Expression-Enhanced Fluorescent Proteins Based on Enhanced Green Fluorescent Protein for Super-resolution Microscopy

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ABSTRACT “Smart fluorophores”, such as reversibly switchable fluorescent proteins, are crucial for advanced fluorescence imaging. However, only a limited number of such labels is available, and many display reduced biological performance compared to more classical variants. We present the development of robustly photoswitchable variants of enhanced green fluorescent protein (EGFP), named rsGreens, that display up to 30-fold higher fluorescence in E. coli colonies grown at 37 °C and more than 4-fold higher fluorescence when expressed in HEK293T cells compared to their ancestor protein rsEGFP. This enhancement is not due to an intrinsic increase in the fluorescence brightness of the probes, but rather due to enhanced expression levels that allow many more probe molecules to be functional at any given time. We developed rsGreens displaying a range of photoswitching kinetics and show how these can be used for multimodal diffraction-unlimited fluorescence imaging such as pcSOFI and RESOLFT, achieving a spatial resolution of ∼70 nm. By determining the first ever crystal structures of a negative reversibly switchable FP derived from Aequorea victoria in both the “on”- and “off”-conformation we were able to confirm the presence of a cis—trans isomerization and provide further insights into the mechanisms underlying the photochromism. Our work demonstrates that genetically encoded “smart fluorophores” can be readily optimized for biological performance and provides a practical strategy for developing maturation- and stability-enhanced photochromic fluorescent proteins.

KEYWORDS: fluorescent proteins - reversible photoswitching - super-resolution fluorescence microscopy - SOFI - RESOLFT - crystal structure determination - rsEGFP - superfolder

Fluorescent proteins (FPs) enable the minimally invasive labeling of intracellular structures in live systems. The discovery and development of “smart photoactive FPs”, with features such as irreversible photoactivation and photoconversion or reversible photoswitching, allowed the development of diffraction-unlimited imaging techniques such as (f)PALM (fluorescence) photoactivated localization microscopy), RESOLFT (reversible saturable optical fluorescence transitions), and (pc)SOFI ((photochromic) stochastic optical fluctuation imaging). These techniques strongly rely on the performance of the fluorophores, and considerable efforts have therefore been dedicated to create optimized “smart labels”. This is exemplified by the continuous optimization and diversification of the EosFP family or the development of Dronpa mutants with different or added photo-physical properties. Probes that combine multiple “smart” behaviors have also been engineered.

On the whole, however, the general acceptance of the FP-based “smart labels” has not quite risen to the high expectations set by the many applications they enable. In some cases this is due to concerns surrounding the biological compatibility of the labels, meaning that the label may interfere with the functioning of the system under study. Many end-users have large bodies of
work, resources, and efforts invested in using one or just a few different fluorescent proteins and are justifi-
cably reluctant to re-engineer their samples using the latest FPs out of concern that this major effort may lead to inconsistent results if the new label has a different biological compatibility. To some extent these concerns are warranted: many FPs derive from corals, which display low sequence homology with the widely used FPs derived from the A. victoria jellyfish. In some cases, “smart FPs” also display a reduced biological performance compared to their ancestor proteins, simply because the evolutionary approach used in the majority of FP development makes it difficult to optimize for multiple target parameters simultaneously, as this converts an essentially one-dimensional search (e.g., optimizing photoswitching) into an n-dimensional search (optimizing photoswitching, brightness, etc).

A recent addition to the list of “smart labels” is rsEGFP (reversibly switchable enhanced green fluorescent protein),26 which displays very fast photoswitching and a high resistance to photodestruction. A direct descendant of monomeric EGFP (mEGFP), rsEGFP holds great promise for the wide range of applications in which other (Aequorea victoria) avGFP-based proteins are routinely used, driven by several advantages of avGFP-based labels such as a low toxicity and a monomeric character.27–32 Intrigued by the optical properties of rsEGFP,26 we set out to use it for our own imaging. However, in our hands mammalian cells cultured at 37 °C and expressing rsEGFP displayed a total fluorescence signal that was much lower than that of cells expressing EGFP, suggesting that many fewer rsEGFP molecules became functional when expressed under identical conditions. We reasoned that this propensity not only limited imaging experiments at this temperature but was also likely to place considerable stress on the expressing cells as a result of the prevalent protein misfolding. We therefore set out to generate expression-enhanced versions of rsEGFP, while also preserving or even creating a range of different photochromic behaviors.

In this work we demonstrate what we believe to be a general strategy to dramatically enhance the folding and maturation of reversibly switchable fluorescent proteins (RSFPs), using a combined rational and evolutionary approach. We shed light on the structural aspects of these improvements using X-ray crystallography and show that the photochromic properties can be directly modulated using targeted mutations, while preserving improvements in the robust formation of the functional label. Given the highly conserved mechanism of photoswitching,33,34 our insights can be applied in the engineering of other photochromic FPs and may expand the range of “smart labels” available for imaging.35

Since the overall exterior structure is almost identical to EGFP, our rsGreens can ideally be introduced in any construct where EGFP is currently used, with minimal perturbations. In combination with its robust photochromic properties, this should expand the range of experiments amenable to diffraction-unlimited imaging and other advanced fluorescence imaging techniques.

During the initial stages of this work, a faster switching mutant of rsEGFP, rsEGFP2, was published and reported to have improved maturation.36 In what follows we include rsEGFP2 for comparison with our rsGreens.

RESULTS AND DISCUSSION

While investigating the use of rsEGFP for our own imaging, we noticed only very dim fluorescence when the protein was expressed in HeLa cells at 37 °C, severely impeding our imaging. Similarly, E. coli colonies transformed with rsEGFP-encoding expression plasmids barely became fluorescent when grown at 37 °C. Incubating colonies at 4 °C for prolonged periods of time (3–4 days to 2 weeks) resulted in a brighter fluorescence emission, as did growing the E. coli at lower temperatures (∼20 °C), although it was still below what could be expected given the spectroscopic properties of rsEGFP. In all cases, the detectable fluorescence was low compared to other known green RSFPs such as Dronpa16 and members of the mGeos14 series (data not shown). We concluded that rsEGFP displays a strongly impaired folding/maturation pathway and/or reduced structural stability.

Mutagenesis. We set out to improve the rsEGFP formation and stability while preserving the photoswitching properties. For this purpose, the fluorescence formation of rsEGFP was first further disturbed by creating a genetic fusion to an interfering bait peptide, bullfrog red cell ferritin subunit-H.37,38 This poorly soluble peptide imposes an additional load on the folding of its fusion partner. Expression of this fusion construct (pRSETb:: ferritin-rsEGFP) in E. coli at 37 °C resulted in an even more reduced fluorescence signal (Figure S1, Supporting Information). We used this ferritin fusion construct for all mutagenesis and initial screening and afterward subcloned the resulting fluorescent proteins to an empty plasmid for expression and all further characterization. Initially, we introduced the folding-enhancing mutations reported during the evolution of cycle-3 GFP39 and superfolder GFP38 (sfGFP), resulting in a mutant with seven amino acid replacements, named rsGreen0.6 (rsEGFP-S30R/Y39N/F99S/N105T/Y145F/M153T/I171V). This bright and switchable clone was subjected to further site-directed mutagenesis, this time focusing on mutations reported in other works (Table S1 provides a complete list of the tested mutations, Supporting Information). This resulted in an additional two mutations (S72A/T105Y) that further increased in-colony fluorescence compared to rsGreen0.6. We named this rationally improved switchable FP rsGreen0.7 (rsEGFP-S30R/Y39N/S72A/F99S/N105Y/Y145F/M153T/I171V).
We took care not to mutate any of the residues reported to be important for the photochromicity (L69, A150, S163, and S205). The monomerization mutation A206K, introduced during the development of rsEGFP, was also kept to ensure the monomeric character of the FPs.

DNA shuffling of rsGreen0.7 and rsEGFP did not identify redundant mutations. Although rsGreen0.7 is an efficient photoswitcher, further characterization revealed a slightly slower off-switching and increased susceptibility to photoswitching fatigue compared to rsEGFP. We attributed this to a single mutation, Y145F. The photoswitching behavior of rsEGFP was restored in rsGreen0.7b (rsGreen0.7-F145Y), but at a loss of fluorescence intensity in *E. coli* colonies compared to rsGreen0.7.

We continued the directed evolution with several rounds of random mutagenesis, starting from the clone with the highest in-colony fluorescence, rsGreen0.7. Screening efforts were primarily focused on a more efficient photoswitching performance, this time preserving the fluorescence level. After two rounds of mutagenesis and screening of (50–100) × 10^3 colonies, we selected a single clone, named rsGreen0.8 (rsGreen0.7-K162R/A227S), and two additional interesting clones. In an attempt to further improve rsGreen0.8, we directly introduced the beneficial mutations from the other two clones, yielding rsGreen0.9 (rsGreen0.8-L44M/K101E/H169L), which showed an improved photoswitching contrast and speed. Two final clones were identified after screening of approximately 35 × 10^3 colonies from a final random mutagenesis library. rsGreen1 (rsGreen0.9-N149D) provided improved in-colony fluorescence, while rsGreenF (rsGreen0.9-F145L) displayed both improved in-colony fluorescence and faster photoswitching with a higher contrast (or lower residual off-state fluorescence) compared to rsGreen0.9. Figure S2 (Supporting Information) shows an amino acid sequence alignment of all rsGreens with EGFP, sfGFp, rsGFp, and rsEGFP2.

During the mutagenesis of rsEGFP to the different rsGreens, a faster switching mutant of rsEGFP, rsEGFP2, was published and reported to have improved maturation.36 We therefore included both rsEGFP and rsEGFP2 as benchmarks during the characterization of the rsGreens.

**Fluorescence Intensity in Prokaryotic and Eukaryotic Cells.**

After the mutagenesis and initial screening of the ferritin-FP constructs, all rsGreens were subcloned in pRSETb. Expression of the generated FPs without fusion partners in *E. coli* at 37 °C showed fluorescence intensity increases in line with those of the ferritin-FP constructs used during the mutagenesis. Measured at the colony level, bacterial cells expressing rsGreen0.7 are approximately 20 times brighter than rsEGFP, while bacteria expressing rsGreen0.7b are only 10 times more fluorescent than rsEGFP. Expression of the other members of the rsGreen series in *E. coli* resulted in almost 30-fold more fluorescence emission than the ancestor protein, rsEGFP, and up to 4 times more fluorescence signal than rsEGFP2 (Figure 1A and Table 1: *E. coli* fluorescence). The relative increase in fluorescence intensity was less drastic at lower temperatures, mostly because of an increased performance of rsEGFP. At these lower temperatures, rsEGFP performs almost on par with the rsGreen mutants (Figure 1A).

To verify that this performance increase was not limited to FPs expressed in prokaryotic systems, we transfected HEK293T cells with pcDNA3::FP constructs encoding untargeted expression of the FPs and assayed the overall fluorescence intensity of individual cells using flow cytometry.41 Healthy cells were selected based on morphology and 7-AAD death cell staining. Between 20% and 25% of living cells expressed fluorescent proteins. Once again, all generated mutants performed considerably better than rsEGFP
d, with the increase in cell fluorescence ranging from 2.5 to 4.5 times that of rsEGFP and 1.5 to 3 times that of rsEGFP2 (Figure 1B and Table 1: HEK293T fluorescence). Taken together, our probes thus result in a strong overall increase in fluorescence signal when expressed...
in E. coli and HEK293T cells, with a stronger relative improvement in E. coli.

**Spectroscopic Properties and pH Dependence.** All of the developed proteins are green FPs absorbing maximally between 485 and 489 nm, with maximal emission between 509 and 511 nm (Figure 2 and Table 1: $\lambda_{ex}$ and $\lambda_{em}$). All rsGreens display a characteristic absorbance peak with a maximum between 390 and 396 nm when switched to the nonfluorescent state through irradiation with light (Table 1: $\lambda_{abs\_off}$ and Figure S3, Supporting Information). We determined three different extinction coefficients: the intrinsic extinction coefficient of the on-state chromophore ($\epsilon_{on}$), the overall extinction coefficient at pH 7.4 ($\epsilon_{on\_overall}$), which describes the absorption at the excitation maximum as a function of the total (analytical) FP content at pH 7.4 ($\epsilon_{on\_overall}$/C using in vitro renaturation rates. $\epsilon_{on\_overall}$ Maturation rates measured at 37 °C using in vitro expression. Residual fluorescence of the off-state ([off/on] × $\epsilon_{off}$) for purified proteins and HeLa cells. Residual fluorescence in the off-state after 500 completed switching cycles. Time at which the fluorescence is reduced to half its initial value. Quantum efficiency of OFF-switching and ON-switching. NA = not applicable. ND = not determined. Values between square brackets are taken from the cited literature. The Methods section contains further details on the listed parameters.

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\begin{table}[h]
\centering
\caption{Spectroscopic Properties of EGFP, rsEGFP, rsEGFP2, and the rsGreens} \label{tab:1}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
 & \multicolumn{2}{c|}{EGFP} & \multicolumn{2}{c|}{nEGFP} & \multicolumn{2}{c|}{nGreen} \\
\hline
$\lambda_{ex}$ (nm)$^a$ & 489 & 491 & 478 & 487 & 489 & 485 & 486 & 485 \\
$\lambda_{em}$ (nm)$^b$ & 509 & 510 & 503 & 511 & 511 & 509 & 509 & 511 \\
$\lambda_{abs\_off}$ (nm)$^c$ & NA & 396 & 408 & 396 & 396 & 396 & 396 & 390 \\
$\epsilon_{on}$ (10$^3$ M$^{-1}$ cm$^{-1}$)$^d$ & 54 & 57 & 53 & 62 & 53 & 61 & 66 & 61 & 52 \\
$\epsilon_{on\_overall}$ (10$^3$ M$^{-1}$ cm$^{-1}$)$^e$ & 52 (33)$^f$ & 46 (47)$^g$ & 52 (61.3)$^h$ & 48 & 44 & 49 & 63 & 58 & 41 \\
$\epsilon_{off}$ (10$^3$ M$^{-1}$ cm$^{-1}$)$^i$ & NA & 17.9 & 20.3 & 20.4 & 19.7 & 18.7 & 20.5 & 19.5 & 18.2 \\
QY$_{on}$ (%)$^j$ & 61 (60)$^k$ & 42 (36)$^l$ & 34 (30)$^m$ & 40 & 43 & 38 & 41 & 42 & 39 \\
pK$_a$ & 5.9 (5.8)$^n$ & 6.7 (6.5)$^o$ & 5.7 (5.8)$^p$ & 6.7 & 6.7 & 6.7 & 5.7 & 5.9 & 6.7 \\
molecular brightness$^q$ & 100 & 61 & 56 & 61 & 60 & 59 & 81 & 77 & 50 \\
E. coli fluorescence$^r$ & 100 & 14 & 100 & 104 & 142 & 374 & 343 & 392 & 405 \\
HEK293T fluorescence$^s$ & 100 & 14 & 24 & 39 & ND & 59 & 36 & 42 & 36 \\
renaturation (10$^{-3}$ s$^{-1}$)$^t$ & 5.5 & 7.2 & 7.0 & 9.2 & 9.6 & 9.6 & 12 & 12 & 11 \\
maturation (10$^{-3}$ s$^{-1}$)$^u$ & 14 & 0.002 & 0.5 & 1.4 & ND & ND & 1.4 & 0.9 & 1.1 \\
resid off-state fluorescence, pure FP (%)$^v$ & 100 & 0.3 & 0.7 & 5.6 & 0.3 & 5 & 0.9 & 0.9 & 0.3 \\
resid off-state fluorescence, cell (%)$^w$ & ND & 1.1 & 2.3 & 4.7 & 1.0 & 4.4 & 1.5 & 1.6 & 0.6 \\
switching fatigue (% resid fluor)$^x$ & NA & 52 & 95 & 32 & 51 & 29 & 46 & 35 & 50 \\
switching half-time (s)$^y$ & NA & 9.71 & 5.78 & 20.10 & 10.56 & 17.04 & 10.13 & 10.10 & 6.66 \\
QY$_{on}$ (10$^{-3}$)$^z$ & NA & 5.5 & 8.9 & 2.6 & 5.3 & 3.0 & 3.8 & 4.2 & 8.7 \\
pK$_a$ & 1.7 & 1.2 & 1.2 & 1.6 & 1.5 & 1.3 & 1.4 & 1.4 & 1.8 \\
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\end{tabular}
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The first group had a pK$_a$ value of 5.7–5.9, close to the pK$_a$ of EGFP (5.9), and comprised rsEGFP2, rsGreen0.9, and rsGreen1. The pK$_a$ of the second group, which

![Figure 2](image.png)
However, this experiment describes only the refolding differences between the spectroscopic properties ("QYfluo") and only slightly slower than the folding behavior of the studied FPs. Dots represent measured data, and solid lines show the calculated double-exponential fits.

The return of fluorescence was followed in time. (B) Initial kinetics of the renaturation show only minor differences between the studied FPs. Dots represent measured data, and solid lines show the calculated double-exponential fits.

Figure 3. (A) Renaturation kinetics after denaturation in urea and dilution in renaturation buffer. The return of fluorescence was followed in time. (B) Initial kinetics of the renaturation show only minor differences between the studied FPs. Dots represent measured data, and solid lines show the calculated double-exponential fits.

Biological Performance. Because there are no major differences between the spectroscopic properties (e.g., QYfluoro, ε, õ), of the switchable FPs used here, the improved fluorescence signal in cells can be explained only by large changes in the population of fully functional fluorophores. To get a feel for the formation efficiency of the FPs used, we calculated the ratio of the measured fluorescence emission in E. coli and HEK293T cells to the molecular brightness (εon, overlap × QYfluoro) and scaled it relative to the same ratio for EGFP (Table S2, Supporting Information). Assuming that the spectroscopic properties of the FPs do not change radically when expressed in cells, a higher ratio corresponds to a greater number of successfully formed FPs and thus a more effective formation and/or stability.

We find a marked improvement in our rsGreen mutants in E. coli (ratios between 2.4 and 8.0) and HEK293T cells (ratios between 0.4 and 1.0) compared to rsEGFP (both 0.2) and rsEGFP2 (1.8 in E. coli and 0.4 in HEK293T cells). Interestingly, we found that several of our photochromic labels outperform EGFP in E. coli.

We analyzed the folding and maturation behavior of the different FPs to verify if these were indeed the cause of the improved fluorescence emission when expressed at 37 °C. Renaturation experiments revealed only minor differences in the folding kinetics of the different probes (Figure 3, Table 1: renaturation and Table S2, Supporting Information). The amplitude-weighted average rate constant of rsEGFP (7.2 × 10^{-3} s^{-1}) is very similar to that of EGFP (5.5 × 10^{-3} s^{-1}) and only slightly slower than the folding behavior of the "superfolding" rsGreen variants (9.2–12 × 10^{-3} s^{-1}). However, this experiment describes only the refolding of the protein with an already-formed chromophore and does not capture the complete folding and chromophore formation process.

We used the PURExpress in vitro protein expression kit to mimic the complete formation process of FPs and followed the increase in fluorescence in time. The results are summarized in Figure 4 and Table 1: maturation. While an increase in fluorescence was apparent for all proteins, it was difficult to obtain quantitative information for the rsEGFP formation due to the very low signal. However, the process is clearly slower than that of the rsGreen mutants. The reduced biological performance of rsEGFP was further exemplified by SDS-PAGE analysis of E. coli lysates, showing very little rsEGFP protein content compared to the rsGreens or EGFP under identical growth conditions (Figure S6, Supporting Information). Likewise, the rsEGFP content of E. coli cultures grown at 37 °C was much lower than in rsEGFP cultures grown at 20 °C. These results suggest an efficient degradation of the formed rsEGFP polypeptides. While EGFP outperforms the rsGreens in terms of formation rate, the presence of the superfolder mutations overcomes the deteriorating effects of the rsEGFP mutations, leading to comparable formation rates of the rsGreens measured in this work and sfGFP, reported in the study of lizuka and co-workers.

To verify that the renaturation and in vitro expression experiments were not influenced by off-switching of the fluorophores due to the probing light, we repeated the experiments using purified, nondenatured RSFPs at similar concentrations using identical illumination schemes. We confirmed that the used settings did not cause detectable off-switching (data not shown).

Taken together, our folding/renaturation and in vitro expression experiments do not fully explain the observed live-cell fluorescence signals shown in Figure 1. For example, EGFP displays the fastest in vitro expression kinetics (Figure 4), yet displayed lower fluorescence levels compared to our rsGreens when expressed in...
Figure 5. (A) Photoswitching of purified fluorescent protein solutions. Protein solutions were illuminated with 488 nm (off-switching, ∼0.5 W/cm²) or 405 nm (on-switching, ∼0.035 W/cm²) laser light, and absorbance and emission spectra were recorded every 5 s. Displayed traces represent the integrated emission spectra for each time point. (B) Photoswitching fatigue assay the resistance to photoswitching fatigue. Displayed traces represent the residual fluorescence after 500 switching cycles used to assay the resistance to photoswitching fatigue. Displayed traces represent the residual fluorescence after the indicated amount of photoswitching cycles.

E. coli grown at 37 °C (Figure 1). In particular, other effects such as rates of protein expression or protein degradation pathways, possibly activated by protein misfolding or aggregation and influenced by the folding-assisting machinery, are likely to play an important role in living cells.47–50

Because all our rsGreens are based on mEGFP (EGFP-A206K), we expect that they are monomeric at physiologically relevant concentrations,40 which was confirmed for rsGreen0.7 and rsGreen0.7b by size exclusion chromatography (Figure S7, Supporting Information). As further controls we created constructs in which the FPs were fused to tubulin and expressed in HeLa cells. Tubulin localization is known to be strongly affected by oligomerization of the FP.51 We found that all proteins localized as expected and showed typical monomeric behavior in fusion constructs with α-tubulin (Figure S8, Supporting Information). We further confirmed this in N-terminal fusions of the FPs to the NH2-terminal residues of the Lyn kinase (Lyn tag), whose targeting to sphingolipid- and cholesterol-enriched microdomains in the plasma membrane is also disturbed by fusions to oligomeric FPs.40,52

Photochromic Behavior. We characterized the photochromic behavior of the proteins both in purified protein solutions and in HeLa cells. Investigating the photoswitching of the purified proteins allowed the identification of their intrinsic properties such as the quantum efficiency for off- and on-switching.25

rsGreen0.7 and rsGreen0.8 show the lowest efficiency in terms of off-switching, approximately 2 times lower QY_OFF than rsEGFP, rsGreen0.7b, rsGreen0.9, and rsGreen1. rsEGFP2 is the most efficient photoswitcher, switching off almost twice as efficiently as rsEGFP in this assay, but is very closely followed by rsGreenF. The quantum efficiencies of off-switching also correspond nicely with the off-switching half-times and contrast values (Table 1: resid off-state fluor, pure FP; switching half-time; QY_OFF; Figure 5A and Figures S3 and S9, Supporting Information).

rsGreen0.7, the least efficient rsGreen, displays an off-switching quantum efficiency that is 16 times higher than that of Dronpa, while the off-switching quantum efficiency of rsGreenF is almost 55 times higher. This range illustrates the possibility of tweaking the photoswitching speed to satisfy the needs of the experiment, optimizing for instance photoswitching speed versus the number of photons emitted per cycle.53

In terms of on-switching, the quantum efficiencies are remarkably similar for all RSFPs (Table 1: QY_ON), including Dronpa,54 indicating that this is a very efficient and fast process in all negative-type RSFPs.

To assay the resistance to photoswitching fatigue, we performed experiments on transfected HeLa cells illuminated using a wide-field microscope. All proteins were subjected to 500 photoswitching cycles using the same illumination settings. After 500 cycles, rsEGFP2 showed the least signs of photoswitching fatigue. From the rsGreen series, the slower switchers, rsGreen0.7 and rsGreen0.8, are the least resistant, while several other members display similar performance to rsEGFP in terms of switching fatigue resistance. The contrast values obtained from these measurements correspond nicely with the data from the purified proteins (Table 1: resid off-state fluor, cell; switching fatigue; Figure 5B and Figure S10, Supporting Information).

Since we characterized multiple closely related FPs for this study, we also investigated the presence of correlations between the different spectroscopic parameters. We found that the resistance to photoswitching fatigue correlates well with the off-switching rate and efficiency. This correlation suggests that faster
off-switchers are more resistant to switching fatigue, a finding that is reasonable considering that these probes undergo fewer excitation cycles before converting to the weakly absorbing or nonabsorbing off-state, in which they are protected from photodegradation. In addition, this correlation appeared to be linear (Figure S11, Supporting Information), which suggests that it is simply the switching efficiency that determines the fatigue resistance of the probes, rather than an intrinsic resistance to the bleaching process. We also observed a negative correlation between the extinction coefficient of the off-state proteins and the quantum yield of on-switching, which might be explained by an increased stabilization of the nonfluorescent state (lower QYON) with consistent increase in extinction coefficient (Table 1: $\varepsilon_{\text{off}}$ and $\varepsilon_{\text{ON}}$ and Figure S11, Supporting Information).

**Structure Determination.** To gain more insight into the photochromic and biological behavior, we determined the crystal structure of rsGreen0.7 in the fluorescent on-state and the nonfluorescent off-state to a resolution of 1.25 and 1.20 Å, respectively (Table S4, Supporting Information). rsGreen0.7 shows a similar overall structure to other GFP-like FPs, with a typical 11-stranded barrel fold and a central helix holding the chromophore in place (Figure S12, Supporting Information). All mutations affecting the photoswitching behavior are located near the chromophore and are oriented inward. This includes the original rsEGFP mutations (Q69L, V150A, V163S, and S205N) and the additional Y145F mutation. Mutated residues mainly affecting the formation and/or stability are located further away from the chromophore, on the outside of the $\beta$-can and near the loops (Figure S12, Supporting Information).

The chromophore of rsGreen0.7, which comprises threonine 65, tyrosine 66, and glycine 67, adopts a cis-conformation in the fluorescent state, analogous with the vast majority of fluorescent proteins. The high resolution of the crystallographic data reveals a heterogeneous chromophore conformation, as multiple conformers of the $p$-hydroxyphenyl group can be fitted to the electron density, here modeled with two conformations. Anisotropic refinement using a single conformer further demonstrates an increased heterogeneity of the $p$-hydroxyphenyl group compared to sfGFP and EGFP, in which the anisotropically refined $B$-factors have similar sizes over the entire chromophore (Figure 6). This suggests a significant increase in the flexibility of the chromophore in the on-state compared to EGFP and sfGFP, which is a likely explanation for the reduced fluorescence quantum yield. At the same time, we speculate that this increased flexibility is a consequence of destabilizing the interaction between the chromophore and its environment and ultimately favors the photoswitching process.21,55

![Figure 6](Image)

**Figure 6.** (A) Two modeled conformations of the on-state chromophore in rsGreen0.7 (left) versus one conformation for the EGFP chromophore (PDB ID: 4EUL, right) as observed in the 2$F_o$ − $F_c$ electron density map contoured at 1.0σ. (B) Displacement ellipsoids drawn at the 50% probability level after anisotropic refinement with a single chromophore conformation for rsGreen0.7 (left) and EGFP (right).

The cis-conformation of the on-state chromophore isomerizes into a trans-conformation when the protein switches to the nonfluorescent state (tilt and twist angles of the cis-conformation: 10.7° and −11.3°, respectively; tilt and twist angles of the trans-conformation: −161.4° and −60.9°, respectively). In this conformation the original position of the $p$-hydroxyphenyl moiety is occupied by the imidazole moiety of histidine 148 hydrogen bonded to water molecule 562 (Figure 7). However, due to steric hindrance of phenylalanine 165, the chromophore is unable to isomerize in its original plane, but makes an angle of approximately 62° compared to the plane of the on-state chromophore (Figure S13, Supporting Information).

The large chromophore rearrangement associated with the photochromism is made possible by the rsEGFP mutations at positions 69 and 150. When the off-state chromophore of rsGreen0.7 is modeled in the (nonmutated) EGFP chromophore environment, unfavorably close contacts appear with the glutamine at position 69 (~2.1 Å) and the valine at position 150 (1.4–1.8 Å), which effectively prohibits this from occurring. The Q69L mutation in the rsGreen0.7 increases the distance from this residue to the chromophore to a minimum of 3.3 Å, while the introduction of an alanine at position 150 increases the distance to the $p$-hydroxyphenyl moiety to 3.0 Å, providing ample space for the isomerization of the chromophore (Figure S13, Supporting Information).

A striking feature of the off-state is the presence of a water molecule (W510) that allows for a hydrogen-bonding network between the chromophore, serine 163, and glutamine 183, stabilizing the off-state chromophore (Figure S14, Supporting Information), facilitated by the introduction of serine 163, a key
while maintaining the hydrogen network with a water molecule, the backbone shifts back to the original position occupied in EGFP and sfGFP when rsGreen0.7 is switched off. This backbone shifting can act on the photoswitching behavior in different ways. The outward shift in the on-state potentially creates more room for the chromophore to isomerize, leading to efficient off-switching. When the backbone is shifted back inward in the off-state, the presence of the larger amide moiety might create an additional barrier that the chromophore has to overcome to switch back on, increasing the stability of the off-state.

In rsGreen0.7, we found a slowing effect on the photoswitching caused by the Y145F mutation, which was confirmed by reverting this residue to the original tyrosine (rsGreen0.7b). The importance of residue 145 in the photoswitching process is further exemplified by the fast photoswitching of rsGreenF compared to rsGreen0.9, differing only by the F145L mutation. Because in rsEGFP and rsGreen0.7b, position 145 is occupied by a tyrosine, which can be incorporated into polar interactions with neighboring amino acids, such as His148 or Asn205, we speculate that the phenylalanine present in rsGreen0.7 is unable to make these interactions, while remaining bulky, and may therefore result in a reduced photochromism. In the case of rsGreenF, Leu145 is also unable to make polar interactions, but is more flexible than phenylalanine and may thus allow faster photoswitching. Interestingly, residue 145 also affects the observed $pK_a$ of the FPs with a pronounced difference between rsGreen0.9 (fast switcher, Phe145, $pK_a = 5.7$) and rsGreenF (very fast switcher, Leu145, $pK_a = 6.7$). However, the photoswitching rate is not solely affected by chromophore $pK_a$, as illustrated by the comparison of rsGreen0.7 (slow switcher, Phe145, $pK_a = 6.7$) with rsGreen0.7b (fast switcher, Tyr145, $pK_a = 6.7$).

The structural mechanisms responsible for the superfolding behavior are difficult to determine since a static crystal structure provides no information on the maturation process. However, multiple mechanisms likely influence both the rate of the formation and the formation efficiency. Most of the mutations that we introduced in our rsGreens are located on the outside of the protein, away from the chromophore, and therefore enhance the biological performance rather than the intrinsic spectroscopic parameters. This may involve the creation of electrostatic networks that stabilize the barrel structure or a reduction of the overall hydrophobicity and proneness to aggregation. Such a reduction in aggregation could explain why our rsGreens form slower than EGFP, yet achieve a higher relative fluorescence signal (higher functional FP content) when expressed in E. coli. A complete discussion of the superfolder mutations can be found in the Supporting Information.

**Subdiffraction Microscopy.** We evaluated the use of the developed RSFPs for subdiffraction microscopy by...
employing these labels in pcSOFI\textsuperscript{8,65} and RESOLFT\textsuperscript{26,61} experiments. pcSOFI experiments were successful in TIRF mode using the Lyn tag described above (Figure 8 and Figure S17, Supporting Information) and in epi mode using fusions of the RSFPs targeted to the outer membrane of mitochondria (Figure S18, Supporting Information).\textsuperscript{66} We found that a 2-fold resolution enhancement was readily achievable in both TIRF and epi illumination mode, yielding a subdiffraction spatial resolution together with improved contrast and background reduction. These second-order SOFI calculations could be performed on small data sets, achieving a temporal resolution of less than 7.5 s (Figure 8). Several samples also allowed analysis using a third-order cumulant, further improving the resolution enhancement and providing additional pixels, created by
the calculation of cross-cumulants. This allows the more detailed visualization of fine structures present in the samples. Because the third-order SOFI analysis is more susceptible to noise, not all samples were suitable for this analysis, and most samples achieving 3-fold improved resolution were acquired using TIRF mode excitation.

We reasoned that rsGreenF, which almost rivals rsEGFP2 with respect to switching speed, should also facilitate RESOLFT nanoscopy. To test this, we recorded RESOLFT images of HeLa cells transfected with vimentin-rsGreenF (Figure 9). We measured filament widths of down to ∼70 nm in living cells. Although the obtained resolution is less than previously demonstrated with rsEGFP2, which is presumably due to the reduced switching resistance of rsGreenF, we could discern details entirely obscured in the corresponding diffraction-limited confocal image.

We conclude that the rsGreen proteins represent a versatile family of expression-enhanced RSFPs, which can be used for different super-resolution approaches depending on the question addressed.

CONCLUSION

In conclusion, we have developed a strategy to enhance the formation and biological tolerance of photochromic fluorescent proteins while retaining their spectroscopic properties. We suggest performing two rational and evolutionary optimizations in parallel, one focusing on introducing photochromism while the other focuses on enhancing folding, maturation, and stability (biological performance). This reduces a difficult two-dimensional optimization problem into two much simpler one-dimensional optimizations, which can be combined to achieve both, as this work shows. Using this generic approach, we developed a series of new RSFPs, which we named rsGreens. These EGFP-based proteins exhibit a range of photochromic properties and overall improved biological performance compared to the related proteins rsEGFP and rsEGFP2.

To gain more knowledge about the requirements for reversible photoswitching, we determined the first ever crystal structures of a negative reversibly switchable αvGFP-based protein. The crystal structures of the fluorescent and nonfluorescent state of rsGreen0.7 provided insights into the improved formation efficiency and the photoswitching process. We were able to identify several important factors for the introduction of reversible photoswitching in αvGFP-based FPs, including the creation of space around the chromophore to facilitate its isomerization and the introduction of stabilizing interactions for the nonfluorescent state through hydrogen bonding. We further show that these labels are well suited to subdiffraction microscopy such as pSOFI, achieving a resolution down to ∼80 nm, and RESOLFT, obtaining a resolution of ∼70 nm. rsGreen0.7 and rsGreen0.8 show the potential for efficient but slower switching EGFP-based RSFPs. The final mutants, rsGreen1 and rsGreenF, show fast and efficient reversible photochromism, with rsGreenF coming close to rsEGFP2 in terms of photoswitching speed, but demonstrating less resistance against photoswitching fatigue. All rsGreens do exceed rsEGFP and rsEGFP2 in terms of in-cell fluorescence, indicating an increase in expression and formation of functional FPs. This combination of robust biological performance and photochromism suitable for subdiffraction microscopy makes the rsGreens ideally suited for use in demanding experiments across a wide range of advanced fluorescence imaging applications.

METHODS

Cloning, Mutagenesis, Expression, and Purification. The gene encoding rsEGFP in a pQE31 expression vector was purchased from Abberior GmbH. The ferritin-rsEGFP fusion construct was created by inserting the PCR-amplified rsEGFP with an N-terminal AGSAAGSG peptide linker between KpnI and HindIII sites of a pRSETb vector. An EcoRI restriction site was included in the linker sequence for easy cloning during the mutagenesis. The DNA sequence encoding bullfrog red-cell ferritin subunit-H was ordered from Integrated DNA Technologies (IDT), PCR-amplified, and cloned between the BamHI and KpnI restriction sites.

Directed mutagenesis of selected residues was performed according to a modified QuikChange protocol. All primers used for directed mutagenesis (Table S3, Supporting Information) were designed using the QuikChange Primer Design tool (Agilent Technologies) and ordered from IDT. For site-saturation purposes, the target codon was replaced with an NNK sequence (Agilent Technologies) and ordered from IDT. For site-saturation purposes, the target codon was replaced with an NNK sequence (Agilent Technologies) and ordered from IDT. For site-saturation purposes, the target codon was replaced with an NNK sequence (Agilent Technologies) and ordered from IDT. For site-saturation purposes, the target codon was replaced with an NNK sequence (Agilent Technologies) and ordered from IDT. For site-saturation purposes, the target codon was replaced with an NNK sequence (Agilent Technologies) and ordered from IDT.

DNA shuffing of rsGreen0.7 and rsEGFP was carried out according to the staggered extension process (SSEP). A total of 10 ng of combined plasmid DNA was used to generate short PCR fragments in 100 cycles of 30 s at 94 °C and 5 s at 55 °C. The length of the generated DNA fragments was checked using agarose gel electrophoresis. A 2 μL amount of the generated fragments was treated with DpnI (Thermo Scientific) to reduce background clones and subsequently used as a template in a second PCR, generating full-size, shuffled genes, which were used to replace the rsEGFP sequence in the ferritin-rsEGFP plasmid, using EcoRI and HindIII restriction sites.

The screening and selection of interesting clones was carried out as described in Supporting Method 1 (E. coli fluorescence).

For recombinant expression purposes all fluorescent protein coding sequences were cloned between BamHI and EcoRI restriction sites of a pRSETb plasmid in frame with a polyhistidine sequence. All plasmid transformations were carried out using a sonication protocol. Chemocompetent cells were incubated with plasmid DNA on ice for 5–15 min, placed in a Branson 2210 ultrasonic cleaner for 15 s, incubated with SOC medium at 37 °C for 20 min, and plated on suitable bacterial agar plates.

Expression and purification were carried out as described in Supporting Method 2. Eukaryotic expression vectors were created as follows. PCR-amplified RSFP genes were inserted between BamHI and EcoRI restriction sites of empty pcDNA3, pcDNA3-Lyn, and
pcDNA3-3′-AKAP1 to generate untagged, membrane-targeted, and mitochondria-targeted expression, respectively. For the visualization of human α-tubulin, the mVen irrepressible DNA was replaced by the PCR-amplified promoter encoding the desired RSFP. In a pcDNA3: mVen expression vector, using restriction cloning (Hind III and BamHI sites), preGreenF–Vimentin was created by exchanging the mKate2 sequence in the plasmid pmKate2-vimentin with the PCR-amplified rsGreenF sequence using AgeI and NotI restriction sites. All primer sequences are listed in Table S3 (Supporting Information). All generated constructs were validated by sequencing (LGC Genomics).

Spectra, Molecular Brightness, and pKₐ Determination. Absorbance spectra were recorded with a Shimadzu UV-1650PC spectrophotometer. Fluorescence excitation \( \lambda_{	ext{ex}} \) and \( \lambda_{	ext{em}} \) and emission spectra \( \lambda_{	ext{em}} \) were determined with a PTI Quanta-Master fluorimeter with slits set to 2 nm. Spectra were measured in PBS (pH 7.4). Extinction coefficients were measured according to Ward’s method using the literature values of EGFP, rsEGFP, rsEGFP2, and Dronpa as a reference. The on-state extinction coefficient \( \varepsilon_{\text{on}} \) is reported at pH 9 to ensure complete deprotonation of the chromophore. The overall on-state extinction coefficient \( \varepsilon_{\text{on, overall}} \) is reported at pH 7.4. The off-state extinction coefficient \( \varepsilon_{\text{off}} \) was determined by comparing the absorbance at \( \lambda_{	ext{ex}} \) of the on-switched RSFPs with the absorbance at 405 nm for the off-switched RSFPs. The quantum yield of fluorescence (QY fluor) was determined relative to fluorescein \( (0.48 \text{ W/cm}^2) \) for 100 s in steps of 5 s, while on-switching \( (0.034 \text{ W/cm}^2) \) for 100 s in steps of 5 s, while on-switching. QY fluor is calculated as described earlier. The off-state fluorescence after repeated switching was detected. Excitation was performed by a 20 mW 488 nm laser, and emission was measured at 510 nm in an Edinburgh 920FLS fluorimeter for 10 000 s at 490 nm excitation (480 nm for rsEGFP2). Emission was measured at 510 nm every 30 s.

Photostability, Efficiency and Photowhite Fatigue. The photo-switching behavior of purified RSFPs was investigated as previously described. Briefly, proteins were diluted in 1 mL of TN buffer (100 mM Tris, 300 mM NaCl at pH 7.4), to an optical density of less than 0.2 at the absorption maximum. The cuvette (Hellma) was kept at 4 °C and continuously stirred. For off-switching, the samples were irradiated with 488 nm laser light (0.48 W/cm²) for 100 s in steps of 5 s, while on-switching used 405 nm light (0.034 W/cm²) and the same time protocol. After every step, an absorbance and emission spectrum were recorded. The quantum yield of photowhite (QY fluor) and QY fluor was calculated as described earlier.

Photostability and Photowhite Fatigue. Photostable and high-performance photowhites were measured on a setup consisting of a Sola Light Engine (Lumencor) coupled to an inverted microscope (Olympus IX71) equipped with a 2488rdc dichroic mirror (Chroma) and a 10× objective (UplanSApo, Olympus). Fluorescence images were recorded using an EMCCD camera (iXon, Andor). The system was used to image HeLa cells transfected with pcDNA3::RSFP for untagged expression. The cell culture and transfection were described in Supporting Method 3. The proteins were completely switched off in 5 steps of 0.5 s with cyan light (20%) and switched back on in 5 steps of 0.5 s with violet light (0.5%). This cycle was repeated 500 times to assess the decrease in on-state fluorescence after repeated switching (switching fatigue). The first switching cycle also provided information about the switching behavior under more intense illumination. For this purpose, the off- and off-state were compared after subtraction of the background signal (resid-off-state fluorescence). For the measurement of Dronpa, the illumination scheme was adjusted to 5 steps of 3 s cyan light (100%) for off-switching and 5 steps of 0.5 s violet light (50%) for on-switching. This cycle was repeated 80 times. SDS-PAGE, Renaturation, and in Vitro Expression. To determine the FP expression levels with SDS-PAGE analysis, a single colony from a fresh culture plate was inoculated in 2 mL of LB medium supplemented with ampicillin and grown overnight in a shaking incubator at 37 °C. A 25 μL amount of this starting culture was used to inoculate 2 mL of fresh LB medium supplemented with ampicillin and grown for 4 h at 37 °C, after which 500 μL of this culture was used to inoculate 1.5 mL of fresh LB medium supplemented with ampicillin. After overnight growth, the E. coli were collected by centrifugation, washed twice with 500 μL of PBS, and finally resuspended in 500 μL of PBS supplemented with 1 mg/mL lysozyme and 25 U/mL DNase. Cells were opened by repeated freezing and thawing. Cellular debris was collected by centrifugation and discarded. A 15 μL amount of supernatant was mixed with 15 μL of 2 × SDS loading buffer and placed in boiling water for 5 min. A 25 μL amount of this sample was loaded and separated on a SDS-PAGE gel (4% stacking/12% resolving) according to the manufacturer’s instructions.

Denaturation–renaturation experiments were carried out as described earlier. Briefly, 1 μL of a fluorescent protein solution in PBS was added to 9 μL of denaturation solution (8 M urea, 1 mM DTT) and incubated at >95 °C for 5 min. Subsequently, the denatured protein was added to 990 μL of renaturation solution (35 mM KCl, 2 mM MgCl₂, 50 mM Tris, 1 mM DTT, pH 7.4). The recovery of fluorescence emission (detection at 510 nm) upon 488 nm excitation was followed for 500 s in a PTI QuantaMaster fluorimeter. A maximal value was obtained by measuring the fluorescence emission of an identical dilution of non-denatured protein.

The maturation speed was measured, based on a previously described method, making use of the PURExpress in vitro protein synthesis kit (NEB). Plasmid pDNA (250 ng) was mixed with solution A and B according to the manufacturer’s guidelines, and was diluted to a total volume of 25 μL. The samples were incubated at 37 °C for 15 min before being placed on ice to stop the reaction. The sample was diluted in 150 μL of air-saturated maturation buffer (50 mM Tris-HCl, 2 mM MgCl₂, 35 mM KCl, 5 mM β-mercaptoethanol, and 0.3 mg/mL chloramphenicol at pH 7.5) at 37 °C and immediately measured in an Edinburgh 920FLS fluorimeter for 10 000 s at 490 nm excitation (480 nm for rsEGFP2). Emission was measured at 510 nm every 30 s.

Flow Cytometry. HEK293T cells were cultured, transfected, and prepared as described in Supporting Method 3.

The brightness of HEK293T cells was measured on a FACSCanto II (BD Biosciences) flow cytometry machine ~2 h post-transfection. Viable cells were gated based on morphology (FSC vs SSC), and green and red fluorescence (FP and 7-AAD, respectively) was recorded until 10 000 ‘living green cells’ were detected. Excitation was performed by a 20 mW 488 nm laser, and fluorescence emission was recorded through a 670 LP filter for the PerCP channel (7-AAD detection) and through a 530/30 BP filter for the FITC channel (FP detection). Data analysis was performed with Flowing Software 2 (http://www.flowingsoftware.com/). Raw data were imported, and living cells were gated based on both morphology (SSC vs FSC) and 7-AAD death-cell staining. Green fluorescence was gated above the cell background level measured for the negative control.

Microscopy. pcSOFI experiments were carried out according to the protocol steps described in ref 65 on a commercial cell tiff microscope (Olympus) described elsewhere. For all experiments, the camera acquisition times were varied between the minimum exposure time (14.2 and 31 ms for the ImageX2 and the ImageEM camera, respectively, at full frame) and 100 ms, while the direct EM gain was adjusted (4–900) for optimal dynamic range. Image series of 300–10 000 frames were recorded for pcSOFI analysis, which was done with the Localizer package for IgorPro. For comparison, the average of the time series of each channel was calculated as the diffusion-limited image. Conventional, static wide-field images of tubulin-labeled cells were acquired using simultaneous 405 and 488 nm illumination and an acquisition time of 0.2–1 s.

RESOLFT nanoscopy was performed using a modified RESOLFT Quad P microscope (Abbeior Instruments, Göttingen, Germany). The microscope provides three coaligned focal spots: two normally focused beams at wavelengths of 488 and 405 nm for fluorescence excitation and on-switching, respectively, and one doughnut-shaped focal intensity distribution with a central minimum (‘zero’) for off-switching at the focal periphery in the xy-plane. The RESOLFT image was recorded by applying a pulse scheme, pixel by pixel, with 3-fold line averaging. The following

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pulse scheme was used: (i) on-switching with 405 nm (30 μs; 2 μW measured at the back focal plane of the objective lens); (ii) off-switching at the focal periphery with 488 nm (450 μs; 18 μW); and (iii) reading out fluorescence with 488 nm (20 μs; 6 μW). The diffraction-limited confocal image was recorded in exactly the same manner, but omitting the off-switching step. No background subtraction or image processing was applied.

Cryoprotection. rsGreen0.7 cultures were grown and purified as described above. Afterward, the rsGreen0.7 sample for crystallization was further purified from small impurities and imidazole by size-exclusion chromatography using a HiLoad Superdex 200pg 6/60 column coupled to an Akta Purifier by size-exclusion chromatography using a HiLoad 26/60 column coupled to an Akta Purifier. 0.15 M NaBr and 30% PEG 2000 MME at 16 °C was used as cryoprotectant. For the pH 6.5 and 25% PEG 3350 at 16 °C, 2 μL of precipitant in the well. The rsGreen0.7 green-on cryoprotected crystal presented here was grown in 6 months in 0.1 M Bis-Tris pH 6.5 and 25% PEG 3350 at 16 °C. Prior to the X-ray diffraction experiment, it was flash frozen in liquid nitrogen without any cryoprotectant. For the off-state, an on-state crystal was grown in 0.15 M NaBr and 30% PEG 2000 MME at 16 °C for 12 months and was irradiated with 491 nm light before flash freezing. 25% PEG 400 was used as cryoprotectant. X-ray diffraction data acquisition, analysis, and structure refinement are described in Supporting Method 4.

Conflict of Interest: The authors declare no competing financial interest.

Acknowledgment. This work was supported by the Research-Foundation Flanders (FWO-Vlaanderen) via grants 1521915N, 1502314N, and 1525113N and grants GO484.12 and GO494.15, the Flemish government long-term structural funding ‘Methusalem’ grant METIV/08/04 CASAS, and the Hercules Foundation (HER/08/021). S.D., E.D.Z., V.G., B.M., and W.V. thank the Flemish government for a postdoctoral fellowship and the KULeuven for a BOF-ZAP grant METH/08/04 CASAS, and the Hercules Foundation (HER/08/021). V.G. thanks the Research Foundation-Flanders (FWO-Vlaanderen) via grants 1521915N, 1502314N, and 1525113N and grants GO484.12 and GO494.15, the Flemish government long-term structural funding ‘Methusalem’ grant METIV/08/04 CASAS, and the Hercules Foundation (HER/08/021). V.G. thanks the Flemish government for a postdoctoral fellowship and the KULeuven for a BOF-ZAP grant METH/08/04 CASAS, and the Hercules Foundation (HER/08/021). V.G. thanks the Flemish government for a postdoctoral fellowship and the KULeuven for a BOF-ZAP grant METH/08/04 CASAS, and the Hercules Foundation (HER/08/021).

Supporting Information Available: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b04129.

Discussion of superfolder mutations. Supporting Method 1: Mutagenesis screening. Supporting Method 2: Expression and purification. Supporting Method 3: Eukaryotic cell culture. Supporting Method 4: X-ray data collection, analysis, and refinement. Table S1, mutations introduced through directed mutagenesis. Table S2, additional properties of the developed FPs. Table S3, primers used in this work. Table S4, crystallographic data collection and refinement statistics. Table S5, truncations of the on-state chromatograph of known RSFPs. Figure S1, folding disruption by ferritin. Figure S2, AA sequence alignment of the developed rsGreens, EGFP, rsEGFP, rsEGFP2, and sfGFP. Figure S3, absorbance and emission spectra during photoswitching. Figures S4 and S5, pH-dependence of rsGreens, EGFP, rsEGFP, and sfGFP. Figure S6, SDS-PAGE results of E. coli culture extracts. Figure S7, size exclusion chromatography results of rsEGFP, rsGreen0.7, and rsGreen0.7b. Figure S8, rsGreen-tubulin images. Figure S9, photoswitching behavior of rsEGFP, rsEGFP2, and the rsGreens. Figure S10, initial 25 photoswitching cycles of rsEGFP, rsEGFP2, and the rsGreens. Figure S11, correlation length. Figures S12 and S18, SOFI images obtained with rsGreen-labeled proteins (PDF)

REFERENCES AND NOTES


