Evolutions in Science Triggered by Green Fluorescent Protein (GFP)

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Green Fluorescent Protein (GFP)

Green fluorescent protein (GFP) was discovered in the marine jellyfish *Aequorea victoria* as a side product after purification of aequorin, a chemiluminescent protein. Emission of blue light by aequorin leads to excitation of its companion protein GFP, thereby resulting in green fluorescence.[1] 30 years later, when the sequence of GFP was elucidated,[2] this molecule started to be developed into a valuable tool for various scientific applications, as it became possible to apply cloning approaches and to use GFP either as a reporter molecule or as a fluorescent tag for fusion proteins. However, just after optimization of its fluorescence properties, which led to enhanced versions of GFP, it started to revolutionize many fields of science, especially as a marker in living cells (for review, see ref. [3]).

GFP is a small protein of 28 kDa with a barrel-like structure composed of 11 β sheets slightly twisted around the central axis, designated as a β-can structure[4,5] (see Figure 1 for the similar structure of enhanced GFP (EGFP)).

GFP fluorescence is caused by three cyclized and oxidized amino acids located in the center of the molecule. The process of fluorophore formation and maturation requires molecular oxygen for the generation of oxidized intermediate states of these amino acids. For this reason, GFP can only be expressed under aerobic conditions, but as soon as GFP maturation is completed, O_2 is no longer needed for fluorescence. Wild-type GFP exhibits two distinct excitation wavelengths due to the coexistence of both neutral and anionic amino acids in the chromophore. It has a major absorption maximum at 397 nm and a minor excitation peak at 475 nm.

The scientific potential of a fluorescent protein was rapidly recognized after cloning of GFP. However, some of the properties of wild-type GFP were not satisfactory with respect to fluorescence intensity, folding properties, the kinetics of fluorophore formation, and the biphasic excitation spectrum. Therefore, many efforts were undertaken to optimize this protein by point mutations, which finally led to the generation of a considerably improved GFP, with faster generation of the fluorophore, brighter fluorescence, correct folding at 37 °C, and a single excitation peak at 488 nm. In addition, many silent mutations were introduced to change the codon usage from that of the jellyfish towards the one preferred by vertebrates, in order to improve translation and expression in mammalian cells. This variant of GFP was designated as enhanced GFP (EGFP), the most commonly used GFP variant nowadays (Figure 1).

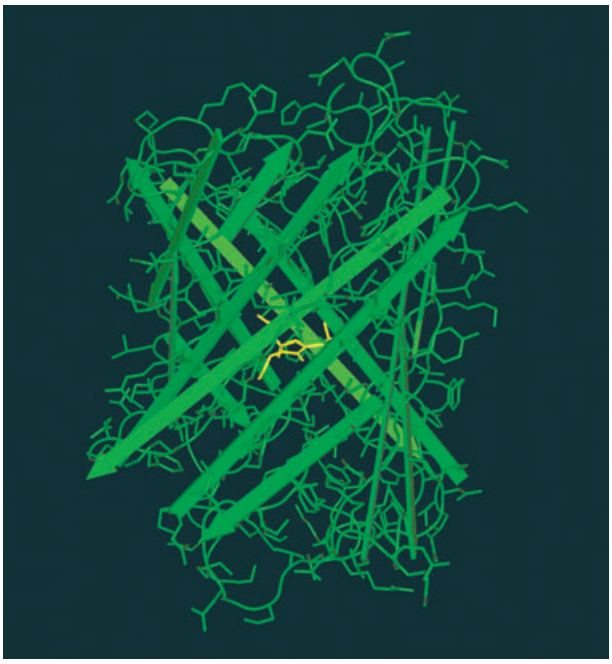

Figure 1. The three-dimensional structure of EGFP is shown, as calculated from the coordinates of atoms derived from X-ray crystallography (protein data bank number 1S6Z[65]). The chromophore is highlighted in yellow. The structure is depicted with Cn3D software, release 4.1, from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/entrez/).

Variants of GFP

In parallel to improvements of the fluorescence properties of GFP, various point mutations also led to the creation of spectral variants of EGFP emitting blue, cyan, or yellow fluorescence (EBFP, ECFP, and EYFP, respectively; Table 1). Later on, even further ameliorations were achieved, thereby leading to the creation of Cerulean (a 2.5-fold brighter variant of ECFP[6]) and Citrine, a variant of EYFP with lower pH and chloride sensitivity and better photostability and expression in organelles.[7]

After development of these spectral variants of GFP, many efforts were undertaken to extend the range of fluorescence further into the red part of the spectrum. This turned out to be a difficult task, which has not been achieved by mutation of GFP so far; however it was achieved just by discovery and cloning of a red fluorescent protein from a different organism,

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Table 1. Some important fluorescent protein (FP) variants are listed in the order of their fluorescence color (emission wavelength).

FP[a]	Ex. peak[b]	Em. peak[b] [nm]	QY[b] [nm]	EC[b]	QY × EC[b]	Relative brightness[b]	Ref.
EBFP	380	440	0.18	31 000	5580	0.17	[3, 66][c]
ECFP	433 (453)	475 (501)	0.4	26 000	10 400	0.32	[3, 66][c]
Cerulean	433	475	0.62	43 000	26 660	0.81	[6]
EGFP	488	507	0.6	55 000	33 000	1.00	[3, 66][c]
wt GFP	397 (475)	509	0.77	27 600	21 252	0.64	[3]
EYFP	513	527	0.61	84 000	51 240	1.55	[3, 66][c]
Citrine	516	529	0.76	77 000	58 520	1.77	[7]
mOrange	548	562	0.69	71 000	48 990	1.48	[13]
dTomato (tandem)	554	581	0.69	138 000	95 220	2.89	[13]
DsRed	558	583	0.29	22 500	6525	0.20	[8]
DsRed2	563	582	0.55	43 800	24 090	0.73	[67][c]
mStrawberry	574	596	0.29	90 000	26 100	0.79	[13]
mCherry	587	610	0.22	72 000	15 840	0.48	[13]
HcRed1 (tandem)	588	618	0.04	160 000	6400	0.19	[68]
PA-GFP	400[d]	515[d]	0.13	20 700	2691	0.08	[15]
	504 (397)[e]	517[e]	0.79	17 400	13 746	0.42	
PS-CFP	400[d]	468[d]	0.16	34 000	5440	0.16	[19]
	490[e]	511[e]	0.19	27 000	5130	0.16	

[a] FP abbreviations: EBFP = enhanced blue fluorescent protein, ECFP = enhanced cyan fluorescent protein, EGFP = enhanced green fluorescent protein, wt GFP = wild-type green fluorescent protein, EYFP = enhanced yellow fluorescent protein, DsRed = *Discosoma species* red fluorescent protein, HcRed = *Heteractis crispa* red fluorescent protein, PA-GFP = photoactivatable GFP, PS-CFP = photoswitchable CFP. [b] Ex. peak = excitation peak, Em. peak = emission peak, QY = quantum yield, EC = molar extinction coefficient. These values can differ slightly between different sources. The given values were taken from the references indicated. The brightness is described by the product of QY and EC and is also given as relative value with the brightness of EGFP set to 1.0. [c] Additional information is available from BD Biosciences Clontech (http://www.bdbiosciences.com/clontech/index.shtml). [d] Before activation. [e] After activation.

namely, *Discosoma* sp., a coral of the anthozoa family. This protein was designated as DsRed (or drFP583[8]) and has a three-dimensional structure that is very similar to that of GFP.[9] While this protein possesses satisfactory fluorescent properties with maximum excitation at 558 nm and emission at 583 nm, it suffers from the significant disadvantage that it has to be present as a tetramer in order to be fluorescent. Furthermore, it has a tendency for aggregation and it exhibits a complex and slow fluorophore maturation with green intermediates.[10] Attempts to improve its properties by amino acid substitutions resulted in a variant with six point mutations, termed DsRed2, with lower aggregation and faster maturation; it was however still a tetramer (BD Clontech).

Further efforts to generate an improved red fluorescent protein lead to the development of the dimeric and even more red-shifted fluorescent protein HcRed1, which was generated by multistep mutagenesis of a red chromoprotein from the coral *Heteractis crispa*.[11] Its property of being fluorescent as a dimer made it possible to apply an elegant trick to prevent oligomerization of a tagged protein of interest. By linking two copies of HcRed1 with the coding sequence of the target protein, one can obtain a fluorescent fusion protein without dimerization. However, it has to be considered that the fluorescent tag is then about 50 kD in size.

Later on, a monomeric variant of a red fluorescent protein was described, designated as mRFP1, which was generated by targeted mutagenesis of DsRed to eliminate oligomerization (resulting in loss of fluorescence) and subsequent mutagenesis to rescue fluorescence.[12] mRFP1 shows an excitation peak at 584 nm and an emission peak at 607 nm, but its fluorescence properties with respect to quantum yield and molar extinction coefficient are not satisfactory for standard applications. A bright monomeric red fluorescent protein would represent a very valuable tool and a perfect companion for GFP because many excitation light sources in microscopy are designed for green and red fluorescence, such as that seen with fluorescein and rhodamine. Therefore, the hunt for the ideal red fluorescent protein went on, and very recently it guided the way to the development of a whole panel of new fluorescent proteins, from a very interesting monomeric orange-red variant (mOrange) to various monomeric red versions (mStrawberry, mCherry) and an extremely bright dimeric far-red variant termed dTomato (Table 1).[13] The latter can also be applied as a tandem construct as in the case of HcRed1.

Besides the generation of spectrally different fluorescent proteins, which can be used for simultaneous tracking of two, three, or even more distinct target proteins in living cells, other fascinating variants of GFP were also developed. One of these is a mutant of DsRed that changes the fluorescence slowly from green to red during maturation, a feature that can be used to trace time-dependent expression and promoter activity.[14]

Another promising GFP variant, designated as photoactivatable GFP (PA-GFP),[15] specifically changes its fluorophore properties by a photoactivation process from a nearly nonfluorescent form (at 488 nm excitation) to a fully fluorescent one that is about 100 times more fluorescent. This photoactivation phenomenon was already known for wild-type GFP, which has an excitation peak at 397 nm and another small one at 475 nm, apparently resulting from neutral phenol and anionic phenolate chromophore populations. Upon intense illumination at around 400 nm, the chromophores undergo a photoconversion and shift to the anionic form, thereby producing an increase in excitation at the higher wavelength and a corresponding increase in fluorescence at 488 nm excitation of about threefold.

Based on the fact that mutations of threonine residue 203 reduce the higher excitation wavelength peak while maintaining the 400 nm excitation, Patterson and Lippincott-Schwartz found that mutation to histidine at this very position (T203H in a mammalian codon optimized wild-type GFP) practically eliminates the excitation at 488 nm while still maintaining the peak excitation at 400 nm and also the photoactivation phenomenon.[15] By this means, a fluorescent protein was generated that is practically nonfluorescent at 488 nm excitation and that can

be activated to bright fluorescence by intense illumination at around 400 nm. This can also be achieved by 413 nm laser excitation, thereby bringing in all the advantages of confocal laser scanning microscopy including the possibility of bleaching clearly defined regions of interest. This allows the tagging of a fusion protein in living cells in a timed and spatially controlled fashion and opens up several possibilities: For instance, it is possible to label only a spatially defined subset of PA-GFP-tagged chimeric proteins and to follow their subsequent transport to other intracellular compartments or regions. Furthermore, this method can also be used as a fluorescence microscopy based alternative to radioactive pulse/chase experiments: For a certain time period, molecules can be made visible and their subsequent dynamics and turnover can be followed in vivo. Molecules synthesized after the photoactivation event will not be visible, in analogy to the chase with nonradioactive amino acids in pulse/chase experiments.

Another powerful and promising development is the generation of photoconvertible fluorescent proteins, which change their fluorescence color after intense illumination and which have the advantage that they are also detectable, at a different wavelength, before photoconversion. One of the first photoconvertible proteins described was the tetrameric fluorescent protein variant termed "Kaede", which turns its fluorescence from green to red upon illumination.[16] A similar, but improved photoconvertible fluorescent protein was very recently reported by the same group; this newer protein shows better brightness and photoconversion by both conventional and two-photon excitation.[17] However, this protein is still oligomeric and this hampers its use as a marker in chimeric proteins. For a different fluorescence protein exhibiting green to red photoconversion, the tetrameric form could be converted into a monomeric one by introducing two point mutations, thereby leading to the very promising variant EosFP.[18] At about the same time, another highly interesting monomeric fluorescent protein variant was described, which changes its fluorescence from cyan to green by photoconversion upon intense illumination at 405 nm and which was therefore named "photoswitchable CFP".[19,20]

Applications of GFP and Its Variants

In principle, fluorescent proteins can be applied in two ways: First, they can be used as a tracer (for example, for detection of labeled cells in vivo); second, they can be covalently linked to a protein of interest by combination of the coding sequence of GFP or one of its variants with that of a specific protein followed by transient or stable transfection of the chimeric construct.

Applications in which GFP variants are not linked to another protein have become increasingly popular in studies of transgene animals, where specific cells (for example, with cell-type specific transgenes) have to be tracked in the context of the intact organism.[21,22] Due to the availability of spectral variants of GFP, different cell types can be monitored synchronously to allow complex studies of cell behavior and interactions in the organism. In addition, nonchimeric GFPs are also used to monitor promoter activities in reporter gene assays in vitro or in vivo.

However, most of the studies with GFP molecules involve the use of fusion proteins of GFP variants with other proteins of interest, where the fluorescent protein is serving as a tag.

Despite problems of potential artefacts due to overexpression of the chimeric molecule and/or alteration of the function by the covalent linkage with the GFP tag, these applications have provided useful and important insights in many biological systems. Certainly, it has to be considered that fluorescent fusion proteins might behave differently from their wild-type counterparts due to the considerable size of the tag and its nature as a protein. However, it turned out that GFP chimeras are, in most cases, functional and that the GFP tag is rather inert. Nevertheless, the functional integrity should be tested for every fusion protein before it is used as a model system. Potential artefacts by overexpression should be minimized by using moderate expression (for instance, by choosing appropriate stable transfectants) or by using single-cell detection systems and focusing on low-expressing cells. Future approaches might also use GFP variants under the control of endogenous promoters in order to avoid potential problems with overexpression.

Time-Lapse Microscopy and Confocal Laser Scanning Microscopy Including Spectral Imaging

Standard cuvette-based spectrofluorometry or microtiter-plate fluorescence measurements are possible with GFP and its variants as with other fluorophores, but the main application of fluorescent fusion proteins from the beginning was live-cell microscopy. The possibility of visualizing proteins in their intact cellular environment revolutionized many fields of cell biology and life science. This was further supported by technological innovations, such as cooled charge-coupled-device (CCD) cameras as detection devices for conventional epifluorescence microscopes, as well as improvements in confocal laser scanning microscopy and digital image analysis.

Furthermore, the generation of spectral variants of GFP allowed simultaneous tracing of distinct GFP mutants exhibiting sufficient differences in their fluorescence properties (for example, ECFP and EYFP) simply by filter-based discrimination. The possibility of tracking several different fluorescent proteins in parallel was recently significantly extended by spectral imaging systems (such as the Zeiss LSM510 META system or Leica confocal microscopes) that allow the recording of wavelength emission curves from image data. By using reference spectra and a mathematical algorithm termed "linear unmixing" (or "emission fingerprinting"), even strongly overlapping emission curves (such as those of EGFP and EYFP) can be distinguished.[23] Our own results indicate that at least five different fluorescent protein variants (ECFP, EGFP, EYFP, DsRed2, and HcRed1) can be discriminated by this method with the usual Ar- and He/Ne-laser light sources.

The acquisition of a limited number of images of living cells is rather simple with normal fluorescence microscopes, where-

as it is still a challenge to acquire movies of live cells under physiological conditions. Apart from the need to heat the cells on the microscope stage without evaporation of the medium (or changes in buffer composition or pH value) and without losing the focal plane, repetitive or continuous illumination of cells can pose severe problems of bleaching and/or phototoxicity. However, these problems can be approached by low-light-level excitation, as realized in spinning-disk confocal microscope systems[24] or with neutral grey filters to minimize excitation light power in conventional epifluorescence microscopes. In addition, rapid computer-controlled shutters or filter wheels that are triggered by the CCD-camera software can minimize the bleaching effect by controlled and short-term excitation of the specimen during image acquisition. Alternatively, monochromatic light sources can be used, which have a narrow bandwidth of about 10 nm and therefore only a limited bleaching effect and which furthermore provide the possibility of switching between the excitation wavelength and a non-exciting wavelength (serving as shutter) within a few milliseconds. These monochromatic light sources can also be synchronized with CCD cameras; since the cameras usually just require about 100 milliseconds to acquire a decent image, the bleaching effect is usually significantly below that of a confocal laser scanning system, which often requires several seconds of averaging to obtain the same image quality.

Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP)

About 30 years ago,[25] techniques were developed to study the mobility and diffusion of fluorescent molecules in living cells. This was achieved by brief and intense bleaching of a subset of fluorophores in a defined region of the cell and monitoring of fluorescence recovery, which occurs in this area due to diffusion of unbleached fluorophores from outside into the bleaching region. Initially, this technique, termed fluorescence recovery after photobleaching (FRAP) analysis, found only limited application because the insides of cells were hardly accessible for fluorescent markers, except by sophisticated approaches such as microinjection (for example, of fluorescently labeled antibodies). This limitation ceased with the development of optimized fluorescent proteins, EGFP, and other related fluorescent proteins turned out to be ideal tools for this method as they can be easily attached by molecular-biology means to a protein of interest and expressed in various cells by transfection methods. Moreover, EGFP and EYFP are perfectly excited by Ar lasers, which are the standard lasers in most of the commercially available confocal microscopes. Although FRAP analysis is, in principle, also applicable in conventional epifluorescence microscopes, the intense laser light and the scanning features of confocal microscopes render them especially suited for this method. In most cases, the data-acquisition software of confocal microscopes allows a convenient experimental setup, with a prescan with weak excitation light, followed by a number of repetitive bleaching scans at full laser power (usually 70–100 scans), and the subsequent capture of a time series to record the fluorescence recovery in the bleaching area at low laser power. At least for nonmembrane proteins, which diffuse quite quickly, it is recommended that the postbleaching time series is captured just for the bleached area in order to obtain a reasonable time resolution. An example of a FRAP data set and the equation for fitting the data is given in Figure 2A. Since a certain fraction of the total amount

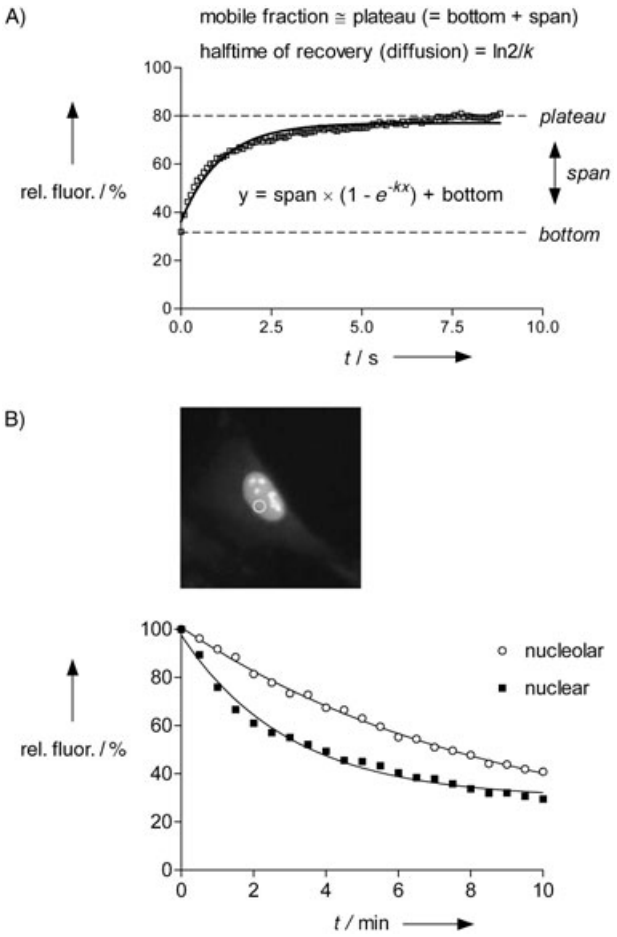

Figure 2. Examples of FRAP and FLIP experiments. A) FRAP: Bleaching an area of interest causes a reduction of fluorescence in the bleached area to the "bottom" level. The subsequent increase in fluorescence due to diffusion of GFP-chimeric proteins into the bleached area is shown. The raw data can be fitted with a single exponential increase algorithm as indicated by the equation. Fluorescence increases to a plateau value of "bottom + span", which indicates the percentage of mobile molecules. The half-time value of recovery is a measure of the diffusion rate. B) FLIP: Upper panel: Repetitive bleaching of fluorescent fusion proteins in one area of the cell (for example, the nucleoplasm; depicted in the fluorescence image as circle) can result in a concomitant decrease of fluorescence in another area of the cell (for example, a nucleolus) if the molecules from the second area (or compartment) are constitutively transported or diffusing into the bleached area. Lower panel: The quantification of mean fluorescence intensities over time shows a decrease both in the nucleoplasm and in the nonbleached nucleoli.

of fluorophores is destroyed during bleaching in the small region of interest, it is necessary to quantify the loss in total fluorescence. The fluorescence values in the bleach region during the fluorescence recovery have to be related to the corrected total fluorescence with the slight decrease of total fluo-

rescence taken into account. Fitting of the raw data of the fluorescence increase with single exponential algorithms reveals two important values. First, the plateau to which the fluorescence recovers is a direct number for the percentage of mobile fluorescent molecules; this means that if fluorescence recovers to 90% of the initial fluorescence in the bleach region, 10% of the molecules are immobile due to binding to nondiffusing cellular elements such as the cytoskeleton. The second important parameter that can be derived is the half-time value of diffusion into the bleach region, which is dependent on the diffusion coefficient of the fluorescent molecule. By always using the same bleaching region, or by normalizing the half-time value of recovery to the area of the bleaching region, it is rather simple to obtain relative and comparative data for different fluorophores. However, calculation of correct diffusion coefficients is more challenging and requires more sophisticated models of diffusion under the respective experimental setups. While mathematical models for two-dimensional diffusion within membranes were developed quite early on,[25] it is still a complex task to calculate three-dimensional diffusion coefficients. Mathematical models that take the special experimental conditions into consideration were developed just recently.[26]

Another powerful application of GFP is the so-called fluorescence loss in photobleaching (FLIP) approach. In this case, a certain region of a living cell expressing a GFP-chimeric protein is repetitively bleached and the loss of fluorescence in a different region is monitored over a longer time period (Figure 2B). By this means, transport processes between different compartments of the cell (for example, the nucleus and cytosol) can be assessed and it can be determined whether a molecule shuttles between different compartments, even if the fluorescence appears constant under steady state conditions.[27] By using modern laser scanning microscopy, these techniques can be scaled down to even small structures, such as nucleoli, and the dynamic distribution of a protein between the nucleoli and the nucleoplasm can be determined.[28]

Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is another powerful application of fluorescent proteins for analyzing the dynamics of molecules. In this method, fluorescence-intensity fluctuations are measured in a minute volume (in the femtoliter range); this is usually achieved by a confocal laser scanning microscopy setup with fast and sensitive photodiode detectors, followed by autocorrelation analysis. The autocorrelation function describes the normalized variance of fluorescence fluctuations and gives information on the diffusion coefficient, the concentration of the fluorophore, and other parameters such as interactions between molecules or the microenvironment of the fluorophore.[29,30] This general principle can also be applied to fluorescent-protein chimeras. Interestingly, FCS revealed that EGFP and other GFP variants exhibit "blinking" characteristics with short dark states between the fluorescent states. This blinking depends on the protonation status of the fluorophore and can even be applied to measure the pH value in the microenvironment of the fluorescent protein.[31,32]

Fluorescence Resonance Energy Transfer (FRET)

The availability of spectrally distinct fluorescent proteins opened up another large field of potential applications based on the quantum physical phenomenon of energy transfer between two different fluorophores, which was first described in 1948.[33] The most important prerequisites for this phenomenon to occur are that the fluorophores are in close proximity to each other (closer than 10 nm for most fluorophores) and that the emission curve of one fluorophore (the energy donor) overlaps with the excitation curve of the second fluorophore (the energy acceptor). The energy is transferred by a dipole–dipole interaction (not by emission of photons) and leads to a decrease in donor fluorescence and an increase in acceptor fluorescence. Since fluorescence resonance energy transfer (FRET) declines with the sixth power of the distance, it is virtually undetectable at fluorophore distances larger than 10 nm. This makes FRET an ideal tool for monitoring macromolecular interactions, because energy transfer can practically only be observed if the two fluorescent molecules interact with each other. The second condition, the spectral overlap, can be realized with several of the different fluorescent protein variants. Earlier studies used a combination of blue and green fluorescent proteins; however, the inferior fluorescence properties of blue fluorescent protein made it difficult to detect FRET. Therefore, ECFP and EYFP became the most popular pair of fluorescent proteins for FRET applications as they exhibit the required fluorescence properties and spectral overlap while still being distinguishable by appropriate microscope filter sets.[34–36]

However, most confocal laser scanning microscopy systems, as well as flow cytometry equipment, use Ar and He/Ne lasers as light sources, in which the 458 nm line of the Ar laser is the lowest available excitation light. This is not optimal for excitation of ECFP, especially in FRET applications. Therefore, there has always been considerable interest in getting access to a monomeric red fluorescent protein with suitable properties, which can be excited at the 543 nm line of the He/Ne laser and which would be a perfect FRET acceptor with EGFP as the donor. Unfortunately, the combination of EGFP and red fluorescent proteins has been problematic, because previously available red fluorescent proteins were either dimers or tetramers and, moreover, showed green intermediates in the course of chromophore generation. The very recent development of novel monomeric orange and red fluorescent proteins with good quantum yields[13] will probably end this limitation and these proteins will most likely find broad application in FRET microscopy and flow analysis.

The combination of EGFP and EYFP, although having a very good overlap of EGFP emission and EYFP excitation, is usually not used as these proteins cannot easily be separated by filter technology. However, spectral imaging or wavelength scanning fluorometry are suited to detect FRET between EGFP and EYFP.[37]

The main application of FRET analysis is in the monitoring of protein interactions by using chimeras of proteins of interest. The advantages of using FRET analysis to determine protein interactions is that the interactions can be monitored in living cells and that the intracellular localization of an interaction and potential temporal or spatial changes can be visualized. As FRET is not easily detectable by the presence or absence of a distinct signal but by a relative decrease of donor fluorescence and an increase in acceptor emission with the problem of overlapping emission and excitation curves, it is usually monitored by specialized microscopy methods. One important technique is the so-called three-filter method: images are acquired with a specific donor filter, a specific acceptor filter (clearly discriminating acceptor from donor fluorescence), and a third filter, termed the FRET filter, which combines donor excitation and acceptor emission. Samples containing either donor or acceptor alone are used to determine the spill-over of fluorescence into the FRET channel by calculating the fraction of non-FRET fluorescence in the FRET filter for a given donor or acceptor fluorescence, respectively. Another very important and elegant method to determine FRET by including an internal control is to monitor donor recovery after acceptor photobleaching (DRAP). In this case, an image is acquired with the donor filter, then the acceptor is bleached with intense illumination at its own excitation peak, and a second image is acquired with the donor filter. If photodestruction of the acceptor fluorophore results in brighter donor fluorescence, this is a clear indication that FRET had occurred before acceptor bleaching and that energy had been transferred to the acceptor for as long as the acceptor fluorophore was functional. The different methods of FRET microscopy are discussed in more detail elsewhere.[34–36]

A special field of FRET applications arose with the development of various FRET-based biosensors, which are usually designed in such a way that a sensory domain is situated between ECFP and EYFP, thereby giving intramolecular FRET (Figure 3). A conformational change of the sensory domain leads to a change in the distance between ECFP and EYFP and thus to a change in the FRET signal. The sensory domain can be, for instance, a calmodulin domain for measuring calcium concentrations[38,39] or a consensus substrate site for a protein kinase in combination with a flexible linker and a domain that binds to the phosphorylated substrate region. These and similar biosensor principles were developed for protein tyrosine kinases,[40] protein kinase A,[41] protein kinase B/Akt,[42] phospholipase C,[43] phosphatidylinositol-3,4,5-triphosphate (PIP3),[44,45] cyclic adenosine monophosphate (cAMP),[46] cyclic guanosine monophosphate (cGMP),[47,48] protein kinase C,[49] Ras,[50] and Rho,[51] as summarized in ref. [52]. In certain cases, the sensory domain is not responding by a conformational change but instead contains a cleavage site for specific proteolytic enzymes (such as caspases to monitor apoptosis[53]). The increasing number of FRET biosensors indicates that this general concept will find broader application. An important feature of the biosensor approach is that endogenous enzymes or molecules of interest are monitored (and not artificially overexpressed molecules).

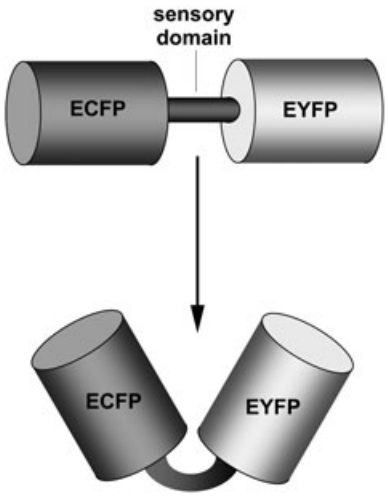

Figure 3. Principle of FRET biosensors. A sensory domain is situated in between ECFP and EYFP (or other appropriate FRET fluorophores). Upon conformational change of the sensory domain due to a cellular parameter affecting the sensory domain, the distance between ECFP and EYFP is changed, thereby resulting in a corresponding change of the FRET signal.

Bimolecular Fluorescence Complementation and Split GFP

It was reported earlier that certain enzymes such as β-galactosidase or β-lactamase lose their activity when they are split into two parts and that they can regain their activity when the two halves are brought together again by linking them to two interacting proteins. The same principle can be realized for fluorescent proteins, such as EYFP, by splitting them into two parts with a resulting loss of fluorescence. Linkage of the two halves to two different proteins results in a restoration of fluorescence if the two proteins interact with each other tightly (Figure 4).[54] This so-called bimolecular fluorescence complementation could be further extended to distinct spectral variants of fluorescent proteins, thereby resulting in multicolor fluorescence complementation analysis, a method suited to visualizing several different interaction processes simultaneously in living cells. Moreover, this method also allows a compari-

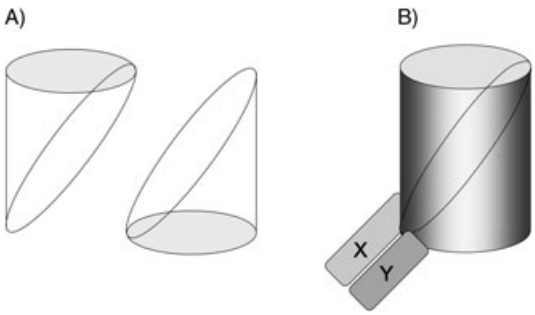

Figure 4. Principle of bimolecular fluorescence complementation. A) A fluorescent protein such as EYFP is split into two halves, which by themselves are nonfluorescent. B) Linking the split parts to two interacting molecules, X and Y, results in complementation and the subsequent formation of a functional chromophore between the two halves of the fluorescent protein.

son of the efficiencies of molecular associations of a given protein with different competing interaction partners.[55] However, it has to be noted that the formation of a functional chromophore between the two halves of fluorescent proteins generates a covalent linkage, and thus a rigid association of the two interacting "host" proteins, thereby eliminating the dynamics of complex assembly and dissociation that are expected to occur with physiological interaction processes. Similarly to the fluorescence complementation approach, protein interactions can also be detected and visualized through reconstitution of split EGFP by protein splicing and intein technology.[56–58] In this context, it is interesting to note that circular permutations, such as rearrangements in the GFP sequence or insertions at certain points, quite often do not destroy the fluorescence but instead lead to fluorescent proteins with new properties suited to biosensors and indicators.[59,60]

Flow Analysis and Fluorescence-Activated Cell Sorting (FACS)

While the intrinsic fluorescence of GFP and some of its variants renders them well suited for flow analysis, the application of fluorescent proteins in this area of methodology appears to be less frequent than in the field of microscopy. This is mainly because flow analysis cannot give the spatial resolution that microscopy can provide. However, the strength of flow analysis is the quantification of fluorescence intensities on a single-cell level for several thousands of cells, combined with the huge possibilities of statistical evaluation. An important application of fluorescent proteins in cytometry is the marking of transiently or stably transfected cells in combination with flow analysis of various cellular parameters, such as apoptosis, cell proliferation, or the presence of specific molecules stained by immunofluorescence techniques.

Besides that, flow cytometry and fluorescence-activated cell sorting (FACS) have become increasingly important in the analysis of transgene mice, where specific cells and their fates in the organism can be tracked by marking them with fluorescent proteins. These applications became even more powerful with the possibility of distinguishing different spectral variants of fluorescent proteins. This can be achieved with appropriate filter sets and it was demonstrated that ECFP, EGFP, and EYFP can be discriminated by flow analysis with 458 nm single-laser excitation.[61] Even four-color flow analysis by including DsRed can be achieved by using a second laser excitation line at 568 nm.[62] Importantly, flow analysis is not only useful for the detection of one or more fluorescent proteins or for their quantification; it can also be applied to study protein interactions based on the FRET effect[63] and specialized software solutions were even developed for that purpose.[64] Until recently, FRET applications in flow cytometry usually required special hardware setups with respect to laser excitation and emission filters. These limiting requirements will most likely overcome with the development of new monomeric orange and red fluorescent proteins,[13] which should represent ideal FRET acceptors with EGFP as the donor, thereby allowing rather simple detection with standard laser and filter sets.

Besides analytical possibilities of fluorescent proteins in cytometry, fluorescence-activated cells sorting (FACS) of cells expressing various fluorescent proteins opens up an additional spectrum of possibilities. The separation of cells of interest (labeled by fluorescent proteins) from other cells allows, for instance, purification before subsequent analysis of proteins, DNA, or other cell components. Moreover, sorting of labeled living cells followed by recultivation enables evolutionary mutation approaches or the enrichment of rare cells, for example, in screening protocols.

We can conclude that GFP and other fluorescent proteins have already proved to be powerful tools in the life sciences but that there is still great potential for novel applications, which have become possible with the occurrence of novel variants and innovative methods to apply them.

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Keywords: analytical methods · cells · fluorescence · fluorescent proteins · proteins

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