Improving the photostability of bright monomeric orange and red fluorescent proteins

Nathan C Shaner[1,5], Michael Z Lin[1,2], Michael R McKeown[1,2], Paul A Steinbach[1,2], Kristin L Hazelwood[4], Michael W Davidson[4] & Roger Y Tsien[1–3]

All organic fluorophores undergo irreversible photobleaching during prolonged illumination. Although fluorescent proteins typically bleach at a substantially slower rate than many small-molecule dyes, in many cases the lack of sufficient photostability remains an important limiting factor for experiments requiring large numbers of images of single cells. Screening methods focusing solely on brightness or wavelength are highly effective in optimizing both properties, but the absence of selective pressure for photostability in such screens leads to unpredictable photobleaching behavior in the resulting fluorescent proteins. Here we describe an assay for screening libraries of fluorescent proteins for enhanced photostability. With this assay, we developed highly photostable variants of mOrange (a wavelength-shifted monomeric derivative of DsRed from *Discosoma* sp.) and TagRFP (a monomeric derivative of eqFP578 from *Entacmaea quadricolor*) that maintain most of the beneficial qualities of the original proteins and perform as reliably as *Aequorea victoria* GFP derivatives in fusion constructs.

Substantial progress has recently been made in developing monomeric or dimeric fluorescent proteins covering the visual spectrum[1–13], but although brightness and wavelength have been primary concerns, photostability has generally been an afterthought (with the notable exception of mTFP1; ref. 12). Consequently, many new fluorescent protein variants have relatively poor photostability. The first-generation monomeric red fluorescent protein, mRFP1 (ref. 1), although reasonably bright, was less photostable than its ancestor, *Discosoma* sp. DsRed[14]. In subsequent generations of mRFP1 variants (the 'mFruits'), we observed serendipitous enhancement in photostability in some variants[2], leading us to believe that it would be possible to apply directed evolution strategies to this property as well.

To extend the utility of fluorescent proteins, having optimized them for many other properties, we developed a screening method that additionally assays photostability in a medium-throughput format during directed evolution. Using a high-intensity light source, we photobleached entire 10-cm plates of bacteria expressing the fluorescent proteins of interest and selected those that maintained the most brightness. This approach allowed us to screen libraries containing up to 100,000 clones reliably with no observed false-positive hits and to select simultaneously for the most photostable mutants that also maintained an acceptable level of fluorescence emission at the desired wavelength, minimizing the tradeoff of desirable properties that frequently results from single-parameter screens. We applied our photostability screening assay to the directed evolution of variants derived from the bright red monomeric red fluorescent protein TagRFP and the fast-bleaching monomeric orange fluorescent protein mOrange. The resulting variants, TagRFP-T and mOrange2, were ninefold and 25-fold more photostable than their respective ancestors, and both made excellent fusion partners when expressed in mammalian cells.

RESULTS

Photostability assay and rationale

To photobleach large numbers of bacterial colonies, we used a solar simulator, which produces a collimated beam approximately 10 cm in diameter with light intensities of 95 or 141 mW/cm^2 with 525–555 (540/30) or 548–588 (568/40) nm bandpass filters, respectively. This intensity, although approximately 100-fold lower than that produced by unattenuated arc lamp illumination and 10^5-fold lower than instantaneous intensities during confocal laser illumination, was sufficient to photobleach the photolabile fluorescent protein mOrange to 50% initial intensity after approximately 10 min. This reasonably short time allowed us to quickly screen bacterial libraries of up to 100,000 clones on plates. We minimized the heating of plates by placing them on a custom-built water-cooled aluminum block. At wavelengths necessary to photobleach orange and red fluorescent proteins, we found no substantial decrease in bacterial viability after 2 h of illumination.

Evolution of a brighter photostable red monomer

To create a better red monomer, we initially undertook a rational design approach, drawing on analysis of mCherry's enhanced photostability and mOrange's higher quantum yield relative to mRFP1. Six generations of directed evolution with constant photostability selection yielded the variant 'mApple', which, though

[1]Department of Pharmacology, [2]Howard Hughes Medical Institute and [3]Department of Chemistry and Biochemistry, University of California at San Diego, 9500 Gilman Drive, La Jolla, California 92093, USA. [4]National High Magnetic Field Laboratory and Department of Biological Science, The Florida State University, 1800 East Paul Dirac Drive, Tallahassee, Florida 32310, USA. [5]Present address: The Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, California 92037, USA. Correspondence should be addressed to R.Y.T. (rtsien@ucsd.edu).

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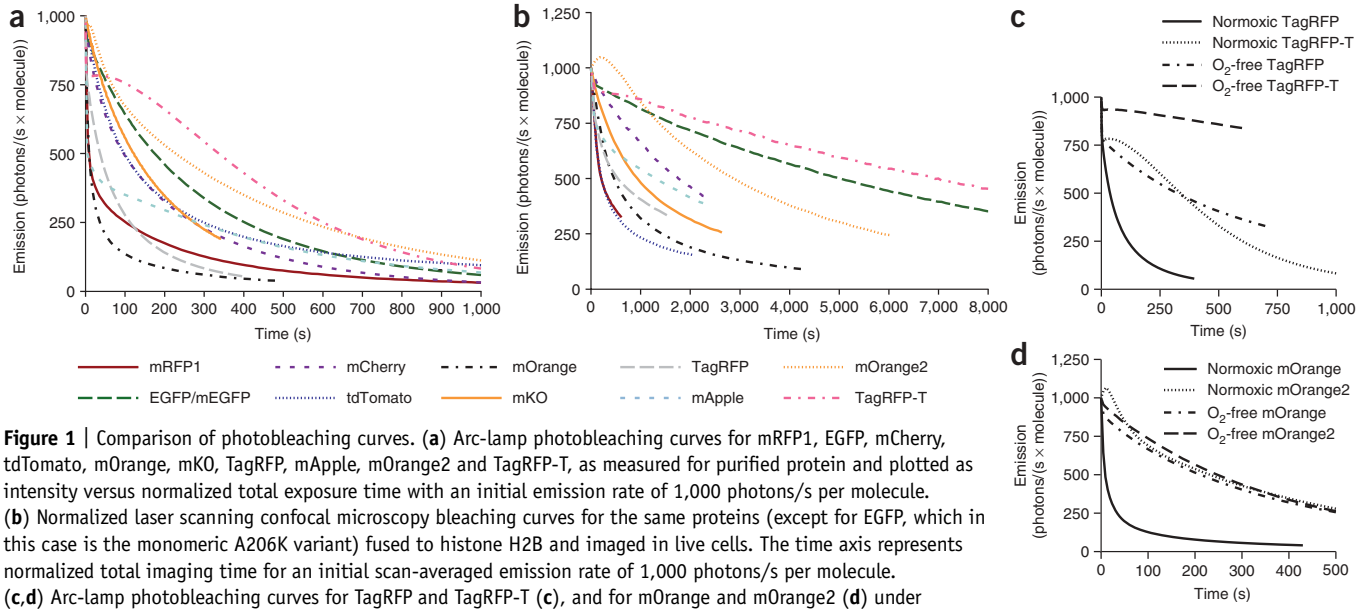

Figure 1 | Comparison of photobleaching curves. (**a**) Arc-lamp photobleaching curves for mRFP1, EGFP, mCherry, tdTomato, mOrange, mKO, TagRFP, mApple, mOrange2 and TagRFP-T, as measured for purified protein and plotted as intensity versus normalized total exposure time with an initial emission rate of 1,000 photons/s per molecule. (**b**) Normalized laser scanning confocal microscopy bleaching curves for the same proteins (except for EGFP, which in this case is the monomeric A206K variant) fused to histone H2B and imaged in live cells. The time axis represents normalized total imaging time for an initial scan-averaged emission rate of 1,000 photons/s per molecule. (**c**,**d**) Arc-lamp photobleaching curves for TagRFP and TagRFP-T (**c**), and for mOrange and mOrange2 (**d**) under normoxic and O_2-free conditions (normalized as in **a**).

substantially brighter than mCherry, displayed complex photoswitching behavior (**Fig. 1** and **Tables 1** and **2**, and **Supplementary Fig. 1** and **Supplementary Note 1** online). This behavior was more pronounced with continuous wide-field than with laser-scanning illumination and could be largely eliminated by excitation at alternate wavelengths or by intermittent illumination. However, given our later results using the brighter TagRFP as starting material, we chose not to pursue mApple any further.

Although the recently developed orange-red monomer TagRFP[13] exhibits remarkable brightness, we have found that its photostability is still far from optimal. In both our standard arc-lamp photobleaching and laser-scanning confocal assays, we determined that TagRFP bleaches approximately threefold faster than mCherry (**Fig. 1a**,**b** and **Table 1**). Thus, we chose this protein as another starting point for improvement of photostability. We first attempted rational design of a mutant library guided by the crystal structure of the closely-related protein eqFP611 (ref. 15). With the rationale that chromophore-interacting residues could influence photostability, we performed saturation mutagenesis of Ser158 and Leu199, two residues proximal to the TagRFP chromophore. We then screened this library in bacteria with our solar simulator–based assay, using the 540/30 nm bandpass filter and exposure times of 120 min per plate, imaging the plates before and after bleaching to select those colonies that displayed high absolute brightness and a high ratio of post-bleach to pre-bleach fluorescence emission.

From this directed library, we identified one clone, TagRFP S158T (designated TagRFP-T), which had a photobleaching half-time of 337 s by our standard assay, making it approximately ninefold more photostable than TagRFP (**Fig. 1a–c** and **Table 1**). TagRFP-T, which we further modified by appending GFP-like N and C termini, possesses identical excitation and emission

Table 1 | Physical and optical properties of new photostable fluorescent protein variants

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient (M^{-1} cm^{-1})	Fluorescence quantum yield	Brightness[a]	pK_a	$t_{1/2}$ for maturation at 37 °C	$t_{1/2}$ bleach (arc lamp)[b] (s)	$t_{1/2}$ bleach (O_2-free)[c] (s)	$t_{1/2}$ bleach (confocal)[d] (s)
mRFP1	584	607	50,000	0.25	13	4.5	<1 h	8.7	ND[e]	210
mCherry	587	610	72,000	0.22	16	<4.5	15 min	96	ND	1,800
mOrange	548	562	71,000	0.69	49	6.5	2.5 h	9.0	250	460
DsRed	558	583	75,000	0.79	59	4.7	10 h	326	ND	ND
tdTomato	554	581	138,000	0.69	95	4.7	60 min	98	ND	210
mKO	548	559	51,600	0.60	31	5.0	4.5 h	122	ND	930
TagRFP[f]	555	584	98,000	0.41	40	3.1	100 min	37	323	550
EGFP or mEGFP	488	507	56,000	0.60	34	6.0	ND	174	ND	5,000
mOrange2	549	565	58,000	0.60	35	6.5	4.5 h	228	228	2,900
mApple	568	592	75,000	0.49	37	6.5	30 min	4.8	ND	1,300
TagRFP-T	555	584	81,000	0.41	33	4.6	100 min	337	>>600	6,900

[a]Brightness of fully mature protein, (extinction coefficient × quantum yield)/1,000. [b]Time to bleach to 50% emission intensity under arc-lamp illumination, at an illumination level that causes each molecule to emit 1,000 photons/s initially, as measured in our lab. See reference 16 for details. [c]With arc lamp illumination, equilibrated under O_2-free conditions. [d]Time to bleach to 50% emission intensity measured during laser scanning confocal microscopy, at an average illumination level over the scanned area that causes each molecule to emit an average 1,000 photons/s initially, as measured in our lab. A 543-nm laser line was used for all proteins except mEGFP, which was bleached with a 488-nm laser (see **Supplementary Methods** for detailed description of normalization). [e]ND, not determined. [f]All measurements were performed in our lab.

Table 2 | Mutations of new photostable fluorescent protein variants

Protein	Mutations
mApple	mOrange R17H,G40A,T66M,A71V,V73I,K92R,V104I,V105I,T106H, T108N,E117V,S147E,G159S,M163K,T174A,S175A,G196D,T202V
mOrange2	mOrange Q64H,F99Y,E160K,G196D
TagRFP-T	TagRFP S158T

wavelengths, quantum yield and maturation time to TagRFP, with only a slightly lower extinction coefficient (81,000 versus 98,000 M^{-1} cm^{-1}) and a higher fluorescence pK_a, the pH value at which the fluorescent protein exhibits half-maximal fluorescence emission (4.6 versus 3.1). We expect that the benefit of increased photostability should offset the small decrease in brightness and increase in acid sensitivity in most applications. Additionally, TagRFP-T matures to apparent completion and has virtually no emission in the green region of the spectrum (**Supplementary Fig. 1**), making it suitable for co-imaging with green fluorescent proteins. We verified that TagRFP-T remains monomeric by gel filtration (data not shown). Because the S158T mutation is in the interior of the folded protein, we anticipated that TagRFP-T would perform nearly identically to TagRFP when used as a fusion tag. Indeed, live-cell imaging confirmed that TagRFP-T does not interfere with localization of any fusions tested (**Fig. 2**).

Photobleaching of TagRFP and TagRFP-T under oxygen-free conditions revealed that TagRFP-T's photobleaching remains oxygen-sensitive (**Fig. 1c** and **Table 1**). However, the oxygen-free bleaching half-time for TagRFP is similar to the ambient oxygen bleaching half-time for TagRFP-T. We next compared TagRFP and TagRFP-T as fusions to histone H2B expressed in living cells under confocal illumination (**Fig. 1b** and **Table 1**). TagRFP-T had a photobleaching half-time approximately ninefold greater than that of TagRFP, consistent with the results obtained for purified proteins under continuous wide-field illumination.

Evolution of a photostable orange monomer

We next attempted to engineer a photostable variant of mOrange, which is the brightest of the previously engineered mRFP1 variants but exhibits relatively fast bleaching. Because substitutions at position 163 improved photostability during the evolution of mCherry and mApple, we initially tested the M163Q mutant of mOrange, but found that improved photostability was accompanied by undesirable decreases in quantum yield and maturation efficiency. The M163K mutant of mOrange exhibited enhanced photostability and matured very efficiently, but suffered from increased acid sensitivity (pK_a of ~7.5). Because another orange fluorescent protein, mKO (derived from *Fungia concinna*)[6], is both highly photostable[16] and possesses a methionine at the position equivalent to 163, we reasoned that other pathways must exist for increasing photostability.

To explore alternative photostability-enhancement evolution pathways, we used iterative random and directed mutagenesis and selection using the solar simulator. Initially we screened a randomly mutagenized library of mOrange by photobleaching with 540/30 nm light for 15–20 min per plate (a time sufficient to bleach mOrange to ~25% of its initial brightness) and selecting the brightest post-bleach clones by eye. This screen identified a single

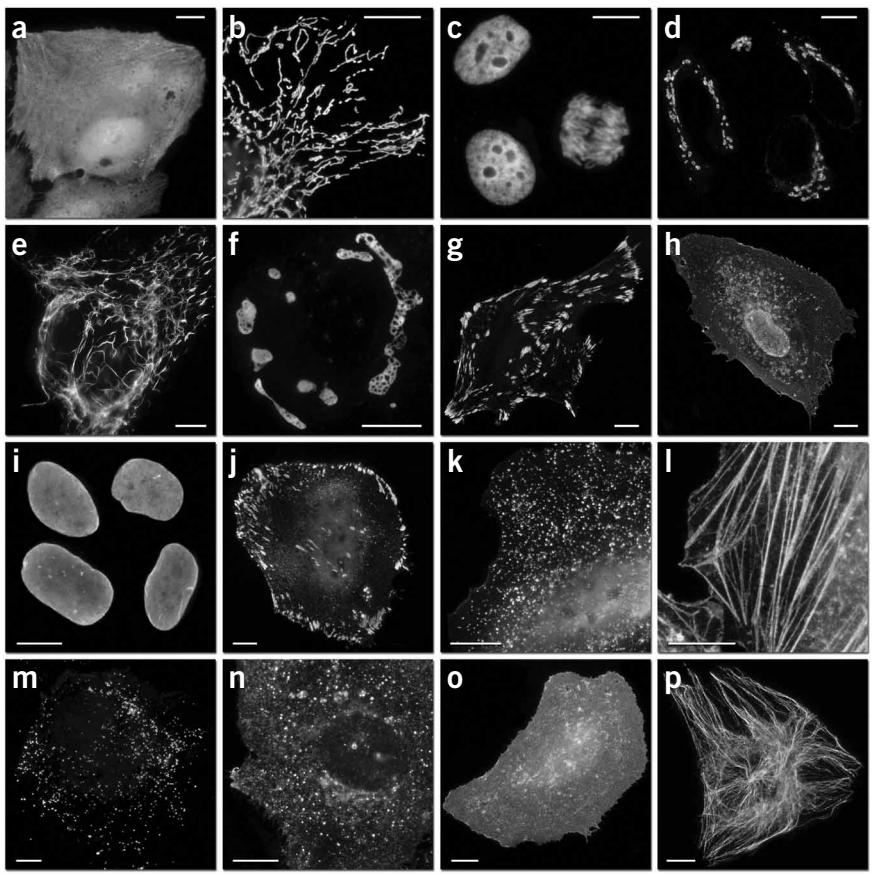

Figure 2 | Fluorescence imaging of TagRFP-T subcellular targeting fusions. (**a**–**g**) N-terminal fusion constructs (linker amino acid length indicated by the numbers): TagRFP-T–N1 (**a**; N-terminal fusion cloning vector; expression in nucleus and cytoplasm with no specific localization); TagRFP-T–7–cytochrome *c* oxidase (**b**; mitochondria human cytochrome *c* oxidase subunit VIII); TagRFP-T–6–histone H2B (**c**; human; showing two interphase nuclei and one nucleus in early anaphase); TagRFP-T–7–β-1,4-galactosyltransferase (**d**; golgi; N-terminal 81 amino acids of human β-1,4-galactosyltransferase); TagRFP-T–7-vimentin (**e**; human); TagRFP-T–7-Cx43 (**f**; rat α-1 connexin-43); and TagRFP-T–7-zyxin (**g**; human). (**h**–**p**) C-terminal fusion constructs (linker amino acid length indicated by the numbers): annexin (A4)–12–TagRFP-T (**h**; human; illustrated with ionomycin-induced translocation to the plasma and nuclear membranes); lamin B1–10–TagRFP-T (**i**; human); vinculin-23–TagRFP-T (**j**; human); clathrin light chain–15–TagRFP-T (**k**; human); β-actin–7–TagRFP-T (**l**; human); PTS1-2–TagRFP-T (**m**; peroximal targeting signal 1); RhoB-15–TagRFP-T (**n**; human RhoB GTPase with an N-terminal c-Myc epitope tag; endosome targeting); farnesyl-5–TagRFP-T (**o**; 20-amino-acid farnesylation signal from c-Ha-Ras); and β-tubulin–6–TagRFP-T (**p**; human). All TagRFP-T fusion vectors were expressed in HeLa (CCL-2) cells. Scale bars, 10 μm.

clone, mOrange F99Y, which had approximately twofold improved photostability (data not shown). Saturation mutagenesis of residue 99—and residues 97 and 163, which we imagined could have synergistic interactions with residue 99—did not yield additional improvements.

We then constructed a randomly mutagenized library of mOrange F99Y and screened with a longer illumination time of 40 min per plate. This round of screening identified an additional mutation, Q64H, which conferred about a tenfold increase in photostability over the mOrange F99Y single mutant. Again, saturation mutagenesis of residues 64 and 99 along with neighboring residues 97 and 163 did not produce clones that were improved over the original clone identified in the random screen. Additionally, we found that the Q64H mutation alone did not confer substantially enhanced photostability but required the presence of the F99Y mutation (data not shown). Two additional rounds of directed evolution with continued selection for photostability (540/30 nm filter, 40 min per plate) improved the folding efficiency with mutations E160K and G196D, giving the final clone, mOrange2 (**Table 2**).

The highly desirable increase in photostability achieved in mOrange2 is balanced by a modest decrease in quantum yield (0.60 versus 0.69) and extinction coefficient (58,000 versus 72,000 M^{-1} cm^{-1}), together corresponding to a 30% decrease in brightness compared to mOrange. It also exhibits slightly shifted excitation and emission peaks (549 nm and 565 nm) and an increased maturation half-time (4.5 h versus 2.5 h; **Table 1**). However, its photostability under arc-lamp illumination is over 25-fold greater than that of mOrange (**Fig. 1d**), making it nearly twice as photostable as mKO[6], the previously most photostable known orange monomer[16], approximately sixfold more photostable than TagRFP[13] and about 1.3-fold more photostable than enhanced GFP (EGFP)[16] (**Fig. 1** and **Table 2**). During laser-scanning confocal imaging, mOrange2 was approximately sixfold more photostable than mOrange and threefold more photostable than mKO (**Fig. 1b**). Notably, the brightness and maturation time of mOrange2 are quite similar to those for mKO. mOrange2 remains acid-sensitive with a pK_a of 6.5, making it undesirable for targeting to acidic compartments, but attractive as a possible marker for exocytosis or other pH-variable processes[17]. Also, because it contains a small fraction of immature (but nonfluorescent) chromophore (**Supplementary Fig. 1**), mOrange2 may not be an ideal FRET acceptor. As with TagRFP-T, we verified that mOrange2 remained monomeric using gel filtration (data not shown). We then investigated the role of the key photostability-enhancing mutations present in mOrange2, tested it in a wide range of fusion constructs, and compared its performance with that of mKO and tdTomato (**Fig. 3** and **Supplementary Note 2** online).

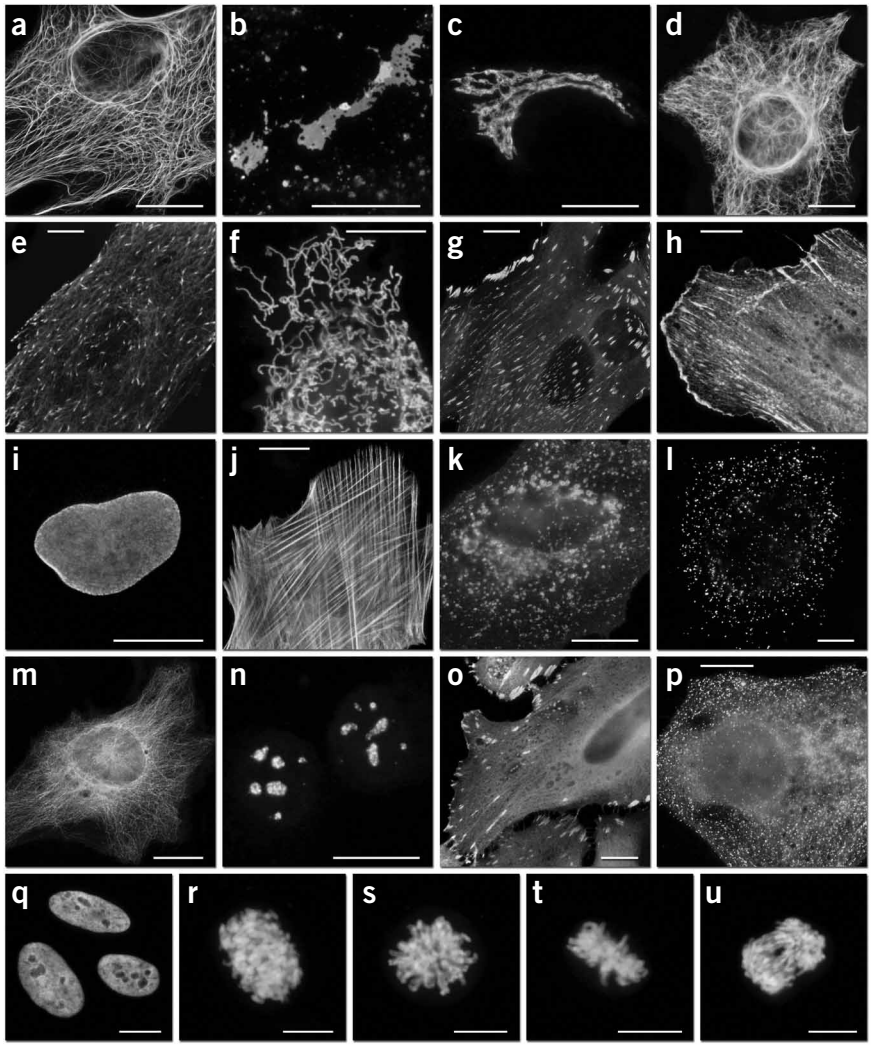

Figure 3 | Widefield fluorescence imaging of mOrange2 subcellular targeting fusions. (**a**–**h**) N-terminal fusion constructs (linker amino acid length indicated by the numbers): mOrange2-17-keratin (**a**; human cytokeratin 18); mOrange2-7-Cx26 (**b**; rat β-2 connexin-26); mOrange2-7–β-1,4-galactosyltransferase (**c**; golgi; N-terminal 81 amino acids of human β-1,4-galactosyltransferase); mOrange2-7-vimentin (**d**; human); mOrange2-7-EB3 (**e**; human microtubule-associated protein; RP/EB family); mOrange2-7–cytochrome *c* oxidase (**f**; mitochondria; human cytochrome *c* oxidase subunit VIII); mOrange2-22-paxillin (**g**; chicken); and mOrange2-19–α-actinin (**h**; human non-muscle). (**i**–**p**) C-terminal fusion constructs (linker amino acid length indicated by the numbers): lamin B1–10-mOrange2 (**i**; human); β-actin–7-mOrange2 (**j**; human); glycoprotein 1–20-mOrange2 (**k**; rat lysosomal membrane glycoprotein 1); peroxisomal targeting signal 1–2-mOrange2 (**l**); β-tubulin–6-mOrange2 (**m**; human); fibrillarin-7-mOrange2 (**n**; human); vinculin-23-mOrange2 (**o**; human); and clathrin light chain-15-mOrange2 (**p**; human). (**q**–**u**) Laser scanning confocal images of HeLa cells expressing histone H2B–6-mOrange2 (N-terminal fusion; human) progressing through interphase (**q**), prophase (**r**), prometaphase (**s**), metaphase (**t**) and early anaphase (**u**). The cell line used for expressing mOrange2 fusion vectors was Gray fox lung fibroblast cells (FoLu) in **e** and **j**, and human cervical adenocarcinoma cells (HeLa) in the remaining panels. Scale bars, 10 μm.

Evaluation of reversible photoswitching

Because of concerns that our screening method might select for photoswitching behavior, we tested our selected variants as well as other commonly used fluorescent proteins using both widefield and confocal imaging. Nearly all had some degree of reversible photoswitching, which we observed as a recovery of up to 100% of pre-bleach fluorescence intensity when the fluorescent protein was bleached to ~50% of its initial intensity and then observed again after 1–2 min without illumination. In fact, several commonly used *A. victoria* GFP variants including EGFP, Cerulean and Venus, displayed reversible photoswitching[18] more severe than that observed for the variants we identified. A summary table of the results of these experiments along with representative traces for TagRFP, TagRFP-T, EGFP and Cerulean are available in **Supplementary Note 3** online. These results suggest that our screen is not selecting specifically for photoswitching, which is no worse in the new proteins (except for mApple) than in well-established fluorescent proteins.

Although our observation of reversible photoswitching in such a broad range of fluorescent proteins certainly raises concerns about the potential for previously undetected experimental artifacts, it is beyond the scope of this study to determine how common or severe this phenomenon may be. Of particular concern is the implication that fluorescence recovery after photobleaching experiments may be prone to artifacts that would confound data interpretation. We performed a limited evaluation of this possibility using histone H2B fusions to EGFP and EYFP expressed in mammalian cells and imaged on a laser-scanning confocal microscope. When we bleached these proteins to near completion with full laser power and then observed for recovery, we observed a negligible amount of reversible photoswitching (data not shown). However, an in-depth investigation is warranted to rule out such an effect in other fluorescent proteins and under more varied experimental conditions.

DISCUSSION

Although the precise kinetics of photobleaching for a given fluorescent protein are strongly dependent on illumination intensity and temporal regimen, we found that improvements in photostability at ~0.1 W/cm^2 usually qualitatively predict improved performance under typical conditions for wide-field and laser scanning microscopy. The exceptions were mApple's reversible photoswitching (**Supplementary Note 1**) and tdTomato's poor performance under laser scanning confocal illumination (**Fig. 1b**). Also, our screen used bacteria to express fluorescent protein libraries, but all proteins produced from these studies behaved similarly when later tested in purified form or expressed in mammalian cells, consistent with our previous experience.

Fluorescent proteins had been photobleached using an array of LEDs during the evolution of mTFP1 to select against unacceptable photolability or photoswitching, resulting in a protein with a bleaching half-time 110 s[12]. We applied photostability as a primary criterion to improve multiple fluorescent proteins, and our results demonstrate that high photostability is a selectable phenotype. Moreover, a solar simulator takes advantage of the strong mercury lines at 546, 577 and 579 nm and allows greater flexibility in the choice of excitation wavelength than would be possible with LEDs.

Although it is difficult to draw strong conclusions about exact mechanisms of photobleaching from the mutations that confer photostability to mOrange2, specific regions proximal to the chromophore appear to influence the modes of photobleaching the protein is able to undergo. DsRed, when illuminated by a 532-nm pulsed laser, undergoes decarboxylation of Glu215, as well as *cis*-to-*trans* isomerization of the chromophore[19]. Such chromophore isomerization has been implicated in the photoswitching behavior of Kindling fluorescent protein (KFP)[20,21] and Dronpa[5,22] as well as predecessors to mTFP1 (refs. 12 and 23). Decarboxylation of the corresponding glutamate (position 222) in *A. victoria* GFP also leads to changes in optical properties[24–26]. However, our observation that oxidation is important in mOrange, TagRFP and TagRFP-T photobleaching suggests that chromophore isomerization and Glu215 decarboxylation may have only a minor role for such proteins under normoxic conditions. Additionally, we found no evidence by mass spectrometry that photobleaching using the solar simulator led to any detectable decarboxylation of Glu215 in mOrange (data not shown). Under some conditions mOrange2 shows an initial photoactivation of about 5% (**Fig. 1a,d**) before bleaching takes over. At present we have no molecular explanation for this effect or the reversible photoswitching that is common to most fluorescent proteins (**Supplementary Note 3**).

For mRFP1 variants, we observed the importance of residue 163 in influencing photostability (**Supplementary Note 1**) but also observed somewhat context-specific effects of residue 163 and surrounding residues on different wavelength-shifted variants. This region, composed of residues 64, 97, 99 and 163, appears to be important in determining photostability. However, of these, only residue 163 is in direct contact with the chromophore. It may be that the mutations Q64H and F99Y together lead to a rearrangement of the other side chains in the vicinity of the chromophore so as to hinder a critical oxidation that leads to loss of fluorescence.

Discrepancies in tubulin and connexin localization (**Supplementary Note 2**) when fused to mOrange2 versus mKO or tdTomato can probably be attributed to the three-dimensional structure of the fluorescent protein and potential steric hindrance in the fusions. mOrange2 contains extended N and C termini derived from EGFP to improve performance in fusions, whereas the much shorter protein, mKO (218 versus 236 amino acids), may experience steric interferences that lead to poorer performance in similar fusions. The fused dimeric character of tdTomato effectively doubles its size compared to the monomeric orange fluorescent proteins, so steric hindrance is the most likely culprit in preventing tubulin localization. For most fusions, however, we observed little or no difference in performance between mOrange2 and mKO, suggesting that many proteins are more tolerant of fusion partners than tubulin or connexins.

Though it already possessed reasonably good photostability, TagRFP was still amenable to improvements by our photostability selection method. From a saturation-mutagenesis library of two chromophore-proximal residues (consisting of 400 independent clones), we selected a single clone with substantially enhanced photostability. The selected mutant, TagRFP-T, should prove to be a very useful addition to the fluorescent protein arsenal, as it is the most photostable monomeric fluorescent protein of any color yet described under both arc-lamp and confocal laser illumination.

As the applications of genetically encoded fluorescent markers continue to diversify and become more complex, the demand for greater photostability than is now available in fluorescent proteins has likewise continued to grow. We expect our screening method to

be applicable to any of the existing fluorescent proteins and, with modifications, to be useful in selecting for more efficient photoconvertible and photoswitchable fluorescent proteins as well[3,5,10,20,27–31]. Possible enhancements to this selection technique could include time-lapse imaging of bacterial plates during bleaching to enable direct selection for kinetics (independent of absolute brightness) and the use of higher-intensity illumination from other light sources (such as lasers) during screening to select for or against nonlinear photobleaching behavior. Ideally, a selection scheme that allows true simulation of microscopic imaging light intensities while maintaining a medium-to-high throughput should allow selection of fluorescent proteins with the most beneficial properties for imaging applications.

METHODS

Mutagenesis. As the initial templates for library construction by random mutagenesis we used cDNA encoding mOrange[2] and TagRFP (Evrogen)[13], both of which had been previously human codon–optimized. We performed error-prone PCR using the GeneMorph II kit (Stratagene) following the manufacturer's protocol, using primers containing *Bam*HI and *Eco*RI sites for mOrange variants or *Bam*HI and *Bsr*GI sites for TagRFP variants. We digested products of error-prone PCR products with appropriate restriction enzymes and ligated the fragments into a modified pBAD vector (Invitrogen) or a constitutive bacterial expression vector pNCS, both of which encode an N-terminal 6His tag and linker identical to that found in pRSET B (Invitrogen). We performed site-directed mutagenesis using the QuikChange II kit (Stratagene) following the manufacturer's protocol or by overlap-extension PCR. Sequences for all primers used in this study are available in **Supplementary Methods** online. We transformed chemically competent or electrocompetent *Escherichia coli* strain LMG194 (Invitrogen) cells with libraries and grew them overnight at 37 °C on LB-agar supplemented with 50 μg/ml ampicillin (Sigma) and 0.02% (wt/vol) L-arabinose (Fluka) (for pBAD-based libraries).

Library screening. For each round of random mutagenesis, we screened 20,000–100,000 colonies (10–50 plates of bacteria), a number sufficient to sample all possible single-site mutants and a limited number of double mutants. For each round of site-directed mutagenesis, we screened approximately threefold more colonies than the expected library diversity (for example, 1,200 colonies for a 400-member library) to ensure full coverage. We photobleached whole plates of bacteria for 10–120 min (determined empirically for each round of directed evolution) on a Spectra-Physics 92191–1000 solar simulator with a 1,600 W mercury arc lamp equipped with two Spectra-Physics SP66239-3767 dichroic mirrors to remove infrared and ultraviolet wavelengths. Remaining light was filtered through 10-cm square bandpass filters (Chroma Technology Corp.) appropriate to the fluorescent protein being bleached (540/30 nm (B540/30; 525–555 nm) for mOrange- and TagRFP-based libraries or 568/40 nm (B568/40; 548–588 nm) for mApple libraries). We measured final light intensities produced by the solar simulator by a miniature integrating-sphere detector (SPD024 head and ILC1700 meter, International Light Corp.) to be 95 mW/cm^2 for the 540/30 filter and 141 mW/cm^2 for the 568/40 filter. We maintained the temperature of the bacterial plates at 20 °C during solar simulator bleaching using a home-built water-cooled aluminum block. For mOrange mutant selection, we examined the plates by eye as previously described[32] using a 150 W xenon lamp equipped with a 540/30 nm excitation filter and fiber optic light guides to illuminate the plates and 575 nm long pass filter to visualize emission. For TagRFP mutant selection, we imaged the plates before and after bleaching on an imaging system (UVP) using 535/45 nm (512.5–557.5 nm) excitation and 605/70 nm (570–640 nm) emission filters. In either case, we grew colonies that maintained bright fluorescence after photobleaching and/or those with high post- to pre-bleach fluorescence ratios for 8 h in 2 ml of LB medium supplemented with 100 μg/ml ampicillin and then increased the culture volume to 4 ml with additional LB supplemented with ampicillin and 0.2% (wt/vol) L-arabinose to induce fluorescent protein expression and grew the cultures overnight. We extracted protein from a fraction of each cell pellet with B-PER II (Pierce) and obtained spectra using a Safire 96-well plate reader with monochromators (Tecan). When screening for photostable variants, we obtained spectra before and after photobleaching extracted protein on the solar simulator. We extracted plasmid DNA from the remaining cell pellet with a miniprep kit (Qiagen) and used it for sequencing.

Protein production and characterization. We expressed fluorescent proteins from pBAD vectors in *E. coli* strain LMG194, purified them on Ni-NTA agarose (Qiagen) and characterized them as described[2]. Photobleaching measurements were performed on aqueous droplets of purified protein under oil as described[2,16]. To determine whether the presence of molecular oxygen influenced bleaching, we performed our standard bleaching experiment before and after equilibrating the entire bleaching apparatus under humidified N_2.

Additional methods. Primer list, descriptions of mass spectrometry analysis, mammalian expression vectors, live-cell imaging and laser scanning confocal microscopy live-cell photobleaching are available in **Supplementary Methods**.

Accession numbers. GenBank: DQ336159 (mOrange2), DQ336160 (mApple) and EU582019 (TagRFP-T).

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

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AUTHOR CONTRIBUTIONS

N.C.S. designed the photostability selection protocol, performed all directed evolution and physical characterization of mApple and mOrange2, analyzed and organized all data collected by other authors, and prepared the manuscript; M.Z.L. and M.R.M. performed directed evolution and physical characterization of TagRFP-T; P.A.S. designed the home-built components of the solar simulator apparatus and performed photobleaching measurements of purified proteins; K.L.H. and M.W.D. constructed mammalian expression vectors and performed all microscopy experiments involving live cells; R.Y.T. contributed to conceptual development, data analysis and manuscript preparation; all authors contributed to editing the manuscript.

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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