Supplementary Methods

Library Design and Construction

The crystal structure of BFP (PDB: 1BFP)[1] containing Y66H and Y145F mutations was used as the template structure for design. An algorithm for library construction derived from the ORBIT Dead-end elimination (DEE) algorithm[2] was used to guide construction of a twelve-position library (61, 64, 145, 148, 150, 163, 165, 167, 203, 205, 220, 224). This algorithm used DEE over the energetic sum of an ensemble of amino acids at each position to identify an optimum degenerate codon subject to input constraints (TPT, SLM, unpublished data). A library specifying 3.3×10^5 unique variants (DYA TTK KBC CAC DTA RYG TWC ATK RSC KCA MTG RTG) was identified for construction (**Table I**). During library construction a more inclusive codon (RBG) was inadvertently incorporated at position 224 in place of RTG. A set of 44 overlapping oligonucleotides coding for the forward and reverse BFP library sequences, as well as a Shine-Dalgarno sequence and two noncomplementary *Sfi*I sites for cloning was synthesized (Operon). Oligonucleotides coding for the wild-type BFP (GFP+Y66H, Y145F) were used as a control. Oligonucleotides were pooled and assembled using overlapping gene assembly as described[3]. The resulting PCR products were digested with *Sfi*I for 3 hrs at 50 ºC, purified by gel electrophoresis, and ligated overnight at 14 ºC to similarly-digested pBKCm1, a derivative of the pBAD18-Cm vector[4] containing a stuffer fragment flanked by two asymmetric *Sfi*I sequences. The resulting plasmid library was electroporated into *E. coli* MC1061 cells. After one hour incubation in SOC (3 mL), serial dilutions were plated on chloramphenicol (Cm) plates to determine library size, and the library was diluted into 25 mL LB-Cm and allowed to grow to log phase.

Library analysis and sorting using flow cytometry

For screening, a frozen library aliquot of the library was thawed and subcultured 1:100 into 25 mL of media and induced as described above. The cells were scanned and sorted using a FACSAria flow cytometer (BD Biosciences) equipped with a 407 nm diode laser and 450/40 nm and 530/30 nm filters for fluorescence detection, and a 488 nm diode laser for forward and side-scatter detection. Blue fluorescent events were sorted to LB agar plates (2 plates x 100 events/plate) supplemented with Cm and arabinose.

Protein Expression and Purification

Isolated single colonies were grown overnight, and subcultured 1:100 in 5 mL LB-Cm. Cells were grown for 2 hrs at 37 ºC and induced with arabinose (20 % w/v) at a final concentration of 0.2 % for 6 hrs at room temperature. Subsequently, the cells were placed on ice to slow further expression during analysis.

To measure whole-cell fluorescence development at 37 ºC, cells were grown and induced as above and placed in microwell plates in a Safire fluorescence spectrophotometer (Tecan) with shaking at 37 ºC under continued induction. BFP fluorescence was monitored at 383 nm excitation and 450 nm emission, with 2.5 nm excitation and emission slit widths. GFP fluorescence was monitored at 488 nm excitation and 532 nm emission with identical gain and slit widths, and corrected for differences in spectrophotometer detector quantum efficiency using quinine sulfate as a standard[5]. Each

signal was divided by its respective sample absorbance at 600 nm to normalize for cell concentration.

For studies of individual purified BFP variants, selected mutants were re-amplified with a primer containing a C-terminal 6x Histidine tag and ligated into pBKCm1. After expression, clones were centrifuged and lysed using BPER-II Bacterial Protein Extraction Reagent (Pierce). The soluble fraction was collected and purified using Ni-NTA affinity chromatography resin (Qiagen) and dialyzed for 2 hours at 25 ºC and overnight at 4 ºC in 10mM Tris and 10mM EDTA, pH 8.0. Purification was verified with SDS-PAGE and SimplyBlue Coomassie Stain (Invitrogen), and protein concentrations were measured using a bicinchoninic acid (BCA) Assay Kit (Pierce).

Photophysical Characterization of BFP Variants

Fluorescence emission spectra between 400 nm and 600 nm were collected using excitation at 383 nm and 407 nm with a Safire microplate fluorescence spectrophotometer (Tecan). Excitation spectra were collected between 300 nm and 430 nm using emission at 450 nm. Quantum yield and extinction coefficient measurements were performed as described previously,[6] using 1 mM quinine sulfate in 0.1N sulfuric acid as a standard (QY = 0.53)[5].

Fluorescence lifetime measurements were performed using a time-correlated single-photon-counting technique (TCSPC)[5]. 300μM of sample was excited by 383nm laser pulses with a duration of about 120 fs, produced via the second harmonic generation

process from the output of an ultrafast Ti:sapphire laser (Spectraphysics Tsunami). In order to avoid saturation of the chromophore, repetition rate of the excitation pulses was reduced by a home-made acousto-optical pulse picker. The luminescence was dispersed in a monochromator (Acton Research SPC-300) and detected by a micro channel-plate photomultiplier tube (MCP PMT; Hamamatsu R3809U-51). Triggering signal for the correlator board was generated by a fast photodiode illuminated via beamsplitter introduced into the excitation beam. MCP PMT output and triggering signal were connected to an SPC-630 TCSPC board (Becker & Hickl) which performed statistical analysis of the photon flux and restored the fluorescence transients. The instrument response function has FWHM ~ 55ps and is determined by the speed of MCP PMT response. Since the instrument response time is much shorter than the photoluminescence life-time in the materials studied, we did not use a deconvolution procedure to improve the temporal resolution of the system.

Mammalian Cell Expression

Genes having mammalian-optimized codon versions of BFP, BFP(F64L/V163A), and BFP.B3 were assembled, cloned into vector pLNCX[7] using *Hind*III and *Cla*I restriction sites, and verified by DNA sequencing. The vectors were transfected using Lipofectamine 2000 (Invitrogen) into human renal epithelial 293T cells cultured in DMEM with 10% fetal bovine serum. Cells were imaged after two days using a Zeiss Axiovert 25CFL with a BFP filter set (Chroma) and analyzed using MetaMorph software.