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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.