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LOV-based reporters for fluorescence imaging

Anthony M Buckley[1], Jan Petersen[2], Andrew J Roe[1], Gillian R Douce[1] and John M Christie[2]

Chromophore-binding domains from plant and bacterial photoreceptor proteins have recently gathered increasing attention as new sources of genetically encoded fluorescent proteins (FPs). In particular, FPs based on the flavin-binding LOV (light, oxygen, or voltage sensing) domain offer advantages over green fluorescent protein (GFP) owing to their smaller size, pH and thermal stability, utility under anaerobic conditions and their ability to generate reactive oxygen species. This review focuses on the potential applications of this emerging class of fluorescent reporters, discusses the advantages and limitations of LOV-based FPs, whilst offering insights regarding the further development of this technology for bioimaging and photodynamic therapy.

Addresses
[1] Institute of Infection, Immunology & Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK
[2] Institute of Molecular Cell and Systems Biology, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Corresponding author: Christie, John M (John.Christie@glasgow.ac.uk)

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Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is widely used throughout cell and developmental biology to study protein dynamics within living cells [1]. Since its introduction, an extensive range of variants and related FPs have been generated through molecular evolution exhibiting improved fluorescence properties, as well as different emission colors that span the entire visible spectrum [2]. However, despite its wide range of applications, GFP and related FPs are reported to have several limitations. For instance, GFP requires molecular oxygen to catalyze the posttranslational cyclization of its fluorophore [3], which can restrict its utility under low-oxygen conditions such as tumor growth [4]. Its relatively large size (~25 kDa) can also lead to steric constraints associated with dysfunctional GFP fusion proteins [5]. Slow maturation of the GFP fluorophore, as well as temperature and pH stability are other factors to consider. Consequently, considerable interest has been directed towards developing alternative genetically encoded fluorescent reporters to overcome these limitations.

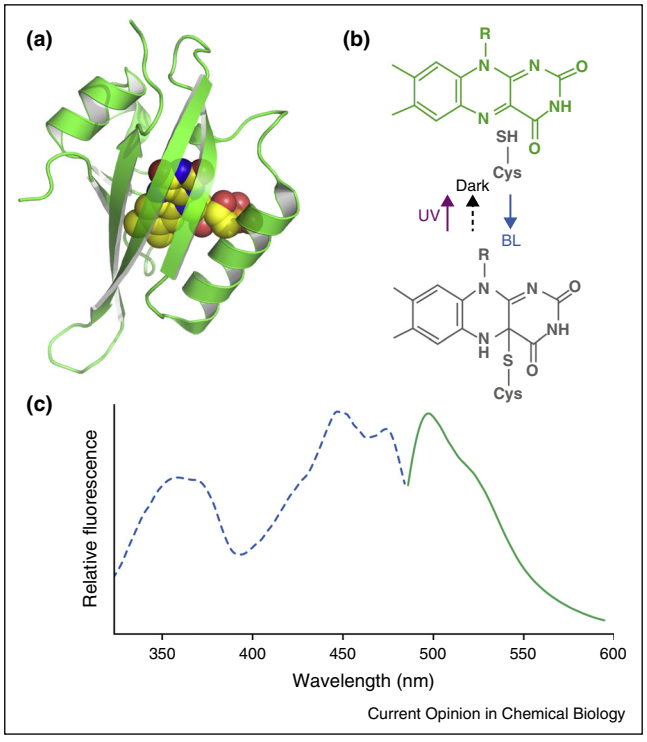
LOV-domain function and fluorescence

LOV domains form a subset of the Per-ARNT-Sim (PAS) domain superfamily and were first identified in proteins regulated either by light, oxygen or voltage, hence the acronym LOV [6]. LOV domains specifically function as photosensory modules and typically bind flavin mononucleotide (FMN) as an ultraviolet (UV)/blue light-absorbing chromophore within the protein that comprises five antiparallel β-sheets and several α-helices (Figure 1a). These relatively small light-sensing moieties (~12–19 kDa; Table 1) are typically linked to a diverse range of effector domains, which together function as blue-light receptors in plants, fungi and bacteria [7]. In this context, the LOV domain serves as a modular photoswitch, placing protein activities, such as kinases, phosphodiesterases and DNA-binding under light control. This modularity of regulation has lately been exploited to create artificial photoswitches for the optical control of biological processes in an emerging field known as optogenetics [8].

The FMN chromophore provides the LOV domain with a yellow color, as well as an intrinsic green fluorescence when excited with UV/blue light [9]. Upon photoexcitation, LOV domains undergo a reversible photocycle involving formation of a covalent adduct between the FMN chromophore and a conserved cysteine residue within the protein (Figure 1b). FMN-cysteinyl adduct formation is accompanied by a loss of fluorescence that subsequently recovers when the protein is incubated in darkness coinciding with photoadduct decay [10,11]. Replacement of this cysteine within the LOV domain abolishes its photochemical activity, improving the fluorescent properties of the protein [12,13]. Mutation of this invariant cysteine is therefore a prerequisite for the generation LOV-based FPs [14•].

Several LOV-based or flavin-based FPs (FbFPs) have now been isolated and are available from a variety of sources (Table 1). These include BsFbFP and its *Escherichia coli* codon-optimized counterpart EcFbFP derived from the bacterial photoreceptor YtvA from *Bacillus subtilis* and PpFbFP, which was developed from a sensory box protein (SB2) from *Pseudomonas putida* [15•]. These

Figure 1



Structure and fluorescence properties of the LOV-based FP iLOV. **(a)** Structure of iLOV (PDB entry 4EES) visualized using PyMOL. The FMN fluorophore is shown as spheres. **(b)** Natural photocycle of the LOV domain. The FMN chromophore in the LOV domain emits green fluorescence upon photoexcitation. Blue light (BL) irradiation induces the formation of a covalent adduct between the FMN chromophore and a conserved cysteine residue that is no longer fluorescent. The photoadduct decays back to the fluorescing state in darkness or in response to near UV irradiation. **(c)** Excitation and emission spectra of iLOV fluorescence. The fluorescence excitation spectrum (dashed blue) was obtained using an emission wavelength of 495 nm, whereas the fluorescence emission spectrum (green) was measured using an excitation wavelength of 450 nm.

LOV-based FPs exhibit a broad wavelength excitation range between 300 and 500 nm with an excitation and emission maximum ~450 nm and ~495 nm, respectively (Figure 1c). An excitation peak is also visible ~380 nm, consistent with the absorbance properties of flavin-based photoreceptors [6]. The requirement for FMN does not restrict its utility as a fluorescent reporter, as this appears to be in plentiful supply in bacterial, plant and human cells [16••] nor limit their targeting to subcellular compartments [16••,17]. Moreover, the fast maturation of LOV fluorescence (with minutes) constitutes one advantage over GFP as a noninvasive real-time reporter of transcriptional activities [18]. However, it is worth noting that LOV-based FPs engineered thus far exhibit fluorescence quantum yields (Q_F) that are significantly lower (~0.2–0.4; Table 1) to that of GFP (0.6) [2], which could impact their utility for certain reporter applications.

Brightness and photostability

Several improvements in the brightness and photostability of LOV-based FPs have been made since they were first introduced in 2007 [15•]. This includes mutation of the invariant cysteine to facilitate reporter function as well as molecular evolution approaches. In addition, targeted mutagenesis of F37 in PpFbFP has produced variants with 2-fold increases in Q_F [19], whilst additional improvements have been made through mutations at Y112 and Q116 [20•]. Whether these mutations can be combined (F37, Y112 and Q116) to enhance the fluorescence of PpFbFP remains to be investigated.

Mutagenesis and DNA shuffling of LOV-coding sequences derived from *Arabidopsis thaliana*, together with fluorescence screening in living plant cells, led to the isolation of an improved LOV-based FP known as iLOV [16••]. Subsequent molecular evolution of iLOV produced variants that are more readily detectable by confocal imaging in living cells due to order-of-magnitude increases in photostability [21] (Figure 2a). However, the Q_F of these photostable iLOV (phiLOV) derivatives appears to be reduced compared those of iLOV and other LOV-based FPs [20•], suggesting that there could be trade-offs to consider when engineering for photostability enhancements. In addition, there are likely to be limits in improving the overall brightness of LOV-based FPs given that their fluorescence is dictated by the flavin cofactor. While small spectral blue shifts in fluorescence emission (~10 nm) have been reported [20•,21], extending these properties beyond the green region of the spectrum will not be possible without replacing the FMN with a different fluorophore.

Natural diversity

In addition to mutagenesis, recent genome searches have uncovered a wide range of genetically diverse LOV-coding sequences that can be further exploited for fluorescence applications. For example, DsFbFP, derived from a LOV-coding sequence isolated from the marine α-proteobacterium *Dinoroseobacter shibae*, exhibits improved fluorescence over PpFbFP [20•], whilst CreiLOV, obtained from a photoreceptor protein from the fresh water alga *Chlamydomonas reinhardtii*, shows enhancements in brightness, photostability and pH tolerance over LOV-based FPs such as iLOV [22]. Thermal stability enhancements in reporter activity have also been reported for CreiLOV, through targeted mutagenesis of EcFbFP [23] and from genome mining for LOV-coding sequences from thermotolerant organisms [8]. Disappointingly, LOV-coding sequences appear to be absent from extremophiles [24], thus limiting the opportunity to exploit this source of genetic diversity.

Utility in low-oxygen environments

One hallmark of LOV-based FPs is the capacity of these proteins to fluoresce under low-oxygen environments

Table 1

Properties of LOV-based FPs

Organism	Protein	FP	Molecular mass (kDa)	Oligomeric state	Quantum yield (Q_F)	Photostability $t_{50\%}$ (min) [20•]	Comments	Refs
Bacillus subtilis	YtvA	EcFbFP	15.1	Dimer	0.34–0.44	2.8	*E. coli* codon optimized version of BsFbFP	[15•,18,20•]
Pseudomonas putida	SB1	Pp1FbFP	16.3	Dimer	0.27	6.8	Increased brightness over Pp2FbFP	[20•]
	SB2	Pp2FbFP	16.3	Dimer	0.17–0.22	2.7		[15•,18,20•]
		Pp2FbFP F37T/S	16.3	Dimer	0.24–0.30		Increased brightness over Pp2FbFP	[19]
		Pp2FbFP Y112L	16.3	Dimer	0.30		Increased brightness over Pp2FbFP	[20•]
		Pp2FbFP Q116V	16.3	Dimer	0.26	1.5	Increased brightness over Pp2FbFP	[20•]
		Pp2FbFP L30M	16.3	Dimer	0.25	1.2	Enhanced O_2 photosensitization over miniSOG	[52]
Arabidopsis thaliana	Phot2	iLOV	12.1	Monomer	0.34–0.44		Smallest LOV reporter	[16••,18,22]
		phiLOV2.1	12.1	Monomer	0.20	13	Improved *in vivo* photostability over iLOV	[20•,21]
		miniSOG	12.1	Monomer	0.37–0.41	2.9	$^1O_2$ photosensitization	[20•,41••]
Chlamydomonas reinhardtii	Phot	CreiLOV	13.0	Monomer	0.51		Enhanced brightness, photostability, thermal and pH tolerance *in vitro*	[22]
Vaucheria frigida	Aureo1	VafLOV	19.4	Dimer	0.23			[22]
Dinoroseobacter shibae	Unknown	DsFbFP	15.2	Dimer	0.35	0.4		[20•]

[15•]. This property makes the application of this technology very attractive for the study of anaerobic cellular processes. For example, studies have shown that EcFbFP could surpass yellow fluorescent protein (YFP) as a fluorescent reporter in anaerobically grown cultures of *E. coli* and *Rhodobacter capsulatus* [15•,25,26], as well as yeast [27] and a range of mammalian cells [28]. In addition, LOV-based FPs have been used to track host cell infections by anaerobic pathogens under physiologically relevant conditions [29–31]. Similarly, our recent work has evaluated the utility of phiLOV2.1 as a translational fusion in the obligate anaerobe *Clostridium difficile*. Genetically fusing phiLOV2.1 to the bacterial cytokinetic protein FtsZ enables efficient monitoring of its localization to the septum during binary fission, where it becomes polymerized to form a characteristic Z-ring at the mid-cell (Figure 2b).

When size matters

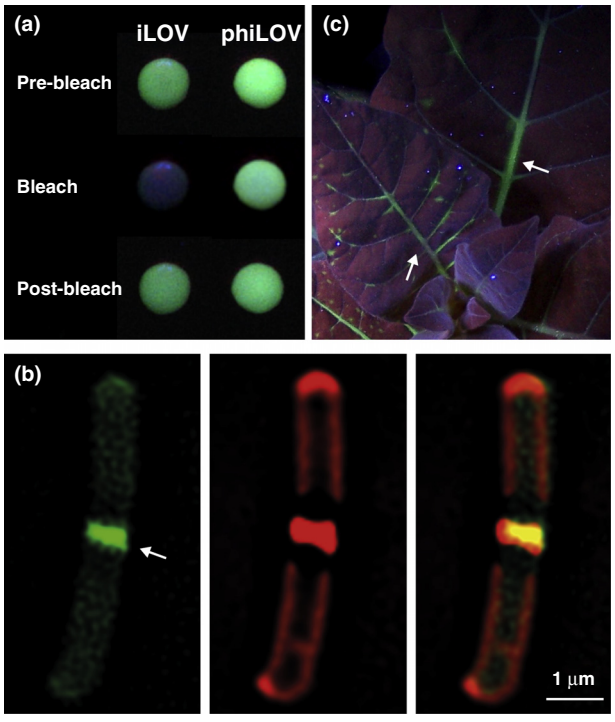
Another benefit of LOV-based FPs over GFP is its smaller size. While some LOV-based FPs form dimers *in vitro*, iLOV, its derivatives and the newly isolated CreiLOV are monomeric (Table 1) and relatively small in size (110–118 amino acids). These latter properties confer advantages in creating translation fusions where inclusion of larger FPs could impact on protein function due to steric constraints [32]. For example, whilst fusion of GFP is known to compromise the functionality of some viral movement proteins, iLOV was found to be less disruptive when fused to the movement protein of tobacco mosaic virus (Figure 2c) and the coat protein of potato mop-top virus [16••]. Furthermore, the coding sequence of iLOV (~300 nt) is considerably smaller than that of GFP (~700 nt) and provides a reduced genetic load with respect to viral packaging constraints. Hence, iLOV is reported to outperform GFP as a real-time reporter of viral infection dynamics and has successfully extended the range of plant and animal viruses that can be fluorescently tagged *in vivo* [16••,33,34].

Biosensor applications

In addition, LOV-based FPs have also been used in the development of several biosensor applications. For instance, iLOV fluorescence is quenched in the presence of copper at physiological pH and is reversible in the presence of the metal ion chelator EDTA [35]. Two asparagine residues within the vicinity of the FMN fluorophore at positions 401 and 425 (based on the amino acid positions in *Arabidopsis* phototropin 2 from which iLOV was derived) are proposed to coordinate copper binding. While mutation of these residues is required to confirm their involvement, these studies clearly demonstrate the potential for LOV-based FPs to serve as intracellular metal sensors.

The flavin-binding properties of LOV-based FPs also make them attractive candidates for developing

Figure 2



Utility of LOV-based FPs for bioimaging. **(a)** Photobleaching properties of iLOV and phiLOV expressed in liquid droplets of *E. coli*. Images were taken before photobleaching, immediately after bleaching and post-recovery after 5 min. Adapted from Christie *et al.* [21]. **(b)** Protein localization in the obligate anaerobic bacterium *Clostridium difficile*. The LOV-based FP, phiLOV2.1 was fused to the cytokinetic protein FtsZ. Green fluorescence from FtsZ-phiLOV2.1 is localized at the mid-cell during bacterial cell division (left). Accumulation of red fluorescence from the lipophilic membrane dye FM4-64 showing increased membrane biogenesis at the mid-cell (centre). Merge of green and red fluorescence overlapping at the mid-cell (right). Arrow denotes the point of cellular division. Adapted from Buckley *et al*. [58]. **(c)** Tobacco mosaic virus (TMV)-based expression of iLOV driven from a viral subgenomic promoter. White arrows show systemic movement of TMV expressing the iLOV reporter in the upper leaves of tobacco at 4 days post inoculation. Adapted from Chapman *et al.* [16••].

fluorescent sensors of photo-induced electron transport (PET), which can be used to monitor pH changes within living cells. For this purpose, iLOV was reengineered to incorporate unnatural tyrosine analogs at position 486 that exhibit a reduced pK_a [36••]. The phenolate anion of this side chain quenches iLOV fluorescence at physiological pH by PET. However, protonation of the tyrosine analog enhances fluorescence by uncoupling the PET pathway. Consequently, *E. coli* cells expressing the variant iLOVU3 show increased fluorescence when the pH of the medium was lowered 7–5. This 'acid turn-on' property of iLOVU3 therefore holds great promise for monitoring cellular pH changes and has been used effectively to track bacterial phagocytosis into macrophages [36••]. Oxidation of the tyrosine analog in iLOVU3 also blocks the PET quenching pathway, opening up new possibilities for using LOV-based FPs as redox sensors [36••]. Moreover, fusion of EcFbFP to YFP has proved successful in generating a Förster resonance energy transfer (FRET)-based biosensor named FluBO for visualizing temporal changes in oxygen levels in *E. coli* [37]. In this case, efficient FRET from the EcFbFP donor to the YFP acceptor is only observed in the presence of cellular oxygen.

Singlet oxygen generation

Aside from their intrinsic fluorescence, flavins offer great potential as photosensitizers for singlet oxygen generation (SOG). Consequently, LOV-based FPs have been developed as phototoxic modules capable of triggering cell death [38•,39•,40]. MiniSOG was the first LOV-based FP engineered for singlet oxygen ($^1O_2$) photosensitization [41••]. In response to light, miniSOG generates a sufficient amount of $^1O_2$ to induce diaminobenzidine photooxidation and polymerization, which is discernible by correlative light and electron microscopy [41••]. Exquisite protein localization and complex protein structures within persevered subcellular organelles have been obtained with high resolution (~8–10 nm) using miniSOG as a genetically encoded tag [42–46]. This photosensitizing ability of miniSOG has therefore created new possibilities for electron microscopy. Additional applications for miniSOG include new methods for optical immunodetection [47], the development of new methodologies for imaging protein–protein interactions in intact cells [48••] and chromophore-assisted light inactivation, a powerful technique where $^1O_2$ photosensitization can be used to selectively interfere with the function of proteins in close proximity [49]. Detailed photochemical characterization indicates that miniSOG initiates other photo-induced electron transfer processes in addition to $^1O_2$ production [50,51]. Further photosensitizer improvements could thus be obtained through protein engineering strategies. Indeed, a Pp2FbFP derivative has recently been reported with a $^1O_2$ quantum yield that is ~3-fold higher than that of miniSOG [52]. Establishing the $^1O_2$ photosensitizing capabilities of existing LOV-based FPs (Table 1) will also be important to minimize any potential phototoxic side effects during live cell imaging.

Super-resolution microscopy

Photoswitchable FPs are of considerable interest for super-resolution microscopy applications. The natural photocycle of the LOV domain has recently been exploited for such purposes. Irradiation with near UV light is reported to accelerate photoadduct decay within the LOV domain [53] (Figure 1b). Similarly, LOV-containing proteins, including YtvA, can photoswitch between their fluorescing, dark state and their non-fluorescing, active state upon exposure to blue light and near UV light, respectively [54]. This photoswitchable property can facilitate the

distinction between neighboring fluorophore molecules by separating their fluorescence emission in time thereby conferring resolution beyond the diffraction limit of light microscopes [55]. Using Fluorescence photoactivation localization microscopy (FPALM), the intracellular location of YtvA molecules within *E. coli* cell was resolved with an average precision of 35 nm [54]. These photochromic properties of the LOV domain will contribute to extending the range of genetically encoded tools available for super-resolution microscopy.

Conclusions

Since the first identification of the flavin-binding property of the LOV domain over 15 years ago [9], much progress has been made in characterizing the biophysical and structural properties of these photosensory modules. Rational and random protein-engineering approaches have proved successful in harnessing the fluorescence potential of these, and other photosensor domains, for specific applications in cell biology and biotechnology [56]. These photosensors are prevalent in bacteria, fungi, plant and algae thereby enhancing the diversity that can be used for these design principles. Further development is now required to identify additional LOV variants with improved fluorescence, photostability and photosensitizing properties. Whether recent computational predictions can be used to incorporate mutations within the fluorophore-binding pocket in order to shift the fluorescence emission maximum to longer wavelengths should also be investigated [57]. With the advantages of this emerging class of FPs now firmly established, a bright future lies ahead in maximizing their utility for a wide range of applications from fluorescence imaging to cancer therapy.

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