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Flavin-based fluorescent proteins: emerging paradigms in biological imaging

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Flavin-based fluorescent proteins (FbFPs) are an emerging class of fluorescent reporters characterized by oxygen-independent fluorescence and a small size — key advantages compared to the green fluorescent protein (GFP). FbFPs are at a nascent stage of development. However, they have already been used as versatile reporters for studying anaerobic biosystems and viral assemblies. Recently, FbFPs with improved brightness and photostability have been engineered. In addition, several FbFPs show high degrees of thermal and pH stability. For these reasons, FbFPs hold strong promise to extend bioimaging to clinically and industrially significant systems that have been challenging to study using GFPs. In this review, we highlight recent developments in the FbFP toolbox and explore further improvements necessary to maximize the potential of FbFPs.

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Introduction

Fluorescent proteins have revolutionized biological studies by enabling imaging of molecular-scale events with high spatial and temporal resolution. In particular, the green fluorescent protein (GFP) and related analogs are widely used as genetically encoded reporters to investigate gene expression, protein localization, macromolecular trafficking, and protein interactions [1,2]. However, despite more than a decade of discovery and engineering, all known GFP variants are strictly dependent on molecular oxygen for maturation of fluorescence [3–7]. For these reasons, GFP-based proteins are dimly fluorescent to non-fluorescent in low-oxygen environments and have limited utility for studying anaerobic biosystems including high-density fermentations, cerebral ischemia, tumor hypoxia, pathogenesis, and biofilm development. Alternative fluorescent probes for imaging under low-oxygen conditions include small molecule organic dyes used in conjunction with biological labeling systems (e.g. SNAP-tags and HaloTags). However, cell-based fluorescence imaging methods that rely on organic dyes can suffer from cytotoxicity, poor cell permeability, and high levels of background fluorescence arising from the need to remove excess unlabeled dye [8,9]. Consequently, the fluorescence reporter toolbox for low-oxygen imaging is severely inadequate for addressing a broad class of foundational and applied problems in anaerobic biology. From this perspective, there is a strong need for the development of new genetically encoded fluorescent proteins that are functional under anaerobic or low-oxygen conditions.

Recently, a new class of flavin-based fluorescent proteins (FbFPs) derived from bacterial and plant photosensory flavoproteins has been developed and shown to fluoresce in an oxygen-independent manner [10••,11••,12]. In this way, FbFPs are promising candidates for addressing the long-standing challenge of developing viable genetically encoded fluorescent probes for imaging in low-oxygen conditions. In this review, we highlight key advances in the development and application of FbFPs and describe ongoing efforts to expand and diversify the FbFP imaging toolbox through protein engineering. In addition, we identify key challenges in FbFP-based imaging and suggest future directions to maximize the general utility and overall scope of FbFPs as an emerging class of fluorescent reporters.

LOV-domain photoreceptors — molecular scaffolds for developing FbFPs

FbFPs are derived from a highly conserved family of blue light photoreceptors known as light, oxygen, and voltage (LOV) sensing proteins. Wild type LOV proteins typically associate with flavin mononucleotide (FMN) to function as light-driven regulators of diverse cellular functions ranging from stress response and virulence in microbes to phototaxis in plants and algae [13,14]. Upon blue light illumination, LOV proteins exhibit a complex photocycle that results in the formation of a covalent adduct between FMN and a cysteine residue located in the FMN-binding pocket. FMN-cysteine adduct

formation induces a conformational change in the LOV domain that is transduced to actuate downstream effector domains, such as kinases, esterases, and DNA binding motifs [15,16]. Although FMN is a fluorescent molecule ($\lambda_{em,max}$ = 525 nm and $\lambda_{ex,max}$ = 450 nm), the light-driven LOV-domain photocycle renders the protein-FMN complex non-fluorescent.

In 2007, a protein engineering approach was used to develop LOV-based reporter proteins with stable (albeit weak) fluorescence emission [10••]. In order to engineer fluorescent LOV proteins, the natural photochemical cycle was broken by introducing a Cys → Ala in the FMN-binding pocket [10••]. The resulting FbFPs containing the Cys → Ala mutation show a hypsochromic shift in fluorescence emission ($\lambda_{em,max}$ = 495 nm) relative to free FMN in solution, while the excitation maximum remains unchanged ($\lambda_{ex,max}$ = 450 nm) (Figure 1a). Using this approach, three LOV proteins were originally engineered as FbFPs: first, BsFbFP, which is based on the N-terminal LOV domain of the *Bacillus subtilis* YTVA protein [10••], second, iLOV, which is derived from the LOV2 domain of the *Arabidopsis thaliana* blue light photoreceptor, phototropin (Phot2) [11••], and third, PpFbFP, which is engineered from a sensory box protein (SB2) from *Pseudomonas putida* [10••]. BsFbFP was subsequently codon-optimized for expression in *Escherichia coli*, which generated a variant known as EcFbFP [10••].

Applications of FbFPs as fluorescent reporters for low-oxygen imaging

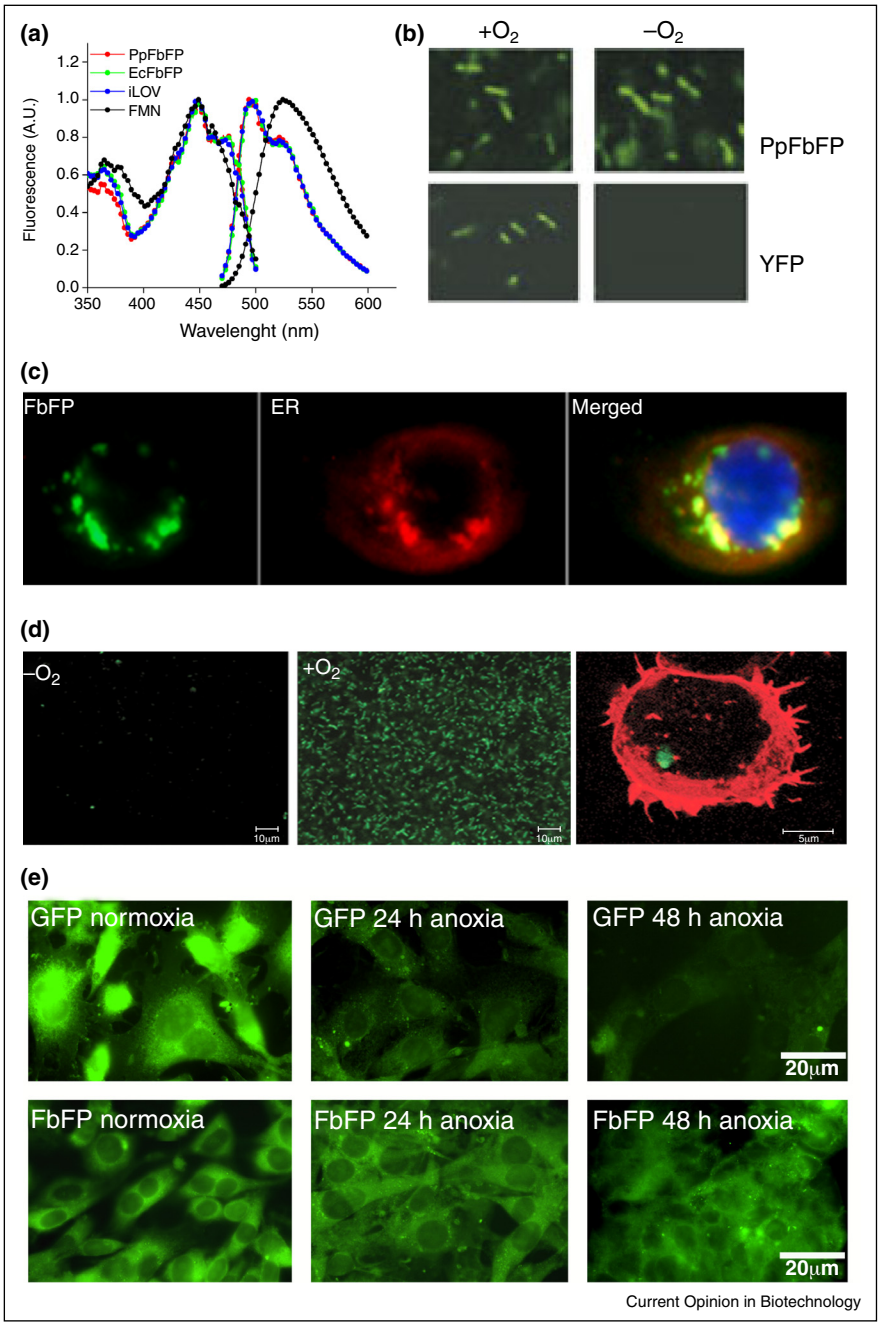
Due to their oxygen-independent fluorescence properties, FbFPs have proved particularly useful for labeling and investigating anoxic and hypoxic biological systems. For example, PpFbFP and EcFbFP have been shown to express and fluoresce in anaerobically cultivated *Rhodobacter capsulatus* and *Escherichia coli* (Figure 1b) [10••]. Furthermore, EcFbFP was shown to outperform YFP as a fluorescent reporter for monitoring dynamic gene expression in high-density *E. coli* cell cultures, which mimic industrial bioprocess fermentation platforms [17]. In this work, it was shown that fluctuations in oxygen tension are associated with different growth regimes (exponential and stationary) in *E. coli*, which resulted in imprecise quantification of gene expression using oxygen-dependent YFP as a transcriptional reporter. In contrast, excellent agreement was observed between mRNA profiles and fluorescence emission trajectories obtained using EcFbFP as a transcriptional reporter.

PpFbFP has also been developed as a reliable and stable fluorescent marker for the gastrointestinal anaerobic symbionts including *Bifidobacterium breve* and *Bifidobacterium longum*, which have recently been pursued as potential probiotics for replenishing the intestinal microbiota [18]. In addition, FbFPs have been used to tag anaerobic pathogens and characterize host-pathogen interactions under physiologically relevant anaerobic conditions. Specifically, BsFbFP was used to demonstrate activation of two key oxidative stress-response genes in *Bacteroides fragilis* cells upon induction of anoxia and during infection in macrophages in low-oxygen conditions (Figure 1c) [19•]. Using an analogous approach, BsFbFP was exploited to demonstrate localization of an obligate anaerobe, *Porphyromonas gingivalis*, in human gingival epithelial cells (Figure 1d) [20•]. EcFbFP has also been used to probe conjugative plasmid transfer between *E. coli* strains under anaerobic conditions, which is of tremendous significance for monitoring transfer of antibiotic resistance genes between anaerobic pathogens [21]. Furthermore, expression and fluorescence of FbFPs in hypoxic conditions have been demonstrated in fungal pathogens such as *Saccharomyces cerevisiae* and *Candida albicans* [22], as well as in hypoxically cultured mammalian cell lines including human embryonic kidney (HEK), Chinese hamster ovary (CHO), and HeLa cells, as well as murine tumor and neuronal stem cells (Figure 1e) [23•]. These applications open up exciting new vistas for probing gene expression in hypoxic disease models including solid tumors and animal models of cerebral hypoxia or stroke. Finally, a translational fusion between oxygen-independent EcFbFP and oxygen-sensitive YFP was utilized as a FRET-based reporter of cytoplasmic oxygen levels in *E. coli* [24••]. Taken together, these early studies highlight the robust versatility of the FbFP imaging toolbox for applications in anaerobic biology.

Applications of FbFPs as small and minimally perturbative fluorescent reporters

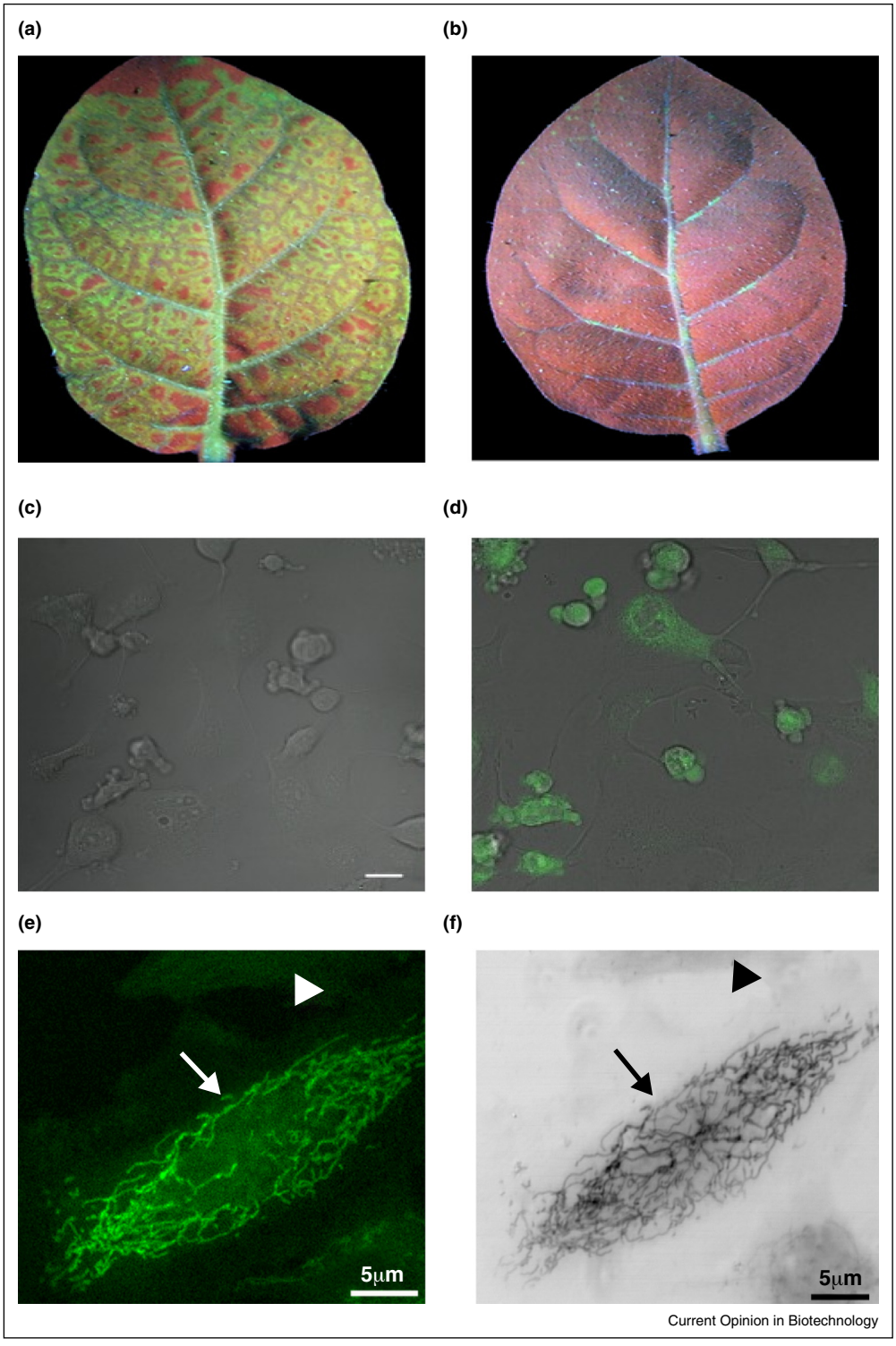
Although GFPs have been extensively used for constructing translational fusions, their large size (~240 amino acids) and complex folding requirements often lead to impaired functionality of fusion protein partners. In contrast to GFP, FbFPs are characterized by a small size ranging from ~110 to 140 amino acids. Chapman *et al.* leveraged this key advantage for constructing translational fusions between iLOV and the movement protein (MP) of a tobacco mosaic virus (TMV) and separately between iLOV and the coat protein (CP) of potato mop-top mosaic virus (PMTV) [11••]. MP-iLOV and CP-iLOV fusions were subsequently employed to track viral infection and localization in tobacco leaves. In sharp contrast to the iLOV fusions, fusions to the bulkier YFP hindered effective viral cell-to-cell trafficking *in planta* (Figure 2a, b). In a similar approach, Seago *et al.* employed iLOV to study a recombinant foot-and-mouth disease viral infection in goat epithelial cells (Figure 2c, d) [25]. Specifically, the authors demonstrated that while the larger GFP mRNA was excised from the viral RNA genome via recombination, the smaller iLOV mRNA was stably integrated, thereby enabling real-time tracking of viral infections.

Figure 1



Application of FbFPs for anaerobic imaging. **(a)** FbFPs (PpFbFP, EcFbFP, and iLOV) are characterized by a fluorescence emission peak at 495 nm, which is blue-shifted relative to the emission peak of the chromophore FMN (525 nm). Reproduced from [38]. **(b)** PpFbFP, expressed in *E. coli* cells displays cyan-green fluorescence in aerobic ($+O_2$) and anaerobic conditions ($-O_2$), in sharp contrast to YFP, which is fluorescent only in the presence of oxygen. Reproduced with permission from [10••]. **(c)** BsFbFP was codon optimized and used to study *Porphyromonas gingivalis* infections in gingival epithelial cells (GEC). FbFP-tagged *P. gingivalis* (green, left) localized to the endoplasmic reticulum (stained red, center) in infected GECs. The overlaid image (right panel) shows the nucleus stained blue using DAPI. Reproduced from [20•]. **(d)** The alkyl hydroperoxide reductase promoter (ahpC) in *Bacteroides fragilis* remains repressed in anaerobic conditions ($-O_2$, left) but is activated upon incubation in aerobic conditions ($+O_2$, right). *B. fragilis* cells expressing codon-optimized BsFbFP under the control of an anaerobically activated ahpC promoter could be detected in infected macrophage cells. Reproduced with permission from [19•]. **(e)** GFP expression in hippocampal tumor cell lines (HT22) is clearly visible in aerobic conditions (left). However, fluorescence is dim after incubation in anoxic conditions for 24 hours (middle panel) or 48 hours (right panel). In contrast, FbFP expression is clearly detectable in normoxic and anoxic conditions. Reproduced from [23•].

Figure 2



FbFPs as versatile fluorescent reporters. **(a)** iLOV was used to tag the viral movement protein (MP) in tobacco mosaic virus (TMV), which was subsequently used to infect tobacco leaves. TMV expressing MP-iLOV fusions showed efficient systemic infection spreading from the central vein to the secondary and even tertiary veins through the plasmodesmata. Reproduced with permission from [11••] (Copyright (2008) National Academy of Sciences, U.S.A.). **(b)** In contrast, TMV expressing MP-YFP fusions failed to efficiently translocate between leaf cells following infection, likely due to the bulkier nature of the fusion. Reproduced with permission from [11••]. **(c)** Goat epithelial cells were infected with a foot-and-mouth-disease virus (FMDV) expressing iLOV. **(d)** Green fluorescence from iLOV was clearly detectable. Under similar conditions, the GFP was gene excised from the viral genome owing to its larger size, therefore rendering GFP unsuitable for viral imaging. Reproduced with permission from [25]. **(e)** An iLOV variant known

In a series of proof-of-principle experiments, FbFPs were used as structurally compact tags for optimizing recombinant protein production in high throughput platforms. In this work, translational fusions between iLOV and 10 distinct recombinant proteins were constructed and demonstrated to retain fluorescence under diverse conditions of protein expression [26]. Strikingly, a related study showed that a translational fusion between EcFbFP and a hydroxynitrile lyase enzyme dramatically improved enzymatic stability and enhanced enzyme catalyzed product turnover in acidic conditions relative to wild type enzyme or enzyme-YFP fusions [27]. Overall, these studies suggest that FbFPs could serve as highly promising alternatives to bulky GFP-based probes for generating fusions with small footprints, particularly in the case of large macromolecular assemblies such as viruses or unstable cellular enzymes.

Applications as singlet oxygen generators for electron microscopy and optogenetics

Aside from their application as fluorescent probes for low-oxygen imaging, FbFPs have also been used as efficient singlet oxygen generators for *in vivo* imaging. In this strategy, FbFPs were engineered for light-induced production of reactive oxygen species (ROS) by energy transfer to oxygen from the excited state FMN chromophore. In a seminal study, Tsien and colleagues engineered an iLOV variant, known as mini super oxide generator (miniSOG), to develop a probe for correlated electron and light microscopy (Figure 2e, f) [28••]. Furthermore, light-driven release of singlet oxygen from miniSOG constitutes the basis for a genetically encoded photosensitizer, which is a powerful and emerging application in the field. In this way, engineered FbFPs can be used to optogenetically inactivate proteins via ROS-mediated oxidation. For example, miniSOG was used to drive light-induced cell death in HeLa cells [29] and in specific neuronal cells in *Caenorhabditis elegans* [30••]. In related work, miniSOG was used to inhibit the presynaptic release of neurotransmitters via inactivation of SNARE proteins using translational fusions [31]. Recently, fusions between miniSOG and a fluorescent protein, IFP1.4, have been used to facilitate an alternative method to Förster Resonance Energy Transfer (FRET) to characterize macromolecular complexes at distances exceeding the FRET limit of ~10 nm, based on the quenching of IFP1.4 fluorescence by singlet oxygen generated by miniSOG [32••]. For most live cell imaging experiments, of course, singlet oxygen generation by iLOV and other FbFPs is undesirable; indeed, we have not observed evidence of phototoxicity using FbFP variants under standard imaging conditions commonly employed in live cell fluorescence microscopy and spectroscopy [33].

Engineering new FbFP variants with improved properties

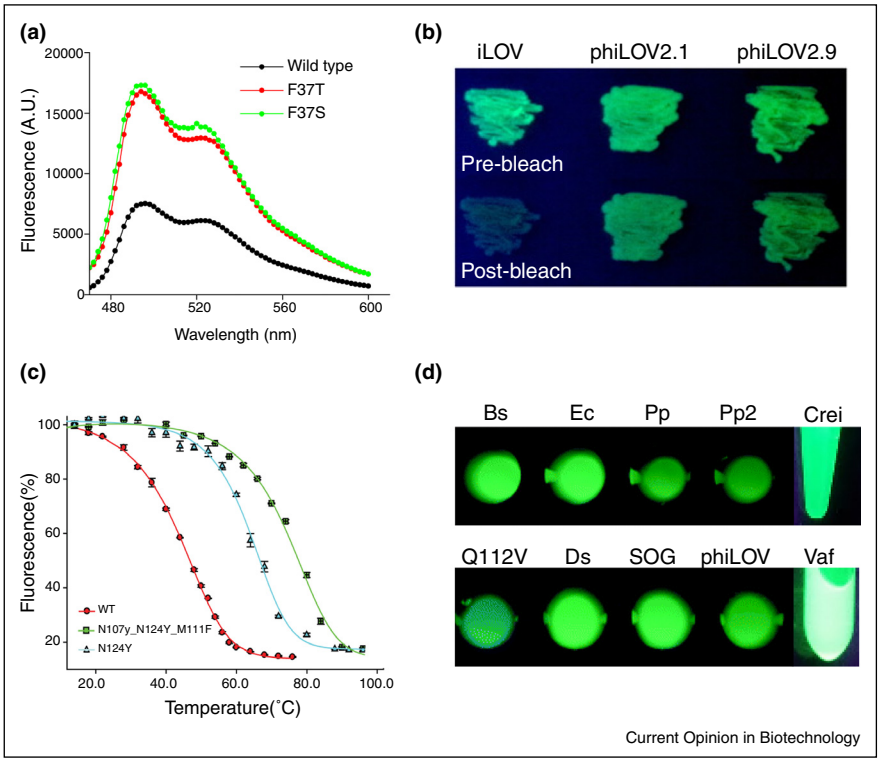
In order to broaden the utility of FbFPs, efforts to expand the FbFP toolbox through the development of improved probes are critically needed. To this end, we have used site saturation mutagenesis of FMN-proximal amino acids to engineer PpFbFP mutants (F37S and F37T) that exhibit a two-fold increase in brightness (Figure 3a) [34••]. In addition, DNA shuffling was recently employed to engineer iLOV to develop a photostable variant known as phiLOV (Figure 3b) [35]. Moreover, Song *et al.* described a molecular dynamics-guided mutagenesis approach to enhance the thermal stability of EcFbFP by engineering mutations in the dimerization interface (N107Y, M111F, N124Y), thereby increasing the melting temperature by 31 °C (Figure 3c) [36]. Recently, we used genome mining to identify two entirely new FbFPs based on photoreceptors from the blue-green algae *Chlamydomonas reinhardtii* (CreiLOV) and *Vaucheria frigida* (VafLOV) [33]. Interestingly, CreiLOV emerged as one of the brightest known FbFPs with a quantum yield that is approximately 1.5-fold greater than the quantum yield of iLOV, measured under identical conditions. Drepper and colleagues have also exploited the natural diversity of LOV proteins to develop two new FbFPs from *Dinoroseobacter shibae* and from *Pseudomonas putida* SB1 protein (known as DsFbFP and Pp1FbFP, respectively; Figure 3d) [37]. Importantly, this work also reported a PpFbFP variant (Q116V) that exhibits a blue shifted fluorescence emission (~10 nm), which could potentially prove useful for multispectral imaging using FbFPs (Figure 3d).

Key challenges and future directions

FbFPs are at a relatively nascent stage of development, and an exhaustive understanding of their properties as practical fluorescent reporters is currently lacking. In a recent and impressive study, Wingen *et al.* addressed this issue by developing a robust platform for precise characterization and comparison of key spectral properties of existing FbFPs [37]. Similarly, in our lab, we have tackled this challenge by comprehensively characterizing biophysical and biochemical properties of FbFPs [38], in tandem with developing new FbFP variants using protein engineering. Strikingly, our results suggest that FbFPs (in particular iLOV and CreiLOV) exhibit multiple advantages as fluorescent reporter probes, including an overall

(Figure 2 Legend Continued) as miniSOG expressed in the mitochondria as a cytochrome C fusion and imaged via fluorescent microscopy. Upon strong illumination, miniSOG releases singlet oxygen species, which catalyzes the oxidation of diaminobenzidine producing a high optical contrast between transfected and non-transfected cells (indicated by arrows) that can be leveraged for imaging via electron microscopy. Reproduced from [28••].

Figure 3



Engineering new FbFPs with improved properties. **(a)** Site saturation mutagenesis was used to engineer two PpFbFP mutants with a nearly twofold increase in overall fluorescence emission (PpFbFP F37S and F37T). Reproduced from [34••] as published by BioMed Central. **(b)** DNA shuffling was used to develop photostable variants of iLOV known as phiLOV2.1 and phiLOV2.9. Reproduced from [35]. **(c)** Computational modeling and optimization were utilized to enhance the thermal stability of EcFbFP by nearly 31 °C by engineering mutations N107Y, M111F, and N124Y. Reproduced from [36]. **(d)** Fluorescence emission of several existing members of the FbFP family including *E. coli* codon-optimized BsFbFP, generally known as EcFbFP (Ec), the original BsFbFP from *B. subtilis* (Bs), PpFbFP from *P. putida* (Pp), a PpFbFP variant derived from sensory box protein 2 also from *P. putida*, and known as Pp2FbFP (Pp2), and CreiLOV that was identified in *Chlamydomonas reinhardtii* via genome mining. Lower panel depicts a blue shifted FbFP (Q112V) that was engineered by introducing Q112V mutation in PpFbFP, a recently identified DsFbFP from *Dinoroseobacter shibae*, an iLOV variant known as miniSOG (indicated as SOG) that is typically used as a singlet oxygen generator, a photostable iLOV variant known as phiLOV, and a recently identified algal FbFP from *Vaucheria frigida* (VafLOV). Reproduced with permission from [37].

small size, oxygen-independent fluorescence, enhanced thermal stability, rapid maturation of fluorescence, and a broad operational pH range.

In order to truly realize the potential advantages of FbFPs as robust fluorescent probes, however, further engineering is required to address improvements in brightness, photostability, and cellular expression. In some cases, we have observed that overexpression of FbFPs may lead to varying degrees of metabolic burden and a concomitant reduction in cellular growth rate [38]. Based on these results, we conjecture that targeted mutagenesis of FMN-binding pocket amino acids or 'insoluble' hydrophobic patches in FbFPs could be particularly useful for optimizing FbFPs for improved intracellular expression and brighter fluorescence. Furthermore, performance of FbFPs as intracellular reporters can conceivably be improved using approaches that enhance cellular FMN concentrations — for example, engineering flavin transporters or supplementing FMN in media in case of cells that naturally transport flavin (e.g. *Bacillus subtilis* or animal cells) [39]. Finally, the abundant representation and natural diversity of LOV-domains in sequenced genomes and metagenomes [40,41] provides a vast and broad natural 'pool' of potential FbFP candidates for biological imaging. In addition, genome mining could provide a powerful method for engineering improved FbFP variants, for example, by motivating sequence homology independent shuffling strategies such as SHIPREC. In summary, further engineering and broader application of FbFPs will be crucial for the development of an improved FbFP toolbox, which promises to 'shine the light' on biological systems of outstanding importance to medicine, environment, clinical, and industrial microbiology.

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- •• of outstanding interest

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