Methods 57 (2012) 308–317

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Fluorogen activating proteins in flow cytometry for the study of surface molecules and receptors

Matthew J. Saunders [a,*], Christopher Szent-Gyorgyi [a], Gregory W. Fisher [a], Jonathan W. Jarvik [a,b], Marcel P. Bruchez [a,b,c], Alan S. Waggoner [a,b]

[a] Molecular Biosensor and Imaging Center, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213, USA
[b] Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213, USA
[c] Department of Chemistry, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213, USA

ARTICLE INFO

Article history:
Available online 16 February 2012

Keywords:
Fluorogen activating protein (FAP)
Fluorescence
Receptor internalization
Fluorescence amplification
Fluorescent proteins
Dose–response

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 extracellular 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 (β2AR) 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.

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

* Corresponding author. Fax: +1 412 268 6571.
E-mail address: matsaun@andrew.cmu.edu (M.J. Saunders).

1046-2023/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2012.02.003

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 quantitative 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 evolutionarily 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