Supplementary Methods

Molecular cloning and mutagenesis

Site directed mutagenesis and error prone PCR (EP-PCR) was performed using plasmids encoding mApple as templates. All site-directed mutagenesis was performed using the Quikchange lightning mutagenesis kit (Agilent), with primers designed according to the manufacturers guidelines. EP-PCR products were digested with XhoI and HindIII restriction enzymes and ligated into pBAD/His B vector (Life Technologies) digested with the same two enzymes. The ligation product was used to transform electrocompetent *Escherichia coli* strain DH10B (Life Technologies), which were then plated on agar plates containing LB medium supplemented with 0.4 mg/ml ampicillin and 0.02% w/v L-arabinose. Plasmids were purified with the GeneJET miniprep kit (Thermo Scientific) as per manufacturer's instruction. All DNA sequencing was done using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

Protein purification and characterization

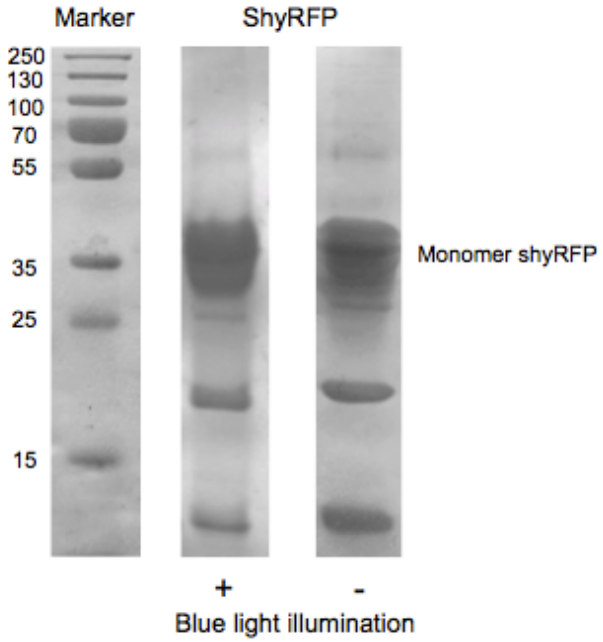
A single colony of *E. coli,* transformed with the pBAD/His B plasmid containing the shyRFP gene, was used to inoculate 4 mL LB supplemented with ampicillin. This culture was incubated at 37° in a shaker incubator (220 rpm) for 12 hours. The liquid culture was then added into 500 mL of LB with ampicillin and incubated a further 4 hours. The inducer L-arabinose was added to a concentration of 0.02% and the culture allowed to grow overnight at 30 °C. The cells were then centrifuged at 8,000 rpm for 10 minutes at 4 °C and resuspended in 1× Tris buffered saline (TBS). The cells were then lysed using a cell disruptor (Constant system). The cell debris was removed by centrifugation at 10,000 rpm for 35 minutes at 4 °C. The protein was then purified using Ni-NTA agarose beads (MC Labs) and buffer exchanged with 1× TBS using a centrifugal device with 10,000 MWCO (Amicon). The shyRFP absorbance profile was obtained by adding 10 μL of purified shyRFP to various pH solutions using the Carmody buffers.[1] Spectra were measured using a UV-Vis spectrometer (Agilent) with the illumination of a 405 nm laser (150 mW, 1200 mW/cm^2, Changchun New Industries Optoelectronics Tech. Co., Ltd.). The photoswitched profile was obtained by unblocking of the laser illumination 30

seconds before each measurement to allow for full activation, and then reblocking of the laser illumination after each measurement to minimize photobleaching of the FP. Activation and decay plots at various temperatures and pHs were obtained by performing a continuous absorbance reading with an integration time of 0.1 s. Light intensity was modified using neutral density filters (Chroma) with the transmittance values 100% (no filter), 10% (ND 1), 5% (ND 1.3), and 3% (ND 1.5). Photoactivation rates were fitted using first order increase equation: $Y = 1 - e^{(-K*X)}$, where Y represents the normalized absorbance change, X represents the time (in seconds), and K is the fitted activation rate. Thermal decay rates were fitted using first order decay equation: $Y = e^{(-K*X)}$, where Y represents the normalized absorbance, X represents the time (in seconds), and K is the fitted decay rate.

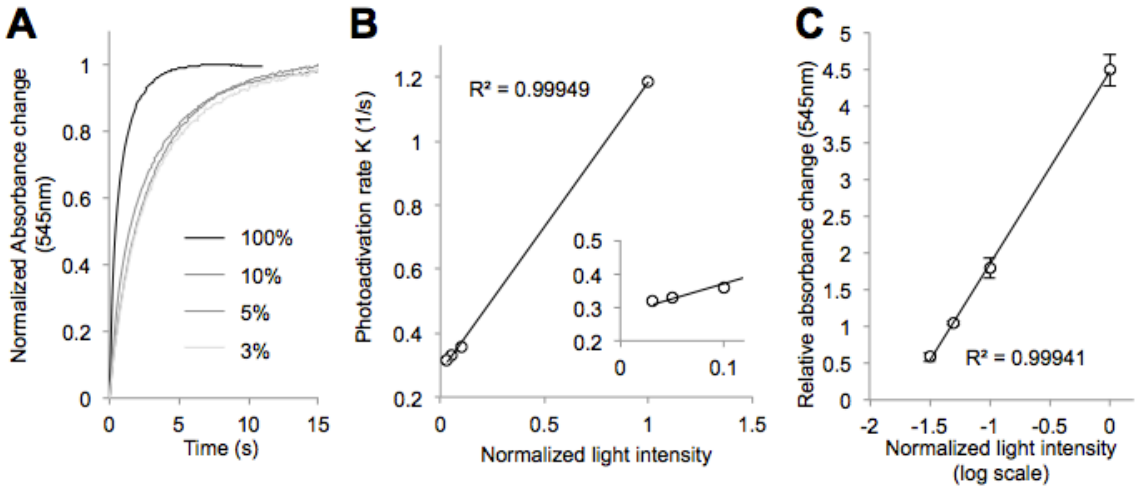
Extinction coefficients were determined by measuring the absorption spectrum of shyRFP using an UV/Vis spectrometer (Beckman Coulter DU 800) at pH 7.25 in 1× TBS and the same concentration of shyRFP in 1 M NaOH. In 1 M NaOH solution, the FP chromophore is assumed to have an extinction coefficient of 44,000 $M^{-1}cm^{-1}$ at 446 nm.[2] Through the Beer-Lambert law this gives a concentration of the alkaline denatured chromophore that can be applied to the pH 7.5 fluorescent protein, allowing the calculation of the yellow state extinction coefficient. The red state extinction coefficient was measured from the absorbance of the shyRFP solution under active 405 nm laser illumination. We assumed that the decrease in yellow state concentration corresponded to the increase in red state concentration. Accordingly, the ratio of yellow state to red state extinction coefficients could be calculated from the relative changes in yellow state and red state absorbance.

Supplementary Figures

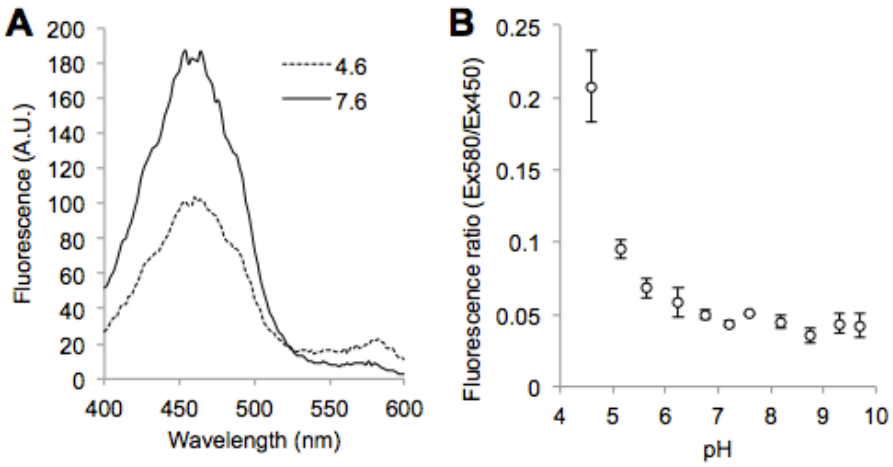
Supplementary Fig. S1. Sequence alignment between shyRFP and mApple. Mutated residues are highlighted with a pink background colour.



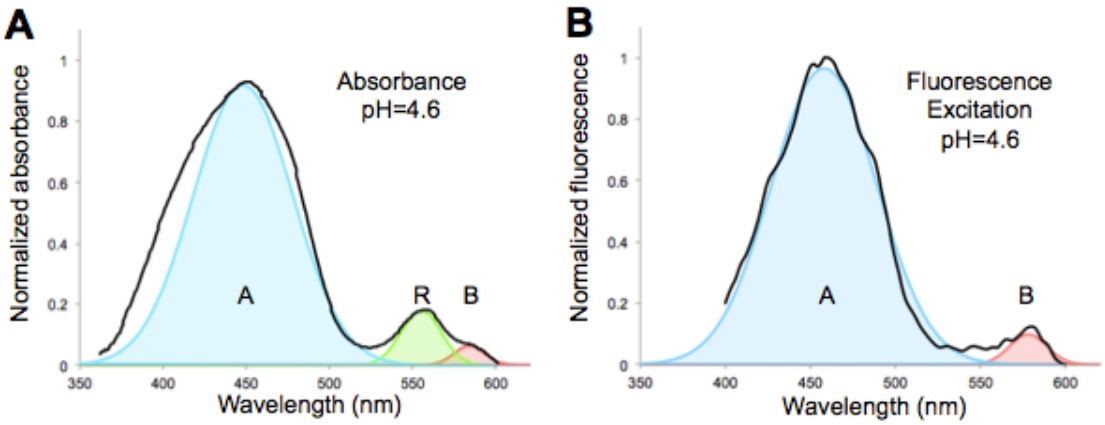
Supplementary Fig. S2. SDS-PAGE analysis of shyRFP under blue light illumination and dark conditions. The lower bands are consistent with the expected ~19 kDa and ~7 kDa fragments produced by hydrolysis of the chromophore imine linkage in RFPs.[2, 3]



Supplementary Fig. S3. Light intensity dependence of photoactivation rate and relative absorbance change. A, photoactivation traces (545 nm absorbance) under different intensities of laser illumination. B, photoactivation rates are linearly correlated with the light intensity. B inset, zoom-in for the first 3 data points in B. C, relative absorbance increase $((A-A_0)/(A_0))$ at 545 nm after illumination under different light intensities.



Supplementary Fig. S4. Fluorescence excitation change at different pH. A, fluorescence excitation spectrum of shyRFP at low pH (4.6) and at neutral pH (7.6). B, excitation ratio (580 nm/450 nm) as a function of pH.



Supplementary Fig. S5. Absorbance and fluorescence excitation at low pH (pH = 4.6). The three distinct species are indicated using solid coloured curves.

References:

1 W. R. Carmody, *J. Chem. Educ.*, 1961, **38**, 559.

2 L. A. Gross, G. S. Baird, R. C. Hoffman, K. K. Baldridge and R. Y. Tsien, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11990.

3 R. E. Campbell, O. Tour, A. E. Palmer, P. A. Steinbach, G. S. Baird, D. A. Zacharias and R. Y. Tsien, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 7877.