided distinct advantages for use[4]. The ability to track protein expression, trafficking and localization in viable cells has made a great impact on many applications in cytometry and microscopy. Protein-based fluorophores label proteins of interest with only one copy of the fluorophore, which makes detection of low copy proteins difficult, and photobleaching of low copy fluorescent proteins can lead to poor quantitate tracking of protein movement [4]. These obstacles are usually addressed by overexpression of fusion protein. However, overexpression can lead to accumulation of target/fluorescent protein in regions of the cell where surface proteins do not normally stably reside, and can also hinder the study of cellular responses to physiological conditions due to the high copy number of the fluorescent target [5]. Given that approximately 20–30% of genes in eukaryotes encode for surface proteins serving many important functions [6], and that fluorescent proteins themselves are not evolutionary adapted for secretion, the use of fluorescent proteins for the study of surface proteins is problematic.

Nonetheless, the use of EGFP and other related protein fluorophores has been immensely popular in flow cytometry, particularly because sorted cells can be recovered and grown in cell culture as opposed to fixed cells, which are not viable. EGFP, however, has significant drawbacks when used in flow cytometry assays and applications intended to monitor surface proteins such as receptors or lineage markers. As mentioned, large amounts of fluorescent protein accumulate inside cells, especially within biosynthetic, secretory and degradative compartments, as well as on the surface of the cell[7]. This is of particular concern in cases where genetic fusion of a protein fluorophore to a surface protein and attendant overexpression cause less efficient or inappropriate sorting into intracellular compartments, or where natural recycling of surface fluorophore cause accumulation in degradative pathways. In flow cytometry based cellular techniques that measure interactions of surface molecules, such as fluorescently tagged receptor internalization studies, this internally

accumulated protein fluorescence will complicate measurements of fluorescence signal associated with surface molecules.

Recently developed fluorogen activating proteins (FAPs) that non-covalently bind to small molecule fluorogens have distinct advantages for the study of cell surface protein fusions by fluorescence. FAPs are able to bind cognate fluorogens only when their protein fusion partner reaches the surface, due to the membrane impermeant nature of the fluorogens used in these methods, and the fact that the fluorogen binding pocket is presented on the external surface of the plasma membrane [8–10]. Fluorogens may be directly added to suspensions of FAP-expressing cells in a variety of buffers or growth media. With optimal FAPs, saturation levels of fluorescence are reached within seconds to a few minutes after adding fluorogen at final concentrations in the low nanomolar to low micromolar range. The non-fluorescent nature of unbound fluorogen eliminates the requirement for wash or separation steps, thereby defining a true homogeneous format protocol. Confinement of fluorogen access to the cell surface or surface-derived endocytotic vesicles prevents accumulation of fluorescence in biosynthetic or recycling pathways often seen when protein-based fluorophores are overexpressed and mis-sorted in the secretory pathway. Internalization of receptor/FAPs via endocytotic vesicles can be followed using a surface fluorescence depletion assay or an internal fluorescence accumulation assay. The fluorescence depletion assay may be tailored to measure kinetics and dose response using FACS by varying the time of addition of fluorogen relative to the time of addition of receptor agonist or antagonist [9].

FAPs were discovered using a large yeast surface display library of human single chain fragment variable antibody (scFvs) [8, 11]. Yeast display technologies have been used for a variety of purposes including selection and affinity maturation of ScFvs, directed evolution of g-protein coupled receptors (GPCRs), increased enzyme stability, antibody epitope mapping, human cDNA library display and metal chelation [12]. Yeast surface display is a powerful tool for selection and improvement of a desired protein function through selection of protein libraries displayed on the surface of cells. Flow cytometric sorting of yeast display libraries incorporating screens for desired protein function (in the case of FAPs, binding fluorogen and becoming fluorescent) selects individual cells, which can be grown and used to sequence the clone, and upon transfer to a secretion vector, conveniently purify and characterize the encoded protein. The yeast display system thus is an ideal platform for both the discovery and characterization of FAPs. The single chain antibodies on which the FAPs are based are derived from germline antibody sequences from non-immunized humans, and are efficiently expressed on the surface of yeast as proteins fused to the Aga2p surface agglutinin that is covalently attached to the cell wall [11]. Not only are these FAPs expressed in high copy number, but fluorescence is limited to the outside of the cell and is therefore not sensitive to intracellular physiology. Conditionally fluorescent yeast displayed FAPs are useful not only as a means to select and affinity mature FAPs, but also as a platform on which to build and characterize FAP-based reagents and sensors, in many cases incorporating biological fusion partners.

1.1 Theory

The molecules thiazole orange (TO) and malachite green (MG) are known fluorogens, with fluorescence activation seen upon binding to DNA [13] or RNA aptamers [14]. In PBS these fluorogens exhibit strong absorbance maxima at 607 nm (malachite green, MG) and 504 nm (thiazole orange, TO), but exhibit extremely low levels of fluorescence. The structure of these dyes indicates that free rotation around chromophore single bonds promotes quenching of the electronic excited state. When held rigid in a specific conformation that does not allow internal conversion via bond rotation, these dyes become intensely fluorescent. When bound to FAPs, these fluorogens exhibit red-shifted excitation maxima that are well matched

to lasers (MG, 633 nm) (TO1, 488 or 514 nm) (Table 1) commonly used in microscopy and flow cytometry.

The non-fluorescent nature of unbound fluorogen can also be exploited to amplify fluorescence signal via intramolecular energy transfer from covalently attached Cy3 molecules. In this approach a single fluorogen is tethered to Cy3 molecules and acts to anchor the complex to the FAP as well as to quench unbound Cy3 dye. Excitation of Cy3 while measuring emission of MG amplifies the signal many fold due to the much stronger extinction of the Cy3 donor(s) and surprisingly efficient energy transfer to MG[15]. This demonstrated fluorescence amplification signal based upon multiple Cy3 energy transfer to MG could lead to dramatically improving the fluorescence signal-to-noise ratio, especially in cases when there are low copy numbers of protein/FAP at the surface of the cell. Order of magnitude fluorescence enhancements were obtained on the yeast cell surface using four donor Cy3 dyes linked to a single MG acceptor.

2 Materials and Methods

Use of FAPs in flow cytometry assays can be done in many ways using a variety of DNA subcloning techniques, cell transformation/transfection protocols and compatible expression systems. The advantages of using FAPs over other fluorescent proteins in the study of surface molecules as described suggests that FAPs may also have advantages in a variety of other applications. Specific protocols will need to be developed and optimized by the user of FAPs for their research purposes and FAP/protein fusion of interest. The simplicity of using FAPS (e.g. binding of fluorogen to FAP, and immediate readout via flow cytometry) will make development of protocols using FAPs relatively straightforward.

2.1 Synthesis of fluorogens and sub-cloning of FAPs

The fluorogen derivatives used in the studies described here, TO1-2p, MG-2p and MG-11p (Figure 1), were synthesized as described in [8]. Unmodified MG and TO fluorogens are commercially available. However, additional chemical modification is required to create the derivative forms that reduce non-specific binding, decrease background fluorescence, and prevent passage across the plasma membrane. Cy3 containing MG dyedrons were synthesized as previously described [15]. Subcloning into plasmids or chromosomal integration using preferred methods to create the appropriate FAP fusion proteins must be employed for specific vector or cell-based applications of FAPs.

2.2 Yeast display of FAPs

FAPs can be amplified by PCR and cloned into plasmids by standard molecular biology techniques. Development of yeast displayed FAPs was carried out using the EBY100 (Leu-, Trp-) or JAR200 (Leu-, Trp-, Ura-, an EBY 100 derivative with G418 resistance, courtesy of James Rakestraw, MIT) strains of yeast transformed with yeast display plasmids (pPNL6 from Pacific Northwest National Laboratory, containing a TRP1 selection marker), with FAPs cloned in frame with the yeast display scaffold and epitope tags. Other yeast display plasmids such as the pYDI yeast display system (formerly available from Invitrogen) could be suitable as well. Transformation can be carried out using a standard methodology of choice (e.g. LiAC chemical transformation or electroporation). For libraries or clonal cultures of displayed FAP, yeast were grown overnight in 1–5 ml of SD + CAA media (5 g/L casamino acids -ade, -ura, -trp, 20 g/L dextrose, 1.7 g/L YNB (Yeast Nitrogen Base w/out ammonium sulfate amino acids Becton Dickinson Difco YNB #233520), 5.3g/L ammonium sulfate, 10.19 g/L Na₂HPO₄·7H₂O, 8.56 g/L NaH₂PO₄·H₂O) in a shaking incubator at 30°C. Yeast were pelleted by centrifugation and re-suspended in SG/R + CAA media (Same as SD + CAA media except substitute the following sugars for dextrose: 20 g/L galactose,

20 g/L raffinose, 1 g/L dextrose) to a cell density of 0.5 OD₆₀₀/ml. Cells were grown at 20°C in SG/R + CAA media for 48–72 hours. For flow cytometry fluorescence assays, cells were spun down and re-suspended in PBS+ (PBS pH 7.4 with 2 mM EDTA and 0.1% w/v Pluronic F-127 Anatrace catalog #P305). However, other buffers or even growth media are suitable as well. Fluorogen was added to a concentration between 10 and 100 nM, and fluorescent signal was quantitated on a Becton Dickinson Biosciences FACSVantage SE with FACSDiVA option flow cytometer using a 488 nm laser and an emission filter of 530/30nm for TO-1p fluorogen, or a 635 nm laser and an emission filter of 685/35nm for MG-2p and MG-11p. To normalize total fluorogen signal to the number of displayed FAPs, the C-terminal c-myc epitope was immunostained as described in [8].

2.3 Dyedron fluorogens in yeast display

Multichromophoric Cy3-MG dyedrons were studied using JAR200 yeast cells displaying the FAP L5 E52D-MG (Table 1) grown as described above but including 20 µg/ml uracil. The Dyedrons were added to cells in PBS+ at a concentration ranging between 1 and 300 nM. Cells were analyzed on a Becton Dickinson Biosciences FACSVantage SE with FACSDiVA option flow cytometer using laser excitation at either 532 or 635 nm and emission detection through a 675/50 band pass filter.

2.4 Surface display on mammalian cells

Protein or receptor of interest is displayed on the surface of mammalian cells using the pDisplay vector (Invitrogen catalog # V66020) with protein cloned in frame with the display sequence and FAP of choice (Table 1.) by standard cloning methodology. For the example of the β 2AR with FAPs HL1.0.1-TO1 and HL4-MG subcloning protocols are detailed in [9]. Construction of target protein/FAP display fusions will be specific to the biological problem at hand, and could potentially be implemented using strategies other than pDisplay, such as overexpression of a transmembrane protein fused to a FAP.

Choice of expression system will be dependent upon the protein of interest and expression levels desired. Mammalian cells NIH 3T3 and U937 cells were transfected using Mirus TransIT[®]-LT1 Transfection reagent (Mirus Bio, Madison, WI product # MIR 2300) following the manufacturer's instructions. Other standard transfection materials and protocols would also be acceptable. Viral transduction can also be performed with the advantage that stable integration into the genome is guaranteed and selection of stable lines is greatly facilitated. NIH 3T3 cells stably expressing β 2AR-FAPs were generated using the Phoenix Ecotropic Packaging System (ATTC product SD 3444; ATTC, Manassas, VA) according to the manufacturer's instructions. U937 cells stably expressing β 2AR-FAPs were generated using the Phoenix-GP Packaging system (ATTC product SD 3514) according to the manufacturer's instruction. Preferred transfection and chromosomal integration techniques for genes encoding target/FAP fusion proteins can be used if compatible with the nature of the assay.

2.5 Receptor/ FAP internalization assays

β 2AR-FAP internalization was induced by exposing NIH 3T3 or U937 cells stably expressing β 2AR-FAPs to 10 µM isoproterenol and observing fluorescence signal by fluorescence microscopy. Microscopy was used to verify internalization of β 2AR-FAP-fluorogen complexes from the surface of the cell to internal structures after stimulation of β 2AR-FAP with 10 µM isoproterenol. Fluorescence microscopy was carried out using an Olympus IX50 microscope equipped with a spinning disc confocal imaging system (Solamere Technology Group, Salt Lake City, UT). For TO1-2p, excitation was done with a 488 nm argon laser and a 500 nm long pass filter for emission (HQ500 LP; Chroma Technologies, Santa Fe Springs, CA) For MG-11p an HBO 100-watt light source was used

with appropriate filters (HQ620/60 excitation and HQ 700/75 emissions; Chroma Technologies).

2.5.1 Surface fluorescence depletion assay by flow cytometry—Cells stably expressing β 2AR-FAP are incubated with 10 μ M isoproterenol for 40 minutes with a control sample receiving no isoproterenol. Membrane impermeant fluorogen is added to both samples to a concentration of 100 nM and cells were immediately analyzed by flow cytometry. HL1.01-TO2p- β 2AR was excited with an Argon-488nm laser and detected with an emissions filter of 530/30nm, while HL4-MG11p- β 2AR was excited with a HeNe 633 nm laser and detection was with an emission filter of 685/35nm using a FACS Diva flow cytometer. Cells treated with isoproterenol were compared to those not treated with isoproterenol to measure differences in fluorescence values. This receptor internalization assay can potentially be used with any receptor fused to FAP for which there is a known agonist that is fluorescent on the surface when expressed and fluorogen is added.

2.5.2 Internal fluorescence accumulation assay by flow cytometry—Cells stably expressing β 2AR-FAP are incubated with 10 μ M isoproterenol in the presence of 100 nM fluorogen for 45 minutes then treated with 1 mg/mL trypsin. A control sample with no isoproterenol with addition of fluorogen is also done and trypsinized after 45 minutes. Cells were analyzed by flow cytometry using the same settings as described in 2.5.1 and fluorescence values between sample and control cells was compared. This internal fluorescence accumulation assay can be performed in a similar manner with any receptor-FAP complex stably expressed on the cell surface which responds to a known agonist.

2.5.3 Receptor internalization kinetics assay—Cells stably expressing β 2AR-FAP on the surface were used in the surface fluorescence depletion assay to measure β 2AR-FAP receptor internalization in kinetic and dose response experiments. Cells were incubated with 10 μ M isoproterenol to initiate β 2AR-FAP internalization. Aliquots of cells were removed at time points and mixed with 100 nM fluorogen and run on the flow cytometer as in 2.5.1.

2.5.4 Agonist dose response measurements by flow cytometry—Dose response measurements of isoproterenol over several orders of magnitude from 100 nM to 500 μ M were done using the surface depletion assay as described in 2.5.1 on cells stably expressing β 2AR-FAP. Mean intensity of fluorescence values was plotted against isoproterenol concentration (log μ M) to estimate an EC_{50} value. Such a dose response assay could be performed with an antagonist against a receptor fused to FAP and displayed on the surface of cells.

2.5.5 Antagonist dose response measurements by flow cytometry—The β 2AR antagonist propranolol was used in the surface depletion assay described in 2.5.1 in increasing concentrations from 100 pM to 10 μ M. β 2AR-FAP expressing cells were incubated with μ M isoproterenol along with propranolol for 45 minutes prior to addition of 100 nM fluorogen. Cells were analyzed by flow cytometry as in section 2.5.1 and mean fluorescence intensity vs. propranolol concentration (log nM) to estimate an IC_{50} concentration. This antagonist assay could be developed for any functional receptor-FAP complex on the cell surface with a known antagonist.

2.6 Critical Aspects of the Methodology

The FAPs and fluorogens discovered and utilized in these studies share some of the same advantages and drawbacks of other protein fusion tags, but have the singular advantage that one has much more control over when and where the fluorescence is generated. Expression

levels of FAPS on the surface of cells can be quantified by simple addition of fluorogen, and verified by fluorescent immunostaining of additional epitopes on the fusion protein.

One important note in using fluorogenic staining is that dead cells may absorb and locally concentrate high levels of fluorogen and thereby generate background signal. Dead cells can be stained by the addition of propidium iodide (PI) to the cells along with fluorogen to verify that cells generating fluorogenic signal are in fact live cells. PI positive cells can be gated out to restrict fluorescence measurements to live cells. Use of PI will also reduce false positive sorts when using FAPs for FACS. Surface quantification is less problematic using FAPs compared to other protein fluorophores because mis-sorted or mis-folded FAPs are inaccessible to membrane impermeant fluorogens and thus won't fluoresce at all. Intracellular overexpression of protein will not create often intense background fluorescence that can saturate the dynamic range of photo detectors and interfere with microscopy or quantitative flow cytometry.

We should also point out that FAPs expressed at the mammalian cell surface have a potential drawback in that they are exposed to the extracellular milieu via the N-terminus of the targeted membrane protein, and any substance or activity in the milieu that might interfere with fluorogen binding to FAP can pose a problem. In our experience this usually does not occur, and our modified fluorogens do not show substantial non-specific staining. As demonstrated in the receptor internalization assay, proteases can release scFvs into the medium and degrade them. One notices this when the medium turns acidic and cells start to die; cells lose all of their surface fluorescence over the course of one to two days. Although this raises the concern that the cells might actually have lost the FAP-encoding gene, this is not the case and they fully recover over one or two days if fresh media is provided. Certainly care must be taken to ensure the good health of cells, and proper growth conditions must be maintained to get consistent results. Residual protease contamination in buffers or media is generally not a concern, and most problems are easily avoided.

3 Results

3.1 Development of FAPs from yeast display libraries

To reduce non-specific binding to DNA, a sulfonated version of TO was synthesized. TO and MG are coupled to a diethylene glycoldiamine to increase solubility and render them membrane impermeant, while maintaining the fluorogenic nature of the dyes. These fluorogens, TO1-2p and MG-2p, are shown in Figure 1. Sulfonated TO1 and MG coupled to biotin polyethylene glycol were used to pre-enrich the PNNL yeast displayed scFv library for fluorogen binders using Miltenyi streptavidin and anti-biotin magnetic beads [8, 11]. The enriched library was then screened for fluorescence gain in the presence of TO1-2p or MG-2p using FACS. From the initial display library of $\sim 10^9$ individual clones comprised of synthetically recombined heavy and light chain variable regions [11] two unique clones that bind TO1-2p and six unique clones that bind MG-2p were isolated. (Table 1)[8]. These fluorogens showed increases in brightness that accompany specific binding to FAPs ranging from 2,700-fold (TO1-2p) to as much as 20,000-fold (MG-2p). The TO1-2p binding HL1-TO1 FAP was further affinity matured through directed evolution and FACS selection to give the FAP HL1.0.1-TO1, which has markedly improved TO1 binding affinity (Table 1.). HL1.0.1-TO1/TO1-2p complexes display EGFP-like brightness with an extinction coefficient of $\epsilon=60,000 \text{ M}^{-1} \text{ cm}^{-1}$ and a quantum yield of $\Phi=0.47$ [8], compared to values of $\epsilon=53,000 \text{ M}^{-1} \text{ cm}^{-1}$ and $\Phi=0.60$ for EGFP [16]. FAP-fluorogen pairs that have been characterized and used in flow cytometry assays to date are listed in Table 1

Some of the MG-2p binding FAPs described in this study were comprised of only the ScFv heavy or light chains, with the smallest FAP, L5-MG, consisting of only 110 amino acids.

Further directed evolution and FACS selection led to the discovery of the L5 E52D-MG point mutant, which increased affinity to MG. An additional point mutation in the L5-MG FAP (L91S) increased the quantum yield of L5-MG, and these mutations in combination confer tighter fluorogen binding and five-fold greater brightness (Table 1)[15]. We are currently pursuing other potential FAP/fluorogen pairs that emit in the purple and orange regions of the spectrum[17]. Eventually fluorogen emission will be extended further into the red. Observation of several FAP-fluorogen pairs reveals relatively low levels of photobleaching as compared to other fluorescent proteins, which may be due to either fluorogen exchange replacing photobleached fluorogen or shielding of fluorogen from reactive oxygen by the FAP.

Recent experiments using intramolecular energy transfer between Cy3 dye molecules coupled to the malachite green fluorogen molecule (termed “dyedrons”) have shown amplification of MG emission signal upon direct excitation of Cy3. The absorbance of light by Cy3 is increased in proportion to the number of Cy3 dyes on the Cy3 dendrimer[15]. High absorbance values and efficient intramolecular energy transfer have been previously observed in multi chromophoric dendrimers[18, 19]. These fluorescent dendrimers, however, were not able to be conjugated to biological molecules such as antibodies and have no inherent protein targeting mechanism. Dyedrons with up to four Cy3 donors emit at the MG emission wavelength (670 nm) only when the dyedron is bound to FAP and the MG acceptor is constrained. Moreover, MG emission increases proportionally according to the number of Cy3 dyes/dyedron (Figure 2). This was shown on yeast cells displaying the FAP L5E52D-MG on their surfaces and using dyedrons containing zero (MG-2p, M) one (CM), two (BCM) or four (TCM) molecules of Cy3 coupled to MG (Table 1, Figure 2). These flow cytometry experiments establish the use of dyedrons to linearly amplify fluorescence of FAP/fluorogen complexes by intramolecular energy transfer, and demonstrate the potential to enhance flow cytometry assay sensitivity for low-abundance proteins. Dyedrons are relatively small compared to the FAPs and can amplify fluorescence signal to that approximately 5 fold higher than EGFP and 10 fold higher than the monomeric red fluorescent protein mCherry[15]. Such amplification is advantageous as compared to increasing signal by either overexpression of fluorescent protein/target fusions or construction of targets fused to multiple fluorescent proteins.

3.2 FAPs secreted from yeast: the basis for exogenous immunoreagents

Through the use of plasmids that secrete FAPs and FAP-based fusion proteins from yeast, soluble FAPs can be purified and studied in solution. Although some purified MG-binding FAPs display lower binding affinities in solution than when displayed on the cell surface [8], solution-based FAP proteins can be used to measure FAP-fluorogen assembly and *in vitro* molecular assembly. Purified FAP-based fusion proteins have a wide variety of potential applications as flow cytometry and microscopy reagents. For example, bispecific scFvs consisting of scFvs fused to a FAP may enable on-demand spectrally configurable visualization after binding to cellular antigens on living or fixed cells. If such a bispecific reagent is used in conjunction with a dyedron where the Cy3 donor is replaced with an environmentally sensitive dye, one can create surface-based biosensors for use on living cells.

3.3 Display systems in Mammalian cells

Display of FAPs can be achieved in mammalian cells using the pDisplay system that directs fusion proteins to the surface of the cell through the use of a murine IgK signal sequence and tethers fusion proteins to the cell surface through a C-terminal trans-membrane domain from platelet derived growth factor. Cloning and expression of FAP to the N terminal extracellular end of the transmembrane domain of pDisplay and EGFP or monomeric red

fluorescent protein (mRFP) on the C-terminus, expressed on the internal side, has shown that FAP fluorescence is entirely on the surface of the cell while fluorescent protein fluorescence is on the inside of the plasma membrane and also accumulated inside of cells [10].

3.4 β 2AR internalization assays

Fusion of FAPs to the N-terminus of the beta2 adrenergic receptor (β 2AR) and expression on the surface of mammalian cells has been demonstrated, with fluorogen activation on the surface as well as receptor internalization upon stimulation, as measured by internalized FAP fluorescence (Figure 3) [9]. In this demonstration of receptor internalization NIH 3T3 and U937 cells were stably transfected with N-terminal FAPs HL1.0.1 and HL4 fused to β 2AR. Addition of the fluorogens TO1-2p to the HL1.01- β 2AR and MG-11p to HL4- β 2AR yields surface fluorescence after FAP-fluorogen binding. HL1.01-TO2p- β 2AR was excited with an Argon-488nm laser and detected with an emissions filter of 530/30nm, while HL4-MG11p- β 2AR was excited with a HeNe 633 nm laser and detection was with an emission filter of 685/35nm using a FACS Diva flow cytometer. Fluorescence microscopy of these cells was performed with similar wavelength excitation and detection confirming that these FAP-receptor fusions were in fact located entirely at the surface of the cell (Figure 3) [9]. Additions of 10 μ M isoproterenol, a known agonist of β 2AR, led to internalization of fluorescence from the cell surface into distinct vesicular structures (Figure 3).

3.4.1 Surface fluorescence depletion assays—Using the information observed by microscopy, a flow cytometry based surface fluorescence depletion assay was developed for β 2AR by incubating cells with 10 μ M isoproterenol for 45 minutes prior to addition of the cell impermeant fluorogen. Fluorescence intensity of these cells was significantly reduced when compared to cells not treated with isoproterenol prior to fluorogen addition, due to the reduced number of receptor-FAP molecules on the surface (Figure 4.). This surface depletion assay was performed by flow cytometry on both NIH 3T3 and U937 cells transfected with either HL1.01-TO2p- β 2AR and HL4-MG11p- β 2AR FAP-receptor complexes excited with 488 nm and 633 nm lasers respectively.

3.4.2 Internal fluorescence accumulation assays—A complementary flow cytometry approach to the surface depletion assay, an internal fluorescence accumulation assay was also developed using FAP- β 2ARs. In this approach cells were incubated with both fluorogen and isoproterenol for 40 minutes prior to addition of trypsin to strip the remaining surface FAPs. When measured by flow cytometry and compared to cells not treated with isoproterenol these cells showed much higher fluorescence values due to the internal FAP- β 2AR complexes. (Figure 4). This internal fluorescence accumulation assay was also performed in both 3T3 and U937 cells using the two FAP-fluorogens described above yielding similar results. Microscopy done on these trypsinized cells (Figure 4 panels E and F) verified that remaining fluorescence was internalized in these cells while surface fluorescence was nearly completely depleted by proteolytic removal of FAPs[9].

3.4.3 Kinetic and dose response experiments using β 2AR-FAP—The surface fluorescence depletion assay was also used to characterize the FAP- β 2AR in kinetic and dose response experiments (Figure 5). U937 expressing FAP- β 2AR cells were incubated with 10 μ M isoproterenol to initiate FAP-receptor internalization. Aliquots of cells were removed at time points and mixed with 100 nM fluorogen and run on the flow cytometer. Fluorescence intensity of cell populations was reduced over approximately 45 minutes and leveled off at about one third of the initial value (Figure 5 panel A)[9]. These findings are consistent with previous observations of relatively slow receptor internalization mechanisms[20]. Dose response experiments with several orders of magnitude of

isoproterenol concentrations were done using the surface depletion assay, resulting in an EC₅₀ value of 65 nM. (Figure 5 panel B) This value is greater than previously published EC₅₀ values observed for isoproterenol response for β 2AR[20], however, those studies measured signaling events and not receptor internalization specifically. Another response of FAP- β 2AR, that of an antagonist, was measured using the surface fluorescence depletion assay as well. A range of propranolol concentrations was used to treat FAP- β 2AR cells along with 10 μ M isoproterenol for 45 minutes prior to fluorogen addition. The mean fluorescence intensity plotted against propranolol concentration yielded an IC₅₀ value of approximately 3.5 nM. (Figure 5 panel C).

3.5 Additional FAP-receptor approaches

FAPs have also been genetically fused to additional transmembrane proteins and successfully expressed at the cell surface as well. The human glucose transporter 4 (GLUT4) has been expressed with N-terminal FAP in C2C12 cells and the cystic fibrosis transmembrane conductance regulator protein (CFTR) with N-terminal FAP has been expressed in HEK 293 cells [10]. Both CFTR and GLUT4 are 12 transmembrane domain proteins, and showed only surface localized fluorescence when expressing FAPs bound to membrane impermeant fluorogen. (data not shown) EGFP C-terminally fused to CFTR, GLUT4 and β 2AR with N-terminally displayed FAPs showed both surface fluorescence of FAPs as well as internal membrane fluorescence and accumulation of fluorescent protein in other cellular compartments [10]. While neither GLUT4 or CFTR expressing cells were analyzed by flow cytometry in these studies, fluorescence microscopy of these cells demonstrated accumulation of EGFP inside of the cells, complicating measurements done by flow cytometry.

4 Discussion

4.1 Use of FAPs as a platform to study surface and membrane proteins

FAPs have an enormous potential for use in flow cytometry cell surface based assays because fluorescence can be limited to proteins that are or have recently been resident on the surface membrane. Use of fluorogen/FAP pairs of different colors will also support multiplex protocols. FAPs that can exchange for more than one color fluorogen or dyedron are being developed for use in pulse-chase experiments involving the cell surface and endocytosis, and new opportunities will surely present themselves as more FAPs, with different binding avidities and colors become available. There is also the possibility of stably entrapping fluorogen/FAP complexes within intravesicular stores originating from the surface, thereby enabling long term tracking of vesicle recycling.

Another area of study to which FAPs could be applied is the insertion of proteins into the plasma membrane. Both GLUT4 and CFTR are membrane proteins that have regulated insertion into the cell surface membrane [21, 22], and have both been expressed as fluorescent FAP fusion proteins. It may be possible to measure and quantify both membrane insertion and removal or recycling using FAP/receptor complexes and fluorogen in a manner similar to the flow cytometry based β 2AR assays already characterized. Furthermore, it may be possible to use FAPs in high-throughput screening applications for compounds or peptides which initiate membrane insertion or removal of proteins.

4.2 Use of FAPs with dyedrons to amplify signal

The use of dyedrons enhances of flow cytometry applications that monitor low copy surface receptors or markers. FAP/receptor complexes are sometimes observed in low copy number even when the fusion protein is overexpressed, most likely due to inefficient protein trafficking. Dyedron signal amplification of low copy molecules on the surface, without

internal fluorescence accumulation, could be of great value in the study of surface molecules by flow cytometry. The use of dyedrons also increases the prospects of better signal to noise ratios because unbound dyedrons, like MG, are essentially non-fluorescent while FAP-bound dyedrons have high fluorescent signal. This enhancement could be of great importance in the field of flow cytometry-based receptor agonist or antagonist screening, where a high signal to noise ratio leads to fewer false positives and a robust screening assay.

Use of dyedrons could potentially be extended to receptor-FAP molecules being expressed under the receptor's physiological or cell type specific promoters, which may lead to biologically relevant low copy numbers on the cell surface as well. Using current fluorescent protein technology, molecules of low copy number are poorly observed and quantified. Dyedron-based signal amplification could enable analysis of low copy number targets using established flow cytometry and microscopy methods and instrumentation.

The spectral separation inherent in dyedrons could lead the way to a series of dye reagents which not only increase fluorescence signal, but also excite at multiple distinct wavelengths, depending on the choice of donor and the spectral breadth of that donor (e.g. Cy3 may be efficiently excited using 514, 532 or 561 nm lasers, Figure 2). A dyedron may also be used in pulse/chase protocols in combination with a bichromophoric dye, where the dyedron acceptor chromophore (e.g. MG) is instead used as a donor that transfers energy to a red-shifted acceptor (e.g. Cy7) within the bichromophore. Such combinations, which can entail pulse/chase excitation/emission separations of over 200 nm, facilitate the use of current lasers with a new generation of near infrared detectors that are further removed from cellular auto fluorescence and thus have better signal-to-noise properties.

4.3 Adaptation of β 2AR–FAP assays to high-throughput screening applications

The surface fluorescence depletion and the internal fluorescence accumulation flow cytometry based assays on β 2AR–FAP compliment each other well. Together with the fact that neither requires any complicated wash steps or handling of cells makes emerging FAP technologies ideally suited for flow cytometry based high throughput screening applications [23] to discover agonists and antagonists of β 2AR. Discovery of such potential drugs could have therapeutic value in the treatment of diseases such as hypertension and asthma[24][25]. The application of high-throughput screening against this specific G-protein coupled receptor (GPCR) using FAPs could potentially be applied to other GPCRs of interest in human health[26]. The use of fluorogen activating proteins in flow-cytometry based high-throughput screening could also be useful to screen for compounds which could up-regulate insertion of protein into the plasma membrane leading in a gain of fluorescence as well in cases where membrane trafficking or insertion is desired.

4.4 Practical Considerations for Extending Labeling to the Cell Cytoplasm

With proper excitation and emission filtering it is possible to study more than one receptor in the surface membrane at a time. Once receptors are labeled at the cell surface they can be followed by microscopy deep into the cell post-endocytic uptake for a surprisingly long times (hrs), thus receptor recycling is ripe for study using the FAP/fluorogen pairs at hand. We've also studied cell behavior in dishes for several days running, with no apparent ill effects of fluorogen in the media. Cell surfaces are bright, and cell divisions do not appear to be impeded in any fashion.

A membrane permeant form of MG (MG ester) is available and will effectively cross all cell membranes and label FAPs. However, there are additional issues that come up when FAPs (scFvs) are expressed in the cytoplasm. Attempts to express intracellular scFvs have shown that an scFv to Huntingtin protein did not express well in the cell cytoplasm because

folding did not occur properly in the cytoplasm the same way as it does in the lumen of the ER or Golgi[27]. This is also true for our FAPs. Generally, two pairs of disulfides in the scFv have to form and are unable to do so in the intracellular reducing environment, leaving the FAPs unable to bind fluorogen. It was also shown that misfolding of the Huntingtin scFv could be obviated by replacing the disulfide cysteines with amino acids that provide the same structure through stronger hydrogen bonding[27]. Currently, one of our FAPs binds MG ester inside the cells as efficiently as when it is expressed on the external side of the surface membrane. HeLa cells with a disulfide free version of the H6 FAP genetically fused to actin showed bright actin fluorescence when treated with the MG ester fluorogen in STED microscopy experiments. It likewise required the kind of genetic engineering described in experiments to engineer intracellular Huntingtin ScFvs. This also points out the versatility of using ScFvs for developing fluorescence signaling reagents. Much of our current work is focused on the discovery of new FAPs that function both inside and outside cells, as well as ones that bind more than one color fluorogen, ones that fluoresce in the UV, in the orange, and others further out in the red, and bind with widely different off rates, and others.

4.5 Availability of fluorogens and FAPs

The fluorogens and dyedrons used in these studies are currently not commercially available. We anticipate the commercial production of these fluorogens and FAP plasmids for wide distribution and general use in the near future. Fluorogens and FAPs can currently be obtained through contacting the Molecular Biosensor and Imaging Center at Carnegie-Mellon University.

5 Conclusion

With applications in flow cytometry and microscopy being developed rapidly, fluorogen activating protein technology exhibits some features that are similar to those of popular fluorescent proteins. While both fluorescent proteins and FAPs are typically used as genetically encoded proteins fused to a protein of interest, FAPs have a number of distinct advantages for the study of cell surface proteins. Fluorogen/FAP binding assays are homogeneous, requiring no wash steps prior to visualization; this greatly simplifies flow cytometric analysis of surface proteins fused to FAPs. Fluorogen binding to most FAPs occurs within seconds of addition, and can be carried out in a near physiological buffer or medium of choice. A major advantage of using FAPs with MG fluorogens is that these FAPs are optimally excited with 633 nm lasers common to many commercial flow cytometry systems, and emit at about 670 nm, far to the red of EGFP or other fluorescent proteins. The use of multichromophoric Cy3-MG/FAP complexes also amplifies the signal as much as 8-fold over that of MG/FAP alone, and enables excitation with commonly available 514, 532, or 561 nm lasers.. FAPs that are fused to other scFvs or proteins of interest, and then expressed and purified, also have potential for development of a wide variety of exogenously applied flow cytometry reagents and biosensors. Future work will determine the applications and limitations of FAPs in this area.

The FAPs discussed here are mainly canonical scFvs that bind a single type of fluorogen at a specific pocket formed by interfacing variable domains of antibody heavy and light chains. These FAPs exhibit fluorescence properties that are strictly a result of binding a fluorogen, and that do not give information protein interactions, alterations, or local environmental conditions. The principal use of these FAPs thus far has been in studying fusion protein localization with respect to important membrane biology. But the FAP-fluorogen technology is inherently modular and adaptable, and the future promises a profusion of new applications. The dyedrons are but one example of what can be done with fluorogens and other chromophores that are linked together by polymers; under development are transgenic

biosensors that incorporate environmentally sensitive chromophores as energy donors. FAPs that bind more than one chemically and spectrally distinct fluorogen, presumably at the same site, have been isolated [17] and are being further characterized biochemically and in cells. Single chain FAPs have also been isolated [8], and studies are only now revealing to what extent fluorogen binding and light production may be dependent on dimerization to produce something more like a canonical scFv. Crystal structures are now available for several of our FAPs. We expect that more FAPs, with different properties and structures, will be discovered. Clearly, many practical applications based on this new knowledge await development. The studies described here, focused on the use of FAPs and fluorogens in flow cytometry, presage a broader future that makes FAPs an exciting emergent fluorescent protein technology.

Highlights

FAPs are proteins that bind small molecule fluorogens to activate fluorescence.
 FAPs can be used as fusion proteins in a similar manner as GFP.
 FAP/fluorogen excitation and emission are well suited to current flow cytometers.
 Impermeant fluorogens will label FAPs at the cell surface but not in the cytoplasm.
 FAPs can measure dose responses of receptor agonists and antagonists.

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Figure 1.
Fluorogen chemical structures, formulas, and molecular weights for the fluorogens used in these studies.

Figure 2.

Fluorescence emissions histograms from flow cytometry analysis of *Saccharomyces cerevisiae* cells displaying L5 E52D-MG bound to dyedrons. Dyedrons with 0 (Malachite Green, M, identical to MG-2p), one (Cy3-Malachite Green, CM), two (Bis-Cy3-Malachite Green, BCM) or four (Tetra-Cy3-Malachite Green, TCM) Cy3 donor chromophores were bound to yeast displaying FAP and analyzed on a FACSVantage SE flow cytometer by excitation of Cy3 (532 nm) or MG (635nm) with MG emission collected through a 675/50 nm band pass filter. Excitation of Cy3 donors (magenta) yields higher intensity emission relative to direct excitation of MG acceptor (cyan) in proportion to the number of donors. Unstained yeast controls (U) illustrate the relative levels of cellular auto fluorescence at the

two excitation wavelengths. All samples contain a sub-population of cells that do not express scFvs due to plasmid loss, and exhibit the same fluorescence emission as unstained controls (peaks marked by thin vertical lines). Figure reprinted with permission from [15]

Figure 3.

Agonist-stimulated internalization of human beta2 adrenergic receptor (β 2AR)-FAP fusion proteins in NIH 3T3 cells. Cells expressing HL1.0.1-TO- β 2AR (A1–A3) and cells expressing HL4-MG- β 2AR (B1–B3) were imaged without (A1, B1) and with stimulation (A2, A3, B2, B3) 10 μ M isoproterenol. Fluorogens (100 nM TO1-2p and 100 nM MG-11p) were added at time zero. Images A2 and B2 were taken 60 minutes after stimulation while images A3 and B3 were taken 90 minutes after stimulation. Figure reprinted with permission from [9].

Surface Fluorescence Depletion Assay

Internal Fluorescence Accumulation Assay

Figure 4.

Surface fluorescence depletion and internal fluorescence accumulation assays. (A) Flow Cytometric analysis of U937 cells expressing the FAP HL1.0.1 that were unstimulated (red histogram) or stimulated (blue histogram) with 10 μ M isoproterenol for 45 minutes prior to addition of membrane impermeant fluorogen TO1. (B) Cells imaged from unstimulated sample. (C) Cells imaged from stimulated sample. (D) Flow cytometry analysis of U937 cells that were unstimulated (red histogram) or stimulated (blue histogram) with 10 μ M isoproterenol for 45 minutes while in the presence of TO1 fluorogen and then treated with trypsin. (E) Images of unstimulated cells. (F) Image of stimulated cells. Figure reprinted with permission from [9].

Figure 5.

Kinetic and dose-response assays using U937 cells stably expressing HL1.0.1-TO1-2p- β 2AR assayed by flow cytometry. (A) Time course showing fluorescence response to cells stimulated with 10 μM isoproterenol. (B) Isoproterenol dose response. Cells were treated with varying concentrations of isoproterenol and after 45 minutes 100 nM TO1-2p was added and fluorescence determined by flow cytometry. Each point represents the mean fluorescence of a 10,000 cell population. (C) Propranolol dose response curve. Cells were treated with 10 μM isoproterenol plus varying concentrations of propranolol and assayed 45 minutes after addition of the drugs. Time points represent the mean fluorescence of a 10,000 cell population. Figure reprinted with permission from [9].

Table 1

Properties of primary and improved FAPs. Primary FAPs were isolated using FACS screens of library of yeast surface displayed scFvs derived from naive human germline IGG sequences [11]. Improved FAPs (*) were obtained by directed evolution of primary FAPs using FACS screens to select displayed scFvs carrying point mutations that increase affinity and/or quantum yield. Spectral properties of FAP/fluorogen complexes were experimentally measured using purified FAPs, except for L5 E52D L91S, where brightness was determined on yeast displayed FAP using plate fluorimetry and flow cytometry. Dyedrons comprised of MG coupled to variable numbers of Cy3 donors are abbreviated as: CM, 1 Cy3; BCM, 2 Cy3; TCM, 4 Cy3. Fluorescence enhancement values for dyedrons were determined using Cy3 excitation at 514 nm. For comparison purposes, values for EGFP and mCherry fluorescent proteins are included.

FAP	Fluorogen	Size kDa	Cell surface K _D (nM)	Excitation max (nm)	Emission max (nm)	Extinction Coef. ϵ (M ⁻¹ cm ⁻¹)	Quantum yield (Φ)	Brightness ($\Phi * \epsilon$) / 10 ³	Fluorescence Enhancement
HL4-MG	MG-2p	26.1	3.2	629	649	133,000	0.16	21.2	15,700
L5-MG	MG-2p	11.5	1.2	640	668	103,000	0.048	4.9	4,100
L5 E52D-MG*	MG-2p	11.5	<1	642	680	79,000	0.06	4.4	ND
L5 E52D-MG*	CM	11.5	<1	552	678	140,000	0.06	8.0	115
L5 E52D-MG*	BCM	11.5	4	551	678	290,000	0.05	14	251
L5 E52D-MG*	TCM	11.5	15	552	676	530,000	0.05	29	287
L5 E52D L91S-MG*	TCM	11.5	2	552	676	ND	ND	160	ND
H6-MG	MG-2p	14.4	7.5	635	656	105,000	0.25	26	18,000
HL1-TO1	TO1-2p	26.1	360	510	527	ND	ND	ND	ND
HL1.0.1-TO1*	TO1-2p	25.9	3.1	509	530	60,000	0.47	28	2,600
HL2-TO1	TO1-2p	26.3	600	516	550	ND	ND	ND	ND
EGFP	N/A	28.8	N/A	488	507	63,000	0.65	32	1
mCherry	N/A	28.8	N/A	587	610	72,000	0.22	16	1