Flavin Mononucleotide-Binding Fluorescent Proteins

# FLAVIN MONONUCLEOTIDE-BINDING FLUORESCENT PROTEINS

Aba Losi, Cristiano Viappiani

## Affiliation

Department of Mathematical, Physical and Computer Sciences University of Parma,
www.unipr.it
Parma
Italy
e-mail: aba.losi@unipr.it;
cristiano.viappiani@unipr.it

Synonims
FP: fluorescent protein
FMN=flavin mononucleotide = riboflavin 5'-phosphate.
FbFPs: FMN-binding fluorescent proteins
LOV (light, oxygen or voltage) domains
$\Phi_F$ = fluorescence quantum yield
GFP = Green Fluorescent Protein

## Introduction

Flavin mononucleotide (FMN)-binding fluorescent proteins (FbFPs) are genetically-encoded reporters for cell microscopy engineered from photoreceptors of the LOV (Light, Oxygen Voltage) superfamily (Buckley 2015). FbFPs exhibit fluorescence in the green region of the spectrum with quantum yield typically around 0.3-0.4 (Table 1). Different to prototypical FP of the green fluorescent protein (GFP) type, FpFBs are small and minimally perturbative fluorescent reporters whose maturation is oxygen independent (Mukherjee 2013). Additional advantages are their pH and thermal tolerance, oligomeric state stability, resistance to strongly reducing conditions, ability to photosensitize reactive oxygen species (ROS) and photochromicity. In the last decade FbFPs have been exploited for diverse applications, lately also as tools for Correlative Light and Electron Microscopy (CLEM)(Shu 2011), as donors in (Forster Resonance Energy Tranfer (FRET) pairs for oxygen (Potzkei 2012) and pH sensing

(Rupprecht 2017), as singlet oxygen generators for biotechnological applications (Souslova 2017), and as heavy metal sensors (Ravikumar 2015).

**Basic characteristics of FbFPs**

FbFPs are enginereed from LOV domains (604), photoresponsive protein units of ca. 110 amino acids in length, where the FMN chromophore (152) is non covalently bound in the dark adapted state and brightly fluorescent with quantum yield $\Phi_F$ ca. 0.2-0.3 (Losi 2017). UVA/Blue-Light excitation leads to the reversible formation of a FMN-cysteine covalent adduct, with complete loss of fluorescence (referred to as $LOV_{390}$, from the absorption maximum). FbFPs have been first introduced as fluorescent reporters of choice for anaerobic or microaerobic environments (Drepper 2007); this aspect still represents their main advantage over GFP (Green Fluorescent Proteins) and structurally related FPs, where chromophore formation implies post-translation maturation and is oxygen-dependent (Thorn 2017). Given that in LOV domains fluorescence is lost upon formation of $LOV_{390}$, this last process can be annihilated by mutating the reactive cysteine (to alanine, glycine or serine), thus yielding a permanently fluorescent molecule, with $\Phi_F$ presently comprised between 0.13 and 0.51 and brightness ($\Phi_F \times \varepsilon$) between 1850 and 6380 $M^{-1}cm^{-1}$(Table 1). In the seminal work of Drepper et al., the bacterial proteins YtvA (*B. subtilis* Uniprot code O34627 ) and SB2 (*P. putida,* UniProt code Q88JB0) were used as starting points to engineer BsFbFP (full length protein, 261 aa) and PpFbFP (aa 3-151) respectively, changing the reactive cysteine into alanine; BsFbFP was further truncated to encompass solely the LOV domain (aa 1-137) and codon-optimized for *E.coli* expression, yielding EcFbFP (Drepper 2007). These novel FP were tested in the facultative aerobe *Rhodobacter capsulatus* and in mammalian cells; as expected they were found to be fluorescent also under $O_2$ depletion. Soon after iLOV was engineered from *At*phot2 -LOV2 and tested for studying the dynamics of viral infections in plants and animal cells (Chapman 2008). iLOV bears six mutations (R386F, S394T, S409G, C426A, I452T, F470L) able to enhance fluorescence to $\Phi_F$ = 0.32 and minimize irreversible photobleaching. Tagging viruses with GFP-derivatives often results in decreased infectivity and loss of FP through recombination events, due to the limited size of viral genomes and high recombination rate; the smaller size of iLOV (ca 11kDa, ca 55% the size of GFP) seems to overcome this problem, allowing optimal packing within the genetic material of a virus(Chapman 2008). Furthermore, different to GFP, post-translational maturation of the FMN fluorescent chromophore is not needed and this allows good visualization of the infection dynamics, within minutes. Additionally the small size permits other applications where steric constraints might impair protein translocation, the concept has been applied to proteins

important during *E. coli* infections (Christie 2012a.). Several variants of iLOV have been developed bearing additional mutations, with the aim of increasing photostability, among which phiLOV2.1 revealed that the N390S and N401Y changes are crucial to this goal, by anchoring and rigidifying the chromophore (Christie 2012b). Extensive characterization of iLOV, Pp2FbFP and EcFbFP demonstrated further useful qualities of LOV-based FP: a broad functional pH range with a considerable percentage of fluorescence retained between pH 4 and 11; a quite high thermal stability, up to 60°C for iLOV; persistence of fluorescence under strongly reducing conditions, up to a reduction potential 0f -660 mV; stability of the oligomeric state, being iLOV a monomer and the other two proteins stable dimers; reliable detection of protein expression thanks to fast and complete maturation, even in bioprocesses that imply semi-aerobic or anaerobic stages (Mukherjee 2013; Shcherbakova 2015).

**Novel FbFPS and novel applications**

Strictly related to iLOV is miniSOG (mini Singlet Oxygen Generator) designed from *At*phot2-LOV2 as a genetically encoded photosensitizer to be employed for CLEM (Correlative Light Electron Microscopy)(de Boer 2015). The basic idea was to combine fluorescence imaging with the high resolution of EM microscopy, provided that miniSOG was able to sensitize sufficient excited singlet state $^1O_2$ ($a^1\Delta_g$) (shortly $^1O_\Delta$) (168) to locally catalyze polymerization of diaminobenzidine (DAB) into a precipitate that can be stained with osmium (Shu 2011). Shu et al. succeeded to discriminate the localization of two closely related synaptic cell-adhesion molecules in cultured neurons and in intact mouse brain. MiniSOG CLEM applications have become countless and this approach appears superior, as for discrimination and resolution, to methods relying on immunolabeling and chemical staining (Ellisman 2012; Rodríguez-Pulido 2016; Follain 2017; Hirabayashi 2017; Lee 2017; Souslova 2017) In particular, miniSOG is substantially smaller than GFP-based FPs, has low toxicity, and produces strong EM contrast (Hauser 2017). A crucial question is the value of $^1O_\Delta$ formation quantum yield ($\Phi_\Delta$) for miniSOG, initially reported as 0.47 (Shu 2011) and later downscaled to 0.03 by direct detection of $^1O_\Delta$ phosphorescence (Ruiz-González 2013). Previous overestimation of $\Phi_\Delta$ was probably due the employement of anthracene-9,10-dipropionic acid as $^1O_\Delta$ sensor, a molecule that can be oxidized also by other reactive oxygen specie (Pimenta 2013). Notwithstanding the relatively low value for $\Phi_\Delta$, E. coli cells expressing miniSOG were successfully use to test a novel probe for intracellular for $^1O_\Delta$ (Ruiz-González 2017). Further LOV-based fluorescent photosensitizers have been later designed with $\Phi_\Delta$ comprised between 0.09 and 0.39 (Torra 2015; Westberg 2015, 2017; Rodríguez-Pulido 2016).

Since their first applications, researchers have tried to further improve FbFPs: the relatively low brightness, generally one order of magnitude below GFP-related FP, has been slightly improved by engineering novel proteins from the algae *Chlamydomonas reinhardtii* (CreiLOV) and *Vaucheria frigida* (VafLOV); CreiLOV has indeed a large $\Phi_F$ = 0.51 and the largest brightness (6375 $M^{-1}cm^{-1}$) so far reported for a FbFP, but the relatively low absorbtion coefficient of flavins in the visible region, < 15000 $M^{-1}cm^{-1}$, intrinsically limits brightness (Mukherjee 2015) (Table 1). Thermal and photo stability could be further improved with novel FbFPs from thermostable bacteria, among which the most promising for applications are derived from *Meiothermus ruber* (MrFbFP) and from a metagenomic sequence from Yellowstone park ("Chocolate Pots", YNP3FbFP). The group of newly characterized FbFPs from thermostable organisms shows a remarkable array of different fluorescence lifetimes, from 1.5 to 4.6 ns, making them promising candidates for multispecies labeling within a fluorescence lifetime image (FLIM) approach.(Mukherjee 2015).

The strongest limitations of FbFP are represented by their relatively low brightness and by the difficulties in tuning their absorption and fluorescence maxima maxima towards the red flank of the visible spectral range, that is considered the most useful for animal cell applications (Shcherbakova 2015). The mutation Q489K in iLOV was predicted to have strongly red shifted absorption and fluorescence spectra (Khrenova 2015), but experimental characterization of this variant has shown instead an 8 nm blue-shift (Davari 2016). Recently it was proposed that FbFPs could be spectrally tuned and enhanced in fluorescence by means of structurally modifed chromophores, such as lumichrome and 7-methyl-8-chloro-riboflavin (8-Cl-RF) (Arinkin 2017). Apoprotein W619_1-LOV from *Pseudomonas putida* (strain W619) bound to lumichrome increased its $\Phi_F$ to 0.4 and was ca. 30 nm blue-shifted. This approach is extremely interesting for elucidating the structural and chemical factors that affects photophysical parameters of FbFPs, but difficult to export to *in vivo* experiments, given that solely riboflavin derivatives such as FMN, are ubiquitous.

Fluorescence applications of FbFPs have been recently extensively reviewed, in most cases they are related to anaerobic and micro-aerobic environments and hypoxic niches (Buckley 2015; Sanford 2017; Rodriguez 2017; Thorn 2017). They have assumed precious roles as real-time reporters for cell processes and host-microbe interactions involving microorganisms of great medical or technological importance, e.g. *Listeria* (Landete 2017), *Porphyromonas gingivalis*, (Choi 2011) *Saccharomyces cerevisiae* and *Candida albicans*,(Eichhof 2016)

*Synechocystis sp.* (Immethun 2017), *Trichomonas vaginalis* (Wang 2017), *Clostridium* (Buckley 2016), *Shigella* and *E. coli* (Gawthorne 2016) (figure 2). Another fluorescence-based FbFP application is the in-cell sensing of heavy metals ions, such as mercury (Ravikumar 2016) and arsenic (Ravikumar 2017).

Given their intrinsic photochromicity (Kennis 2004; Chang 2017) wt-LOV domains can also function as LOV-based FPs. Photochromicity of *Bs*YtvA has been proposed for super-resolution microscopy methods based on photoswitchable fluorescent proteins, using blue- (for complete swith-off of fluorescence) and violet light (random recovery of fluorescence at single molecule level) (Losi 2013; Pennacchietti 2014) (figure 3). Violet and ultraviolet light, by exciting both $LOV_{390}$ and $LOV_{447}$, drive the system into a photoequilibrium, whereas BL fully converts $LOV_{447}$ into $LOV_{390}$ (Song 2013). The *in vivo* relevance of UV/violet-driven photoconversion is unknown for LOV proteins (van der Steen 2015) but this property could be useful to visualize LOV proteins within their natural host without labeling and, furthermore, it could combine the power of super-resolution fluorescence microscopy with optogenetics: different to canonical FbFP, wt LOV domains are in fact able to photoactivate fused effector domains (figure 4).

FbFPs can function as donors in Förster resonance energy transfer (FRET) pairs, an approach that has been used for visualizing intracellular changes in oxygen levels (Potzkei 2012) and pH (Rupprecht 2017). In the former case, FluBO was built with EcFpFB as donor and Enhanced Yellow Fluorescent Protein (EYFP), that become fluorescent only when $O_2$ is present. FluBO was calibrated in *E.coli* cells expressing the fused construct, that changes its fluorescence properties depending on oxygen concentration at the time that the fluorophore of EYFP matures (Potzkei 2012). In a similar approach, EcFbFP was fused EYFP having different $pK_a$: given that EcFbFP fluorescence is tolerant towards acidic pHs ($pK_a \sim 3.2$) EcFbFP as donor domain was fused to EYFPs with $pK_a$ values of 5.7, 6.1 and 7.5. This FRET toolbox was called FluBpH (Rupprecht 2017). Finally a fused protein comprising the LOV domain of *Bs*YtvA-C62S and a bilin-binding, photochromic (red/green absorbing form) GAF domain from a cyanobacteriochrome was recently characterized and found to constitute a good and minimal FRET pair with three-color fluorescence (Simon 2017).

As a whole, LOV-based FbFPs offer certain advantages over GFP-related proteins owing to their smaller size, pH and thermal tolerance, oligomeric state stability, utility under anaerobic conditions and ability to generate singlet oxygen. FbFbs seem nevertheless to have intrinsic applicability limitations due to relatively low brightness and difficult spectral tunability.

**Table 1. Photophysical parameters of FbFPs**

| **Protein** | **$\Phi_F$** | **Brightness/ $M^{-1}cm^{-1}$** | **$Abs_{max}$/ nm** | **$Fluo_{max}$/ nm** | |
|---|---|---|---|---|---|
| BsFbFP | 0.39 | 5420 | 449 | 495 | (Drepper 2007; Wingen 2014) |
| EcFbFP | 0.34-0.44 | 6380, 4250 | 448 | 496 | (Drepper 2007; Mukherjee 2013; Wingen 2014) |
| Pp2FbFP | 0.17, 0.22 | 2125, 3120 | 449 | 495 | (Drepper 2007; Mukherjee 2013; Wingen 2014) |
| Pp2FbFP-F37S[a] | 0.30 | 4260 [b] | 450 | 497 | (Mukherjee 2012) |
| Pp2FbFP-F37T | 0.24 | 3400 [b] | 450 | 498 | (Mukherjee 2012) |
| Pp2FbFP-Q116V | 0.26 | 3930 | 439 | 485 | (Wingen 2014) |
| Pp2FbFP-Y112L | 0.30 | 4200 | 449 | 496 | (Wingen 2014) |
| Pp1FbFP | 0.27 | 3750 | 450 | 496 | (Wingen 2014) |
| DsFbFP | 0.35 | 5000 | 449 | 498 | (Wingen 2014) |
| Pp2FbFP-L30M | 0.25 | 3550[b] | 448 | 494 | (Torra 2015) |
| iLOV | 0.32-0.33 | 4880, 4250 | 447 | 493 | (Chapman 2008; Mukherjee 2013; Davari 2016) |
| iLOV-Q489K | 0.35 | 5630 | 440 | 489 | (Davari 2016) |
| miniSOG | 0.40 | 5820 | 447 | 497 | (Shu 2011; Wingen 2014) |
| phiLOV2.1 | 0.20 | 2500 | 450 | 496 | (Christie 2012b) |
| MrFbFP | 0.22 | 3340 | 448 | 498 | (Wingen 2017) |
| TeFbFP | 0.13 | 1850 | 445 | 494 | (Wingen 2017) |
| YNP1FbFP | 0.31 | 4120 | 446 | 496 | (Wingen 2017) |
| YNP2FbFP | 0.33 | 4690 | 449 | 497 | (Wingen 2017) |
| YNP3FbFP | 0.20 | 2840 | 449 | 498 | (Wingen 2017) |
| YNP3FbFP-Y116F | 0.26 | 3590 | 449 | 498 | (Wingen 2017) |
| YNP4FbFP | 0.33 | 4720 | 446 | 496 | (Wingen 2017) |
| VafLOV | 0.23 | 2875 | 450 | 498 | (Mukherjee 2015) |
| CreiLOV | 0.51 | 6375 | 450 | 498 | (Mukherjee 2015) |

[a]: phenylalanine in this position is conserved in the LOV series (Losi 2014); [b]: calculated using the absorption coefficient of Pp2FbFP, 14200 $M^{-1}cm^{-1}$(Wingen 2014). Organism labels: Bs = *Bacillus subtilis;* Ec = *Escherichia coli*; Pp = *Pseudomonas putida*; Ds = *Dinoreoseobacter shibae*; Mr=*Meiothermus ruber*; Te= *Thermosynechococcus elongatus*; YNP=metagenomic sequences; Vaf = *Vaucheria frigida*; Cre = *Chlamydomonas reinhardtii*.

## Figure captions

**Figure 1.** Confocal fluorescence images of miniSOG-targeted endoplasmic reticulum (A), Rab5a (B), zyxin (C), tubulin (D), β-actin (E), α-actinin (F), mitochondria (G), and histone 2B (H) in HeLa cells; scale bars, 10 μm. Reproduced from (Shu 2011), open access article distributed under the terms of the Creative Commons CC-BY license.

**Figure 2.** Bacterial type III secretion systems, facilitate translocation of effector proteins (such as *E.coli* TIR) into host cells, and the process can be followed with FbFPs. In this figure, imaging of Tir-phiLOV translocation: enterohemorrhagic *E. coli* (EHEC) strains were transformed with Tir-phiLOV and added to bovine embryonic lung (EBL) cells. After 2 h, the bacteria were fixed, and images were obtained and quantified. **a.** WT EHEC transformed with Tir-phiLOV showed expression of the reporter after contact with EBL cells. The bacterial cytoplasm was marked using the chromosomal red fluorescent protein (RFP) reporter and host cell actin stained using labeled phalloidin. **b.** 3D, false-colored projections of the bacteria show that Tir-phiLOV (green) is spatially distinct from the bacterial cytoplasm (red). Areas of correlation between phiLOV and RFP are highlighted using yellow, with pink showing areas where no correlation was measured. Ahigh proportion of the fusion protein was localized directly adjacent to the bacterial cell, in stark contrast to expression of the phi-LOV domain alone, which showed excellent colocalization with the bacterial cytoplasm. Reproduced from (Gawthorne 2016), open access article distributed under the terms of the Creative Commons CC-BY license.

**Figure 3.** Photochromism and nanoscopy FPALM image of an *E. coli* cell expressing wt YtvA soaked in isotonic buffer. The protein is widespread over the whole cell body with some major aggregates at the cell wall. Top: conventional; bottom: superresolution. Experimental conditions: Intensity: 1–2 W/cm$^2$ at 405 nm and 0.2 kW/cm$^2$ at 488 nm; frame rate: 30 Hz and total number of collected frames: 20000. Scale bar: 1 μm. Image modified from (Pennacchietti 2014), open access article distributed under the terms of the Creative Commons CC-BY license.

**Figure 4.** Overview of FbFPs properties and biophysical applications. Constitutively fluorescent FbFPs are engineered from wt-LOV domains, by substituting the substrate cysteine and introducing other mutations to increase $\Phi_F$. They can be used for imaging in fluorescence microscopy, as donors in FRET pairs and as fluorescence-based sensors. Photochromicity of wt-LOV domains can be exploited in cellular super-resolution microscopy, while photoactivation

of proteins bearing a wt-LOV domain leads to optogenetic applications. FbFPs can function as low-yield, genetically-encoded photosensitizers for $^1O_2$, with a range of further applications. Colored arrows indicate excitation with blue or violet light. See text for details and table 1 for relevant photophysical parameters of FbFPs.

## Cross-References

→(152) Flavins
→ (428) Photoactivated Localization Microscopy (PALM)
→ (175) Fluorescence -General aspects
→ (604) LOV proteins photobiophysics
→ (168) Reactive Oxygen Species
→ (184) Optical Fluorescence Microscopy

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