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Theme Issue Article

Fluorescent proteins and fluorescence resonance energy transfer (FRET) as tools in signaling research

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Summary

The advent of fluorescent proteins has revolutionized signaling research, shifting focus from biochemical assays to analysis of live cells, organized tissues and even entire organisms. Modern applications of fluorescent proteins go beyond their use as specific markers of cells or tissues, allowing the researcher to visualize intracellular translocations as well as biochemical reactions. In this mini-review, we summarize the properties of a variety of fluorescent proteins, their detection using fluorescence microscopy and flow analysis, as well as their basic and more advanced applications, including fluorescence resonance energy transfer (FRET) to study signaling dynamics.

Keywords

Endothelial cells, signal transduction, protein trafficking

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A rainbow of fluorescent proteins

A major challenge in vascular biology research is to find model systems which reflect the complex and dynamic physiology of the vascular system and to build up appropriate tools to study them. The emergence of fluorescent protein technology and its applicability to cell-culture and in-vivo systems in recent years has brought us one step closer to this goal. The stage for these developments, which have since revolutionized cell biology research, was set with the elucidation of the DNA-sequence of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in 1992 (1) about 30 years after the discovery of the protein (2). The knowledge of the DNA-sequence allowed further optimization of fluorescence properties by amino acid mutations. The resulting optimized mutant of GFP was termed enhanced GFP (EGFP), which is currently the most commonly used fluorescent protein (3). Parallel to the improvement of fluorescence intensity and expression levels, different point mutations also led to the generation of spectral variants of GFP, which differ from the original molecule in excitation and emission wavelengths. According to the color of the predominant fluorescence light, these new fluorescent protein versions were designated as enhanced blue, cyan and yellow fluorescent proteins (EBFP, ECFP and EYFP, respectively). Further attempts to generate a red fluorescent variant by mutating the GFP molecule were not successful. However, the search for a red fluorescent protein finally led to the identification of a protein in corals of the species *Discosomas*, which was termed DsRed (4). Although the fluorescence properties of this protein were satisfactory, the molecule was not optimal, as it had a tendency to form aggregates and required tetramerization for its fluorescence (5). A mutated variant termed DsRed2 revealed lower aggregation problems but was still a tetramer, while a protein from another organism (*Heteractis crispa*), abbreviated as HcRed1 occurred as a dimer (6). Numerous amino acid mutations and tedious optimization studies then led to the development of a monomeric red fluorescent protein (7), and finally a whole new panel of fluorescent proteins was generated including a monomeric orange-red variant (mOrange), various monomeric red fluorescent proteins (mStrawberry, mCherry) and a particularly bright dimeric far-red variant designated as dTomato (7, 8).

This extended panel of spectrally different fluorescent proteins (see Table 1 and the review by Shaner et al. [9]). finally allows monitoring several different proteins at the same time. Standard microscope filter sets enable the discrimination of at least three different variants, whereas tailored, optimized narrow band filters and spectral imaging approaches further extend this number significantly.

Interestingly, the various mutation efforts not only changed the color of the mature protein, but also some other important

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Table 1: Properties of important fluorescent proteins. Additional information can be found in the review of Shaner et al. (9). For fluorescent proteins marked with an asterisk in the reference column, more information can be found at http://www.clontech.com.

Fluorescent protein	Excitation peak (nm)	Emission peak (nm)	Quaternary structure	Laser for excitation	Reference
EBFP	380	440	monomer	UV or 405 nm	3, 75
ECFP	433 (453)	475 (501)	monomer	405 nm or 458 nm Ar	3, 75
Cerulean	433	475	monomer	405 nm or 458 nm Ar	76
EGFP	488	507	monomer	488 nm Ar	3, 75
wt GFP	397 (475)	509	monomer	405 nm	3
EYFP	513	527	monomer	514 nm Ar	3, 75
Citrine	516	529	monomer	514 nm Ar	77
mOrange	548	562	monomer	543 He/Ne	8
dTomato tandem	554	581	dimer	543 He/Ne or 568 nm Ar/Kr	8
DsRed2	563	582	tetramer	543 nm He/Ne or 568 nm Ar/Kr	78 *
Monomeric DsRed (Clontech)	556	586	monomer	543 nm He/Ne or 568 nm Ar/Kr	
mStrawberry	574	596		543 nm He/Ne or 568 nm Ar/Kr	8
mCherry	587	610		543 nm He/Ne or 568 nm Ar/Kr	8
HcRed1	588	618	dimer	543 nm He/Ne or 568 nm Ar/Kr	79
Convertible fluorescent proteins					
PS-CFP (photoswitchable CFP)	400 before act.	468 before act.	monomer	405 nm	12
	490 after act.	511 after act.		488 nm Ar	
PA-GFP (photo-activatable GFP)	400 before act.	515 before act.	monomer	405 nm	11
	504 (397) after act.	517 after act.		488 nm Ar	

properties like folding kinetics and fluorophore formation. One example is a protein which changes its color from green to red over time (10) and therefore can be used as a molecular visual "timer". Even more striking is the development of fluorescent protein variants that can be activated by UV-light (photoactivatable GFP [11]), or change their color from cyan to green (12, 13), or from green to red (14) upon intense illumination with violet or UV-light. These proteins open up the possibility to perform "pulse/chase" experiments in living cells, visualizing the protein of interest in a defined region of the cell and following its transport and turnover in real time.

However, the complex photochemical processes underlying the phenomena of photoactivation and photoconversion can also cause problems with normal fluorescent proteins leading to potential artifacts under certain circumstances. An example is the finding that intense illumination of EYFP with 514 nm laser light can lead to photoconversion to a protein with ECFP-like fluorescence properties (15), which can be a problem in experiments that are based on bleaching of EYFP.

Expression of fluorescent proteins and applications

One important application of fluorescent proteins in cell biology is their use as fusion partners to visualize and monitor a target protein in living cells. Given the size of GFP (about 30 kD), there were major concerns that chimeric proteins might not be fully functional. However, GFP turned out to be a rather inert molecule which in most cases did not affect the functional integrity of its fusion partner. This might be explained by the compact molecular structure of the GFP-molecule resembling a small cylindrical can with the chromophore buried in the center (Fig. 1 [16–18]). Interestingly, the red fluorescent protein, although originating from a different organism, exhibits a very similar molecular structure (19).

The list of proteins which have successfully been expressed as fluorescent fusion proteins is extremely long and contains a great variety of signaling molecules, structural proteins, transcription factors and enzymes. An important advantage is that they do not only provide information on the steady state localiz-

ation of the target protein, but also monitor intracellular translocation processes and dynamics. Examples in the field of vascular biology are fluorescent fusion protein of eNOS (20), p65 (21), PECAM (22) or TNFR (23), just to name a few.

An important aspect when studying a fluorescent chimeric protein is the expression system that is used. In many studies, scientists applied mammalian expression vectors with rather strong viral promoters such as the CMV promoter and used transient transfection methods to express the fusion protein in the cells of interest. However, strong ectopic expression of a protein can alter the cellular homeostasis in several ways, e.g. by saturating interaction partners or components of cellular localization mechanisms. Moreover, strong overexpression of a protein can activate stress response pathways or occupy the translation machinery. For endothelial cells it was reported that overexpression of GFP can lead to induction of the heat shock protein hsp70 and the downstream target COX-2 (24).

In order to prevent potential artifacts due to strong overexpression it is important to either control the expression level of the fluorescent protein or to focus analyses on moderately expressing cells. Lower expression levels can be achieved by using weaker (and, if possible, endogenous) promoters or by integrating the transgene into genomic DNA, either in gene targeting models or by generating stably transfected cell lines. In addition, inducible expression systems such as the tetracycline-regulated system can be used (25).

For studying transgene expression, another point of consideration is the method of gene delivery. While some cells can be easily transfected by DNA-calcium precipitates or with liposome-based transfection reagents, it is often hard to transfect primary cells especially of the vasculature by using these methods. Although a limited transfection efficiency still allows studies with single-cell detection (such as fluorescence microscopy or flow analysis), it is not suitable for biochemical assays or to illuminate an entire structure in organized tissue. These limitations can be overcome with viral expression systems (e.g. using adenoviral or retroviral approaches (26–30). The applicability of these viral systems could also be demonstrated *in vivo* in a gene therapy model, where endothelial cells were targeted with adenoviral vectors (31).

The development of advanced transgenic mouse technology enabled researchers to express proteins as GFP-chimeras under the control of the endogenous promoter, thereby providing the most elegant and physiologically relevant system for the application of fluorescent fusion proteins. Such transgenic lines have been developed to light up the vasculature *in vivo* – and were applied to monitor angiogenesis in tumor formation (32).

Next to their use in fusion proteins, fluorescent proteins are utilized as tracers to illuminate cells of interest, such as particular cell types, or to mark transfected cells. For the latter application, co-transfections of DNA elements carrying the fluorescent protein have been employed, but an arguably more elegant way is the use of bicistronic vectors expressing a fluorescent protein from an internal ribosomal entry site (IRES). The major advantage is the introduction of the gene of interest in its wildtype form, excluding any possible functional deficiencies of the fusion protein while still retaining the fluorescent label of the transfected cell.

Cell- or tissue-specific delivery of the fluorescent protein can be achieved by retroviral transfection of a given cell type (e.g. bone marrow-derived mononuclear cells) with the fluorescent tracer, followed by injection into mice (33). Alternatively, transgenic mouse technology can be used to place a fluorescent protein downstream of a cell type-specific promoter. Fluorescent

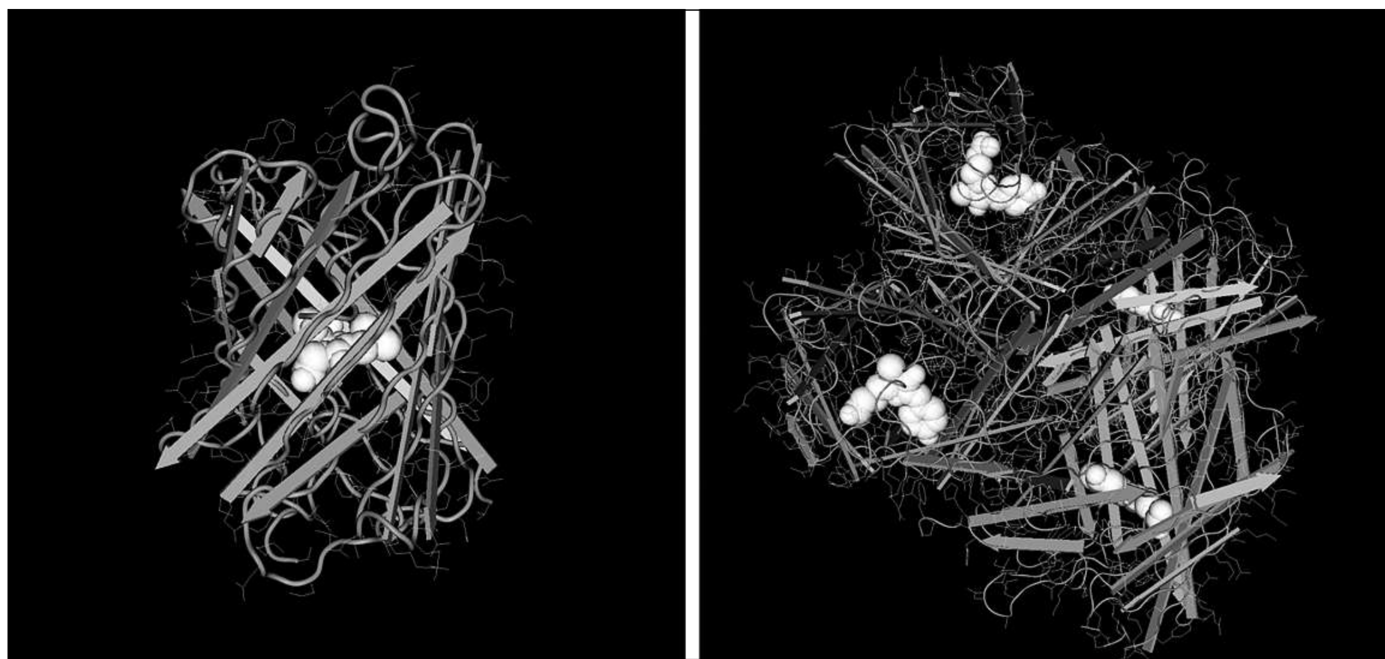

Figure 1: Structures of EGFP and DsRed. Crystallographic data on the structures of EGFP is shown on the left panel and the structure of the DsRed tetramer on the right. The chromophores are indicated in space-fill mode in white. Data is from the NCBI structure files 1S6Z (17) and 1G7K (19), respectively and is visualized with the Cn3D software package from NCBI.

cells or tissues can then be monitored by means of flow analysis, fluorescence microscopy or other imaging techniques.

In addition to applications of fluorescent proteins as tags of effector molecules or tracers of cells, EGFP and variants thereof can also be used as reporter molecule as an alternative to luciferase to monitor the activity of transcription factors or signaling pathways.

Methods to detect and monitor fluorescent proteins

Fluorescence microscopy

The most common method to detect and visualize fluorescent proteins in cell biological applications is fluorescence microscopy in different settings. Conventional epifluorescence microscopes with modern cooled CCD-cameras as detection devices are usually perfectly suited for this purpose. The standard filter sets provided with most microscopes, originally designed for FITC- and rhodamine-like fluorophores, are appropriate for EGFP and red fluorescent proteins, respectively. However, tailored filter sets for ECFP and EYFP as well as some other fluorescent protein variants are available – and should be used for these proteins to prevent overlapping signals. The usual light source in a conventional microscope (most often a mercury lamp), delivers the complete spectrum of visible light for excitation. In contrast, confocal laser scanning microscopes deliver light of defined wavelengths for excitation, depending on the laser that is used. Standard argon lasers provide ideal lines for exciting EGFP or EYFP, but they are not optimal for excitation of the popular ECFP variant. However, ECFP can be efficiently excited by 405 nm solid state lasers, which are an alternative to rather expensive UV-lasers. Finally, red fluorescent proteins can be excited by argon/krypton mixed gas lasers (at 568 nm) or with green helium/neon lasers at 543 nm. Using the appropriate excitation laser line, imaging of fluorescent proteins with confocal scanning microscopes provides a better spatial resolution than conventional microscopy. Furthermore, it opens the way for methods based on laser-driven photobleaching of fluorescent proteins in a defined region of the cell as can be achieved by the scanning process. This can be applied to assess the mobility and dynamics of molecules in living cells based on a technique termed fluorescence recovery after photobleaching (FRAP [34]). The principle of the method is that fluorescent molecules are bleached in a certain region of live cells by intense laser light, leading to a significant darkening in that area. A subsequent time series of pictures then monitors the fluorescence recovery in the bleached region which is due to diffusion of fluorescent molecules from outside. The time course and extent of fluorescence recovery is an indication for the fraction of mobile molecules and their diffusion characteristics. Similar data on diffusion coefficients can be obtained with a technique called fluorescence correlation spectroscopy (FCS), in which the fluctuations of fluorescence intensities in an extremely small volume are measured and mathematical correlation algorithms are employed to calculate the diffusion rate (35, 36).

Another technique based on bleaching strategies is used to determine the dynamic distribution of molecules between two compartments. This method, called fluorescence loss in photobleaching (FLIP), measures changes in fluorescence intensity in a certain compartment (e.g. the nucleus) when fluorescent molecules in another compartment (e.g. the cytosol) are repetitively bleached. A decrease in fluorescence intensity in the non-bleached compartment indicates dynamic distribution between the two compartments (34). All together, these techniques are extremely helpful in elucidating the dynamics of specific proteins in living cells, which is especially important for signaling molecules.

While bleaching – in a controlled manner – is a necessity for FRAP and FLIP methods, it is an unwanted effect in other circumstances and may also lead to phototoxicity. Photobleaching can be significantly reduced by specialized forms of confocal microscopy such as spinning-disk microscopy, in which the excitation light is guided through a series of small pinholes (37), or by an excitation technology termed 2-photon or multi-photon laser scanning microscopy (38–40). The latter relies on the physical phenomenon that two (or more) low energy photons (e.g. with a wavelength in the red or infra-red range) can cooperate to excite a fluorophore (such as GFP), which then emits fluorescence of a shorter wavelength (e.g. in the green range). Two-photon excitation is a non-linear process, as the absorption rate increases with the second power of the excitation light intensity. As a consequence, the excitation occurs only at a small volume in the focal plane of the specimen, where the photon concentration (the excitation light intensity) is high enough. The practical outcome of this phenomenon is an optical sectioning without the need for a pinhole to block fluorescence from locations out of focus. A side effect of this excitation principle is a significantly reduced overall bleaching effect, because the excitation occurs just at the focus. Another important advantage of this technology is that the deep red (or infra-red) excitation light penetrates much deeper (up to approximately 1 mm) into the object due to reduced scattering and absorption by endogenous chromophores, while normal laser scanning devices have a penetration depth of only about 250 µm. This feature allows studying fluorescent molecules in the context of organized tissues such as blood vessels (41).

Flow analysis

Apart from detection of fluorescent proteins by microscopy, a number of other detection principles can be used to measure the fluorescence. An extremely powerful technique is flow analysis or fluorescence-activated cell sorting (FACS). This method uses suspensions of cells that are focused in a way to allow only single cells to pass a detection area excited by appropriate laser light. The excitation light is scattered in different directions giving information on the size and granularity of the cell. Furthermore the light emitted by fluorophores such as fluorescent proteins is quantified by sensitive detectors. While microscopy provides spatial information on the fluorescence, the strength of flow analysis is to quantify fluorescence intensities from single cells for a high number of cells and automatically provide the corresponding statistics. Fluorescent proteins which stain living cells without any further modification are ideal markers for flow analysis, especially if cells have to be cultured after sorting (42). Moreover, GFP and its variants can also serve as fluorescent markers to gate specific cells of interest (e.g. transfected cells) for flow analysis of various biological parameters.

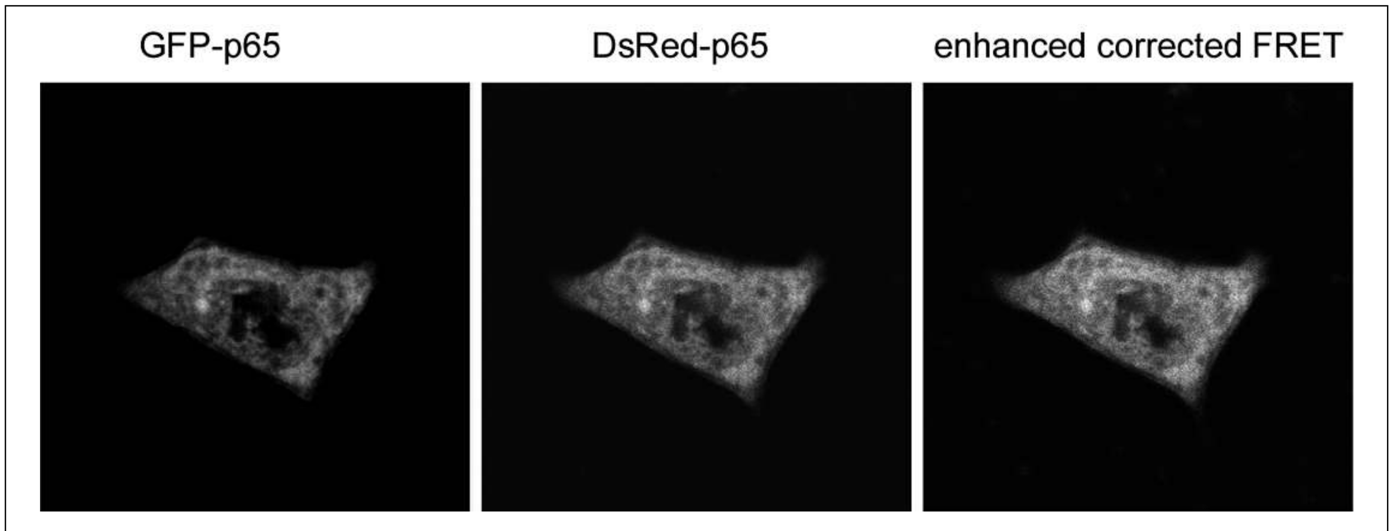

Figure 2: FRET microscopy to visualize the homodimerization of the p65-member of the NF-κB protein family. p65 was expressed as EGFP- and monomeric DsRed-fusion protein, respectively, and FRET microscopy was performed with the three-filter method (44, 80) to calculate a corrected FRET image (3x enhanced). For a negative control (EGFP and monomeric DsRed not linked to p65) the corrected FRET image was black (image not shown).

As with laser scanning microscopy, the laser type defines its adequacy for detecting a given fluorescent protein. While EGFP and EYFP can be efficiently excited with the standard argon laser, the ECFP variant requires a 405 nm laser for sensitive detection, which is usually just available for the newer generation of flow-analysis machines.

In addition to fluorescence microscopy and flow analysis, fluorescent proteins can also be measured by standard fluorometry using protein extracts.

Fluorescence resonance energy transfer (FRET)

An extremely powerful application of fluorescent proteins is their use in FRET, a quantum physical process in which energy is transferred from one fluorophore (the donor) to another (the acceptor) only if the two fluorophores are in close proximity and the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor (43–46). This energy transfer results in a reduction of the donor fluorescence and an increase in acceptor fluorescence. The energy is not transferred by photons, but via a dipole-dipole interaction of the two fluorophores. Thus, the effect decreases with the 6th power of the distance and is therefore usually detectable only up to a distance of 10 nm. These features make FRET an ideal method to monitor protein-protein interactions (Fig. 2) or conformational changes, processes of great importance in signaling. FRET has also been successfully used in vascular biology research as exemplified by studies to measure the binding of the transcription factor NF-κB to DNA (21), the activation of Rac in endothelial cells by shear stress (47) or the interaction between endothelial nitric oxide synthase with calmodulin (48).

Another potent application of the FRET principle is the use of FRET-biosensors, which are usually fusion proteins of ECFP and EYFP (or other appropriate FRET pairs such as EGFP and a monomeric red fluorescent protein) linked by a sensory domain. This domain responds to changes in certain cellular parameters by a conformational change, leading to a change of the FRET signal. A variety of enzymatic activities or biological activities can be determined by the proper choice of the sensory domain. Examples relevant for vascular biology include protein tyrosine kinases (49), protein kinase A (50), protein kinase B/Akt (51), phospholipase C (52), protein kinase C (53), Ras (54) and Rho (55) as summarized by Zaccolo (56).

Next to FRET biosensors appropriate for studying intracellular signalling processes, other sensors have been developed which monitor receptor and signal transduction processes at the cell surface. Some of these FRET-based biosensors are of particular interest in various aspects of vascular biology research and comprise tools to monitor activation of G-protein-coupled receptors (57–59), the interaction between receptors and heterotrimeric G-proteins (59–61), as well as the activation of G-proteins (62–64).

Moreover, FRET sensors have also been developed to measure second messenger molecules such as phosphatidylinositol-3,4,5-triphosphate (PIP3) (65, 66), cAMP (67) or cGMP (68, 69).

Since cAMP and cGMP FRET sensors are particularly important, their design was improved, leading to optimized sensors with better signal-to-noise ratios, faster responses or improved specificities. The original cAMP reporter used the regulatory domain of PKA fused to GFP and the catalytic domain of PKA fused to blue fluorescent protein (67). At low concentrations of cAMP these bind to each other leading to FRET between blue and green fluorescent protein. Upon generation of cAMP, the two domains dissociate and the FRET signal is lost. For second generation sensors, the suboptimal combination of blue and green fluorescent proteins was replaced by cyan (CFP) and yellow (YFP) fluorescent fusion proteins (70). A further improvement was achieved by using a different sensor, which is based on a conformational change of a sensory domain upon binding of cAMP rather than on dissociation (71). An inactive cytosolic variant of the Rap1 guanine nucleotide exchange factor Epac

was fused to CFP and YFP at its N- and C-terminus, respectively, leading to a sensor with increased sensitivity and faster kinetics.

Similarly, the originally developed cGMP FRET sensors, which were based on CFP-YFP fusion proteins of truncated cGMP-dependent protein kinase I (GKI) (68, 69) were recently replaced by improved biosensors. A detailed study comparing the original constructs to new sensor designs identified a cGMP-binding domain of PDE5 fused to CFP and YFP as the currently best cGMP FRET sensor with respect to sensitivity, specificity and response time (72).

It is important to note that FRET can not only be used to measure signalling events, but also to visualize and localize biochemical reactions such as phosphorylations (73). This can be achieved by using a fluorescent fusion protein in combination with a fluorescently tagged antibody binding the modification to be detected (e.g. phospho-tyrosine). An example for a successful application of this principle is the monitoring of autophosphorylation of the EGF receptor on the cell surface (74).

In conclusion, fluorescent proteins have come a long way since the first applications of green fluorescent protein in cell-culture systems. The realization of their potential by pioneers in the field has inspired many researchers to develop sophisticated assays to use fluorescent proteins in various aspects of signaling research, replacing more tedious and less accurate older methods. Exciting new developments appearing more and more frequently in the scientific literature have indicated that there is currently no limit for the rainbow of fluorescent proteins.

References

1. Prasher DC, Eckenrode VK, Ward WW, et al. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 1992; 111: 229–233.
2. Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J Cell Comp Physiol 1962; 59: 223–239.
3. Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998; 67: 509–544.
4. Matz MV, Fradkov AF, Labas YA, et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol 1999; 17: 969–973.
5. Baird GS, Zacharias DA, Tsien RY. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc Natl Acad Sci USA 2000; 97: 11984–11989.
6. Gurskaya NG, Fradkov AF, Terskikh A, et al. GFP-like chromoproteins as a source of far-red fluorescent proteins. FEBS Lett 2001; 507: 16–20.
7. Campbell RE, Tour O, Palmer AE, et al. A monomeric red fluorescent protein. Proc Natl Acad Sci USA 2002; 99: 7877–7882.
8. Shaner NC, Campbell RE, Steinbach PA, et al. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 2004; 22: 1567–1572.
9. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. Nat Meth 2005; 2: 905–909.
10. Terskikh A, Fradkov A, Ermakova G, et al. „Fluorescent timer": protein that changes color with time. Science 2000; 290: 1585–1588.
11. Patterson GH, Lippincott-Schwartz J. A photoactivatable GFP for selective photolabeling of proteins and cells. Science 2002; 297: 1873–1877.
12. Chudakov DM, Verkhusha VV, Staroverov DB, et al. Photoswitchable cyan fluorescent protein for protein tracking. Nat Biotechnol 2004; 22: 1435–1439.
13. Miyawaki A. Fluorescent proteins in a new light. Nat Biotechnol 2004; 22: 1374–1376.
14. Wiedenmann J, Ivanchenko S, Oswald F, et al. EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. Proc Natl Acad Sci USA 2004; 101: 15905–15910.
15. Valentin G, Verheggen C, Piolot T, et al. Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments. Nat Methods 2005; 2: 801.
16. Ormo M, Cubitt AB, Kallio K, et al. Crystal structure of the Aequorea victoria green fluorescent protein. Science 1996; 273: 1392–1395.
17. Rosenow MA, Huffman HA, Phail ME, et al. The crystal structure of the Y66L variant of green fluorescent protein supports a cyclization-oxidation-dehydration mechanism for chromophore maturation. Biochemistry 2004; 43: 4464–4472.
18. Yang F, Moss LG, Phillips GN, Jr. The molecular structure of green fluorescent protein. Nat Biotechnol 1996;14: 1246–1251.
19. Yarbrough D, Wachter RM, Kallio K, et al. Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution. Proc Natl Acad Sci USA 2001; 98: 462–467.
20. van Haperen R, Cheng C, Mees BM, et al. Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice. Am J Pathol 2003; 163: 1677–1686.
21. Schmid JA, Birbach A, Hofer-Warbinek R, et al. Dynamics of NF kappa B and Ikappa Balpha studied with green fluorescent protein (GFP) fusion proteins. Investigation of GFP-p65 binding to DNa by fluorescence resonance energy transfer. J Biol Chem 2000; 275: 17035–17042.
22. Kearney JB, Kappas NC, Ellerstrom C, et al. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. Blood 2004; 03: 4527–4535.
23. Chan FK, Siegel RM, Zacharias D, et al. Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein. Cytometry 2001; 44: 361–368.
24. Zhang F, Hackett NR, Lam G, et al. Green fluorescent protein selectively induces HSP70-mediated up-regulation of COX-2 expression in endothelial cells. Blood 2003; 102: 2115–2121.
25. Meyer-Ficca ML, Meyer RG, Kaiser H, et al. Comparative analysis of inducible expression systems in transient transfection studies. Anal Biochem 2004; 334: 9–19.
26. Giaretta I, Madeo D, Bonaguro R, et al. A comparative evaluation of gene transfer into blood cells using the same retroviral backbone for independent expression of the EGFP and deltaLNGFR marker genes. Haematologica 2000; 85: 680–689.
27. Motoike T, Loughna S, Perens E, et al. Universal GFP reporter for the study of vascular development. Genesis 2000; 28: 75–81.
28. Eslami MH, Gangadharan SP, Sui X, et al. Gene delivery to in situ veins: differential effects of adenovirus and adeno-associated viral vectors. J Vasc Surg 2000; 31: 1149–1159.
29. Garton KJ, Ferri N, Raines EW. Efficient expression of exogenous genes in primary vascular cells using IRES-based retroviral vectors. Biotechniques 2002; 32: 830, 832, 834.
30. Lamfers ML, Wijnberg MJ, Grimbergen JM, et al. Adenoviral gene transfer of a u-PA receptor-binding plasmin inhibitor and green fluorescent protein: inhibition of migration and visualization of expression. Thromb Haemost 2000; 84: 460–467.
31. Breuss JM, Cejna M, Bergmeister H, et al. Activation of nuclear factor-kappa B significantly contributes to lumen loss in a rabbit iliac artery balloon angioplasty model. Circulation 2002; 105: 633–638.
32. Amoh Y, Yang M, Li L, et al. Nestin-linked green fluorescent protein transgenic nude mouse for imaging human tumor angiogenesis. Cancer Res 2005; 65: 5352–5357.
33. Zentilin L, Tafuro S, Zacchigna S, et al. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. Blood 2006; 107: 3546–3554.
34. White J, Stelzer E. Photobleaching GFP reveals protein dynamics inside live cells. Trends Cell Biol 1999; 9: 61–65.
35. Maiti S, Haupts U, Webb WW. Fluorescence correlation spectroscopy: diagnostics for sparse molecules. Proc Natl Acad Sci USA 1997; 94: 11753–11757.
36. Medina MA, Schwille P. Fluorescence correlation spectroscopy for the detection and study of single molecules in biology. Bioessays 2002; 24: 758–764.
37. Maddox PS, Moree B, Canman JC, et al. Spinning disk confocal microscope system for rapid high-resolution, multimode, fluorescence speckle microscopy and green fluorescent protein imaging in living cells. Methods Enzymol 2003; 360: 597–617.
38. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science 1990; 248: 73–76.
39. Denk W, Svoboda K. Photon upmanship: why multiphoton imaging is more than a gimmick. Neuron 1997; 18: 351–357.
40. White N, Errington R. Multi-photon microscopy: seeing more by imaging less. Biotechniques 2002; 33: 298–295.
41. Tozer GM, Ameer-Beg SM, Baker J, et al. Intravital imaging of tumour vascular networks using multi-photon fluorescence microscopy. Adv Drug Deliv Rev 2005; 57: 135–152.
42. Galbraith DW, Anderson MT, Herzenberg LA. Flow cytometric analysis and FACS sorting of cells

based on GFP accumulation. Methods Cell Biol 1999; 58: 315–341.
43. Jares-Erijman EA, Jovin TM. FRET imaging. Nat Biotechnol 2003; 21: 1387–1395.
44. Schmid JA, Sitte HH. Fluorescence resonance energy transfer in the study of cancer pathways. Curr Opin Oncol 2003; 15: 55–64.
45. Förster T. Zwischenmolekulare Energiewanderung und Fluoreszenz. Ann Phys 1948; 2: 57–75.
46. Wallrabe H, Periasamy A. Imaging protein molecules using FRET and FLIM microscopy. Curr Opin Biotechnol 2005; 16: 19–27.
47. Tzima E, Del Pozo MA, Kiosses WB, et al. Activation of Rac1 by shear stress in endothelial cells mediates both cytoskeletal reorganization and effects on gene expression. EMBO J 2002; 21: 6791–6800.
48. Jobin CM, Chen H, Lin AJ, et al. Receptor-regulated dynamic interaction between endothelial nitric oxide synthase and calmodulin revealed by fluorescence resonance energy transfer in living cells. Biochemistry 2003; 42: 11716–11725.
49. Sato M, Ozawa T, Inukai K, et al. Fluorescent indicators for imaging protein phosphorylation in single living cells. Nat Biotechnol 2002; 20: 287–294.
50. Zhang J, Ma Y, Taylor SS, et al. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. Proc Natl Acad Sci USA 2001; 98: 14997–15002.
51. Sasaki K, Sato M, Umezawa Y. Fluorescent indicators for Akt/protein kinase B and dynamics of Akt activity visualized in living cells. J Biol Chem 2003; 278: 30945–30951.
52. van der Wal J, Habets R, Varnai P, et al. Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J Biol Chem 2001; 276: 15337–15344.
53. Violin JD, Zhang J, Tsien RY, et al. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J Cell Biol 2003; 161: 899–909.
54. Mochizuki N, Yamashita S, Kurokawa K, et al. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. Nature 2001; 411: 1065–1068.
55. Pertz O, Hahn KM. Designing biosensors for Rho family proteins--deciphering the dynamics of Rho family GTPase activation in living cells. J Cell Sci 2004; 117: 1313–1318.
56. Zaccolo M. Use of chimeric fluorescent proteins and fluorescence resonance energy transfer to monitor cellular responses. Circ Res 2004; 94: 866–873.
57. Hoffmann C, Gaietta G, Bunemann M, et al. A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. Nat Methods 2005; 2: 171–176.
58. Vilardaga JP, Bunemann M, Krasel C, et al. Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. Nat Biotechnol 2003; 21: 807–812.
59. Nobles M, Benians A, Tinker A. Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. Proc Natl Acad Sci USA 2005; 102: 18706–18711.
60. Gales C, Rebois RV, Hogue M, et al. Real-time monitoring of receptor and G-protein interactions in living cells. Nat Methods 2005; 2: 177–184.
61. Hein P, Frank M, Hoffmann C, et al. Dynamics of receptor/G protein coupling in living cells. EMBO J 2005; 24: 4106–4114.
62. Bunemann M, Frank M, Lohse MJ. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc Natl Acad Sci USA 2003; 100: 16077–16082.
63. Gibson SK, Gilman AG. Gialpha and Gbeta subunits both define selectivity of G protein activation by alpha2-adrenergic receptors. Proc Natl Acad Sci USA 2006; 103: 212–217.
64. Janetopoulos C, Jin T, Devreotes P. Receptor-mediated activation of heterotrimeric G-proteins in living cells. Science 2001; 291: 2408–2411.
65. Sato M, Ueda Y, Takagi T, et al. Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. Nat Cell Biol 2003; 5: 1016–1022.
66. Tanimura A, Nezu A, Morita T, et al. Fluorescent biosensor for quantitative real-time measurements of inositol 1,4,5-trisphosphate in single living cells. J Biol Chem 2004; 279: 38095–38098.
67. Zaccolo M, De Giorgi F, Cho CY, et al. A genetically encoded, fluorescent indicator for cyclic AMP in living cells. Nat Cell Biol 2000; 2: 25–29.
68. Honda A, Adams SR, Sawyer CL, et al. Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. Proc Natl Acad Sci USA 2001; 98: 2437–2442.
69. Sato M, Hida N, Ozawa T, et al. Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase Ialpha and green fluorescent proteins. Anal Chem 2000; 72: 5918–5924.
70. Zaccolo M, Pozzan T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 2002; 295: 1711–1715.
71. Ponsioen B, Zhao J, Riedl J, et al. Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. EMBO Rep 2004; 5: 1176–1180.
72. Nikolaev VO, Gambaryan S, Lohse MJ. Fluorescent sensors for rapid monitoring of intracellular cGMP. Nat Methods 2006; 3: 23–25.
73. Wouters FS, Verveer PJ, Bastiaens PI. Imaging biochemistry inside cells. Trends Cell Biol 2001; 11: 203–211.
74. Verveer PJ, Wouters FS, Reynolds AR, et al. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science 2000; 290: 1567–1570.
75. Patterson G, Day RN, Piston D. Fluorescent protein spectra. Journal of Cell Science 2001; 114: 837–838.
76. Rizzo MA, Springer GH, Granada B, et al. An improved cyan fluorescent protein variant useful for FRET. Nat Biotechnol 2004; 22: 445–449.
77. Griesbeck O, Baird GS, Campbell RE, et al. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. J Biol Chem 2001; 276: 29188–29194.
78. Bevis BJ, Glick BS. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). Nat Biotechnol 2002; 20: 83–87.
79. Fradkov AF, Verkhusha VV, Staroverov DB, et al. Far-red fluorescent tag for protein labelling. Biochem J 2002; 368: 17–21.
80. Youvan DC, Coleman WJ, Silva CM, et al. Calibration of fluorescence resonance energy transfer in microscopy using genetically engineered GFP derivatives on nickel chelating beads. Biotechnology et alia 1997; 3: 1–18.