Fluorescent Protein Variants Generated by Reassembly between Skeleton and Chromophore

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ABSTRACT: Fluorescent proteins (FPs) can be used as intrinsic molecular tags to track the dynamic activity in live cells. To obtain variants in an available and massive manner is always a challenge. Here, we adopted a computer-based microarray synthesis method to realize the reassembly between the chromophore and the skeleton. DNAWorks was used to segment the input FP templates into a set of overlapping oligonucleotides (20−43 mer) with a balanced annealing temperature, G + C content, and codon frequency. The constitution of the chromophore was kept in the same section by switching the divided sites during segmentation and the codon was optimized to further keep the balanced parameters. The designed oligonucleotides were synthesized on photo-programmable microfluidic arrays. Sequence analysis and the subsequent conditional induced expression of FPs revealed that oligonucleotides were highly reassembled. Spectra, photostability, and molecular size detection of randomly selected variants showed that they were distinct monomeric proteins that preserved photoactivity. Our study provides an effective means of obtaining FP variants based on a computer-designed parallel synthesis.

1. INTRODUCTION

Fluorescent proteins (FPs) are powerful tools for tracking biological processes in vivo at the level of single molecules, intact organelles, live cells, and whole organisms.1−4 FPs have undergone several generations of evolution, from natural isolation, site-exhaustive mutation, to gene synthesis nowadays.5−9 Most of the naturally isolated FPs were deserted as molecular tags due to their intrinsic defects in maturation or brightness. Later random mutations and site-directed mutagenesis gave rise to a series of FP variants, mainly with a modified chromophore constitution or structure, little attention being paid to the FP skeleton.10−12 Recent studies have revealed that the quantum yield of the separated chromophore is only one-thousandth that of the overall structure, suggesting a critical role of the skeleton in FP brightness.12 Especially, the findings that cancellation of the π skeleton extension resulted into a red shift of FP emission, suggest a role of skeleton in the emission color of FPs.13

Microfluidic arrays, with the advantage of massively parallel synthesis of oligonucleotides, have been adopted to synthesize several molecules besides FP variants, but still, FP variants have been generated on these microfluidic arrays based on the principle of site-exhaustive mutation.14,15 Attempts have been made to use polypeptide arrays to synthesize FP variants in vitro in order to simplify the whole experiment process. However, these cell-free synthesized proteins were defective in bioactivity or structure, as they lacked essential modifications during protein synthesis in cells.16

To explore the feasibility of generating FP variants in the recombination between chromophore and skeleton and to improve the experimental efficiency, computer-based cutting of skeleton coupled with microfluidic array synthesis and cloning were utilized in this study. The massively parallel synthesis of the light-directed microfluidic arrays had been verified by our de novo synthesis of Saccharomyces cerevisiae cytochrome b5.17 Oligonucleotide design for synthesis was the key part of this study. Although several algorithms have now been made to design combinatorial oligonucleotide pools, few computationally designed oligonucleotide pools have been characterized experimentally.18−20 DNAworks algorithm was used as it has advantages in the balance of highly homogeneous melting temperatures, minimization of hairpin formation, ease of handling, and experimental verification.21 Here, 18 widely used FPs were selected as input templates for computer-based oligonucleotide design, and thermally balanced oligonucleotides were massively synthesized in parallel in our light-directed microfluidic arrays. After PCR, ligase chain reaction (LCR), and cell cloning, aliquots were sampled for testing the
reassembly, diversity, photoactivity, and photostability of FP variants.

2. RESULTS

2.1. Designing the FP Variant Library. Several methods for parallel assembling genes from synthetic oligonucleotides have been developed.\(^\text{22-24}\) Our effort to include gene synthesis in a gene to its skeleton structure prompted us to expand current methods in gene assembly for recombinant FP variant production. The principle is to divide 18 FP nucleotide sequences into small overlapping fragments so that they can be recombined with each other to obtain novel variants (Figure 1). The whole design mainly includes two segmentation steps.

First, each input templates containing flanking sequences was divided into five overlapping sections. These 180 (18 × 5 × 2) fragments were characterized by near-equivalent melting temperatures and balanced G + C contents (0.44–0.64), which increase the success of reassembly. With the length of fragment ranging from 166 to 169 mer, chromophores from each input FP were just in the secondary fragment. Then, each section was further divided into four parts (positive strand) or five parts (negative strand) after stringent evaluation on location of chromophore sites. Therefore, only sequences that contained BamH I recognition sites were amplified with two methods to explore the best assembly conditions. Results showed that two-gradient PCR was more suitable for assembly and amplification than one-gradient PCR, as there were more abundant target products in two-gradient PCR compared with one-gradient PCR (Figure S1). To obtain the full lengths of these assemblies for clone, primers of 18 FP-R and 18 RP-R, flanked with BamH I and EcoR I recognition sites separately, were added to identify and hybridize with fragment containing BamH I and EcoR I recognition sites (Table S2). Therefore, only sequences that contained BamH I and EcoR I recognition sites were amplified. Then, the full-length sequences were cloned into pET28a (+) vectors after BamH I and EcoR I digestion. The total number of kanamycin-resistant clones was 3830, and 96 were randomly selected with a random number generator for further sequence analysis. Among these 96 clones, 46 presented distinct colored fragments containing BamH I and EcoR I recognition sites (Table S2). Error removal was conducted at both the oligonucleotide and fragment stages. To increase the quantity, the oligonucleotide mixtures were amplified by PCR with Mly I primers. After annealing, the hybridization product of oligonucleotides was between lanes of 100 and 200 bp (Figure S1), corresponding to the length of fragment in design (166–169 mer). Further fragment assembly and amplification were conducted with two methods to explore the best assembly conditions. Results showed that two-gradient PCR was more suitable for assembly and amplification than one-gradient PCR, as there were more abundant target products in two-gradient PCR compared with one-gradient PCR (Figure S1). To obtain the full lengths of these assemblies for clone, primers of 18 FP-F and 18 RP-R, flanked with BamH I and EcoR I recognition sites separately, were added to identify and hybridize with fragment containing BamH I and EcoR I recognition sites (Table S2). Therefore, only sequences that contained BamH I and EcoR I recognition sites were amplified. Then, the full-length sequences were cloned into pET28a (+) vectors after BamH I and EcoR I digestion. The total number of kanamycin-resistant clones was 3830, and 96 were randomly selected with a random number generator for further sequence analysis. Among these 96 clones, 46 presented distinct colored after isopropyl-β-D-thiogalactopyranoside (IPTG) induction, so it was concluded that the ratio of overall FP variants was ~48% (1838 out of 3830).

2.3. Varieties and Variation of Skeleton Recombination. Sequencing results from the samples demonstrated that the accuracy of this method was higher than other high-throughput results with improved error removal. The total error frequency of our method was 1.72/kb, including deletion, insertion, and substitution (Table 1). About 48% of positive clones presented colors after IPTG induction and five clones with distinct colors were selected for subsequently assembly analysis and characterization of photoactivity, photostability, and molecular size. According to their colors after purification, these proteins were named Green2, Reseda, Plum, Pink, and

![Figure 1. Overview of the oligonucleotide design process by DNAWorks and reassembly process of full-length of sequences. The input templates were mainly segmented through two steps into oligonucleotide that can be used for synthesis, the “star” represented the chromophore site. The assembly process of full-length sequences just in the reverse direction of oligonucleotide design process. LCR, ligation chain reaction; OligoMix, oligonucleotide mixture.](image)

Table 1. Error Analysis of Reassembled Sequences Among Different Error Removal Methods\(^{\text{a, b, c}}\)

| error type      | non-error removal | multiplex assembly | this study | throughput-improved error removal | Plusion + gel stab |
|----------------|------------------|--------------------|------------|---------------------------------|------------------|
| deletion       | 175              | 4                  | 8          | 4                               | 5                |
| insertion      | 38               | 11                 | 4          | 9                               | 0                |
| substitution   | 229              | 29                 | 105        | 23                              | 1                |
| total errors   | 442              | 44                 | 117        | 36                              | 6                |
| bases sequenced| 31,445           | 24,874             | 68,040     | 16,343                          | 7648             |
| error frequency (error/kb) | 14.05 | 1.77              | 1.72      | 2.2                            | 0.785            |

\(^{a}\)Error removal was conducted at both the oligonucleotide and fragment stages. \(^{b}\)Data from the study of Wen Wan et al. (Sci. Rep., 2017, 7, 6119). \(^{c}\)Data from the study of Matthew C. Blackburn et al. (Nucleic Acid Res., 2016, 44 (7), e68).
Reddle (Figure 2A). To track the recombination process, these five FP sequences were deliberately aligned with input templates in the design step. The results revealed that skeleton sequences from different input templates were successfully recombined. Alignment results demonstrated that the sequence of Green2 was a recombination of EBFP 1−158, 222−298, and 524−550; EGFP 160−221 and 524−550; Cype 299−433, 524−550, 551−654, and 682−714; Topaz 434−460 and 524−550; mCitrine 461−522 and 524−550; and EYFP 655−681; Reseda was a recombination of wtGFP1-74, Cype 75−134 and 678−714, EBFP 135−192 and 678−714, Venus 193−320, EGFP 135−192 and 321−457, Cerulean 458−497, ECFP 498−677, and T-Sapphire 75−134; Plum was a recombination of mStrawberry 1−104 and 208−240, mCherry 105−167 and 321−497, dTomato 168−207, mOrange 208−240 and 596−705, and mBanana 241−320 and 498−595; Pink was a recombination of mCherry 1−61, 96−119 and 498−705, mBanana 62−95 and 168−240, mStrawberry 120−167, and mOrange 241−497; Reddle was a recombination of mCherry1-57, 79−104, 321−383, and 385−404; mBanana 59−77, 105−320, 385−404, and 406−595; and mOrange 596−705 (Figure 2B).

In addition, several substitutions occurred during oligonucleotide synthesis or recombination. Alignment results showed that there were four substitutions in Green2 (G159T, A523G, G528A, and A551T); two in Reseda (T692A, G693T or T681G, C682G); one in Pink (G298A); and four in Reddle (G58T, G78T, G384A, and C405T) (Table S3).

To further evaluate the similarities of these five variants to the 18 input templates, a phylogenetic tree was constructed with MEGA7.0 software. The results revealed that these proteins were mainly organized in three subclasses (Figure 2C). Reseda belonged to the yellow-green FPs and it had the highest similarity with Venus (distance, 0.006) but still had four sites different from Venus (H25Q, L46F, G175S, and Y203T). Green2 belonged to the yellow FPs and was distinct from all FPs in the same clade with a distance of 0.012 from the main branch. Plum, Pink, and Reddle were in the orange-red subclass (Figure 2C). Compared with Pink, Plum and Reddle had high similarities with mOrange but were still distinct from it with distances of 0.012 and 0.024, respectively. Pink was located in the same clade as mCherry with a distance of 0.015 away from it. These results reflect the diversity of our synthesized FPs after oligonucleotide recombination.

2.4. Conserved Photoactive Spectra of FPs. To preserve the accuracy of 3D structural folding and photoactivity, the cell expression method was adopted for these proteins. Spectra were analyzed after the proteins were purified to >99% on a Ni2+-chelating Sepharose FF chromatographic column and fast protein liquid chromatography (FPLC). The elution spectra of FPLC showed that the elution time of green and yellowish-green FPs was eluted earlier than red and orange FPs, which are consistent with their pKα (green vs red = 6.0 vs 4.5). As the elution time of the proteins depended on their relative charge differences, the less negatively charged proteins are eluted earlier at rather low salt concentration, while the
highly charged proteins eluted at higher salt concentration and required much longer time. EGFP, with less negative charge and low molecular weight, was the first to be eluted (Figure S2). Spectra results revealed that the five selected variants were mainly divided into three categories. The variants with absorbed spectra of green and yellowish green, Reseda and Green2, had absorption peaks at 484 and 505 nm, respectively, and both had a shoulder at ~400 nm (Figure 3A). Variants with absorption spectra of the orange red, Plum, Pink, and Reddle, had more complicated absorption curves compared
with mCherry. All these three variants had three absorption peaks in a light span of 300–700 nm (Plum: 384, 504, and 543 nm; Pink: 337, 419, and 539 nm; and Reddle: 385, 501 and 557 nm), which was probably associated with skeletal-induced immature chromophore intermediates. Besides, the absorption wavelengths of these three variants were shorter than mCherry (587 nm) (Figure 3A).

To determine the photoactivity and applicability of these representative variants, excitation and emission spectra were further examined. The results showed that the maximum excitation wavelength of Reseda was red-shifted (emission peak, 513 nm) and exhibited a larger stroke shift (~33 nm) than EGFP (emission wavelength, 510 nm, stroke, ~26 nm) (Figure 3B). Green2 presented excitation and emission spectra (excitation peak: 484 nm; emission peak: 510 nm) similar to EGFP (Figure 3B). The orange-red variants, typified by Plum, Pink, and Reddle, had maximum excitation and emission wavelengths at 541 and 555, 535 and 560, and 540 and 565 nm, respectively (Figure 3B).

2.5. Photostability and Molecular Size. We further assessed the photostability of these representative synthesized FP variants. In vitro photobleaching measurements demonstrated that the photostability half-life of Reseda was 136 s, comparable to that of EGFP (t½ = 150 s), while Green2 was the least stable among these three yellowish-green FPs with a half-time of 46 s (Figure 4A). The photostability of Plum, Pink, and Reddle was 92, 65, and 51 s, respectively (Figure 4B). They were more photostable than most red FPs, though to a lesser degree than mCherry, a widely used red FP reporter.23

We further measured their molecular size by mass spectrometry, which can reflect their conformation in vivo after maturation. The results showed that Reseda and Green2 had a single peak with m/z values of 28,187.08 and 28,057.99 Da, respectively (Figure 5). However, the other three RFP variants, Plum, Pink, and Reddle, had two main peaks with m/z values at about 1.4–2.0, which probably resulted from the disparity of charge during chromophore maturation, as the RFP chromophore can donate one to two electrons when passing through the transitional stage (9). On this basis, the latter peak corresponding to one electron-charged state was speculated to be the protein size of these RFP variants, which were 26,610.22, 26,953.72, and 26,176.38 Da for Plum, Pink, and Reddle, respectively (Figure 5). The mass spectrometry results suggested that all of these variants were monomers after expression, according to the previously calculated mass of EGFP.26

3. DISCUSSION

3.1. Feasibility of Obtaining FP Variants by Recombining Chromophore and Skeleton. FPs contain an internal chromophore constituted of triple amino acids housed within a 11-stranded β-barrel.27 FPs owe their emitted fluorescence to the autocatalytically formed intrinsic chromophore. The main structure of the chromophore constituted by triple amino acids has been estimated to be flexible.3 However, quantum mechanical calculations have demonstrated that the excited state of the chromophore has significant dihedral freedom, which may lead to fluorescence quenching by transforming the absorbed light energy into chemical bond motion.28–30 Further FP quantum yield assessment in a vacuum and molecular dynamics simulations revealed that restriction in the rotational freedom of the chromophore leads to an increase in fluorescence brightness and quantum yield,29 which indicated the role of skeleton in brightness (quantum yield) of FPs. Especially, recent research has disclosed that a rigid FP skeleton can increase the quantum yield of the fluorophore by restraining its rotational freedom.31 Moreover, delicate dynamics studies have examined the electrostatic and quantum mechanical interactions within the protein cavity and found that with cancellation between the π skeleton extensions, the emission was red-shifted, otherwise blue-shifted, and suggested a critical role of the immediate skeleton in determining the emission color of red FPs.32 Based on the importance of the skeleton in the brightness (quantum yield) and emission color of FPs, we proposed that FPs can be converted by recombination between chromophore and skeleton. De novo parallel synthesis of FPs was achieved by coupling microfluidic arrays with cell cloning. In our study, ~48% of clones exhibited colors after maturation, demonstrating the success of skeleton reassembly. The further spectral analysis of the bioactivity and photostability of representative variants demonstrated the feasibility of this method to obtain new variants (Figures 3 and 4).

3.2. Factors Affecting the Diversity of FP Variants by Skeleton Recombination. From tracing sequence assembly and constructing phylogenetic trees, sequence similarity was one of the main determinants of successful reassembly and diversity. The sequence analysis of the representative variants demonstrated that variants were limited to those derived from FPs of the same clade, such as fragments from a green clade were prone to reassemble into green or yellowish-green variants, and fragments from a red clade preferentially
reconstructed into red or orange-red variants (Figure 2C), which was in line with the similarity between templates, as the similarity of the coding sequences in the same clade was more than 90%, while it was only 35% between green FPs and red FPs (Table S4).

Despite the fact that the similarities of input templates affect the reassembly results, the abundance and the length of fragments, the homology between fragments, and the reaction temperature were critical for the assembly, as the reassembly is a competitive fragment-binding process. To increase the diversity of successful assembly, we optimized the oligonucleotide pool to similar melting temperatures by adjusting the length of oligonucleotides and optimized the codon encoding (Table S1). In addition, random mutation was another source of the diversity for obtaining variants. Sequence alignment

Figure 5. MALDI-TOF-MS polypeptide profiling of each variant in linear mode (9900–80,500 Da). A series of different weight of proteins including BSA and EGFP tagged with 6× histidine are selected as the external control. Each variant was measured in triplicate and the values were averaged and normalized to a maximum intensity of 100%.
showed no more than 0.16% random mutation in these variants (Table 1).

3.3. Advantages of Obtaining FP Variants by Skeleton Recombination. The study demonstrated the feasibility of obtaining FP variants based on recombination of the FP skeleton and chromophore. The computer-based design and parallel synthesis method in our study is fast and economical. Above all, this method is easy to manipulate and thus to generate monomer variants with preserved photoactivity and photostability (Figures 3 and 4). Monomericity is important in order to avoid aggregating the linked protein target by self-oligomerization.33 Photoactivity and photostability affect the brightness of fluorescence of FPs, they are the key characteristics of FPs as biological tags, and both of them demonstrated the advantages of this method for engineering FP variants.

Unlike the conventional site-directed mutagenesis and random mutation, multi-dimensional mutations can be achieved at one time using this method (Figure 2). In addition, it has more scalability in design by adding site-specific mutations and codon optimization during synthesis. The method used here provides an available approach to obtaining FP variants, which can further be applied in generating variants of molecules with high homology, such as antibodies and riboswitches.

4. METHODS

4.1. Oligonucleotide Pool Design and Composite. All the 18 input template FPs used in this study were either taken from the NCBI website or from references (Table S5). The coding sequences of these templates were converted to an Escherichia coli (BL21 trxB(DE3)) codon-optimized nucleotide sequence using JCat (Java Condon Adaptation Tool, www.jcat.de).34 The resultant sequences with BamHI and XhoI recognition sites as flanking sequences were loaded into the DNAWorks (version 3.2.4) interface in fasta format. The full length of each template was partitioned into five fragments (166–169 mer) with the key features: annealing temperature ranging from 72 to 82 °C, length of overlap from 29 to 30 mer, G + C content between 0.44 and 0.64, and codon frequency in the range of 0.54–0.59. Fragments (18 × 5 × 2) of both positive and negative chains were further uploaded into DNAWorks and segmented into 810 oligonucleotides (fragment of positive chain was divided into four sections, while fragment of negative chain was divided into five sections) with lengths ranging from 20 to 43 mer, annealing temperature 51 to 93 °C, dH −152 to −364, and the four bases were balanced (proportion of each base < 0.57). The key step in this process was to optimize the length of oligonucleotides to achieve homologous melting temperatures and keep the nucleotides of the chromophore in the same oligonucleotides by testing all possible arrangements of sections within a gene sequence and codon optimization. The length, G + C content, and relative position of matches between sequences is used for calculating the secondary structure score. The output reports included the melting temperatures, oligonucleotide sequences, and potential formation of secondary structures.

4.2. Oligonucleotide Synthesis on Microarray. The microfluidic reactor array device was fabricated as previously reported.35–37 Briefly, the fabrication of microfluidic reactors included structural design, mask fabrication, mold fabrication, alignment, and bonding. After derivatization and washing of the chip, the synthesizer was programmed to deliver reagents and photogenerated acid precursor to the chip for oligonucleotide synthesis. The set of oligonucleotides designed by DNAWorks have undergone light-directed synthesis on microfluidic pico-array reactors as we previously reported.38 In brief, oligonucleotides were synthesized on a photo-programmable 4k microfluidic microchip, with five points for one oligonucleotide. After synthesis, the microarray was hybridized with Cy3- and Cy5-labeled complementary oligonucleotides to detect synthesis quality.32 The oligonucleotides were cleaved by ammonium hydroxide, followed by vacuum evaporation to eliminate excess ammonium hydroxide. The synthesized oligonucleotides were purified with ethanol precipitation.

4.3. Oligonucleotide Mixture Reassembly by LCR and PCR. The cleaned oligonucleotides were amplified by PCR with Mly I primers (Sangon Biotech, Shanghai, China, Table S2). PCR reactions were performed using DeepVent polymerase (M0258L, NEB) as described in the Supporting Information Methods. The amplified products were digested with Mly I and then assembled to fragments with LCR. The fragments were then assembled and joined to form full-length sequence by full-length PCR with 18FP-F and 18FP-R primers (Sangon, Shanghai, China, Table S2). The detailed reactions and procedures were described in the Supporting Information Methods.

4.4. Positive Clone Selection and Sequence Analysis. The full-length FP sequences were inserted into the pET28(+) plasmid through sequential XhoI (R0146V, NEB) and BamHI I (R0136T, NEB) digestion, followed by ligation with T4 ligase (M0202L, NEB). Then, the product was expressed in BL21 trxB(DE3) E. coli host cells (Novagen). Clones that survived on kanamycin plates (10 µg/mL) were selected and inoculated into 96-well plates. After thermostatic oscillation incubation for 12 h, 1 mM IPTG was added to induce the expression of FPs. Clones were randomly selected by a random number generator from colored wells for sequencing via the Sanger method, followed by analysis using Bioedit software (version 7.2.6.1). Sequences of these variants and the input templates were used to construct phylogenetic trees using MEGA7 software with neighbor-joining statistical parameters and the evolutionary distances were computed using the Poisson correction method.39 The sequences and detailed information on these five selected FPs have been uploaded to GenBank with the accession number MN729579–MN729583.

4.5. Protein Purification. Proteins were expressed in BL21 trxB(DE3) E. coli host cells (Novagen) at 37 °C, and cells were harvested and resuspended in PBS (0.1 M, pH 7.4) after IPTG (1 mM) induction for 7 h. Then, cell suspensions were on ice and sonicated for 15 min at 40% power with 0.5 s of pulses and 2 s of pauses. The lysates were centrifuged at 12,000 rpm for 20 min and the supernatants were then subjected to Ni2+-chelating Sepharose FF (Amersham Pharmacia Biotech, Freiburg, Germany) for pre-purification. The elution in 150–250 mM imidazole gradient was collected and applied to FPLC (Amersham Pharmacia Biotech, Freiburg, Germany) for further purification. Sample screening were accomplished by passing through the anion exchange resin DEAE Sephadex A-25 (GE Healthcare, 45 × 3 cm in diameter) by equilibration and elution. First, the resin in the column was equilibrated sequentially with 5 times of column volumes of 10 mM Tris–HCl, pH 7.0, 10 times of column volumes of 10 mM Tris–HCl, pH 7.0, 1 M NaCl, and 5 times of column volumes of 10 mM Tris–HCl, pH 7.0. After loading the protein
solution (~10 mL) onto the column, 10 times of bed volumes of 10 mM Tris—HCl, pH 7.0 was applied to wash at a flow rate of 0.6 mL/min and column pressure was set at not more than 0.3 MPa. The purified proteins were analyzed on 12% SDS-PAGE.

4.6. Characterization of Spectra. The purified proteins were subject to spectroscopic analysis. Absorption was measured with a UV—Vis spectrophotometer (lamda 900 UV/Vis/NIR spectrometer, PerkinElmer Instruments) and the concentration of each protein was 0.5 mg/mL (pH 7.4). Fluorescence spectra were detected by an F-4500 spectrofluorometer (Techcomp Ltd., Japan) at room temperature in 10-mm quartz cuvettes and the concentration of each protein was 0.05 mg/mL. Furthermore, a range of proteins and the standard FPs (EGFP and mCherry) were diluted in PBS (pH 7.4) to achieve absorption at excitation within the 0.01—0.05 OD range.

4.7. Photostability Assessment. In vitro photobleaching was measured as in our previous report.40 Purified proteins in mineral oil droplets were faded by an X-Cite 120-W metal halide lamp (Lumen Dynamics) at 100% neutral density. Mineral oil droplets were faded by an X-Cite 120-W metal halide lamp (Lumen Dynamics) at 100% neutral density. The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05299.

4.8. Molecular Weights Characterized by MALDI-TOF-MS. The molecular weights of FP variants were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Applied Biosystems, 4700). Before mass spectrometry, protein samples were concentrated and desalted by Millipore MWCO 5000 (Millipore). Then, the samples were mixed with a saturated energy-absorbing molecule solution at a volume proportion of 1:1. EAM solution consisted of sinapinic acid (148.67 M, Sigma-Aldrich) in 50% acetonitrile (Sigma-Aldrich) and 0.5% trifluoroacetic acid. Finally, the samples (1 μL per spot) were spotted on the MALDI target plate in triplicate and then air-dried at room temperature. Spectra were collected over an m/z range of 9900 to 80,500 in reflector-positive mode. External calibration used insulin (5734.518 Da), cytochrome c (12,360.974 Da), myoglobin (16,952.306 Da), EGFP tagged with 6× histidine (27.8 kDa, GP0172, Genloci Biotech), and BSA (66,218.98 Da, A0281, Sigma-Aldrich). Mass spectra were exported from FlexAnalysis as text files and data were analyzed with Data Explorer software (version 4.3.0., Applied Biosystems), which included spectral mass adjustment, optional smoothing, spectral baseline subtraction, normalization, and peak picking.41

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05299.

Oligonucleotide mixture (OligoMix) harvest and clean-up, OligoMix PCR, fragment assembly by LCR, overlap PCR, full-length PCR; designed oligonucleotides for synthesis; primers used in amplification and reassembly; mutation of the five selected variants; FP identity matrix; characteristics of the 18 template FPs; oligonucleotide amplification and assembly results; and purification of FP variants (PDF)

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Author Contributions
T.S. and X.G. conceived the project and designed the experiments on clones, protein purification, and investigation of spectra; T.S. and T.L. performed and analyzed the experiments on photostability and phylogenetic trees. T.S. and T.L. wrote and revised the manuscript. All the authors made critical revisions of the manuscript and approved the final version.

Notes
The authors declare no competing financial interest.

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