GREEN FLUORESCENT PROTEIN AND THEIR APPLICATIONS IN ADVANCE RESEARCH

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Abstract

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its homologs from diverse marine animals are widely used in biological science as a universal genetically encoded fluorescent labels. Many laboratories have focused their efforts on identification and development of fluorescent proteins with novel characteristics and enhanced properties, resulting in a powerful toolkit for visualization of structural organization and dynamic processes in living cells and organisms. The discovery of green fluorescent protein (GFP) followed by elucidation of its molecular structure introduced a new and promising marker for biological studies. The diversity of currently available fluorescent proteins covers nearly the entire visible spectrum, providing numerous alternative possibilities for multicolor labeling and studies of protein interactions. This protein is intrinsically fluorescent, has a low toxicity, and allows easy imaging and quantification using fluorescence-activated cell sorting (FACS), confocal microscopy and epifluorescence microscopy which further expand the options for real time studies in living tissues. The review article focuses on the structure, properties and numerous applications of GFP as a reporter gene in biological research, with particular attention to recent techniques.

Keywords: Green Fluorescent Protein, Reporter gene, Fusion tag, Cell biology.

1. Introduction

GFP was discovered by Shimomura *et al.* in 1962 from the Pacific Northwest jellyfish *Aequorea victoria* as a companion protein to aequorin. Its role is to transduce, by energy transfer, the blue chemiluminescence of aequorin into green fluorescent light. GFPs have found to exist in a variety of coelenterates e.g. *Aequorea*, *Obelia*, *Phialidium* and *Renilla*. GFP from *A. victoria* is biochemically well characterized and found to have wide applications in molecular and cellular studies. Hence, GFP of the jellyfish *A. victoria* has been used as a convenient reporter in many applications in prokaryotic and eukaryotic organisms. Detection of GFP in living cells can be performed easily and efficiently and it is amenable to real-time analysis of molecular events. Use of GFP also eliminates the need for fixation or cell permeabilization. The already mentioned remarkable characteristics, combined with availability of GFP cloning vectors, has led to extensive use of GFP-fusion proteins for studying cellular processes such as protein localization, gene expression and more recently in drug development studies.

2. Structure and Properties

GFP is composed of 238 amino acids with an apparent M.W. of about 27 kDa. The wild-type GFP has absorbance/excitation peak is at 395 nm with a minor peak at 475 nm and extinction coefficients of roughly 30,000 and 7,000.m/cm, respectively. The emission peak is at 508 nm (1). GFP is very resistant to denaturation and is stable at a wide pH range of <4.0 or >12.0. Partial to near total renaturation occurs within minutes following

reversal of denaturing conditions by dialysis or neutralization. GFP chromophore is a cyclic tripeptide p-hydroxybenzylideneimidazolinone derived from residues 65-67, which are Ser-Tyr-Gly in the native protein. Chromophore formation is a cyclization reaction and is autocatalytic or use factors that are ubiquitous. Chromophore is fluorescent only when embedded within the fully folded, complete GFP molecule. Nascent GFP is not fluorescent, since chromophore formation occurs post-translationally.

Fig. 1: Three-dimensional structure of GFP consisting of 11-stranded β -barrel with central α -helix [adapted from Deo & Daunert, 2001; *Fresenius J Anal Chem* 369: 258-66 (1)].

Yang *et al.* in 1996 (2) and Ormo *et al.* in 1996 (3) described the crystal structure of recombinant wild-type GFP to a resolution of 1.9 angstrom by multiwavelength anomalous dispersion phasing methods. The protein is in the shape of a cylinder, comprising 11 strands of α -sheet with an α -helix inside and short helical segments on the ends of the cylinder. The fluorophores are protected inside the cylinders. The environment inside the cylinder explains the effects of many existing mutants of GFP and suggests specific side chains that could be modified to change the spectral properties of GFP. The 3D structure of GFP has provided a physiochemical basis for many observed features of the protein, including its stability, protection of its fluorophore, behavior of mutants, and dimerization properties. Overall structure of GFP β -barrel with semitransparent surface is shown from the side and from the top view in figure 1.

3. Applications of GFP

Nowadays, GFP and its variants and homologs of different colors are used in a variety of applications to study the organization and function of living systems (Figure 2). GFP has provided scientists with a rich palette of variants with different biochemical and spectral characteristics, which represent a huge source of potentially powerful molecular tools for numerous applications in the study of complex biological systems. In this section, the basic applications of GFPs are briefly summarized and described. Each of the briefly mentioned applications, however, deserves a separate comprehensive review to summarize all the information relevant for rational experimental planning.

3.1. GFP as Reporter Gene

GFP has been successfully used as a reporter gene to detect gene expression in a variety of cells. GFP was used for tracing *in vivo* expression of *mec-7* gene in *Caenorhabditis elegans*, a small nematode (5). GFP shows promise for monitoring the effectiveness of gene transfer in intact transgenic embryos and animals (6). Advantage of GFP over other reporters is summarized in table 1.

Fig. 2: Main area of application of GFP in Biological science (adapted from Chudakov *et al.*, 2010, [4]).

Table 1: Advantages of GFP over other reporter molecules

- Does not require addition of cofactors or substrate.
- Relatively less toxic to cells.
- Imaging and quantitation easy.
- Stable to pH change, proteases, and temperature (upto 65°C).
- Does not alter the function and localization of the fusion partner.

3.2. GFP as a Fusion Tag

The most successful applications of GFP have been as a genetic fusion partner to host proteins to monitor their localization and fate. The gene encoding for GFP is fused in frame with the gene encoding the endogenous protein and the resulting chimera expressed in the cell or organism of interest. The result is a fusion protein that maintains the normal functions and localizations of the host protein but is now fluorescent. GFP has been targeted practically almost every major organelle of the cell, including plasma membrane, nucleus, endoplasmic reticulum, Golgi apparatus, secretory vesicles, mitochondria, peroxisomes, vacuoles and phagosomes. Fusions can be attempted at either the amino or carboxyl termini of the protein.

3.3. GFP as an Active Indicator

This protein can act as indicator of environment surrounding them or that in the organelle. It is possible to engineer phosphorylation sites into GFP such that phosphorylation produces major changes in fluorescence under defined conditions. The engineered fusion of GFP within potassium channel is the first genetically encoded optical sensor of membrane potential (7). The most general way to make biochemically sensitive GFPs is to exploit FRET between GFPs of different color.

3.4. Fluorescence Resonance Energy Transfer (FRET)

FRET is a quantum-mechanical phenomenon that occurs when two fluorophore are in molecular proximity and the emission spectrum of one fluorophore, the donor, overlaps the excitation spectrum of the second fluorophore, the acceptor. The current advances in fluorescence microscopy, coupled with the development of new fluorescent probes, make FRET a powerful technique for studying molecular interactions inside living cells improved spatial (angstrom) and temporal (nanoseconds) resolution, distance range, and sensitivity and a broader range of biological applications. FRET imaging using GFP spectral mutants provides the ability to localize and monitor ion binding and molecular protein-protein interaction *in vivo* (8). The onset and termination of Ca^{2+} signaling in cytoplasm, nucleus or

endoplasmic reticulum can be observed by measuring the change in the ratio of the fluorescence intensities of acceptor and donor molecules in live cells (9). FRET also finds significant application in membrane fusion assays and real-time PCR assays. With recent advances in fluorescent probes, instrumentation and methodologies, FRET is sure to revolutionize scientific research in the near future.

3.5. GFP-based Ca^{2+} Indicators

These biochemical indicators were first developed independently by Romoser (1997) and Miyawaki (1997). Recently fluorescence Ca^{2+} imaging agent that can be inserted into the chromosome of an organism and on expression used to image Ca^{2+} in the cells without the addition of any external reagents has been described. The method is based on the use of a fusion protein in which two different GFP color mutants are spliced to two opposite ends of the Ca^{2+} binding protein calmodulin and FRET. These Ca^{2+} responsive fluorescent complexes have been used in microscopic imaging of cellular Ca^{2+} , for discovery of new Ca^{2+} -channel regulators by high

3.6. GFP as a Reporter in High throughput Screening

3.6.1. Antimycobacterial agents

GFP is used as a marker of gene expression in *Mycobacterium smegmatis* and *M. bovis* BCG. Collins *et al.* (1998) describe the expression of a mutant form of GFP in *M. tuberculosis* and studied the suitability of GFP microplate assay (GFPMA) for HTS of 12 antimycobacterial agents (10). The plasmid pFPV2 containing the gene for EGFP was transformed into two strains of *M. tuberculosis* H₃₇Ra and H₃₇Rv by electroporation. The expression of EGFP after addition of antimycobacterial agents was monitored by fluorescence emission at 508 nm. The minimum inhibitory concentration values were obtained in 7 days. This method provides rapid and inexpensive way of screening antimycobacterial agents, and at the same time it is biological safe, as the microplate doses not need to be reopened after inoculation.

3.6.2. Platinum-based antitumor drugs

Platinum chemotherapy is routinely used in many cancer treatments. Platinum complexes such as cisplatin and carboplatin analogs are successfully used in cancer chemotherapy. New combinatorial library of cisplatin analogs were screened by Sandman *et al.* (1999) in a high throughput cell-based assay using EGFP as a reporter (11).

3.7. Use of GFP to Monitor Protein Localization

GFP based biosensors are used to monitor events such as

cell surface receptor internalization, transcription factor trafficking, organelle and cytoskeletons dynamics during cell division, and protein kinase translocation. By selective use of GFP color variants, multicolor assays can be designed for screening, lead optimization and target validation to monitor the localization of several different targets in same cell. Furthermore, because of potential of detection of GFP fluorescence in real time, such localization assays can provide dynamic information on both the spatial and temporal distribution of GFP fusion biosensors.

3.8. High-content Screening (HCS)

The most advanced system currently available for the detection of GFP fusion biosensors is the ArrayScan™ system from Cellomics. This fluorescence-imaging platform, together with a collection of HCS assay systems, is suited to exploit fully the utility of GFP technology for drug discovery. Recently a high-content screen for drug-induced human glucocorticoid receptor (hGR) translocation using an hGR-GFP biosensor has been used in HeLa cells. These cells are transiently transfected with a plasmid coding for hGR-GFP chimeric protein. The cells were treated with lead compounds and the translocation of hGR-GFP into the nucleus is quantified over time or at a fixed time point (12). GFP has also been used for *in vitro* screening of compounds against mammalian cells.

3.9. Applications of GFP in Different Organisms

Use of the GFP is a powerful tool for *in situ* monitoring of gene expression because GFP expression does not require any substrate addition. Studies of specific gene expression in prokaryotes and eukaryotes have been greatly facilitated by the use of GFP. The *GFP* gene have been transferred to expressed in a wide range of microbes from broad variety of bacteria (13, 14) to yeast (15). Wild-type GFP or its mutants have been used for various studies in mice (16), filamentous fungus (17), and plant cells (18).

4. GFP Mutants: Construction and Applications

Wild-type GFP has many drawbacks like low fluorescence intensity, multiple absorption and emission maxima, four-hour lag-time between protein expression and full fluorescence. These problems were addressed by the development of viable GFP mutants. While majority of point mutations in wt-GFP lead to non-fluorescent protein, a handful of them have been found to significantly improve the properties of wt-GFP. This increased intensity and the wavelength variation greatly facilitates both single-wavelength and multiple-wavelength detection; the latter not only permits multiple GFP mutants to be observed simultaneously, which in turn should allow the simultaneous imaging of multiple cellular events, but also allows signal ratioing

for improved sensitivity. An additional advantage of several of these mutants is that they undergo oxidation to the fluorophore more rapidly than wt-GFP, which reduces the lag time between cellular expression and maximum fluorescence (Table 2). Recent advances relevant to the application of GFP in drug discovery are the development of mutant form of GFP with more stable and intense expression and more suitable cloning vector (19, 20, 21).

Table 2: Fluorescence properties of enhanced GFP mutants

Mutant	Mutations	Excitation max. (nm)	Emission max. (nm)	Quantum yield
Wt- GFP	-	395 (470)	509	0.77
EGFP	S65T	488	509	0.70
EBFP	Y66H, Y145F	380	440	0.20
EYFP	S65G, V68L, S72A, T203Y	513 (498)	527	0.63
ECFP	T66W	433 (453)	475 (501)	0.24

Abbreviations: EGFP = enhanced green fluorescent protein, EBFP = enhanced blue fluorescent protein, EYFP = enhanced yellow fluorescent protein, ECFP = enhanced cyan fluorescent protein.

4.1. Red-shifted Variants: A number of 'red-shifted' variants of wt-GFP are described by Kain *et al.* (12). They have introduced one or more amino acid substitutions in the chromophore region of the protein. Here, fluorescence excitation peak shifted towards the red, from 395 nm in wt-GFP to 488-490 nm. The emission spectra for such variants are largely unaffected and these mutants still produce green light with a wavelength maximum of 507-511 nm. The two most commonly used red-shifted GFP mutants are S65T, which contain a Ser 65 to Thr substitution in the chromophore, and enhanced GFP (EGFP).

4.2. Blue, Cyan and Yellow Fluorescent Proteins: These proteins produce fluorescence signal that are blue, yellow-green and cyan. These provide good signal intensity in mammalian cells by virtue of elevated molar extinction coefficients and improved expression levels by virtue of the same humanized backbone as that found in EGFP. The combinations of these spectral variants open up a wide range of applications, such as the simultaneous analysis of multiple gene expression cascades and intracellular localization of different proteins e.g. dual-color detection of ECFP and EYFP in the same living cell when these reporter proteins are targeted to the mitochondria and nucleus, respectively.

5. Conclusion and Future Prospects

GFP can visualize particular cell types in whole animals, organs, tissues, and cell cultures. This possibility is particularly important in such fields as immunology, neurobiology, development, carcinogenesis etc. The greatest challenge to the broad application of GFP

technology in drug discovery is that of sensitivity. For cell-based fluorescence assay, the sensitivity provided by GFP sensors is largely dependent on the cellular concentration of GFP and the nature of the detection platform. As the power and versatility of GFP-based assays for drug discovery continue to be appreciated, instrument providers also have to follow with concerted efforts to develop compatible detection platforms. But answers could also lie in the discovery of additional GFPs in bioluminescent species other than *A. victoria*. With the advent of GFP mutants such as destabilized EGFP and the different color variants, continued development of fluorescence instruments for both cellular imaging and microwell detection, and the exciting discovery of new GFPs from other species, the future certainly seems bright for the use of GFP technology in the cellular, molecular and drug discovery studies. GFPs can be applied in a large variety of studies related to various aspects of living systems, and the range of these applications is continuously expanding.

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References

- [1] Deo S.K., Daunert S. (2001). Luminescent proteins from *Aequorea victoria*: applications in drug discovery and in high throughput analysis. *Fresenius J Anal Chem.*; 369(3-4): p. 258-66.
- [2] Yang, F., Moss, L. G. & Phillips, Jr. G. N. (1996). The molecular structure of green fluorescent protein. *Nature Biotechnology* 14, p. 1246-1251.
- [3] Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. & Remington, S. J. (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* 273, p. 1392-1395.
- [4] Hudakov DM, Matz MV, Lukyanov S, Lukyanov KA. Fluorescent Proteins and Their Applications in Imaging Living Cells and Tissues. *Physiol Rev* 90: , 2010, p. 1103–1163.
- [5] Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prashar, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263, p. 802-805.
- [6] Muldoon, R. R., Levy, J. P., Kain, S. R., Kitts, P. A. & Link, C. J. (1997). Tracking and quantitation of retroviral-mediated transfer using a completely humanized, red-shifted green fluorescent protein gene. *Biotechniques* 22, p. 162-167.
- [7] Siegel, M.S. and Isacoff, E.Y. (1997). A genetically encoded optical probe of membrane voltage.

- [8] Sekar, R.B and Periasamy, A. (2003). Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J. Biol. Chem.*, 160, (5), p. 629-633
- [9] Truong, K., A. Sawano, H. Mizuno, H. Hama, K. Tong, T.K. Mal, A. Miyawaki, and Ikura., M. (2001). FRET-based in vivo Ca^{2+} imaging by a new calmodulin-GFP fusion molecule. *Nat. Struct. Biol.* 8: p. 1069–1073.
- [10] Collins L. A., Franzblau S. G. (1997). Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother.* 41:1004–1009.
- [11] Sandman KE, Marla SS, Zlokarnik G, Lippard SJ (1999). Rapid fluorescence-based reporter-gene assays to evaluate the cytotoxicity and antitumor drug potential of platinum complexes. *Chem Biol.* 6: p. 541–551.
- [12] Kain, S.R. (1999). Green fluorescent protein (GFP): applications in cell-based assays for drug discovery. *Drug Discovery Today* 4, p. 304-312.
- [13] Chalfie M, Tu Y, Euskirchen G, Ward W W, Prasher D C. Green fluorescent protein as a marker for gene expression. *Science*. 1994;263: p. 802–805.
- [14] Christensen B B, Sternberg C, Molin S. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequorea victoria* as a marker. *Gene*.1996;173: p. 59–65.
- [15] Lanzer M, Bujard H. Promoters largely determine the efficiency of repressor action. *Proc Natl Acad Sci USA*. 1988, 85, p. 8973–8977.
- [16] Chiocchetti, A., Tolasano, E., Hirsch, E., Silengo, L. & Altruda, F. (1997). Green fluorescent protein as a reporter of gene expression in transgenic mice. *Biochem Biophys Acta* 1352, p. 193-202.
- [17] Fernandez-Abalos, J. M., Fox, H., Pitt, C., Wells, B. & Doonan, J. H. (1998). Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. *Molecular Microbiology* 27, p. 121-130.
- [18] Leffel, S. M., Mabon, S. A. & Stewart, C. N. (1997). Applications of green fluorescent protein in plants. *Biotechniques* 23, p. 912-916.
- [19] Cormack BP, Valdivia RH, Falkow S.1996. FACS optimized mutants of the green fluorescent protein (GFP). *Gene* 173, p. 33–38.
- [20] Collins, L.A., Torrero, M. N. & Franzblau, S. G. (1998). Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 42, p. 344-347.
- [21] Stauber, R. H., Horie, K., Carney, P., Hudson, E.A., Tarasova, N. I., Gaitanaris, G.A. & Pavlakis, G.N. (1998). Development and applications of enhanced green fluorescent protein mutants. *BioTechniques* 24, p. 462-471.