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A Rapidly Maturing Far-Red Derivative of DsRed-Express2 for Whole-Cell Labeling†

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ABSTRACT: Fluorescent proteins (FPs) with far-red excitation and emission are desirable for multicolor labeling and live-animal imaging. We describe E2-Crimson, a far-red derivative of the tetrameric FP DsRed-Express2. Unlike other far-red FPs, E2-Crimson is noncytotoxic in bacterial and mammalian cells. E2-Crimson is brighter than other far-red FPs and matures substantially faster than other red and far-red FPs. Approximately 40% of the E2-Crimson fluorescence signal is remarkably photostable. With an excitation maximum at 611 nm, E2-Crimson is the first FP that is efficiently excited with standard far-red lasers. We show that E2-Crimson has unique applications for flow cytometry and stimulated emission depletion (STED) microscopy.

Far-red FPs[1] are potentially well suited to live-animal imaging because at wavelengths above 600 nm, absorption and light scattering are relatively low in tissues (*1*−*3*). Moreover, far-red FPs are useful for multicolor labeling in conjunction with orange and green FPs. Monomeric FPs are the most versatile, but oligomeric FPs are useful for labeling whole cells or the lumenal spaces of organelles (*4*). Various far-red FPs have been developed, including the monomeric mPlum (*5*), mRaspberry (*5*), mKate (*6*), and mKate2 (*7*), as well as the pseudomonomeric tdKatushka (*7*) and the oligomeric Katushka (*6*), RFP637 (*8*), and RFP639 (*8*). In addition to these GFP homologues, monomeric infrared bacteriophytochrome-based FPs were recently developed (*9*).

Despite these options, an optimal far-red whole-cell label has not yet been described. The brightest of the available far-red GFP homologues are also the least red-shifted (Table 1), and the bacteriophytochrome FPs require an exogenous cofactor for strong fluorescence (*9*). Far-red GFP homologues have excitation spectra that peak around 590 nm and decline rapidly beyond 600 nm, limiting the excitation efficiency with tissue-penetrating wavelengths such as the standard 633 and 635 nm laser lines. Another problem is the cytotoxicity observed with many FPs, including close relatives of the far-red FPs (*4*), suggesting that the available far-red FPs may compromise the viability of labeled cells.

For these reasons, we set out to generate a far-red derivative of DsRed-Express2. This tetrameric red FP displays low cytotoxicity in bacterial and mammalian cells (*4*) and can be modified to produce noncytotoxic color variants (*10*). DsRed-Express2 was subjected to targeted and random mutagenesis (see the Supporting Information for details). The result was a far-red variant termed E2-Crimson, for which both the excitation and emission spectra are red-shifted by ∼55 nm relative to those of DsRed-Express2 (Figure 1A,B).

E2-Crimson contains 13 substitutions relative to DsRed-Express2 (Table S2), but the red-shifted fluorescence is due largely to two mutations: Gln66Phe and Ser197Tyr.

The Gln66Phe mutation alters the first position of the chromophore, and reversion of this mutation blue shifts the fluorescence spectra by ∼20 nm (data not shown). Changing Phe66 to Leu causes an only ∼5 nm blue shift (data not shown), suggesting that hydrophobicity of this side chain is critical. The Ser197Tyr mutation is analogous to the Ser203Tyr mutation that was previously introduced into GFP to generate the red-shifted YFP (*11*). Presumably, Tyr197 in E2-Crimson is involved in $\pi-\pi$ stacking with the chromophore (*12*).

E2-Crimson has an emission maximum at 646 nm and absorbance and excitation maxima at 611 nm (Table 1 and Figure S1A), giving it the furthest red-shifted excitation of any known GFP homologue (*13*). In addition, E2-Crimson is bright due to a very high extinction coefficient of 126000 M^{-1} cm^{-1} and a moderate quantum yield of 0.23 (Table 1). During widefield imaging, E2-Crimson exhibits unusual biphasic photobleaching behavior, maintaining ∼40% of the original signal during extended imaging (Figure 1C). The biphasic bleaching is unexplained, but as described in the Supporting Information, this behavior is not due to photoswitching (Figure S2A,B) or photoconversion (Figure S2C). During confocal imaging, E2-Crimson is photostable and the biphasic bleaching is less pronounced (Figure S1B). In a bacterial viability assay (*4*), E2-Crimson exhibited very low phototoxicity (Figure S3). This combination of brightness, photostability, and low phototoxicity makes E2-Crimson well suited to live-cell imaging.

E2-Crimson matures faster than any previously described red or far-red FP (*4*, *13*), with a half-time for chromophore maturation of 26 min at 37 °C (Table 1 and Figure 1D). For comparison, the red FP mCherry has a maturation half-time of 40 min in the same assay (*4*). Rapid maturation makes E2-Crimson suitable for kinetic experiments that were previously possible only with GFP and its derivatives (*14*). E2-Crimson is also pH stable, with a pK_a of 4.5. No spectral shifts were observed between pH 4 and 10 (data not shown). In this regard, E2-Crimson outperforms mKate2 and Katushka, which have pK_a values in the physiological range (Table 1).

E2-Crimson is derived from DsRed-Express2, which was engineered to reduce cytotoxicity by minimizing higher-order

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[1]Abbreviations: FP, fluorescent protein; sem, standard error of the mean; ER, endoplasmic reticulum; STED, stimulated emission depletion; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Table 1: Fluorescence Properties of Relevant FPs

FP	excitation/ emission maxima (nm)	extinction coefficient (M^{-1} cm^{-1})	quantum yield	relative brightness[a]	extinction coefficient at 633 nm (M^{-1} cm^{-1})	relative brightness with 633 nm excitation	maturation half-time (h)	photobleaching half-time (s)[b]	pK_a[c]
DsRed1[d]	558/583	51500	0.71	1	–	–	11	–;	–
mPlum	587/649	29300	0.10	0.08	2300	0.02	1.6	31 ± 2	5.5
mRaspberry	594/627	42000	0.14	0.16	3600	0.03	2.1	21 ± 2	5
mKate2	586/630	56400	0.39	0.6	2300	0.04	0.8	23 ± 2	6.5
Katushka[d]	584/631	76300	0.32	0.67	4600	0.03	0.6	15 ± 1	7.5
RFP637	585/637	53800	0.22	0.32	4600	0.03	2.4	60 ± 4	4.5
RFP639	587/639	74700	0.21	0.43	6700	0.05	1.7	29 ± 3	4.5
E2-Crimson	611/646	126000	0.23	0.79	55400	1	0.4	26 ± 3	4.5

[a]Brightness was calculated as the product of the extinction coefficient and quantum yield and was normalized to a value of 1 for DsRed1 (wild-type DsRed). [b]Photobleaching half-times during widefield illumination are listed as mean ± sem for three independent replicates. [c]All pK_a values were obtained during this study. [d]Measurements for DsRed1 and Katushka were taken from ref *4*.

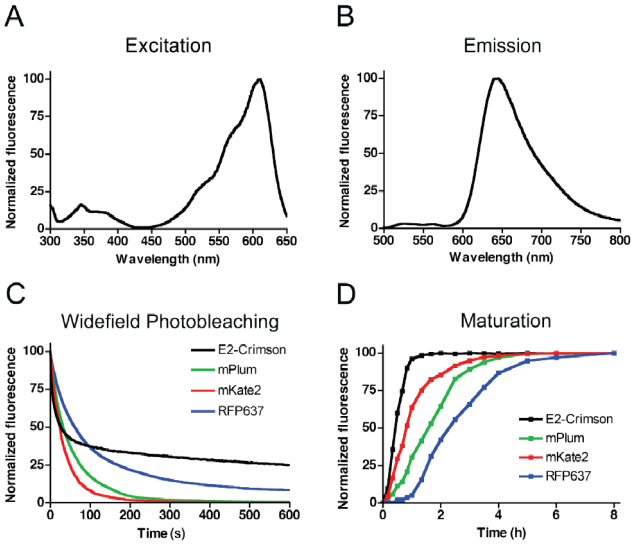

FIGURE 1: Fluorescence properties of E2-Crimson. (A) Excitation spectrum. (B) Emission spectrum. (C) Photobleaching kinetics of E2-Crimson and other representative far-red FPs during widefield fluorescence microscopy with a Texas Red filter set. (D) Maturation kinetics of E2-Crimson and other representative far-red FPs. Data for panels C and D are the means of three independent measurements.

aggregation of the tetramers (*4*). Using a bacterial extraction assay, we found that E2-Crimson is as soluble as DsRed-Express2 and much more soluble than other oligomeric far-red FPs (Figure S4A).

To assess the cytotoxicity of far-red FPs in *Escherichia coli*, cells were transformed to express the desired FP under control of a repressible promoter (*4*). Under repressing conditions, all of the transformants grew robustly (Figure S4B). However, under derepressing conditions, large colonies were obtained only with E2-Crimson. Medium-sized colonies were obtained with mPlum and mKate2; small colonies were obtained with mRaspberry, and tiny colonies were obtained with RFP637 and RFP639. Tiny colonies were also observed for Katushka in a previous study (*4*). As judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), all of the fluorescent proteins exhibited similar expression levels (Figure S4C). Thus, E2-Crimson is less cytotoxic to bacteria than other far-red FPs.

We next examined whether E2-Crimson maintains the low cytotoxicity of DsRed-Express2 in mammalian cells. Indeed, transiently transfected HeLa cells expressing either DsRed-Express2 or E2-Crimson maintained a nearly constant average fluorescence, while cells expressing other far-red FPs declined significantly in average fluorescence throughout the experiment (Figure 2). A similar decline was previously observed for Katushka (*4*). These declines were due to loss of the most strongly expressing cells (Figure S5). We conclude that E2-Crimson is substantially less cytotoxic to mammalian cells than other far-red FPs.

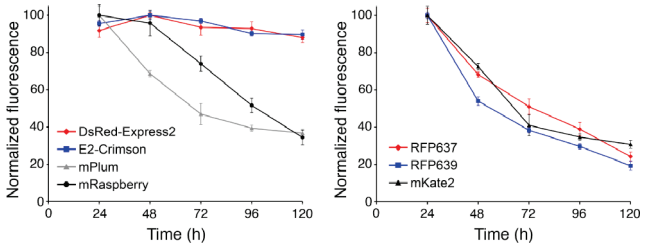

FIGURE 2: E2-Crimson is noncytotoxic to HeLa cells under conditions of standard high-level expression. HeLa cells were transiently transfected for constitutive high-level expression of the indicated FP. Three wells per FP were analyzed daily by flow cytometry. The average brightness of viable fluorescent cells was measured, and the strongest signal for a given FP was normalized to 100 units. Error bars represent sem.

A key benefit of the red-shifted fluorescence spectra of E2-Crimson is efficient excitation with standard far-red lasers. For GFP homologues, excitation efficiency tends to decrease steeply at wavelengths longer than the excitation maxima, so most far-red FPs are excited with less than 10% efficiency at 633 nm (Table 1). By contrast, E2-Crimson excites at 633 nm with 42% of its maximal efficiency.

To demonstrate the utility of strong excitation with a 633 nm laser, *E. coli* cells were induced to express either a far-red FP or no FP and were analyzed by flow cytometry. Cells were binned according to the brightness of far-red emission (Figure S6). Only the cell population expressing E2-Crimson was fully separable from nonfluorescent control cells. In the next brightest population consisting of cells expressing Katushka, fewer than 50% of the fluorescent cells could be resolved from nonfluorescent control cells. Consistent with these data, measurements with purified proteins indicated that other far-red FPs are less than 5% as bright as E2-Crimson when excited with 633 nm light (Table 1).

To confirm that E2-Crimson is suitable for use in multicolor microscopy experiments, a three-color yeast strain was

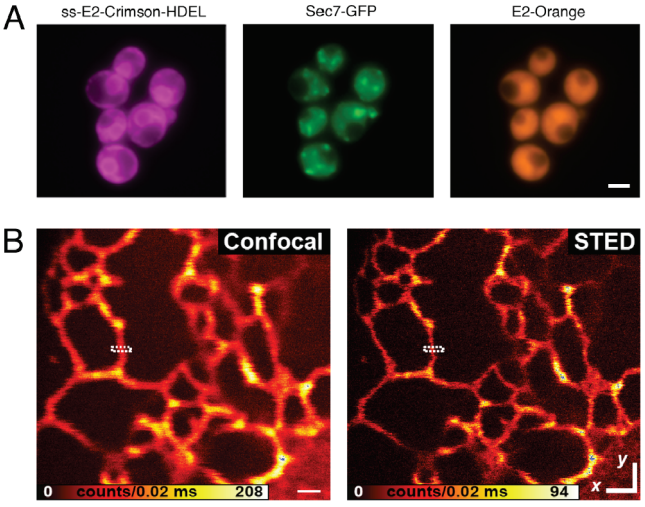

FIGURE 3: E2-Crimson is useful for multicolor and STED microscopy. (A) A three-color yeast strain was generated by labeling the ER with E2-Crimson, late Golgi cisternae with GFP, and the cytoplasm with E2-Orange. The scale bar is 3 μm. (B) The mammalian ER was imaged by conventional confocal microscopy (left) or by STED microscopy (right) with 635 nm excitation and a STED wavelength of 760 nm. Fluorescence from the region designated by white dashed lines is quantified in Figure S7. The scale bar is 1 μm.

generated with GFP-labeled Golgi cisternae, an E2-Orange-labeled cytoplasm (*10*), and an E2-Crimson-labeled endoplasmic reticulum (ER) (Figure 3A). The three fluorescence signals were cleanly resolvable using a widefield microscope with standard filter sets.

Red-shifted excitation also makes E2-Crimson the only FP useful for super-resolution STED microscopy with far-red lasers, such as those used in commercially available STED microscopes. To demonstrate this point, the ER of mammalian PtK2 cells was labeled with E2-Crimson as previously described (*15*) and was visualized using a STED microscope. As expected, STED imaging yielded substantially improved resolution compared to standard confocal imaging (Figures 3B and S7).

In sum, E2-Crimson is noncytotoxic in bacterial and mammalian cells and has favorable fluorescence properties, including brightness, photostability, low phototoxicity, fast maturation, and pH stability. Red-shifted excitation and emission spectra make E2-Crimson uniquely suited to excitation with far-red lasers and useful in multicolor labeling experiments together with orange and green FPs. E2-Crimson should also be an excellent marker for live-animal imaging.

Because E2-Crimson is tetrameric, it should be used to label whole cells or the lumenal spaces of organelles, rather than as a fusion partner. The mutations described here may guide the engineering of new far-red monomeric FPs.

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SUPPORTING INFORMATION AVAILABLE

Supporting results, experimental procedures, and Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

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