A noncytotoxic DsRed variant for whole-cell labeling

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A common application of fluorescent proteins is to label whole cells, but many RFPs are cytotoxic when used with standard high-level expression systems. We engineered a rapidly maturing tetrameric fluorescent protein called DsRed-Express2 that has minimal cytotoxicity. DsRed-Express2 exhibits strong and stable expression in bacterial and mammalian cells, and it outperforms other available RFPs with regard to photostability and phototoxicity.

Fluorescent proteins can be used as whole-cell labels or as fusion tags[1]. Enhanced GFP (EGFP) and its monomeric derivative mEGFP are suitable for either purpose. Oligomeric RFPs have been engineered as whole-cell labels. For example, DsRed was modified to create DsRed-Express[2]; eqFP578 was modified to create TurboRFP[3] and Katushka[4]; and eqFP611 was modified to create RFP611 (ref. 5). Monomeric RFPs have also been engineered. For example, DsRed-Express was modified to create DsRed-Monomer[6] as well as mCherry and tdTomato[7]; Kusabira-Orange was modified to generate mKO2 (ref. 8); TurboRFP was modified to generate TagRFP[3] and TagRFP-S158T[9]; and Katushka was modified to generate mKate[4].

Despite this abundance of options, anecdotal data suggest that the use of RFPs as whole-cell labels has been limited by cytotoxicity. Whole-cell labeling typically employs strong promoters, and cells containing high levels of fluorescent proteins may have growth defects and/or instability of the fluorescent protein markers[10,11]. We therefore engineered an improved RFP for whole-cell labeling.

We first evaluated cytotoxicity for mEGFP and 11 RFPs (**Supplementary Results** online). During constitutive high-level expression in *Escherichia coli*, mEGFP was well tolerated, but the RFPs showed moderate to severe cytotoxicity as judged by colony size (**Supplementary Fig. 1** online). To assess cytotoxicity in HeLa cells, we expressed fluorescent proteins by transient transfection with a plasmid containing the strong *CMV* promoter and analyzed viable fluorescent cells by flow cytometry daily after transfection. For mEGFP, average cellular fluorescence remained nearly constant from 24 to 120 h (**Fig. 1a**). In contrast, average fluorescence of all of the RFPs progressively declined (**Fig. 1a**) because of preferential loss of the most highly expressing cells (**Supplementary Fig. 2** online). Expression of RFPs caused many cells to detach from the growth surface (data not shown), further indicating cytotoxicity.

To generate a noncytotoxic RFP, we modified an existing protein that showed fast maturation and high photostability. Among the 11 RFPs tested, DsRed-Express, Katushka and mCherry matured the fastest (**Supplementary Table 1** and **Supplementary Fig. 3a** online). We assessed photostability using a simple assay involving illumination through an epifluorescence microscope at a fixed light intensity (**Supplementary Fig. 3b** and **Supplementary Methods** online) because this configuration mimics the typical experience for many researchers. DsRed-Express was by far the most photostable of the RFPs (**Supplementary Table 1**). We therefore chose DsRed-Express as the starting point.

The cytotoxicity of DsRed-Express may stem from aggregation[11]. We measured aggregation using an assay in which bacteria expressing a fluorescent protein were lysed and centrifuged[2]. With DsRed-Express, >50% of the fluorescence was in the pellet (**Fig. 1b**). We used directed evolution of the DsRed-Express protein surface to reduce higher-order aggregation as measured by the bacterial extraction assay (**Supplementary Results**, **Supplementary Figs. 4** and **5**, and **Supplementary Tables 2–5** online). One mutation boosted solubility but nullified a change that had increased bacterial expression, requiring re-optimization of bacterial expression (**Supplementary Results**). The final resulting protein, DsRed-Express2, retained the favorable fluorescence properties of DsRed-Express but was as soluble as mEGFP during bacterial extraction (**Fig. 1b**).

DsRed-Express2 is not as bright as wild-type DsRed, so we mutagenized it to create DsRed-Max, which was ~30% brighter than DsRed-Express2 and retained high solubility (**Fig. 1b** and **Supplementary Results**). DsRed-Max had lower green emission than DsRed-Express2 (**Supplementary Fig. 6** online), but was slower to mature and more photolabile (**Supplementary Table 1** and **Supplementary Fig. 7** online). DsRed-Max should be useful when brightness and/or pure red emission are most important.

E. coli cells constitutively expressing DsRed-Express2 or DsRed-Max produced large colonies similar to those obtained with mEGFP (**Fig. 1c**). Transiently transfected HeLa cells expressing DsRed-Express2 or DsRed-Max maintained nearly constant average fluorescence (**Fig. 1d**), and the highly expressing cells remained

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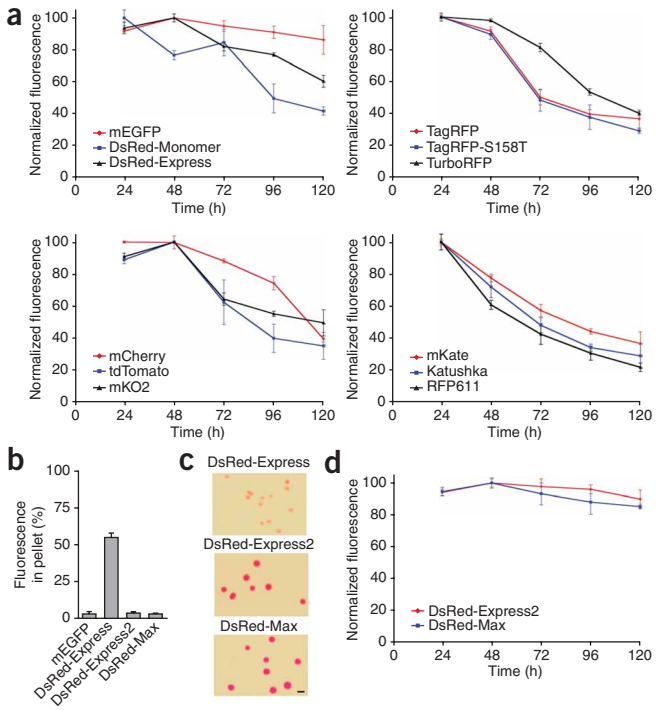

Figure 1 | Cytotoxicity of RFPs. (**a**) To measure RFP cytotoxicity in mammalian cells, HeLa cells were transiently transfected in 6-well plates for constitutive high-level expression of the indicated fluorescent proteins. Daily, three wells for each fluorescent protein were analyzed by flow cytometry to measure the average signal from the viable fluorescent cells. The strongest signal obtained for a given fluorescent protein was defined as 100 units. For clarity, the results are shown in four separate panels. (**b**) To assay higher-order aggregation, for each indicated fluorescent protein, percent fluorescence in the pellet from a bacterial lysate was measured for 8 independent replicates. The signal with mEGFP represents background in the assay. (**c**) To measure cytotoxicity in bacteria, *E. coli* cells were transformed with plasmids for constitutive expression of DsRed-Express, DsRed-Express2 or DsRed-Max and were grown overnight on adjacent sectors of a Petri dish. Equally sized representative areas are shown. Scale bar, 1 mm. (**d**) To measure cytotoxicity of DsRed-Express2 and DsRed-Max in mammalian cells, HeLa cells were transiently transfected for expression of these two proteins and analyzed as in **a**. Error bars represent s.e.m.

viable (**Supplementary Fig. 2**). Thus, with both bacterial and mammalian plasmid expression vectors, DsRed-Express2 and DsRed-Max are tolerated better than any of the other RFPs tested.

For mammalian cells, an alternative to plasmid-based expression is retroviral transduction, which yields long-term expression at moderately high levels[12]. We used a commercial lentiviral system to compare DsRed-Express2 with five other RFPs and mEGFP. We transduced HeLa cells using the same lentiviral titer for each fluorescent protein and then analyzed viable fluorescent cells by flow cytometry after 3 and 10 d. At day 3, the fluorescence signal over background was stronger for DsRed-Express2 than for any other RFP (**Fig. 2a**). At day 10, the average fluorescence intensity was nearly unchanged for mEGFP and DsRed-Express2, but had dropped substantially for the other fluorescent proteins, presumably owing to a loss of the most highly expressing cells. This interpretation was supported by counting the percentage of viable cells that were fluorescent. Between days 3 and 10, this percentage was nearly unchanged for mEGFP and DsRed-Express2 but dropped substantially for the other fluorescent proteins (**Fig. 2b**). Even at day 3, we observed relatively few fluorescent cells with DsRed-Monomer, TagRFP and TurboRFP, possibly reflecting cytotoxicity at early stages of expression. In a separate experiment, lentivirally transduced HeLa cells containing DsRed-Express showed a lag in growth compared to cells containing DsRed-Express2 or mEGFP (**Fig. 2c**).

Next we tested DsRed-Express2 in a context where fluorescent protein cytotoxicity had previously hindered an experimental analysis. A suitable system was suggested by a report[11] in which mouse bone marrow cells had been retrovirally transduced to express either EGFP or DsRed-Express, and then grown *in vitro* under culture conditions that favored the preservation and growth of hematopoietic stem and progenitor cells. The cells containing EGFP grew robustly whereas those containing DsRed-Express did not. To test DsRed-Express2 in this system we transduced mouse

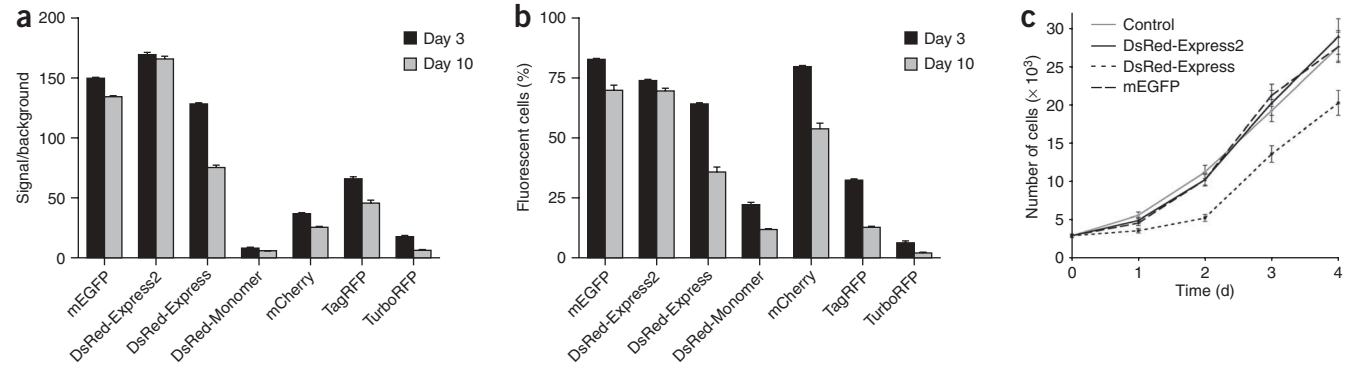

Figure 2 | Fluorescent protein cytotoxicity after lentiviral transduction. (**a**) HeLa cells were transduced with lentiviruses encoding the indicated fluorescent proteins or with a control lentivirus lacking a fluorescent protein gene. At 3 and 10 d after transduction, three wells for each fluorescent protein were analyzed by flow cytometry. Fluorescence from viable cells was detected using a 488-nm laser and a FITC filter set (mEGFP) or a 543-nm laser and a phycoerythrin (PE) filter set (RFPs). Plotted are the average fluorescence signals relative to the control lentivirus background. (**b**) The data presented in **a** were used to determine the percentage of viable cells that were fluorescent. (**c**) HeLa cells were lentivirally transduced to express the indicated fluorescent proteins. At 3 d after transduction, fluorescent cells were sorted and grown in culture, in parallel with unsorted cells that had been transduced with a control lentivirus lacking a fluorescent protein gene. The number of cells was counted by microscopy daily. Three wells were analyzed for each data point. Error bars indicate s.e.m.

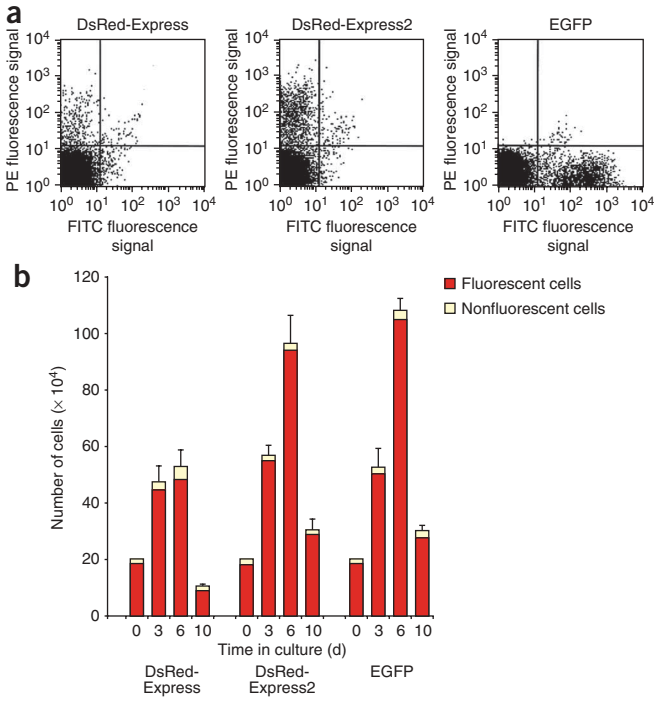

Figure 3 | Robust growth of mouse bone marrow hematopoietic stem and progenitor cells expressing DsRed-Express2. (**a**) Mononuclear bone marrow cells were transduced with retroviral vectors encoding DsRed-Express, DsRed-Express2 or EGFP, and fluorescent cells were sorted after 87 h. Red and green fluorescence signals were detected using PE and FITC filter sets, respectively. The lines represent gates defined by analyzing untransduced cells. (**b**) Sorted cells from **a** were cultured under conditions favoring preservation and growth of hematopoietic stem cells, and the cultures were analyzed by flow cytometry after 3, 6 and 10 d. Three wells were analyzed for each data point. Error bars represent s.d. The decrease in total cell number at day 10 is not fluorescent protein–related but reflects senescence that is routinely observed under *in vitro* culture conditions[11].

bone marrow cells with DsRed-Express–, DsRed-Express2– or EGFP-expressing lentiviral vectors. We collected viable fluorescent cells by flow cytometry at 87 h after transduction (day 0) and then started individual cultures with 20,000 cells. For each culture we recorded the total number of viable cells and the number of fluorescent cells after 3, 6 and 10 d of growth. At day 0, the cells containing DsRed-Express2 were more abundant and were on average twice as bright as those containing DsRed-Express (**Fig. 3a**). The cells containing DsRed-Express2 proliferated to the same degree as those containing EGFP, but the cells containing DsRed-Express proliferated much less (**Fig. 3b**). These data are similar to the results with HeLa cells. We conclude that DsRed-Express2 is minimally cytotoxic in a variety of cell types.

DsRed-Express2 has additional advantages. It is excited efficiently by both blue and green lasers that are routinely used in flow cytometers. In bacteria, DsRed-Express2 is expressed from the endogenous start codon much more strongly than DsRed-Express (**Supplementary Fig. 4**). Another important property is phototoxicity, which has only rarely been measured for engineered fluorescent proteins. Our results indicate that DsRed-Express2 has low phototoxicity (**Supplementary Results** and **Supplementary Fig. 8** online). Thus, for whole-cell labeling, DsRed-Express2 is the RFP of choice.

DsRed-Express2 is likely to be well-tolerated in transgenic organisms. Although the previously available RFPs have been used successfully to generate fluorescent animals[4,13,14], those findings probably reflect a threshold effect in which a cytotoxic fluorescent protein is tolerated up to a certain concentration. We suspect that the transgenes in earlier studies were expressed at relatively low levels and may sometimes have caused residual cytotoxicity. DsRed-Express2 should facilitate the production of healthy transgenic organisms with strong and stable red fluorescence. Similar improvements can be expected when fluorescent cells are transplanted into animal hosts[15]. For example, when retrovirally transduced mouse hematopoietic stem cells had been transplanted into mice, cells expressing EGFP were maintained, but those expressing DsRed-Express were progressively lost[11]. This result suggests that cytotoxicity in cultured cells can predict the behavior of a fluorescent protein *in vivo*.

Because DsRed-Express2 and DsRed-Max are tetramers, they are best suited to labeling whole cells rather than making fusion proteins. Future modifications of the chromophore environment of these fluorescent proteins should enable us to create additional noncytotoxic whole-cell labels.

Sequences were deposited in GenBank for DsRed-Express2 (FJ226077) and DsRed-Max (FJ226078). The crystal structure of DsRed-Max was solved and deposited in the Protein Data Bank (2V4E).

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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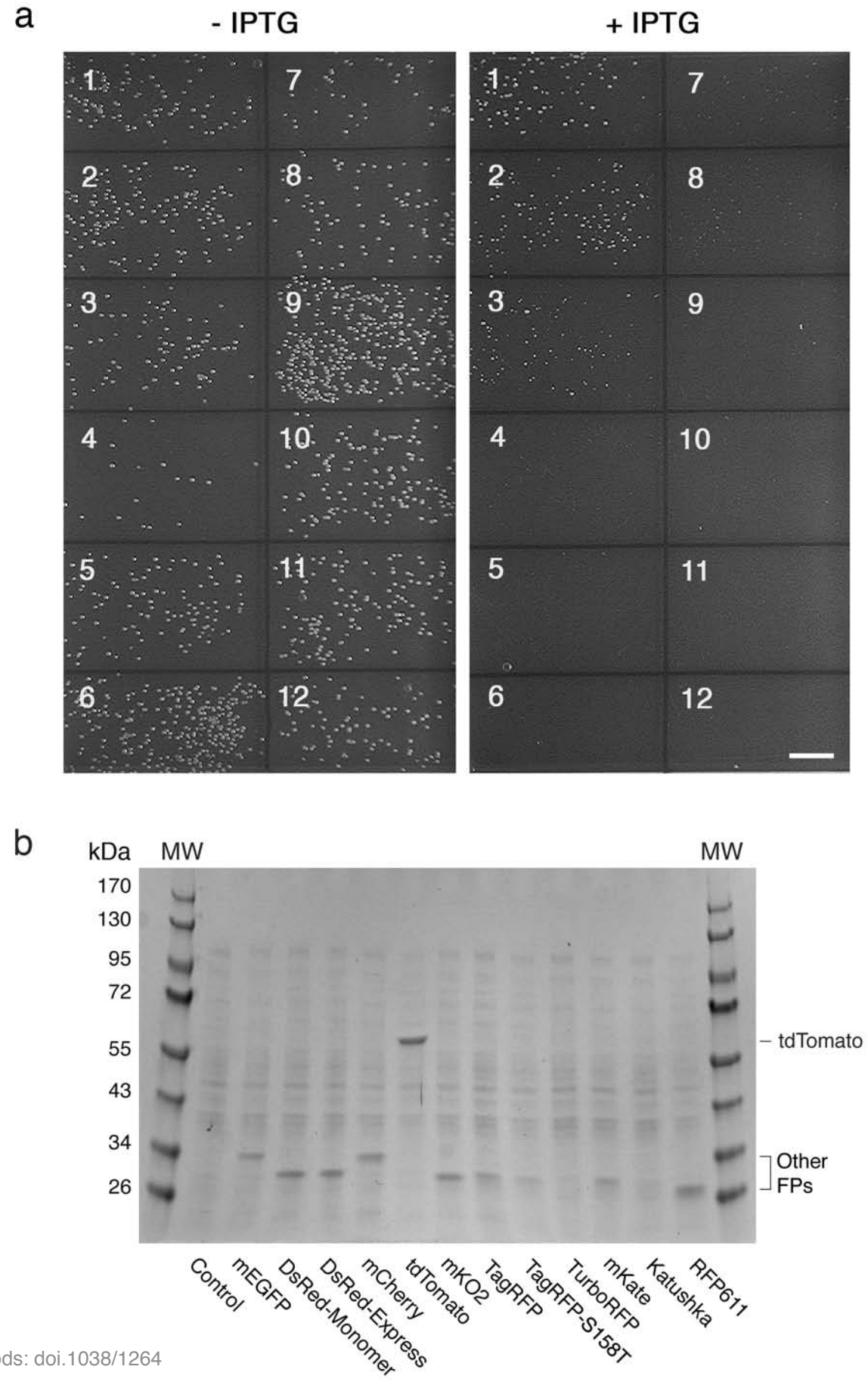
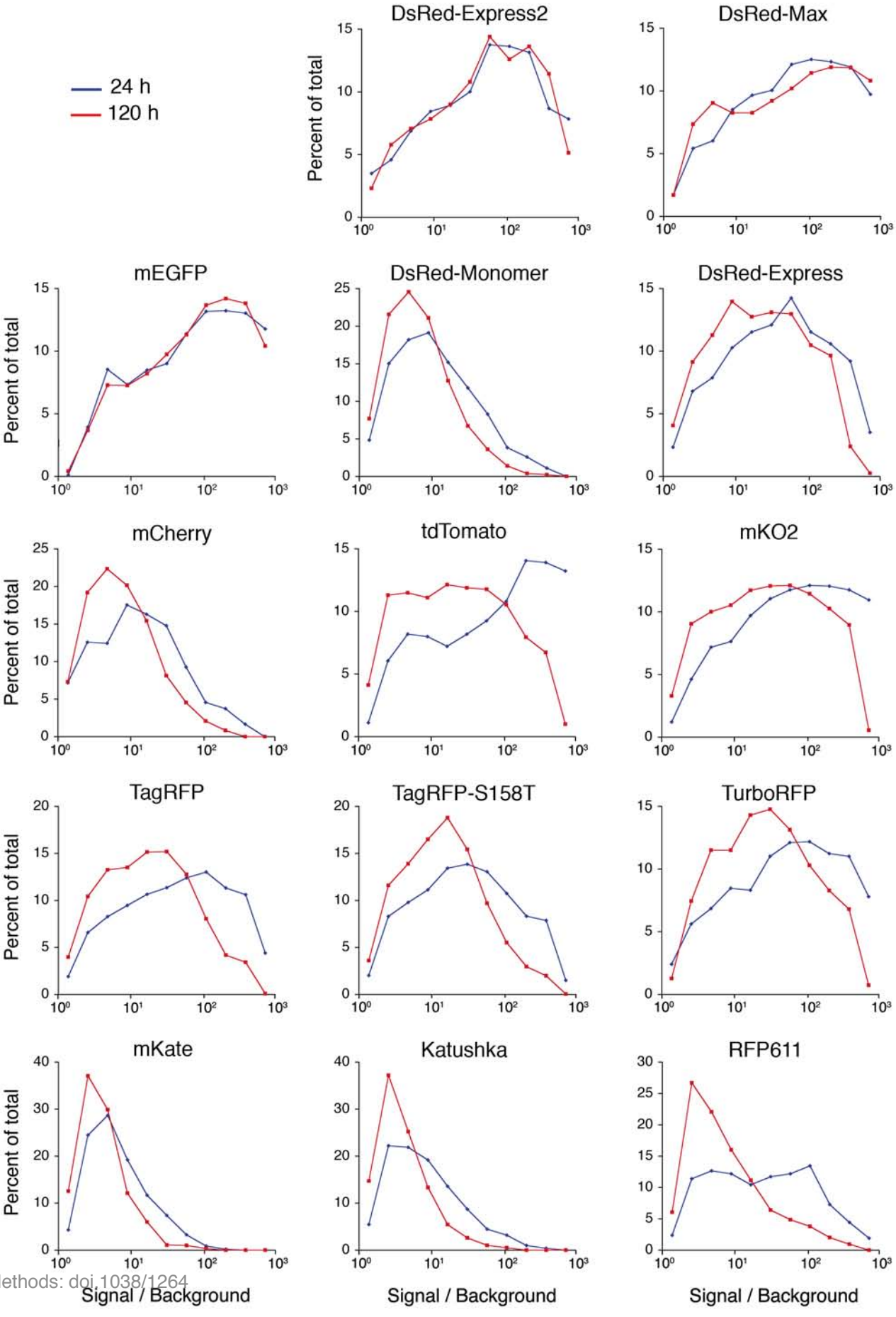
Supplementary Figure 1 | High-level expression of red fluorescent proteins is toxic to bacteria.



E. coli DH10B cells containing the pREP4 repressor plasmid were transformed with pQE-60NA encoding the following fluorescent proteins. 1: mEGFP. 2: DsRed-Monomer. 3: DsRed-Express (modified as described in **Supplementary Results** to enhance bacterial expression). 4: mCherry. 5: tdTomato. 6: mKO2. 7: TagRFP. 8: TagRFP-S158T. 9: TurboRFP. 10: mKate. 11: Katushka. 12: RFP611. (**a**) Cytotoxicity assay. Half of each transformation mixture was plated under repressing conditions in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG), the other half under derepressing conditions in the presence of IPTG. Scale bar, 1 cm. (**b**) Analysis of fluorescent protein expression under derepressing conditions. Cells were grown to an OD_{600} of ~0.5 and then treated with IPTG for 4 h. Whole-cell lysates were separated by SDS-PAGE, and proteins were visualized with Coomassie Blue. Control, cells transformed with empty pQE-60NA; MW, molecular weight standard. Numbers represent marker sizes in kDa.

Supplementary Figure 2 | Fluorescence intensity distributions of HeLa cells transiently transfected to express fluorescent proteins.



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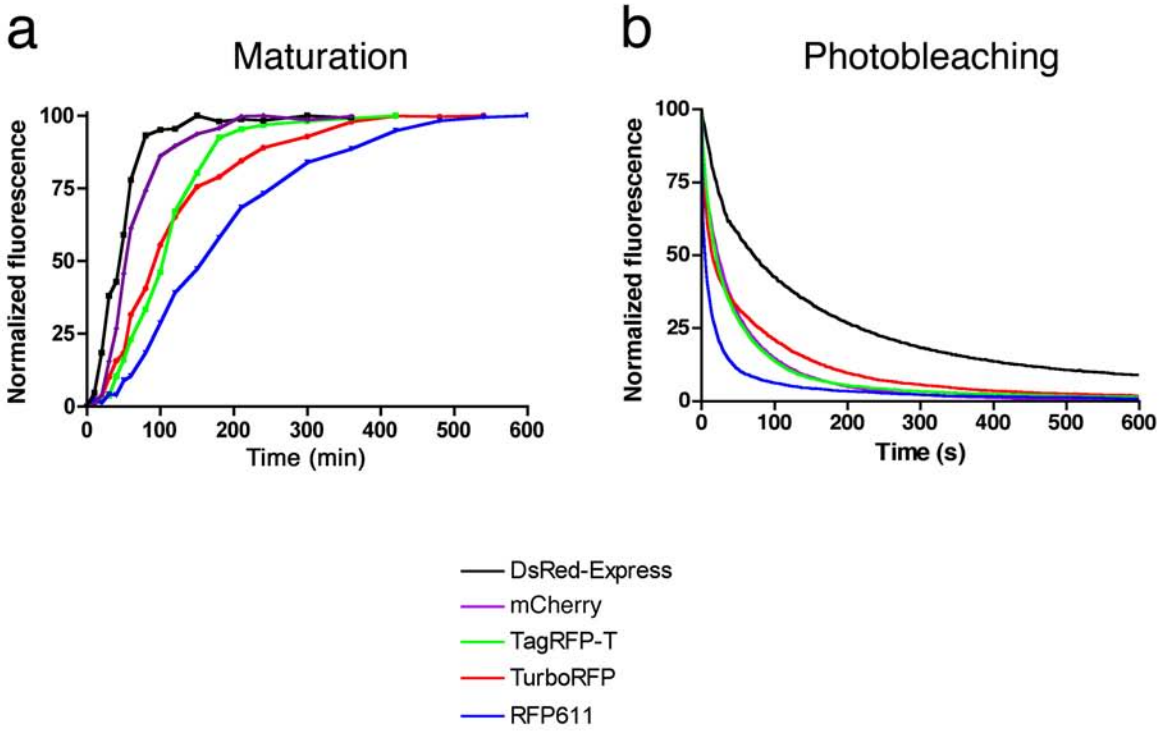
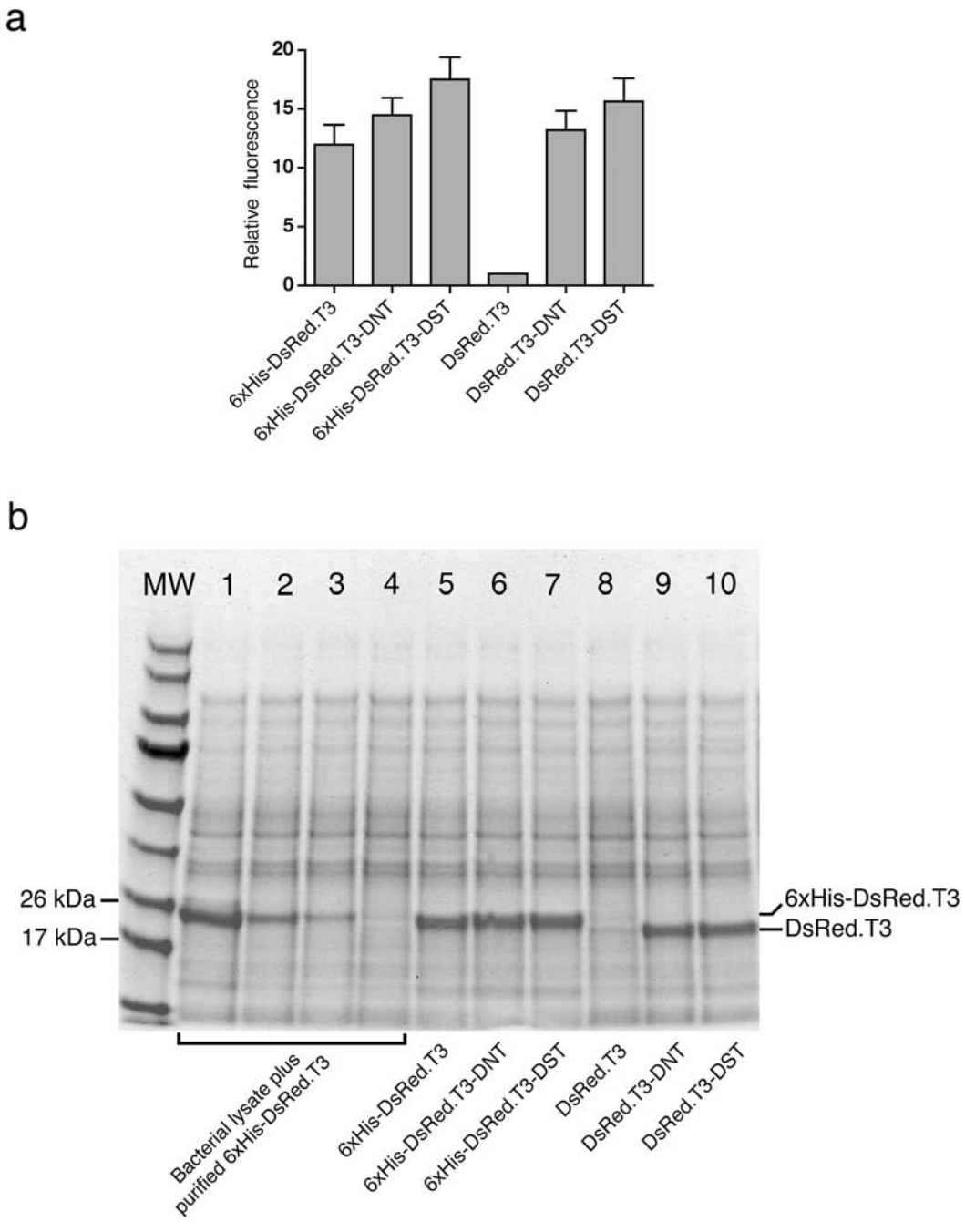
Data are from the experiment shown in **Fig. 1a** and **Fig. 1d**. The horizontal axis indicates cellular fluorescence intensity relative to background. Each data point is a binned value representing the percent of the viable fluorescent cells giving the indicated fluorescence signal at 24 h (blue) or 120 h (red) after transfection.

Supplementary Figure 3 | Fluorescence maturation and photobleaching kinetics of five representative red fluorescent proteins.



The legend is applicable to both parts of the figure. (**a**) Kinetics of chromophore maturation at 37 °C in *E. coli*. Dots represent mean values of three independent samples. (**b**) Photobleaching in *E. coli* during constant epifluorescence illumination through a Texas Red filter. Fluorescence signals were recorded every 2 sec for 10 min. Lines represent average fluorescence values for three independent samples, and were normalized to 100% at time zero.

Supplementary Figure 4 | Substitutions in codons 2-4 increase DsRed expression in *E. coli*.

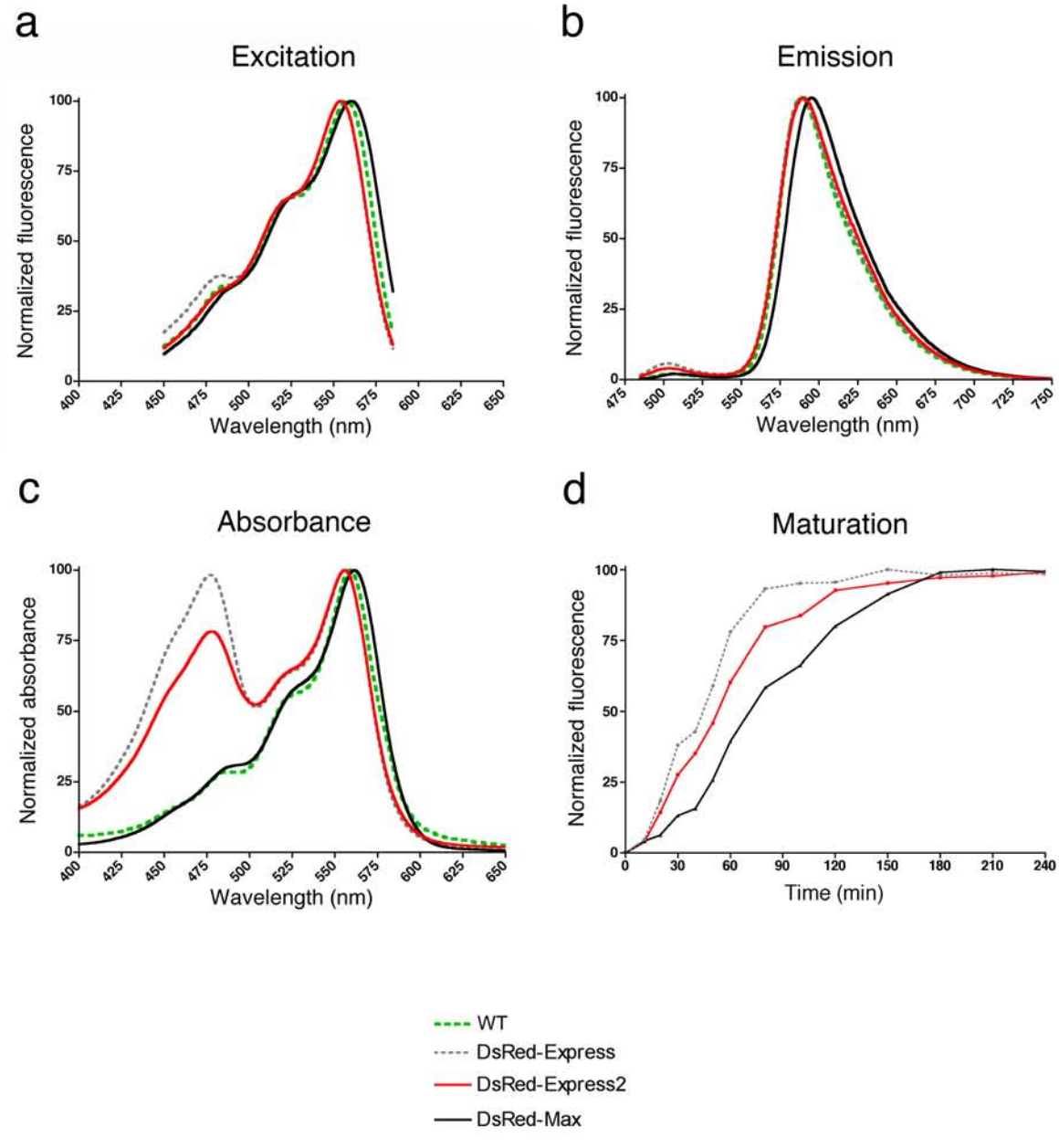


(**a**) The expression levels of DsRed.T3 variants as indicated by whole-cell fluorescence. Bars represent the fluorescence signal relative to DsRed.T3. Data are shown as mean with s.e.m. for three independent experiments. (**b**) The expression levels of DsRed.T3 variants as indicated by SDS-PAGE. Whole-cell lysates from the cells used in part (a) were normalized according to OD_{600} and separated by SDS-PAGE, and proteins were visualized with Coomassie Blue. Lanes 1-4 show control cell lysates supplemented with 2, 1, 0.5, or 0 μg purified hexahistidine-tagged DsRed.T3 (6xHis-DsRed.T3), respectively. Lanes 5-7 show hexahistidine-tagged DsRed.T3, DsRed.T3-DNT, and DsRed.T3-DST, respectively. Lanes 8-10 show untagged DsRed.T3, DsRed.T3-DNT, and DsRed.T3-DST, respectively. MW, molecular weight standard.

Supplementary Figure 5 | Crystal packing arrangements of DsRed tetramers.

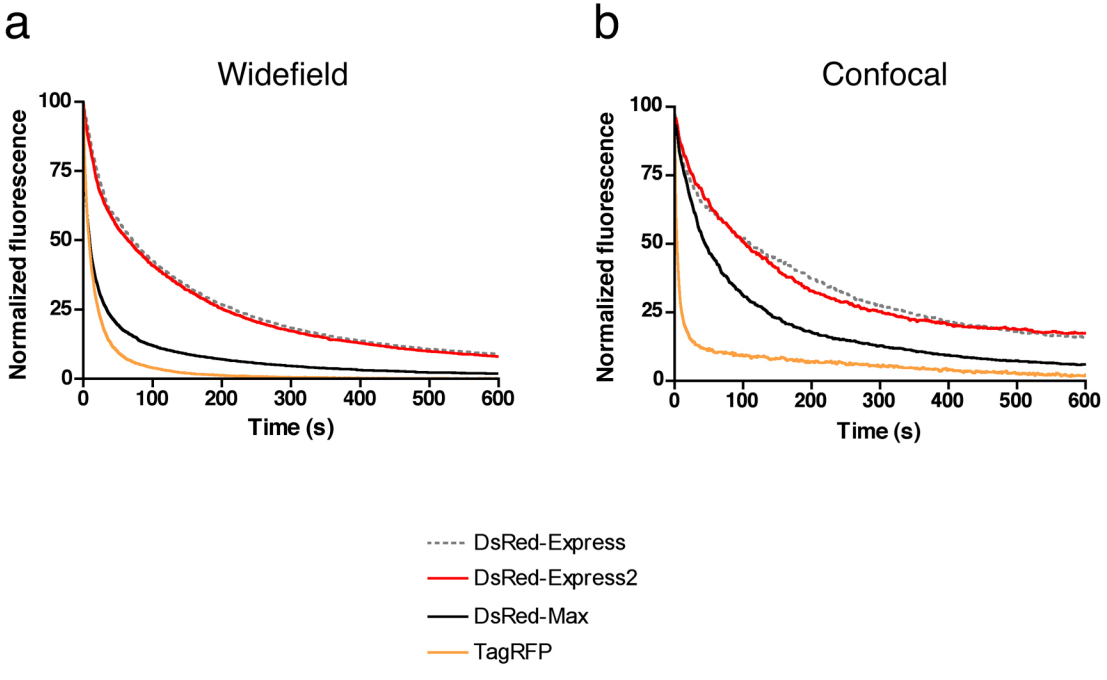
Symmetry mates of (**a**) wild-type DsRed (PDB code 1ZGO), (**b**) DsRed.T4 (PDB code 2VAE), or (**c**) DsRed-Max (PDB code 2V4E) were generated using PyMOL (http://www.pymol.org). Tetramers are colored individually and images are scaled to protein size.

Supplementary Figure 6 | Fluorescence properties of the new DsRed variants.



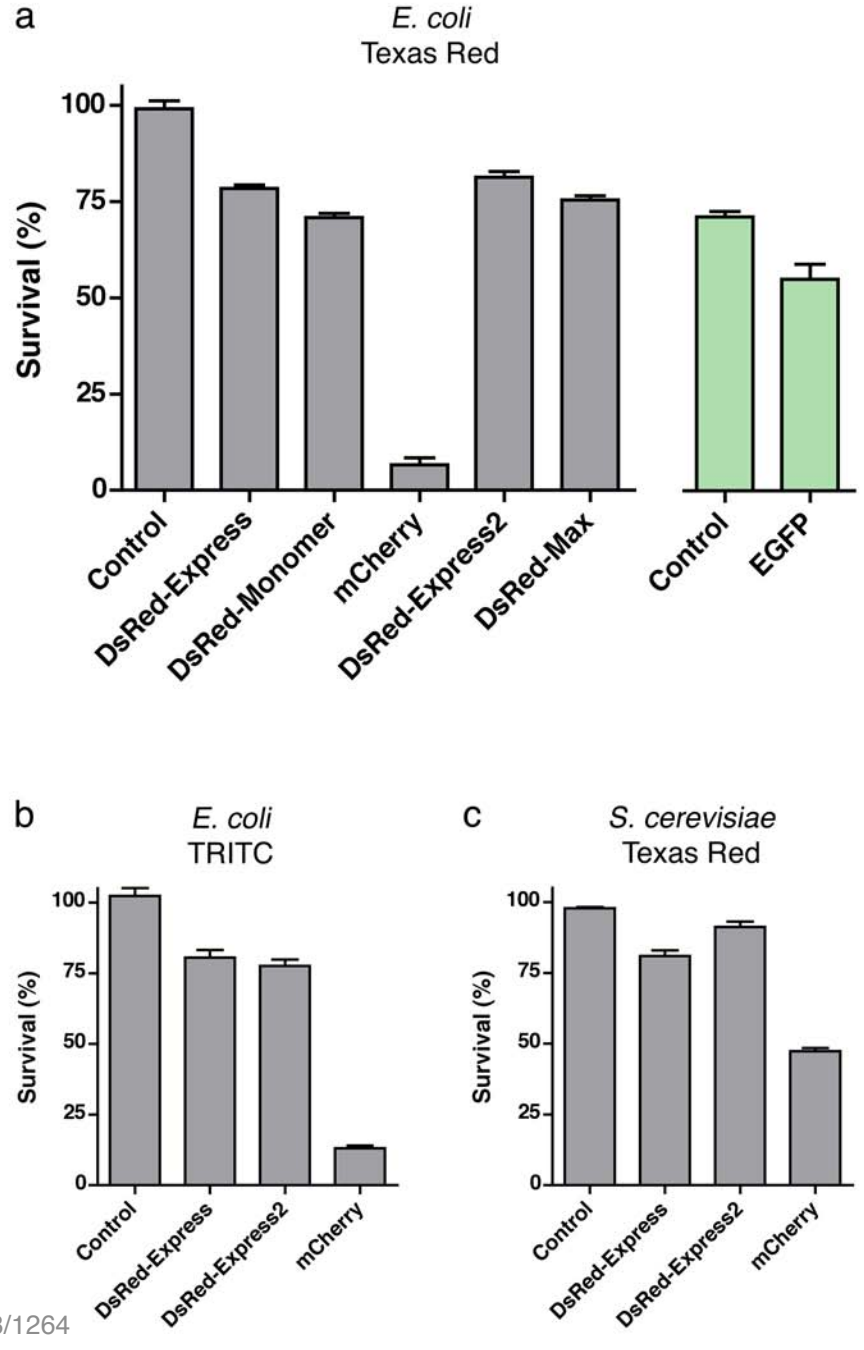
Wild-type (WT) DsRed and DsRed-Express are included for reference. The legend is applicable to all parts of the figure. (**a**) Excitation spectra of purified proteins. Emission was measured at 600 nm. (**b**) Emission spectra of purified proteins. Excitation was at 470 nm. (**c**) Absorbance spectra of purified proteins. (**d**) Kinetics of chromophore maturation at 37 °C in *E. coli*.

Supplementary Figure 7 | Photobleaching kinetics of the new DsRed variants.



DsRed-Express is included for reference, and TagRFP is included as an example of a red fluorescent protein that bleaches rapidly. The legend is applicable to both parts of the figure. (**a**) Photobleaching during widefield imaging with a Texas Red (535-585 nm) filter. (**b**) Photobleaching during confocal imaging with a 543-nm excitation laser.

Supplementary Figure 8 | Phototoxicity of fluorescent proteins in *E. coli* and yeast.



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(**a**) *E. coli* cells expressing no fluorescent protein (Control) or the indicated fluorescent protein were illuminated through a Texas Red (535-585 nm) filter (gray bars) or a GFP (470-510 nm) filter (green bars) for 15 min. This time point was chosen because light-induced killing was progressive and was largely complete at 15 min (data not shown). Parallel samples were treated identically but not illuminated. Cells were then plated and grown overnight. Percent survival was calculated as the number of colonies from the illuminated sample divided by the number from the non-illuminated sample. Bars represent mean and s.e.m. for three independent replicates. A comparison of the two "Control" samples indicates that the blue light used to excite GFP fluorescence caused some fluorescent protein-independent lethality. (**b**) Same as (a), except that a TRITC (530-560 nm) filter was used. (**c**) *S. cerevisiae* cells expressing nuclear-targeted DsRed-Express, DsRed-Express2, or mCherry were illuminated through a Texas Red filter set for 10 min. This time point was chosen because fluorescent protein-independent phototoxicity became notable after 10 min of illumination (data not shown). Percent survival was calculated after 2 days of growth.

Supplementary Table 1 | Properties of red fluorescent proteins

Variant	Excitation / Emission maxima	Extinction coefficient	Quantum yield	Relative brightness[a]	Maturation half-time (h)	Photobleaching half-time (s)[b]
Wild-type DsRed[c]	558 / 583	51,500	0.71	1	11	---
DsRed-Express	554 / 586	33,800	0.44	0.41	0.6	71 ± 3
TurboRFP[d]	550 / 573	---	---	---	1.5	32 ± 1
Katushka	584 / 631	76,300	0.32	0.67	0.6	15 ± 1
RFP611	555 / 606	109,700	0.6	1.8	2.7	7 ± 2
DsRed-Monomer[e]	557 / 592	27,300	0.14	0.10	1.3	15 ± 1
mCherry[f]	585 / 609	66,400	0.23	0.42	0.6	18 ± 1
tdTomato[f]	553 / 581	85,700	0.69	1.6	2.0	5 ± 1
TagRFP	554 / 582	77,000	0.47	0.98	1.5	8 ± 4
TagRFP-S158T	554 / 584	67,800	0.40	0.73	1.6	20 ± 2
mKate	584 / 632	45,500	0.33	0.41	1.3	15 ± 2
mKO2	549 / 563	54,300	0.82	1.2	1.8	5 ± 1
DsRed-Express2	554 / 591	35,600	0.42	0.41	0.7	64 ± 4
DsRed-Max	560 / 589	48,000	0.41	0.54	1.2	9 ± 1

Unless otherwise indicated, all measurements were obtained during the present study using standardized procedures.

[a] Brightness was calculated as the product of extinction coefficient and quantum yield, and was normalized to a value of 1 for wild-type DsRed.

[b] Photobleaching half-times during widefield illumination are listed as mean ± s.e.m. for three independent experiments.

[c] The maturation rate of wild-type DsRed was taken from Supplementary Ref. 1. Photobleaching of wild-type DsRed *in vivo* could not be reliably measured due to slow maturation.

[d] Because TurboRFP showed very poor solubility during extraction from bacteria, we were unable to perform brightness measurements for this protein.

[e] For DsRed-Monomer, all of the data except the photobleaching half-time were taken from Supplementary Ref. 2.

[f] The initial report describing mCherry and tdTomato listed substantially faster maturation rates[3]. Our measurement for mCherry is more consistent with a subsequent paper[4].

Supplementary Table 2 | Substitutions made during the creation of DsRed-Express2 and DsRed-Max[a]

Construct	Residues differing from DsRed.T3
Round 1	A2D, S3N, S4T, D6N, R36K, K47Q, C117T, K121H, M141L, K168E, D169G, I210V, G219A, L225Q
Round 2	A2D, S3N, S4T, D6N, E10P, R17H, R36K, K47Q, C117T, K121H, M141L, K168E, D169G, Q188K, I210V, G219A, L225Q
Round 3	A2D, S4T, D6N, E10P, R17H, R36K, K47Q, C117T, K121H, M141L, D169G, Q188K, I210V, G219A, L225Q
DsRed-Express2	A2D, S4T, D6N, E10P, R17H, R36K, K47Q, C117T, K121H, M141L, D169G, Q188K, I210V, T217A, G219A, L225Q
DsRed-Max	A2D, S4T, D6N, E10P, R17H, R36K, K47Q, Q66M, V73T, C117T, K121H, M141L, D169G, V175C, Q188K, I210V, G219A, L225Q

Construct	Residues differing from DsRed-Express
DsRed-Express2	A2D, S4T, D6N, E10P, R17H, R36K, K47Q, S117T, K121H, M141L, A145P, D169G, Q188K, I210V, G219A, L225Q
DsRed-Max	A2D, S4T, D6N, E10P, R17H, R36K, K47Q, Q66M, V73T, S117T, K121H, M141L, A145P, D169G, V175C, Q188K, I210V, A217T, G219A, L225Q

[a] The initial optimization started with DsRed.T3 rather than DsRed-Express because DsRed.T3 is the brightest of the rapidly maturing DsRed tetramers[1]. Based on subsequent analysis, we mutated the interior of the improved protein to match that of DsRed-Express.

Supplementary Table 3 | Residues targeted from crystal packing analysis

Residue	Wild-type amino acid	Other amino acids tested[a]	DsRed-Express2 amino acid
9	K	A	K
10	E	P	P
13	R	Q	R
17	R	H, Y	H
29[b]	I	V	I
58[b]	W	F, Y	W
77	A	E	A
85	L	Q	L
115	D	G	D
116	G	D	G
166	K	E, L, T	K
188	Q	E, K	K
198	K	E, S, T	K
203	S	K, E, D	S
206	E	A, S	E
209	T	S	T

[a] The amino acids tested were chosen based on the physical properties of the side chains and homology alignments with other fluorescent proteins. The wild-type residue was also tested in all cases.

[b] Residues 29 and 58 are internal, but seem to be important for creating a prominent cleft in the protein[5].

Supplementary Table 4 | Data collection and refinement statistics for DsRed-Max

Data collection	
Space group	$P2_12_12$
Unit cell dimensions	a = 115.5, b = 122.6, c = 164.9 Å $\alpha = \beta = \gamma = 90.0^o$
Molecules per asymmetric unit	8
X-ray source	APS 14-BM-C
Wavelength (Å)	0.900
Resolution range (Å)	50 - 2.40 (2.49 - 2.40)
Total/unique observations	597,626/91,172
Completeness (%)	98.0 (84.8)[a]
<I/sigI>	14.3 (2.2)
Rsym (%)	14.2 (61.4)
Refinement and model statistics	
Rcryst (%)	22.3
Rfree (%)	27.1
Non-hydrogen atoms (solvent)	14,710 (491)
Average B-factor ($Å^2$)	35.3
RMS Bond lengths (Å)	0.011
RMS Bond angles (^{o})	1.4

[a]Values in parentheses are for the high-resolution shell.

Supplementary Table 5 | Protein crystal statistics for DsRed tetramers

Protein (PDB code)	Solvent content (%)	Buried surface area (Å^2)[a]
Wild-type DsRed (1ZGO)	35	4,900
Wild-type DsRed (1G7K)	39	2,900
Wild-type DsRed (1GGX)	38	3,500
DsRed.T4 (2VAE)	47	3,400[b]
DsRed-Max (2V4E)	58	1,700 [b]

[a] For a given tetramer, the total buried surface area of inter-tetramer interfaces was calculated from PISA analysis as the sum of all packing interfaces larger than 100 Å^2. Intra-tetramer interfaces were excluded.

[b] The average buried surface area per tetramer is listed for structures with two tetramers per asymmetric unit.

SUPPLEMENTARY RESULTS

Cytotoxicity of red fluorescent proteins in *E. coli*

To assess cytotoxicity in *E. coli*, cells were transformed with expression vectors derived from pQE-60, which contains the strong T5 promoter[6]. With vectors of this type, researchers typically use regulated expression because the production of foreign proteins can inhibit cell growth. However, the pQE vectors are relatively low-copy plasmids, and we have found that constitutive expression of a non-cytotoxic protein such as mEGFP allows the *E. coli* cells to grow robustly. This observation provided a way to compare the cytotoxicities of different fluorescent proteins.

In **Supplementary Fig. 1a**, expression was blocked by a second plasmid that encoded a repressor. As expected, large colonies were obtained regardless of which fluorescent protein was encoded in the pQE vector. However, when expression was derepressed by including IPTG in the medium, large colonies were obtained only with mEGFP. Medium-sized colonies were obtained with DsRed-Monomer, and small colonies were obtained with DsRed-Express. Very small colonies were obtained with mCherry, TagRFP, and TagRFP-S158T. Tiny "pinprick" colonies were seen with tdTomato, mKO2, TurboRFP, Katushka, mKate, and RFP611. As judged by SDS-PAGE, all of the fluorescent proteins showed similar expression except for TagRFP-S158T, TurboRFP, and Katushka, which were present at lower levels (**Supplementary Fig. 1b**). We conclude that many red fluorescent proteins show moderate to severe cytotoxicity in *E. coli*.

Optimization of bacterial expression

In previous work, we expressed DsRed variants in *E. coli* with an N-terminal hexahistidine tag[1]. Expression from the endogenous DsRed start codon was much weaker due to mRNA secondary structure[7, 8]. To enhance bacterial expression from the endogenous start codon, we started with DsRed.T3, which is the brightest of the rapidly maturing tetramers[1]. Expression was enhanced by the substitutions A2D, S3N, and S4T, yielding DsRed.T3-DNT[9]. These same substitutions were incorporated during the engineering of DsRed-Monomer[2].

During the creation of DsRed-Express2, the N3S reversion (see below) boosted solubility but nullified a change that had increased bacterial expression. To restore high expression while maintaining high solubility, all combinations of synonymous codons for Asp2, Ser3, and Thr4 were screened for bacterial expression of DsRed.T3. The brightest colonies contained the sequence GAT/AGC/ACT at codons 2-4, yielding the variant DsRed.T3-DST. Bacteria expressing DsRed.T3-DST were slightly brighter than those expressing DsRed.T3-DNT and 15-fold brighter than those expressing DsRed.T3 (**Supplementary Fig. 4a**). These differences in brightness were due to intracellular fluorescent protein levels, because SDS-PAGE of bacterial lysates showed a much stronger fluorescent protein band from cells expressing DsRed.T3-DNT or DsRed.T3-DST than from cells expressing DsRed.T3 (**Supplementary Fig. 4b**).

A random mutagenesis screen for enhanced brightness in bacteria also yielded a silent mutation, Ala164(GCC→GCG). These various codon optimizations were incorporated into DsRed-Express2.

Engineering of DsRed-Express2 and DsRed-Max

Our goal was to reduce the higher-order aggregation of DsRed-Express. As a preliminary step, amino acids 2-4 were modified to boost expression in *E. coli* (see above). Based on our earlier work with DsRed-Monomer[2], we introduced ten additional substitutions to stabilize the protein and eliminate potentially interactive surface residues (**Supplementary Table 2**). The resulting variant was termed Round 1. With the bacterial extraction assay, the Round 1 variant showed a small but reproducible decrease in aggregation (data not shown).

To identify additional residues that promote aggregation, we reasoned that inter-tetramer interactions might be visible in the high-concentration environment of a protein crystal. Packing interactions in published DsRed crystal structures[2, 5, 10, 11] were analyzed with the Protein Interfaces, Surfaces and Assemblies (PISA) server[12]. Certain residues were consistently found to make inter-tetramer contacts. Sixteen residues identified using PISA (**Supplementary Table 3**) were mutagenized combinatorially with Round 1 as the template. The substitutions E10P, R17H, and Q188K reduced aggregation. These

three substitutions were combined to create the Round 2 variant, which had ~15% of its fluorescence in the pellet (data not shown).

Round 2 was then subjected to random mutagenesis[13] followed by screening with the bacterial extraction assay. This screen led to the substitutions N3S and E168K, both of which reverted mutations that had been introduced into Round 1. The resulting variant was termed DsRed-Express2. Compared to DsRed-Express, DsRed-Express2 showed almost identical brightness and photostability, marginally slower maturation, and less contaminating blue absorbance and green emission (**Supplementary Table 1, Supplementary Figs. 6** and **7**).

DsRed-Express2 is less bright than wild-type DsRed (**Supplementary Table 1**). We conjectured that the interior of DsRed-Express2 could be modified to enhance brightness while preserving high solubility. Targeted mutagenesis of key residues led to the brighter variant DsRed-Max, which contained the substitutions Q66M[3, 11], V73T, and V175C. Unfortunately, the Q66M substitution rendered DsRed-Max much less photostable than DsRed-Express2 during widefield imaging, although this problem was less pronounced during confocal imaging (**Supplementary Table 1**, **Supplementary Fig. 7**, and data not shown). DsRed-Max is vividly colored, making it useful as a marker for directly visualizing labeled cells.

A DsRed variant with reduced aggregation might be expected to show fewer crystal contacts. This prediction was confirmed by solving a crystal structure of DsRed-Max to 2.4 Å resolution (**Supplementary Table 4**). When compared to the previously analyzed crystals of wild-type DsRed and DsRed.T4, the DsRed-Max crystal showed a higher solvent content and about a 50% decrease in surface area of inter-tetramer contacts (**Supplementary Fig. 5** and **Supplementary Table 5**).

Phototoxicity

Excitation of fluorescent proteins *in vivo* can cause phototoxicity[14, 15]. To quantify this effect, *E. coli* cells expressing a fluorescent protein were illuminated for 15 min with an epifluorescence microscope using a low-power objective, and the percent survival was measured relative to a non-illuminated sample. When the excitation light was passed through a Texas Red (535-585 nm) filter, no phototoxicity was seen with control cells

containing an empty vector, but some cytotoxicity was seen with DsRed-Express (78% survival), DsRed-Monomer (71% survival), DsRed-Express2 (81% survival), and DsRed-Max (75% survival) (**Supplementary Fig. 8a**). A direct comparison with green fluorescent proteins is difficult, but the phototoxicity of variants such as DsRed-Express2 appears to be similar to that of EGFP (**Supplementary Fig. 8a**). mCherry was considerably more phototoxic (7% survival). The greater phototoxicity of mCherry was not due to higher protein expression (data not shown), nor was it due to stronger excitation because similar results were obtained with a TRITC (530-560 nm) filter (**Supplementary Fig. 8b**), which should give more efficient excitation of the non-red-shifted DsRed variants than of the red-shifted mCherry[3].

To test phototoxicity in a eukaryotic system, we labeled the yeast S*accharomyces cerevisiae* by using the constitutive *TPI1* promoter to drive expression of nuclear-localized DsRed-Express, or DsRed-Express2, or mCherry. Nuclear localization enhanced fluorescent protein phototoxicity (data not shown), presumably by facilitating DNA damage. Relatively low phototoxicity was seen with DsRed-Express (81% survival) and DsRed-Express2 (91% survival), whereas mCherry showed higher phototoxicity (47% survival) (**Supplementary Fig. 8c**). The combined data imply that phototoxicity is of practical importance and that DsRed-Express2 is a good choice in this regard.

SUPPLEMENTARY METHODS

Fluorescent proteins

EGFP, DsRed-Express (also known as DsRed.T1[1]), and DsRed-Monomer were obtained from Clontech. mEGFP was created by introducing the A206K mutation[16] into EGFP. For experiments with *E. coli*, DsRed-Express was modified as described in **Supplementary Results** to enhance bacterial expression. TurboRFP, Katushka, TagRFP, and mKate were obtained from Evrogen. TagRFP-S158T[17] was generated by site-directed mutagenesis of TagRFP. RFP611[18] was provided by J. Wiedenmann (University of Ulm). mCherry and tdTomato[3] were provided by R. Tsien (University of California at San Diego). mKO was obtained from MBL International, and was modified by site-directed mutagenesis to create mKO2[19].

Expression of fluorescent proteins in *E. coli*

Fluorescent protein genes were subcloned into *E. coli* expression vectors (Qiagen). pQE-81 was used to add an N-terminal hexahistidine tag. A derivative of pQE-60 termed pQE-60NA was used to express an untagged fluorescent protein from its endogenous start codon. In strains containing the *lacI*^{q} gene, expression was derepressed by adding IPTG.

Fluorescent protein cytotoxicity in bacteria was examined as follows. For **Supplementary Fig. 1**, *E. coli* DH10B cells containing the repressor plasmid pREP4 (Qiagen) were transformed with pQE-60NA encoding the relevant fluorescent protein. Each transformation mixture was split in half and spread on sectors of two Luria broth (LB) plates containing 50 μg/ml carbenicillin plus 30 μg/ml kanamycin, and either lacking or containing 1 mM IPTG. The plates were incubated for 14 h at 37 ^{o}C. For **Fig. 1c**, DH10B cells were transformed with pQE-60NA encoding the relevant fluorescent protein, and the transformants were plated on adjacent sectors of an LB plate containing 100 μg/ml ampicillin. After growth for 14 h at 37 ^{o}C, the plate was stored at 4 ^{o}C for 2 days to enhance colony color.

HeLa cell cytotoxicity and growth assays

For transient transfection assays, the fluorescent protein genes were subcloned into pDsRed1-N1 (Clontech). Identical wells of HeLa cells at ~50% confluence were transfected using Lipofectamine 2000 (Invitrogen). At intervals of 24 h after transfection, three wells for each fluorescent protein were analyzed with an LSR II flow cytometer (BD Biosciences), using either a 488-nm laser for mEGFP or a 543-nm laser for the red fluorescent proteins. Between 20-50% of the viable cells were detectably fluorescent at 24 h after transfection. Data were processed using FlowJo software (Treestar Inc.).

For lentiviral expression assays, HeLa cells expressing the desired fluorescent protein were generated by lentiviral gene transfer using the Lenti-X HT Packaging System with associated vectors (Clontech). A fluorescent protein gene with a Kozak sequence upstream of the start codon was subcloned into pLVX-DsRed-Monomer between BamHI and NotI. pLVX-Puro was used as a no-insert control. To generate viral particles, HEK 293T/17 cells (ATCC No. CRL-11268) in a 10-cm dish at 50% confluence were transfected with ~6 μg of the appropriate vector. Viral particles were collected 48-72 h post-transfection and frozen in aliquots at -80 ^{o}C. Viral particle concentration was determined using the QuickTiter Lentivirus Quantitation Kit (Cell Biolabs). HeLa cells at 50% confluence in a 10-cm dish were transduced by adding 3.5 x 10^{10} viral particles to the medium together with polybrene (4 μg/ml final concentration). The medium was changed after 24 h, and cells were grown in the absence of drug selection. Viable fluorescent cells were analyzed at 3 or 10 days post-transduction using an LSR II flow cytometer (BD Biosciences) with 488-nm excitation and a FITC (525/15 nm) filter (green fluorescence) or 543-nm excitation and a PE (585/15 nm) filter (red fluorescence).

To measure the growth of cells expressing a given fluorescent protein after lentiviral transduction, 3000 fluorescent HeLa cells were cultured in each of 12 wells in a 96-well plate. On Days 1, 2, 3, and 4, cells from three wells were trypsinized and counted with a hemocytometer. As a control, viable cells were cultured and counted after transduction with lentiviral particles generated using pLVX-Puro.

Hematopoietic stem and progenitor cell growth assay

This assay was carried out essentially as described[20]. Briefly, murine stem cell virus-based bicistronic retroviral constructs encoding EGFP, DsRed-Express, or DsRed-Express2 were transfected into the Phoenix-Eco packaging cell line to make ecotropic retroviral vectors. Low-density bone marrow mononuclear cells isolated from 8- to 12-week-old female C57BL/6J mice were cultured for 2 days with a cytokine cocktail (100 ng/ml murine stem cell factor, 100 ng/ml murine FLT3-ligand, and 100 ng/ml murine thrombopoietin) and then transduced with the retroviral vectors. At 87 h after transduction, the cells were sorted by flow cytometry. For each fluorescent protein, three wells with 20,000 fluorescent cells each were cultured in the presence of the cytokine cocktail. At selected time points, an aliquot from each well was analyzed by flow cytometry to determine total cell number and the percentage of fluorescent cells.

Aggregation assay

Fluorescent protein aggregation was measured using a modification of an earlier assay[1]. DH5α *E. coli* cells were transformed with fluorescent protein-encoding plasmids, and after overnight growth, colonies were transferred to 96-well plates. Each well contained 175 μl Terrific broth or LB, supplemented with 100 μg/ml carbenicillin. Cultures were grown for 2-4 h at 37 ^{o}C and then induced with IPTG for 6-12 h. The plates were centrifuged, and each bacterial pellet was resuspended in 100 μl BPER II lysis reagent (Pierce) for 15 min at 37 ^{o}C. Lysates were centrifuged for 5 min at 3000 x g, and the supernatants were transferred to black 96-well plates. Each pellet was resuspended in 100 μl BPER II and transferred to another black 96-well plate. Fluorescence was measured on a Tecan Safire II Microplate reader with 470 ± 10 nm excitation and 510 ± 10 nm emission for green fluorescent proteins, or 550 ± 10 nm excitation and 595 ± 10 nm emission for red fluorescent proteins. The percentage of the total fluorescence in the pellet was then calculated.

In the targeted mutagenesis screen for variants with reduced aggregation, ~2000 clones were analyzed. For the random mutagenesis screen, ~30,000 colonies were pre-screened for fluorescence using a visual assay[1], and ~3000 of the fluorescent clones were analyzed for aggregation.

Optimization of DsRed expression

To screen for optimized expression in *E. coli*, all synonymous substitutions encoding residues 2-5 (Asp-Ser-Thr-Glu) were tested by annealing two pairs of phosphorylated oligonucleotides: 5'-CATGGAYTCNACNGARAACGT-3' plus 5'-TYTCNGTNGARTC-3', and 5'-CATGGAYAGYACNGARAACGT-3' plus 5'-TYTCNGTRCTRTC-3'. These annealed oligo pairs were subcloned separately into the DsRed.T3 gene[1] in pQE-60NA between NcoI and AatII. The mutant libraries were transformed into DH10B cells, and ~4000 colonies were screened for each library using the slide projector assay described below. The brightest colonies from each library were then directly compared, and the DNA sequences of the brightest clones were determined.

To quantify expression in *E. coli*, DsRed.T3 variant genes in pQE-60NA were generated with the following sequences at codons 2-4: GCC/TCC/TCC (Ala-Ser-Ser; DsRed.T3), GAC/AAC/ACC (Asp-Asn-Thr; DsRed.T3-DNT), or GAT/AGC/ACT (Asp-Ser-Thr; DsRed.T3-DST). These constructs were transformed into DH10B/pREP4 cells, and the transformants were grown in LB plus 100 μg/ml ampicillin and 30 μg/ml kanamycin to an OD_{600} of 0.5-0.6. A pulse of protein synthesis was initiated by inducing with 1 mM IPTG for 2 h at 37 ^{o}C. Translation was then inhibited by adding 30 μg/ml tetracycline and 170 μg/ml chloramphenicol, and the cells were chased for 6 h at 37 ^{o}C to allow complete chromophore maturation. Cell density was normalized to an OD_{600} of 1.0 in LB, and whole-cell fluorescence was measured using a Tecan Safire II Microplate reader. Fluorescence was excited at 550 ± 10 nm and emission was measured at 595 ± 10 nm. Data were normalized to the signal obtained with DsRed.T3. As a control, expression was also initiated from the non-native start codon preceding the hexahistidine tag in pQE31 (Qiagen). Experiments were performed in triplicate, and the results were averaged.

To confirm that the fluorescence readout in this experiment reflected differences in protein concentration, 1 OD_{600} unit of each sample was collected after the 6-h chase, centrifuged, resuspended in 400 μl SDS-PAGE loading buffer, boiled for 10 min, and centrifuged again for 5 min at 16,000 x g. Twenty μl of supernatant was loaded per lane of an 8-12% polyacrylamide gel. As a reference standard, 0-2 μg of purified

hexahistidine-tagged DsRed.T3 was added to a lysate from cells containing an empty vector.

An additional synonymous substitution, Ser179(TCC→TCA), was included in DsRed-Express2 and DsRed-Max because it reportedly increases expression in mammalian cells[21]. We did not verify this effect.

Library construction and screening

Combinatorial gene libraries with targeted mutations were built by overlap extension PCR[22] using primers with wobble bases at the appropriate positions. Combinatorial gene libraries with random mutations were built by error-prone PCR[13]. The PCR product mixtures were subcloned into pQE-60NA, and then the mutant libraries were transformed into *E. coli*. Strain DH5α was used for aggregation screens, and strain DH10B was used for brightness screens.

Brightness screens

Qualitative screens for fluorescence brightness of DsRed variants were carried out using the slide projector assay as previously described[1, 23]. Briefly, fluorescence from bacterial colonies constitutively expressing red fluorescent proteins was excited by passing light from a slide projector through a 520 ± 20 nm bandpass filter, and fluorescence emission was detected visually using laboratory goggles covered with a Kodak Wratten filter #22, which passes wavelengths >550 nm. Approximately 60,000 colonies were screened from a targeted mutant library, and approximately 120,000 colonies were screened from a random mutant library.

Photobleaching assays

Photobleaching during widefield illumination was measured as follows. DH10B/pREP4 cells that had been transformed with pQE-60NA encoding the relevant fluorescent protein were grown to an OD_{600} of ~0.5. After a 2-h derepression with IPTG, translation was inhibited with 30 μg/ml tetracycline plus 170 μg/ml chloramphenicol, and the cells were incubated for 4 h at 37 ^{o}C to allow chromophore maturation. One OD_{600} unit of cells were then pelleted and resuspended in 500 μl of 0.5% low-melt agarose in

phosphate buffered saline at 37 ^{o}C. 1.5 µl of this cell-agarose mixture was placed on a glass slide and covered with a coverslip. Photobleaching was carried out with a Zeiss AxioPlan2 epifluorescence microscope using a 100 W mercury arc lamp, a 40X 0.75-NA air objective, and a Texas Red filter set (Chroma) with bandpass excitation at 535-585 nm. Cells were continuously illuminated for 10 min, and images were collected every 2 sec. Five representative cells were chosen for analysis. The total fluorescence signal above background from these cells was quantified in each frame using Zeiss AxioVision 4.6 software. The fluorescence signals were normalized to 100% at time zero and the data were averaged from the 5 cells in each of three independent experiments. Bleaching half-time was defined as the point at which the signal reached 50% of its initial value.

Photobleaching during confocal imaging was measured for *E. coli* cells prepared in the same manner, using a Zeiss LSM 510 scanning confocal microscope with a 40X 1.3-NA oil objective and 543-nm laser excitation. Images were collected every 2 sec over the course of 10 min. The laser intensity was adjusted to a level that would produce substantial bleaching during this time period. Data analysis was performed as described above.

Phototoxicity assays

To measure phototoxicity in *E. coli*, DH10B cells were was transformed with pQE-81 encoding either a DsRed variant or EGFP, or with empty pQE-60NA as a control. Cells were grown to an OD_{600} of ~0.5 and induced with IPTG for 4 h. A 1:10,000 dilution of each culture in LB gave 150-200 cells/µl. For light treatment, a 2-µl drop of diluted cells was placed on a coverslip in a Petri dish that contained wet paper wipes to maintain humidity. The lid of the dish had a 1.5-cm hole to accommodate the microscope objective. Cells were exposed to 15 min of illumination from a 100 W mercury arc lamp using a Zeiss Axioplan 2 epifluorescence microscope with a 5X 0.16-NA objective and a filter (Chroma) for either Texas Red (535-585 nm), TRITC (530-560 nm), or GFP (470-510 nm). Light intensities measured using a Newport Power Meter Model 2931-C with a silicon photodiode detector were 777 mW/cm^2 for the Texas Red filter, 449 mW/cm^2 for the TRITC filter, and 156 mW/cm^2 for the GFP filter. Control samples

were placed on a coverslip in an identical Petri dish but were not illuminated. Each sample was resuspended in 100 μl H_2O and then plated on LB plus 100 μg/ml ampicillin for overnight growth. Percent survival was measured by comparing the number of colonies from an illuminated sample versus the matched non-illuminated sample.

To measure phototoxicity in *S. cerevisiae*, we created yeast strains expressing nuclear localized mCherry, DsRed-Express, or DsRed-Express2. The appropriate fluorescent protein gene was amplified by PCR and subcloned using EcoRI and BamHI into a YIplac204[24] derivative between the *S. cerevisiae TPI1* promoter and *CYC1* terminator[25]. Phosphorylated oligonucleotides encoding the simian virus 40 large T antigen nuclear localization signal[26] (5'-AATTCAAAAAATGCCAAAAAAGAAAAGAAAGGTCGGC-3' and 5'-AATTGCCGACCTTTCTTTTCTTTTTTGGCATTTTTTG-3') were annealed and then ligated into the EcoRI site. In the case of mCherry, two tandem signals were added to achieve efficient nuclear localization. These constructs were transformed into haploid *S. cerevisiae* JK9-3d cells[27]. The phototoxicity assay was performed as described above, except that the illumination time was 10 min and the cells were plated on yeast extract/peptone/dextrose media.

Fluorescent protein purification and analysis

Hexahistidine-tagged DsRed variants were purified from bacteria as previously described[1]. Purified protein was dialyzed into 50 mM Na^+-HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA in the dark at room temperature. Corrected excitation and emission spectra[1,2] were obtained on a Horiba Fluoromax-3 spectrofluorometer. Absorbance spectra were collected on a Shimadzu UV-1650PC spectrophotometer. Quantum yields were determined using ethanolic Rhodamine 101 as a reference[28, 29]. For this analysis, excitation was at 535 nm and fluorescence emission was integrated from 550-800 nm. Protein concentrations were determined using the BCA method or by amino acid analysis at the Molecular Structure Facility, University of California, Davis. Molar extinction coefficients were determined at the absorbance maxima using a Spectronic Unicam GENESYS 10 UV spectrophotometer.

The kinetics of chromophore maturation were determined as previously described[2]. Briefly, DH5α cells were transformed with pQE-81 encoding the relevant fluorescent protein. A 15-min pulse of protein synthesis was initiated with 2 mM IPTG, and was stopped by adding 30 μg/ml tetracycline and 170 μg/ml chloramphenicol to inhibit translation. Whole-cell fluorescence was measured at regular intervals up to 4 h after translation inhibition. The fluorescence value at time zero (immediately following translation inhibition) was subtracted from each subsequent value. Maturation half-time was determined by plotting data averaged from three samples.

Crystallography

DsRed-Max was expressed in DH10B from a modified version of pQE31 encoding a tobacco etch virus (TEV) cleavage site between the start codon and hexahistidine tag, and was purified as previously described[1]. After elution from Ni^{2+}-NTA agarose, the hexahistidine tag was removed by incubation for 12 h at 30 ^{o}C with hexahistidine-tagged TEV protease[30]. Cleaved DsRed-Max was then collected in the flow-through from a second Ni^{2+}-NTA agarose column. The purified protein was buffer exchanged and concentrated to ~12 mg/ml in 5 mM Na^{+}-HEPES, pH 8.0, 100 mM NaCl, 1 mM Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl), and stored at 4 ^{o}C.

Initial crystallization conditions were identified using Crystal Screen HT and Index HT screens (Hampton Research) at room temperature in 96-well sitting drop vapor diffusion format. Final crystals were grown in 24-well, hanging drop format using equal volumes of protein and reservoir solution containing 100 mM Bis-Tris, pH 5.5, 200 mM ammonium sulfate, and 16% PEG-8000. Crystals were mounted in nylon loops after briefly transferring them to cryoprotectant containing 100 mM Bis-Tris, pH 5.5, 300 mM ammonium sulfate, 16% PEG-8000, and 20% ethylene glycol. Diffraction data were collected at 100K at APS beamline 14BM-C (Advanced Photon Source, Argonne, IL) on an ADSC Quantum 315 detector.

Diffraction data were processed as previously described[2]. DsRed.T4 (PDB code 2VAE)[2] with the chromophore and waters omitted was used as the search model for molecular replacement with Phaser[31]. After two rounds of coordinate and isotropic B-factor refinement, difference maps suggested the presence of the red form of the

-MYG- chromophore, as expected[11]; due to the relatively limited and anisotropic diffraction from these crystals, we only modeled the red chromophore and refined it using tight geometric restraints. Coot[32] and REFMAC5[33] were used for model building and crystallographic refinement. See **Supplementary Table 4** for refinement statistics.

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