SUPPLEMENTARY MATERIAL

Table 1S Rationale for design of the synthetic gene library.

Residue number	Mutation	Codon[a]	Rationale
His42	His, Asn, Gln, Lys	MAS	Mutations beneficial to tetrameric DsRed variants [1]. Not conserved in coral CFPs.
Leu44	Leu, Val, Ala, Pro	SYC	Mutations beneficial to tetrameric DsRed variants [1]. Not conserved in coral CFPs.
Gln66 (residue of chromophore)	Gln, Lys, Met, Leu	MWG	Mutations beneficial to *Aequorea* GFP [2] and monomeric RFPs [3]. Lys or Gln present in coral CFPs.
Arg70	Lys, Arg	ARG	Mutations beneficial to monomeric RFPs [4]. Lys or Arg present in coral CFPs.
Ala71	Ala,Val	GYC	Mutations beneficial to monomeric RFPs [4]. Ala or Cys present in coral CFPs.
Leu72	Leu, Phe, Ile	HTC	Phe in 2 coral CFPs and Leu in 1 coral CFP.
Phe83	Leu, Phe, Ile	HTC	Mutations beneficial to monomeric RFPs [3, 4]. Phe in 2 coral CFPs and Leu in 1 coral CFP.
Ile104	Ile,Thr	AYC	May disrupt A-B interface interactions
Phe124	Leu, Phe, Ile	HTC	Mutations beneficial to dimeric RFPs [4]. Strict conservation in coral CFPs.
Asp125	Lys,Arg	ARG	Likely to disrupt A-B interface interactions.
Met127	Lys, Arg	ARG	Likely to disrupt A-B interface interactions.
Met150	Met, Leu	MTG	Mutations beneficial to monomeric RFPs [4]. Met in 2 coral CFPs and Leu in 1 coral CFP.
His163	His, Gln	CAS	Mutations beneficial to dimeric and monomeric RFPs [3, 4]. His in 2 coral CFPs and Ala in 1 coral CFP.
Ser179	Ser, Thr	WCC	Mutations beneficial to dimeric RFPs [4]. Thr in 2 coral CFPs and Ser in 1 coral CFP.

[a]Single letter codes for bases are as follows: A = adenosine, C = cytidine, G = guanosine, T = thymidine, H = A or C or T, M = A or C, R = A or G, S = C or G, W = A or T, and Y = C or T.

Table 2S Mutations in dimeric and monomeric TFP variants.

Variant	Library construction strategies	Mutations
dTFP0.1	See Supplementary Table 1.	Inside: H42N, L44V, L72F, F124L, M150L, S179T A-B interface: D125K, M127K
dTFP0.2	2 generations of random mutagenesis.	Inside: D81N Outside: S226P
mTFP0.3	Site-directed mutagenesis at 162 and 164. Saturation mutagenesis at 163.	A-C interface: S162K, S164K
mTFP0.4	Saturation mutagenesis at 66. Semi-saturation mutagenesis at 175.	Inside: Q66C, C175V
mTFP0.5	3 generations of random mutagenesis.	Inside: S62T, C66G Outside: A80P, N216S A-B interface: K127E, K182R
mTFP0.6	Saturation mutagenesis at 66+163, 66+197. Semi-saturation mutagenesis at 66+147, 66+213.	Inside: G66A, L213V Outside: S2N Replace 223-228 with TG
mTFP0.7	2 generations of random mutagenesis.	Inside: V44I, Y173H Outside: V186A A-B interface: R123H Mutate: N2S
mTFP0.8	Semi-saturation mutagenesis at 62, 63, 64, 65, and 66 with screening for photostability.	Inside: N63T
mTFP0.9	Semi-saturation mutagenesis at 142, 144, 145, 149, 150, and 161 with screening for photostability.	Inside: L150M (reversion to wt), I161V Outside: K142G A-C interface: E144D, P145A, I149R
mTFP1	2 generations of random mutagenesis with screening for photostability.	Outside: L141T, V158K, Y221N, G224D

Table 3S Summary of crystallographic statistics.

Statistic	mTFP1
Total reflections	842,305
Unique reflections	65,393
Cell dimensions (a, b, c), Å	89.83, 38.02, 61.12
Resolution, Å	35 - 1.19
Highest resolution shell, Å	1.22 - 1.19
Completeness, * %	98.0 (97.4)
Average I/σ *	26.2 (3.0)
R_{merge} *†	0.034 (0.364)
R_{work} ‡	0.137
R_{free}	0.207
R factor (all data)	0.149
Average *B* factors, Å^2	23.2
Protein atoms	20.6
Solvent	37.3
Rmsd bond lengths, Å	0.014
Rmsd bond angles, °	0.031

*Values in parentheses indicate statistics for the highest-resolution shell.

†$R_{merge} = \sum_i\sum_j(I_{ij} - \langle I\rangle_i)/\sum_i\sum_j\langle I\rangle_I$, where I_{ij} is the amplitude of the *j*th observation of reflection *i* and $\langle I\rangle_I$ is the mean value of observations I_{ij}.

‡R factor = $\sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure amplitudes.

Table 4S Experimentally determined relative fluorescence intensities for identical concentrations of mTFP1, mCerulean, and mCitrine imaged on an epi-fluorescence microscope with xenon arc lamp illumination.

Protein	Excitation filter[a]	Beamsplitter[a]	Intensity relative to mCerulean imaged with a HQ436/20 excitation filter and D480/40 emission filter[a]	
			D480/40	HQ495/30
mTFP1	D436/20	455DCLP	1.3	1.5
	HQ445/30	470DCXR	ND[b]	2.6
mCerulean	D436/20	455DCLP	1.0	0.8
	HQ445/30	470DCXR	ND[b]	1.3

Protein	Excitation filter[a]	Beamsplitter[a]	TFP or CFP emission filter[a]	Relative intensity passed by YFP emission filter[a,c]	
				HQ535/30	HQ545/30
mTFP1	D436/20	455DCLP	HQ495/30	0.39	0.30
	HQ445/30	470DCXR	HQ495/30	0.39	0.30
mCerulean	D436/20	455DCLP	D480/40	0.35	0.30
	HQ445/30	470DCXR	HQ495/30	0.41	0.34
mCitrine	HQ500/20	Q515LP	ND[b]	1	0.91
	D436/20	455DCLP	ND[b]	0.12	0.07
	HQ445/30	470DCXR	ND[b]	0.15	0.09

[a]All filters and beamsplitters were purchased from Chroma Technology Corp. Filters are designated with Chroma part numbers. [b]Not determined. [c]For mTFP1 and mCerulean, intensities are relative to the intensity in the indicated TFP or CFP emission channel. For mCitrine, all intensities are relative to the intensity obtained with a HQ500/20 excitation filter and HQ535/30 emission filter.

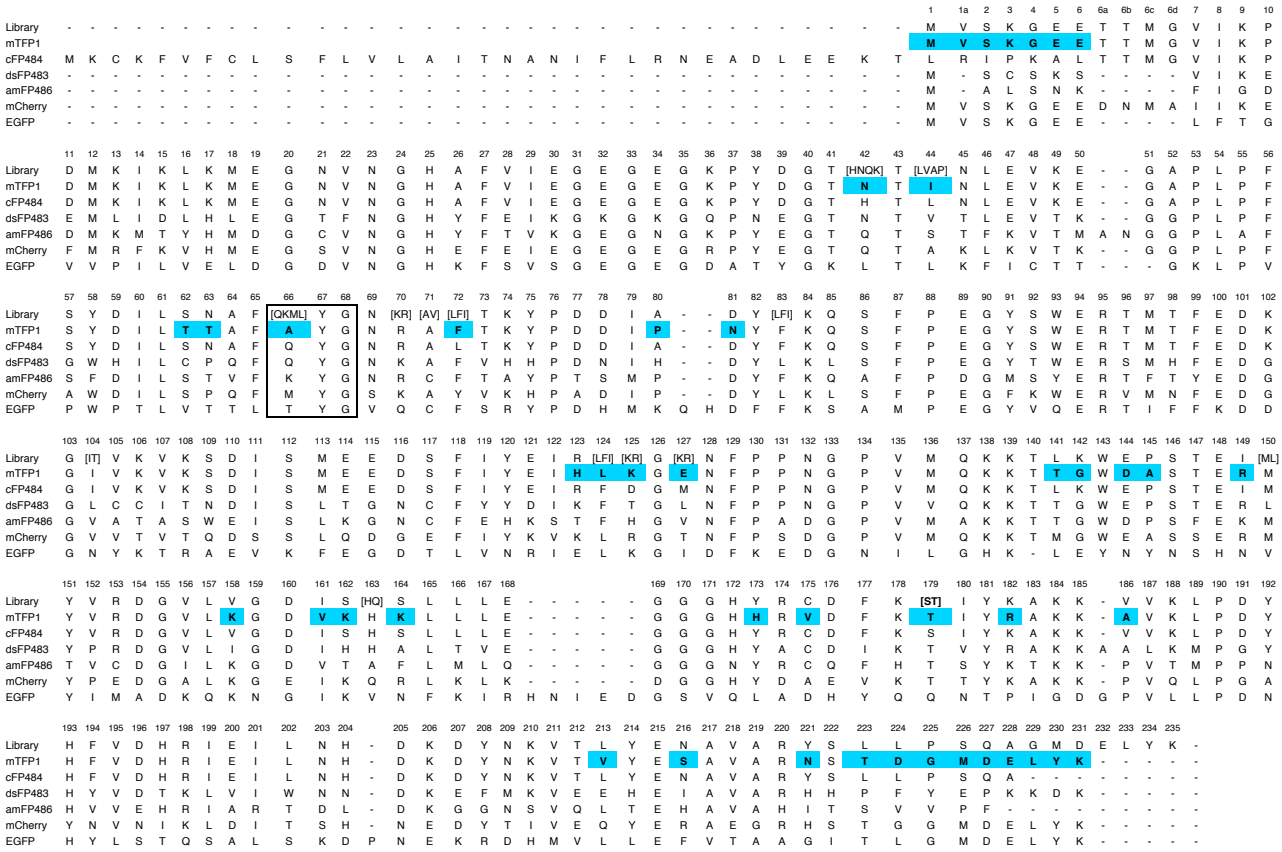

Figure 1S Protein sequence alignments of mTFP1, coral CFPs, and homologues.

The aligned sequences are the designed synthetic gene library, mTFP1, cFP484 from *Clavularia sp*. (Genbank accession AAF03374), dsFP483 from *Discosoma striata* (Genbank accession AAF03370), amFP486 from *Anemonia majano* (Genbank accession Q9U6Y6), mCherry derived from *Discosoma striata* dsFP583 (Genbank accession AAV52164), and EGFP derived from *Aequorea* GFP (from Clontech vector pEGFP) [3, 5]. Residues shaded cyan are changes or point mutations that are present in mTFP1. Residues 66, 67, and 68 (enclosed in black box) are the precursors of the chromophore. The rationale behind the design of the gene library is summarized in Supplementary Table 1S. Numbering is consistent with DsRed and its monomeric variants that have no internal insertions or deletions relative to cFP484.

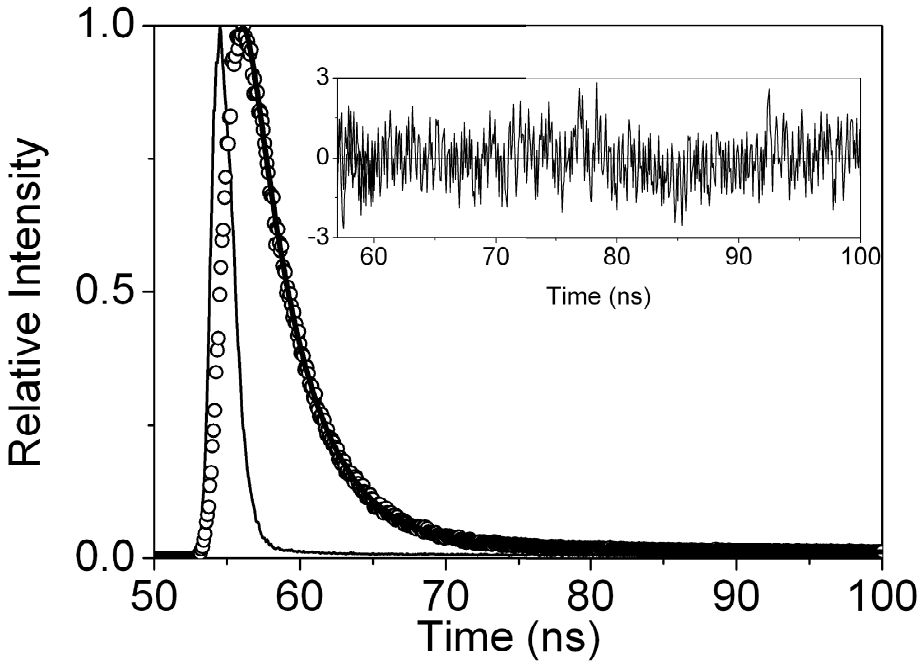

Figure 2S Fluorescence lifetime decay of mTFP1.

Shown is the experimental data for the lifetime decay (○) of 5 nM mTFP1 in buffer fit with a single exponential decay (thick black line) convolved with the instrument response function (thin black line). The inset is the residuals of the best single exponential fit for mTFP1 (τ = 3.2 ns, χ^2 = 1.0).

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