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Application of Green Fluorescent Protein in Immunoassays

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Abstract

Green fluorescent protein (GFP) is a protein that emits green fluorescence when exposed to a radiation of ultraviolet wavelength range, even without the addition of substrate and cofactor. Because of such characteristics, the usage of GFP is widespread in both *in vivo* and *in vitro* applications. In addition, recent advances in biotechnology have enabled GFP to be expressed in various hosts, including bacteria, yeast, plants, animals, and even living-cells, for multiple purposes. Currently, GFP is a subject of great interest in the analytical sciences, especially in immunoassays for qualitative and quantitative analyses, when it is fused with an antibody because of the high sensitivity of GFP and antigen-binding specificity of antibodies. Recently the fluobody, which is a fusion protein of GFP with single-chain variable fragment antibody (scFv), has become a useful tool in various fields. We review here the applications of GFP as fluobodies in immunoassays.

Keywords

GFP, Immunoassay, Fluobody

1. Introduction

Green fluorescent protein (GFP) was first isolated from the jellyfish *Aequorea victoria* in the early 1960s [1]. The fluorescence spectra of GFP exhibit two absorption peaks at 395 nm and 475 nm (excitation), which individually emitted maximum at 508 nm and 503 nm (emission). The absorption peaks at 395 nm and 475 nm re-

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sult from two different chromophore species: a neutral protonated form and an anionic deprotonated form, respectively [2] [3]. The primary structure of GFP was elucidated three decades after its first discovery, and the GFP gene was found to encode 238 amino acid residues with a calculated molecular weight of 26,888 Da [4]. Thereafter, the use of GFP increased widely and dramatically. The first expression of GFP was successfully performed in nematode *Caenorhabditis elegans* [5], and the results suggested that GFP can be used as a marker for gene expression. In 1996, two groups successfully crystallized GFP and found that the protein fold consists of an 11-stranded β-barrel with a coaxial helix, with the chromophore forming from the central helix [6] [7]. Currently, GFP is an indispensable tool as a reporter gene for the study of gene expression and subcellular localization of proteins in plant, bacterial, and mammalian systems [8]-[10]. This widespread use of GFP as a biological tool is attributed to three characteristics. First, GFP is highly stable. The fluorescence of GFP is species-independent and remains stable under a wide variety of conditions [11]. Second, GFP maturation requires only oxygen [12]. Third, GFP is noninvasive, and therefore the fluorescence can be monitored in living cells, or in the entire organism in case of transparent organisms [13].

Thus far, various applications of GFP in both *in vivo* direct monitoring of the target protein and *in vitro* analyses have been reported. Fluorescence resonance energy transfer (FRET) is one of the most widely used applications of GFP. FRET is the nonradiative exchange of energy from an excited donor fluorophore to an acceptor fluorophore within 10 - 100 Å. Because the efficiency of FRET depends on the distance between fluorophores, protein-protein interactions can be investigated using this method *in vivo* and *in vitro*. GFP can be used as an intercellular molecular sensor in this case. The applications of GFP in FRET have recently been reviewed [14]-[18]. The use of GFP has been expanded to photobleaching, which can be used to explore the lateral mobility of fluorophores on surface of cells. The fusion of GFP with a target protein makes it possible to investigate the protein dynamics within a cell [19]-[21]. Two different types of photobleaching processes can be used to explore molecular motion or diffusion: fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). In the FRAP method, a region of interest is bleached once by high-intensity illumination, and then recovery of the resultant fluorescence loss is monitored, which reveals the relative mobility of the GFP chimera protein [20]. FLIP can be used to study the transport of GFP chimera proteins between different organelles. After a region has been repeatedly bleached, the loss of fluorescence in an area away from the bleached region is monitored. The FLIP method can be used to confirm FRAP results because the photobleaching process in FRAP can photo damage the protein of interest and alter its dynamic characteristics, whereas FLIP assesses the mobility of fluorescent proteins adjacent to the bleached region [22].

A fluobody, which is a chimera protein of GFP combined with antibody, has become a useful tool in both *in vivo* and *in vitro* applications. In *in vivo* applications, a fluobody can be used to "tag" the target protein by utilizing binding specificity of an antibody when the fluobody is expressed in a living host. Li *et al*. recently found that the camelid-derived homodimeric heavy-chain antibody (VHH), which is composed of a single-variable domain, can across the blood-brain barrier (BBB) without any treatment [23]. The VHH antibodies against the astrocyte-specific marker—human glial fibrillary acidic protein (GFAP), were genetically fused with GFP to produce a fluobody, and this fluobody was expressed to label the GFAP in murine brain. As a result, the fluobody could pass the BBB and label GFAP. This study revealed the ability of the fluobody to be used as a specific transporter. Another application has been demonstrated in plants [24], where the investigators constructed a fluobody directly against phytohormone, which is a precursor of gibberellin (GA24) that regulates growth and influences various developmental processes [25]. This fluobody was expressed in *Arabidopsis thaliana*. The results revealed the localization of scFv, which is bound to GA24. In this case, the fluobody was applied as an *in vivo* tool to elucidate the mechanism of the appearance of a GA-deficient phynotype. When the scFv antibody targets the cancer-cell receptor, which is associated with cell proliferation, it can be used in molecular targeted therapy [26]-[28]. Huang *et al*. constructed a fluobody against HepG2 hepatocellular carcinoma and injected the fluobody into mice to elucidate the localization in HepG2 [29]. The fluorescence derived from the fluobody was detected 24 hours after injection, which demonstrates the potential use of fluobodies in *in vivo* molecular targeted therapy.

With respect to *in vitro* applications, fluobodies have been widely used as probes in immunoassays for qualitative/quantitative analysis. In an analytical application, a highly sensitive probe is generally required; thus, the conjugation between fluorescent labels and antibodies has conventionally been performed by chemical conjugation of organic fluorophores [30]; fluorescein isothiocyanate (FITC) is the most commonly employed fluorophore in various fields. Because FITC can bind to the free amino group of the proteins and peptides to form sta-

ble thiourea bond, FITC-labeled antibodies have been used primarily for flow cytometry and immunohistochemical staining for more than 50 years [31] [32]. This procedure, however, requires a large amount of purified protein. Moreover, the fluorophores may bind to the paratope of an antibody, which results in the antibody's partial or complete loss of reactivity. A fluobody can overcome these disadvantages because once the genes are constructed in the expression vector, the resultant protein is always expressed in a one-to-one ratio between the fluorochrome and scFv. Currently, the usage of fluobodies *in vitro* has expanded from the immunolabeling of cancer cells, fluorescence-activated cell sorter (FACS), and diagnosis, to fluorescent-linked immunosorbent assay (FLISA) for the qualitative/quantitative analyses [33]-[37].

The *in vitro* applications of GFP as a fluobody are reviewed in this article.

2. Applications of Fluobody in Immunoassays

2.1. Immunolabeling

The fluobody possesses two characteristics: strong emission intensity and an antibody that binds to a target antigen. Immunolabeling using fluobodies is convenient method for investigating the subcellular protein and oncoprotein in living cells. When the cells are treated with the fluobody and washed, they are ready to be observed under a fluorescence microscope. Schwalbach *et al*. successfully expressed a fluobody against the E6 protein of human papillomavirus type 16 (E6 protein) in *Escherichia coli* (*E. coli*) and revealed that the subcellular localization and movement of E6 protein transfected in COS-7 cell lines [38]. Around the same period, Casey *et al*. expressed a fluobody specific to hepatitis B surface antigen (HepBsAg) in the periplasmic fraction of *E. coli* and revealed that the fluobody retains the functional form to recognize HepBsAg even though it is expressed as a periplasmic protein [33]. In addition, a fluobody against a series of cluster of differentiation (CD) antigen [35], p24 (human immunodeficiency virus 1) CB4-1 [39], single-chain T-cell receptor (scTCR) [40], and B-cell-activating factor of the TNF family (BAFF) [36] were successfully applied for one-step immunolabeling. More recently, a new, multi-colored newly fluobody was suggested by Markiv *et al*. [41]. They constructed a fluorescent antibody, where humanized scFv against $p185^{HER\text{-}2\text{-}ECD}$ (Trastuzumab, Herceptin, 4D5), which is widely used in breast cancer immunotherapy [42], was fused with four kinds of monomeric red, blue, cerulean and citrine fluorescent proteins (RFP, BFP, CER, and CIT, respectively) as bridging molecules. A cell staining study using SK-BR-3 breast carcinoma cells revealed that 4D5-RFP and 4D5-CIT were found to specifically recognize $p185^{HER\text{-}2\text{-}ECD}$ and effectively accumulate on the surface of the cells. Notably, a fluorescent protein other than GFP was used as a fluorescent probe. The results of this study open up the possibility of fluobodies that can be used in immunolabeling because of the various measurement wavelengths of fluorochrome.

2.2. Fluorescence-Activated Cell Sorter

Peipp *et al*. prepared scFv fused with two fluorescent proteins (GFP and DsRed); the fused product exhibited spectral properties that are ideal for dual-color experiments with GFP [35] [43] in which it is applied as a fluorescence-activated cell sorter (FACS). One common problem frequently encountered with a FACS is the background associated with the antigen-independent interaction between the Fc-protein and Fc receptors on various cells. These interactions result in a diminished intensity of the specific signal and subsequently cause a decrease in the signal-to-noise ratio, which complicates the collection of experimental date [44] [45]. Therefore, Peipp *et al*. used a fluorescent antibody because scFv antibody is composed of minimal variable regions for antigen-binding activity without Fc regions. They successfully reduced the background and enhanced the signal-to-noise ratio by a factor of 5 - 10 compared with the value obtained using conventional antibodies. Their study demonstrates the possible use of fluobodies in FACS, especially when treating with cell populations expressing Fc receptors.

2.3. Fluorescence-Linked Immunosorbent Assay

FLISA can be used for both large molecules and small molecules [46] [47]. Indirect FLISA (iFLISA) is suitable for large molecules such as proteins and peptides, whereas indirect competitive FLISA (icFLISA) is appropriate for small molecules such as natural products, agrochemicals, pesticides, and herbicides. The advantages of FLISA over the conventional enzyme-linked immunosorbent assay (ELISA) are its rapidity and sensitivity. Conventional ELISA using an antibody (*i.e*., monoclonal antibody, polyclonal antibody, or scFv antibody) re-

quires the following five steps, and the assay requires approximately 4.5 hours: 1) fixation of a coated antigen, 2) a blocking step to prevent the plate from adsorbing non-specific proteins, 3) the primary antibody reaction, 4) the secondary antibody reaction, and 5) the enzyme-substrate reaction. However, in the case of FLISA performed using a fluobody, the assay is completed within 3 hours because the time-consuming and costly secondary antibody reaction and the subsequent enzyme-substrate reaction can be avoided. In addition, sensitive FLISA can be performed by taking advantage of the strong fluorescence intensity of GFP [48]. Up to date, ic-FLISA can be developed to detect/determine small molecules, which include picloram [49], s-triazine [50], 5-methyl 2'-deoxycytidine [51] and bioactive natural product, plumbagin (PL) and ginsenoside Re (GRe) as previously described in our group [52]-[54]. The icFLISA for PL and GRe shows a lower limit of determination than conventional icELISA. In our study, we assessed the formation of a fluobody has been assessed by constructing two chimera proteins of scFv fused at the C-terminus of GFP (C-fluobody) and the N-terminus of GFP (N-fluobody). In both cases, the fluorescence intensity of the C-fluobody was superior to that of the N-fluobody. These results are supported by the findings of other groups [33] [51]. The difference in fluorescence intensity between the two can be accounted for the flexibility of the linker peptide fusing GFP and the scFv antibody. The 10 amino acids (30 bp) of the C-terminus of the GFP are well known to be flexible sequence [6] and can function as an additional linker in the C-fluobody fusing scFv at the C-terminus of GFP. Because the length of the linker peptide of fluobody was designed to contain 10 amino acids of $(Gly_4Ser)_2$ encoded by 30 bp, the linker sequence of the C-fluobody is equivalent to twice as long as that of the N-fluobody. Our results showed that the flexibility of the linker strongly affects the function of GFP in the fluobody.

Recently, a new type of FLISA has been developed by Ferrara *et al*. [55]. In their fluobody, scFv was expressed with a tag composed of a small domain of GFP (strand 11, residues 215 - 230; GFP11) with retaining its antigen-binding activity. The complementary GFP fragment (1 - 10, residues 1 - 214) is expressed separately. Neither fragment alone is fluorescent. Only when they are mixed, the small and large GFP fragments spontaneously associate, resulting in reconstitution of the fluorophore and fluorescence. This approach allows both the antigen and the scFv concentrations to be determined.

3. Conclusion

In this review, the applications of GFP as a fluobody have been described. Although the fluobody was defined as a chimera protein of GFP with scFv in this article, variations of fluobodies can be designed for different purposes. The type of fluorescent protein used can be expanded from GFP to UV, blue, cyan, yellow, orange, and red fluorescent proteins. The varieties of known fluorescent proteins have been well reviewed [56]. In addition, the antibody can be changed from scFv to fragment antigen-binding (Fab) (~55 kDa), Fab_2 (bispecific; ~110 kDa), Fab_3 (trispecific; ~165 kDa), diabody (bispecific; ~50 kDa), triabody (trivalent; ~75 kDa), tetrabody (tetravalent; ~100 kDa), bis-scFv (bispecific; ~55 kDa), and minibody (bivalent; ~75 kDa) antibodies [57]. These combinations, in conjunction with different linker designs, open up the use of fluobodies more in various fields.

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