Introduction to Fluorescent Proteins

Based on the Introduction chapter of Daniel Thedié's PhD thesis with input from Virgile Adam, Romain Berardozzi, Dominique Bourgeois, Martin Byrdin, Oleksandr Glushonkov, Angela Mantovanelli, Lukas Rane and Jip Wulffele (spring 2021)

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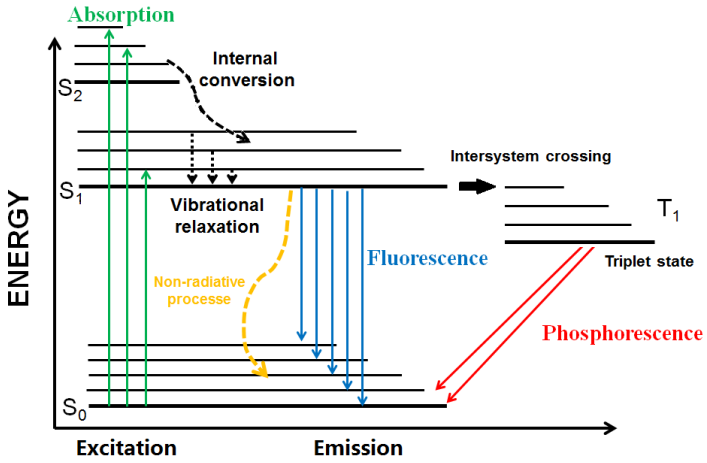
Summary

Fluorescence

Fluorescence is a process by which an electronically excited molecule or atom spontaneously emits a photon in order to relax to its ground state (Ref 1). As shown on the figure (so called Jablonski diagram), when a molecule (e.g. a fluorescent protein's chromophore) in the electronic ground state absorbs a photon of suitable wavelength, it accesses the first electronically excited state. This state is typically short-lived (a few nanoseconds), and rapidly decays back to the ground state, either through the emission of a photon (fluorescence), or by non-radiative relaxation. Alternatively, the singlet excited state can undergo inter-system crossing to the triplet state, which will relax back to the singlet ground-state typically on a millisecond timescale (Ref 2). The unpaired electrons of the triplet state are highly reactive and constitute an important entry point to chemical modification of the chromophore (Ref 3).



Alexandre Jablonski, a Polish physicist, proposed in 1935 a diagram to explain the mechanism of fluorescence. (Ref 4) The thin horizontal lines represent vibrationally excited states.

The fluorescence quantum yield (QY) is defined as the probability of emitting a fluorescence photon upon absorption of a photon. For example a quantum yield of 0.77 means that on average for 100 absorbed photons, 77 fluorescence photons are emitted. The higher the extinction coefficient, the easier the molecule will absorb photons, and thus enter the excited state. Molecular brightness (MB) is defined as the product of the QY and Extinction coefficient, and therefore represents capacity of a fluorescent molecule to emit photons under a given intensity and colour of excitation light. For FPs, one often compares the MB to that of enhanced Green Fluorescent Protein (EGFP), which is about 30000 $M^{-1}.cm^{-1}$

Fluorescence is an essentially background-free signal, to the point that single fluorescent molecules can be observed. Thus, fluorescence forms the basis of many techniques in biological sciences.

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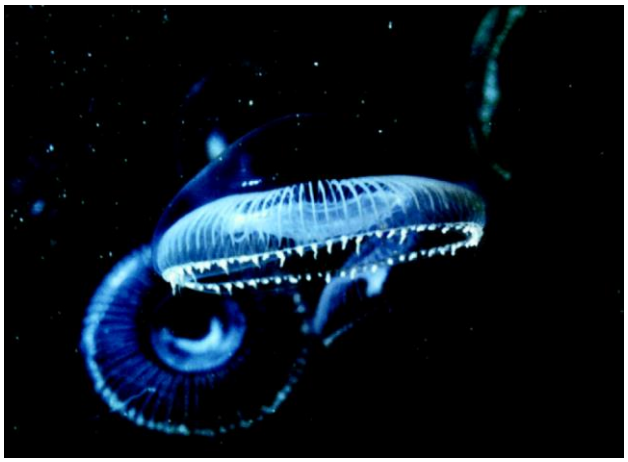
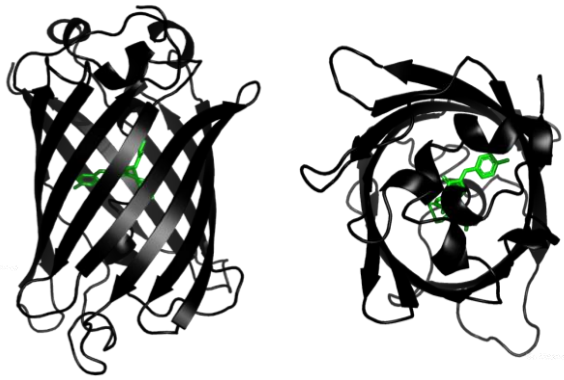
The Green Fluorescent Protein (GFP): Discovery & Structure

In 1962, while trying to isolate the bioluminescent protein Aequorin from the jellyfish *Aequorea victoria*, Japanese researcher O. Shimomura discovered the first known FP: the Green Fluorescent Protein (GFP) (Ref 5). In the jellyfish, in the presence of Calcium ions, Aequorin can to turn into an excited form that relaxes with the emission of blue light that in turn excites the GFP, which ultimately emits green light (Ref 5) giving *Aequorea victoria* its color that, hence, varies from blue to green .



In 2008, the Nobel Prize in chemistry was awarded to O. Shimomura, M. Chalfie and R. Tsien « for the discovery and development of the green fluorescent protein, GFP ». The first structure of the GFP was obtained in 1996 (Refs 7-9) and is shown on the figure below. The protein is made of 238 amino acids, and measures approximately 40 by 20 Ångströms. It is composed of 11 beta-strands arranged as a barrel, inside which an alpha helix bears the three amino acids of the chromophore (Ser65-Tyr66-Gly67). After maturation, these three amino acids form a *para*-HydroxyBenzylideneDimethylImidazolinone (*p*-HBDI) structure, responsible for the light absorption and emission properties of the protein.



View of the structure of the GFP showing the chromophore inside the barrel structure. PDB: 1emb

The barrel structure produces a very tight packing of the interior of the protein, so that the chromophore and water molecules are largely fixed into place by hydrogen bonds, with limited (but not impossible) diffusion of ions or small molecules. This constraining of the chromophore is essential for fluorescence; in fact, the free molecule of p-HBDI is very weakly fluorescent, due to its high flexibility (Ref 10).

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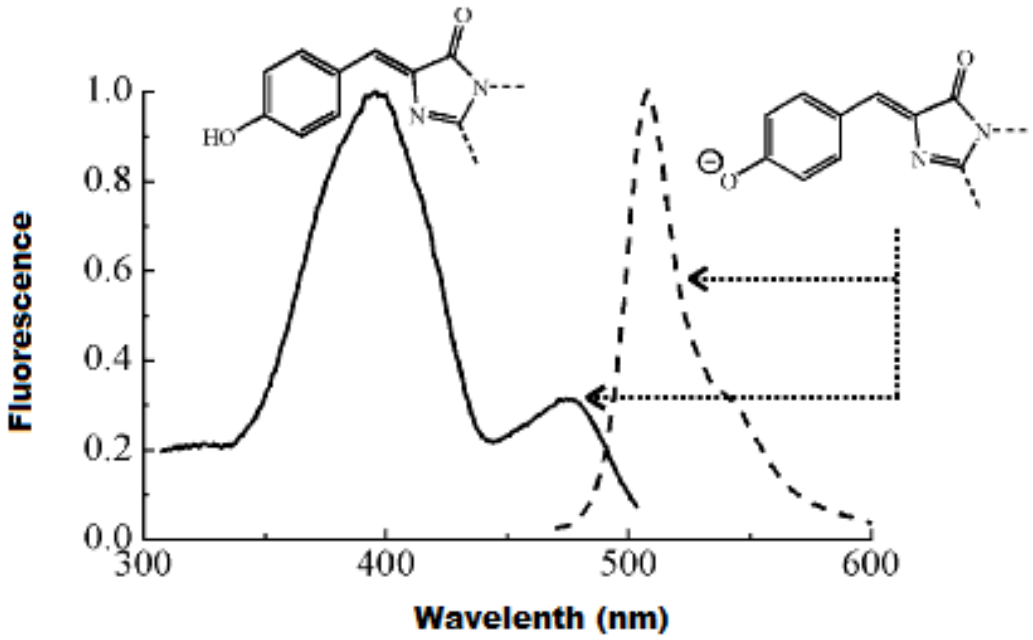
Chromophore: Maturation & Spectral properties

One of the most interesting features of the GFP chromophore is that it does not require a catalytic system to be formed, but is autocatalytically assembled after protein production, requiring only molecular oxygen as a cofactor for its full maturation. The maturation is initiated thanks to the protein environment, which constrains the three amino acids of the chromophore (Ser, Tyr, Gly) in a sharp turn. This strongly favors a nucleophilic attack of the nitrogen of Gly67 on the carbonyl of Ser65 (Ref 11, 12). This is followed by an oxidation of the newly formed ring and a dehydration connecting its electron-conjugation system with that of Tyr66. Whereas Tsien originally proposed that the maturation process should feature dehydration followed by oxidation (Ref 13, 14), recent studies proposed that the dominant pathway would in fact be oxidation followed by dehydration (Ref 15), but this is still debated in the community. In both models, oxidation is described as the rate-limiting step of the reaction, with full maturation occuring in 2 to 4 hours at 37 C°.

While Gly67 is essential for chromophore formation, the other two amino acids (Ser65 and Tyr66) can be substituted, the effect of which will be discussed below.



The excitation spectrum (solid) of GFP has a major band around 395 nm (protonated chromophore) and a minor band around 480 nm (deprotonated chromophore). Excitation at both bands results in green fluorescence emission around 509 nm (dashed).

Interestingly, the protonated chromophore hardly fluoresces by itself. Upon excitation at 395 nm, the protonated residue Tyr66 of the chromophore becomes very acid and it transfers its proton to Glu222 by an H-bond chain creating a deprotonated and fluorescent chromophore. This process is called excited-state proton transfer (ESPT). It is completed by backtransfer of the proton in the ground state (Ref 16).

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Extending the palette: Fishing & Engineering

Following the discovery of GFP, it quickly became clear that the large number of applications and techniques derived from it could greatly benefit from variant proteins with improved or different properties. This has been achieved both by mutations, and by isolating GFP homologs from diverse marine organisms such as jellyfishes, anemones or corals.

The search for protein homologs in other organisms resulted in the discovery of a number of FPs in various organisms such as anemones and stony corals (Ref 17). Among these discoveries were the first red-emitting FP DsRed, as well as the various PhotoTransformable Fluorescent Proteins (PTFPs), which will be described in more detail below.

Mutating FPs to change their properties

Engineering of FPs by introducing mutations in their amino-acid sequence can be done by directed or random mutagenesis, or a combination of both.

Directed mutagenesis is used to change a specific characteristic, based on mechanistic studies. It is a high-risk/high-gain approach: even though rational design can enhance a property of interest, its overall effects are difficult to predict. It often results in the modification of other properties of the protein, or even in the disappearance of fluorescence.

Random mutagenesis on the other hand can be a more cost- and time-effective solution. It can be used to find FP variants with new or improved properties, or applied after directed mutagenesis to rescue lost properties. However, it is not always easy to implement, since it requires a high-throughput screening method for the characteristic of interest.

An intermediate solution is semi-rational engineering, for example by using saturation mutagenesis on a single residue of interest.

To keep track of the multitude of newly found/made FPs, a useful tool has been introduced in 2018 by Talley Lambert at Harvard Medical School. "fpbase.org" is a web-based, user-alimented database which gives rapid access to most FPs interrelations and key properties (Ref 21).

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Key parameters: colour, Brightness, Colour, Monomericity, pKa

Brightness

As mentioned above, the molecular brightness of an FP is defined as the product of its fluorescence quantum yield (QY) and extinction coefficient (EC). Modification of either of these two properties will affect the brightness of the FP.

The QY depends on the mechanical stability of the chromophore. The more constrained the chromophore, the less non-radiative relaxation can occur, and the higher the QY. In FPs, the protein barrel holds the chromophore in a sterically constrained environment, forcing it to have a much higher QY than when isolated in solution.

One drawback of wild-type GFP for microscopy is its limited brightness when excited at 488-nm, due to a large part of the chromophore being in the neutral state (395-nm absorption peak). Even though bright fluorescence could be obtained by excitation at 400-nm through ESPT, this wavelength is phototoxic, and not all microscopes are equipped with the appropriate filter sets. One of the very first published engineering of GFP was therefore the S65T mutation, whichcompletely abolished the neutral form at physicological pH, to the benefit of the anionic one (Ref 22). The resulting protein was called EGFP, for enhanced GFP, and was highly fluorescent when excited at 488-nm. This was a decisive step in making GFP the powerful and widely used marker that it is now.

The S65T mutation has succesfully been applied to other GFP mutants with the same effect (Ref 23), and to a humanised version of GFP designed for transfection into human cells (Ref 24). FP brightness continues to be a major concern for microscopists, as fluorescent proteins are typically 10 times less bright than the best organic dyes on the market. Very bright GFPs have recently been engineered or discovered in nature, such as mNeonGreen (Ref 25) and the superbright AausFP1 from *Aequora Australis* (Ref 26).

Colour

The first colour variations of fluorescent proteins came from the team of R. Tsien in 1994, from mutations of the chromophore's hydroxybenzylidene (Ref 27). Mutating the corresponding pre-maturation tyrosine residue changes the length of the electron conjugation system, and therefore the absorption and emission properties: the GFP Y66H mutant emits blue light, and the Y66W cyan light. Other variants that were generated had slightly red-shifted emission (Ref 7).

In 1999 the first natural red FP was discovered (Ref 17). DsRed was found in a coral and is an obligate tetramer, a property that initially limited its use in biology. However, major engineering of DsRed led to the monomeric mRFP1, which opened the way to a broader use (Ref 28). Later on, mRFP1 was further engineered to yield the mFruit collection of fluorescent proteins, a set of monomeric FPs covering a broad range of colors (image below, Ref 29).

The first and most obvious use of different colors of FPs is to perform multi-color imaging. Distinct emission peaks allow simultaneous tagging of several proteins of interest and opens the way to colocalisation studies. Different colors also open more possibilities for the study of protein-protein interactions using Förster Resonance Energy Transfer (FRET).

Finally, using FPs with red-shifted excitation has proven useful for imaging of living cells, since longer wavelengths are less phototoxic. Their use hence constitutes an advantage for imaging at the tissue or organism level, since red light penetrates better in biological tissues. Therefore, efforts have been made to develop bright, near-infrared emitting FPs (Ref 30, 31).

Monomericity

GFP has a tendency to dimerise at high concentration, due to its compact shape, and the presence of aliphatic residues at its surface, as suggested by its dimeric crystal structure (Ref 8). The dissociation constant (K_d) for the dimer was found to be 0.11 mM for the YFP variant by Analytical Ultracentrifugation (AUC) (Ref 32). Such dimerisation tendency leads to bulkier labels that can influence the function and dynamics of the target proteins. Such effects or aggregate formation cause artefacts in in-vivo studies, especially when looking at abundant proteins, or membrane proteins for which diffusion is constrained in a 2D space. Therefore, Zacharias et al. engineered the S206K mutation in GFP, which perturbed the dimerisation interface by introduction of a positive charge (Ref 32).

A similar, though more difficult problem came up with the discovery of DsRed, which was an obligate tetramer in its wild-type form. Introduction of arginine residues at its surface succesfully converted the tetrameric protein to a dimer, and then to a monomer, but destroyed its fluorescence properties. These could be rescued by extensive random and directed mutagenesis to generate mRFP1 (Ref 28). Of note, the dimeric intermediate generated during the process was further developed as a genetic dimer, which gave rise to tdTomato, still one of the brightest existing FPs.

Since then, monomerisation has been applied to a large number of newly discovered and engineered FPs, and is still one of the prime concerns when generating new FP variants.

pKa

The hydroxybenzylidine moiety of the chromophore exists in two main forms, anionic and neutral, which are visible as different peaks in the absorption spectrum. The respective stability of the two forms depends on the conformation of the chromophore and the surrounding amino acids (which is generally different for ground and excited state chromophore). The pH value at which the neutral and deprotonated forms coexist at equal probability is called the pKa value of the chromophore. As the neutral form is generally less fluorescent than the deprotonated one, this is also the pH value at which fluorescence “appears”. In order to function as visible markers, GFP variants should hence have a pKa value lower than the pH of the studied milieu. Even if the original GFP has a pKa of 4.5, extended engineering has allowed to create FP variants of all colors with pKa values covering the whole physiological range.

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Photostability: Blinking & Bleaching

Fluorescence is not the only way FPs react to light excitation. Under certain circumstances, the excited chromophore can undergo side reactions that lead to a loss of fluorescence. If this fluorescence turn-off is reversible, we speak of fluorophore "blinking". Blinking is immediately visible at the single-molecule level, but not at the ensemble level. If the loss of loses fluorescence is irreversible, the term is "bleaching". Both processes can be regarded as nuissances but have also been put to service in various imaging applications.

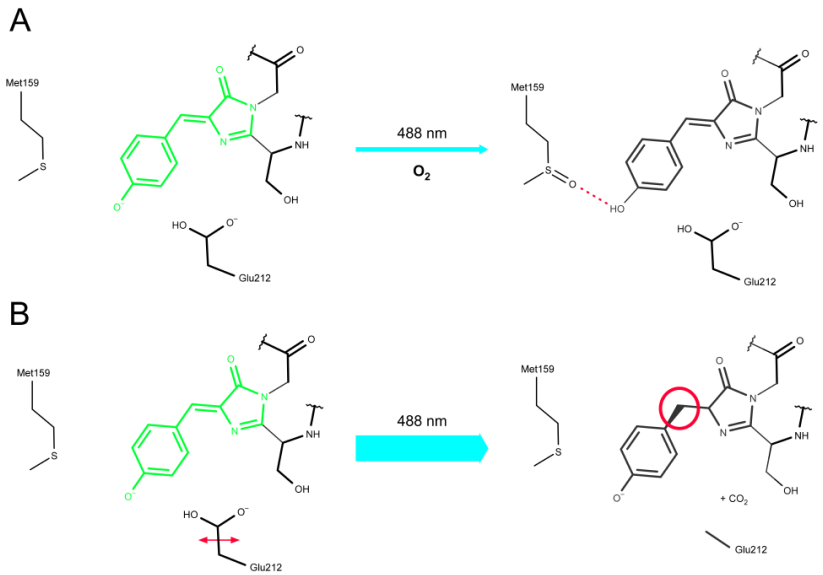
Photobleaching

In microscopy, the loss of fluorescence caused by photobleaching depends on the fluorophore itself and on its nanoenvironment as well as the experimental conditions. Oxygen, temperature, light excitation intensity or wavelength, pH and chemical environment of the chromophore can all affect the photobleaching behavior. The total number of photons a fluorophore can emit before photobleaching is called "photon budget".

It has been reported that at equal illumination intensities, different FPs have very different bleaching half-times (Ref 33). Furthermore, for many tested FPs the photobleaching rate is not linearly dependent on illumination intensity, but has a « supralinear » behaviour. As a consequence, FP photobleaching is usually much faster under high illumination intensities as compared to what would be expected from a linear dependence. The reasons for such behaviour are still debated. One proposed mechanism is that a second photon absorption occurs in one of the excited states of the chromophore, typically the triplet state. Also, several parallel photobleaching pathways may co-exist.

We recently found (Ref 34) that the interplay of available photons and oxygen as a potent triplet quencher can influence the outcome of the bleaching reaction in the phototransformable fluorescent protein Iris FP.

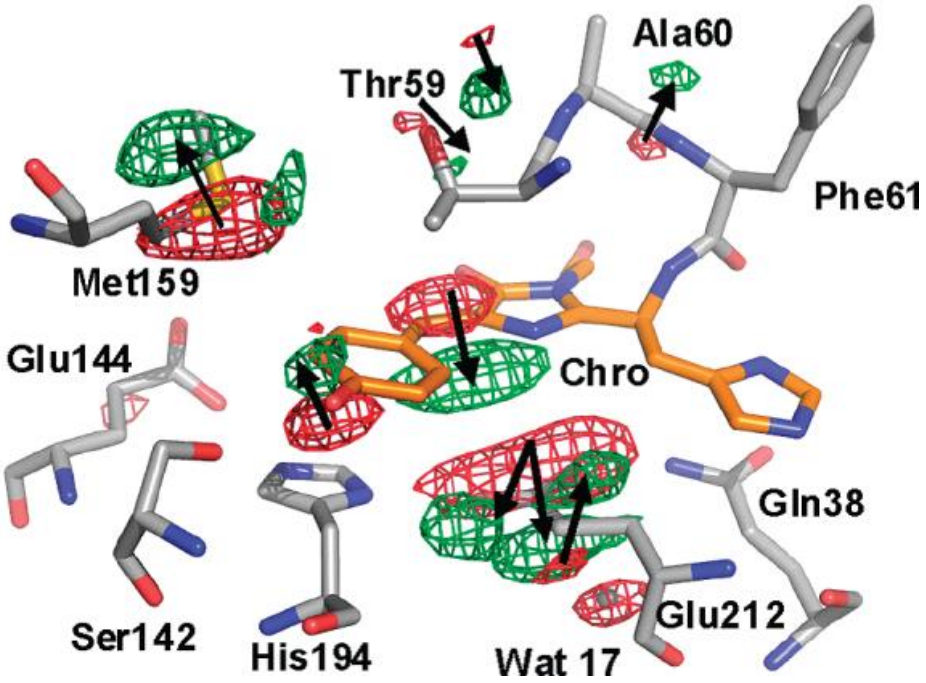
At low excitation-light intensities (< 100 W/cm², Panel A in the picture below), photobleaching is oxygen-dependent, via the sulfoxidation of Met159, located in the chromophore pocket. This results in a locking of the chromohore in the non-fluorescent protonated form. Sulfoxidation is caused by singlet oxygen generated in the chromophore triplet state quenching process (Ref 3).



In contrast, at higher excitation light intensities (> 100 W/cm², Panel B in the picture below), which are typically used in super-resolution microscopy, another, oxygen-independent, mechanism predominates. In this pathway, Glu212 undergoes decarboxylation, resulting in a modification of the hydrogen-bond network around the chromophore, and an sp2- to- sp3 hybridization change of the alpha carbon of the chromophoric hydroxybenzylidene. This hybridization change breaks the conjugation of the electron system, and therefore shifts absorption, preventing visible fluorescence emission.

Photoblinking

At the single molecule level, fluorescent proteins blink upon continuous illumination, switching randomly between a fluorescent on-state and a non-fluorescent off-state. Blinking was first reported by Dickson et al. in 1997, in their observation of single GFP molecules embedded and immobilized in a gel matrix (Ref 35). Like photobleaching, blinking mechanisms are incompletely understood. We initially proposed (see image below) that in IrisFP, a distorted chromophore, potentially due to a coupled electron and proton transfer reaction might be at the origin of blinking (Refs 36, 37). Meanwhile, further studies have yielded a more intricated picture (see more detail below in the Phototransformable FP section).



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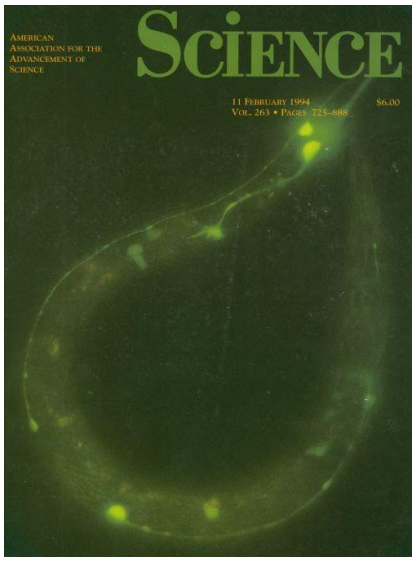
Applications: Sensors & Imaging

After the discovery of GFP in 1962, 30 years passed before it started being used in biological research. This began in 1992, when D. Prasher cloned the gfp gene (Ref 38), and M. Chalfie introduced it in the nematode *C. elegans* (Figure below) (Ref 39). This was a new breakthrough: it showed that GFP can be expressed in foreign organisms, and confirmed that the protein was able to mature without the intervention of a specialised enzymatic machinery.



Finally, this achievement showed that fusing GFP to another protein did not impair GFP fluorescence, nor the function of the target protein. Although this last point varies depending on the targeted protein and should always be a matter of concern when producing new GFP fusions, this "uncomplicated nature" made GFP a tag of choice for a growing number of applications. This section will give an overview of some of the popular uses of FPs in biological research.

Fusing GFP to different target proteins greatly improved the imaging of cellular processes. Thanks to the specific fluorescence emission, the distribution of small and previously undetectable particles could be probed. Furthermore, the genetic encoding and auto-catalytic maturation of GFP made it an outstanding tool for live-cell imaging. This, combined with specific techniques based on GFP properties, such as Fluorescence Recovery After Photobleaching (FRAP), multi-color imaging, or two-photon excitation, enabled the imaging of dynamic cellular processes; for example, secretory and endocytic vesicle trafficking (Ref 40), and bacterial division (Ref 41).

At the time of these first studies, a major bottleneck of protein-tagging with GFP was the insertion of the gfp gene in the cells or organism of interest. The easiest and most common approach was transient transfection, which has the major inconvenience of often causing expression of the protein of interest largely above the endogenous level. Recent progress in molecular biology has made it more and more accessible and cost-effective to perform genome-editing, e.g. using CRISPR-Cas9, enabling endogenous labelling of proteins. Although such a modification still requires more work than a simple transfection, it constitutes an invaluable tool for the study of proteins *in-vivo*, under physiological conditions.

Markers for microscopy: Fluorescent proteins or organic dyes?

In parallel to FPs, organic dyes have been developed and are widely used for fluorescence microscopy. These small molecules are not genetically encoded, but they are brighter and generally more photostable as compared to FPs. So, why still use FPs?

The fact that FPs are genetically encoded constitutes a very big advantage for all *in-vivo* applications and is almost indispensable for imaging on whole organisms or tissues. Using organic dyes often requires permeabilising the cells and using specific buffers that are not always compatible with living cells. Finally, organic dyes do not provide the ideal specificity and one-to-one labelling that FPs do.

In conclusion, although organic fluorophores combine very good imaging characteristics with a wealth of tunable properties, FPs remain markers of choice for labelling of living material, and applications where labelling specificity is crucial. It is therefore expected that FP development will still be of major interest to the fluorescence microscopy community in the coming years.

FP-based biosensors

The fluorescence of GFP constitutes not only a potent marker for biological imaging, but also a useful reporter for different cellular processes and properties. This section will present some of the sensor applications that use FPs.

Gene expression

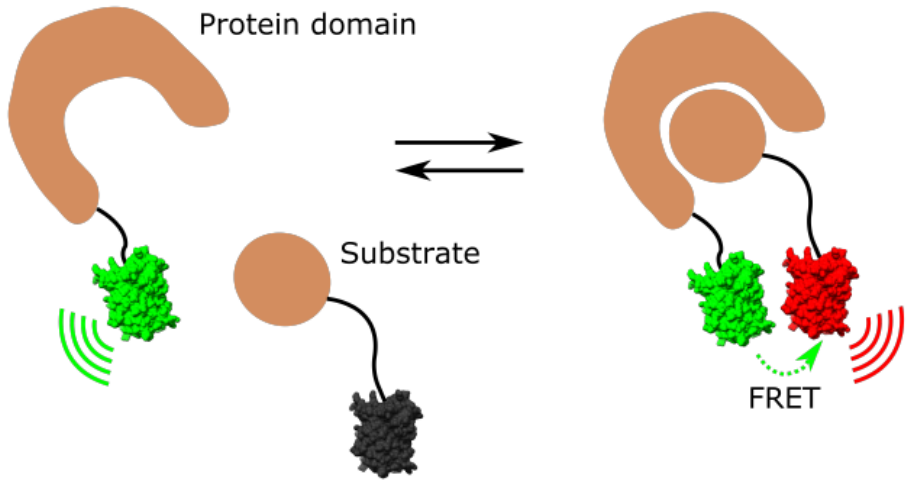
Fusing the gfp gene with a gene of interest is the most straightforward application of GFP to probe the production of a specific protein (even if *in situ* maturation may be an issue). Such labeling can give valuable information on the temporality of gene expression (e.g. during embryonic development), its localisation (specific tissues within an organism, or cells within tissues), or its response to external factors (environmental stress, ingestion of a drug...) (Ref 42).

Protein-protein interactions

Using differently colored FPs allows probing protein-protein interactions. A basic way of assessing such interactions is to fuse each protein of interest to a different-colored FP, and estimate colocalisation of the fluorescence signals. Such an approach, however, does not allow differentiation of protein colocalisation and interaction, and can be challenging if the proteins of interest are present at high concentrations. This method can therefore be suitable to show colocalisation of proteins within a region of interest (e.g. an organelle), but establishing direct interactions between them requires an independent proof.

Probing direct protein interactions has been facilitated by the development of Förster Resonance Energy Transfer (FRET) (Refs 43, 44). FRET relies on the overlap between the emission spectrum of oene fluorescent molecule with the excitation spectrum of another. If this overlap is sufficiently large, one fluorophore can excite the other through a resonance process, which decreases with the 6-th power of the distance between the two probes. Therefore, the FRET signal is maximal when the two fluorophores are in close proximity (< 10 nm), and vanishingly small if they are further apart, making it a valuable detector of close interactions of proteins within living cells. Two main types of FRET experiments can be distinguished. Intermolecular FRET probes the interaction between two proteins by tagging one with the acceptor, and the other with the donor. Intramolecular

FRET probes conformational changes of a protein by labelling it with both acceptor and donor. Intramolecular FRET is rarely performed using FPs due to their size, and the difficulty to label different sites within a protein without altering its function. There exist several FRET based sensors : eg for calcium, IP3, ATP (see next section).



Schematic representation of intermolecular FRET between a protein domain and its substrate. The association brings the two FPs in close proximity, which generates a FRET signal.

Calcium concentration

Calcium ion signalling is implicated in the regulation of a large number of cellular processes (Ref 45). Hence, probing calcium concentration inside tissues, cells or organelles may help to understand their functioning. Some of the first calcium sensors used the calcium-sensitivity of aequorin and led to a number of discoveries (Ref 46). The calcium-sensing abilities of aequorin are however limited: first by its reduced sensitivity range (~100 nM to 10 mM), and secondly by its unsuitability to be targeted to certain compartments, such as the endoplasmic reticulum (one of the major calcium-regulating organelles in the cell). Thus, alternatives were sought, and one of the most used system has been a 4-component fusion construct between a blue- or cyan-emitting FP, calmodulin, the calmodulin-binding peptide M13, and a green- or yellow-emitting FP. Upon calcium binding, calmodulin undergoes a conformational change, and wraps around the M13 peptide, bringing the two FPs close together, which produces a FRET signal (see schematic above). This system has proved to be reliable and usable in different organelles, with a broader calcium-sensitivity range (10 nM to 10 mM) (Ref 47). Since then, calcium sensors have continuously been improved, and applied to biological research (Refs 48, 49).

pH measurements

The pH-sensitivity of the FP chromophore's fluorescence has been exploited to design *in-vivo* pH sensors.

Such an FP-based sensor needs to have a pKa within the range of pH studied, and detectable fluorescence emission from both the neutral and anionic states of the chromophore. If these conditions are met, the pH of diverse cellular compartments can be inferred by calculating the ratio of the fluorescence produced under excitation in each absorption band of the chromophore (Ref 50).

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Smart labels: Phototransformable fluorescent proteins (PTFPs)

Three different types of PTFPs

PTFPs are GFP-like fluorescent proteins that display photochromism properties, meaning that their spectral properties can change under specific illumination. Three main types of PTFPs can be distinguished:

PhotoActivatable Fluorescent Proteins (PAFPs) irreversibly change from a non-fluorescent to a fluorescent state

Reversibly Switchable Fluorescent Proteins (RSFPs) reversibly switch between a fluorescent and a non-fluorescent state

Photo-Convertible Fluorescent Proteins (PCFPs) irreversibly convert between fluorescent states with different absorption and emission colours

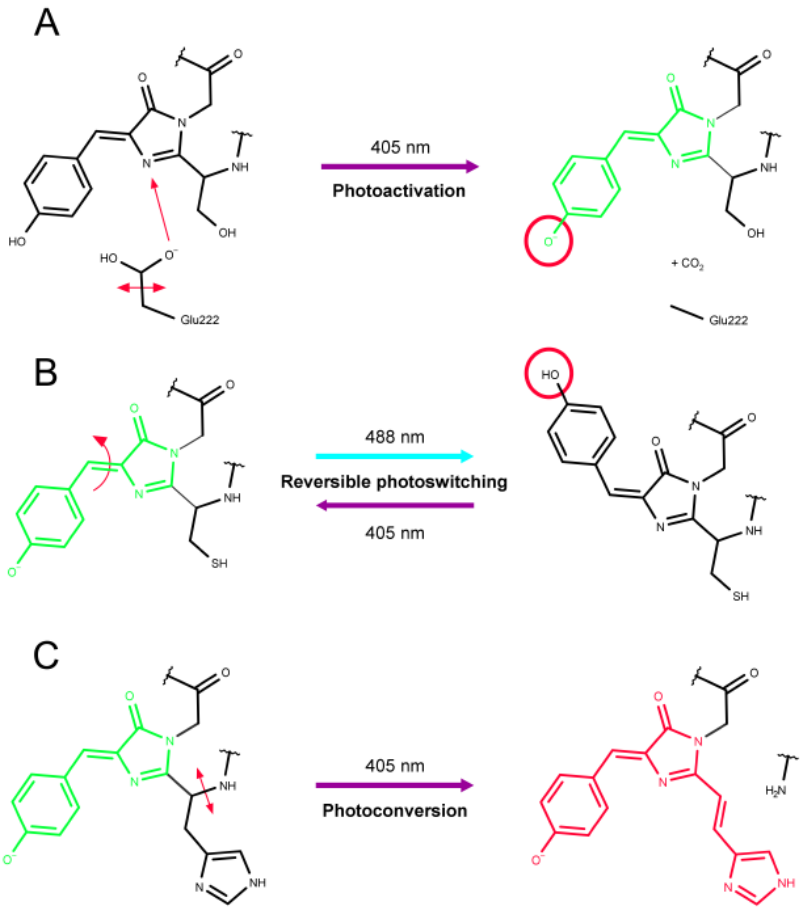
Below we give an overview of the basic characteristics of PTFPs from these three classes.

Discovery

Photochromism in FPs was first observed in the early times of GFP development. In 1997, it was shown that under anaerobic conditions and blue light excitation, GFP changed its emission color from green (508 nm) to red (600 nm) (Ref 51). A similar process termed oxidative redding was later reported, where green-to-red photoconversion occured in the presence of oxidant molecules (Ref 52).

Despite these early findings, photochromism was rarely studied, and perceived more as a nuisance than an asset. However, a few years later, the discovery of coral and anemone FPs with photochromism properties, and the first engineering of PTFPs, changed the situation.

Among these new FPs were the first RSFPs, asFP595 and 22G (engineered to Dronpa) (Ref 53), as well as the PCFPs Kaede and EosFP (Refs 54, 55). Around the same period, GFP was engineered to make the first PAFP, PA-GFP (Photo-Activatable GFP) (Ref 56). This, together with the first applications in super-resolution microscopy triggered huge interest in PTFPs and their engineering.



Categories of PTFPs according to their mechanism. (A) Photoactivatable FPs undergo decarboxylation of Glu222 under illumination at 405 nm, which favors the anionic, fluorescent form of the chromophore over the neutral, non-fluorescent form. (B) Reversibly switchable FPs undergo reversible switching between the fluorescent cis-anionic conformation of the chromophore, and a non-fluorescent trans-protonated conformation. The reaction shown here corresponds to a negative photoswitcher (e.g. Dronpa). (C) In photoconvertible FPs, breakage of a main chain peptide bond under 405-nm illumination results in the extension of the electron-conjugation system to the histidine of the chromophore, and thus to a shift in emission colour. Adapted from Ref 57.

Photoactivatable fluorescent proteins (PAFPs)

The design of the first PAFP was based on the observation that wild-type GFP possessed two main absorbance peaks corresponding to the neutral and anionic chromophore, as described above. Upon UV-irradation however, the neutral form can convert to the anionic form, giving rise to an approximately 3-fold increase in fluorescence upon 488-nm excitation. Furthermore, it had been previously reported that mutation of Threonine 203 affected the equilibrium between the neutral and anionic peaks (Ref 27). Therefore, G. Patterson and J. Lippincott-Schwartz conducted random mutagenesis at this position, seeking to amplify this behaviour. The result of this study was the GFP T203H mutant, termed PA-GFP, which was almost completely non-fluorescent when produced (upon excitation with 488-nm light), and underwent a 100-fold increase in fluorescence upon UV-irradiation (Ref 56).

Some years later, the first photoactivatable red fluorescent protein was engineered from mCherry using a combination of saturation mutagenesis at a few sites of interest, random mutagenesis, and extensive screening of

the produced variants (Ref 58) The resulting protein was called PA-mCherry and enabled two-color applications with PAFPs.

PA-GFP and PA-mCherry have partially similar activation processes, where absorption of near-UV light induces decarboxylation of Glu222, and deprotonation of Tyr66 to form the fluorescent anionic chromophore (Refs 57, 58). This process is irreversible, and once activated, the protein cannot revert to its inital dark state (but will eventually photobleach).

Reversibly switchable fluorescent proteins (RSFPs)

Unlike photoactivation, photoswitching is a reversible process. It generally relies on the combined isomerisation and (de)-protonation of the chromophore to switch the protein between a non-fluorescent and a fluorescent state. Most often, the fluorescent state is the cis anionic state, whereas the trans protonated state is non-fluorescent. Different categories of RSFPs exist depending on whether photon absorption at the peak excitation wavelength induces:

off-switching (negative switchers such as Dronpa (Ref 53)

on-switching (positive switchers such as Padron (Ref 59)

no switching (decoupled switching as in Dreiklang (Ref 60)

Important engineering efforts have been made to improve RSFPs, in terms of brightness, switching efficiency, switching contrast (the ratio between the maximum and minimum fluorescence attainable upon on- and off-switching, respectively), and folding properties (Refs 61-63).

Photoconvertible fluorescent proteins (PCFPs)

The first PCFPs discovered were Kaede (Ref 54) and EosFP (Ref 55) from the stony corals *Trachyphyllia geoffroyi* and *Lobophyllia hemprichii*, respectively. Both of these proteins, upon near-UV illumination, undergo cleavage of a peptide bond linking the chromophore to the protein backbone, which results in an extension of the electron-conjugated system of the chromophore, shifting the main absorbance peak from blue to yellow, and the fluorescence emission from green to red. This is made possible by the composition of the chromophore, which features the amino-acids His-Tyr-Gly in all known green-to-red PCFPs. Like photoactivation, photoconversion is an irreversible process, and photoconverted proteins cannot return to the green-emitting state.

A historical inconvenience of PCFPs was their obligate tetrameric state. However, this initial hurdle was overcome by several rounds of mutagenesis, successively breaking the tetramers into dimers and monomers. Nevertheless, because of this initial tetrameric assembly, some of the monomerised PCFPs kept a tendency to oligomerise at high concentrations. Therefore, some of the subsequent studies focused on designing truly monomeric PCFPs (Ref 64). Popular PCFPs include mEos2 (Ref 65), Dendra2 (Ref 66), Kaede (Ref 54) and mMaple (Ref 67).

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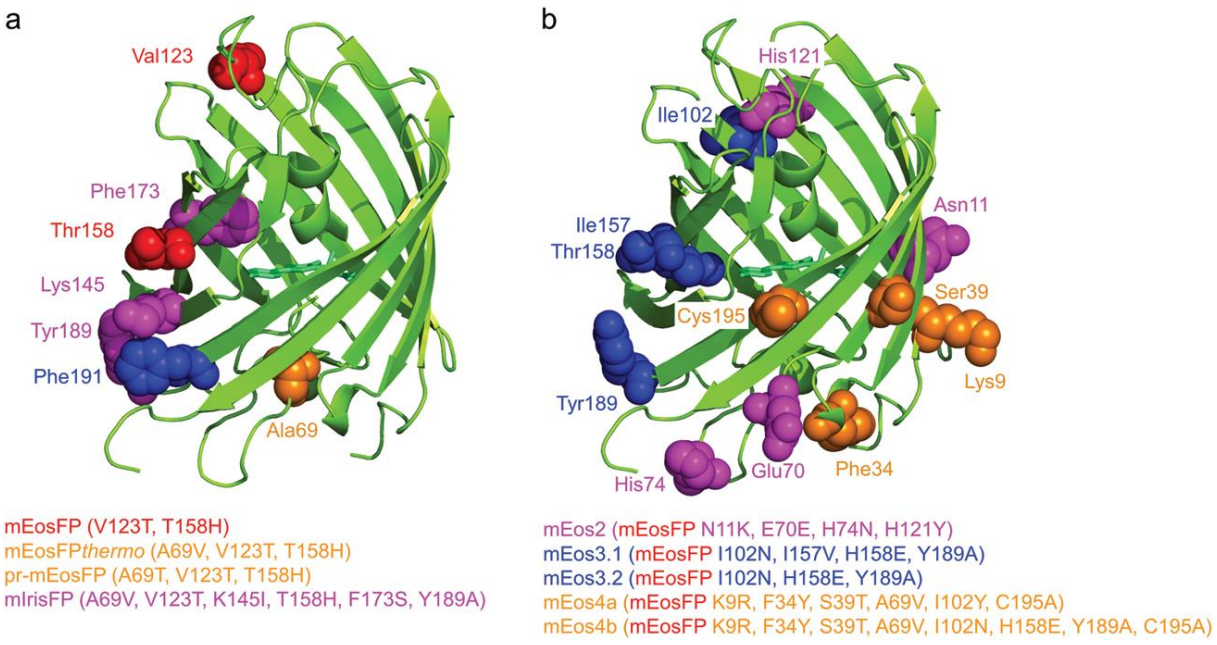
Our working horse: The Eos familiy

Discovery and developments

As mentioned previously, EosFP was one of the first discovered PCFPs, and was soon engineered to a monomeric form called mEosFP (Ref 55). However, mEosFP still suffered from improper folding at 37 C°, which practically prevented its use in mammalian cells. This prompted the development of mEos2, which folded properly at 37 C°, while correctly targeting various cellular structures of interest (e.g. tubulin, histones, or intermediate filaments) (Ref 65). The next development was introduced when it became apparent that mEos2 was not fully monomeric and still induced protein aggregation, in particular in the limited space of membrane-anchored proteins. This led to the design of mEos3.1 and mEos3.2, two truly monomeric variants (Ref 64) as well as that of the robust mEos derivative named PCStar (Ref 67a). Finally, the most recent step came when researchers tried to use Eos proteins in correlative light and electron microscopy, where the FPs need to resist to resin embedding and OsO_4 fixation. This was achieved starting from mEos2, and without damaging other properties such as maturation rate, folding properties or brightness. The resulting protein was named mEos4b (Ref 68). Recently, another mEos derivative that resists Eppon resin embedding was also engineered, named mEosEM (Ref 68a). Mutations introduced in the developed EosFP variants are summarised in the figure below.



(a) Modifications of EosFP to obtain monomeric mEosFP (red), the thermostable mEosFPthermo and the primable pr-mEosFP variants (orange), and mIrisFP (magenta). (b) Additional modifications of mEosFP to obtain mEos2 (magenta), mEos3.1 and mEos3.2 (blue) and mEos4a and mEos4b (orange). With permission from Ref 69 (« Published by The Royal Society of Chemistry »).

All along their development, Eos proteins have been very popular for microscopy, and especially super-resolution approaches. This is mainly due to the fact that they are brighter than PAFPs and other PCFPs (as shown on the table below for a few popular PA- and PCFPs). Moreover, their green fluorescence can be used to locate features of interest before imaging of the red state, which is an advantage over PAFPs. As a result, mEos2,

and now its derivatives, have been extensively used in diverse microscopy experiments requiring photochromism.

FP	Extinction coefficient ($M^{-1}.cm^{-1}$)	Fluorescence QY	Brightness
mEos2 (red)	46000	0.66	30.36
mEos3.2 (red)	32200	0.55	17.71
mEos4b (red)	55500	0.71	39.41
PA-GFP	17400	0.79	13.75
PA-mCherry2	24000	0.53	12.72
Dendra2 (red)	35000	0.55	19.25
Kaede (red)	60400	0.33	19.93
mMaple (red)	30000	0.56	16.8

Comparison of FP brightnesses between Eos variants, PAFPs and other PCFPs. For PCFPs, numbers are given for the red form, which is the one typically imaged in super-resolution. Data obtained from FPbase (fpbase.org). QY: Quantum Yield.

IrisFP: combining properties

IrisFP, designed from EosFP, was the first engineered biphotochromic protein. On top of retaining the photoconversion ability of its parent EosFP, it was also able to undergo efficient reversible switching, in both its green and red forms. This became possible by the F173S mutation, which resulted in the creation of two cavities near the chromophore, and thus allowed its isomerisation (Ref 70). This protein however retained its tetrameric character, until a variant with similar properties was engineered directly from mEosFP and named mIrisFP (Ref 71). Interestingly, another biphotochromic variant (pcDronpa) was later designed using the RSFP Dronpa as a template and enabling it to photoconvert to a red-emitting state (Ref 72). Like IrisFP, it is tetrameric, but a noteworthy difference is that is does not photoswitch in the red state.

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Our dayly bread: Understanding the molecular mechanisms underlying phototransformations

The study of photochromism brought renewed interest to structural studies on fluorescent proteins. Solving the structure of new FPs and their different states by crystallography helped understand how light-induced structural modifications could lead to reversible and irreversible spectral changes.

More recently, setups have been designed to record absorbance and fluorescence spectra on protein crystals, thus allowing a direct correlation between spectroscopically observed changes and differences in protein structure (Ref 73). In parallel, time-resolved crystallography allowed deciphering the dynamic processes behind photochromic behaviours and was recently pushed to the picosecond timescale using X-Ray Free Electron Lasers (XFELs) (Ref 74). Finally, NMR has recently been shown to be very useful complementary tool to study PTFPs structural dynamics in solution (Refs 75, 75a). Such studies are still ongoing and will surely bring more insights into the functionning of PTFPs, and clues for their improvement.

PCFPs display complex photophysical behaviours

As stated previously, PCFPs such as mEos2, mEos3.2 or mEos4b are widely used in advanced microscopy experiments. However, their photophysics are in fact not as straightforward as was initially thought, and it appeared that several light-induced processes occurr beyond green-to-red photoconversion. Understanding these processes can help to take into account their effects in imaging experiments, and to avoid related artefacts. This section aims at giving an overview of such behaviour in PCFPs of the Eos family, including incomplete photoconversion, blinking, switching, and conversion to the triplet state.

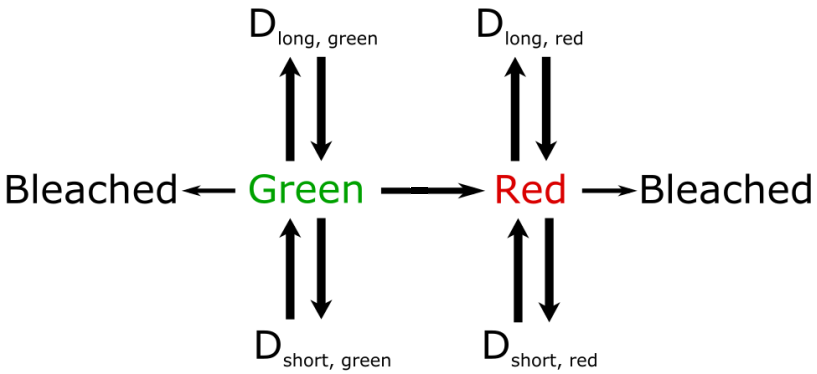
Incomplete photoconversion

A commonly reported issue with PCFPs is the incomplete photoconversion of the protein, which results in a fraction of the labels remaining undetected in fluorescence images of the red state. For example, mEos2 showed a photoconversion efficiency of ~ 60%; meaning that 40% of the FP labels were not seen (Ref 76). Reasons for this include incorrect folding of the FP, non-maturation of the chromophore, or early photobleaching in the green or red states (i.e. before the protein has been photoconverted or has emitted enough red photons to be detected).

PCFP Blinking is "incomplete switching"

Since its first observation in gel-embedded GFP, blinking has been observed in different fluorescent proteins including PCFPs such as mEos2 and Dendra2 (Refs 77-79). In PCFPs, two blinking regimes have been characterised, both in the green and the red states: a short-lived one, with durations up to ~100 ms, and a longer-lived one, which can last for several seconds. The corresponding photophysical model is depicted on the scheme below. In the red form, the short-lived dark-state is thought to be a non-absorbing radical state, and therefore recovers to the fluorescent state by thermal relaxation, with a rate constant of 15-20 s^{-1} (Refs 80, 81). The nature

of the long-lived dark-state, on the other hand, has been reported to be sensitive to 405-nm illumination (Refs 78, 79) and has recently been shown, in the case of mEos4b, to be caused by « frustrated » cis-trans isomerization coupled to protonation of the chromophore, essentially similar to photoswitching in RSFPs (Ref. 80). A similar frustrated isomerization has been characterized also for the green form of mEos4b (Ref 81a).



Basic photophysical scheme for blinking in PCFPs (adapted from Ref 79). Once photoconverted to the red state, the protein can enter a short-lived dark-state (responsible for fast blinking), a long-lived one (responsible for slow blinking), or irreversibly photobleach. Similar dark states exist in the green state of the protein.

Different PCFPs have different blinking properties

Although few comparative studies exist, previous work in the lab by former PhD student Romain Berardozzi has shown that different PCFPs have different blinking behaviours, like in the case of Dendra2 and mEos2 (Ref 79). These two proteins were found to be different in that aspect: while both showed blinking, mEos2 had a high propensity to blink towards long-lived dark states, and was relatively resistant to photobleaching, while Dendra2 was more prone to photobleaching, which resulted in a lower apparent blinking propensity. Interestingly, another finding in that project was that a single point mutation (A69T in mEos2 or T69A in Dendra2) was enough to invert completely the properties of both proteins, reducing blinking and increasing photobleaching in mEos2 while increasing blinking and decreasing photobleaching in Dendra2.

Dependence of blinking on the experimental conditions

Not only the intrinsic properties of the fluorophores, but also the experimental conditions have been reported to affect blinking of FPs, the main factors investigated being illumination intensity (at different wavelengths) and the physicochemical environment.

Light-sensitivity of blinking. Several blinking processes in different FPs have been reported to be light-induced, including recovery from dark-states and transition from the fluorescent to blinked states (Refs 35, 78, 79, 81). This light sensitivity arises from the fact that non-emissive states are typically reached through the S1 excited state. Recovery to the fluorescent state can happen thermally if the dark state is of higher energy than the S0 ground state. If the dark state absorbs light, its transition to an excited state can speed up considerably its recovery to the fluorescent state. The rate of transition from one state to another will depend on: (i) illumination intensity, (ii) absorbance of the starting state at the illumination wavelength, and (iii) probability of the transition occuring per absorbed photon (also called phototransformation quantum yield, not to be confused with the fluorescence quantum yield described earlier). In analogy to the brightness of a fluorophore, one can refer to the

phototransformation brightness, which is the product of the absorption coefficient at the illumination wavelength, and the phototransformation quantum yield.

Sensitivity to the chemical environment. Some studies have probed the behaviour of FPs in buffers with different chemical compositions. Notably, reducing agents have been found to increase blinking in mEos2 and mEos3.2 (Refs 82, 83). Even though only small molecules are able to diffuse in the beta-barrel of FPs to directly interact with the chromophore, bigger molecules can initiate long-range electron transfer reactions, thus also affecting FPs photophysical behaviour. Further studies of the influence of buffer composition on FPs could be of interest, given the strong dependence of organic dyes photophysics on these parameters (Ref 84, 85).

Conversion to the triplet state

As mentioned earlier, FPs can undergo inter-system crossing from the S1 singlet excited state to the T1 triplet state. Different values have been reported for the yield of inter-system crossing (0.1 % (Ref 86) to 1% (Ref 3)), as well as for the lifetime of the triplet state (from µs (Ref 86) to ms (Ref 3)). In any case, the lifetime is much shorter than typical exposure times in widefield fluorescence microscopy, meaning transition to the triplet state is not directly observed as intermittencies in fluorescence emission, contrary to the case of blinking.

Even though this process is not directly observable in microscopy experiments, it can still be highly detrimental. Its most straightforward consequence is the reduction of the total number of photons emitted by a protein during a single camera exposure, reducing the apparent brightness of the fluorophore. A recent study in the lab has furthermore proposed that the triplet state could serve as an entry point for further chemical reactions, including photobleaching and blinking: the findings suggest that electron acceptors can abstract an electron from the chromophore in the triplet excited state, yielding a chromophore radical state (Ref 3). Such a radical state was previously proposed to be at the source of short-lived blinking in PCFPs (Ref 40).

However, triplet-mediated photochemistry is not necessarily detrimental to microscopy experiments. In 2015, a study demonstrated the possibility to photoconvert the Dendra2 PCFP by using a combination of 488- and 642- or 730-nm light instead of the traditional 405-nm light (Ref 87). This technique, called "primed photoconversion", allows photoconverting certain PCFPs without using potentially phototoxic 405-nm light. A later study by the same authors suggested that primed photoconversion proceeds from a triplet state (Ref 88).

Overall, the discovery of PTFPs and the progressive understanding of their photophysics opened the way to the use of FPs in SMLM, but also points at the extreme difficulty to precisely understand the fluorescence traces recorded from single molecules and to extract quantitative information from them.

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FP function: A dog walking on his hind legs?

In a 2015 paper dedicated to the incomplete maturation of FPs, Yewdell and colleages paraphrased Samuel Johnson: “A fluorescent protein is like a dog walking on his hind legs. It is not done well; but you are surprised to find it done at all” (Ref 89).
Most FP-harbouring species live in a low light environment (shallow to deep water) where little sunlight penetrates and a change of colour as produced by the redshift of fluorescence with respect to shorter-wavelength excitation, can make a difference for being seen or not seen, depending on the spectral sensitivity of the observer. Accordingly, proposed FP functions of biological relevance include sexual or prey attraction, or vice versa, camouflage.

Interestingly, it appeared that not all organisms expressing FPs did so for bioluminescence purposes. Some marine organisms growing in shallow water seem to use them for protection against short-wavelength irradiation (Ref 18, image below), whereas others growing in deeper water (where mostly blue light is available) may use them to produce longer-wavelength light for their photosynthetic symbiotes (Refs 19, 20).

Moreover, photoconversion in PCFPs also appears to be an evolved process, and to be playing a role in stony corals - contrarily to PAFPs and RSFPs, which rather result from the engineering of properties largely shared by the different FP families. Stony corals are symbiotic organisms, made from the association of a cnidarian host, and a unicellular alga. They grow in shallow waters, which means they are exposed to strong sunlight irradiation. In this context, PCFPs would be produced as a means to protect the algae's photosynthetic system from strong irradiation.

The tetrameric structure has an important role here: under the natural UV exposition from sunlight, some of the proteins in the tetramer convert to the red-emitting form and can thus serve as FRET acceptors for the green-emitting proteins. This has the net effect of absorbing the high-energy and potentially harmful blue light, and re-emitting red light, which is less damaging and more useful for photosynthesis.

From a broader perspective, the relevance of the FP architecture (« self-forming chromophore in a protective barrel ») serving as a scaffold for endlessly sophisticated marker constructs can hardly be overestimated for its use in biological research and beyond.

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