Engineering and characterizing monomeric fluorescent proteins for live-cell imaging applications

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Published online 20 March 2014; doi:10.1038/nprot.2014.054

Naturally occurring fluorescent proteins (FPs) cloned from marine organisms often suffer from many drawbacks for cell biology applications, including poor folding efficiency at 37 °C, slow chromophore formation and obligatory quaternary structure. Many of these drawbacks can be minimized or eliminated by using protein engineering and directed evolution, resulting in superior probes for use in live-cell fluorescence microscopy. In this protocol, we provide methods for engineering a monomeric FP, for enhancing its brightness by directed evolution, and for thoroughly characterizing the optimized variant. Variations on this procedure can be used to select for many other desirable features, such as a red-shifted emission spectrum or enhanced photostability. Although the length of the procedure is dependent on the degree of optimization desired, the basic steps can be accomplished in 4–6 weeks.

INTRODUCTION

One of the most notable advances in FP technology, second only to the original cloning and recombinant expression of *Aequorea victoria* green FP (avGFP)[1–3], was the discovery and cloning of a variety of new colors of FP from Anthozoa species such as reef corals[4]. Unfortunately, most Anthozoa FPs can be applied to only a limited subset of live-cell imaging applications owing to their inherent undesirable properties (see **Table 1** for favorable features of FPs for live-cell imaging applications). For example, Anthozoa-derived FPs typically have an obligate tetrameric structure that can perturb fusion protein localization in many live-cell imaging applications. In addition, Anthozoa FPs may suffer from a variety of other potential issues, which may include slow and/or incomplete folding, slow and/or incomplete chromophore formation, inadequate intrinsic brightness for properly formed chromophores and poor photostability.

Here we detail how to use protein engineering and directed evolution to alter the properties of a wild-type FP in order to create an improved and potentially useful new fluorescent probe (**Fig. 1**). This process involves the use of molecular biology to make structure-guided changes in the protein that are expected to disrupt the oligomeric protein-protein interfaces. To achieve improvements in FP brightness, we provide a detailed directed evolution protocol in which large libraries of randomly generated variants are created and screened in the context of bacterial colonies. After the selection of a monomeric and improved FP variant, the protein is purified and fully characterized in terms of oligomeric structure, extinction coefficient, quantum yield, pH sensitivity and photostability. Finally, we provide detailed methods for characterizing the performance of new FPs in protein fusions expressed and imaged in live cells. These procedures have been successfully used by the authors and co-workers to develop several of the now-popular FPs, including mCherry[5,6], mTFP1 (ref. 7) and mPapaya1 (ref. 8).

Application of protein engineering and directed evolution to FPs

The majority of the Anthozoa FPs are obligate tetramers and thus are unsuitable for applications that involve the creation of chimeras between the FP and a second protein of interest[9]. When a tetrameric Anthozoa FP is genetically fused to a cellular protein, the resulting fusion protein is a tetramer and will commonly display aggregation-related artifacts. If the protein of interest is itself a monomer, this forced tetramerization may or may not be compatible with normal biological function[5,10–12]. As imaging of fusion protein localization is arguably the most common use of FPs, this issue must be addressed before a FP can achieve widespread utility. Accordingly, researchers have used protein engineering combined with an accelerated and directed process of evolution in the laboratory to create bright, stable, monomeric probes from wild-type FPs.

The first Anthozoa FP subjected to such an effort was a red FP (RFP) cloned from the coral *Discosoma sp.*[4]. By using techniques analogous to those described in this protocol, a lengthy process of directed evolution was used to engineer a new variant, known as mRFP1, in which the most crucial shortcomings of *Discosoma* RFP were addressed[5]. Further, directed evolution of mRFP1 for improved brightness, color diversification and photostability eventually produced what is now known as the mFruit series of monomeric FPs[6,13]. Similar protein engineering efforts have now been applied to a growing number of naturally tetrameric FPs including GFPs from *Galaxeidae*[14] and lancelet[15], a cyan FP (CFP) from *Clavularia sp.*[7], a yellow FP (YFP) from *Zoanthus sp.*[8], an orange FP from *Fungia concinna*[16], photoconvertable FPs from both *Lobophyllia hemprichii*[17] and *Dendronephthya sp.*[18], a photoactivatable GFP from *Pectiniidae*[19] and a chromoprotein from *Montipora sp.*[20]. It should be emphasized that well over a hundred wild-type FPs are known[21,22], yet only a small percentage of these have been adapted for imaging applications to date.

TABLE 1 | Features of FPs generally favorable for live-cell imaging applications.

FP features	Comments
Monomeric structure	Monomeric FPs are less likely to perturb the normal localization of the fusion protein, which is commonly perturbed by oligomerization
High fluorescence brightness	Less excitation light can be delivered to cells to obtain adequate signal intensity, resulting in reduced photodamage and phototoxicity
High folding efficiency at 37 °C	Most live mammalian cell studies are done at 37 °C
Narrow absorption and emission peaks	Higher fractions of the excitation or emission peak will be covered by a standard band-pass filter, leading to improved brightness and less bleedthrough when using multiple FPs or fluorescent dyes
Resistance to photobleaching	Time-lapse studies require FPs with good photostability. For fluorescence recovery after photobleaching and fluorescence loss in photobleaching experiments, FPs with mediocre photostability should be used to reduce light dosage. Similarly, to quantify FRET by acceptor photobleaching, it is preferable to use an acceptor FP with low-to-mediocre photostability
Resistance to pH changes	Most imaging applications benefit from using FPs with low sensitivity to pH changes. However, FPs that are sensitive to pH are useful as genetically encoded pH sensors
Single exponential lifetime	This property is not relevant to intensity-based measurement. FPs with single-exponential fluorescence lifetime decay are preferable for fluorescence lifetime imaging microscopy

Comparisons with other methods

The method described in this protocol involves a combination of structure-guided mutagenesis and either manual or digital image–based screening of FP libraries presented in hundreds of individual colonies of *Escherichia coli* grown on solid growth medium. FP libraries have been screened in cell types other than *E. coli*, but this adds a number of additional challenges that cannot be addressed with the scope of this protocol. Some key examples include screening of FP-based libraries by yeast surface display[23] and screening in hypermutating mammalian[24] or avian[25] cell lines. For these nonbacterial screening approaches, colony-based imaging is not feasible, and therefore FACS is the method of choice for interrogating large libraries of individual cells and for selectively sorting out the brightest or most hue-shifted variants. Notably, there are only a few examples to date in which FACS has been used to sort FP libraries expressed in *E. coli*[26–28]. The primary advantage of FACS-based screening is that much larger libraries can be interrogated in order to find improved variants. However, this increased throughput comes with an associated cost in terms of the additional time required to prepare the library in a suitable vector and the requirement of expensive instrumentation.

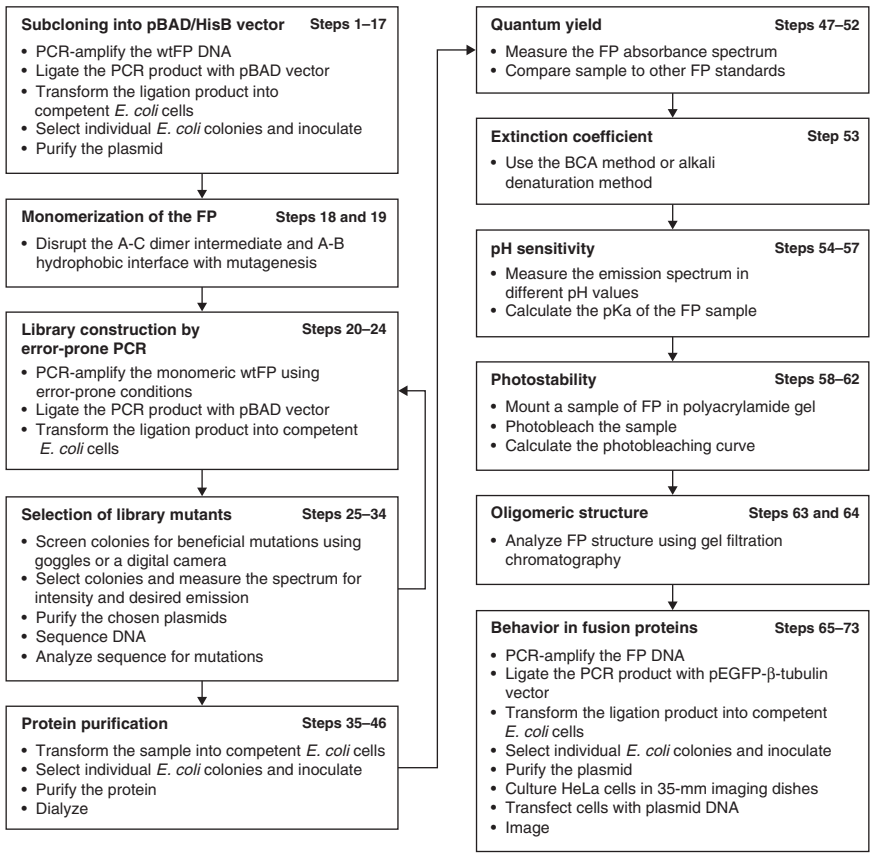

Figure 1 | Steps used to engineer a new FP. Starting with a wild-type FP (wtFP), PCR is used to subclone the FP into a protein expression vector. The wtFP is then monomerized, mutated and screened for additional beneficial characteristics. The new FP is then characterized by a series of assays designed to test its performance.

Although this method will not be discussed in this protocol, there are many relevant resources that are available for the interested researcher[24,26–28].

Yet another approach, which is in its infancy yet holds great promise, is the computationally assisted design of improved FPs. One example of such an approach is the use of software-generated sequence alignments of many FPs to guide the design of a consensus FP that incorporates the most frequently occurring amino acid at judiciously chosen positions within the sequence[29,30]. A more sophisticated example is the use of computational structural modeling for the design of high-quality libraries of FP variants that are enriched for variants that are likely to fold properly and are designed to have particular properties[31,32]. In this case, the computational library design replaces the use of random mutagenesis for library creation. A high-throughput library screening method is still required, and this can be performed by using either FACS[28] or manual screening, as described in this protocol.

Experimental design

Obtaining an initial FP template gene. Once a wild-type FP of interest has been identified, a mammalian codon-optimized gene can be designed (Reagent Setup). Online tools for back-translation can facilitate the construction of a gene that is codon-optimized for the eventual target organism of interest. Genes optimized for mammalian codon usage will typically express reasonably well in *E. coli*, and thus the engineering and directed evolution methods described in this protocol remain feasible. Although several protocols for gene assembly are available[33,34], total gene synthesis by one of the growing number of companies that offer such services is typically the most rapid and cost-effective route to obtaining the initial FP template gene. Sequence alignments with engineered homologs such as EGFP[35,36], mCherry[6] and mTFP1 (ref. 7) can reveal whether the FP of interest has particularly long unstructured polypeptide extensions at either the N or C terminus compared with other known variants. If so, the N-terminal extension can be partially truncated to give a protein of similar length to the engineered variants mentioned above. However, care must be taken not to truncate residues that extend into the β-barrel domain. For Anthozoa FPs, truncations at the C terminus should not be undertaken until later in the directed evolution process, as the C-terminal residues are often involved in protein-protein interactions at the so-called 'A-C dimer' interface[37]. On receiving the synthetic gene, it can be amplified by PCR and inserted into an appropriate expression vector (e.g., pBAD/His B) to enable bacterial expression and subsequent purification (Steps 1–17).

Oligonucleotide design. For this protocol, we follow the common practice of appending the seven N-terminal residues (MVSKGEE encoded by ATGGTGAGCAAGGGCGAGGAG) and the seven C-terminal residues (GMDELYK encoded by GGCATGGACGAGCTGTACAAG) of avGFP to the normal N- and C termini of the engineered FP. By adding these sequence extensions, all avGFP PCR primers with 21 bases or fewer of overlap will also amplify the newly designed FP of interest. This is not necessary for a successful FP, but a matter of convenience to avoid ordering multiple primers. In this particular example, we will insert the FP gene between the XhoI and EcoRI restriction sites of a pBAD/His B plasmid. The designed DNA sequence should include an XhoI restriction at the 5′ end of the gene (GGTAACTCGAGA) and a stop codon followed by an EcoRI site at the 3′ end of the gene (TAAGAATTCGGC). The extra 'A' after the XhoI sequence at the 5′ end is necessary to maintain the reading frame with the leader sequence that contains the hexahistidine (His_6) affinity purification tag of the pBAD/His B expression vector. The extra bases preceding the XhoI site (GGTAA) and following the EcoRI site (GGC) are necessary to increase the efficiency for restriction enzyme cleavage close to the end of the synthetic DNA fragment.

Engineering dimeric and monomeric FPs. As essentially all coral FPs are tetramers, altering the oligomeric structure such that the FP exists as a monomer at physiologically relevant concentrations is one of the first priorities. Converting a tetramer into a monomer is a two-step process that proceeds through a dimeric intermediate. In the first step, the tetrameric FP is converted into the A-C dimer intermediate (**Fig. 2**). To create the A-C dimer of a tetrameric Anthozoa FP, the hydrophobic A-B interface must be disrupted by the introduction of large positively charged amino acids in place of hydrophobic amino acids normally located as close as possible to the axis of symmetry near the center of the interface. Specifically, we will replace the residues that are structurally aligned with residues 126 and 128 of avGFP (as numbered in Protein Data Bank (PDB) ID 1EMA)[38], and with the equivalent residues 125 and 127 of *Discosoma* RFP (as numbered in

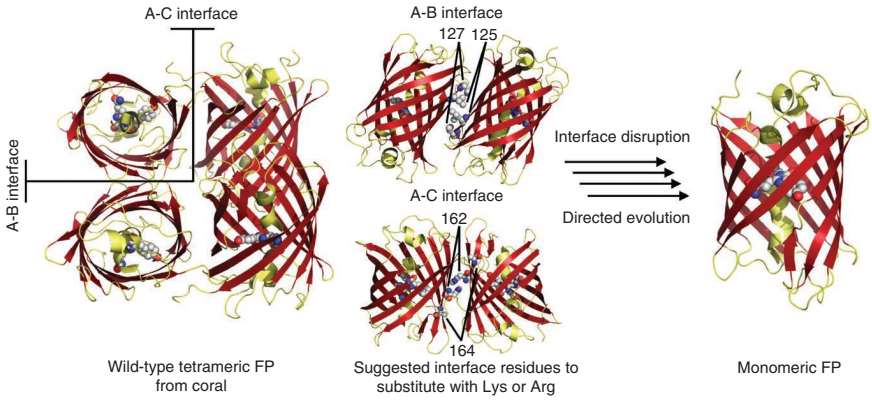

Figure 2 | Conversion of a naturally tetrameric FP to an engineered monomeric FP. To convert a tetrameric FP into a monomeric FP, two different protein-protein interfaces must ultimately be disrupted. Disruption of the first interface produces a dimer intermediate and subsequent disruption of the second interface produces a monomer. Anthozoa FPs tend to tolerate disruption of the hydrophobic A-B interface much better than they tolerate disruption of the hydrophilic A-C interface. Accordingly, the A-B interface should be the first to be disrupted to generate a dimer. Subsequent disruption of the A-C interface will generate a monomer. Interface-disrupting mutations are typically detrimental to the correct folding and chromophore maturation of the intermediate dimer or target monomer, and these variants must be rescued by directed evolution. Cartoon structures are based on PDB IDs 1G7K (ref. 37) and 2H5Q (ref. 57).

PDB ID 1G7K)[37], with lysine or arginine. These substitutions can be introduced by using the QuikChange mutagenesis protocol (Step 19), or by introducing a semi-degenerate 'ARG' codon (where R = A and G) at both positions. This procedure will result in a four-member library that should be screened to find the preferred combination (if any) of lysine and arginine residues at the two positions. To disrupt the A-C interface, the residues that are structurally aligned with residues 166 and 168 of avGFP (as numbered in PDB ID 1EMA) and with 162 and 164 of *Discosoma* RFP (as numbered in PDB ID 1G7K) should be replaced with lysine or arginine, as described above for the A-B interface. In most cases, these interface-disrupting mutations will decrease the folding efficiency and/or the efficiency of chromophore maturation.

After experimental confirmation of the successful dimerization or monomerization of a FP, an extensive process of directed evolution can be used to recover some or all of the fluorescent brightness lost during the interface disruption. This procedure is obviously crucial at the final monomer stage, as the end goal is to produce a monomeric FP with high brightness. However, it is also critical at the dimer stage as a dimeric variant engineered for high folding and chromophore maturation efficiency is much more likely to 'survive' the critical and crippling disruption of the remaining A-C interface. To rescue the brightness, large libraries of variants should be created by error-prone PCR of the whole gene and then screened in order to identify rare variants with high brightness. In this protocol, we rely on the use of error-prone PCR with Taq polymerase (Steps 20–23) for the creation of libraries of randomly mutated genes. Briefly, by performing PCR with Taq polymerase in the presence of Mn^{2+}, altered ratios of dNTPs and increased concentration of Mg^{2+}, a tunable frequency of random mutation can be introduced into an amplified PCR product[39,40]. The error rate can be adjusted by altering the conditions, but typically a rate of approximately three mutations per 1,000 bp is desirable. Under such conditions, approximately half of the clones will retain fluorescence, and those that do will exhibit a substantial amount of diversity in terms of their intrinsic brightness. Lower rates will result in a greater proportion of library members with indistinguishable changes compared with the template DNA. Higher rates of mutation will result in a substantial proportion of library members with little to no fluorescence. A variation on this approach is to use the GeneMorph random mutagenesis kit (Stratagene), which contains a blend of two error-prone polymerases and gives a broader spectrum of mutations than does the *Taq* polymerase alone.

Library creation can also be achieved by mutagenesis of one or more specific codons using an overlap-extension PCR method[41,42] or by using the Quikchange protocol (Step 19). This targeted approach enables the exploration of further diversity at those specific positions at which beneficial substitutions were discovered during the screening of libraries generated by error-prone PCR. In either case, degenerate (encoding all 20 amino acids) or semi-degenerate (encoding >1 but <20 amino acids) codons are introduced at specific locations within the gene. Detailed protocols for library construction by both mutagenesis methods have been provided elsewhere[43].

Library screening. After the successful creation of a library and its expression in *E. coli*, colonies must be screened on Petri dishes to identify those with improved brightness relative to the library template. Two screening options for increased brightness (Steps 25 and 26) are visual screening with long-pass goggles with appropriate wavelength cutoffs and digital image–assisted screening by using a camera equipped with band-pass filters. There are several options for goggles available with a variety of wavelength cutoffs (e.g., wrap-around filter goggles from Forensics Source and macro-viewing goggles from Chroma Technology). We have found the Forensics Source goggles to be a cost-effective option; however, they are only available in a few wavelength cutoffs (~490, ~530 and ~580 nm). The macro-viewing goggles from Chroma are more expensive, but they are customizable and available in more discrete wavelength ranges. Obtaining a good match between the transmitted wavelengths of the goggles and the desired emission spectrum of the engineered FP is important when screening for hue-shifted variants. For example, it tends to be difficult to distinguish CFPs (~480–490-nm maximal emission) from GFPs (~500–515-nm maximal emission), even when using goggles with an ~490-nm cutoff. In cases such as this, a digital imaging system equipped with high-quality band-pass emission filters, such as the custom-built device described in this protocol (Equipment Setup), is much more effective.

In a single round of screening, the brightest clones are picked (i.e., 1 out of 500–1,000), cultured, and then collected for the isolation of the plasmid DNA. The clone, or pool of clones, identified in the first round is then used as the template for the second round of library creation by error-prone PCR[40]. Improvements in brightness tend to be relatively modest in any given round, and it is only through the use of multiple iterative rounds of library creation and screening that dramatic improvements in brightness can be achieved. One advantage of maintaining a pool of clones, rather than moving forward with a single clone, is that error-prone PCR can be combined with, or alternated with, rounds of staggered extension PCR[44,45], which can serve to recombine beneficial mutations and accelerate the overall directed evolution process[46].

Although this protocol focuses on screening for improved brightness, there are several other properties that can be screened for in the context of colonies. Specifically, through the use of arrays of bright LED light sources, it is possible to photobleach[7] or photoconvert[30] FPs expressed in bacterial colonies. Accordingly, screens for improved photostability or improved photoconversion are made possible with the introduction of an illumination step in the screening protocol. The most effective implementation of such a step is in the context of a three-step procedure in which a digital fluorescence image of a Petri dish is acquired, the dish is then evenly illuminated with an appropriate light source for photoconversion or photobleaching, and then a second image of the plate is acquired. Through the use of software macros and digital-image processing, the fluorescence intensities of each colony before and after illumination can be determined and the top clones identified. Yet another variation on this protocol is to screen for improved Förster resonance energy transfer (FRET) efficiency between a genetically fused pair of FPs[27,47].

***In vitro* characterization.** Once a clone with suitable improvements in brightness and/or other selected characteristics has been identified, it must be subjected to a thorough *in vitro* characterization. Although standards of reporting the characteristics for new FP variants will differ from journal to journal,

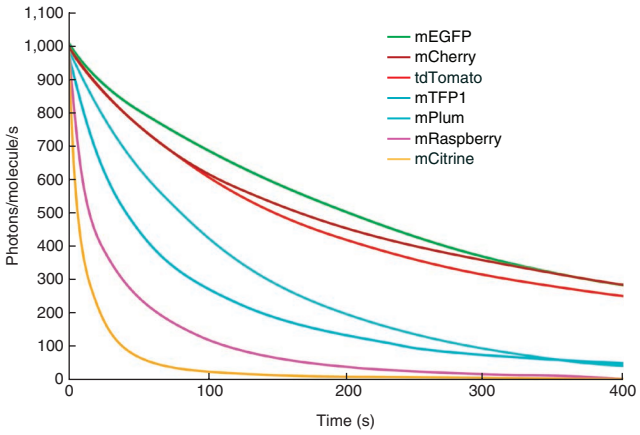

Figure 3 | Typical photobleaching kinetics of FPs. Decay curves are plotted after normalization of each FP to an initial emission rate of 1,000 photons/molecule/s. The time required to photobleach each FP to an emission rate of 500 photons/molecule/s is referred to as the $t_{1/2}$, and can be used to compare the photostability between FPs.

a useful guideline is the minimal reporting standard recently posted on the *Nature Methods* Methagora blog (http://blogs.nature.com/methagora/2012/12/our-reporting-standards-for-fluorescent-proteins-feedback-wanted.html). These standards, which were drafted in consultation with members of the FP-development community, are paraphrased here: (i) full absorbance spectrum; (ii) fluorescence excitation and emission spectra (possibly including two-photon excitation data); (iii) quantum yield (Φ); (iv) extinction coefficient (ε); (v) pH dependence of fluorescence; (vi) gel filtration analysis to determine oligomeric structure; (vii) FP maturation time data; (viii) Photostability data under wide-field and confocal illumination conditions; (ix) imaging of representative fusion proteins (e.g., α-tubulin) expressed in cells; and (x) some measure of cytotoxicity relative to EGFP.

Much of the FP characterization is performed *in vitro*, necessitating the expression and purification of a substantial quantity of protein for analysis (Steps 35–46). Once a purified protein is obtained, typically as a His_6-tagged protein isolated by using Ni-nitrilotriacetic acid (NTA) affinity chromatography, a series of standard spectroscopic measurements can be used to determine the key photophysical properties of the variant (Steps 47–62). One of the most challenging measurements listed above is the determination of the photobleaching kinetics. The actual rate is dependent on the power (W/cm^2) and spectral profile of the light source, the transmission profile of the filters and the light throughput of the microscope optics, and is thus highly instrument-dependent. Methods for correcting this instrument-to-instrument variation have been described[48]. However, in the absence of instrument correction, by using the same instrument and identical conditions, the rate of photobleaching can be compared with other well-characterized FPs. These measurements are usually sufficient to provide a preliminary estimate of the photostability of the engineered FP. Representative photobleaching traces for several well-studied FPs are included below (**Fig. 3**). In addition to the *in vitro* characterization of the purified protein, a new FP must also be analyzed in the context of live cells by creating a cellular expression vector. Three of the most commonly used proteins to test FP fusion behavior, β-actin, α-tubulin and connexin-43, can be ligated to the new FP, allowing for a quick visual assay to determine whether the FP has any oligomeric structure in cells by judging how well the FP is incorporated into the cytoskeleton and gap junctions of living cells. In this protocol we will specifically describe how to test the FP as a fusion partner for live-cell microscopy by using a commercially available α-tubulin plasmid; however, the same procedure would be applicable to a variety of target proteins.

Limitations

An oft-repeated truism of directed evolution is that "you get what you screen for." Accordingly, FPs engineered by directed evolution will rarely have favorable characteristics that were not specifically selected for. The application of the engineering and directed evolution protocols described here is practically certain to lead to the development of FPs that are brighter when expressed in colonies of *E. coli*. Although some of the improved brightness will be attributable to improvements in the intrinsic photophysical properties (i.e., Φ and ε), much of the improvement will be due to improved folding and chromophore maturation efficiency in *E. coli*. It is well established that improvements in FP performance in *E. coli* typically translate into improvements in performance when expressed in mammalian cells[49]. However, the fact that the FP has been optimized in an organism that is so distant from a mammalian cell remains a limitation of this approach that should always be kept in mind. One approach for minimizing unanticipated effects from switching organisms is to perform occasional tests of several different FP variants in mammalian cells during the process of directed evolution.

Yet another major limitation of the directed evolution approach described here is that it is focused on optimizing a single property at a time (i.e., brightness). Brightness alone is not sufficient for a FP to be useful for live-cell imaging. At the very least, the FP must also be monomeric and relatively resistant to photobleaching. In a simple screen for brightness, neither of these properties is being selected for; thus, reversions to a higher-order oligomeric state[30] or diminished photostability[7] can often arise. As with tolerance to expression in mammalian cells, the oligomeric state should occasionally be determined during the process of directed evolution, especially when DNA sequencing reveals a beneficial mutation in the former A-B or A-C interfaces. Similarly, photostability can be occasionally tested during the process of directed evolution or, preferably, rounds of selection for brightness can be alternated with rounds of selection for photostability.

MATERIALS

REAGENTS

- pBAD/His B expression vector (Life Technologies, cat. no. V43001)
- Custom oligonucleotide primers are available from commercial suppliers (primer sequences used to generate example data are supplied in **Table 2**)
- FP synthetic cDNA template (Integrated DNA Technologies)
- dNTPs mixture of dATP, dTTP, dGTP and dCTP; 10 mM (Life Technologies)
- Individual dATP, dTTP, dGTP and dCTP; 10 mM (Life Technologies)
- Pfu DNA polymerase (Thermo Scientific, cat. no. EP0571)

TABLE 2 | Primer sequences.

Primer name	Nucleotide sequence
avGFP_XhoIfwd	5′-GGTAACTCGAGCATGGTGAGCAAGGGCGAGGAG-3′
avGFP_EcoRIrev	5′-GCCGAATTCTTACTTGTACAGCTCGTCCATG-3′
pBADfwd	5′-ATGCCATAGCATTTTTATCC-3′
pBADrev	5′-GATTTAATCTGTATCAGG-3′
avGFP_Nhe1fwd	5′-GATAAGCTAGCGCTACCGGTCGCCACCATGGTGAGC AAGGGCGAGGAG-3′
avGFP_XhoIrev	5′-ATTACCTCGAGATCTGAGTCCGGACTTGTACAGCTCG TCCATGCC-3′

- Pfu polymerase buffer; 10× (Thermo Scientific, included with DNA polymerase)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- Analytical grade agarose (Promega, cat. no. V3121)
- Tris-acetate-EDTA buffer; 10× TAE buffer (Sigma-Aldrich, cat. no. T8280)
- GeneRuler 1 kb DNA ladder plus (Thermo Scientific, cat. no. SM1332)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- Ethidium bromide solution; 10 mg/ml (Bio-Rad, cat. no. 161-0433)
  ! CAUTION Ethidium bromide is a known mutagen and a suspected carcinogen. When handling it, use gloves at all times and avoid skin contact.
- Gel loading dye, blue; 6× (New England Biolabs, cat. no. B7021S)
- T4 DNA ligase buffer; 5× (Life Technologies, cat. no. 46300-018)
- T4 DNA ligase (Life Technologies, cat. no. 15224-017)
- Taq DNA polymerase (New England Biolabs, cat. no. M0273S)
- Taq polymerase buffer; 10× (New England Biolabs, cat. no. M0273S)
- QuikChange Mutagenesis Kit with DpnI and XL10-Gold cells (Agilent Technologies, cat. no. 200518)
- ElectroMAX *E. coli* strain DH10B (Life Technologies, cat. no. 18290-015 (electrocompetent))
- One Shot *E. coli* strain TOP10 (Life Technologies, cat. no. C4040-03 (electrocompetent))
- QIAprep spin miniprep kit (Qiagen, cat. no. 27104)
- Ampicillin; 100 mg/ml (Sigma-Aldrich, cat. no. A5354)
- L-Arabinose (Sigma-Aldrich, cat. no. A3256)
- Appropriate restriction enzymes (New England Biolabs)
- B-PER bacterial protein extraction reagent (Thermo Scientific, cat. no. 78248)
- Plasmid midi kit (Qiagen, cat. no. 12143)
- Ni-NTA resin (Thermo Scientific, cat. no. PI-88221)
- Pierce bicinchoninic acid (BCA) protein assay (Thermo Scientific, cat. no. 23227)
- DYEnamic ET DNA sequencing kit (GE Healthcare, cat. no. US81050)
- HeLa cells (ATCC, cat. no. CCL-2)
- DMEM medium (Life Technologies, cat. no. 12491-015)
- FBS (Sigma-Aldrich, cat. no. 12107C)
- Linear polyethylenimine molecular weight ~25,000 Da (Polysciences, cat. no. 23966-2)
- OptiMEM (Life Technologies, cat. no. 31985)
- HBSS (Life Technologies, cat. no. 14024-076)
- Ultrapure Agarose (Life Technologies, cat. no. 15510-019)
- Agar (Fisher Scientific, cat. no. BP1423)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. 05038)
- Bacto tryptone (Fisher Scientific, cat. no. BP1421)
- Bacto yeast extract (Fisher Scientific, cat. no. BP1422)
- Ammonium persulfate (Sigma-Aldrich, cat. no. A3678)
- TEMED (Sigma-Aldrich, cat. no. T9281)
- Acrylamide/bis-acrylamide; 30% (wt/vol) solution (Sigma-Aldrich, cat. no. A3699) **! CAUTION** Acrylamide is acutely toxic when touched, ingested or inhaled. When handling the reagent, use gloves and a fume hood at all times and avoid skin contact.
- Imidazole buffer; 1 M solution (Sigma-Aldrich, cat. no. 68268)
- Phosphate buffer; 10× (Sigma-Aldrich, cat. no. P7059)
- Cytoseal (Richard Allen Scientific, cat. no. 8310-4)
- Quinine sulfate (Sigma-Aldrich, cat. no. 22640)
- Fluorescein (Sigma-Aldrich, cat. no. 32615)
- Rhodamine (Sigma-Aldrich, cat. no. 79754)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045)
- Manganese chloride ($MnCl_2$; Sigma-Aldrich, cat. no. 450995)
- Sterile deionized H_2O, 18 MΩ
- Ethanol (99.5% molecular biology grade; available from multiple suppliers)
- pEGFP-α-tubulin (Clontech, cat. no. 6117-1)
- Tris buffer (Reagent Setup)

EQUIPMENT

- PCR thermocycler (Bio-Rad, cat. no. 186-1096)
- Thin-walled 0.2-ml PCR tubes (Eppendorf, cat. no. E0030-124-260)
- Microcentrifuge (Eppendorf, model no. 5430R)
- Microcentrifuge tube, 0.6 ml (Sigma-Aldrich, cat. no. T5149-500EA)
- Electrophoresis equipment for agarose gel electrophoresis (e.g., Owl EasyCast electrophoresis system)
- UV transilluminator
- Glassware (bottles, flasks and tubes)
- Incubator (37 °C)
- Shaking incubator (37 °C)
- Titer plate shaker
- Water bath (37 °C)
- Microwave
- Razor
- Parafilm
- Petri plates
- Wood applicator sticks (Fisher Scientific, cat. no. 22029491)
- Syringe filter, 0.2-μm pore size (Nalge-Nunc, cat. no. 09-741-07)
- Gold Seal beveled-edge microslides (Thermo Scientific, cat. no. 3061-002)
- Microcentrifuge tube, 1.5 ml (Fisher Scientific, cat. no. 02-681-320)
- Corning cover glass, 18 mm (Corning, cat. no. 12-520A)
- Corning cover glass, 22 mm (Corning, cat. no. 2875-22)
- 96-well plate black clear-bottom plates (Corning, cat. no. CLS3904)
- Imaging dishes, 35 mm (Mattek, cat. no. P35G-1.5-10-C)
- Sterile pipette tips
- Polypropylene column, 6 ml (Qiagen, cat. no. 34924)
- Ice bucket
- Magnetic stirrer
- Pipettes
- Vortex
- Sonicator
- Bio-Rad MicroPulsor electroporator (Bio-Rad, cat. no. 165-2100)
- High-speed centrifuge tube, 50 ml (Fisher Scientific, cat. no. 06-443-18)
- Centrifuge bottle, 250 ml (Nalgene, cat. no. 3140-0250)
- Electroporation cuvettes with 0.2 cm gap (Bio-Rad, cat. no. 165-2082)
- Quartz cuvettes (Sigma-Aldrich, cat. no. Z276898)
- Wrap-around viewing goggles (Lightining Powder Company, Chroma Technology, etc.)
- Waterproof marker
- Biotech snakeskin tubing; 16 mm (Thermo Scientific, cat. no. 88243)
- Snakeskin dialysis tubing clips (Thermo Scientific, cat. no. 68011)
- Slide-a-Lyzer G2 dialysis cassettes (Thermo Scientific, cat. no. 87730)
- Lambda LS 175W xenon arc lamp (Sutter Instruments)
- Lambda 10-3 filter wheel changer and two filter wheels (Sutter Instruments)
- Appropriate band-pass filters (Chroma Technology, Omega Filters, Semrock, etc.)
- Fiber bundle focusing assembly (Newport, cat. no. 77800)
- Oriel bifurcated fiber optic bundle (Newport, cat. no. 77533 or 77565)
- LEDs (Lumiled) or LED arrays (Opto Diode)
- Monochrome 12-bit CCD camera for colony imaging (QImaging)
- C-mount fixed focal length lens (Tamron)
- Personal computer with Image Pro Plus 6.0 (or later) image capture and image processing software (Media Cybernetics)
- Safire2 plate reader equipped with monochromators (Tecan)
- DU-800 UV-visible spectrophotometer (Beckman)
- QuantaMaster spectrofluorometer (Photon Technology International)
- Zeiss Axiovert 200M epifluorescence inverted microscope with a xenon arc lamp
- Monochrome Retiga 2000R 12-bit cooled CCD camera (QImaging)

• µManager image capture and microscope control software (http://www.micro-manager.org/)
• AKTAbasic liquid chromatography system (GE Healthcare)

REAGENT SETUP

Luria-Bertani/agar plates Dissolve 15 g of agar, 10 g of Bacto tryptone, 10 g of NaCl and 5 g of yeast extract in 1,000 ml of deionized water. Adjust the pH to 7.0 and autoclave the medium. Cool it to 50 °C and add 1 ml of ampicillin solution. Pour the medium into plates. Plates can be stored at 4 °C for up to 4 weeks. Plates should be dried in a flow bench before use.

Luria-Bertani (LB) liquid medium Dissolve 10 g of Bacto tryptone, 10 g of NaCl and 5 g of yeast extract in 1,000 ml of deionized water. Adjust the pH to 7.4 and pour 500 ml into flasks. Autoclave the medium. Flasks can be stored at 4 °C for up to 4 weeks.

L-Arabinose, 20% (wt/vol) Add ddH_2O to 20 g of L-arabinose to a total volume of 100 ml. Mix the solution by using a benchtop vortex until the L-arabinose is dissolved completely. Filter it with a 0.2-µm syringe filter. To prevent repeated freeze-thaw cycles, aliquot it into sterile 0.6-ml tubes. The solution can be stored for ~1 year at −20 °C.

Tris buffer Dissolve 6.05 g of Tris base in 30 ml of ddH_2O. Adjust the pH to 7.5 with 5 M HCl. Adjust the final volume to 50 ml with ddH_2O. Tris buffer can be stored at room temperature or at 4 °C for up to 2 weeks.

Agarose gel,1.5% (wt/vol) Dissolve 1.5 g of agarose in 100 ml of 0.5× TAE buffer. Microwave the mixture until the agarose is dissolved. Gels are stored at room temperature, in a sealed container to prevent water evaporation, for up to 2 weeks.

Imidazole wash buffer To prepare 20 mM wash buffer, add 90 ml of 10× stock PBS solution to 1.26 g of imidazole, and then add ddH_2O to a volume of 800 ml. Adjust the pH to 8.0, and then add ddH_2O to a final volume of 900 ml. Filter the solution through a 0.22-µm filter and store it at 4 °C. Wash buffer can be stored for up to 2 weeks.

Imidazole elution buffer To prepare 250 mM elution buffer, add 10 ml of 10× stock PBS solution to 1.7 g of imidazole, and then add ddH_2O to a volume of 50 ml. Adjust the pH to 7.8, and then add ddH_2O to a final volume of 100 ml. Filter the solution through a 0.22-µm filter and store it at 4 °C for up to 2 weeks.

Binding buffer To prepare binding buffer, add 50 ml of 10× stock PBS solution to 450 ml of ddH_2O. Store it at 4 °C for up to 2 weeks.

Polyethylenimine transfection reagent To prepare 1 µg/µl transfection reagent, mix 5 ml of 0.1 M HCl with 10 mg of linear polyethylenimine. After dissolving the solid, add 500 µl of 1 M NaOH to neutralize the solution. Add ddH_2O to a final volume of 10 ml. Filter the solution through a 0.22-µm filter and store it at −20 °C for up to 1 year.

EQUIPMENT SETUP

Macroscopy Setup A photograph of the digital macroscope used for colony imaging is provided in **Figure 4**. The light from a 175-W xenon arc lamp is passed through an appropriate band-pass filter held in a filter wheel (the excitation filter wheel) and passed into a fiber bundle focusing assembly. In the implementation shown in **Figure 4**, the light from the focusing assembly is projected on to a mirror that reflects the light on to the Petri dish holder. An alternative implementation is to attach a bifurcated fiber-optic bundle to the focusing assembly and position the two ends ~25 cm above and to the sides of the Petri dish, such that it is illuminated as evenly as possible. The Petri dish is held in a slight recession in a matte-black board, which ensures reproducible placement of Petri dishes in subsequent experiments. Positioned ~45 cm directly above the Petri dish is a 12-bit CCD camera connected via a firewire interface to a personal computer on which the Image Pro Plus (Media Cybernetics) software package and camera drivers are installed. The camera is fitted with a C-mount fixed focal length lens and is located immediately behind a second filter wheel (the emission filter wheel) that contains an appropriate band-pass emission filter. Filters used for screening should have a spectral bandwidth of 30–40 nm to successfully screen for the correct fluorescent color in the colonies. Both the excitation and emission filter wheels are controlled by a filter wheel changer (Lambda 10–3, Sutter Instruments) that is in turn connected to the serial port of the personal computer. With the exception of the lamp and excitation filter wheel, the whole system should be enclosed in a box or shrouded in black fabric to minimize interference from outside light sources.

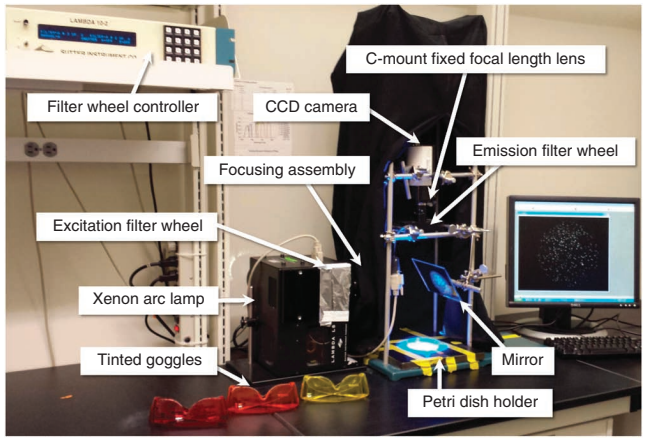

Figure 4 | Digital macroscope for acquisition of fluorescence images of colonies of bacteria grown on solid medium in a Petri dish.

PROCEDURE

Subcloning into pBAD/His B vector ● TIMING 4–5 h

1| To obtain sufficient template DNA for cloning and to add appropriate restriction enzyme sites, set up a PCR in a 0.2-ml thin-walled PCR tube by adding the following components:

Component	Amount (per 100-µl reaction)	Final concentration
Pfu polymerase buffer (10×) stock solution	10 µl	1×
dNTPs mix (10 mM) stock solution	2 µl	200 µM
Forward primer (100 µM) avGFP_XhoIfwd	0.5 µl	0.5 µM
Reverse primer (100 µM) avGFP_EcoRIrev	0.5 µl	0.5 µM
Template gene (dilute to 50 ng/µl)	1 µl	50 ng
Pfu polymerase (2 U/µl)	1 µl	2 U
dH_2O	Add to final volume of 100 µl	

▲ **CRITICAL STEP** Use RNase- and DNase-free materials whenever possible.

2| Mix the contents by gently pipetting up and down several times, and then centrifuge briefly to bring down the contents. Place the tube in a thermocycler with a heated lid. If the PCR machine does not have a heated lid, add ~30 µl of mineral oil on top of the reaction to prevent evaporation of the tube contents.

3| Thermocycle by using the program listed below. After the PCR cycle has been completed, cool the reaction to 4 °C.

Cycle number	Denaturing	Annealing	Extension
1	95 °C, 2 min		
2–25	95 °C, 20 s	55 °C, 20 s	72 °C for 1 min
26			72 °C for 4 min

▲ **CRITICAL STEP** Extension time is determined by the length of the template DNA and the polymerase used. New users should refer to the manufacturer's recommended guidelines and modify the existing PCR method to reflect the length of their template gene.

4| Combine 5 µl of the PCR product with 1 µl of 6× DNA loading buffer in a microcentrifuge tube. Gently mix and then load it into a 1.5% (wt/vol) agarose gel alongside a DNA ladder. Stain the gel with ethidium bromide and confirm that a PCR product of ~700 bp has been produced by using a UV transilluminator to illuminate the band.
! **CAUTION** Ethidium bromide is a known mutagen and a suspected carcinogen. When handling it, use gloves at all times and avoid skin contact.
▲ **CRITICAL STEP** A bright band should be clearly visible on the gel. Use the DNA ladder to determine whether the PCR product has the anticipated size. If there are multiple products or the fragment is an unexpected size, consider altering the PCR conditions or redesign the PCR primers.
? **TROUBLESHOOTING**

5| Purify the remainder of the PCR product by using the QIAquick PCR cleanup kit according to the manufacturer's instructions.
■ **PAUSE POINT** Purified PCR products can be stored at –20 °C for ~1 year.

6| Perform two separate restriction digestion reactions, one on the purified PCR product and one on 5 µg of pBAD/His B vector, by using XhoI and EcoRI. Follow the enzyme manufacturer's instructions.

7| On completion of digestion, add the 6× DNA loading buffer to each of the digested products and load the contents into separate 1.5% (wt/vol) agarose gels. Stain the gels with ethidium bromide. Use a UV transilluminator to visualize the band.
▲ **CRITICAL STEP** Exposure to UV light could lead to DNA damage and reduced efficiency of ligation (Step 9). Long-wavelength UV and minimal illuminating time should be used.
▲ **CRITICAL STEP** When performing a restriction enzyme digest on the pBAD vector stock there should be two bands visible on the gel, provided that the smaller band has not migrated off the bottom of the gel. The largest molecular-weight band is the linear pBAD vector fragment to be purified.

8| Cut the relevant band from each gel with a new sharp clean razor blade and purify the PCR product and pBAD vector by using the QIAquick gel extraction kit according to the manufacturer's instructions.
! **CAUTION** Ethidium bromide is a known mutagen and a suspected carcinogen. When handling it, use gloves at all times and avoid skin contact.
? **TROUBLESHOOTING**
■ **PAUSE POINT** Linear DNA products can be stored at –20 °C for ~1 year.

9| Set up the ligation reaction in a 0.6-µl tube for the digested PCR product and pBAD/His B vector as shown in the following table:

Component	Amount (per 20-µl reaction)	Final concentration
Digested PCR product	50 ng	2.5 ng/µl
Digested vector	50 ng	2.5 ng/µl
T4 ligase buffer (5×)	4 µl	1×
T4 ligase (1 U/µl)	1 µl	1 U
dH_2O	Add to final volume of 20 µl	

Mix the contents by gently pipetting up and down several times, and then centrifuge briefly to bring down the contents. Incubate the reaction for 2–4 h at room temperature (~22 °C) or 16 °C overnight.
▲ CRITICAL STEP A typical ligation reaction has an insert-to-vector molar ratio of between 3:1 and 6:1; however, if the concentration of the insert or vector is low, alternative ratios can be used to improve ligation efficiency.

Transformation of *E. coli* ● TIMING 15–18 h

10| Thaw an aliquot of electrocompetent *E. coli* strain DH10B on ice.
▲ CRITICAL STEP Competent cells must be kept at 4 °C after removal from –80 °C to prevent them from becoming unstable.

11| Add 2 µl of ligation mixture (from Step 9) directly into 60 µl of DH10B-competent cells. Gently tap to mix the solution, and then immediately place it back on ice.

12| Gently transfer the mixture to a 2-mm electroporation cuvette that has been prechilled on ice.
▲ CRITICAL STEP Do not excessively mix the solution with the pipette, as this will cause the competent cells to become damaged.

13| Electroporate the cells with a Bio-Rad MicroPulser at 2 kV as per the manufacturer's instructions. Add 1 ml of LB medium into the cuvette immediately and transfer the solution to a sterile culture tube; incubate it at 37 °C with shaking at 225 r.p.m. for 1 h to allow cell recovery and initial expression of the antibiotic resistance gene.

14| Depending on the transformation efficiency of the cells used, plate a volume of 5–100 µl of the LB medium on LB/agar plates supplemented with ampicillin (0.4 mg/ml) and L-arabinose (0.02% (wt/vol)). Incubate plates overnight at 37 °C before inspection and fluorescence imaging.

15| View and screen the fluorescence of the *E. coli* colonies expressing the FP as described in Steps 25 and 26.
▲ CRITICAL STEP Even for the initial subcloning of the wild-type FP, it is important to view the colonies to establish that there are no severe detrimental mutations in the gene that resulted in a loss of fluorescence. Depending on the transformation efficiency of the competent cells, there should be ~100 colonies on the agar plate. If there are only a few colonies (<10), try increasing the volume of cells plated during the transformation.
? TROUBLESHOOTING
■ PAUSE POINT Plates can be sealed with Parafilm and stored at 4 °C for 2–3 weeks.

Plasmid purification ● TIMING 1 d

16| By using a sterile loop, pipette tip or wooden stick select a single fluorescent colony from the plate and add it to 5 ml of sterile LB supplemented with ampicillin (0.1 mg/ml). Grow the culture overnight at 37 °C with shaking at 225 r.p.m.

17| The following day, isolate plasmid DNA by using the plasmid miniprep kit (Qiagen) according to the manufacturer's recommended protocol. The resulting plasmid DNA is used as the template for further manipulations and library creation (Steps 19–24).
▲ CRITICAL STEP The DNA concentration can be quantified by using a NanoDrop spectrometer, a microcuvette coupled with a regular UV-visible spectrometer, or a microvolume plate coupled with a microplate reader. The DNA concentration from a miniprep will vary on the basis of the vector copy number. As the pBAD vector is a low copy number plasmid, we will normally have a DNA concentration of 0.05–0.2 µg/µl per sample.
■ PAUSE POINT Plasmid DNA can be stored at –20 °C for several years.

Monomerization of the FP ● TIMING 3–5 d

18| To identify the residues involved in A-B and A-C interfaces, first perform an online protein structure search at the Research Collaboratory for Structural Bioinformatics (RCSB)'s PDB website (http://www.rcsb.org/pdb/home/home.do)[50] to check whether a crystal structure of the protein of interest, or a close homolog, is available. If an informative crystal structure is available, follow option A to visualize the structure and identify key residues. If a crystal structure is not available, use option B (sequence alignment) to identify the key residues. Alternatively, computer-assisted protein structure modeling and docking can be used to obtain a predicted structure and to guide the selection of oligomeric interface residues, as was done for LanYFP from *Branchiostoma lanceolatum*[15].

(A) When a crystal structure is available

(i) Use molecular structure viewing software, such as Jmol (http://jmol.sourceforge.net/) or PyMol (http://www.pymol.org/), to visualize the structure.

(ii) Zoom in to the oligomeric interfaces of the tetrameric structure to enable identification of those residues that are making key interactions at the A-B and A-C interfaces.

(B) When a crystal structure is not available

(i) Align the protein sequence with avGFP or *Discosoma* RFP by using appropriate software such as ClustalW (http://www.clustal.org/).

(ii) Identify residues aligned with residue 126 and 128 of avGFP (as numbered in PDB ID 1EMA)[38], and with the equivalent residues 125 and 127 of *Discosoma* RFP (as numbered in PDB ID 1G7K)[37] (**Fig. 2**). Owing to the structural and sequence similarity among FPs discovered to date, it is highly likely that these residues are engaged in stabilizing interactions in the A-B interface. Similarly, residues aligned with residues 166 and 168 of avGFP (as numbered in PDB ID 1EMA) and with 162 and 164 of *Discosoma* RFP (as numbered in PDB ID 1G7K) are likely involved in stabilizing interactions in the A-C interface.

19| Disrupt the interfaces by mutating the residues identified at Step 18 into the positively charged amino acids lysine or arginine, by using the QuikChange protocol according to the manufacturer's guidelines or by overlap-extension PCR using previously published methods[41–43].

(A) When QuikChange is used

(i) Perform the reaction by following the manufacturer's protocol, and then transform eletrocompetent cells (as described in Steps 10–14 above) by using 2–3 µl of the Dpn1-treated QuikChange reaction.

(B) When the overlap-extension PCR method is used

(i) Purify the PCR product by QIAquick gel extraction kit according to the manufacturer's recommended protocol, and then digest and ligate into predigested plasmid (as in Steps 6–9 above). Use the ligation product to transform electrocompetent *E. coli* (as described in Steps 10–14 above).

▲ **CRITICAL STEP** Disruption of an interface will likely result in a marked decrease in FP brightness. Performing the disruption in a stepwise manner is advised. We recommend disrupting the A-B interface first, followed by the A-C interface. After successful disruption of the A-B interface, a few rounds of directed evolution (Steps 20–34) to rescue the brightness of A-C dimer can be done before monomerization. Hopefully, the fully monomerized protein will retain some degree of fluorescence, in order to facilitate early rounds of subsequent directed evolution for improved fluorescence. If the fluorescence is particularly dim and not visible after overnight growth at 37 °C, the LB agar plates with colonies can be stored at 4 °C for several more days before screening by fluorescence.

Library construction by error-prone PCR ● TIMING 2–3 d

20| Set up four PCR 0.2-ml thin-walled reaction tubes, one for each different dNTP. To each PCR tube, add the following, by pipetting up and down to mix solution:

Component	Amount (per 100-µl reaction)	Final concentration
Taq PCR buffer with Mg^{2+} (10×) stock solution	10 µl	1×
$MgCl_2$, 50 mM	10 µl	5 mM
$MnCl_2$, 10 mM	1.5 µl	150 µM
dNTPs mix (10 mM) stock solution	2 µl	200 µM
dNTPs (10 mM) individual stock solutions (one dNTP per tube)	2 µl	
Forward primer (100 µM)		
avGFP_XhoIfwd	0.5 µl	0.5 µM
Reverse primer (100 µM)		
avGFP_EcoRIrev	0.5 µl	0.5 µM
Template gene (5 ng)	5 ng	5 ng
Taq DNA polymerase (5 U)	1 µl	5 U
dH_2O	Add to final volume of 100 µl	

▲ **CRITICAL STEP** Use RNase- and DNase-free materials whenever possible. This protocol produces two or three mutations per 1- kb gene. Error rate can be increased or decreased, by increasing or decreasing the concentration of Mn^{2+}, respectively.

21| Place the four PCR tubes in a thermocycler and run the following program:

Cycle number	Denaturing	Annealing	Extension
1–35	94 °C, 30 s	55 °C, 30 s	72 °C, 60 s

After the PCR cycle has been completed, cool the reaction to 4 °C.

22| Combine 5 µl of each of the PCR products with 1 µl of DNA loading buffer on a 1.5% (wt/vol) agarose gel alongside a DNA ladder. Stain the gel with ethidium bromide and confirm that a PCR product of ~700 bp has been produced by using a UV transilluminator to resolve the band.
! CAUTION Ethidium bromide is a known mutagen and a suspected carcinogen. When handling it use gloves at all times and avoid skin contact.
▲ CRITICAL STEP A bright band should be clearly visible on the gel. Use the DNA ladder to determine whether the PCR product is approximately correct in size.
? TROUBLESHOOTING

23| Purify each of the PCR products by using the QIAquick PCR cleanup kit according to the manufacturer's instructions.
■ PAUSE POINT Purified PCR products can be stored at –20 °C for ~1 year.

24| Digest the purified PCR products by using XhoI and EcoRI restriction endonucleases and ligate into the XhoI/EcoRI predigested pBAD/His B vector from Step 6. The ligation product is used to transform electrocompetent *E. coli* as described in Steps 10–14.

Library screening ● TIMING 3–4 d per round, 3–10 rounds
25| Turn on the Lambda LS light source and Lambda 10–3 filter wheel controller and change the excitation and emission filter wheels to the desired positions. Light of the appropriate color should be illuminating the target area. Option A is preferred at early stages of the directed evolution when most colonies are dim and bright colonies can be easily distinguished. At later stages of the directed evolution when most colonies are bright, Option B is preferred as smaller differences in brightness are more easily distinguishable in pseudo-colored digital images.

(A) If using tinted goggles

(i) Put on tinted goggles that block the excitation light yet allow the desired color of fluorescence to pass to your eyes. The light exiting the bifurcated bundle should no longer be visible.
(ii) Remove the lid of the Petri dish and scan all of the colonies for the ones that appear brighter than the neighboring colonies.
(iii) Once a colony of interest has been identified, use a fine-tipped waterproof marker to circle the bottom of the plastic dish to indicate the position of the colony.

(B) If using digital acquisition

(i) Turn on the digital camera and computer and open the Image Pro software. Select 'Video/digital capture' from under the 'Acquire' menu. The exact image acquisition settings will depend on the brightness of the fluorescence and the sensitivity of the camera, but a reasonable starting point is to set the gain to 1.0 and the exposure time to 1 s.
(ii) Click on 'Preview' in the 'Video/digital capture' panel to see a real-time image of the Petri dish as viewed by the camera.
(iii) Adjust the position of the Petri dish (in its holder) to the center of the camera's field of view and adjust the exits of the fiber optic bundles to provide even illumination of the whole 10 cm radius of the Petri dish.
(iv) With the aperture of the camera lens open to its widest setting, adjust the camera lens zoom such that the dish just fills the field of view.
(v) Adjust the exposure time and/or gain such that there are no saturated pixels (i.e., an intensity of 4,095 or less on a 12-bit camera).
(vi) Turn off 'Preview' and click 'Snap' to acquire a single image. Once the monochrome grayscale image is acquired, process it with the tools available in the Image Pro software package.
(vii) To facilitate the identification of the brightest colonies, first convert the grayscale image into a pseudo-colored image by using a lookup table (LUT). This is done using the 'Pseudo-color' command under the 'Process' pull-down menu. Picking a blue-to-red LUT results in a coloring of the image that, in the opinion of the authors, is somewhat intuitive. The brightest pixels appear red and the dimmest pixels appear blue.

(viii) Threshold the image by using the 'Display range' tool under the 'Enhance' menu to find the colonies containing the brightest pixels. Drag the upper boundary to the highest pixel value (4,095 on a 12-bit image) and then slowly drag it back to lower pixel values. Colonies with the brightest fluorescence will be the first to turn red.

(ix) Once a colony of interest has been identified in the digital image, the same colony must be identified on the actual plate. Use a fine-tipped waterproof marker to write on the bottom of the plastic dish and circle the position of the colony of interest. Identifying the actual colony on the basis of the image can be tricky, and it is often facilitated by viewing the fluorescence with tinted goggles as described in option A.

▲ **CRITICAL STEP** When examining images to find the brightest colonies, keep in mind the imperfect and uneven illumination of the plate, which could cause the colonies in the middle of the viewing fields to appear much brighter than the colonies on the edge. The most effective way to avoid these illumination artifacts is to compare only neighboring colonies with each other (1–2-cm radius). A beneficial mutation is a rare event, only occurring in ~1 in 1,000 colonies. The use of such a small radius will enable the single bright colony with improved mutations to markedly stand out. To check whether the plate is being evenly illuminated with excitation light, obtain a fluorescence image of a Petri dish containing a solution of fluorescein and compare the fluorescence intensity from center to edge. If the illumination is found to be uneven, the fluorescein image can be used as a scaling factor to adjust the digital image of the colony image. Briefly, the colony image can be divided by the fluorescein image by using the 'Operations' function under the 'Process' pull-down menu. To allow pixel values to take on non-integer values, the output should be set to a 'Floating point' format. At later stages of directed evolution, the improvements from one round to the next will diminish and the identification of improved variants becomes increasingly difficult. Accordingly, picking and culturing larger number of colonies is crucial for finding improved variants.

? TROUBLESHOOTING

26| Repeat this screening process for all the plates. For each round of error-prone PCR library, 5–10 plates with 500–1,000 colonies each should be screened.

27| Once all the plates have been screened and colonies of interest identified, pick the colonies and transfer them into liquid culture. Select the colony of interest with a sterile plastic pipette tip and drop it into a sterile culture tube by using appropriate sterile procedures. For every colony to be picked, a sterile culture tube containing 4 ml of LB medium supplemented with ampicillin (0.1 mg/ml) and L-arabinose (0.2% (wt/vol)) is prepared. Tubes should be numbered with a waterproof marker.

28| Once all the cultures have been inoculated, incubate the culture tubes overnight at 37 °C with shaking at 225 r.p.m.

29| On the following day, pellet 2 ml of the bacterial culture by centrifugation. Extract the proteins from the pellets by using B-PER bacterial protein extraction reagent according to the manufacturer's protocol.

30| For each culture, dispense 10 µl of protein extract into 100 µl of Tris buffer in individual wells of a clear-bottom 96-well plate. For each variant, record the full emission spectrum with a Safire2 microplate plate reader or a similar plate reader equipped with monochromators. Label each well with the culture tube number to indicate the position of the samples.

▲ **CRITICAL STEP** The B-PER extraction can sometimes be skipped and 100 µl of bacterial culture can be directly dispensed into individual wells of 96-well plates for fluorescence characterization. Although this approach can provide reliable spectra for bright and red-shifted FPs, for dim or blue-shifted FPs, the emission spectrum will be obscured and unreliable due to autofluorescence and scattering from LB medium and the intact bacteria. Therefore, we suggest that protein extracts should be prepared.

31| Identify the variants with the desired color and most intense fluorescence emission by examination of the resulting emission spectra. Note that higher fluorescence intensity is a result of not only the improved brightness of FP but also of faster FP maturation time, higher FP expression level and faster bacterial growth. However, all of these features are desirable characteristics to select for during the evolution of a FP. Indeed, faster cell growth can be indicative of lower FP cytotoxicity[51]. By using the remaining 2 ml of the cultures that correspond to the variants with the most promising properties, perform plasmid minipreps (Qiagen) according to the manufacturer's instructions.

32| Use the plasmids (from Step 31) isolated after colony screening as the template for DNA sequencing. We use the pBADfwd and pBADrev sequencing primers (**Table 2**) and the DYEnamic ET kit as per the manufacturer's instructions.

33| Translate the DNA sequence of the FP variant by using the online Translate tool (web.expasy.org/translate/)to give the protein sequence, and then align the sequence with the wild-type FP protein with ClustalW (http://www.clustal.org/). This procedure will quickly reveal the location and identity of the mutations (if any) in the FP variants that have been picked from the screening procedure. Translating the sequence of the FP is crucial to tracking both beneficial and detrimental mutations.

34| Use the plasmids containing mutations as a template for further library construction (Steps 20–24) and screening (Steps 25–34).
▲ **CRITICAL STEP** Multiple rounds of library construction and screening should be performed to accumulate beneficial mutations. Mutants identified from a given round can be compared with its template plasmids. *E. coli* harboring the corresponding plasmids can be plated onto LB agar plates supplemented with 0.02% (wt/vol) L-arabinose. After overnight incubation at 37 °C, the fluorescence intensities of the colonies can be visualized as described in Step 25. The directed evolution cycles can be stopped at the point when no further improvement can be achieved.

Protein purification ● TIMING 12–18 h
35| Transform electrocompetent *E. coli* strain DH10B or TOP10 with the pBAD/His B expression vector containing the FP gene of interest (from Step 31) as described in Steps 10–14.

36| Pick a single colony from the plate and use it to inoculate a 4 ml culture of LB supplemented with ampicillin (0.1 mg/ml) and then incubate it overnight at 37 °C with shaking at 225 r.p.m.

37| On the following day, inoculate 1 liter of LB medium containing ampicillin (0.1 mg/ml) and L-arabinose (0.2% (wt/vol)) with the 4-ml culture from Step 36.
▲ **CRITICAL STEP** Some wild-type FPs, and essentially all FPs that have undergone some laboratory optimization, fold well at 37 °C. However, if the protein yield is low, the bacterial culture can be transferred to a lower temperature (~22–33 °C) and shaken for a longer time period (24–48 h).

38| Grow the culture at 37 °C with shaking at 225 r.p.m. for ~12 h. Next, divide it equally into four 250-ml centrifuge bottles on ice. Cool the bottles to 4 °C.

39| Pellet the bacterial cells by centrifugation at 4,000*g* for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 5 ml of binding buffer (Reagent Setup).

40| Place a 50-ml centrifuge tube on ice to cool to 4 °C. Transfer the contents of the bottles to the centrifuge tube to cool. Then sonicate the sample for 10 s, followed by a 20 s rest on ice. Repeat this sonicate-rest cycle 15 times to release the protein from the cells. Keep the suspension on ice at all times.
▲ **CRITICAL STEP** Excessive foaming is an indication of protein denaturation and can markedly decrease protein yield. If the solution begins to foam during sonication, immediately discontinue and proceed forward with the remainder of the protocol.

41| Centrifuge the lysate for 20 min at 20,000*g* at 4 °C to pellet the cell debris.

42| During Step 41, prepare the purification column. Gently invert the Ni-NTA resin to resuspend the solution and then add 3 ml to the column. Allow it to settle completely then gently remove the supernatant. Resuspend the resin with 6 ml of ddH_2O, allow it to settle, and then remove the supernatant. Repeat this procedure with 6 ml of binding buffer (Reagent Setup).

43| Remove the sample from the centrifuge and add the protein supernatant to the column. Wrap the column in foil to protect the protein from light exposure and then place it on ice. Gently agitate the mixture for 60 min to allow the resin to bind to the protein.

44| Remove the column from foil and allow the resin to settle. Remove the supernatant and then resuspend the resin with 6 ml of wash buffer (Reagent Setup). Allow the solution to settle and then remove the supernatant. Repeat for a total of three washes.

45| Add 1 ml of imidazole elution buffer (Reagent Setup) and collect the protein solution in a sterile 1.5-ml centrifuge tube.
▲ **CRITICAL STEP** If protein concentration is high, more than 1 ml of elution buffer may be needed to elute the protein from the resin.
? TROUBLESHOOTING

46| Dialyze the eluted protein into 50 mM Tris (pH 7.5) by using snakeskin tubing or a dialysis cassette according to the manufacturer's instructions. Once dialyzed, the protein can be stored at 4 °C if it is being used immediately.
■ **PAUSE POINT** If long-term storage is required, protein supplemented with 10% (vol/vol) glycerol can be stored at −80 °C indefinitely. However, it is worth noting that some slowly maturing FPs (e.g., mPlum) can continue to mature during storage.

Quantum yield determination ● TIMING 2 h

47| Choose an appropriate reference fluorophore that is well matched to the excitation profile of the FP being engineered. Suitable reference fluorophores include quinine sulfate in 0.1 M H_2SO_4 for blue FPs[52], fluorescein in 10 mM NaOH for GFPs[53] and rhodamine B or rhodamine 101 in ethanol for RFPs[54]. Prepare a solution of the FP in 5.0 mM Tris, pH 7.5.

48| To precisely measure the full absorbance spectra, use a step size of 0.5–1 nm and 1-cm quartz cuvettes. Record the spectra of the blank solutions (one for the standard and another for the FP) for baseline subtraction.

49| Identify a particular wavelength at which both the standard and the FP show substantial absorption, and use this wavelength as the excitation wavelength to determine the quantum yield of the FP.
▲ **CRITICAL STEP** As UV-visible spectrophotometers respond accurately to absorbance ranging from 0.3 to 1, the concentrations of the standard and FP solutions need to be adjusted accordingly.

50| Prepare a series of solutions of the diluted standard. To minimize the inner filter effect, dilute the standard to make five solutions with absorbance values of <0.05 at the excitation wavelength. The intensity of the absorbance (I_A) derived from Step 49 and the dilution factor (D) are used for calculating the absorbance values of the final solutions. For example, if an absorbance of 0.43 is obtained for protein diluted by 25-fold, the final concentration is 0.43/25 = 0.0172. Also prepare a serial dilution of FP in 5.0 mM Tris buffer (pH 7.5).
▲ **CRITICAL STEP** Thoroughly mix the FP sample before measurement. Proteins do not quickly solubilize in Tris buffer and a heterogeneous sample can lead to an unusually high emission spectrum.

51| By using the excitation wavelength identified in Step 49, measure the emission spectra for the FP solutions and the standard solutions. Also measure the emission spectra for the two blank solutions. Do not change the settings on the instrument.

52| Integrate the entire emission spectra to give the total fluorescence intensities. Subtract the total fluorescence intensities by the emission integration of the corresponding blank solutions. Plot a graph of corrected fluorescence intensities versus absorbance. Apply linear progression to the two data sets (intercept = 0), and derive the slopes for both the FP and the standard (S_{FP} and S_{ST}, respectively). The quantum yield of the FP is calculated by using the formula:

$$\Phi_{FP} = \Phi_{ST}\left(\frac{S_{FP}}{S_{ST}}\right)\left(\frac{R_{FP}^2}{R_{ST}^2}\right)$$

in which R_{FP} and R_{ST} are refractive indexes of the buffers used for the standard and the FP.
▲ **CRITICAL STEP** Make sure to account for the differences in refractive indexes when different buffers are used for the standard and the FP. They are usually very similar in value; however, it is still necessary to account for the differences for an accurate measurement.

Extinction coefficient determination ● TIMING 1 h

53| Determine the concentration of the FP stock solution by using a BCA protein analysis kit (Thermo Scientific) according to the manufacturer's instruction. Convert the concentration into a molar concentration (C) value by using the calculated molecular weight for the FP. To calculate the extinction coefficient (ε; units of $M^{-1}cm^{-1}$), the following formula is used:

$$\varepsilon = \frac{I_A * D}{C}$$

where I_A is the intensity of the absorbance at the given wavelength and D is the dilution factor (Steps 48–50). Alternatively, if the extinction coefficient for the alkali-denaturated chromophore is known, the alkali denaturation method can be used[6,55].

pH sensitivity determination ● TIMING 2 h

54| To determine the pH-dependence of the fluorescence emission of an engineered FP, dialyze a stock solution of 1 μM of the FP in 5.0 mM Tris (pH 7.5) (Step 46).

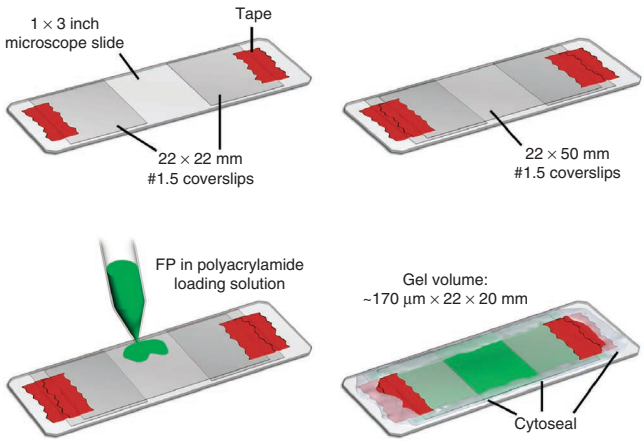

Figure 5 | Mounting procedure for photobleaching proteins. Photobleaching slides are prepared by attaching two 22 × 22 mm (#1.5) coverslips ~1.5 cm apart with a precut section of tape. A 22 × 50 mm coverslip is placed on top of the attached coverslips to create a space between the bottom slide and upper coverslip. A homogenous mixture of the FP and polyacrylamide solution is prepared and catalyzed as described in the protocol. This solution (75 µl) is immediately dispensed underneath the 22 × 50 mm coverslip and then left to polymerize for 3–5 min. The edges of the slide are then sealed with Cytoseal to prevent dehydration of the gel.

55| Prepare a series of buffers containing 200 mM citric acid (or citrate) and 200 mM boric acid (or borate) with pH values ranging from 2 to 10. Dispense 0.1 ml of each different buffer into separate wells of a black 96-well plate with a clear bottom.

56| To each well containing a buffered solution, add 10 µl of the stock FP solution. Allow the plates to incubate at room temperature for 10 min. Collect the full emission spectra for each well with a Safire2 plate reader.

57| Determine the relative fluorescence at each pH at the peak wavelength and plot it as a function of pH. The pK_a of the FP sample will be the pH value at which 50% of the molecules are fluorescent.

Photostability determination ● TIMING 1 d

58| To photobleach purified proteins, mount a coverslip onto two spacer coverslips ~1 cm apart with adhesive tape to create space underneath for the gel (**Fig. 5**).

59| Prepare a polyacrylamide gel by mixing a 1 µM solution of the purified FP in 20% (wt/vol) acrylamide/bis-acrylamide solution (Reagent Setup) in a 1.5-ml centrifuge tube. Add 3 µl of ammonium persulfate and 0.5 µl of TEMED to catalyze the reaction. Polymerization takes 3–5 min, so the following steps must be performed quickly. Pipette ~80 µl of the solution underneath the upper coverslip and then seal the edges with a paintable sealant, such as Cytoseal, to prevent evaporation. Allow the slides to rest for ~12 h before imaging.

! CAUTION Acrylamide is acutely toxic when touched, ingested or inhaled. When handling it, use gloves and a fume hood at all times and avoid skin contact.

60| Place the glass slide on the stage of a wide-field microscope equipped with a xenon arc lamp illumination source and a 60× objective. Add neutral density filters between the light source and the sample in order to decrease the light intensity to the minimum required for visualization of individual droplets of the protein solution.

61| Position the stage such that the gel in the field of view is evenly illuminated. Remove the neutral density filters from the excitation light path just before the start of the acquisition. By using a time series acquisition, continuously collect images from the sample using identical settings. It is critical that the shutter remains open at all times. Collect images until the intensity of the fluorescence is <50% of its maximum intensity and ideally <10% of its maximum intensity to allow for the greatest amount of data points.

▲ CRITICAL STEP For a typical photobleaching experiment, collect 15 sets of data at each power intensity measured. Remove the highest and lowest $t_{1/2}$ collected and average the remaining 13 data points for the mean $t_{1/2}$ of the FP sample.

62| Process images in a software package such as Image Pro Plus. Fluorescence intensity in an area of interest on the gel is plotted as a function of time (**Fig. 3**). The resulting curve approximates an exponential decay with time zero being the instant at which the neutral density filter was removed from the light path.

? TROUBLESHOOTING

Gel filtration analysis ● TIMING 2–3 h

63| Determine the oligomeric structure of FP variants by gel filtration chromatography. In this procedure we use a HiLoad 16/60 Superdex 75-pg gel filtration column on an AKTAbasic liquid chromatography system with a multiple-wavelength absorbance detector. The most suitable molecular weight standards to compare with the engineered FP are similar FPs of known oligomeric structure. Good examples of FP variants that are available in various oligomeric states are the tetrameric *Discosoma* RFP, dimeric dTomato and monomeric mCherry[6]. Express and purify each protein (Steps 10–17) for comparison to the new FP.

64| Analyze each of the FP standards, as well as the purified FP sample, by gel filtration chromatography per the manufacturer's instructions; construct a linear calibration curve by plotting the logarithm of molecular weight versus elution volume (V_e) divided by void volume (V_o). By using the experimentally determined V_e/V_o, the molecular weight (and oligomeric structure) can be read from the calibration curve.

Behavior in fusion proteins ● TIMING 2–3 d

65| To create the FP fused to an α-tubulin mammalian expression plasmid, PCR-amplify the FP gene with avGFP_Nhe1fwd and avGFP_XhoIrev (**Table 2**). Then enzyme-digest and ligate (Step 9) the PCR product into a pEGFP–α-tubulin vector that has been previously digested with the same restriction enzymes in order to excise the EGFP coding sequence.

66| Purify samples by using a plasmid miniprep kit (Qiagen) according to the manufacturer's recommended protocol. As mentioned in Step 17, the DNA concentration from a miniprep will vary on the basis of the vector copy number. A mammalian expression vector such as the one in this protocol is a high-copy-number plasmid and will normally have a DNA concentration of ~1.0 μg/μl of a 50-μl miniprep sample.

67| Culture HeLa cells in DMEM (Life Technologies) supplemented with 10% (vol/vol) FBS (Sigma) at 37 °C. Seed 2 × 10^5 cells into 35-mm imaging dishes the day before transfection (Steps 68–71). Incubate the cells in the humidified incubator at 37 °C with 5% CO_2.

68| Dilute 4 μg of plasmid DNA from Step 66 in 0.5 ml of OptiMEM (Life Technologies). To the mixture, add 10 μl of polyethylenimine transfection reagent (Reagent Setup). Incubate the mixture at room temperature for 30 min.

69| Take the imaging dishes containing HeLa cells from the incubator. Remove the DMEM medium. Add the DNA/PEI/OptiMEM mixture from Step 68 to the imaging dishes.

70| Return the imaging dishes to the humidified incubator at 37 °C with 5% CO_2.

71| Remove the DNA/PEI/OptiMEM transfection medium after 1 h. Add 1.5 ml of fresh DMEM containing 10% (vol/vol) FBS into the imaging dishes. Return the imaging dishes to the humidified incubator at 37 °C with 5% CO_2.

72| Approximately 16–48 h after Step 71 and just before imaging (Step 73), exchange the medium for HBSS containing no calcium chloride, magnesium chloride, magnesium sulfate or phenol red (Life Technologies).
▲ CRITICAL STEP HeLa CCL2 cells that are transiently transfected with an expression plasmid are best to image ~16–48 h after transfection. As the strong cytomegalovirus (*CMV*) promoter drives expression, longer incubation leads to overexpression and cell death.

73| Transfer the imaging dish with transfected HeLa cells to the stage of a Zeiss Axiovert 200M epifluorescence inverted microscope equipped with a xenon arc lamp, a monochrome Retiga 2000R 12-bit cooled CCD camera (QImaging), and a filter set that is appropriate for the engineered FP. Compare digital fluorescence images obtained with the engineered FP with images obtained with analogous EGFP fusions expressed in the same cell type. Example images for α-tubulin, α-actin and other common fusions are available in the primary literature[56].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4, 22	No band appears on the gel	No PCR product	Check the primer and template concentrations Redesign new forward and reverse primers with higher melting temperature Use freshly prepared dNTPs Use a gradient PCR machine to simultaneously test a range of annealing temperatures

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
8	More or fewer than the expected number of bands appear	Restriction enzyme digest was not successful	Make sure there are not multiple restriction enzyme sites in the plasmid or FP Double-check that the correct restriction enzyme and buffers are used Shorten the incubation time of the digestion reaction
15	No or few colonies on the plate	Ligation was unsuccessful due to low concentration of the insert or vector	Consider using a different ratio of insert to vector for the ligation reaction
		Transformation was unsuccessful	Check competent cell transformation efficiency using a control plasmid
		Low transformation efficiency of competent cells	Purchase or prepare new competent cells Extend the incubation time for ligation reaction Perform control ligation with and without the previously validated insert sequence to check the quality of cut plasmid Decrease or dilute the ligated DNA used for electroporation to prevent arcing
25	Many nonfluorescent but very few fluorescent colonies are on the plate	Error rate was too high during PCR amplification	Decrease the concentration of $MnCl_2$ or leave it out altogether. Decrease the concentration of $MgCl_2$
	Practically all colonies on the plate are fluorescent	Error rate was too low during PCR amplification	Increase $MnCl_2$ (up to 150 μM maximum) and $MgCl_2$ concentration (up to 5 mM greater than what is already in the Taq buffer (1.5 mM))
45	No protein elutes from the column	Protein purification was unsuccessful	If using an expression plasmid with a C-terminal His-tag, ensure that a stop codon has not been inadvertently inserted before the tag Culture bacteria for longer time period at lower temperature. Some proteins require lower temperatures to fold correctly
62	Abnormal photobleaching curves	Because of abnormalities introduced in the optical setup (filters, light source, etc.), photophysical data may have artificial artifacts	Photobleach the sample at multiple powers and use the log-log of power versus $t_{1/2}$ when comparing the photostability of one FP to another and not actual $t_{1/2}$ times

● TIMING

Steps 1–9, subcloning into pBAD/His B vector: 4–5 h
Steps 10–15, transformation of *E. coli*: 15–18 h
Steps 16 and 17, plasmid purification: 1 d
Steps 18 and 19, monomerization of the FP: 3–5 d
Steps 20–24, library construction by error-prone PCR: 2–3 d
Steps 25–34, Library screening: 3–4 d per round, 3–10 rounds
Steps 35–46, protein purification: 12–18 h
Steps 47–52, quantum yield determination: 2 h
Step 53, extinction coefficient determination: 1 h
Steps 54–57, pH sensitivity determination: 2 h
Steps 58–62, photostability determination: 1 d
Steps 63 and 64, gel filtration analysis: 2–3 h
Steps 65–73, behavior in fusion proteins: 2–3 d

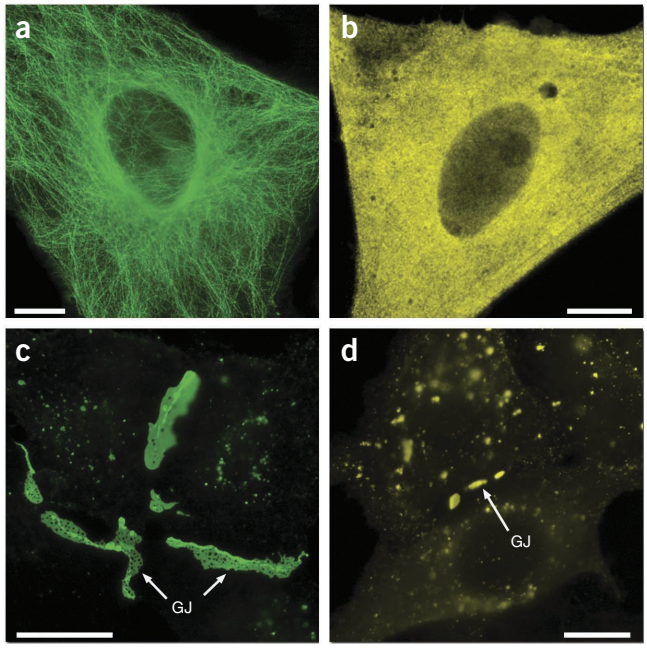

Figure 6 | Comparison of good and poor localization of FP fusions in similar constructs. (**a**–**d**) mEmerald FP (**a**) and mKusabira Orange FP (**b**) fused to human α-tubulin; mEmerald FP (**c**) and mKusabira Orange (**d**) FP fused to rat connexin-43, which forms gap junctions (GJs). When an FP fusion is used to label a particular protein or target an organelle, the degree of oligomerization often becomes apparent through FP aggregation and incorrect targeting. In particular, α-tubulin and connexin-43 can be used to demonstrate the marked differences in localization between monomeric, dimeric or tetrameric FPs. In both examples shown, the highly monomeric FP, mEmerald, is giving the expected localization (**a**,**c**). Specifically, α-tubulin is visible as distinct microtubules (**a**) and connexin-43 is localized in highly defined and well-organized GJs (**c**). (**b**,**d**) In contrast, mKusabira Orange has substantial dimeric character, and therefore it exhibits poor localization in both fusions. Specifically, discrete microtubules of α-tubulin are not apparent (**b**), and the GJ plaques are small and poorly defined (**d**). Scale bars, 10 μm.

ANTICIPATED RESULTS

Shown in **Figure 3** are representative results obtained after Step 62. Each FP is expected to respond differently to light illumination. FPs such as EGFP, mCherry and mTFP1 are relatively photostable, whereas YFP and its derivatives such as mCitrine are less photostable. The light intensity, system setup (e.g., widefield or confocal microscopy) and the duration of light illumination all have an impact on photobleaching. It is suggested to perform the photobleaching experiment under both wide-field and confocal conditions. The same experiment should also be repeated in the presence of different light intensities.

Illustrated in **Figure 6** are two sets of identical fusions incorporating two high-performance FPs, mEmerald (an avGFP derivative) and mKusabira Orange (a bright, monomerized coral derivative). Similarly to EGFP, mEmerald localizes well in most constructs. In contrast, mKusabira Orange does not localize well in many constructs including fusions with α-tubulin (using the Clontech-style vector described above) or connexins (constructed with an N-terminal Clontech vector). Note the intricate microtubule network with mEmerald (**Fig. 6a**) in contrast to the punctate, nonspecific localization of an identical fusion substituting mKusabira Orange (**Fig. 6b**). Similarly, mEmerald forms functional gap junction plaques when fused to connexin-43 (**Fig. 6c**), whereas the plaques observed with an identical mKusabira Orange construct (**Fig. 6d**) are much smaller.

ACKNOWLEDGMENTS This research was made possible with financial support from the University of Alberta, the Canada Foundation for Innovation, the Natural Sciences and Engineering Research Council of Canada, and Alberta Ingenuity (scholarship to Y.S. and a New Faculty Award to R.E.C.). R.E.C. holds a Canada Research Chair in Bioanalytical Chemistry.

AUTHOR CONTRIBUTIONS M.W.D. and M.A.B. acquired data and images shown in **Figures 3** and **6**. H.A., M.A.B., Y.S., M.W.D. and R.E.C. developed the protocols and wrote and edited the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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