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A non-cytotoxic DsRed variant for whole-cell labeling

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Abstract

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

Fluorescent proteins can be used as whole-cell labels or as fusion tags¹. Enhanced GFP (EGFP) and its monomeric derivative mEGFP are suitable for either purpose. Oligomeric red fluorescent proteins have been engineered as whole-cell labels. For example, DsRed was modified to create DsRed-Express²; eqFP578 was modified to create TurboRFP³ and Katushka⁴; and eqFP611 was modified to create RFP611⁵. Monomeric red fluorescent proteins have also been engineered. For example, DsRed-Express was modified to create DsRed-Monomer⁶ as well as mCherry and tdTomato⁷; Kusabira-Orange was modified to generate mKO2⁸; TurboRFP was modified to generate TagRFP³ and TagRFP-S158T⁹; and Katushka was modified to generate mKate⁴.

Despite this abundance of options, the usage of red fluorescent proteins 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 show growth defects and/or instability of the fluorescent protein markers^{10,11}. We therefore set out to engineer an improved red fluorescent protein for whole-cell labeling.

Cytotoxicity was evaluated for mEGFP plus 11 red fluorescent proteins. During constitutive high-level expression in *E. coli*, mEGFP was well tolerated but the red fluorescent proteins

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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 standard plasmid containing the strong CMV promoter. Viable fluorescent cells were analyzed by flow cytometry at daily intervals after transfection. For mEGFP, average cellular fluorescence remained nearly constant from 24 h to 120 h (Fig. 1a). By contrast, all of the red fluorescent proteins showed progressive declines in average fluorescence (Fig. 1a) due to preferential loss of the most highly expressing cells (Supplementary Fig. 2 online). Expression of red fluorescent proteins caused many cells to detach from the growth surface (data not shown), further indicating cytotoxicity.

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

The cytotoxicity of DsRed-Express may stem from aggregation¹¹. We measured aggregation using an assay in which bacteria expressing a fluorescent protein were lysed and centrifuged². With DsRed-Express, >50% of the fluorescence was in the pellet (Fig. 1b). Directed evolution of the protein surface (Supplementary Figs. 4 and 5 and Supplementary Tables 2-5 online) yielded DsRed-Express2, which retained the favorable fluorescence properties of DsRed-Express but was as soluble as mEGFP during bacterial extraction (Fig. 1b).

Further mutagenesis produced DsRed-Max, which was ~30% brighter than DsRed-Express2 while retaining high solubility (Fig. 1b). Compared to DsRed-Express2, DsRed-Max had even lower green emission (Supplementary Fig. 6 online), but was slower to mature and more photolabile (Supplementary Fig. 7 and Supplementary Table 1 online). DsRed-Max should be useful when brightness and/or pure red emission are of paramount importance.

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

For mammalian cells, an alternative to plasmid-based expression is retroviral transduction, which yields long-term expression at moderately high levels¹². We used a commercial lentiviral system to compare DsRed-Express2 with five other red fluorescent proteins plus mEGFP. HeLa cells were transduced using the same lentiviral titer for each fluorescent

protein, and then viable fluorescent cells were analyzed by flow cytometry after 3 or 10 days. At Day 3, the fluorescence signal over background was stronger for DsRed-Express2 than for any other red fluorescent protein (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 due to loss of the most highly expressing cells. This interpretation was supported by counting the percentage of viable cells that were fluorescent. Between Day 3 and Day 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, relatively few fluorescent cells were observed 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). Our data confirm that with standard cell culture expression vectors, fluorescent protein cytotoxicity is an important problem that can be overcome by using DsRed-Express2.

We wanted to test DsRed-Express2 in a context where fluorescent protein cytotoxicity had previously hindered an experimental analysis. A suitable system was suggested by a report¹¹ in which murine bone marrow cells were retrovirally transduced to express either EGFP or DsRed-Express, and were 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 while those containing DsRed-Express did not. Here, murine bone marrow cells were retrovirally transduced with DsRed-Express, DsRed-Express2, or EGFP. Viable fluorescent cells were collected by flow cytometry at 87 h post-transduction (Day 0), and then individual cultures were started with 20,000 cells. For each culture we recorded the total cell number and the number of fluorescent cells after 3, 6, and 10 days. 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 the 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 online). 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 Fig. 8 online). Thus, for whole-cell labeling, DsRed-Express2 is the red fluorescent protein of choice.

DsRed-Express2 is likely to be well tolerated in transgenic organisms. Although the previously available red fluorescent proteins 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 hosts¹⁵. For example, when retrovirally transduced mouse hematopoietic stem cells were transplanted into mice, cells expressing EGFP were maintained while those expressing DsRed-Express were progressively lost¹¹. 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 non-cytotoxic whole-cell labels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overcoming the cytotoxicity of red fluorescent proteins. **(a)** Many red fluorescent proteins are cytotoxic. HeLa cells were transiently transfected for constitutive high-level expression of the indicated fluorescent proteins. At daily time points, 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. Bars represent s.e.m. For clarity, the results are shown in four separate panels.

(b) Eliminating the higher-order aggregation of DsRed-Express. For each indicated fluorescent protein, percent fluorescence in the pellet from a bacterial lysate is plotted as mean and s.e.m. for eight independent replicates. The signal with mEGFP represents background in the assay. **(c)** The new fluorescent proteins are minimally cytotoxic 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 plate. Equally sized representative areas are shown. Scale bar, 1 mm. **(d)** The new fluorescent proteins are minimally cytotoxic in mammalian cells. As in (a), HeLa cells were transiently transfected for expression of DsRed-Express2 or DsRed-Max.

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 days 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 PE filter set (red fluorescent proteins). Plotted are the average fluorescence signals relative to the control lentivirus background, with s.e.m. **(b)** The data in (a) were used to determine the percentage of viable cells that were fluorescent. **(c)** In a separate experiment, cells were lentivirally transduced to express the indicated fluorescent proteins. At 3 days 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 at daily intervals. Bars indicate s.e.m.

Figure 3.

Robust growth of murine bone marrow hematopoietic stem and progenitor cells expressing DsRed-Express2. **(a)** Flow cytometry of retrovirally transduced cells. 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 days. Bars represent mean and s.d. Green and red indicate fluorescent cell number, and yellow indicates non-fluorescent cell number. The decrease in total cell number at day 10 is not fluorescent protein-related, but reflects senescence that is routinely observed under *in vitro* conditions¹¹.