Characterization of Flavin-Based Fluorescent Proteins: An Emerging Class of Fluorescent Reporters

Arnab Mukherjee1, Joshua Walker1*, Kevin B. Weyant1, Charles M. Schroeder1,2,3,4*

1 Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America, 2 Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America, 3 Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America, 4 Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

Abstract
Fluorescent reporter proteins based on flavin-binding photosensors were recently developed as a new class of genetically encoded probes characterized by small size and oxygen-independent maturation of fluorescence. Flavin-based fluorescent proteins (FbFPs) address two major limitations associated with existing fluorescent reporters derived from the green fluorescent protein (GFP)—namely, the overall large size and oxygen-dependent maturation of fluorescence of GFP. However, FbFPs are at a nascent stage of development and have been utilized in only a handful of biological studies. Importantly, a full understanding of the performance and properties of FbFPs as a practical set of biological probes is lacking. In this work, we extensively characterize three FbFPs isolated from Pseudomonas putida, Bacillus subtilis, and Arabidopsis thaliana, using in vitro studies to assess probe brightness, oligomeric state, maturation time, fraction of fluorescent holoprotein, pH tolerance, redox sensitivity, and thermal stability. Furthermore, we validate FbFPs as stable molecular tags using in vivo studies by constructing a series of FbFP-based transcriptional constructs to probe promoter activity in Escherichia coli. Overall, FbFPs show key advantages as broad-spectrum biological reporters including robust pH tolerance (4–11), thermal stability (up to 60°C), and rapid maturation of fluorescence (~3 min.). In addition, the FbFP derived from Arabidopsis thaliana (iLOV) emerged as a stable and nonperturbative reporter of promoter activity in Escherichia coli. Our results demonstrate that FbFP-based reporters have the potential to address key limitations associated with the use of GFP, such as pH-sensitive fluorescence and slow kinetics of fluorescence maturation (10–40 minutes for half maximal fluorescence recovery). From this view, FbFPs represent a useful new addition to the fluorescent reporter protein palette, and our results constitute an important framework to enable researchers to implement and further engineer improved FbFP-based reporters with enhanced brightness and tighter flavin binding, which will maximize their potential benefits.

Background
Beginning with the discovery of the green fluorescent protein (GFP) and its subsequent heterologous expression in diverse organisms, fluorescent reporter proteins have been established as integral components of the molecular biology toolkit [1–4]. GFP-based fluorescent proteins have been identified, isolated, and characterized from several distinct sources, including Aequorea victoria, Renilla reniformis, Anthozoa sp., and Discosoma sp., [2,5–9]. However, all GFP-based reporters show oxygen-dependent fluorescence [1,10–12]. Consequently, GFP-based fluorescent proteins are dimly fluorescent to nonfluorescent in low-oxygen environments [13–16], which has precluded their application for investigating bioprocesses that are regulated by hypoxia and anoxia such as microbial fermentation, bioremediation, anaerobic sewage treatment, tumor metastasis, development, and inflammation [17], cerebral hypoxia-ischemia, microbial pathogenesis, and biofilm formation.

Recently, a new class of oxygen-independent fluorescent reporters based on bacterial and plant photosensory flavoproteins has been developed [18,19]. These photosensory proteins share a highly conserved light, oxygen, or voltage (LOV) sensing domain that binds flavin mononucleotide (FMN), which is a UV-A/blue light sensing fluorescent cofactor [20,21]. Excitation of these reporter proteins with blue light at 450 nm results in cyan fluorescence emission with a peak at 495 nm (Figure 1). Specifically, three members of the LOV-family of proteins have been identified as FMN-based fluorescent proteins (FbFPs): (1) PpFbFP, which is based on an uncharacterized sensory box protein from P. putida [19], (2) EcFbFP, which is an Escherichia coli codon-optimized gene [19] derived from the N-terminal LOV domain of the Bacillus subtilis YTVA protein implicated in
regulating stress response [22,23], and (3) iLOV [18], which comprises the LOV2 domain of the Arabidopsis thaliana blue light photoreceptor phototropin (Phot2) that mediates diverse light driven adaptive responses including phototropism, stomatal opening, and chloroplast translocation [24,25].

In their native context, LOV-domain proteins function as blue light photoreceptors that exhibit an elaborate photocycle upon absorption of blue light, involving: (1) conversion of the buried FMN to an excited singlet state, followed by immediate conversion to an excited triplet state by intersystem crossing with a red-shifted absorption peak at 660 nm, (2) decay of the excited triplet state to a singlet state that forms a covalent adduct between FMN and a neighboring cysteine residue in the protein, which results in a blue-shifted absorption peak at 390 nm, (3) eventual return to the dark ground state by thermal relaxation [26-29]. Repeated cycling of the photostimulated protein between ground and excited states results in bleaching of fluorescence [18,30]. In order to engineer fluorescent variants of the LOV proteins, the photochemical cycle was abolished through mutation of the active site cysteine residue to alanine. The Cys to Ala mutation prevents the formation of the covalent adduct between the protein and excited state FMN, thereby providing a molecular framework to engineer FbFPs [18,19].

FbFPs from P. putida (PpFbFP) and B. subtilis (codon optimized for expression in E. coli) and subsequently designated EcFbFP) have been shown to express and fluoresce in anaerobically cultivated E. coli cells under conditions in which the GFP-based yellow fluorescent protein (YFP) was nonfluorescent [19]. Furthermore, EcFbFP was shown to outperform YFP as a fluorescent reporter for real-time monitoring of gene expression in E. coli cells cultivated using rich media. Specifically, fluctuations in oxygen levels occurred as the E. coli cells transitioned from actively respiring exponential phase of growth to the stationary phase, thereby resulting in inaccurate assessment of promoter activity by the oxygen-dependent fluorescence of YFP. In contrast, excellent agreement was obtained between fluorescence levels of EcFbFP (derived from B. subtilis and codon-optimized for expression in E. coli) and mRNA measurements by reverse transcription PCR [30]. Heterologous expression and fluorescence of FbFPs has been demonstrated in facultative and obligate anaerobes including Rhodobacter capsulatus [19], Pseudomonas gingivalis [31], Bacteroides fragilis [32], Roseobacter, Photobacter spp. [33], Candida albicans, and Saccharomyces cerevisiae [34], as well as in animal cells [35]. In a recent study, a translational fusion between oxygen-independent EcFbFP and oxygen-sensitive YFP was utilized as a real-time intracellular reporter of oxygen concentration in the cytoplasm of E. coli cells [36].

In addition to oxygen-independent fluorescence, FbFPs are also characterized by small size (~100–140 amino acids), which is a significant advantage over bulkier GFP-based probes (~240 amino acids) for generating minimally perturbative translational fusions. Chapman et al. capitalized on this key advantage by using the A. thaliana FbFP (iLOV) to track the development of local and systemic infection by the tobacco mosaic virus in tobacco leaves [18]. In this study, the authors observed that fusions to bulkier GFP molecules hindered effective viral invasion leading to the development of localized viral lesions. Finally, spectrally improved variants of existing FbFPs have been engineered using directed evolution. Christie et al. employed DNA shuffling to enhance brightness and photostability of iLOV [18,37]. Recently, we discovered brighter mutants of PpFbFP by saturation mutagenesis of chromophore-proximal amino acids [38]. In this work, substitution of a chromophore proximal aromatic amino acid at position 37 (phenylalanine) by serine or threonine (Phe37Ser and Phe37Thr mutants) enhanced brightness of fluorescence emission by nearly 74%. Specifically, the mutations improved quantum yield by relieving fluorescence quenching interactions between FMN and Phe37 and strengthening association between the protein and the FMN chromophore.

In order to expand the scope and enhance the utility of FbFPs as practical fluorescent reporters, a comprehensive evaluation of the
performance properties of FbFPs is critically required. In this work, we systematically evaluated the existing set of FbFPs with respect to quantum yield, oligomeric state, holoprotein fraction, and maturation time. Moreover, we investigated the effects of pH, temperature, and reducing conditions on FbFP fluorescence. Finally, we assessed the utility of FbFPs as reporters of promoter activity in wild type *E. coli* MG1655 cells expressing transcriptional fusions between individual FbFPs and inducible or constitutive bacteriophage promoters. Aside from the oxygen-independent function of these reporter proteins, we identified several advantages to using FbFPs as reporters, including thermal tolerance (up to 60°C), fluorescence emission over a broad pH range (4–11), and rapid maturation of fluorescence (∼10–30 minutes in GFP vs. 1 minute in FbFPs). Based on our results, iLOV emerged as a suitable transcriptional reporter for *E. coli* and showed good agreement with transcriptional profiles determined using a YFP reporter as a benchmark. Overall, we anticipate that our results will enable broader application of FbFPs, as well as providing a basic biochemical framework to further engineer and optimize FbFPs as robust biochemical reporters.

### Results and Discussion

#### Purification of FMN-based Fluorescent Proteins (FbFPs)

In this work, we characterized the performance of FbFPs under a wide range of experimental conditions. In particular, we studied three distinct FbFPs originally derived from three different organisms: (1) PpFbFP isolated from *P. putida*, (2) EcFbFP isolated from *B. subtilis* and codon optimized for expression in *E. coli*, and (3) iLOV isolated from *A. thaliana*. FbFPs were purified using two-stage chromatography consisting of immobilized nickel affinity and anion exchange chromatography. Upon excitation with blue light at 450 nm, all three FbFPs displayed their characteristic emission spectra with a peak at 495 nm and a shoulder at 525 nm (Figure 1). In all cases, FbFPs were purified to greater than 95% homogeneity (Figure 2). Freshly purified FbFPs (average concentration 1–2 mg/mL) were used for all characterization experiments, and purified preparations were stable for at least a week at 4°C, as verified by fluorescence measurements on freshly prepared and stored samples.

#### Quantum Yield of FbFPs

In a first set of experiments, we determined quantum yields (QF) for all three FbFPs using FMN as a standard with known quantum yield (QF_{FMN} = 0.27) [39]. Specifically, quantum yield was calculated by integrating the fluorescence emission spectrum between 480 nm and 600 nm and by normalizing the integrated emission by absorbance at the excitation wavelength (450 nm). In this way, quantum yield quantifies the efficiency with which flavin-bound FbFP molecules (holoprotein) convert excitation light into fluorescence emission. Quantum yields are provided in Table 1 and are in agreement with previously determined values of quantum yields for these fluorescent proteins [18,19]. We further determined the brightness of these fluorescent proteins, where brightness is defined as the product of quantum yield and molar extinction coefficient of flavin (ε = 12,500 M^{-1} cm^{-1}) [19]. Based on these values, we identify EcFbFP and iLOV (brightness = 4250) as the brightest members of the FbFP family.

#### Fraction of Fluorescent Holoprotein

FbFP fluorescence is facilitated by an FMN fluorophore that is noncovalently associated with the protein via an extensive network of hydrogen bonds, coulombic forces, and van der Waals interactions [37]. As a result, maturation of fluorescence in FbFPs relies on the availability of a sufficient pool of intracellular FMN. Moreover, the brightness of FbFPs is also a function of the strength of association between the protein and FMN. Therefore, in addition to the quantum yield and molar extinction coefficient, an accurate assessment of FbFP brightness needs to account for the fraction of FbFP in solution that is fluorescent, i.e., the FMN-bound holoprotein fraction. We verified that apo-FbFPs do not substantially contribute to absorbance at 450 nm (*Text S1*). Furthermore, we found that the concentration of free FMN in a solution of purified FbFP is negligible (Text S1). Therefore, absorbance at 450 nm can be specifically attributed to the holoprotein species in solution. Based on these assumptions, we determined the fraction of fluorescent holoprotein in solution, defined as the ratio of holoprotein concentration to the total protein concentration (holo+apoprotein, estimated by the Bradford assay) (*Materials and Methods*). In recent work, we used an analogous approach to estimate holoprotein fractions in wild type and brightness enhanced mutants of PpFbFP [38].

| Protein | Quantum yield | Molar extinction coefficient (M^{-1} cm^{-1}) | Brightness |
|---------|---------------|----------------------------------------------|------------|
| PpFbFP  | 0.17          | 12500                                        | 2125       |
| EcFbFP  | 0.34          | 12500                                        | 4250       |
| iLOV    | 0.34          | 12500                                        | 4250       |

### Table 1. Quantum yield and brightness of FbFPs.

Brightness is quantified as the product of quantum yield and molar extinction coefficient of the fluorophore (flavin mononucleotide). Brightness values are reported for monomeric units of PpFbFP and EcFbFP, which exist as functional dimers.

doi:10.1371/journal.pone.0064753.t001

| Protein | F_{holo} |
|---------|----------|
| PpFbFP  | 0.60±0.02|
| EcFbFP  | 0.68±0.02|
| iLOV    | 0.39±0.01|

| Protein | F_{holo} |
|---------|----------|
| PpFbFP  | 0.60±0.02|
| EcFbFP  | 0.68±0.02|
| iLOV    | 0.39±0.01|

The fraction of FbFP that exists in an FMN-bound holom form (F_{holo}) is calculated based on absorption at 450 nm by the holoprotein.

doi:10.1371/journal.pone.0064753.t002
absence of a crystal structure for EcFbFP, we used the crystal structure of a closely related protein, YtFA from Bacillus subtilis (PDB ID: 2PR5). The YtFA crystal structure was solved in the dark state conformation of the protein in which the FMN ring is noncovalently buried in the binding pocket. Therefore, the dark structure can be considered to closely resemble the EcFbFP structure in which covalent association between FMN and the protein is abolished through a C62A mutation. We defined the FMN binding cavity to include amino acids that are directly involved in hydrogen bonding or hydrophobic interactions with the isoalloxazine ring of FMN, and these amino acids were identified using Ligand Explorer version 4.0 (Text S2). Despite a high degree of similarity between amino acids comprising the binding sites in the two proteins, the binding cavity in iLOV had a considerably higher average B-factor (Bavg = 23.29) relative to the binding cavity in EcFbFP (Bavg = 17.18). B-factor values (also known as temperature factor) represent the extent of disorder associated with the spatial locations of amino acids in protein structures. High values of B-factor indicate uncertainty in precisely locating the coordinates of amino acid side chains in the crystal structure of a protein, which is usually the case for side chains that are relatively less restrained, such as surface exposed residues. In particular, protein engineering techniques that specifically target high B-factor residues for mutations (relative to lower B-factor amino acids) have been shown to improve stability of enzymes [40,41]. In the context of EcFbFP and iLOV, a higher B-factor in the iLOV binding site may indicate a binding cavity that is less rigid relative to the chromophore-binding cavity in EcFbFP. For example, an arginine residue at position 58 in iLOV, which is directly involved in hydrogen bonding with FMN, has a substantially large B-value of 46.61. The corresponding arginine in EcFbFP (R79) has a B-value of 17.75. The significantly larger B-value associated with a key residue involved in hydrogen bonding with FMN ring may constitute a structural basis for the lower holoprotein fraction in iLOV.

**Oligomeric State of FbFPs**

Monomeric fluorescent reporter proteins are desirable for generating translational fusions with minimal imprint. Multimeric fluorescent proteins often impair the folding and functionality of proteins to which they are translationally fused. Furthermore, for applications involving Förster resonance energy transfer (FRET), oligomerization of fluorescent reporters can significantly misconstrue interpretation of protein-protein interactions. FbFPs exhibit an overall small size (∼130 amino acids), which provides a considerable advantage over the bulkier GFP-family proteins (∼240 amino acids) in constructing translational fusions with small imprint. To this end, we characterized the oligomeric states of these proteins using size exclusion chromatography (Table 3). Using this approach, we determined that iLOV is a monomer, whereas EcFbFP and PpFbFP are predominantly dimers (Figure S1). We found that the oligomeric state of FbFPs was relatively insensitive to ionic strength, and the oligomeric states remained intact in both high salt (1 M and 0.5 M NaCl) and lower salt concentrations (150 mM NaCl). iLOV is the smallest protein in the FbFP family, consisting of only 110 amino acids. Therefore, based on its size and monomeric structure, iLOV has an exceedingly small imprint as a fluorescent tag.

**Effect of pH on FbFP Fluorescence**

Fluorescent proteins with a broad functional pH range are desirable for intracellular imaging in alkaliophilic life forms and for studying acidic cellular environments such as endosomes, lysosomes, and plant vacuoles. We characterized the pH sensitivities of FbFPs by incubating purified fractions of FbFPs in buffers of pH 2, 4, 10, and 11 for several hours. We verified that PpFbFP and EcFbFP fluoresce maximally at pH 7, while iLOV has maximum fluorescence emission at pH 6 (Figure S2). Therefore, we normalized peak fluorescence emission intensity (measured at 495 nm) at a particular pH to the maximum fluorescence emission intensity measured at pH 7 (PpFbFP and EcFbFP) or pH 6 (iLOV) (Figure 3). Changes in overall quantum yields of FbFPs at different pH values are provided as supporting information (Text S3). We found that both EcFbFP and iLOV exhibit fluorescence over a broad pH range (pH 4 to 11), generally retaining 60–70% of their maximum fluorescence up on prolonged incubation at pH 4. Furthermore, iLOV and EcFbFP strongly fluoresce in highly alkaline conditions and retain nearly 60% of their maximum fluorescence when incubated at pH 11 for over two hours. Moreover, PpFbFP is stable at pH 4 (53% of maximum fluorescence), but readily loses fluorescence at pH 11. Finally, all three FbFPs rapidly lose fluorescence at very low pH values, conditions under which the fluorescence emission peak at 495 nm disappears and the FMN-specific emission spectrum begins to emerge (Figure 3, Figure S3). In contrast to FbFPs, YFP has a pKₐ of 5.5–6.5 [42] and readily loses fluorescence in acidic conditions, retaining less than 10% of its maximum fluorescence up on incubation at pH 4 (Figure S4).

**Thermal Stability of FbFPs**

Thermophilic microorganisms are promising host platforms for several bioprocess and biotechnology applications. Thermostable fluorescent proteins extend the scope of fluorescent reporters to investigate biological processes in thermophiles. Wild type GFP matures very slowly at temperatures exceeding 20°C; however, several folding mutations have been identified to improve the thermal tolerance of GFP-based proteins. In this way, GFP mutants have been optimized for fluorescence at elevated temperatures up to 80°C [43,44] (Figure S4). However, oxygen-dependent maturation of fluorescence in GFP-based reporters precludes the application of thermostable GFP variants to vast majority of thermophilic microbes, which are commonly obligate anaerobes. In order to assess the ability of FbFPs to function in high temperature conditions, we determined the temperature dependence of FbFP fluorescence using purified protein preparations (Figure 4). Specifically, we determined peak fluorescence emission intensity (measured at 495 nm) at a particular temperature and normalized this value to the peak fluorescence emission intensity measured at room temperature (25°C). Changes in overall quantum yields of FbFPs at different temperatures are provided as supporting information (Text S3). We found that iLOV significantly outperforms PpFbFP and EcFbFP at elevated temperatures and retains more than 80% and

---

**Table 3. Oligomeric state of FbFPs.**

| Protein   | 0.15 M NaCl | 0.5 M NaCl | 1 M NaCl | Oligo state | Mol. mass (kDa) |
|-----------|-------------|------------|----------|-------------|----------------|
| PpFbFP    | 1.9         | 1.7        | 1.5      | dimer       | 32.4           |
| EcFbFP    | 1.9         | 1.7        | 1.6      | dimer       | 30             |
| iLOV      | 0.9         | 0.7        | 0.8      | monomer     | 12             |

Oligomeric states of FbFPs were assessed in buffers of varying ionic strengths using size exclusion chromatography. Values represent the mean of duplicate experiments. Ionic strength was regulated by adjusting the concentration of NaCl in the various buffers. doi:10.1371/journal.pone.0064753.t003

---

**Figure 3.** Changes in overall quantum yields of FbFPs at different pH values are provided as supporting information (Text S3). We found that both EcFbFP and iLOV exhibit fluorescence over a broad pH range (pH 4 to 11), generally retaining 60–70% of their maximum fluorescence up on prolonged incubation at pH 4. Furthermore, iLOV and EcFbFP strongly fluoresce in highly alkaline conditions and retain nearly 60% of their maximum fluorescence when incubated at pH 11 for over two hours. Moreover, PpFbFP is stable at pH 4 (53% of maximum fluorescence), but readily loses fluorescence at pH 11. Finally, all three FbFPs rapidly lose fluorescence at very low pH values, conditions under which the fluorescence emission peak at 495 nm disappears and the FMN-specific emission spectrum begins to emerge (Figure 3, Figure S3). In contrast to FbFPs, YFP has a pKₐ of 5.5–6.5 [42] and readily loses fluorescence in acidic conditions, retaining less than 10% of its maximum fluorescence up on incubation at pH 4 (Figure S4).

---

**Figure S1.** We found that the oligomeric state of FbFPs was relatively insensitive to ionic strength, and the oligomeric states remained intact in both high salt (1 M and 0.5 M NaCl) and lower salt concentrations (150 mM NaCl). iLOV is the smallest protein in the FbFP family, consisting of only 110 amino acids. Therefore, based on its size and monomeric structure, iLOV has an exceedingly small imprint as a fluorescent tag.

---

**Figure S2.** Changes in overall quantum yields of FbFPs at different pH values are provided as supporting information (Text S3). We found that both EcFbFP and iLOV exhibit fluorescence over a broad pH range (pH 4 to 11), generally retaining 60–70% of their maximum fluorescence up on prolonged incubation at pH 4. Furthermore, iLOV and EcFbFP strongly fluoresce in highly alkaline conditions and retain nearly 60% of their maximum fluorescence when incubated at pH 11 for over two hours. Moreover, PpFbFP is stable at pH 4 (53% of maximum fluorescence), but readily loses fluorescence at pH 11. Finally, all three FbFPs rapidly lose fluorescence at very low pH values, conditions under which the fluorescence emission peak at 495 nm disappears and the FMN-specific emission spectrum begins to emerge (Figure 3, Figure S3). In contrast to FbFPs, YFP has a pKₐ of 5.5–6.5 [42] and readily loses fluorescence in acidic conditions, retaining less than 10% of its maximum fluorescence up on incubation at pH 4 (Figure S4).
60% of its room temperature fluorescence upon prolonged incubation at 50°C and 60°C, respectively. Moreover, EcFbFP retains approximately 80% of its room temperature fluorescence upon incubation at 50°C. However, higher temperatures lead to rapid loss of fluorescence in EcFbFP. In all cases, continued incubation of FbFPs at 70°C leads to protein precipitation along with gradual reduction of the 495 nm emission peak and the appearance of a smoother emission spectrum with a peak at 525 nm, which is characteristic of free FMN (Figure 3).

Fluorescence Recovery after Denaturation

Maturation of fluorescence in GFP is mediated by oxidation of the cyclic tripeptide chromophore, which requires more than an hour at room temperature [10]. Despite the development of fast-maturing GFP variants such as venus [45], chromophore oxidation and maturation of fluorescence requires nearly 10–40 minutes for recovery of half maximal fluorescence even in the fastest maturing variants [42]. This constitutes a major bottleneck in applying GFP to study dynamic biological events. Fluorescence maturation kinetics is typically monitored by observing the recovery of fluorescence emission following renaturation of denatured and sodium dithionite-reduced GFP preparations. We adopted an analogous approach to investigate the maturation of fluorescence in FbFPs by first denaturing FbFPs at high temperature, followed by rapid cooling of the proteins to room temperature (Figure 5). Protein unfolding and refolding were monitored using fluorescence spectroscopy, where we used the distinct signatures of the fluorescence emission spectra of the FbFP holoprotein and free FMN to distinguish apo-protein from intact FbFP holoprotein [46]. First, FbFPs were readily denatured by heating at 90°C for 25 minutes, whereupon we observed that the unfolded proteins aggregated into visible precipitates. In the case of EcFbFP, fluorescence was readily restored upon rapid cooling of samples back to room temperature (Figure 5, Figure S6A). However, PpFbFP and iLOV were irreversibly denatured after the high temperature cycle at 90°C, and cooling failed to restore fluorescence for these proteins. On the other hand, reversible denaturation of iLOV could be achieved by thermal denaturation at a lower temperature of 70°C instead of 90°C (Figure 5). For EcFbFP and iLOV, recovery of fluorescence was rapid and appeared to occur within less than 2–3 minutes, which is

Figure 3. Effect of pH on FbFP fluorescence. Histograms depict the fraction of peak fluorescence (measured at 495 nm at pH 7 for PpFbFP and EcFbFP and pH 6 for iLOV) retained by A) PpFbFP, B) EcFbFP, and C) iLOV after incubation in buffers of pH 2, 4, 10, and 11 for 2.5 h. D) FbFPs are readily denatured by incubation at pH 2 and are characterized by the appearance of a flavin-spectrum with a peak at 525 nm, as is depicted for iLOV. doi:10.1371/journal.pone.0064753.g003
approximately equal to the time required by the temperature controlled fluorescence spectrometer to cool from 90°C (or 70°C) to room temperature (25°C). In contrast, thermally denatured YFP recovered ~46% of its fluorescence in the same time in which EcFbFP fluorescence was restored to greater than 90% (Figure S6B). Furthermore, we note that apparent rate of fluorescence recovery in YFP in this experiment is substantially faster than the actual rate of natural fluorescence maturation because the functional YFP chromophore is not chemically reduced in the course of thermal denaturation. In reality, acquisition of fluorescence in YFP involves cyclization of the tripeptide chromophore followed by oxidation, which substantially slows the overall kinetics of fluorescence maturation.

Effect of Strongly Reducing Conditions on FbFP Fluorescence

The GFP chromophore is readily quenched by high concentrations of strong reductants such as sodium dithionite [12]. As a result, fluorescence in GFP-based proteins is sensitive to reducing conditions, which precludes the use of GFP-based reporters in highly reducing environments as are typically encountered during the cultivation of several obligate anaerobes such as methanogens. We investigated the sensitivity of FbFP fluorescence to reducing conditions by treating purified FbFPs with sodium dithionite at ~1000-fold excess molar concentrations (Figure S7). Under these conditions, the reduction potential of the solution is ~−660 mV, which establishes a very strong reducing environment. In this experiment, we recorded fluorescence emission over a period of 3 hours. We determined that fluorescence emission from all three FbFPs was insensitive to reducing conditions, which could in part reflect the general insensitivity of the FMN chromophore to reductants such as sodium dithionite or dithiothreitol in aerated conditions [46]. It is noteworthy that under similar conditions, the GFP chromophore has been reported to be readily reduced to a nonfluorescent form [42,45].

FbFPs as Real Time Reporters of Gene Expression in E. coli

Fluorescent reporters are widely used to quantify gene expression by constructing transcriptional fusions between the reporter and a promoter of interest. Experimental estimates of promoter activity can be integrated with mathematical modeling to discern regulatory mechanisms underlying genetic networks, as well as to investigate the response of a gene network to...
in addition, steady state promoter dynamics was further validated using Western blot measurements to estimate the production of YFP at three time points corresponding to exponential phase of growth (Figure S11). In the case of transcriptional fusions between FbFPs and the T5 promoter, only iLOV accurately captured the expected steady state dynamics of the T5 promoter and revealed good agreement with the YFP data (Figures 6–7, S9–S10) over a wide range of IPTG concentrations. In contrast, EcFbFP and PpFbFP fluorescence revealed a steadily decreasing trend in E. coli, particularly during growth in glycerol (Figure 7). Furthermore, enhancing the ClpXP protease dependent in vivo degradation rates of EcFbFP (and iLOV) through the construction of LVA-tagged variants did not substantially alter the fluorescence profiles (Figure S12). Based on Western blot experiments, the decreasing fluorescence observed in the case of PpFbFP and EcFbFP can be explained in part by intracellular degradation of these proteins upon overexpression (Figure S11). Progressive loss of fluorescence with time may also be a consequence of incomplete formation of PpFbFP and EcFbFP holoproteins, if the rate of freshly synthesized cellular proteins far exceeds the available intracellular FMN pool, particularly in the case of cells grown using a poor carbon source such as glycerol. Several intracellular mechanisms can explain the differences in expression profiles between iLOV and the bacterial FbFPs. For example, expression of PpFbFP and EcFbFP may impose a greater burden on cellular flavin resources owing to their dimeric nature or stronger affinity for FMN compared to iLOV (Table 2). As a result, progressive depletion of cellular flavin would lead to in vivo synthesis of EcFbFP and PpFbFP existing in predominantly apo-forms. In this scenario, the decreasing fluorescence trend would be the result of dilution by cell growth of previously synthesized holo-EcFbFP and PpFbFP.

Finally, regarding cell doubling times, we observed that EcFbFP and iLOV expressing cells showed comparable exponential-phase growth rates as YFP expressing cells in glucose and glycerol-based media (Table 4). However, we observed retardation in the log-phase growth rates of PpFbFP expressing cells compared to cells expressing YFP, in both glucose and glycerol-based growth media (Table 4). Our results suggest that expression of EcFbFP and iLOV impact cellular growth in a manner analogous to YFP or GFP, whereas PpFbFP expression clearly affects cellular growth rates, which could arise due to an additional metabolic burden of PpFbFP on E. coli cell growth.

FbFPs as Real Time Reporters: Constitutive Promoters & Anaerobic Gene Expression

In order to further evaluate the scope of iLOV as a robust reporter of gene expression, we constructed a transcriptional fusion between iLOV and a constitutive phage lambda Pl promoter [52]. The promoter-reporter cassette was integrated in a low copy promoter probe plasmid constructed in our lab (Materials and Methods). The phage lambda promoter contains operator sequences recognized by the tetracycline repressor TetR protein. In the absence of TetR in E. coli MG1655, the promoter behaves as a constitutive promoter. E. coli cells harboring the above expression constructs were grown in M9 medium using glucose or glycerol as the carbon source. We compared gene expression profiles in the logarithmic phase of cell growth using iLOV and GFP as reporters. Promoter activity measurements and growth curves over the entire cell growth period are provided as supporting information (Figure S13). Similar to results obtained with the T5 promoter, we observed good agreement between gene expression assayed using iLOV and GFP (Figure 8). Furthermore, growth rates were also comparable...
in the case of *E. coli* cells expressing iLOV and GFP from the phage lambda promoter (Table 4). Overall, iLOV significantly outperforms EcFbFP and PpFbFP as a real-time reporter of transcriptional activity in *E. coli*. Finally, we verified cellular expression and fluorescence of iLOV under anaerobic conditions of *E. coli* culture (Figure 9). Previously, anaerobic expression of PpFbFP and EcFbFP was demonstrated by Drepper and colleagues, as well as in our lab [19,30,38]. However, iLOV revealed close agreement with the YFP expression profile over a broad range of IPTG concentrations. Promoter activities are depicted corresponding only to the logarithmic phase of cell growth (0.4 < A600nm < 0.8). As the duration of the logarithmic phase varies for *E. coli* cells expressing distinct transcriptional reporter constructs, so does the time frame over which the promoter activity is depicted in the figures.

doi:10.1371/journal.pone.0064753.g006

Figure 6. FbFPs as transcriptional reporters of T5 promoter activity in *E.coli* grown in M9-glucose. FbFPs and YFP were expressed in *E. coli* MG1655 cells using an IPTG-inducible T5 promoter. Fluorescence and optical density were recorded over the logarithmic phase of cell growth (typically after ~2 hours of lag phase following re-inoculation of overnight culture) in M9 medium supplemented with glucose at 20 mM concentration as the carbon source. IPTG concentrations were varied to span different levels of transcriptional activity of the T5 promoter. Fluorescence was divided by the optical density at 600 nm. Normalized fluorescence values are depicted for A) PpFbFP, B) EcFbFP, C) iLOV, and D) YFP. Steady state promoter activity was verified using YFP as a reporter. PpFbFP and EcFbFP deviated considerably from the expected steady state promoter dynamics. However, iLOV revealed close agreement with the YFP expression profile over a broad range of IPTG concentrations. Promoter activities are depicted corresponding only to the logarithmic phase of cell growth (0.4 < A600nm < 0.8). As the duration of the logarithmic phase varies for *E. coli* cells expressing distinct transcriptional reporter constructs, so does the time frame over which the promoter activity is depicted in the figures.

Conclusions

In this work, we demonstrate that FbFPs represent an emerging class of fluorescent reporters with several useful characteristics for application in a broad range of biological systems. Previous applications of FbFPs have predominantly focused on imaging in...
hypoxic and anoxic environments. However, in this work, we demonstrate that in addition to oxygen-independent fluorescence, FbFPs integrate several key properties that render them immensely useful as biological probes. In particular their small size (<55% the size of GFP), broad pH range, thermal, and redox tolerance make them robust reporters with a potential to address several biological systems of outstanding importance that are poorly suited to investigation using GFP. For example, fluorescent reporter proteins are routinely employed for on-line detection of recombinant protein production in order to identify optimal bioprocess parameters to ensure maximum productivity. The use of GFP and analogous reporters in such conditions is rendered difficult by the incomplete and slow maturation of the GFP-chromophore as semi-anaerobic to anaerobic conditions often prevail in bioprocess production platforms (e.g., fermentation) [57]. In addition, the large size of GFP has been shown to frequently interfere with the folding of partner fusion proteins, e.g., in the case of secreted proteins [16]. FbFPs exhibit an overall small size (Table 3), robust folding (Figure 5), and oxygen-independent fluorescence, all of which makes these probes function as superior fluorescent reporters under these conditions. Strikingly, rapid and robust refolding of FbFPs (specifically, iLOV and EcFbFP) in 2–3 minutes following thermal denaturation is suggestive of fast maturation of fluorescence in this class of proteins (Figure 5). This constitutes a significant advantage over GFP-based probes, because even the fastest maturing GFP variants (notably, venus and Tag-GFP2) require 10–40 minutes for maturation of half-maximal fluorescence [42,45,58]. As fast maturing reporters, FbFPs may be immensely advantageous for precisely resolved temporal studies such as monitoring short-lived proteins and early detection of promoter activation. Moreover, although the intrinsic pH-sensitivity of the GFP chromophore has been exploited to design pH-responsive probes [59], the rapid loss of GFP fluorescence at low pH complicates fluorescence measurements of acidic biological processes such as endocytosis, synaptic vesicle fusion, and periplasmic or extracellular protein secretion. In this sense, the
anaerobic conditions (of transcriptional activities of bacteriophage promoters). Tantalizingly iLOV emerges as a suitable noninvasive real-time reporter. A broad pH tolerance of iLOV and EcFbFP, spanning pH 4–11 (Figure 3), makes them ideally suited to monitor such physiological events. Based on our results, we identify iLOV as the most promising member in the FbFP family with its substantially smaller size (110 amino acids, monomeric) as well as robust fluorescence under extremes of pH and temperature (retaining ~60% of maximum fluorescence at pH 4 and 11, and at 60°C). Importantly, iLOV emerges as a suitable noninvasive real-time reporter of transcriptional activities of bacteriophage promoters (Figures 6–8). As iLOV fluorescence is detectable even under anaerobic conditions (Figure 9), it can serve as a valuable probe for promoter activity under anaerobic conditions. As an example, there is considerable interest in the development of promoters that are tunably regulated by environmental cues commonly encountered in industrial-scale biotechnological applications such as oxygen depletion and the onset of anaerobiosis [60]. The application of GFP for screening oxygen-induced promoters requires post-inoculation of anaerobically cultivated cells in well aerated conditions to initiate chromophore oxidation and maturation in GFP [61]. FbFP-based reporters such as iLOV can substantially ease and intensify the development of such promoter cassettes. Finally, the limited brightness (product of quantum yield and molar extinction coefficient) of FbFPs is currently a major bottleneck restricting their versatile and widespread utilization as practical reporters. Indeed, the brightest FbFPs (iLOV and EcFbFP, brightness = 4250) are only 7–12% as bright as spectrally similar GFP-based probes such as mTFP1 (brightness = 54,400), EGFP (brightness = 33,600), and Venus (brightness = 52,554). Therefore, future work at developing FbFP-based fluorescent reporters should focus on enhancing brightness through improvements in fluorescence quantum yield or tighter binding with the flavin chromophore [38]. Overall, we expect this study to furnish an important characterization framework to encourage the continued development of FbFPs as well as encourage their application to fundamental and applied biological studies.

| Table 4. Doubling times of E. coli MG1655 expressing FbFPs, GFP, and YFP. |
|----------------------------------|-------|-------|-------|-------|
| **Expression conditions** | **PpFbFP** | **EcFbFP** | **iLOV** | **YFP (GFP)** |
| P₅₁ glucose (0.1 mM IPTG) | 3.81±.04 | 2.36±.02 | 2.01±.01 | 2.20±.15 |
| P₅₁ glucose (1 mM IPTG) | 2.46±.06 | 1.76±.03 | 2.04±.02 | 2.20±.03 |
| P₅₁ glucose (10 mM IPTG) | 2.88±.04 | 2.00±.08 | 2.17±.14 | 2.19±.09 |
| P₅₁ glycerol (0.1 mM IPTG) | 5.32±.38 | 2.78±.04 | 2.29±.04 | 3.57±.03 |
| P₅₁ glycerol (1 mM IPTG) | 5.72±.18 | 2.41±.05 | 3.17±.04 | 3.39±.13 |
| P₅₁ glycerol (10 mM IPTG) | 4.72±.20 | 3.99±.67 | 3.61±.19 | 3.33±.06 |
| Pᵢ | n.d. | n.d. | 1.95±.14 | 1.70±.04 |
| Pᵢ (constitutive) | n.d. | n.d. | 2.38±.07 | 2.89±.22 |

PpFbFP, EcFbFP, iLOV, and YFP were expressed in E. coli MG1655 using an IPTG-inducible phage T5 promoter harbored in a medium copy plasmid (pQE80L). In addition, iLOV and GFP were expressed using a constitutive phage lambda promoter in a low copy plasmid (pAM06-tet). Cells were grown in M9 media using glycerol or glucose as the carbon source and doubling times were estimated over the logarithmic phase of cell growth corresponding to 0.4 A₆₀₀nm < 0.8. doi:10.1371/journal.pone.0064753.t004

Figure 8. iLOV as a reporter of phage lambda promoter activity in E. coli. GFP and iLOV were expressed in E. coli MG1655 cells using a constitutive phage lambda promoter. Fluorescence and optical density were recorded over the logarithmic phase of cell growth (typically after ~2 hours of lag phase following re-inoculation of overnight culture) in M9 medium supplemented with glucose at 20 mM or glycerol at 0.5% as the carbon source. Fluorescence was divided by the optical density at 600 nm and normalized to the maximum value reached over 16 h. of cell growth. Steady state promoter activity was verified using GFP as a reporter. We generally observed good agreement between the iLOV and GFP expression profiles. Promoter activities are shown corresponding only to the logarithmic phase of cell growth (0.4<A₆₀₀nm<0.8). As the duration of the logarithmic phase varies for E. coli cells expressing the GFP and iLOV reporter constructs, so does the time frame over which the phage λ promoter activity is depicted in the figures. doi:10.1371/journal.pone.0064753.g008

Figure 9. Expression of iLOV in anaerobically cultured E. coli MG1655. E. coli MG1655 cells were grown in M9 medium supplemented with 20 mM glucose (carbon source) and 20 mM potassium nitrate (electron acceptor). Anaerobic conditions were established by growing the cells in air-tight stoppered Balch tubes filled completely with growth media and evacuated for 30 minutes using vacuum suction. Anaerobic cultures were grown without shaking. Cells were induced with 0.5 mM IPTG for 12 hours, resuspended in phosphate buffered saline and scanned using fluorescence spectrometry (470 nm excitation). doi:10.1371/journal.pone.0064753.g009
Methods

Bacterial Strains and Growth Media
E. coli DH5α cells were routinely used for cloning and propagation of the wild type FMM-based fluorescent protein (FbFP) genes from Pseudomonas putida, Bacillus subtilis, and Arabidopsis thaliana. E. coli BLR (DE3) expression strains (EMD Chemicals) were used for protein expression. Transcriptional assays by fluorescence spectroscopy were performed using wild type E. coli MG1655 cells. Cells were grown with vigorous shaking (200 r.p.m.) at 37°C in Lennox broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) or on solid 1.5% agar plates containing Lennox medium and incubated at 37°C for 20 hours. Ampicillin at a concentration of 100 μg/mL was used for maintenance of the pQE80L plasmids. Kanamycin at a concentration of 30 μg/mL was used for maintenance of the pAM06-tet and pAM09-tet plasmids. For gene expression experiments, cells were grown in M9 minimal medium (12.8 g/L Na2HPO4⋅7H2O, 3 g/L plasmids. For gene expression experiments, cells were grown in M9 minimal medium (12.8 g/L Na2HPO4⋅7H2O, 3 g/L KH2PO4, 0.5 g/L NaCl, and 1 g/L NH4Cl) supplemented with 0.1% casamino acids and 0.5% glycerol or 20 mM glucose as the carbon source. For anaerobic cultivation, M9-glucose medium was supplemented with 20 mM potassium nitrate as the electron acceptor. Anaerobic culture conditions were established by growing cells in air-tight stoppered Balch tubes completely filled with growth media and evacuated using vacuum suction for 30 minutes. Anaerobic cultures were grown without shaking. Lennox broth, ampicillin, casamino acids, and agar were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (Pittsburgh, PA).

Cloning of FbFP Genes
FbFP genes were synthesized by GenScript (Piscataway, NJ) or Integrated DNA Technologies (Coralville, IA) and the sequences are provided as supporting information (Text S4). The gene for the yellow fluorescent protein (YFP) was amplified from a CRIM promoter probe plasmid [62], which was a kind gift from Prof. Christopher Rao (University of Illinois at Urbana-Champaign). The genes were cloned in the pQE80L expression vector from Qiagen (Valencia, CA) using BamHI and HindIII restriction enzymes. The constructs were respectively digested pQE80L-PpFbFP, pQE80L-EcFbFP, pQE80L-iLOV, and pQE80L-YFP. PCR amplification, restriction digestion, and ligation were accomplished using standard protocols [63]. Briefly, PCR was carried out in 50 μL reaction volume using 10 ng of template DNA and 0.5 μM primers, 0.2 mM dNTPs and 2.5 units Taq DNA polymerase. The PCR cycle consisted of an initial denaturation at 94°C for 2 minutes followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s (1 minute for YFP). A final extension step at 72°C for 10 minutes was employed to complete synthesis of full-length templates. Amplicons weredigested with 10 units each of BamHI and HindIII restriction endonucleases at 37°C for 1 hour, purified by gel electrophoresis and subsequently ligated into pQE80L expression vector digested with BamHI and HindIII using similar reaction conditions. Ligation reactions were performed using 400 units T4 DNA ligase in a 20 μL reaction volume at room temperature for 1 h. Promoter probe plasmids pAM06-tet and pAM09-tet were constructed based on a previously reporter pUA139 promoter probe vector that expresses GFP cycle 3 mutant under the control of an upstream promoter of choice [50]. We engineered the plasmid to include a 3-frame stop codon and a strong synthetic ribosome binding site between the GFP gene and the upstream promoter. In order to make the promoter probe vector modular, we incorporated NheI and HindIII restriction sites flanking the GFP reporter gene. The ribosome binding site was flanked by KpnI and NheI restriction sequences. We constructed a synthetic phage lambda P1-tetO hybrid promoter [52] by annealing complimentary oligonucleotides. The promoter was ligated in the plasmid construct using BamHI and NheI restriction enzymes and T4 DNA ligase. An rnR2 transcriptional terminator was included upstream of the promoter to prevent divergent transcription. The modified promoter probe plasmid was designated as pAM06-tet. A promoter probe plasmid harboring iLOV was constructed by digesting pAM06-tet with NheI and HindIII and ligating iLOV, which had been digested with the same enzymes. This construct was designated pAM09-tet. All plasmid constructs were propagated by transformation in E. coli DH5α cells using heat shock at 42°C. Cells were plated on LB-agar supplemented with ampicillin or kanamycin for selection. Plasmids were isolated from E. coli DH5α transformants (Qiagen Miniprep kit) and used to transform E. coli BLR (DE3) cells for protein expression or E. coli MG1655 cells for gene expression studies. Plasmid constructs pQE80L-PpFbFP, pQE80L-EcFbFP, pQE80L-iLOV, and pQE80L-YFP were employed for protein expression as well as promoter activity measurements of the IPTG-inducible phage T5 promoter. Plasmid constructs pAM06-tet and pAM09-tet were utilized for assaying promoter activity of the constitutive phage lambda promoter. Table 5 contains a list of oligonucleotide sequences corresponding to the primers, phage lambda promoter, synthetic ribosome binding site, and transcriptional terminator. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA), and primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Protein Expression and Purification
Single colonies of E. coli BLR (DE3) transformants expressing the pQE80L-FbFP or pQE80L-YFP constructs were inoculated in 5 mL Lennox broth-ampicillin medium and grown for 16 hours. Cells from the overnight culture were diluted in 500 mL medium in a 2 L shake flask. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the culture reached an optical density of 600 nm (A600) of 0.4-0.6. FbFP expression was continued for 5–6 hours at 37°C, followed by harvesting of the cells by centrifugation at 5000 g for 15 minutes at 4°C and resuspension in 10–15 mL lysis buffer (20 mM Tris hydrochloride, 200 mM sodium chloride, pH 8.0). The cells were lysed by addition of lysozyme at a concentration of 1 mg/mL and treated for 30 minutes at room temperature followed by ultrasonication (5 cycles of ten 1-second pulses of 17–20 W each). The lysate was clarified by removal of cell debris by centrifugation at 10,000 g for 20 minutes at 4°C, and the supernatant was incubated with 4 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) on a rocker for 1 hour at 4°C. The Ni-NTA resin and supernatant were loaded onto a gravity flow chromatographic column (Fischer Scientific) and washed with 50 mL of Ni-NTA wash buffer (20 mM Tris hydrochloride, 200 mM sodium chloride, 40 mM imidazole, pH 8.0) to remove nonspecifically bound protein. FbFPs were eluted with 20 mL Ni-NTA elution buffer (20 mM Tris hydrochloride, 200 mM sodium chloride, 500 mM imidazole, pH 8.0). FbFP fractions, which were visibly fluorescent, were pooled and loaded onto a 5 mL HiTrap Q Sepharose anion exchange column (GE Healthcare) using an automated AKTA FPLC system. The bound protein was washed with 5 column volumes (25 mL) of anion exchange wash buffer (20 mM Tris hydrochloride, 200 mM sodium chloride, pH 8.0), and eluted in 5 column volumes of anion exchange elution buffer (20 mM Tris hydrochloride, 1 M sodium chloride, pH 8.0). At NaCl concentration of 200 mM,
PpFbFP and EcFbFP were strongly bound to the anion-exchange column. iLOV and YFP failed to bind to the anion exchange resin at or above 200 mM NaCl, so the salt concentrations in the Ni-NTA elution buffer and anion exchange wash buffer were reduced to 20 mM NaCl for purifying iLOV and YFP. Protein fractions were assayed for homogeneity by denaturing polyacrylamide gel electrophoresis (SDS PAGE) and fluorescence spectrometry. Protein concentrations were estimated using the Bradford assay. In calculating the concentration of holoprotein in solutions of purified FbFPs to be negligible (Text S1). Therefore, the holoprotein concentration to be calculated from the Beer-Lambert equation as follows:

\[ C_{\text{holo}} = \frac{A_{450} \lambda}{\varepsilon} \]

where \( C_{\text{holo}} \) is the concentration of the holoprotein, \( \varepsilon \) is the molar extinction coefficient of FMN (12500 M\(^{-1}\)cm\(^{-1}\)), and \( \lambda \) is the cuvette path length. The fraction of fluorescent holoprotein in solution was determined by normalizing the holoprotein concentration by the total protein concentration estimated from the Bradford assay. In calculating the concentration of holoprotein in this way, we have verified that the concentration of free FMN in solutions of purified FbFPs to be negligible (Text S1).

### Determination of Oligomeric State

Oligomeric states of FbFPs were determined by gel filtration chromatography using a Superdex 200 column in an AKTA FPLC system (GE Healthcare). The column was calibrated with globular proteins standards, including bovine thyroglobulin (670 kDa), bovine \( \gamma \)-globulin (158 kDa), chicken ovalbumin (44 kDa), and horse myoglobin (17 kDa). Purified FbFPs were loaded in the column and washed with 50 mL phosphate buffered saline or anion exchange elution buffer (20 mM Tris hydrochloride, 1 M NaCl, pH 8.0) (Figure S1). Emission volumes corresponding to peaks in the 280 nm absorption chromatogram were recorded and molecular mass was estimated from a calibration graph of the logarithm of molecular mass of standards plotted against corresponding peak elution volumes. Molecular mass of a
Effect of pH on FbFP Fluorescence

Buffers of varying pH were diluted using 200 mM sodium hydrogen phosphate/100 mM sodium citrate (pH 2-8) and 100 mM sodium carbonate/100 mM sodium bicarbonate solutions (pH 9-11). These solutions were titrated with 4 mM Na2HPO4/98 mM sodium citrate, pH 4 (77.1 mM Na2HPO4/61.45 mM sodium citrate), pH 6 (126.3 mM Na2HPO4/36.85 mM sodium citrate), pH 8 (194.5 mM Na2HPO4/2.75 mM sodium citrate), pH 10 (60 mM Na2CO3, 40 mM NaHCO3), and pH 11 (90 mM Na2CO3, 10 mM NaHCO3). Purified FbFP preparations in anion exchange elution buffer (pH 8.0) were diluted 1:30 in 450 μL sample volumes were added to each well of a flat-bottom 96-well plate (Nunc). We verified that the buffer pH remained constant at the desired value subsequent to titration of FbFP using a pH meter (Accumet, ColeParmer). FbFPs were incubated for 2.5 hours in the respective buffers and emission spectra were recorded between 480 and 600 nm every 30 minutes using a fluorescence spectrometer (Tecan M200) with gain setting manually set at 50. Excitation and emission slit widths were set to 9 nm and 20 nm respectively. In the case of YFP, excitation wavelength was set at 505 nm and emission was recorded at 528 nm. At each pH, fluorescence intensities corresponding to the emission wavelength (495 nm) were normalized by the maximum emission intensity recorded at physiological pH. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Effect of Temperature on FbFP Fluorescence

In order to assess the thermal stability of FbFPs, 300 μL volumes of purified protein preparations were added to 6×50 mm borosilicate glass culture tubes (Kimble Chase, Vineland, NJ) housed in custom-made aluminum holders. The glass tube/holder assembly was then placed in a fluorescence spectrometer (Cary Varian), which was heated to the desired temperature using a temperature controlled water bath (Cary Varian). Evaporative loss was minimized by sealing the mouth of the glass tubes with heat resistant tape or adding 10 μL BacLight mounting oil (Invitrogen) to the surface of the protein solution in the cuvette. FbFPs were incubated at different temperatures (40°C, 50°C, 60°C, and 70°C) for up to 2.5 hours, and fluorescence emission spectra were recorded between 470 and 600 nm every 30 minutes. Excitation and emission slit widths were set at 5 nm and the photomultiplier tube gain was set to medium (600 V). In the case of YFP, excitation was provided at 500 nm and emission spectra were scanned between 515 and 600 nm. For each temperature, the fluorescence intensity corresponding to the emission wavelength (495 nm) was normalized to the emission intensity at 495 nm measured at room temperature.

Fluorescence Recovery after Denaturation

300 μL volumes of purified FbFPs were added to 6×50 mm borosilicate glass culture tubes (Kimble Chase, Vineland, NJ) housed in custom-made aluminum holders. The glass tube/ aluminum assemblies were then placed in a fluorescence spectrometer (Cary Varian), which was heated to 90°C or 70°C. FbFPs were denatured by heating at 90°C or 70°C for 25 minutes. Denaturation was monitored by the loss of peak fluorescence emission at 495 nm and the appearance of a 523 nm emission peak characteristic for free FMN. Following denaturation, protein samples were cooled to 25°C, and renaturation was monitored by recording the emission spectra between 470 and 600 nm. Excitation and emission slit widths were set at 5 nm and the photomultiplier tube gain was set to medium (600 V).

Effect of Strong Reducing Agents on FbFP Fluorescence

In order to probe the sensitivity of FbFPs to reducing conditions, 200 μL of protein samples were added to the wells of a 96-well plate and treated with a strong reductant (sodium dithionite) at 25 mM concentration. Protein solutions were excited with 450 nm light in a fluorescence spectrometer (Tecan M200), and emission spectra were recorded between 480 and 600 nm every 30 minutes for a period of 2.5 hours with gain setting manually set at 50. Excitation and emission slit widths were set to 9 nm and 20 nm respectively. Fluorescence intensities corresponding to the emission wavelength (495 nm) were normalized by the fluorescence intensity at 495 nm of purified proteins incubated without sodium dithionite.

Monitoring Promoter Activity in E. coli Using FbFPs as Reporters

FbFPs were evaluated as potential reporters for real-time measurements of transcriptional activity from IPTG-inducible phage T5-lacO [49] and constitutive phage lambda-tetO [52] hybrid promoters in E. coli MG1655 cells. Single colonies of E. coli cells expressing the FbFP of interest were inoculated in M9 minimal medium supplemented with 20 mM glucose or 0.5% glycerol as the carbon source and grown for 16 hours with vigorous shaking (200 r.p.m.) at 37°C. Following overnight growth, cells were inoculated at 1% dilution in 250 μL of the relevant medium (M9-glucose, or M9-glycerol) in an optically clear, flat-bottom, 96-well plate (Nunc). The growth medium was supplemented with IPTG to express the T5-lacO promoter. We employed an IPTG concentration that yielded the highest levels of normalized fluorescence for the respective reporters - specifically, 1 mM (for iLOV), 0.1 mM (for PpFbFP and EcFbFP) and 10 mM (for venus). In the case of the phage lambda transcriptional constructs, IPTG was eliminated because the promoter is constitutive in wild type E. coli. The 96-well plate was incubated with shaking (3.5 mm amplitude, linear shaking) in a fluorescence spectrometer (Tecan M200) at 37°C. Media evaporation was minimized through the use of covered 96-well plates. E. coli cells in each well were periodically illuminated with 450 nm light and transcription from the promoter was followed by measuring fluorescence intensity at the emission wavelength (495 nm) every 15-20 minutes over a period of 16 hours. Cell growth was simultaneously monitored by measuring absorbance at 600 nm (A600nm). Excitation and emission slit widths were set at 9 nm and 20 nm respectively and gain was manually adjusted to 50. Fluorescence measurements from the induced cultures were normalized by the corresponding A600nm values and normalized background fluorescence measured in uninduced cells was subtracted. In an analogous fashion, promoter activities were also determined using YFP or GFP. E. coli cells expressing YFP were excited at 505 nm and emission intensity was recorded at 530 nm. E. coli cells expressing GFP were excited at 485 nm and emission intensity was recorded at 510 nm. Promoter activity profiles elucidated using FbFPs as reporters were compared with transcriptional profiles derived using YFP or GFP. We restricted our analysis of gene expression to the exponential growth phase of E. coli cells, which corresponds to the optical density range from 0.4 to 0.8, determined by absorbance at 600 nm. Exponential growth was simultaneously monitored by measuring absorbance at 600 nm.
Doubling times were calculated using the equation:

\[ T_d = \frac{\ln(2)}{\mu} \]  

### Supporting Information

- **Figure S1** Size exclusion chromatograms of FbFPs.
- **Figure S2** Emission spectra of FbFPs at pH 6–7.
- **Figure S3** Denaturation of PpFbFP and EcFbFP at pH 2.
- **Figure S4** Effect of pH and temperature on fluorescence in YFP.
- **Figure S5** Denaturation of PpFbFP and EcFbFP at 70°C.
- **Figure S6** Denaturation/renaturation of EcFbFP and YFP monitored using fluorescence emission.
- **Figure S7** Effect of strong reducing conditions on FbFP fluorescence.
- **Figure S8** Doubling times of *E. coli* expressing FbFPs from inducible and constitutive promoters.
- **Figure S9** Complete transcriptional profiles of PT5-lacO promoter in *E. coli* cells grown in M9-glucose.
- **Figure S10** Complete transcriptional profiles of PT5-lacO promoter in *E. coli* cells grown in M9-glycerol.

### References

1. Tsien RY (1998) The green fluorescent protein. Annual Review of Biochemistry 67: 509-544.
2. Ai HW, Henderson JN, Remington SJ, Campbell RE (2006) Directed evolution of a monomeric, bright and photostable version of clavularia cyan fluorescent protein: Structural characterization and applications in fluorescence imaging. Biochemical Journal 400: 531-540.
3. Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, et al. (1995) Understanding, improving and using green fluorescent proteins. Trends in Biochemical Sciences 20: 440-455.
4. Shaner NC, Patterson GH, Davidson MW (2007) Advances in fluorescent protein technology. Journal of Cell Science 120: 4247-4260.
5. Shaner NC, Lin MZ, Mckeeen MR, Steinbach PA, Hazelwood KL, et al. (2008) Improving the photostability of bright monomeric orange and red fluorescent proteins. Nature Methods 5: 545-551.
6. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, et al. (2002) A monomeric red fluorescent protein. Proceedings of the National Academy of Sciences of the United States of America 99: 7877-7882.
7. Delagrange S, Hawtin RE, Silva CM, Yang MM, Youvan DC (1995) Red-shifted excitation mutants of the green fluorescent protein. Bio/Technology 13: 151-154.
8. Matz MV, Fridkovich AF, Labas YA, Savitsky AP, Zaraisky AG, et al. (1999) Fluorescent proteins from nonbioluminescent anthozoans. Nature Biotechnology 17: 969-973.
9. Wiedermann J, Iwanchenko S, Oswald F, Nienhaus GU (2004) Identification of GFP-like proteins in nonbioluminescent, azooxanthellate anthozoa opens new perspectives for bioprospecting. Marine Biotechnology 6: 270-277.
10. Heim R, Prasher DC, Tsien RY (1994) Wavelength mutations and posttranslational oxidation of green fluorescent protein. Proceedings of the National Academy of Sciences of the United States of America 91: 12501-12504.
11. Remington SJ (2006) Fluorescent proteins: Maturation, photochemistry and photophysics. Current Opinion in Structural Biology 16: 714-721.
12. Reid BG, Flynn GC (1997) Chromophore formation in green fluorescent protein. Biochemistry 36: 6786-6791.
13. Corall C, Cemazar M, Kanthou C, Tozer GM, Dachu GU (2001) Limitations of the reporter green fluorescent protein under simulated tumor conditions. Cancer Research 61: 4784-4790.
14. Vordermark D, Shibata T, Brown JM (2001) Green fluorescent protein is a suitable reporter of tumor hypoxia despite an oxygen requirement for chromosome formation. Neoplasia 3: 527-534.
15. Scott KP, Mester DK, Glover LA, Flint HJ (1998) The green fluorescent protein as a viable marker for lactic acid bacteria in complex ecosystems. FEMS Microbiology Ecology 26: 219-230.
16. Hansen MC, Palmer RJ Jr, Ulkonen C, White DC, Molin S (2001) Assessment of GFP fluorescence in cells of streptococcus gordonii under conditions of low pH and low oxygen concentration. Microbiology 147: 1303-1391.
17. Geiger K, Leisberger A, Mierdlein A, Stark N, Geller-Rhomberg S, et al. (2011) Identification of hypoxia-induced genes in human SGBS adipocytes by microarray analysis. PLoS ONE 6.
18. Chapman S, Faulkner C, Kaiser B, García-Mata C, Savchenko EI, et al. (2008) The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. Proceedings of the National Academy of Sciences of the United States of America 105: 20038-20043.
19. Drreper T, Eggert T, Carcolone F, Heck A, Krauf F, et al. (2007) Reporter proteins for in vivo fluorescence without oxygen. Nature Biotechnology 25: 443-445.

### Author Contributions

Conceived and designed the experiments: AM CMS. Performed the experiments: AM KBW JW. Analyzed the data: AM CMS. Wrote the paper: AM CMS.
26. Möglich A, Mo¨glich A, Moffat K (2007) Structural basis for light-dependent signaling in the flavin mononucleotide-binding domains of the photosynthetic complexes. Biochemistry 46: 5851–5860.

21. Losi A (2007) Flavin-based blue-light photosensors: A photobiophysics update. Photochemistry and Photobiology 83: 1293–1300.

20. Herrero J, Crossen S (2011) Function, structure and mechanism of bacterial photosynthetic LOV proteins. Nature Reviews Microbiology 9: 713–723.

25. Drepper T, Huber R, Bende O, Losi A, et al. (2009) In vivo mutational analysis of YtvA from bacillus subtilis. Mechanism of light activation of the general stress response. The Journal of Biological Chemistry 284: 24956–24964.

24. Avila-Perez M, Heilingwerf KJ, Kort R (2006) Blue light activates the signal-dependent response of bacillus subtilis via YtvA. Journal of Bacteriology 188: 6411–6414.

23. Kasahara M, Swartz TE, Olson MA, Onodera A, Mochizuki N, et al. (2002) Photochemical properties of the flavin mononucleotide-binding domains of the chlorophylls from arabidopsis, rice, and chloramidonas reinhardtii. Plant Physiology 129: 762–773.

22. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, et al. (2001) Arabidopsis nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast relocation. Proceedings of the National Academy of Sciences of the United States of America 98: 6969–6974.

21. Moglich A, Mo¨glich A, Moffat K (2007) Structural basis for light-dependent signaling in the dimeric LOV domain of the photosynthetic complex. Journal of Molecular Biology 373: 112–126.

19. Pru¨trigkeit T, Kompa CK, Salomon M, Rudiger W, Michel-Beyerle ME (2003) Primary photophysics of the FMN binding LOV2 domain of the plant blue light receptor phototropin of avena sativa. Chemical Physics 294: 501–506.

18. Losi A, Quest B, Gutter W (2003) Listening to the blue: The time-resolved thermodynamics of the bacterial blue-light receptor YtvA and its isolated LOV domain. Photochemical and Photobiological Sciences 2: 759–766.

17. Swartz TE, Corchon SE, Johnson AL, Zundl I, et al. (2001) The photochrome of a flavin-binding domain of the blue light photoreceptor phototropin. Journal of Biological Chemistry 276: 36493–36500.

16. Drepper T, Huiber R, Beck A, Circolone F, Hinneste AK, et al. (2010) Flavin mononucleotide-based fluorescent reporter proteins outperform green fluorescent protein-like proteins as quantitative in vivo real-time reporters. Applied and Environmental Microbiology 76: 5990–5994.

15. Choi CH, DeGuzman JV, Lamont RJ, Vilmann A (2011) Genetic transformation of an obligate anaerobe, P. gingivalis, with FMN-green fluorescent protein expression in studying host-microbe interaction. PLoS ONE 6: e18499.

14. Lobo LA, Smith CJ, Rocha ER (2011) Flavin mononucleotide (FMN)-based fluorescent protein (PFP), as reporter for gene expression in the anaboreae bacterides. FEMS Microbiology Letters 317: 67–74.

13. Peikarski T, Buchholz I, Drepper T, Schobert M, Wagner-Doehler I, et al. (2009) Genetic tools for the investigation of roseobacter clade bacteria. BMC Microbiology 9.

12. Tielker D, Eichhof I, Jaeger K-E, Ernst JF (2009) Flavin mononucleotide-based fluorescent protein as an oxygen-independent reporter in candida albicans and saccharomories cerevisiae. Eukaryotic Cell 10: 913–915.

11. Walter J, Hausmann S, Drepper T, Puls M, Eggert T, et al. (2012) Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS ONE 7.

10. Potzer J, Kanze M, Drepper T, Gesch T, Jaegger K-E, et al. (2012) Real-time determination of intracellular oxygen in bacteria using a genetically encoded FRET-based biosensor. BMC Biology 10: 28.

9. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, et al. (2012) Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS ONE 7.

8. Potzer J, Kanze M, Drepper T, Gesch T, Jaegger K-E, et al. (2012) Real-time determination of intracellular oxygen in bacteria using a genetically encoded FRET-based biosensor. BMC Biology 10: 28.

7. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, et al. (2012) Tuning of the structural fluorescence of the flavin mononucleotide molecule. Journal of Biological Chemistry 287: 22393–22394.

6. Mukherjee A, Weyant KB, Walker J, Schroeder CM (2012) Directed evolution of flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS ONE 7.

5. Potzer J, Kanze M, Drepper T, Gesch T, Jaegger K-E, et al. (2012) Real-time determination of intracellular oxygen in bacteria using a genetically encoded FRET-based biosensor. BMC Biology 10: 28.

4. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, et al. (2012) Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS ONE 7.

3. Potzer J, Kanze M, Drepper T, Gesch T, Jaegger K-E, et al. (2012) Real-time determination of intracellular oxygen in bacteria using a genetically encoded FRET-based biosensor. BMC Biology 10: 28.

2. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, et al. (2012) Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement. PLoS ONE 7.

1. Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, et al. (2012) Tuning of the structural fluorescence of the flavin mononucleotide molecule. Journal of Biological Chemistry 287: 22393–22394.

0. Mukherjee A, Weyant KB, Walker J, Schroeder CM (2012) Directed evolution of bright mutants of an oxygen-independent flavin-binding fluorescent protein. Journal of Biological Chemistry 287: 22393–22394.