Review TRENDS in Biotechnology Vol.23 No.12 December 2005

Fluorescent proteins as a toolkit for *in vivo* imaging

Dmitriy M. Chudakov, Sergey Lukyanov and Konstantin A. Lukyanov

Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, Moscow 117997, Russia

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and its mutant variants, are the only fully genetically encoded fluorescent probes available and they have proved to be excellent tools for labeling living specimens. Since 1999, numerous GFP homologues have been discovered in Anthozoa, Hydrozoa and Copepoda species, demonstrating the broad evolutionary and spectral diversity of this protein family. Mutagenic studies gave rise to diversified and optimized variants of fluorescent proteins, which have never been encountered in nature. This article gives an overview of the GFP-like proteins developed to date and their most common applications to study living specimens using fluorescence microscopy.

Introduction

Today, fluorescent labeling is of paramount importance to biological studies and a great number of chemical dyes are used extensively to label biological specimens; however, these dyes must be added exogenously, which can be incompatible with living systems. For such applications, genetically encoded fluorophores – members of the GFP family – are more suitable. These fluorescent proteins (FPs) are comprised of β-barrels of ~25 kDa and require no external cofactors (except oxygen) to form the chromophore within the protein globule. Thus, standard genetic-engineering techniques make it possible to label proteins, subcellular compartments, cells of interest and specific tissue-regions using the protein expression system of the cell.

GFP was discovered more than 40 years ago [1,2], but it was not until 1994, after cloning [3] and successful heterologous expression of the *gfp* gene (GenBank accession no. U17997) [4], that this protein attracted attention. GFP and its mutants soon became popular tools for cell and molecular biology and, during the past few years, the great spectral and phylogenetic diversity of GFP-like proteins has been characterized in marine organisms; furthermore, several useful mutant variants of FPs have been generated. Consequently, a panel of fluorescent proteins is now available that covers almost the whole visible spectrum, each possessing different biochemical characteristics. The development of various sophisticated FPs, such as photoactivatable FPs [5–12], Timer [13–16], a series of fluorescent sensors [17–20] and split GFPs [21,22] has opened up novel applications for *in vivo* fluorescent labeling, such as: studies of protein-expression; -interaction; -activity; -movement; and -turnover; direct measurement of cell parameters and state; organelle function; and cell motility studies.

Corresponding author: Lukyanov, K.A. (kluk@ibch.ru).

Evolutionary diversity

Four decades ago, GFP was discovered in the hydroid jellyfish *Aequorea victoria* [1,2] where it acts as a secondary emitter in a bioluminescent system based on the Ca^{2+}-dependent photoprotein aequorin [23]. Although some other bioluminescent Cnidaria contain GFPs [24], bioluminescence and GFP-based coloration are generally independent phenomena: not only do the overwhelming majority of bioluminescent organisms lack GFPs, but also most animals expressing GFP homologues are non-bioluminescent.

Until recently, GFP-like proteins were identified in only two classes of Cnidaria: Hydrozoa (hydroid polyps and medusae) and Anthozoa (scleractinian corals, sea anemones, sea pens). These use GFP-like proteins extensively for fluorescent and non-fluorescent body coloration, and in some cases, in their bioluminescent systems (Figure 1). Recently, we have reported several GFPs derived from evolutionary-distant marine organisms of the Pontellidae species. (Arthropoda: Crustacea: Maxillopoda: Copepoda: Pontellidae) [25]; paradoxically, although many copepods are bioluminescent, those that contain GFPs are not. Visual mate-recognition can be important in Pontellidae, which typically show sexual dimorphism in their eye design [26,27]; therefore, pronounced differences in fluorescence localization between the species indicate that Pontellidae GFPs might have a role in the recognition of potential mates.

The phylogenetic distribution of the GFP family is unusual because Cnidaria and Arthropoda are very distant groups in evolutionary terms. Excluding direct horizontal-gene-transfer from jellies or corals to copepods, it can only be concluded that GFP-like proteins evolved before the separation of Bilateria and Cnidaria and thus almost every animal taxon can potentially contain GFP homologs. Remarkably, a close structural homolog of GFP, a protein-binding domain of nidogen [28] and related Bilateria-derived proteins, probably belong to the same gene superfamily as GFP. The whole evolution of this putative superfamily requires further investigation.

Color diversity

The natural diversity of the spectral properties of GFP-like proteins was first discovered in non-bioluminescent

 0167-7799/$ - see front matter doi:10.1016/j.tibtech.2005.10.005

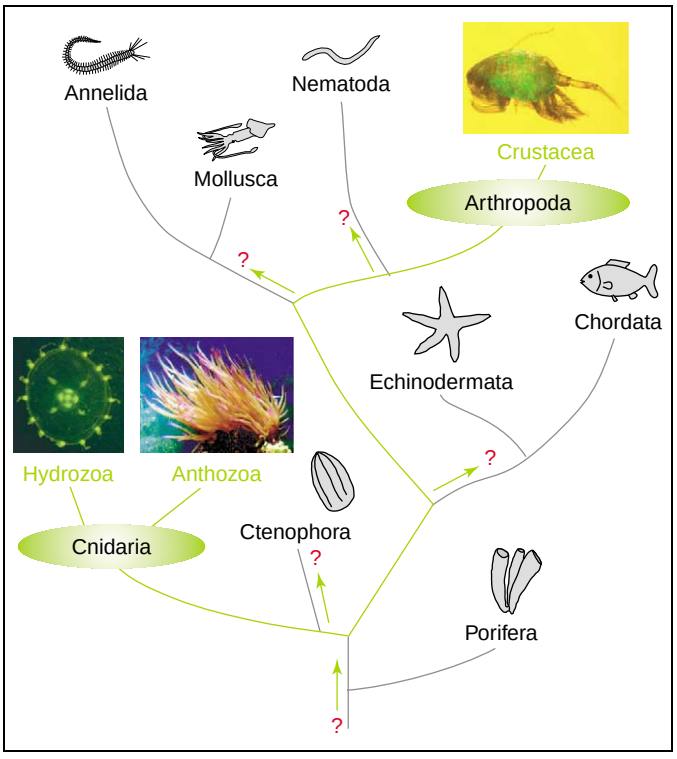

Figure 1. Positional relationship of the fluorescent protein-producing organisms on the phylogenetic tree. The phyla Cnidaria and Arthropoda (where GFP genes were found) and the branches connecting these phyla are highlighted in green. Photos show organisms representative of each phylum expressing GFP-like proteins: jellyfish *Phialidium* showing yellow fluorescence; sea anemone *Anemonia sulcata* with purple tentacle tips; and a copepod displaying green fluorescence. Question marks indicate possible, but unexplored, pathways of the evolution of FPs.

Anthozoa species [29,30]. Five main color classes have been identified: cyan, green, yellow and orange-red, in addition to non-fluorescent purple-blue chromoproteins. Recently, a yellow FP and a purple chromoprotein were also cloned from Hydrozoa jellyfishes [25] revealing a similar spectral diversity to that of Anthozoa GFP-like proteins. In Copepoda, only green FPs have been found to date [25]. Together with engineered mutant variants, FPs are now available for the entire visible spectrum: from ~450 to 650 nm (Figure 2a).

In parallel with the development of new FPs from different sources, considerable progress has been made in the improvement of *Aequorea victoria* GFP cyan- and yellow-shifted mutants (CFP and YFP, respectively) and faster maturating, less chloride- and pH-sensitive yellow mutant variants, Venus and Citrine, have been described [31,32]. A bright cyan-fluorescent-protein named Cerulean was also developed [33].

Furthermore, improvements are constantly being made across the spectrum of existing FPs. In the orange–red part of the visible spectrum, the palette has been recently expanded by the inclusion of the true yellow phiYFP [25], orange mKO1 [34] and a whole series of monomeric mutant variants of DsRed [35]. A novel green-FP – the non-aggregating mutant of fast maturating Copepoda GFP – named TurboGFP (Evrogen, www.evrogen.com), and the monomeric mutant mAG1 (Azami Green) [36] of *Galaxeidae* coral GFP have also been produced recently.

The blue fluorescent variants of *Aequorea victoria* GFP, reported to date, are characterized with low brightness and low photostability [37]. Alternatively, a photoswitchable protein, PS-CFP2, can be used for labeling in the blue

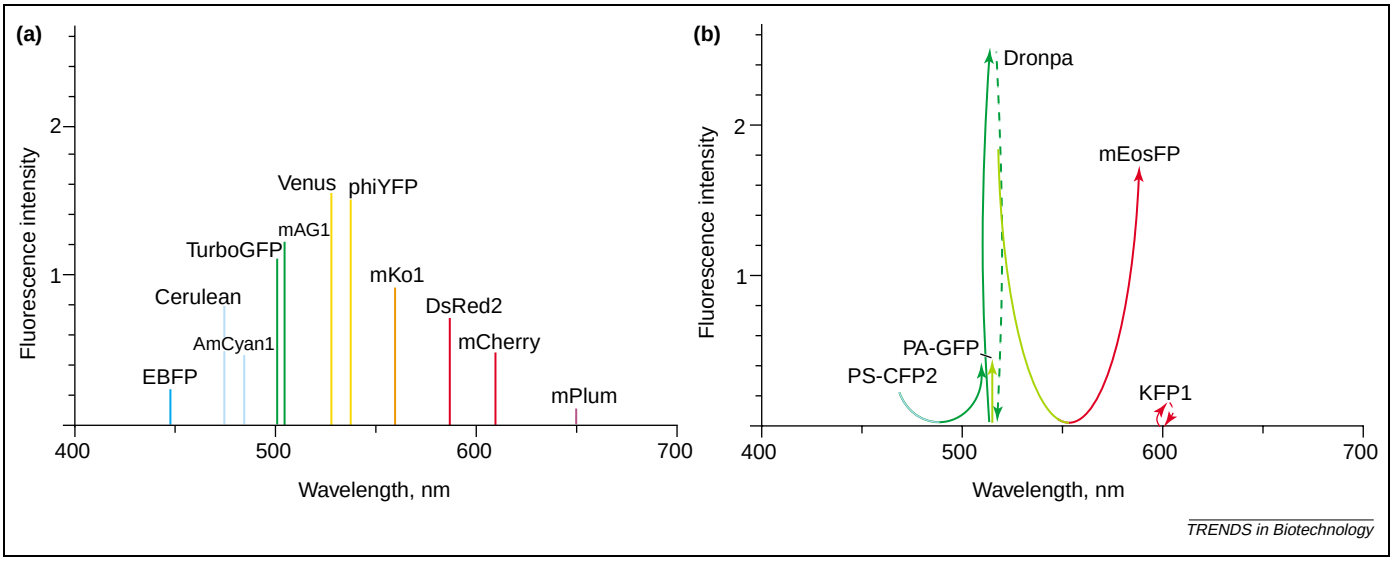

Figure 2. Spectral diversity of fluorescent proteins. **(a)** Emission maxima (x-axis) and relative brightness (y-axis) of some fluorescent proteins, shown as vertical lines. **(b)** Emission changes in photoactivatable fluorescent proteins. The start- and end-points of the arrow indicate the relative brightness and emission maximum of the corresponding protein before and after photoactivation. For both panels, fluorescence brightness values were estimated as a product of extinction coefficient and quantum yield taken from original publications for each protein and normalized per EGFP brightness (extinction coefficient 55 000 $M^{-1}cm^{-1}$, quantum yield 0.6 [37]). EBFP – enhanced blue fluorescent mutant of *A. victoria* GFP [37]; Cerulean – improved ECFP [33]; AmCyan1 – enhanced *Anemonia majano* cyan fluorescent protein (Clontech); TurboGFP – non-aggregating mutant of copepod green fluorescent protein (Evrogen); mAG1 – Azami Green, monomeric mutant of *Galaxeidae* green fluorescent protein [36] (MBL International); Venus – improved EYFP [32]; phiYFP – enhanced variant of *Phialidium* yellow fluorescent protein (Evrogen); mKO1 – Kusabira Orange, monomeric mutant of *Fungia concinna* orange fluorescent protein [34] (MBL International); DsRed2 – nonaggregating mutant of *Discosoma* red fluorescent protein [97] (Clontech); mCherry [35] and mPlum [40] – monomeric mutants of *Discosoma* red fluorescent protein. PS-CFP2 – monomeric photoswitchable cyan fluorescent protein (Evrogen); Dronpa – monomeric reversibly photoactivatable green fluorescent protein [47] (MBL International); PA-GFP – monomeric photoactivatable fluorescent protein [5]; mEosFP – monomeric mutant of EosFP; *Lobophyllia hemprichii* green-to-red photoconvertible protein [11]; KFP1 – kindling fluorescent protein [7].

part of the visible spectrum (Figure 2b). Photoactivation of this cyan FP demands very intense (400 nm) light irradiation; therefore, at conventional light intensities that cause zero or negligible photoactivation, it can be used as a routine violet-light-excited blue-cyan fluorophore.

Great efforts are being made to find or create FPs as far-red-shifted as possible. The provision of these will expand the palette of fluorescent proteins available and improve efficiency as light-scattering intensity drops off as the wavelength increases. Furthermore, these will provide a spectral window favorable for tissue light-penetration (~650–1100 nm): determined by the efficiency of photon absorption, with shorter wavelengths absorbed by blood hemoglobin, and longer wavelengths by water. This will make them suitable for whole-body mapping and other medical applications. A natural FP emitting at 611 nm was cloned from *Entacmea quadricolor* [38] and a more far-red fluorescent mutant, HcRed (emission maximum: 645 nm), was developed from a non-fluorescent red-absorbing chromoprotein [39]. Recently, a monomeric far-red FP named mPlum that fluoresces at 649 nm was created on the basis of the natural red FP DsRed [40]. Despite these advances, there are still no FPs that cover the 650–700 nm wavelengths.

The spectral diversity of FPs allows easy visualization of up to 4–5 colors simultaneously, for example, a combination of Cerulean (excitation/emission maxima: 433/475 nm), Venus (515/528 nm), mStrawberry [35] (574/596 nm) and mPlum (590/649 nm) is possible. Alternatively, a combination of PS-CFP2 (excitation/emission maxima: 400/468 nm), one of the green FPs (~490/510 nm), phiYFP (525/537 nm) or mKO1 (548/559 nm), mStrawberry (574/596 nm) and mPlum (590/649 nm) can also be applied.

Applications

FPs are widely used as noninvasive probes to study different biological models – from individual cells to whole organisms. The use of FPs enable the tracking of every step of the protein of interest: expression, localization, movement, interaction and activity in the cell, tissue or organism. The main applications of FPs are: visualization of target-gene promoter up- and down-regulation, protein labeling, detection of protein–protein interactions, tracking protein movement and monitoring cellular parameters using FP-based fluorescent sensors.

Monitoring of gene expression

Detection of promoter activity is perhaps the simplest of FP applications. The gene encoding a FP is cloned under the control of the target promoter, whereby activity of the promoter can be monitored by the magnitude of the fluorescent signal (Figure 3). While this approach has a reduced sensitivity compared with enzyme-based assays, it has certain advantages and a much wider range of applications when using specially designed FP variants.

The oligomeric state of an FP is important when applied to protein labeling but it does not have a negative effect on monitoring of promoter activity. Therefore, many new FPs are suitable for these applications, making it possible to detect the activity of several different promoters with up to 4–5 distinct fluorescent colors, simultaneously.

Moreover, FPs allow time-scale monitoring of promoter activity. The first approach is to use destabilized FPs (i.e. proteins with short turnover) [41] to obtain a fluorescent signal only during the period of promoter activity. Here, fast-maturating FPs are desirable to provide a minimal delay between the promoter activation and fluorescent signal appearance. The second approach is to use the so-called Timer FP, which is capable of a gradual change in fluorescence color over time – from blue to green, and then to red [13,14]; therefore Timer provides retrospective information about the length of time the promoter is active.

Recently, a novel technique to detect promoter activity has been developed using a so-called split FP. This is a FP expressed as two separate parts but capable of reconstituting to the whole functional protein when cloned under two promoters of interest; the fluorescent signal occurs only when both promoters are active. Moreover, by combining separate halves, each carrying point mutations responsible for spectral shifts, one can obtain information about the combinations of promoters of interest active in a system [22].

Protein labeling and FP oligomeric state

The most widely used FP application is probably protein tagging, achieved by cloning a FP in frame with the target protein at either its N- or C-terminus (Figure 3). Numerous experiments with GFP mutants have demonstrated that most fusion proteins created this way are fully functional; however, in each particular case, the researcher must determine whether the function of the FP-tagged protein remains natural.

The formation of oligomers is the Achilles' heel for the majority of the GFP-like proteins cloned to date, most of which are tetrameric. An oligomeric state becomes crucial when fusing GFP-like proteins to a protein of interest and, in most cases, attempts to use tetrameric FPs to label cellular proteins result in aggregation of the chimera and disturbance to the target protein function and localization. Indeed, it should be taken as a stroke of good fortune that the first cloned fluorescent protein, *Aequorea victoria* GFP, is essentially monomeric. Dimerization of this protein, or of its mutants, is very weak (negligible for most applications) and this can be eliminated by point mutations, as was shown for CFP and YFP [42].

Although several other solutions have been proposed to avoid this complication [43–46], the answer lies in developing monomeric FPs with the desired spectral characteristics. Consequently, several monomeric variants have been generated by extensive mutagenesis of naturally tetrameric FPs. As a result, monomeric orange, red and far-red FPs, as well as novel photoactivatable FPs, have been made available [11,34–36,47].

Photoactivatable fluorescent proteins

Over the past few years, considerable progress has been made in developing the so-called photoactivatable FPs. These proteins are capable of a many-fold increase in

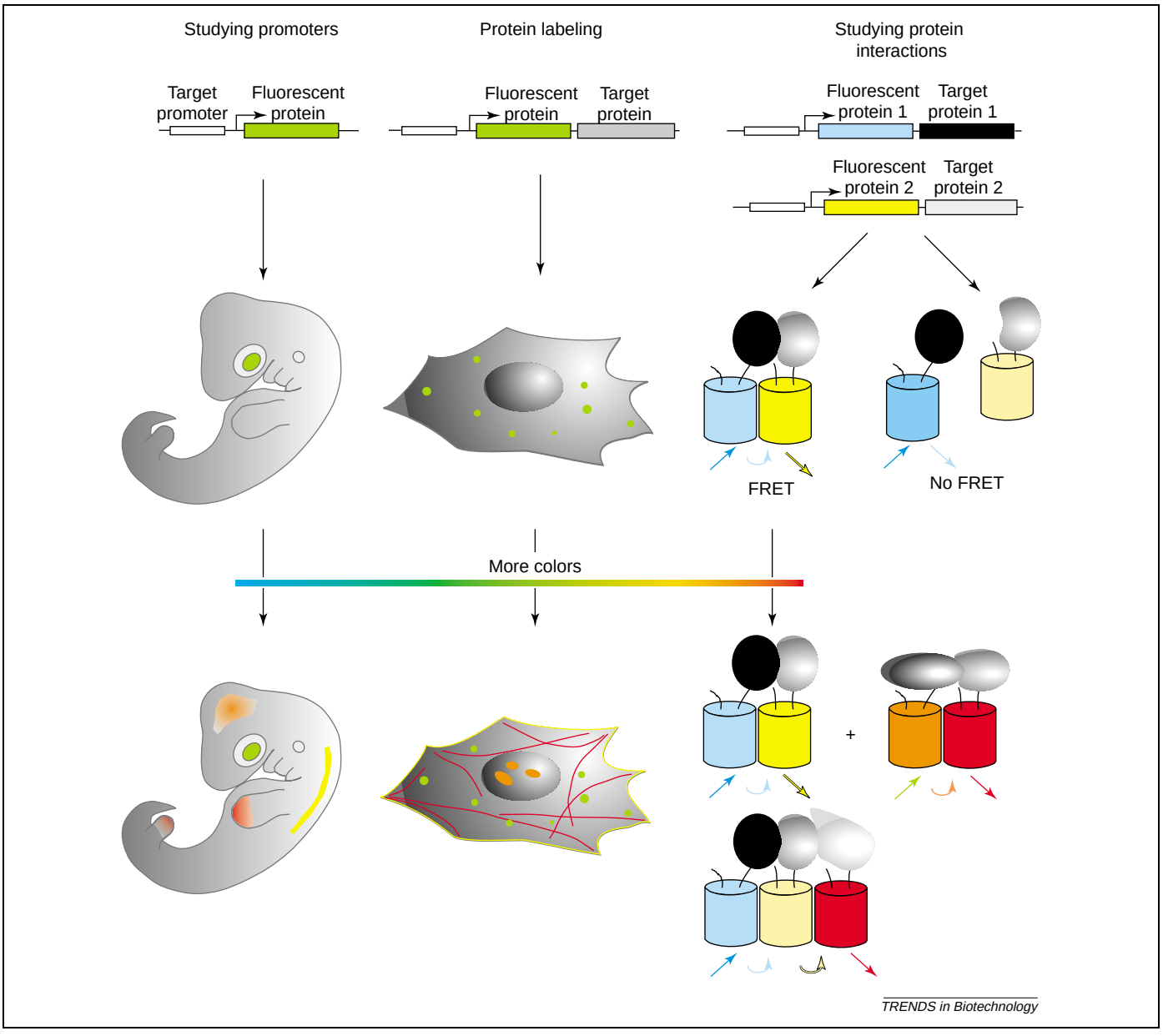

Figure 3. Applications of fluorescent proteins. Investigation of gene promoter activation (left), protein labeling (middle) and detection of protein–protein interactions using FRET (right) are shown schematically. DNA constructs for protein expression in living objects are shown in the upper part. Visualization of green fluorescent signal in an organism or cell is presented in the middle part. Multicolor labeling is shown at the bottom. To illustrate FRET-based detection of protein–protein interactions, fluorescent proteins and target proteins are shown as colored barrels and gray or black ovals, respectively.

fluorescence intensity at certain excitation/emission wavelengths, in response to irradiation with specific light. This property can be used to 'switch-on' a fluorescent signal, using a beam of focused light, and then track the movement of labeled cells, organelles or individual proteins. Until recently, photobleaching techniques [48], such as fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP), were the major tools to study protein mobility. Photoactivatable FPs provide a more precise, direct and less damaging way to study movement of proteins.

Several distinct types of photoactivation have been described (Figure 2b). The first photoactivatable protein reported, PA-GFP, is a mutant of *Aequorea victoria* GFP, capable of a 100-fold increase in green fluorescence at 517 nm (excitation maximum: 500 nm) in response to irradiation with UV to violet light (350–420 nm) [5]: the basis for this photoconversion is the transition of the protonated chromophore to a deprotonated form. The same mechanism probably underlies photoactivation of cyan fluorescent PS-CFP [10], a mutant of a colorless monomeric GFP-like protein cloned from *Aequorea coerulescens* [49]. Upon intense (400 nm) light irradiation, both the fluorescence excitation and emission of PS-CFP change, resulting in a conversion from a cyan (emission maximum: 468 nm) to a green (emission maximum: 511 nm) fluorescent form [10]. Optical contrast between the non-activated and activated states (i.e. change of the green-to-cyan fluorescence ratio) reaches 1500-fold for PS-CFP and 2000-fold for its improved version, PS-CFP2.

The development of another group of photoactivatable proteins was possible following the discovery of a natural

green-to-red photoconvertible protein named Kaede, obtained from the stony coral *Trachyphyllia* [6]. In the dark, this protein matures to a green-fluorescent state; then, following a brief irradiation of this green form with UV–violet light, it undergoes an irreversible transition to the red-fluorescent state. Optical contrast between the Kaede ground- and activated-states (i.e. change of the red-to-green fluorescence ratio) reaches more than 2000-fold. Whereas Kaede is a tetrameric protein, the recently described mEosFP is a Kaede-like monomeric mutant, making it suitable for protein photolabeling and tracking [11].

Reversible photoactivation is a characteristic of another group, derived from a non-fluorescent chromoprotein obtained from the sea anemone *Anemonia sulcata*, which is naturally capable of photoactivation [30]. A kindling fluorescent protein (KFP1), and other photoactivatable variants, have been developed subsequently [7,8]. In response to irradiation with green light, KFP1 red fluorescence (emission maximum: 600 nm) increases 70-fold. Upon more intense green-light irradiation, KFP1 undergoes irreversible photoconversion, making it the only known protein that can be photoactivated either reversibly or irreversibly.

While KFP1 and similar reported proteins are tetrameric, a reversibly photoactivatable monomeric protein, named Dronpa, was developed recently [47]. Dronpa is a bright green FP that can be quenched by irradiation with intense blue-light and converted back to the fluorescent state with a pulse of UV–violet light. In a similar way to that of *Anemonia sulcata*, Dronpa photoconversion can be repeated many times with a minimal loss in fluorescence intensity; therefore, reversibly photoactivatable fluorescent proteins can be used for multiple 'label and track' events, providing a detailed map of protein movement within a single cell.

Besides photolabeling and tracking experiments, the ability to switch fluorescence using a beam of light opens up novel possibilities to develop advanced microscopy techniques, which combine photoactivation with fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and various other established protocols.

Detection of protein–protein interactions

The spatial resolution of light microscopy is too low to indicate whether two differently labeled proteins interact or just co-localize in a cellular compartment. To detect protein interactions in a living cell, methods based on FRET are widely used. FRET is the non-radiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore, which is in close (<10 nm) proximity to the donor and has an excitation spectrum that overlaps with the donor emission spectrum. Because FRET results in the quenching of the donor fluorescence and enhanced fluorescence of the acceptor, this effect can be used to track the interaction of two proteins of interest when they are fused with FPs of different colors by monitoring the changes in the ratio of acceptor–donor fluorescence intensity (Figure 3). This approach is widely used, both for the study of protein interactions, and for the development of FRET-based genetically encoded fluorescent sensors [19].

The development of novel color FP variants [32,33,34,50] will considerably extend the scope of FRET-based techniques. Expansion of the palette of monomeric FPs [35] makes it possible to distinguish between two FRET pairs within a single cell and to monitor the interaction of several proteins simultaneously. Moreover, it allows the application of a three-fluorophore FRET to reveal ternary interactions within a single complex [51–53].

Fluorescence lifetime imaging microscopy (FLIM) is an advanced method of FRET detection [54–56], whereby protein–protein interactions can be studied by measuring the lifetime of donor fluorescence, which decreases during FRET because of a higher probability of energy transfer for the longer-lived excited states. By contrast to filter-based FRET measurements, FLIM is independent of the fluorophore concentrations and photobleaching. It is becoming widely used because of the expansion of the FPs available and advances in microscopy techniques.

Another recently proposed method to measure FRET is fluorescence polarization microscopy [57]. In the absence of FRET, the fluorescence emission from the donor FP is highly polarized because of its relatively large size and slow rotation, whereas in the presence of FRET, the fluorescence emission is depolarized, enabling FRET detection with a high dynamic range.

Recently, an approach to detect protein–protein interactions, based on the reassembly of split FP fragments, has been proposed as an alternative to FRET [58,59]. In brief, FPs can be split into two non-overlapping fragments comprising their N- and C-terminal halves. When fused to proteins of interest and co-expressed, these halves will reassemble to form a functional FP upon interaction, if any, of the target proteins. As the reassembly is irreversible, this complementation assay could be useful in detecting transient interactions.

Genetically encoded sensors

Currently, efforts are targeted at developing genetically encoded fluorescent sensors to detect various analytes (Ca^{2+}, pH, Cl^{-}, membrane potential, specific proteins, etc.), or to measure the activity of specific enzymes. By contrast to adding chemical probes exogenously, these sensors can be expressed within a stable cell-line or transgenic animal, targeted at a specific organelle in a cell or expressed within a specific tissue in an animal, thus expanding the possibilities for cell, developmental and physiological studies and for high-throughput screening. Eventually, it is hoped that such sensors can be developed for all analytes or molecular events. Current GFP-based genetically encoded sensors can be divided into three types:

Sensors employing a single FP molecule

This type of sensor includes single FPs, fluorescence brightness (Figure 4a), spectrum (Figure 4b), or localization; all of which are sensitive to the environment in a living cell. Depending on the protonation of the chromophore, the fluorescence brightness or spectrum of GFP can be pH sensitive [60] and several mutant variants of

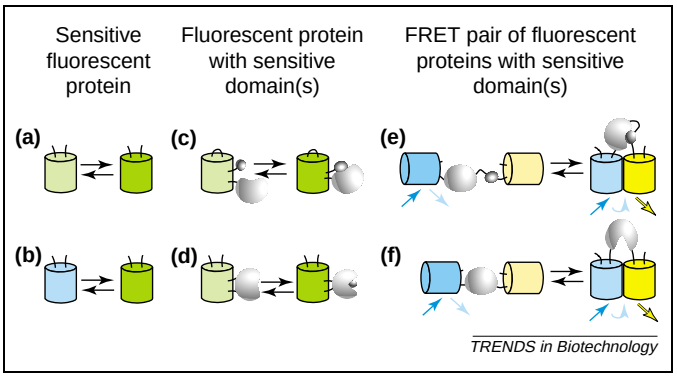

Figure 4. Main types of genetically encoded fluorescent sensors. Fluorescent proteins and sensitive domains are shown as colored barrels and gray ovals, respectively. **(a,b)** Sensors based on a single fluorescent protein that changes brightness **(a)** or emission color **(b)** depending on the environment. **(c,d)** Sensors based on a circularly permuted fluorescent protein fused to sensitive domains **(c)** or fluorescent protein with an inserted sensitive domain **(d)**. **(e,f)** Sensors based on FRET between two fluorescent proteins fused to sensitive domain(s).

GFP – synapto-pHluorin [61,62], deGFPs [63,64], mtAlpHi [65] – demonstrate essential pH-dependent spectral changes. Furthermore, Cl^- sensitivity was observed in some of the yellow FP mutant variants [66]. Redox-sensitive GFPs, with surface-exposed residues replaced by cysteines [67,68], can be also added to this group.

Several single FP-based sensors have been generated, which are capable of translocation between cell compartments upon certain types of stimulation or intracellular events [18]. Here, the fluorescence emission of the sensor is invariable, while its localization within a cell serves as a reporter for the intracellular events, for example, a fluorescent mitosis sensor [69] translocates from the nucleus to the plasma membrane upon prometaphase-associated nuclear envelope breakdown.

Sensors representing chimeric constructs between single FP and analyte detector protein(s)

In this approach, GFP is fused to a detector protein, which undergoes structural rearrangement in the presence of the analyte. This rearrangement forces a rearrangement of GFP, causing changes to its fluorescent properties (Figure 4c,d). Such sensors can be constructed by combining a FP with a detector protein (e.g. Flash – a membrane potential sensor constructed by inserting GFP into a voltage-dependent Shaker K^+ channel [70,71]); by inserting detector domains into a FP (e.g. the Ca^{2+} sensors termed Camgaroo [31,72] with calmodulin insertion); fusing detector domains to the so-called 'circularly permuted' FP (e.g. Pericam [73], GCaMP [74] – calmodulin and M13 peptide fusion, MAPK activity sensor [75]); or constructing other chimeric variants [72]. The most widely used approach employs circular permutation of an FP, which requires construction of a protein with novel N- and C-termini, while the native termini are united using a flexible linker. Permutation of a FP places fusion-sensitive domains closer to the chromophore, thus facilitating the transmission of any conformational changes to the chromophore environment. For example, the GCaMP [74] calcium sensor was constructed by fusing Calmodulin and the M13 peptide to a circularly permuted GFP. In the presence of Ca^{2+}, calmodulin binds to the M13 peptide, causing conformational changes in the vicinity of the chromophore and thus influencing the GFP fluorescence.

Interestingly, fusion of *Aequorea victoria* GFP with the *Aequorea victoria* photoprotein aequorin produced a ready-to-use calcium sensor, which can also be added to this group. It was discovered that the GFP fluorescence excitation spectrum is influenced by aequorin in a Ca^{2+}-dependent manner [76].

Sensors based on the FRET effect between two FPs in the constructs including one or several detector proteins or peptides

In these constructs, changes in the fluorescence emission spectra are brought about by the changes in FRET efficiency within a donor–acceptor pair of FPs. In response to the binding of a particular ligand, or upon specific modification, the intramolecular interaction of domains leads to a change in the spatial orientation and distance between the FPs, resulting in visible changes in the FRET effect (Figure 4e,f). For example, the Cameleon sensor comprises CFP-calmodulin bound to YFP-M13 peptide in the presence of Ca^{2+} ions, thus increasing FRET between CFP and YFP when complexed [77]. FRET-based sensors have also been developed for measuring kinase activity [78–82], glucose [83,84], maltose [85], cAMP [86], cGMP [87,88] and chloride [89]. Because the principal of sensor construction is relatively simple and reliable, this list is growing rapidly [17–20]. In addition, FRET-based protease assays using FPs have been developed [34,90], as well as assays that use a combination of a chemical probe and an FP [91]. The maximum dynamic range of this sensor group is limited by the FRET efficiency and depends on the FP–FRET pairs available. Significant progress in the development of FP–FRET pairs has been achieved [33,34,50,51,57,92], with further progress in FRET-based sensor design anticipated.

In general, the GFP-based genetically encoded sensors developed thus far have a significant limitation because of their low dynamic range: routinely, this range lies between 1.1 and ~3–5-fold [17]. The FRET-based indicator with the highest contrast is the calcium sensor YC3.60 [92], with a 6.6-fold change in ratiometric fluorescence. Some of the circularly permuted GFP-based calcium sensors are reported to reach a maximum of 8–10-fold contrast; although this increase is essentially diminished by the low pH-stability of these sensors [73,74]. This level of sensitivity is insufficient for high-throughput screening and reliable single-cell measurements – a recent comparison, in neurons, between several FP-based calcium sensors and a chemical probe showed that a higher dynamic range was achieved using the probe [93].

Photoactivatable proteins, however, are capable of ~100–2000-fold change in fluorescence; these changes probably occur as a result of the rearrangement of the chromophore [8], the surrounding amino acids [94] and the network of hydrogen bonds around the chromophore [5]. Because sensors based on a single FP fused to a sensitive domain induce rearrangements in the chromophore surroundings, the possibility exists of creating

Table 1. Commercially available fluorescent proteins

Company	Fluorescent proteins available				
	Blue, Cyan	Green	Yellow	Red	Photoactivatable
Amaxa (www.amaxa.com)		pmaxFP-Green[a]	pmaxFP-Yellow[a]	pmaxFP-Red[a]	
BD Biosciences Clontech (www.clontech.com)	AmCyan1	AcGFP1 ZsGreen1	ZsYellow1	DsRed2 DsRed-Express DsRed-Monomer Timer AsRed2 HcRed1	
Evrogen (www.evrogen.com)	PS-CFP2	TurboGFP	phiYFP	JRed	KFP-Red PS-CFP2
Invitrogen (www.invitrogen.com)	BFP CFP	EmGFP	YFP		
Lux Biotechnology (www.luxbiotech.com)		RmGFP PtGFP RrGFP			
MBL International (www.mblintl.com)	Midoriishi-Cyan	Azami Green		Kusabira-Orange	Dronpa Green Kaede KikGR
NanoLight Technology (www.nanolight.com)		RmGFP PtGFP RrGFP			
Promega (www.promega.com)		Monster Green			
Stratagene (www.stratagene.com)		hrGFP			

[a]pmaxFP-Green, pmaxFP-Yellow, and pmaxFP-Red are other names of TurboGFP, phiYFP, and JRed proteins, respectively.

high-contrast genetically encoded sensors and this remains an important task for the future.

Another disadvantage of sensor application is that the high level of expression necessary for reliable visualization can interfere with the normal functions of the cell. The sensitive domains fused to FPs in genetically encoded sensors are commonly functional enzymes, domains binding an analyte of interest or substrates modified by cellular enzymes. In all these cases, the sensor influences cell biochemistry either through its enzymatic activity, its buffering effect, or by competing with an endogenous substrate [19]. To minimize the influence of the sensor, it should be targeted to points in the biochemical pathways where it will be adequately diluted by endogenous molecules. Obviously, high brightness and photostability of a fluorescent sensor provide for better visualization at lower expression levels and, therefore, these are also highly desirable features of the future FP sensors.

Fluorescent proteins on the market

Several years ago, Clontech (www.clontech.com) was the only company to supply plasmids encoding fluorescent proteins – mutants of *Aequorea victoria* GFP. This situation changed drastically in terms of both the number of different fluorescent proteins available and 'demonopolization' of the market. Today, an increasing number of companies are offering fluorescent proteins of different colors, photoactivatable fluorescent proteins and destabilized FPs (Table 1), suitable for a wide range of applications.

Concluding remarks

In summary, the wide palette of available FPs, and their sophisticated variants, form the basis of a huge number of fluorescent assays and multi-parameter imaging. At the cellular level, FPs can provide information about promoter activity, protein-localization, -motility, -activity and interactions with other proteins, organelle movement, continuity, fusion and fission events and analyte concentration. At the organism level, FPs can give precise information about the movement of cells in a tissue, during metastasis, morphogenesis and inflammatory processes. Design of novel FPs, along with significant progress in whole-body imaging techniques [95], provide for multicolor labeling of cells and tissues, as well as monitoring of promoter activity and cellular parameters in living transgenic animals.

It is hoped that the next generation of FPs will include bright far-red or even infrared FPs for use in whole-body imaging and other clinical applications – for example, tumor visualization [96]. Additionally, novel bright and monomeric fluorescent markers in the blue region of the visible spectrum are in high demand because they would expand the scope for multicolor labeling and FRET applications. High-sensitivity applications require novel monomeric photoactivatable fluorescent proteins, especially in the red part of the visible spectrum, whereas high-contrast FP-based sensors would be of great use for reliable measurements of various cellular and tissue parameters and high-throughput drug screenings.

Extensive research in the development of FPs ensures that these, and other, objectives will be attained in the coming years, facilitating studies of living systems and opening-up possibilities for the clinical application of FPs.

Acknowledgements

We are grateful to Natalia Elina, Maria Bulina and Maria Buzdalina for the help in manuscript preparation and T.W.J. Gadella for valuable advice. The authors are supported by grants from European Commission FP-6 Integrated Project LSHG-CT-2003–503259, Russian Academy of Sciences for the Molecular and Cell Biology program and from the National Institutes of Health, GM070358.

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