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MINI-REVIEW

Use of Green Fluorescent Protein (GFP) and Its Homologs for *in vivo* Protein Motility Studies

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Abstract—Green fluorescent protein (GFP) and its homologs are widely used as fluorescent markers of gene expression and for determination of protein localization and motility in living cells. In particular, based on GFP and GFP-like proteins a number of techniques have been developed that can be used either to estimate protein mobility in living cells, or to introduce a distinctive fluorescent signal in order to track the movement of labeled molecules directly. Considerable progress in the development of such technologies in the last two or three years motivates us to reevaluate the present scope of biotechnological instruments in studies of protein movement in cells.

Key words: green fluorescent protein, GFP homologs, photoactivation, photoconversion, mobility of molecules

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was cloned in 1992 [1]. The structure of the protein was later solved by X-ray analysis [2, 3]. It was shown that GFP has the structure of a β-barrel with 11 antiparallel strands, and the chromophore is formed inside the barrel by the cyclization of the protein backbone. The environment of the chromophore in the GFP molecule determines its effective absorption in the violet (396 nm) and blue (476 nm) parts of the spectrum and emission of lower energy green photons. The quantum yield of the system is very high, about 0.8.

Soon after cloning, GFP became used as a fluorescent marker of gene expression and of protein localization and motility in living cells. It turned out that virtually any protein could be tagged with GFP by placing its sequence within the same reading frame. The obtained chimera usually retains the studied protein localization and function when expressed in cells. The possibilities opened for studies of intracellular processes made GFP (and later homologous proteins) the favorite fluorescent marker for experiments *in vivo*.

As the popularity grew (by the year 2000 the number of articles where GFP was applied exceeded 6000), technologies for applications of fluorescent proteins developed and improved. The pallet of the fluorescent color labels expanded. Mutant GFP variants were obtained—blue (BFP), cyan (CFP), and yellow (YFP)—with fluorescence emission maxima at 445, 477, and 527 nm, respectively [4]. Since 1999 a great number of GFP-like fluorescent proteins have been cloned from corals, with the emission spectra maxima ranging from 484 to 611 nm [5-7]. Furthermore, homologous nonfluorescent coral chromoproteins were cloned. Based on these mutants fluorescent forms were obtained with emission maxima reaching the far red part of the spectrum (up to 645 nm) [8]. Today fluorescent protein diversity allows the concurrent labeling and localization of several objects in a living cell, and also the extensive use of FRET (Fluorescence Resonance Energy Transfer) microscopy to determine physical interactions of proteins.

On the basis of GFP-like proteins sensor molecules have been developed capable of changing fluorescence intensity and spectra in response to the changes in the environment—Ca^{2+} concentrations [9-11], pH [12-14], membrane potential [15], etc.

Besides, based on GFP and homologous proteins a range of techniques has been developed allowing either to estimate the motility of molecules in a living cell or to introduce a fluorescent signal optically and to track the movement of labeled molecules directly.

In this review, we will trace the evolution of technologies that use GFP and GFP-like proteins as fluorescent labels in studies of the movement of proteins and organelles in living cells.

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FLUORESCENCE PHOTOBLEACHING TECHNIQUE

This technique is used for studies of fluorophore dynamics [16, 17]. It is based on the photobleaching of the fluorescent label within the region of interest by intense irradiation with light. Then exchange of fluorophores between the bleached and unbleached areas is monitored.

GFP turned out to be a very suitable tag for the experiments that use fluorescence photobleaching technique. It produces bright, stable fluorescence and does not fade at low illumination of the excitation light. With high illumination level, the GFP fluorophore can be photobleached, and the light intensity used for photobleaching does not noticeably perturb living cells [18]. GFP is much more stable than the known small fluorescent dyes, enabling long-term observations and repeated experiments with the same cell.

Finally, as mentioned above, the cell itself synthesizes GFP, constituting a whole with the studied protein. Until recently, precise labeling of a certain protein *in vivo* was almost impossible for techniques using low molecular weight fluorescent dyes (this situation improved with the work of Gaietta et al. [19], see below). This consideration is especially important, and it is also valid for other approaches where GFP and homologs are used for protein localization and dynamics studies in living cells.

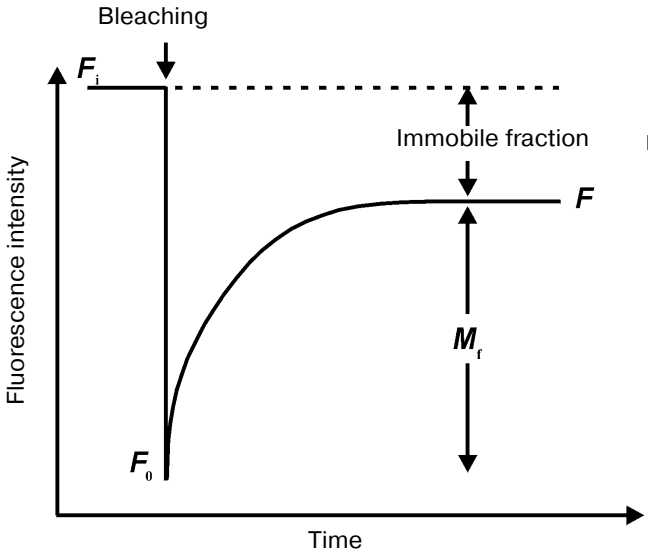

Fig. 1. FRAP technique. Plot of the fluorescence intensity in a region of interest versus time after bleaching a fluorophore. Comparison of signal intensity before photobleaching (F_i) and the asymptote of the recovery (F) can be used to estimate the ratio between mobile (M_f) and immobile fractions.

Interpretation of D and M_f (from Lippincott-Schwartz et al., 2001)

Deviations from predicted *D*
Increase in D
Non-diffusive behavior such as flow-directed movement by motor proteins
Decrease in viscosity of the environment
Decrease in D
Formation of large aggregates or complexes (10-100-fold increase in molecular weight)
Increase in viscosity of the environment
Transient interaction with large or fixed molecules
Changes in M_f (immobile protein fraction)
M_f is 100%
Protein is not restricted in ability to diffuse freely
Increase in M_f
Protein is released from a restricted compartment
Protein is released from a fixed macromolecular complex
Decrease in M_f
Protein binds to fixed molecules or forms aggregates that are restricted in movement
Protein is confined to a compartment that cannot contribute to fluorescence recovery

Here we will describe two main variations of photobleaching technique—FRAP and FLIP.

FRAP. The FRAP (Fluorescence Recovery After Photobleaching) technique is based on monitoring of fluorescence recovery in the bleached zone. A region of a living cell is irradiated by intense light, making GFP nonfluorescent. Time and degree of fluorescence recovery within the bleached region gives information about diffusion rate and mobile protein fraction (Fig. 1).

The information obtained from the fluorescence recovery curve (from F_0 to F) can be used to determine diffusion constant (D) of the fluorescent protein (see the table).

FLIP. The FLIP (Fluorescence Loss In Photobleaching) technique is based on the fluorescence loss outside the bleached region. A selected region in a living cell is irradiated repeatedly or over a long period of time. The rate and degree of fluorescence loss in other parts of the cell gives information about fluorophore exchange rate between bleached and unbleached areas.

This type of photobleaching technique can be used to reveal continuities and discontinuities in intracellular organelles and compartments. FLIP can also be used to measure kinetics of protein binding and release in living cells.

LIMITATIONS OF PHOTOBLEACHING TECHNIQUES

Fluorophore photobleaching within the region of interest can be used to trace either the rate of filling of this region by the fluorophores from the other regions (FRAP) or the exchange rate between irradiated region and non-irradiated parts of the cell (FLIP). These technologies can be used to estimate chromophore motility, but they do not enable direct tracking of the movement of a chosen object (protein group, organelle, living cell) using its fluorescent signal.

For such tracking, it was necessary to develop a photoactivated fluorescent label instead of photobleaching. This problem has recently received intense interest, resulting in the development of a diversity of techniques using GFP-like proteins capable of photoactivated fluorescent signal formation. For the most part, these activated labels are initially fluorescent. Particular wavelengths and intensity of light irradiation causes more or less significant changes of their excitation/emission fluorescence parameters. The uniqueness of these parameters for the irradiated label fraction allows direct tracking of its movement in time and space.

Below we will describe the development of such photoactivated labels.

FLAP TECHNIQUE

FLAP (Fluorescence Localization After Photobleaching) [20] is an improved version of the photobleaching technique. Two fluorophores are used concurrently for fluorescent labeling. One of them is photobleached, and the other is used as a fluorescent control. FLAP signal is calculated as the difference between the intensity of the two signals. In comparison with FRAP and FLIP technologies, the use of an additional non-bleached fluorophore can be used to track molecular redistribution more accurately—taking into account both "dark" redistribution and positive fluorescent signal of the additional fluorophore.

However, similar to the FRAP and FLIP techniques, the photobleaching cannot be used to form new fluorescent signal directly, but only to calculate FLAP signal. Its disadvantage is that two fluorescent colors are required, which impedes the concurrent use of several fluorescent labels.

DsRed "GREENING" TECHNIQUE

A GFP-like red fluorescent protein DsRed was cloned from the *Discosoma* coral in 1999 [5]. Today it is used widely in biotechnology. This protein is tetrameric, and it has been shown that a portion of the four monomers (about 50%) is green fluorescent [21, 22]. However, the greater part of the green photon energy passes to the neighboring red fluorescent monomers due to effective FRET (Fluorescence Resonance Energy Transfer). As a result, red fluorescence dominates in the DsRed tetramer emission spectrum.

In 2001 it was discovered that the "red" monomers of tetrameric DsRed could be selectively bleached by three-photon infrared laser irradiation [23]. Therefore, the green chromophores lose their acceptor, and the green fluorescence quantum yield grows up to 30-fold. This technique, named "DsRed greening", can be used for precise green fluorescent photolabeling, to label cells, organelles, and proteins *in vivo*. Its disadvantages are the tetrameric protein nature, two fluorescent colors engaged, and three-photon laser needed for photobleaching.

KAEDE PROTEIN

In 2002 a fluorescent protein from the stony coral *Trachyphyllia geoffroyi* named "Kaede" was cloned [24]. Initially this protein is green fluorescent. If left in sunlight, Kaede transforms into a bright red fluorescent protein. Further studies showed that this transformation is caused by ultraviolet light irradiation. The resulting contrast before and after such photoactivation (considering both green peak decrease and red peak growth) reaches more than 2000-fold.

Disadvantages of this protein as a photoactivated fluorescent label are its tetrameric nature, two fluorescent colors needed, and UV irradiation required for the photoactivation.

KINDLING FLUORESCENT PROTEINS

Based on several coral chromoproteins, in our lab we developed a group of photoactivated fluorescent proteins—KFPs (Kindling Fluorescent Proteins) [25, 26]. Initially nonfluorescent, these proteins become brightly fluorescent for several minutes ("kindle" reversibly) in response to irradiation with intense light of a particular wavelength. More intense irradiation causes irreversible kindling of most KFPs. Kindled KFPs are fluorescent in the red part of spectrum (fluorescence excitation about 580 nm, emission from 600 to 630 nm for different KFPs). Contrast between the kindled and initial KFP reaches 50-100-fold for the best variants (Fig. 2).

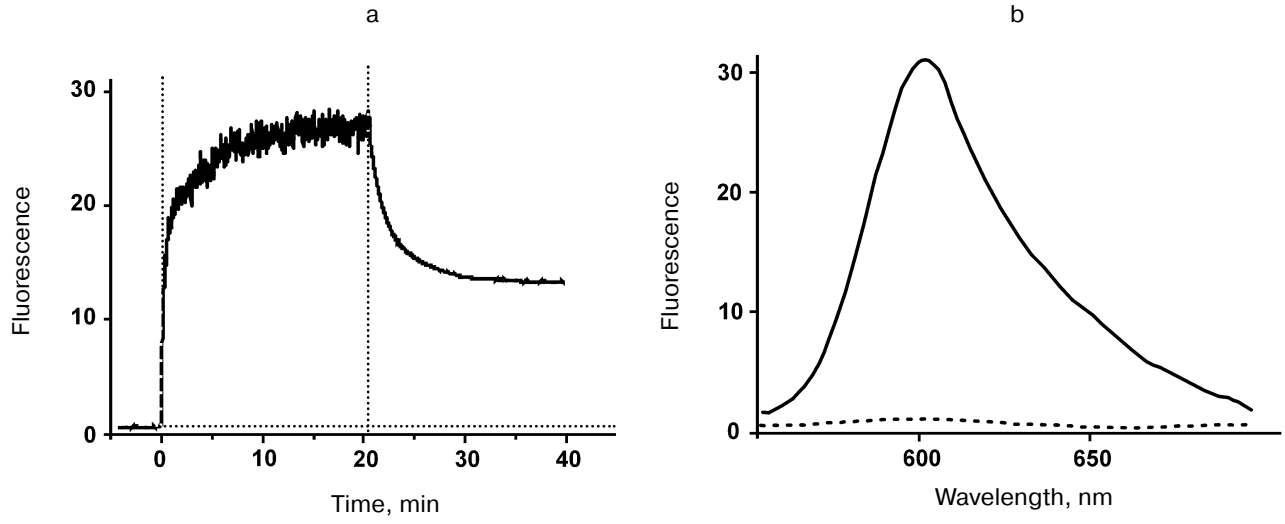

Fig. 2. Irreversible photoactivation of KFP gives a bright fluorescent signal. a) Kindling and partial relaxation of the kindled KFP. Remaining signal can be tracked as irreversibly photoactivated fluorescent label. b) Fluorescence spectra of the irreversibly kindled (solid line) and initial (dotted line) KFP.

The main disadvantage of KFPs is the tetrameric nature of these proteins, which hampers their use as a label for cellular protein tracking (see below). However, as experiments showed, KFP can be successfully used for photolabeling and tracking of cell organelles and living cells. Red light penetrates living tissues better and it is preferable as a fluorescent signal in studies of cell migration processes, such as morphogenesis, metastasis, and inflammation.

The capability for reversible fluorescence kindling is the unique property of KFPs, which can be very useful in a number of applications.

FLUORESCENT LABEL MINIMIZATION

The tetrameric nature of the overwhelming majority of GFP-like coral proteins cloned is their main and serious drawback. In many cases, attempts to use tetrameric proteins for cellular protein labeling resulted in aggregation of the chimera and entire disruption the function and localization of the studied protein. In that case, the main problem is not even fourfold size (comparing to GFP) of the label, but the development of a tangled web of interacting proteins, leading to the formation of large complexes and aggregation. Today this important problem finds its solutions [27-30].

Anyway, it is evident that the fluorescent label used for protein studies *in vivo* should be as small as possible so that the function of the studied protein will be least disturbed. Until recently, small chemical fluorescent dyes could not be selectively attached to the chosen proteins expressed in living cells. Only in 2002, a technique was developed that made such labeling possible by selective binding of the fluorescent dye with a small linker of several amino acids [19]. However, today photoactivated fluorescent label cannot be developed using this approach.

Attempts to decrease GFP molecule size without considerable loss in fluorescent brightness have not been successful, and today the best fluorescent label based on a GFP-like protein is the monomeric GFP-like protein. Thus, the best photoactivated fluorescent label that can be developed based on a GFP-like protein should be capable of direct activation of fluorescent signal within the monomer.

ACTIVATION OF RED FLUORESCENCE IN GFP

In 1997 it was discovered that blue light (488 nm) irradiation of GFP under anaerobic conditions makes it red fluorescent [31, 32]. This technique allowed red fluorescent signal activation and direct tracking of cell protein diffusion *in vivo*. This method has been successfully applied [33], but the necessity for anaerobic conditions restricts the use of this technique.

PHOTOACTIVATION OF WILD TYPE GFP

The GFP fluorescence excitation spectrum has two peaks (with maxima at 396 and 476 nm) that correspond to two close emission peaks (with maxima at 503 and 508 nm). The two excitation peaks, whose ratio is six to

one, correspond to protonated (neutral) and anionic chromophore forms [34]. In 1996 it was shown [35] that UV irradiation (365 nm) causes reproportioning of excitation peaks in favor of the 476 nm peak.

Hence, it turned out that GFP could be used as a photoactivated fluorescent label. Unfortunately, the resulting contrast between the irradiated and initial GFP populations (excited at 476 nm) reached only threefold at best. This contrast is insufficient for most experiments, and this technique has not been widely used.

However, in 2002 in the laboratory of Lippincott-Schwartz [36] a mutant GFP was obtained which contains mostly the protonated chromophore (excitation at 396 nm). Intense blue light irradiation (413 nm) causes effective chromophore conversion to the anionic form (excited at 496 nm). For this mutant, named PA-GFP (PhotoActivated-GFP), the resulting contrast between fluorescence (excited at 496 nm) before and after irradiation reaches more than 100-fold.

Thus, irradiation parameters required (blue light instead of UV) and the resulting contrast were improved considerably for this new mutant protein compared to the wild-type GFP. It is very important also that PA-GFP inherits monomeric GFP nature, and therefore can be used widely for photolabeling proteins in living cells.

To date, PA-GFP is one of the best photoactivated fluorescent markers for studies of protein motility in cells. It can be also used widely for the photolabeling of organelles, single cells, and groups of cells. Today, the development of a monomeric photoactivated red fluorescent marker remains a relevant problem. Such a marker could be used jointly with PA-GFP for the concurrent tracking of two different protein groups *in vivo*, and also for local FRET. The mentioned KFP monomerization may provide the solution for this problem.

In the past few years a great number of the GFP-like proteins have been cloned, and today in different laboratories of the World their novel properties are being developed both purposefully and casually. Owing to some of the recently developed techniques, today the arsenal of biotechnology is replenished with novel powerful tools which can be used to introduce a fluorescent signal precisely in a living cell or its part, and then track the movement of the photolabeled proteins, organelles, and cells. Research activity in the field makes it likely that in the next few years these novel tools will be perfected or at least improved, allowing advances both in studies of intracellular processes and cell migration processes in living tissues.

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