

# Engineering and Characterization of New LOV-Based Fluorescent Proteins from *Chlamydomonas reinhardtii* and *Vaucheria frigida*

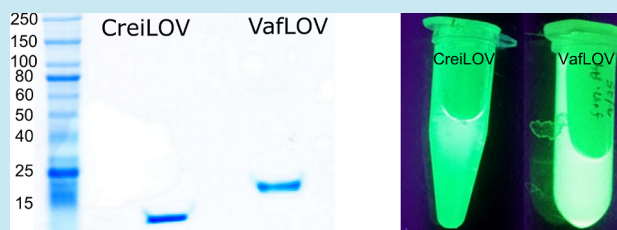
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## Supporting Information

**ABSTRACT:** Flavin-based fluorescent proteins (FbFPs) are a new class of fluorescent reporters that exhibit oxygen-independent fluorescence, which is a key advantage over the green fluorescent protein. Broad application of FbFPs, however, has been generally hindered by low brightness. To maximize the utility of FbFPs, there is a pressing need to expand and diversify the limited FbFP library through the inclusion of bright and robust variants. In this work, we use genome mining to identify and engineer two new FbFPs (CreiLOV and VafLOV) from *Chlamydomonas reinhardtii* and *Vaucheria frigida*. We show that CreiLOV is a thermostable, photostable, and fast-maturing monomeric reporter that outperforms existing FbFPs in brightness and operational pH range. Furthermore, we show that CreiLOV can be used to monitor dynamic gene expression in *Escherichia coli*. Overall, our work introduces CreiLOV as a robust addition to the FbFP repertoire and highlights genome mining as a powerful approach to engineer improved FbFPs.

**KEYWORDS:** fluorescent reporters, LOV domains, flavin-based fluorescent proteins, genome mining



The green fluorescent protein (GFP) and related analogues are nearly ubiquitous as reporter proteins for characterizing genetic circuits in systems and synthetic biology. Maturation of fluorescence in GFP, however, strictly depends on molecular oxygen for the oxidation of an internal tripeptide to form a *p*-hydroxybenzylidene chromophore.<sup>1–3</sup> In recent years, a sizable library of GFP-based reporters has been generated by protein engineering. However, all known GFP-based probes show oxygen-dependent fluorescence, which presents challenges for fluorescence-based imaging in anaerobic or microaerobic conditions.<sup>4–6</sup> Furthermore, maturation of fluorescence in GFP-based probes is generally slow and rate limited by chromophore oxidation. Nearly all GFP analogues exhibit a half-time for fluorescence on the order of several minutes ( $T_{1/2} \sim 10\text{--}40$  min), even for fast maturing variants such as Venus.<sup>3,7</sup> In addition, GFP is a relatively large protein tag ( $\sim 240$  amino acids), which can present challenges for generating translational fusions. Overall, there is a strong need for the development of new genetically encoded fluorescent reporters with improved biochemical and biophysical properties.

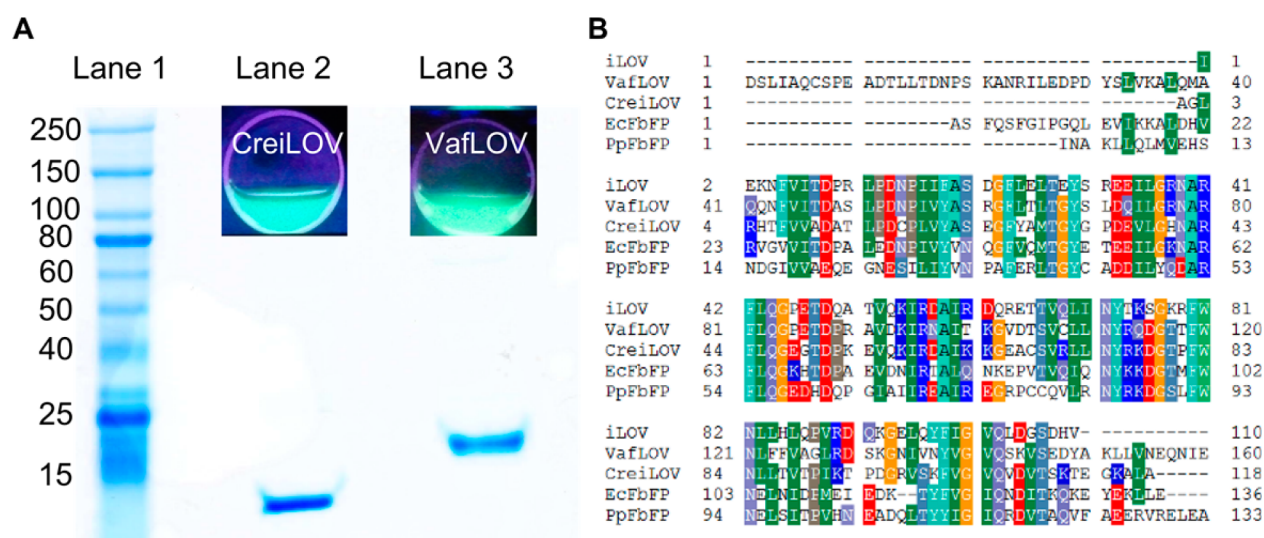
Recently, a new class of oxygen-independent and small ( $\sim 110\text{--}140$  amino acids) fluorescent reporters based on natural flavin-binding photosensory proteins was discovered.<sup>8,9</sup> Flavin-based fluorescent proteins (FbFPs) belong to a highly conserved family of photoreceptors known as light, oxygen, and voltage (LOV) sensing proteins.<sup>10–12</sup> Although LOV proteins have been extensively studied as biophysical models<sup>13</sup> as well as

for designing optogenetic actuators for applications in synthetic biology,<sup>14</sup> only recently have LOV-based proteins been pursued as fluorescent reporters for biological studies. Fluorescent variants of LOV proteins (generally known as FbFPs) were engineered by mutating a key cysteine residue in the FMN-binding pocket to alanine, which abrogates the native photocycle and results in cyan-green fluorescence emission ( $\lambda_{em,max} = 495$  nm and  $\lambda_{ex,max} = 450$  nm).<sup>8,9</sup> Importantly, FbFPs have been shown to express and fluoresce anaerobically, thereby demonstrating a major advantage over GFP-based probes for imaging in low-oxygen conditions.<sup>15–19</sup> Furthermore, we recently demonstrated that several FbFPs are thermostable and fast-maturing probes with a broad operational pH range.<sup>20</sup> Despite their considerable promise as fluorescent tags for studying bioprocesses that are intractable to GFP-based imaging, the FbFP library contains only a handful of proteins derived from *Pseudomonas putida* (PpFbFP), *Bacillus subtilis* (EcFbFP), and *Arabidopsis thaliana* (iLOV).<sup>8,9</sup>

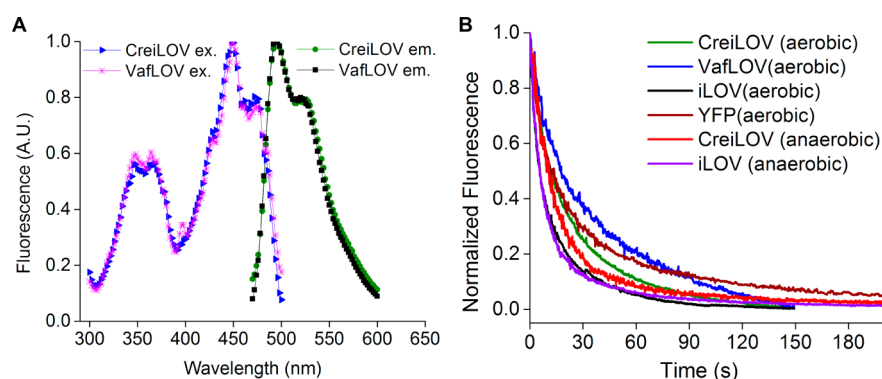
Efforts to expand the limited library size of the FbFP toolbox have mainly focused on directed evolution using DNA shuffling<sup>8,14</sup> and site saturation mutagenesis.<sup>21</sup> However, use of directed evolution to achieve *pareto* optimality, whereby amino acid sequence diversity is maximized while preserving functional and structural integrity, can be particularly challenging.<sup>22,23</sup> For example, in prior work we successfully

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**Figure 1.** Identification of CreiLOV and VafLOV as new oxygen-independent fluorescent proteins. (A) Denaturing polyacrylamide gel electrophoresis of purified CreiLOV and VafLOV. Lane 1: Protein markers (molecular weights indicated in kDa), Lane 2: CreiLOV, Lane 3: VafLOV. Inset: Purified CreiLOV and VafLOV proteins emit cyan-green fluorescence upon excitation using UV light. (B) Amino acid sequence alignment between CreiLOV, VafLOV, PpFbFP, EcFbFP, and iLOV. Amino acids that are conserved in at least 50% of the FbFPs are shaded using identical colors. Although CreiLOV and VafLOV show similar spectral characteristics as the remaining FbFPs, amino acid sequences are highly divergent.



**Figure 2.** Biophysical characterization CreiLOV and VafLOV. (A) Fluorescence excitation and emission spectra of purified FbFPs. Excitation spectra were obtained by monitoring emission at 540 nm while scanning the excitation wavelength from 300 to 520 nm. Emission spectra were obtained by illuminating purified protein samples at a wavelength of 450 nm while recording fluorescence emission between 470 and 600 nm. (B) Fluorescence microscopy was used to study the photobleaching kinetics of CreiLOV and VafLOV in fixed *E. coli* cells, which were continuously illuminated with 488 nm laser excitation. Our results were compared with photobleaching profiles for iLOV and YFP, which were expressed and imaged under identical conditions. In addition, CreiLOV and iLOV photobleaching were also characterized in anaerobically cultured *E. coli* cells. Mean fluorescence intensities were fitted to single exponential decay kinetics. The data represent the mean fluorescence intensities of  $\sim 12$  single cells.

used site saturation mutagenesis to generate brighter mutants of PpFbFP, known as F37S and F37T. However, despite several attempts, we were unable to further engineer brighter variants of F37S PpFbFP or iLOV using random mutagenesis.<sup>21</sup> In order to fully realize the potential of FbFPs, there is a pressing need to identify new FbFPs with diverse properties.

Natural LOV domains are abundant in bacterial, eukaryal, and archaeal organisms.<sup>24</sup> However, individual LOV proteins exhibit large degrees of variability in primary sequence, which generally precludes the application of directed evolution using homology-dependent family shuffling strategies. On the basis of the wide representation and sequence diversity of naturally occurring LOV proteins, we hypothesized that wild type LOV proteins offer a natural repertoire of sequence diversity that can be exploited to develop new FbFPs using genome mining. On the basis of sequence homology to iLOV or to a multiple sequence alignment between PpFbFP, EcFbFP, and iLOV, we

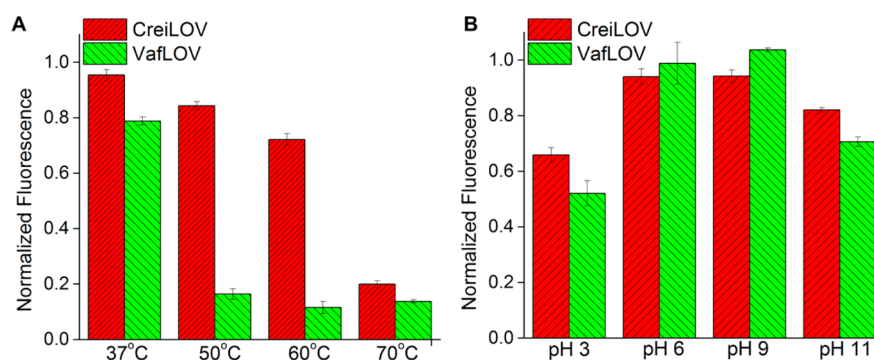
identified 21 LOV proteins in 15 genomes to screen for fluorescence (Table S1, Supporting Information (SI)). The sequences were cloned using gene-specific primers (Table S2 (SI)). In some cases, wild type LOV domains contained a cysteine residue in the FMN-binding pocket; for these proteins, we incorporated a Cys  $\rightarrow$  Ala mutation to avoid the natural LOV photocycle that generally results in loss of fluorescence.<sup>8,9,11,13</sup>

Following cloning and expression, we screened the LOV-domain protein products for fluorescence emission upon excitation with UV or blue light (450 nm). In this way, all 21 LOV domains were successfully expressed at appreciable levels in *Escherichia coli*, though 19 LOV domains showed no significant fluorescence emission. Importantly, 2 LOV domains corresponding to blue light photoreceptors in the freshwater algae *Chlamydomonas reinhardtii*<sup>25</sup> and the yellow-green algae *Vaucheria frigida*<sup>26</sup> were fluorescent under UV illumination.

Table 1. Biophysical Properties of CreiLOV and VafLOV<sup>a</sup>

protein	QY	brightness	photobleaching time constant <i>in vitro</i> (s)	photobleaching time constant in fixed cells (s)	photobleaching time constant in fixed cells (s) (anaerobic)	oligomeric state
CreiLOV	0.51	6375	33.9 ± 1.5	26.3 ± 0.7	20.2 ± 2.0	monomer
VafLOV	0.23	2875	52.8 ± 2.1	40.2 ± 0.8	N.D.	dimer
iLOV	0.34	4250	24.3 ± 0.8	16.8 ± 0.6	14.1 ± 2	monomer

<sup>a</sup>Relative quantum yields (QY) were calculated on the basis of comparison with FMN, which has a known quantum yield of 0.27. Our experimental approach for calculating quantum yield is described in the Supporting Information. Brightness is defined as the product of quantum yield and molar extinction coefficient of the fluorophore (FMN). In accordance with previous studies, brightness of FbFPs is calculated assuming the molar extinction coefficient of FbFP-bound FMN to be identical to free FMN (12 500 M<sup>-1</sup> cm<sup>-1</sup>). Alternate approaches to rigorously quantify molar extinction coefficients of protein-bound FMN that account for the incomplete maturation of FbFPs upon overexpression in *E. coli* are described in detail in the Supporting Information, and the values of molar extinction coefficients calculated using different approaches are listed in Table S5 (SI).



**Figure 3.** Thermal and pH stability of CreiLOV and VafLOV. (A) Fraction of peak fluorescence (relative to fluorescence emission at 495 nm at 25 °C) retained by CreiLOV and VafLOV following incubation at 37, 50, 60, and 70 °C for 2.5 h. (B) Fraction of peak fluorescence (relative to fluorescence emission at 495 nm and pH 7) for CreiLOV and VafLOV after incubation in pH-buffered solutions with pH 3, 6, 9, and 11 for 2.5 h. Each data point represents the mean of at least 3 independent replicates, and error bars indicate the standard error of mean.

These proteins were purified to near homogeneity (Figure 1A), and we found that the purified proteins exhibited bright, cyan-green fluorescence (Figure 1A, inset). Following these identifications, we named FbFPs from *Chlamydomonas reinhardtii* and *Vaucheria frigida* as CreiLOV and VafLOV, respectively. Amino acid sequence alignment between the existing set of FbFPs, CreiLOV, and VafLOV revealed fewer than 50% conserved amino acids (Figure 1B).

Following identification of CreiLOV and VafLOV, we characterized the photophysical properties of these proteins using fluorescence spectroscopy and microscopy. CreiLOV and VafLOV exhibit finely structured fluorescence excitation spectra, with peaks in the UV-A (350–370 nm) and blue (450 nm) regions of the spectrum, and emission spectra with a peak at 495 nm and a shoulder at 525 nm (Figure 2A).<sup>20</sup> We determined the quantum yields for CreiLOV and VafLOV as 0.51 and 0.23, respectively. On the basis of our results, CreiLOV is the brightest known FbFP, with a quantum yield that is a factor of ~1.5-fold larger than iLOV, which was previously identified as the brightest known FbFP (Table 1). In prior work, we demonstrated that fluorescence emission of an FbFP from *Pseudomonas putida* (PpFbFP) can be enhanced through the inclusion of F37S or F37T mutations at an FMN-proximal phenylalanine aromatic amino acid. We were, however, unable to enhance brightness in CreiLOV or VafLOV using similar F → S substitutions, which in the case of CreiLOV and VafLOV resulted in significant loss of fluorescence emission (Figure S1 (SI)).

We characterized the photostability of CreiLOV and VafLOV by observing photobleaching kinetics of purified protein preparations that were immobilized in thin pads of agarose on a glass coverslip and continuously illuminated using a 488

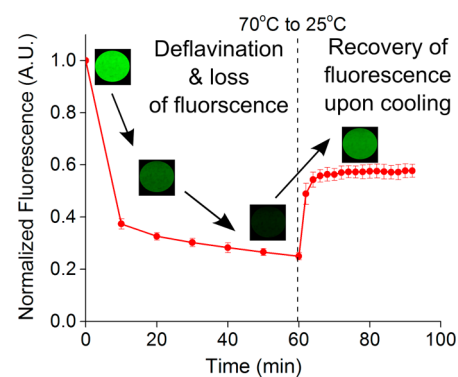
nm laser line until photobleached. We observed that CreiLOV and VafLOV are irreversibly photobleached with fluorescence decays described by single exponential kinetics, with first order time constants  $\tau = 33.9$  s and  $\tau = 52.8$  s for CreiLOV and VafLOV, respectively (Table 1 and Figure S2 (SI)). In a second set of experiments, photobleaching was characterized by expressing CreiLOV and VafLOV in *E. coli* cells that were fixed, continuously illuminated with a 488 nm laser line, and imaged using epifluorescence microscopy. Fixed cell photobleaching kinetics generally agreed with *in vitro* results on purified proteins. Photobleaching was irreversible and characterized by single exponential decay, with first order time constants  $\tau = 26.3$  s and  $\tau = 40.2$  s for CreiLOV and VafLOV, respectively (Table 1 and Figure 2B). Under identical conditions, a GFP-variant (YFP) photobleaches with a time constant  $\tau = 48.6$  s, while iLOV is less photostable with a first order time constant  $\tau = 16.8$  s (Figure 2B). In addition, we also characterized photobleaching under hypoxic conditions by expressing CreiLOV and iLOV in *E. coli* cells that had been grown under anaerobic conditions, fixed, and imaged as described above. CreiLOV and iLOV-expressing cells appeared to photobleach slightly faster under anaerobic conditions with first order time constants,  $\tau = 14.1$  s for iLOV and 20.2 s for CreiLOV (Figure 2B). We note that we also characterized photobleaching in fixed cells using biexponential fits as originally employed by Drepper and colleagues for characterizing photobleaching in PpFbFP and EcFbFP.<sup>9</sup> Biexponential models improved the overall goodness of fit only marginally and closely agree with time constants derived using a monoexponential fit. Photobleaching time constants derived from biexponential fitting are listed in Table S3 (SI).



We further characterized the oligomeric states of CreiLOV and VafLOV using size exclusion chromatography and native polyacrylamide gel electrophoresis. We determined that CreiLOV is a monomer and VafLOV predominantly exists as a dimer (Figure S3 (SI)). Our results directly show that CreiLOV (119 amino acids) is comparable in size to iLOV (110 amino acids), which is the smallest known FbFP. From this perspective, CreiLOV potentially provides a considerable advantage for generating translational fusions with smaller footprints compared to the  $\sim 2$ -fold larger GFP-family proteins.<sup>8,27</sup> Considering the entirety of the results based on brightness, size, and photostability (Table 1), CreiLOV emerges as a promising new FbFP probe with superior biophysical properties.

In prior work, we demonstrated that some FbFPs (iLOV and EcFbFP) are characterized by a high degree of thermal stability and a broad operational pH range.<sup>20</sup> Therefore, we conjectured that CreiLOV and VafLOV might similarly display high levels of thermal and pH stability. Indeed, we found that CreiLOV is comparable to iLOV in thermal stability, generally retaining 84 and 72% of room-temperature fluorescence upon prolonged incubation (2.5 h) at 50 and 60 °C (Figure 3A). Interestingly, and in contrast, VafLOV readily loses fluorescence at high temperatures, retaining only 11% of its room-temperature fluorescence at 60 °C (Figure 3A). The poor thermostability of VafLOV relative to CreiLOV (Figure 3B) is in agreement with a statistical estimate of protein stability based on the occurrence of specific destabilizing dipeptides in a protein sequence. In particular, CreiLOV is predicted to be stable (instability index,  $I_i = 21.5$ ), whereas VafLOV is predicted to be unstable ( $I_i = 40.9$ ). In addition to high levels of thermostability, CreiLOV is fluorescent over a broad pH range (pH 3–11), retaining 82 and 66% of fluorescence emission upon prolonged incubation at pH 11 and 3, respectively (Figure 3B). The pH tolerance of CreiLOV is superior to both EcFbFP and iLOV. Furthermore, VafLOV also shows substantial robustness to extreme pH conditions, retaining  $\sim 70$  and 52% of fluorescence at pH 11 and 3, respectively (Figure 3B). Though, several pH-stable GFP variants have been developed, the pH stability of CreiLOV and VafLOV sharply contrasts with the pH-sensitivity of early GFP(s) as well as certain GFP-based probes such as YFP, which readily lose fluorescence in acidic conditions, retaining  $< 10\%$  of its maximum fluorescence upon incubation at pH 4.<sup>1,5,28</sup> From an applications perspective, the thermal stability (up to 60 °C) and broad operational pH range (pH 3–11) of CreiLOV make it a promising candidate as a versatile and robust fluorescent probe naturally suited for unprecedented applications in extremophilic conditions involving thermophiles, acidophiles, and alkaliphilic microbes, several of which also tend to be obligately anaerobic.

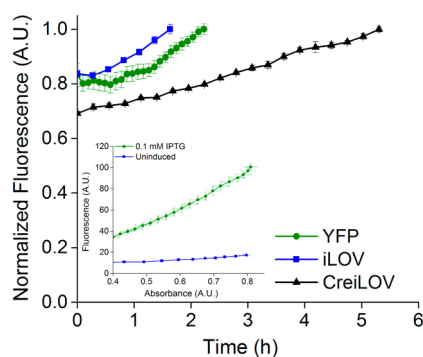
Maturation rates are a key property of genetically encoded fluorescent reporters. In prior work, we introduced an approach to characterize fluorescence maturation kinetics in FbFPs by first deflavinating the proteins at 70 °C, which results in loss of the 495 nm emission peak, followed by rapid cooling of the sample to room temperature, which results in reappearance of the 495 nm peak.<sup>20</sup> Using this approach, we observed that fluorescence emission was rapidly restored for CreiLOV upon cooling of protein samples to room temperature after heating (Figure 4). Fluorescence recovery was fast and half-maximal recovery appeared to occur within  $\sim 2$  min. Fluorescence of renatured CreiLOV saturated to approximately 60% of its initial fluorescence prior to defflavination. In contrast, VafLOV was



**Figure 4.** CreiLOV shows reversible fluorescence emission upon thermal denaturation/renaturation. Fluorescence maturation in FbFPs can be monitored by recording acquisition of fluorescence upon cooling thermally defflavinated FbFP samples, as illustrated schematically in the figure. Using this approach, CreiLOV was denatured by incubation at 70 °C for 1 h, which is accompanied by a progressive loss of peak fluorescence emission at 495 nm (defflavination phase). Following thermal treatment, temperature was rapidly ( $\sim 2$  min) lowered from 70 to 25 °C using a temperature-controlled fluorescence spectrometer. Upon cooling to 25 °C, CreiLOV rapidly regains  $\sim 60\%$  of its fluorescence, indicative of fast renaturation and fluorescence recovery. Each data point represents the mean of  $> 3$  independent replicates, and error bars indicate the standard error of mean.

irreversibly denatured by heat (Figure S4 (SI)). We previously demonstrated that iLOV and EcFbFP are also characterized by rapid acquisition of fluorescence following thermal defflavination and subsequent renaturation. However, CreiLOV is unique in that it simultaneously integrates fast maturation with small size, improved brightness, and robust photostability. Fast-maturing robust reporters such as CreiLOV could prove useful for monitoring biological events with turnover times faster than typical time scales of GFP maturation, such as early events in gene expression and protein translation.

A major application of fluorescent reporters in systems biology, metabolic engineering, and synthetic biology involves the construction of transcriptional fusions between regulatory elements (such as promoters) and fluorescent probes to noninvasively monitor gene expression dynamics in real-time. Therefore, as a proof-of-principle demonstration of the utility of the newly discovered fluorescent proteins, we used CreiLOV and VafLOV to monitor gene expression dynamics from a synthetic T5-lacO hybrid bacteriophage promoter.<sup>29</sup> Wild type *E. coli* MG1655 cells expressing transcriptional fusions between the T5 promoter and CreiLOV or VafLOV were grown in defined minimal medium using glucose as the principle carbon source and gene expression was induced using IPTG. In prior work, we demonstrated the utility of iLOV as a viable reporter for gene expression based on comparative analysis against benchmark transcriptional reporters such as YFP.<sup>20</sup> Here, we compare our results using CreiLOV as a reporter for gene expression from the T5 promoter to those derived using iLOV as a reporter under identical conditions. Analysis was restricted between time points corresponding to  $0.4 < A_{600\text{nm}} < 0.8$  to measure promoter activity (expressed as  $F/A_{600\text{nm}}$ ) in the exponential phase of growth. We found that CreiLOV fluorescence increased almost linearly with cell growth over the exponential phase, which is similar to gene expression profiles derived using YFP and iLOV (Figure 5, inset).<sup>20</sup> Analogous to promoter activity trends derived using iLOV and YFP as transcriptional reporters, activity of the T5-lacO



**Figure 5.** CreiLOV as a transcriptional reporter of promoter activity in *E. coli*. Fluorescence at 495 nm ( $F_{495\text{nm}}$ ) and absorbance at 600 nm ( $A_{600\text{nm}}$ ) were measured for *E. coli* MG1655 cells expressing T5-CreiLOV and grown in M9 medium supplemented with glucose at 20 mM concentration and compared with cells expressing iLOV and YFP under similar conditions of growth. Traces depict normalized promoter activity (measured as  $F/A_{600\text{nm}}$ ) versus time for cells induced using IPTG. Normalized fluorescence plots for iLOV and YFP are provided for comparison; promoter activity profiles display an overall similar trend for CreiLOV, iLOV, and YFP. Analysis is restricted to time points corresponding to the logarithmic phase of cell growth ( $0.4 < A_{600\text{nm}} < 0.8$ ). As CreiLOV expressing cultures grew slower than iLOV or YFP expressing cultures, the duration of the log phase for cells expressing CreiLOV is longer than for iLOV or YFP. Inset:  $F_{495\text{nm}}$  versus  $A_{600\text{nm}}$  for uninduced cells (blank) as well as cells induced for CreiLOV expression using IPTG. Each data point represents the mean of 3 independent replicates corresponding to *E. coli* liquid cultures inoculated using distinct single colonies.

promoter (quantified as  $F/A_{600\text{nm}}$ ) derived using CreiLOV displayed a slowly increasing trend over the exponential growth phase (Figure 5), which suggests that CreiLOV could be useful as a viable reporter for dynamic gene expression studies. Interestingly, fluorescence of cells expressing T5-VafLOV was only marginally enhanced over background fluorescence of uninduced cells even at higher concentrations of IPTG (Figure S5 (SI)). The low cellular fluorescence of VafLOV cultures can be explained in part by the  $\sim 50\%$  reduced quantum yield of VafLOV compared to CreiLOV and iLOV (Table 1) and/or poor solubility. Finally, we verified that CreiLOV is fluorescent in *E. coli* cells grown under anaerobic conditions using glucose as a carbon source and potassium nitrate as an electron acceptor (Figure S6 (SI)).

CreiLOV and VafLOV depend on an essential cellular metabolite (flavin) for fluorescence. Therefore, it behooves us to assess the effect of intracellular expression of these FbFPs on cell growth. We found that T5-CreiLOV expressing cells cultured in glucose grew slower (doubling time,  $T_D \sim 5$  h) than cells expressing T5-iLOV or T5-VafLOV ( $T_D \sim 2$  h) under similar conditions. Furthermore, in the case of high levels of induction or growth using a relatively poor carbon source (glycerol), T5-CreiLOV expressing cells displayed a substantially longer lag phase compared to T5-iLOV expressing cells, indicating metabolic burden associated with medium to high levels of expression of CreiLOV (Table S4 (SI)). Furthermore, in the case of high levels of induction or growth using a relatively poor carbon source (glycerol), T5-CreiLOV expressing cells displayed a substantially longer lag phase compared to T5-iLOV expressing cells, indicating metabolic burden associated with medium to high levels of expression of CreiLOV (Table S5 (SI)). Recently, an iLOV variant known as miniSOG, has been shown to efficiently generate singlet

oxygen species in cells upon intense illumination,<sup>30–34</sup> which prompted us to investigate whether slow growth in CreiLOV-expressing cells could be explained on the basis of phototoxicity and/or production of reactive oxygen species (ROS). However, under our specific experimental conditions (periodic illumination using low intensity light and with short exposure times), we did not detect observable phototoxicity or ROS production in FbFP-expressing cells (Figures S7 and S8 (SI)). Performance of CreiLOV and VafLOV as intracellular reporters can conceivably be improved further using approaches that enhance cellular FMN concentrations, including engineering flavin transporters or supplementing FMN in media in case of cells that naturally transport flavin (e.g. *Bacillus subtilis* or animal cells).

In summary, we engineered a new and robust member of the FbFP family (CreiLOV). Strikingly, CreiLOV embodies multiple beneficial properties in a single FbFP variant, an outcome that is analogous in principle to the construction of an optimal chimera *via* shuffling of homologous genes. In this way, our work highlights the application of genome mining for identifying new FbFPs. In prior research, genome mining has been applied to discover novel enzymes,<sup>35</sup> genetic control elements,<sup>36</sup> and GFP variants,<sup>37</sup> and the present work has further capitalized on this approach for engineering FbFPs as fluorescent reporters. In addition, we demonstrate that CreiLOV can be used as a fluorescent reporter for studying dynamic gene expression, particularly at low to medium levels of transcriptional activity. GFP-based proteins are extensively used as reporters for noninvasive quantification of genetic circuit performance. From this perspective, new FbFPs such as CreiLOV can be used as reporters to interrogate and tune gene networks for applications in systems and synthetic biology. In this way, our work serves as a guide for engineering LOV-based fluorescent proteins, with an overall goal of extending fluorescence imaging to an exciting and extensive class of biological systems (e.g. extremophiles and human gastrointestinal microbiota) that remain intractable to study using GFP-based probes and related analogues.

## ■ MATERIALS AND METHODS

**Genome Mining and Screening LOV Domains for Fluorescence.** LOV proteins were identified for screening based on sequence homology to the existing set of FbFPs. In particular, we used BLAST (using iLOV as query) and HMMER (using a ClustalW alignment between iLOV, EcFbFP, and PpFbFP as query) to interrogate the NR and UniProt databases for homologous proteins. Protein regions corresponding to LOV domains were identified using Pfam. Genes corresponding to these LOV domains were synthesized by Integrated DNA Technologies (Coralville, IA) or amplified directly from the genome. Molecular cloning, protein expression, and purification were accomplished as previously described. In brief, LOV-domains were cloned in a pQE80L expression vector using *Bam*HI and *Hind*III restriction enzymes. Plasmids were propagated in *E. coli* DH5 $\alpha$  and proteins were expressed in *E. coli* MG1655 cells and purified using nickel-chelating and anion exchange chromatography. Purified LOV-proteins were screened for fluorescence using a UV transilluminator.

**Characterization of CreiLOV and VafLOV.** Quantum yield and effects of pH and temperature on fluorescence emission were measured as described previously.<sup>20</sup> Protein photostability measurements were performed by immobilizing

FbFPs in a thin pad of 1% agarose or by protein expression in *E. coli* cells, which were subsequently fixed using 2.5% (v/v) paraformaldehyde. Imaging was performed using an inverted microscope equipped for epifluorescence imaging (Olympus IX71) using a 40X 0.9 numerical aperture (NA) objective lens (*in vitro* measurements) or an oil immersion 100X 1.4 NA objective lens (fixed cells). Samples were illuminated using solid-state blue laser (488 nm), and images were recorded using a 488 nm long pass emission filter (Chroma). Excitation power at the coverslip surface was measured to be  $\approx 0.5$  mW and the exposure time for detection on the EMCCD camera (Andor iXon) was 30 ms.

**Gene Expression Experiments.** Wild type *E. coli* MG1655 cells expressing the FbFPs were inoculated in M9 minimal medium supplemented with 20 mM glucose and grown for 16 h at 37 °C. Overnight cultures were inoculated in a 96-well plate at 1% dilution in fresh medium, supplemented with IPTG. The 96-well plate was incubated with shaking in a fluorescence spectrometer (Tecan M200) at 37 °C. Wells were illuminated using 450 nm light, and transcription from the promoter was followed by periodically measuring absorbance at 600 nm ( $A_{600\text{nm}}$ ) and fluorescence emission at 495 nm (F).

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Detailed protocols for cloning, protein expression, quantification of quantum yields and photostabilities, and assays for phototoxicity and ROS production are provided. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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