Engineering red fluorescent proteins

Bindels, D.S.

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Chapter 2

mScarlet: a novel bright monomeric red fluorescent protein for cellular imaging

Daphne S. Bindels1,*, Lindsay Haarbosch1,*, Laura van Weeren1, Marten Postma1, Katrin E. Wiese1, Marieke Mastop1, Sylvain Aumonier2,3, Guillaume Gotthard2,3, Antoine Royant2,3, Mark A. Hink1 and Theodorus W.J. Gadella Jr1

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1 Section of Molecular Cytology and van Leeuwenhoek Centre for Advanced Microscopy, Swammerdams Institute for Life Sciences, University of Amsterdam, The Netherlands
2 European Synchrotron Radiation Facility, Grenoble, F-38043, France
3 Institut de Biologie Structurale, Université Grenoble Alpes, France

* Contributed equally to this work
Abstract
The engineering of a truly monomeric red fluorescent protein, with a record brightness, quantum yield (70%) and fluorescence lifetime (3.9 ns), mScarlet is reported. mScarlet was obtained starting from a consensus synthetic template followed by improved spectroscopic screening techniques and its crystal structure reveals a planar and rigidified chromophore. mScarlet clearly outperforms existing monomeric red fluorescent proteins and is especially useful as FRET-acceptor in ratiometric imaging experiments.
Fluorescent proteins (FPs) have become indispensable in biological research. After the cloning of GFP from the jellyfish *Aequorea victoria* several spectral variants were obtained through mutagenesis, including blue, cyan and yellow FPs. The field was greatly expanded after the cloning of red FP (RFP) homologs from corals and other Anthozoa species. However, all Anthozoa RFPs form obligate tetramers, which can seriously interfere with the localization and function of RFP-fusion proteins. Unfortunately, monomerization of tetrameric RFPs required extensive mutagenesis and was accompanied by a serious deterioration of the brightness and incomplete and/or partial green maturation of the resulting monomer. After the development of the first monomeric RFP, mRFP1, several improved mRFPs have been reported, such as mCherry, mApple, TagRFP-T, mKate2, mRuby2, mRuby3 and FusionRed (for reviews). But all of these mRFPs are still less bright as compared to their tetrameric ancestors, they all have quantum yields below 50% and several of these mRFPs still harbor additional problems due to incomplete or partial green maturation and a residual tendency to dimerize.

Spectral variants of FPs can be applied in FRET-based biosensors to probe molecular interactions, conformational changes and metabolite concentrations within living cells with high spatiotemporal resolution. While very good FP-based FRET pairs are available with cyan FP (CFP) as a donor and a yellow FP (YFP) as acceptor, it can be highly advantageous to use (additional) FRET pairs with YFP as donor and an RFP as acceptor. This allows higher excitation wavelengths, is less harmful/toxic for cells, induces less autofluorescence and enables multiplexing of several FRET sensors to monitor multiple molecular events in cells simultaneously. Unfortunately, the aforementioned drawbacks of current mRFPs seriously limit their use in FRET experiments. In view of these limitations we focused on engineering a novel bright monomeric RFP with a high quantum yield and complete maturation. Rather than evolving from a tetrameric natural ancestor, we designed a synthetic gene template to start the development of a new monomeric RFP. We based the template on mCherry and multiple other naturally occurring RFPs and chromo proteins, and used knowledge about residues at the outer barrel surface to break the dimerization interfaces. We dubbed this template mRed7 (Supplementary Note 1 and Supplementary Fig. 1).

Remarkably, mRed7 was fluorescent when expressed in bacteria. However, it exhibited a very low fluorescence lifetime and quantum yield. We performed two rounds of multiple site-directed mutagenesis using the OmniChange method, targeting the simultaneous change of multiple amino acid residues in the interior of the beta-barrel in an unbiased manner (Supplementary Note 2, Supplementary Table 1). The resulting libraries of new RFP variants were initially mainly screened for increased fluorescence lifetime in bacteria. Because the fluorescence lifetime (τ) is proportional to the quantum yield but not dependent on expression level, sample thickness or maturation efficiency, it allows for direct
screening of the intrinsic brightness of FPs as shown previously for the evolution of mTurquoise(2)\textsuperscript{22,23}.

Following the initial site-directed mutageneses, the RFPs were subjected to multiple rounds of random mutagenesis and screened for both increased brightness and proper maturation (i.e. minimal dead end green chromophore formation\textsuperscript{24}) by ratiometric screening using a co-translated mTurquoise2 protein as reference (Supplementary Fig. 2). The rationale behind random mutagenesis was to find the supporting (folding) mutations that enhance maturation into the previously selected enhanced spectroscopic state. The ratio of red over cyan fluorescence was used to determine the relative brightness of the RFP, and the ratio of red over green fluorescence is a measure of correct maturation (Supplementary Fig. 2). Eventually, we obtained three monomeric RFPs with distinct properties: a very bright mRFP with a high quantum yield that we dubbed mScarlet, and two variants with a single amino substitution, called mScarlet-I and mScarlet-H (Supplementary Fig. 1).

The normalized absorbance emission spectra indicate that the mScarlet variants are genuine RFPs (Fig. 1a) with absorbance and emission maxima for mScarlet of 569 nm and 594 nm, respectively (Supplementary Fig. 3). Of note, the fluorescence lifetime of mScarlet is 3.9 ns, the highest value recorded to date for monomeric RFPs (Fig. 1b and Table 1), and it shows mono-exponential decay (Supplementary Fig. 4, Supplementary Table 2). The quantum yield of mScarlet is 0.70; much higher than the quantum yield of other (monomeric) RFPs (Table 1, Supplementary Table 2). The extinction coefficient of mScarlet is 100,300 M\textsuperscript{-1} cm\textsuperscript{-1}, resulting in the highest calculated brightness in the mRFP spectral class, with a >3.5 fold increase relative to mCherry. Brightness analysis in mammalian cells confirmed that mScarlet is an extremely bright mRFP (Table 1, Supplementary Table 2, Supplementary Fig. 5a-j), especially when compared to mRuby2, mRuby3 and TagRFP-T. The pK\textsubscript{a} of 5.3 indicates that mScarlet is very tolerant to acid environments (Table 1, Supplementary Fig. 6). The maturation speed of mScarlet in cells favorably compares to that of existing mRFPs (Table 1, Supplementary Fig. 7). The single amino acid substitution T74I found in mScarlet-I results in a marked maturation acceleration in cells (Supplementary Fig. 7), yet at

Figure 1 | Characteristics of the mScarlet variants. (a) Absorbance spectra normalized to the extinction coefficient (ε) (black lines, left axis) and fluorescence emission spectra (area normalized to the quantum yield (QY), red lines, right axis) of purified protein of mScarlet of 569 nm and 594 nm, respectively (Supplementary Fig. 3). Of note, the fluorescence lifetime of mScarlet is 3.9 ns, the highest value recorded to date for monomeric RFPs (Fig. 1b and Table 1), and it shows mono-exponential decay (Supplementary Fig. 4, Supplementary Table 2). The quantum yield of mScarlet is 0.70; much higher than the quantum yield of other (monomeric) RFPs (Table 1, Supplementary Table 2). The extinction coefficient of mScarlet is 100,300 M\textsuperscript{-1} cm\textsuperscript{-1}, resulting in the highest calculated brightness in the mRFP spectral class, with a >3.5 fold increase relative to mCherry. Brightness analysis in mammalian cells confirmed that mScarlet is an extremely bright mRFP (Table 1, Supplementary Table 2, Supplementary Fig. 5a-j), especially when compared to mRuby2, mRuby3 and TagRFP-T. The pK\textsubscript{a} of 5.3 indicates that mScarlet is very tolerant to acid environments (Table 1, Supplementary Fig. 6). The maturation speed of mScarlet in cells favorably compares to that of existing mRFPs (Table 1, Supplementary Fig. 7). The single amino acid substitution T74I found in mScarlet-I results in a marked maturation acceleration in cells (Supplementary Fig. 7), yet at
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**a**

![Graph showing the absorption and emission spectra of mScarlet.](image)

**b**

![Intensity and lifetime images of mScarlet showing emission in different wavelengths.](image)

**c**

![Structural diagram illustrating the packing angles and interactions in the mScarlet protein.](image)
the cost of a moderate decrease in fluorescence quantum yield (to 0.54) and fluorescence lifetime (average of 3.1 ns), although both of these are still higher than the values for all previously engineered bright mRFPs (Table 1). mScarlet bleaches with simple kinetics (Supplementary Figs. 8 and 9) and can be used for prolonged imaging at normal light doses ($<0.5\, \text{W} \cdot \text{cm}^{-2}$). Screening for a more photostable mScarlet variant yielded mScarlet-H, which differs from mScarlet by a single amino acid substitution (M164H). This alteration resulted in a $>2$-fold improvement in photostability as compared to mScarlet (Table 1, Supplementary Fig. 8), albeit accompanied by a serious decrease in quantum yield and fluorescence lifetime. Using the organized smooth endoplasmic reticulum (OSER) approach$^{25}$, the mScarlet series proved to be monomeric in mammalian cells (see Supplementary results and Supplementary Fig. 10, 11), whereas mKate2, mRuby2 and mRuby3 did not pass the OSER test, with the latter two accumulating in the Golgi apparatus (Supplementary Fig. 12).

To investigate the structural basis for the high quantum yield and fluorescence lifetime, the crystal structure of mScarlet was solved at 1.47 Å at the near-physiological pH of 7.8 (Supplementary Table 3). As compared to the mCherry crystal structure, the most
striking difference is the relative positioning of the two conjugated rings of the chromophore. The 5-membered ring (imidazolinone) is very well-stabilized in both mCherry and mScarlet due to lone-pair/π electron interactions to a water molecule from one side and to the carbonyl group of P64 from the other side (Fig. 1c), which amount to strong hydrogen bonds. The difference is at the 6-membered ring (phenolate) which is out-of-plane in mCherry and almost perfectly in-plane in mScarlet: the two dihedral angles controlling their planarity are as high as 26° and -13° in mCherry, and only 1° and -2° in mScarlet (Fig. 1c). The two key mutations to explain this are M164 on the side, and R198 on top of the phenolate moiety, which provides two sets of strong van der Waals interactions (Fig. 1c) that are virtually absent in mCherry. In addition, the introduction of the bulky F178 induces an upward movement of residue 164, which in turns forces the chromophore to adopt a planar conformation. The final striking feature is that the stabilizing R198 is held in place particularly well by a cobweb composed of numerous hydrogen bonds connecting water molecules and a number of hydrophilic residues that are different from mostly hydrophobic residues in mCherry (Fig. 1c). This cobweb ensures that both the water molecules, R71 and R198 are rigidly held against the chromophore. We assume that the planarity of the chromophore and the rigidified surrounding cobweb are the key structural features causing the high quantum yield of mScarlet.

Consistent with its high brightness, monomeric behavior and low pKₐ, the mScarlet series performs well in fusion constructs and as fluorescent label for a variety of cell organelles (See Figure 2a-f, Supplementary results, Supplementary Fig. 13). Furthermore, a cytotoxicity-test in HeLa cells shows that the mScarlet series belong to the least cytotoxic mRFPs and clearly outperform mRuby2, mKate2 and mCherry (Supplementary Fig. 14). Importantly, the profound difference in fluorescence lifetime between mScarlet and mScarlet-H (with equal fluorescence emission spectra) can be used for lifetime unmixing experiments (see Supplementary results and Supplementary Fig. 15).
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**Figure 2** | mScarlet(-I) as fusion tag and enhanced acceptor in ratiometric FRET in living cells. U-2 OS cells (a-d, f) or HeLa cells (e) were transfected with plasmids encoding constructs of (a) LifeAct–7aa–mScarlet (F-Actin), (b) MTS1–4aa–mScarlet (mitochondria), (c) mScarlet–7aa–Giantin (Golgi apparatus), (d) mScarlet–1aa–H2A (nuclei), (e) mScarlet–7aa–α-tubulin (microtubules), and (f) mScarlet–SRL (peroxisomes). Scalebars are 10 μm. Fluorescence intensities were pseudo-colored with the Scarlet-LUT (middle). (g) Donor-normalized emission spectra from live U-2 OS cells expressing various RFPs fused to YFP: mScarlet (red solid), mScarlet-I (red dotted), mScarlet-H (red dashed), mRuby2 (green), mKate2 (magenta), TagRFP-T (dark yellow), mApple (light blue) and mCherry (dark blue). (h) Net sensitized emission normalized to mCherry (red bars, Supplementary Fig. 16) of the FRET experiment displayed in (g) and detected photochromic amplitude (blue bars, Supplementary Fig. 17). Error bars represent 95% confidence intervals. (i) RhoA activity detected by novel intramolecular ratiometric GR-RhoA FRET biosensors with either mCherry (blue trace) or mScarlet-I (red trace) as acceptor in HeLa cells. RhoA activity is stimulated by histamine (black arrow) and antagonized by mepyramine (red arrow), reflected by the RFP sensitized emission increase to donor (SGFP2) fluorescence ratio. (j) Histamine-triggered (black arrow) hydrolysis of PtdIns(4,5)P2 in U-2 OS cells detected by intermolecular FRET biosensors containing Pleckstrin homology domain of phospholipase C fused to YFP (PH-PLCβ1–YFP) and PH-PLCβ2–RFP. Biosensors with mScarlet, mScarlet-I or mCherry are displayed with a solid, dotted or dotted-dashed line, respectively. Loss of PtdIns(4,5)P2 (and FRET) is detected by increased YFP fluorescence (yellow traces) and decreased RFP sensitized emission (red traces) (Supplementary Movie 2).

To evaluate whether the new mScarlet mRFPs are advantageous for ratiometric FRET microscopy in living cells, we transfected U-2 OS cells with direct fusions of RFPs (the mScarlet variants and a set of reference RFPs) to YFP, and recorded single cell fluorescence emission spectra (Fig. 2g). The direct-excited donor and acceptor fluorescence and the sensitized acceptor contributions were determined (Supplementary Fig. 16). Clearly the RFP fluorescence contribution in the FRET spectra is significantly increased for both mScarlet and mScarlet-I as compared to all other mRFPs (Fig. 2g). The sensitized emission component is largest for mScarlet-I (330% of mCherry), followed by mScarlet (270% of mCherry) (Fig. 2h red bars, Supplementary Fig. 16). Also notable is the > 2.5-fold increased sensitized emission of mScarlet and mScarlet-I compared to mRuby2, which was previously described as a preferred FRET acceptor for green/yellow FPs\(^8\). Third in line is mApple (Fig. 2g, h), consistent with its relatively high brightness and quantum yield when compared to other mRFPs. However, mApple shows severe photochromic behavior\(^8\) (Fig. 2h blue bars, Supplementary Fig. 17). Since this is a highly undesirable feature for FRET acceptors, the photochromic behavior of the new mScarlets and existing RFPs was further examined by alternately exciting the RFPs with 556 nm and 448 nm light (Fig. 2h, Supplementary Fig. 17). Negligible photochromic behavior was measured for the mScarlet variants, while TagRFP-T, mRuby2, mRuby3 and mApple showed 15%, 19%, 41%, and 51% photochromic behavior, respectively. Hence, extreme care must be taken when using the latter four RFP variants as acceptor in FRET studies, since a photochromic effect is easily confused with a changed FRET ratio or sensitized emission, especially if one considers that the typical FRET contrast in many sensors is in the range of only 5-20%. The photochromic behavior can also interfere with proper characterization of FPs, like determination of the photostability (Supplementary Fig. 8) or brightness (Supplementary Fig. 5).
sensors from the DORA-RhoA sensor\textsuperscript{27}. Following histamine-triggered GPCR signalling, RhoA is activated by guanine exchange, which is reflected by enhanced FRET. Notably, the GR-RhoA sensor with mScarlet-I yields a much higher change in sensitized emission compared to GR-RhoA sensor with mCherry (Fig 2i, Supplementary Fig. 18).

Finally, the performance of mScarlet was evaluated in a multimeric ratiometric FRET biosensor reporting on phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P\textsubscript{2}) levels in cells\textsuperscript{28}. Histamine-triggered GPCR signaling can decrease PtdIns(4,5)P\textsubscript{2} levels in seconds (Fig. 2j) and indeed a substantial FRET ratio change was observed upon activation. While YFP (de)quenching is roughly equal, a much higher sensitized emission change (>2.5-fold) was detected for mScarlet and mScarlet-I when compared to mCherry, even at 660/100 nm detection, again underlining the superior behavior of mScarlet and mScarlet-I as acceptors in FRET biosensors (Fig. 2j, Supplementary Movie 2).

In conclusion, (see Supplementary Discussion) we describe the development of three new mRFPs: mScarlet, the brightest and highest quantum yield mRFP in its spectral class, and two variants, mScarlet-I with enhanced maturation and mScarlet-H with improved photostability. All three mScarlets show great performance in protein fusions and in cellular functional imaging. mScarlets do not show complications due to photochromicity, cytotoxicity, unwanted residual dimerization in cells or incomplete maturation. Therefore, mScarlets are the preferred FPs in the RFP spectral class for cellular microscopy and quantitative functional imaging.
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Methods

General
All red fluorescent proteins (RFPs) that were used in this study were cloned into a Clontech C1 plasmid by using the AgeI and BsrGI restriction sites. The AgeI and BsrGI restriction sites were added to RFPs that did not contain these sequences in the original plasmid. If applicable, the AgeI and/or BsrGI recognition sites within sequence that encoded the DNA of the RFP were removed by using a silent mutation at the amino acid level. mRuby3 was ordered from Integrated DNA Technologies as a synthetic human codon optimized DNA sequence, mRuby2 was obtained from the plasmid pcDNA3-mRuby2 (40260, Addgene). mKate2 was a kind gift from Dmitriy Chudakov. TagRFP-T was created by introducing the S158T point mutation into a plasmid containing TagRFP. mApple was cloned from the plasmid Myo1E-pmApple-C1 (27698, Addgene). mCherry and tandem dTomato were kind gifts from Roger Tsien. The dTomato plasmid was created from the tandem dimer Tomato (tdTomato) by cloning only one Tomato sequence in the C1 Clontech plasmid.

A general purpose dual expression system with two FPs was created, containing mTurquoise2 and an RFP of interest (Supplementary Fig. 2a). The gene is regulated by a CMV promoter (70823-3, Merck Millipore) and rhamnose-inducible promoter (49011, Lucigen corporation) for mammalian and bacterial expression, respectively. The CFP and RFP are linked with a large linker in bacteria and not linked in mammalian cells due to a P2A sequence. This expression vector was used for the fluorescence lifetime imaging (FLIM) screen, the ratiometric screen (RFP brightness and green component), the photostability assay using widefield microscopy, the assay for measuring photochromic behavior, brightness in mammalian cells, and maturation kinetics. The RFP sequences were cloned into this plasmid by using AgeI and BsrGI restriction sites. The mTurquoise2-, large spatial linker-, and P2A-sequence were removed from the plasmid by using NheI restriction sites. The plasmid with only RFP was used for protein isolation, FLIM measurements in bacteria and as template for random mutagenesis. In this situation a 5xH-tag is fused to the N-terminus of the RFP.

Quantum yield and side mutagenesis libraries were constructed with the OmniChange method using the primers shown in Supplementary Table 1. Random mutant libraries were constructed using Genemorph II (200550, Agilent), up to 4 amino acid changes per FP size (232 amino acids). PCRs were performed with Pfu DNA Polymerase and Phusion High-Fidelity polymerases according to manufacturer’s protocol (EP0502 and F532S, Fermentas). All primers were ordered from Integrated DNA Technologies.

For bacterial screening and protein isolation chemically competent E. coli 5-alpha (Lucigen corporation) were used and transformed according to the manufacturer’s heat shock protocol. Super optimal broth (SOB, 0.5% (w/v) yeast extract, 2% (w/v) Tryptone, 10 mM NaCl, 20 mM MgSO4, 2.5 mM KCl, 100 mg/ml kanamycin, and 0.2% (w/v) Rhamnose to
induce transcription) was used as bacterial growth medium. SOB medium was supplemented with 1.5 % (w/v) agar for agar plates.

Most mammalian cell imaging was conducted with U-2 OS cells (HTB-96, ATCC), unless stated differently. Mammalian cells were grown on round cover slips (Menzel, #1, 24 mm diameter, Thermo Fisher Scientific), in DMEM (61965059, Thermo Fisher Scientific) containing 10% Fetal Bovine Serum (10270106, Thermo Fisher Scientific) and 100 U·ml\(^{-1}\) Penicillin-Streptomycin (15140122, Thermo Fisher Scientific) under 7% humidified CO\(_2\) atmosphere at 37 °C. Polyethylenimine in ddH\(_2\)O (1 mg/ml, pH 7.3, PEI, 23966, Polysciences) was used as a transfection reagent, unless stated otherwise. The transfection mixture was prepared in Opti-MEM (31985047, Thermo Fisher Scientific) with 3 - 4.5 µg PEI and 200-800 ng plasmid. The transfection mixture was incubated for 20 – 45 minutes. Cover slips were mounted in an AttoFluor cell chamber (A7816, Thermo Fisher Scientific) and microscopy medium (140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM glucose, 20 mM HEPES, pH 7.4) was added.

**Bacterial screening**

The RFP library was transformed into bacteria and plated on SOB agar plates. The bacteria were grown overnight at 37°C. To further complete maturation the petri dishes were left at 4°C for several days.

**FLIM screen**

A 561 nm continuous wave laser of 200 mW (Genesis MX 561-500 STM, Coherent) was used for excitation. The excitation light was modulated at 75.1 MHz as previously described\(^{31}\). Additional hardware and analysis of the FLIM screening set-up has been previously described\(^{22}\). For the detection of RFP fluorescence, a dichroic mirror 595 nm (600dcxr, Chroma Technology Corporation) and a 630/60 nm emission bandpass filter (HQ630/60, Chroma Technology Corporation) were used.

**Ratiometric screen**

A Petri dish adapter (tube, diameter, 100 mm; and length, 760 mm) was installed on the stage of an Eclipse Ti-E (Nikon) equipped with an Intensilight Epi-fluorescence Illuminator (Nikon). One of the objectives was replaced with an 80 cm focal lens (FL 800, Melles Griot). For cyan detection the excitation light was filtered using a 448/20 nm bandpass (FF01-448/20, Semrock). The excitation light was directed to the sample with a 466 nm dichroic mirror (Di02-R442, Semrock) and the emission light was filtered with a 482/25 nm bandpass (FF01-482/25, Semrock). For green detection a 500/24 nm excitation bandpass (FF01-500/24, Semrock), a 523 nm dichroic mirror (Di02-R514, Semrock), and a 542/27 nm emission bandpass (FF01-542/27, Semrock) were used. For red detection, a 556/20 nm excitation bandpass (FF01-556/20, Semrock), a 577 nm dichroic mirror (Di02-R561, Semrock), and a 609/54 nm emission bandpass (FF01-609/54, Semrock) was used. The microscope stage and filter wheels were controlled by a ProScan III unit (ProScan H31, Prior
Images were recorded by an ORCA-Flash4.0 V2 Digital CMOS camera (C11440-22CU, Hamamatsu Photonics). Three images using red, cyan, and green detection settings were sequentially acquired of each Petri dish. These three images were also recorded of an empty SOB agar plate, and stored as background image in the NIS-elements software (Nikon). The background was subtracted, after which the red/cyan and red/green fluorescence ratios were determined by NIS-elements software. Colonies with increased fluorescence ratios and therefore producing improved RFP variants, were selected for further optimization.

**Protein purification**

Bacteria were transformed and 100 µl of recovery culture medium was transferred to 50 ml growth medium (see general methods). The cultures were grown overnight (200 rpm, 37°C) and an additional 6 hours incubation at 21 °C was applied to improve maturation. The cultures were washed twice with buffer-A (20 mM Tris-HCl, 200 mM NaCl, pH 8.0) and the pellets were stored at -20°C. The pellets were defrosted and incubated on ice with 5 ml buffer-A supplemented with lysozyme (1 mg/ml, L7651, Sigma-Aldrich) and benzoase nuclease (5 unit/ml, Merck/Millipore, 71205-3). The lysate was centrifuged (30 min, 40,000g, 4°C). The supernatant was added to Ni²⁺ loaded His-Bind resin (Novagen, 69670-2) and incubated for at least 1 hour at 4 °C. The resin was washed 3 times with buffer-A and eluted with 0.2-0.3 M imidazole (in buffer-A). The eluent was filtered (0.22 µm) and the protein solution was dialyzed overnight in 20 mM Tris-HCl pH 8.0 using 3.5 kD membrane tubing (132720, Spectrum Laboratories). Proteins were short-term stored at 4 °C, or flash frozen and stored at -80 °C for long-term storage.

**Spectroscopy**

**Extinction coefficient**

Purified proteins were diluted in PBS (50 mM Na₂HPO₄ – NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Absorbance spectra were acquired with a spectrophotometer (Libra S70, Biochrom). The spectra were recorded in the wavelength range of 260-700 nm, with a step size of 1 nm. PBS was used as a background reference. The samples were diluted such that the absorbance of the red chromophore peaked between 0.2 and 0.3. To denature the RFPs 10 - 30 µl 10 M NaOH was subsequently added to the samples as well as to the reference cuvette, which was directly mixed by pipetting. Spectra were acquired continuously after addition of the sodium hydroxide until the absorbance spectra showed a complete loss of the absorbance peak associated with the red chromophore and displayed only the peak associated with the green chromophore at 457 nm. This absorbance spectrum was used for further analysis, and if necessary, the average absorbance value in the wavelength range 670 - 680 nm was subtracted from the spectra, in order to correct for a minor offset. The concentration of the denatured green chromophore was calculated assuming an extinction coefficient of 44,000 M⁻¹cm⁻¹ at 457 nm for the green chromophore in the denatured RFP. Based on the
concentration of the red chromophore the extinction coefficient for the red chromophore was determined at the maximum absorbance wavelength. The above procedure was repeated three times per RFP variant and the average extinction coefficient and the average RFP absorbance spectra (Fig. 1a, Supplementary Fig. 3) were calculated.

**Quantum yield**

Purified proteins were diluted in PBS (50 mM Na₂HPO₄ – NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Absorbance spectra were recorded with a spectrophotometer (Libra S70, Biochrom) in the wavelength range 260 - 700 nm with a step size of 1 nm. PBS was used as a background reference. Four dilutions of each RFP variant were prepared with an absorbance at 540 nm ($A_{540}$) of $0.005 < A_{540} < 0.05$. Fluorescence emission spectra were taken from the same sample cuvette with a fluorimeter (LS 55 with red sensitive PMT R928 detector, Perkin Elmer). The excitation wavelength was set at 540 nm, the emission spectrum was recorded from 550 to 800 nm with a step size of 0.5 nm at a scan speed of 150 nm·min⁻¹. The excitation as well as the emission slits were set at 2.5 nm. The average RFP emission spectra were calculated from the four dilutions (Fig. 1a, Supplementary Fig. 3).

Absorbance spectra were corrected for a minor offset by subtracting the average absorbance at 670 - 680 nm from the entire spectrum, if applicable. Fluorescence spectra were background corrected by subtracting a spectrum measured with PBS. The emission spectra were corrected for spectral sensitivity and the spectral area ($I_{em}$) was obtained by integrating from 550-800 nm. The absorbance at 540 nm ($A_{540}$) was plotted versus the area under the emission spectrum, subsequently the slope of the line was determined using linear regression. The regression lines were constrained to go through the origin, hence $I_{em} = a \cdot A_{540}$.

**Equation 1** was used to calculate the quantum yield 34:

$$QY_s = QY_r \frac{a_s}{a_r}$$

In **Equation 1** $QY$ denotes the quantum yield ($s$ and $r$ denote sample and reference RFP, respectively) and $a$ corresponds to the acquired slope. The effect of unequal refractive indices is omitted from **Equation 1**, because all measurements were performed with water as solvent. dTomato was used as a reference with a quantum yield of 0.697.

**Fluorescence lifetime**

Fluorescence lifetime measurements were performed at an Olympus FV1000 confocal microscope equipped with a PicoHarp 300 TCSPC module (PicoQuant, Germany). Purified RFP proteins (section protein purification) were diluted in 50 mM phosphate buffer at pH 7.4 and measured in a blackened glass-bottom cell imaging plate with 96 wells (0030741030, Eppendorf) placed at the microscope. Samples were excited using a 561 nm picosecond pulsed diode laser (LDH-D-TA-560, PicoQuant) operating at 20 MHz, using an Olympus
UPLS Apo 60x water NA1.2 objective lens. The fluorescence signal was detected for 180 seconds in confocal mode with the pinhole diameter set at 150 µm at a fixed position. The fluorescence passed through a 455/570 dichroic mirror (ZT445/561rpc-UF2, Chroma Technology Incorporation) and was filtered by a 593/40 nm emission filter (FF01-593/40, Semrock) and detected with an avalanche photodiode (MPD) at 16 ps resolution. The decay curves were fitted over the whole time span using a single or a sum of two exponential functions and were reconvolved with the instrument response function, $IRF(t)$, in the SymphoTime software (Picoquant). The general model for single ($n=1$) or double ($n=2$) decay curves, $h(t)$ was:

$$h(t) = bg + IRF(t) \otimes \sum_i^n A_i / \tau_i \exp\left(\frac{-t}{\tau_i}\right) \quad \text{Eq. 2}$$

where $bg$ denotes the background intensity, $A_i$ denotes the amplitude parameter in total photon counts for fluorescence lifetime component $i$, and $\tau_i$ denotes the fluorescence lifetime of component $i$. By visual inspection of the fit, the fit residuals and the reduced chi-square the fit quality was evaluated. Two measurements were performed per RFP variant, which were highly reproducible. The estimated parameter values and standard errors of one fitted measurement are reported in Supplementary Table 2.

**pH dependence of RFP fluorescence intensity**

Fluorescence emission and absorbance spectra were acquired using a Synergy MX plate reader (BioTek). Purified RFPs (section protein purification) were diluted in a black µ-clear 96 wells plate (655090, Greiner). The samples were diluted to four different concentrations in a pH series ranging from pH 3.0 to 10.8 using 50 mM citric acid – sodium citrate buffer (pH 3.0 - 5.0), 50 mM phosphate buffer (pH 6.0 – 8.0) or 50 mM sodium carbonate – sodium bicarbonate buffer (pH 9.2 - 10.8). Absorbance was measured between 450 and 700 nm in steps of 4 nm to check protein concentration. Fluorescence emission spectra were generated using excitation at 540 nm and detection between 560 and 700 nm, using 4 nm steps. Both excitation and emission slits were set at 9 nm. The spectra were background corrected using the spectra of the corresponding buffers and the integrated area under the emission spectrum for each pH value was used as a measure for intensity at that particular pH. In order to remove the effect of dilution on the overall intensity the dose-response curves were scaled to the mean dose-response curve. The dose-response curves of pH versus normalized integrated fluorescence of the four dilution series, $F(pH)$, (Supplementary Fig. 6) was fitted using the Hill-function, Equation 3 in order to estimate the apparent $pKa$ of the RFP and the Hill-coefficient $n$:

$$F(pH) = F_{min} + \frac{F_{max} - F_{min}}{1 + 10^n(pKa-pH)} \quad \text{Eq. 3}$$
After fitting, the curves and $F_{min}$ were normalized with the estimated $F_{max}$. The normalized intensity at low pH then becomes $f_{min} = F_{min}/F_{max}$.

**Determination of the mScarlet protein structure**

**Crystallization of mScarlet**

mScarlet was isolated from a 1 l culture as described in the section protein purification. The protein solution was concentrated to $A_{569} \pm 70$ using Amicon Ultra centrifugal filters (UFC801096, Merck Millipore). Trypsin-digested (1/100 w/w ratio for 2 h at 20 °C) mScarlet was crystallized using the hanging drop method at 20 °C, at a concentration of 8-10 mg ml⁻¹, in 40 % PEG300, 100 mM sodium phosphate/citrate buffer pH 4.2. Crystal quality was improved using the micro-seeding technique. In order to obtain a structure at a pH close to physiological conditions, a crystal was soaked for 45 min in 40% PEG300, 100 mM sodium phosphate/citrate buffer pH 7.8.

**X-ray diffraction data collection and structure refinement**

X-ray diffraction data were collected at 1.47 Å resolution on beamline ID29 of the ESRF synchrotron in Grenoble. The dataset was integrated, merged and scaled in the C2 space group using the XDS package suite. The structure of mScarlet was solved by the molecular replacement method with Phaser using the structure of PAmCherry1 (3kcs, PDB) as a starting model. The complete model of mScarlet was built in Coot and refined with Refmac5. Data collection and refinement statistics can be found in Supplementary Table 3. The mScarlet structure has been deposited in the Protein Data Bank under ID 5lk4.

**Photostability**

**Screening for photostability in mammalian cells**

An RFP mutant library was transformed into bacteria as described in the general methods. DNA was isolated of individual RFP mutants. U-2 OS cells were grown in Ibitreat µ-plate 96-well (89626, Ibidi). Each well was transfected with 300 ng plasmid and 1 µg PEI in 20 µl Opti-MEM. The culture medium was replaced by microscopy medium directly before imaging. The same microscope setup as described in the section ratiometric screen was used. Here the sample was placed directly in the stage holder and a 20× CFI Plan Apochromat NA 0.75 (MDR00205, Nikon) was used. The sample was excited with a mercury lamp line filtered at 556/20 nm resulting in a local light density above the objective of 6.9 W cm⁻² and the red settings were used for detection. A time series was acquired for each well during 3 minutes, recording an image every 3 seconds. The acquisition was fully automated by NIS-elements software (Nikon).

The images were background subtracted. The mean intensity of all cells was calculated per frame and normalized to 1 at the initial time point ($t_0$). The time point where the fluorescence intensity was decreased to 50% of the initial fluorescence intensity was calculated ($t_{1/2}$). Also the percentage of final fluorescence intensity after 3 minutes was
determined. The mutants with a prolonged $t_{1/2}$ and elevated percentage of final fluorescence intensity were selected for further optimization.

**Widefield bleaching**

U-2 OS cells were cultured, transfected, and mounted after one day of transfection as described in the general methods. The same microscope setup as described in the ratiometric screen was used. Here the sample was placed directly in the stage holder and the light was focused using a 20 × CFI Plan Apochromat NA 0.75 objective (MDR00205, Nikon).

Cells were positioned and focused using cyan settings to prevent any red pre-bleaching (0.36 W·cm$^{-2}$). For the photobleaching acquisition, red settings were used. Only the center part of the camera chip was used to ensure homogenous illumination, resulting in 3-8 imaged cells per acquisition. The first 10 seconds the camera acquired images at high speed (~ 8 ms per frame), during the following 3 minutes every 3 seconds one image was acquired. The sequence was finalized by acquiring one image per 5 seconds. The total acquisition time was adjusted for each RFP variant in order to reach at least 50% decrease of the initial intensity at the final image. The continuous excitation light was manually switched on within the first 5 seconds of the camera acquisition in order to fully capture fast kinetics. The light intensity above the objective at the focal plane corresponded to 6.9 W·cm$^{-2}$.

**Confocal spinning disk bleaching**

mTurquoise2 from the Histone2A containing plasmid pmTurquoise2-H2A (36207, Addgene) was replaced with RFP variants (mScarlet, mScarlet-I, mScarlet-I, mRuby2, mKate2, TagRFP-T, mApple, mCherry or dTomato) by digestion with AgeI and BsrGI. U-2 OS cells were cultured as described in the general methods. The cells were co-transfected with 4.5 µg PEI and 100-500 ng of each plasmid (pmTurquoise2-H2A and pRFP-H2A). One day after transfection cells were mounted as described in the general methods.

Images were acquired on an Eclipse Ti-E (Nikon) microscope equipped with 405 nm and 561 nm lasers (for cyan and red detection, respectively) and a Yokogawa CSU X-1 spinning disk unit (operating at 5000 rpm). The excitation light was directed to the sample via a custom made dichroic mirror 405/488/561/640 through a 100x APO TIRF 100x 1.49 oil objective (Nikon). For cyan and red detection, the emission signal was filtered with a 482/25 nm bandpass (FF01-482/25, Semrock) and 500-535/580-690 bandpass (FF01-512/630m, Semrock), respectively. The signal was recorded by a iXon 897 EMCCD camera (Andor). Cells were positioned and focused using cyan settings to prevent any red pre-bleaching. The red setting was used for the acquisition. The illumination was continuous and switched on automatically from the first image. The illumination intensity was 1.35 W·cm$^{-2}$ measured above the objective at the focal plane. The first 20 seconds images were acquired at maximum speed (~ 200 ms per frame), after which images were recorded every 3 seconds for the remaining time of the acquisition. The total acquisition time was adapted for each variant until the initial intensity was decreased at least 50%.
Chapter 2

Analysis

The time when the illumination was switched on was set to $t_0$. The mean intensity was determined per cell and the background was subtracted. The intensity at $t_0$ was normalized to 1000 photons·s$^{-1}$·molecule$^{-1}$ and the time-axis was adjusted according to the method described by Shaner to correct for RFP specific excitation and emission properties. All cells were averaged and average $t_{1/2}$ values (500 photons·s$^{-1}$·molecule$^{-1}$) are given in Table 1 ($t_{1/2}$ values are based on multiple cells from 1-3 acquisitions). The individual $t_{1/2}$ values obtained from all cells are displayed in Supplementary Figure 9.

Photochromic behavior

The sample preparation and microscope setup was described in the photostability widefield section. Cells were positioned and focused using cyan settings (0.36 W·cm$^{-2}$) to prevent any red pre-bleaching. The cyan excitation light converted the photochromic variants to a brighter state. For acquisition, the excitation light was directed into the sample by a 577 nm dichroic mirror (Di02-R561, Semrock) and the signal was filtered with a 609/54 nm emission bandpass filter (FF01-609/54, Semrock). The acquisition was executed at maximum speed, at $t_0$ the illumination shutter was opened with a 556/20 nm excitation filter (FF01-556/20, Semrock). The excitation light was manually alternated between 556/20 nm at 6.9 W·cm$^{-2}$ and 448/20 nm at 11.5 W·cm$^{-2}$ every 2 seconds for three illumination cycles.

The mean intensity was determined per cell and was background subtracted. The photochromic behavior was determined from the mean intensity immediately after ($I_2$) and immediately before ($I_1$) 448/20 nm induced switching. The photochromic amplitude ($Ph chr$) was calculated using Equation 4 and is depicted with the blue arrow in Supplementary Figure 17.

$$Ph chr = \frac{I_2 - I_1}{I_2} \times 100\%$$

Eq. 4

Two to four acquisitions were performed per RFP variant. The average photochromic amplitudes are presented in Table 1. The number of illumination cycles used to calculate the average photochromic amplitudes were: mScarlet, $n=6$; mScarlet-I, $n=12$; mScarlet-H, $n=12$; mRuby3, $n=12$; mRuby2, $n=12$; mKate2, $n=12$; TagRFP-T, $n=12$; mApple, $n=12$; mCherry, $n=12$; dTomato, $n=9$.

Brightness in mammalian cells

U-2 OS cells were grown as described in the general methods. The cells were transfected with 4.5 µg PEI, 450 ng carrier-DNA (empty cloning vector) and 35 ng mTurquoise2-P2A-RFP (Supplementary Fig. 2a). The low plasmid concentration was used to prevent bystander FRET (this was confirmed by fluorescence lifetime measurements of mTurquoise2). The cells were imaged two days after transfection. The mounting method and
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microscope setup have been described in the sections general methods and photostability widefield.

An RFP and a CFP image were recorded sequentially with excitation power 0.053-0.11 W·cm⁻² and 0.21 W·cm⁻², respectively. Multiple positions (9 or 25) on the same coverslip were imaged; care was taken to avoid pre-excitation. The red/cyan fluorescence intensity ratio was determined per cell (more than 800 cells per RFP variant). The RFP fluorescence intensities were corrected for the spectral differences per RFP variant, e.g. the RFP fluorescence intensities were divided with the relative absorbance at excitation wavelengths and the relative emission at emission filter wavelength. The median of the ratio was calculated per variant, and the values were subsequently normalized to the ratio of mCherry. The normalized values of two experiments were averaged.

For Supplementary Figure 5k an RFP-T2A-mTurquoise2 was compared with a mTurquoise2-P2A-RFP, resulting in an RFP with or without additional polypeptides fused at the C- and N-terminus (the RFP-T2A-mTurquoise2 plasmid was cloned in a similar fashion as 60494 from Addgene). The samples were prepared and recorded as described above. The photochromic behavior can be a severe issue in quantitative assays. For Supplementary Figure 5l the same field of view of a sample with TagRFP-T was recorded 4 times (sequentially red and cyan settings as described above).

**Maturation kinetics**

U-2 OS cells were seeded in an Ibitreat µ-plate 96-well (89626, Ibidi). After one night the culture medium was replaced by microscopy medium and each well was transfected with 300 ng plasmid (Supplementary Fig. 2a) and 1 µg PEI in 20 µl Opti-MEM. Directly after transfection the 96-well plate was placed on the set-up described in the section brightness in mammalian cells. In this experiment the sample was kept at 37°C. An RFP and a CFP image were acquired every 15 minutes for a total duration of 48 hours.

The time course of fluorescence intensity in individual cells was determined in background corrected RFP and CFP images. Using ImageJ regions of interest (ROIs) were carefully drawn around single cells or two cells that exhibited the same time course. Typically, cells only started to produce fluorescent proteins after cell division. The time trace was interactively checked for artifacts. All ROIs were stored and processed in Matlab; time traces were extracted by calculating the mean fluorescence intensity in the ROIs and all curves were subsequently visually inspected and the time point was determined where the signal in the CFP channel surpassed the baseline. All curves were registered using these starting time points in order to synchronize the RFP and CFP time traces. Average curves were calculated both for the CFP and the RFP channel, and were subsequently normalized to the fluorescence level at $t = 16$ h. Only traces that were 16 h long or longer were used. For each average time trace a tangent line was determined at the time point with the maximum slope. Subsequently, the time where the tangent line intersected the time-axis was calculated both for the CFP and RFP channel. This time point was used as measure for apparent delay
observed in the time course of the increase CFP and RFP fluorescence. The difference in apparent delay between RFP and CFP was used as a comparative measure for maturation kinetics in mammalian cells. The time range used to calculate the tangent line at the maximal slope were adjusted for each RFP variant in order to make sure the average time trace comprised the inflection point. The exact number of curves used to estimate the tangent line and maturation delay were: mScarlet, \( n = 28 \); mScarlet-I, \( n = 26 \); mScarlet-H, \( n = 45 \); mRuby3, \( n = 12 \); mRuby2, \( n = 20 \); mKate2, \( n = 32 \); TagRFP-T, \( n = 45 \); mApple, \( n = 45 \); mCherry, \( n = 30 \); dTomato, \( n = 32 \). The number of curves used to calculate the average time trace <16 h are listed in Supplementary Figure 7.

**OSER assay**

Emerald-CytERM-N-17 (56290, Addgene) was digested with *AgeI* and *BsrGI* to replace Emerald with SGFP2, dTomato, mCherry, mApple, TagRFP-T, mKate2, mRuby2, mRuby3, mScarlet, mScarlet-I or mScarlet-H. U-2 OS cells were cultured and transfected as described in the general methods. One day after transfection, the samples were mounted as described in the general methods.

Images were acquired on a spinning disk setup as described in the section photostability confocal spinning disk. In addition, the spinning disk setup was equipped with a 488 nm laser to excite GFP. For SGFP2 detection, the emission was filtered by a 500 nm longpass filter. The signal was acquired by an iXon 897 EMCCD camera (Andor) or an ORCA-Flash4.0 V2 Digital CMOS camera (C11440-22CU, Hamamatsu Photonics). Tilescans (5x5) with 4-8 times averaging were acquired using NIS-elements software (Nikon).

Cells were visually inspected and scored for correct ER structure and localization. Cells that showed fragmented nuclei were excluded from the analysis. For better determination of OSER structures, dTomato was included as a positive control for dimerization and both SGFP2 and mCherry were included as negative controls as these are stated to be true monomers\(^{25}\). Cells with OSER structures, nuclear envelope (NE) thickening and/or incorrect localization (to other organelles) were scored 0. Cells with normal ER structure were scored 1. Per fluorescent protein the percentage of cells with normal looking ER was calculated. For a more accurate determination of the monomeric state, including a correction for total fluorescent protein production among cells, a ratio of intensities within a cell was measured\(^{25}\). The ratio of the mean intensity of the OSER structures over the mean of three NE regions was calculated.

For checking possible Golgi localization of CytERM-fusions, the Giantin DNA coding sequence of FRB-ECFP(W66A)-Giantin (67903, Addgene) was cloned into pmTurquoise2-C1 (60560, Addgene) using the restriction sites *BsrGI* and *BamHI*. U-2 OS cells were cultured and co-transfected with 250 ng of mScarlet-CytERM-N-17 or mRuby3-CytERM-N-17 or mRuby2-CytERM-N-17 and 250 ng of pmTurquoise2-Giantin. One day after transfection, the samples were mounted as described in the general methods. Images
were acquired on a spinning disk setup as described in the section photostability confocal spinning disk. mTurquoise2 was excited at 405 nm and the cyan emission was filtered by a 435-490 nm bandpass filter (FF02-447/60, Semrock).

**Organelle localization**

The Giantin DNA coding sequence of FRB-ECFP(W66A)-Giantin (67903, Addgene) was cloned into pmScarlet-C1, pmScarlet-I–C1 and pmScarlet-H–C1 using the restriction sites BsrGI and BamHI. The following constructs pLifeAct-mTurquoise2 (36201, Addgene), pmTurquoise2-Mito (36208, Addgene), pmTurquoise2-H2A (36207, Addgene), pmTurquoise2-Tubulin (36202, Addgene), and pmTurquoise2-Peroxi (36203, Addgene) were digested with AgeI and BsrGI to exchange mTurquoise2 for mScarlet, mScarlet-I or mScarlet-H. Cells were seeded as described in the general methods. For Golgi apparatus and the peroxisomes labelling, U-2 OS cells were transfected with 200-500 ng plasmid and 1 µl Lipofectamine 2000 (11668027, Thermo Fisher Scientific) in order to obtain a moderate protein production. For F-actin, nuclei and mitochondria labeling the protein production level is less critical, for these samples U-2 OS cells were transfected with 200-500ng plasmid and 4.5 µg PEI. For visualization of microtubules, HeLa cells (CCL-2, ATCC) were transfected with 200-500 ng plasmid and 4.5 µg PEI. One day after transfection, all samples were mounted and imaged as described in the OSER assay section.

**Cytotoxicity in HeLa cells**

The RFP-C1 plasmids as described in the general section were used for transfection. FusionRed (FP433, Evrogen) was cloned into pEGFP-C1 (Clontech) by using AgeI and BspEI restriction sites. HeLa cells (CCL-2, ATCC) were cultured in T25 flasks. On day 0, they were transiently transfected with 4 µg expression vectors coding for the indicated RFP variants, EGFP as non-cytotoxic control, or an empty C1 cloning vector as negative control. The cells were passaged on day 2 and 4. On day 2 and 6, the percentage of fluorescent cells was analyzed on a cell sorter (BD FACSAria III, BD Biosciences), using a 100 µm nozzle at 20 psi with a flow rate of 1.0. Prior to acquisition, cell pellets were washed with HBSS (14175129, Thermo Fisher Scientific) containing 2 % Fetal Bovine Serum (10270106, Thermo Fisher Scientific) and 1 µg·ml⁻¹ DAPI (D1306, Thermo Fisher Scientific) to exclude dead cells from the analysis and resuspended in HBSS containing 2 % Fetal Bovine Serum. At least 30,000 viable cells per sample were recorded.

RFPs were excited with a 561 nm laser and fluorescence emission was filtered with either a 582/15 nm bandpass filter (for detecting mScarlet, mScarlet-I, mScarlet-H, mRuby3, mRuby2, TagRFP-T, mApple and dTomato) or a 610/20 nm bandpass filter (for detecting FusionRed, mKate2 and mCherry). DAPI and EGFP were measured using a 405 nm laser with a 450/50 nm bandpass emission filter and a 488 nm laser with a 530/30 nm bandpass emission filter, respectively.

Raw data were analyzed with FlowJo v10.1 (FlowJo, LLC). The percentage of living fluorescent cells was determined for each time point and normalized according to
EGFP. Data are represented as mean +/- standard deviation of three independent biological replicates.

**FLIM unmixing**

Construct pmScarlet-H2A was used as described in the photostability confocal spinning disk section. The digestion enzymes AgeI and BsrGI were used to exchange mTurquoise2 in the construct pmTurquoise2–NES (36206, Addgene) to obtain pmScarlet-H–NES. Cells were seeded as described in the general methods. The cells were co-transfected with 200-400 ng of each plasmid (pmScarlet–H2A and pmScarlet-H–NES) and 4.5 µg PEI. The control cells were transfected with 300-600 ng plasmid (pmScarlet–H2A or pmScarlet-H–NES) and 4.5 µg PEI. One day after transfection, the sample was mounted as described in the general methods. The sample was placed on an Eclipse Ti-E (Nikon) equipped with a LIFA system (Multi-Led illumination and LI^2^CAM)(Lambert Instruments). The modulated 532 nm LED excitation light passed through a 534/20 excitation filter (FF01-534/20, Semrock) and was directed to the sample by a 577 nm dichroic mirror (Di02-R561, Semrock) and focused using a 60× CFI Plan Apochromat NA 1.4 oil objective (MDR01605, Nikon). The emission was filtered by a 609/54 nm emission bandpass (FF01-609/54, Semrock). The LI-FLIM software (Lambert Instruments) recorded 18 phase steps (with 3 times averaging) in pseudo-random order at a frequency of $f = 40$ MHz. Erythrosin B (198269, Sigma-Aldrich) dissolved in ddH$_2$O was used as reference dye (fluorescence lifetime 0.086 ns, 10 times averaging for reference stack). $\tau_\varphi$ and $\tau_M$ images were calculated by the LI-FLIM software.

Two component fluorescence lifetime-unmixing was performed as previously described$^{22,41,42}$. In frequency domain FLIM, two average fluorescence lifetimes can be obtained from the phase shift ($\varphi$) and from the demodulation ($M$) of the fluorescence emission relative to the excitation light ($\tau_\varphi$ and $\tau_M$, respectively) according to Equation 5, in which $\omega$ is the angular frequency of modulation ($= 2\pi f$):

\[
\begin{align*}
G &= M \cos \varphi \\
S &= M \sin \varphi
\end{align*}
\]

\[
\begin{align*}
\tau_\varphi &= \frac{1}{\omega G} \\
\tau_M &= \frac{1}{\omega \sqrt{\frac{1}{s^2 + g^2} - 1}}
\end{align*}
\]

Eq. 5

From the phase and modulation lifetimes the polar coordinates $G$ and $S$ can be calculated in the polar plot or phasor plot using the following equation:

\[
\begin{align*}
G &= \frac{1}{\sqrt{(1+\omega^2\tau_\varphi^2)(1+\omega^2\tau_M^2)}} \\
S &= \omega G \tau_\varphi
\end{align*}
\]

Eq. 6
In case a fluorophore exhibits multi-exponential decay with \( n \) single exponentially decaying components of which the \( i \)-th component has a lifetime \( \tau_i \) and a fractional contribution to the steady state fluorescence \( \alpha_i \), then in the frequency domain the fluorescence decay can be represented by a single coordinate \((G, S)\) in the polar plot as follows:

\[
\begin{align*}
G &= \sum_{i=1}^{n} \frac{\alpha_i}{1 + \omega^2 \tau_i^2} \\
S &= \sum_{i=1}^{n} \frac{\alpha_i \omega \tau_i}{1 + \omega^2 \tau_i^2}
\end{align*}
\]

Eq. 7

Equation 7 is linear with respect to the fractional steady state contributions \( \alpha_i \). Consequently, the coordinate in the polar plot of a mixture of two fluorophores one with polar coordinates \((G_1, S_1)\) and the other with polar coordinates \((G_2, S_2)\) can be described by:

\[
\begin{align*}
G &= f_1 G_1 + f_2 G_2 \\
S &= f_1 S_1 + f_2 S_2
\end{align*}
\]

Eq. 8

In Equation 8 \( f_1 \) and \( f_2 \) are the fractional contributions of fluorophore 1 and 2 to the total steady state fluorescence with \( f_2 = 1 - f_1 \).

In the polar or phasor plot, the coordinates of the mixture \((G, S)\) therefore lies on the line connecting the polar coordinates of the individual fluorophores \((G_1, S_1)\) and \((G_2, S_2)\) and \( f_1 \) is the relative distance from \((G, S)\) to \((G_2, S_2)\) as compared to the distance from \((G_1, S_1)\) to \((G_2, S_2)\).

For two-component lifetime-unmixing, the single pixel \((G, S)\) coordinates first were projected perpendicularly on the line connecting \((G_1, S_1)\) and \((G_2, S_2)\) and subsequently, the fractions \( f_1 \) and \( f_2 \) were calculated. These fractions multiplied by the DC steady state fluorescence image yielded the unmixed component images. The polar coordinates \((G, S)\) for the individual fluorophores were calculated with Equation 6 using single fluorophore \( \tau_\phi \) and \( \tau_M \) lifetime values measured in triplicate from single transfected cells with exactly the same FLIM settings. For mScarlet-H–NES \( \tau_\phi = 1.179 \pm 0.017 \) ns and \( \tau_M = 1.353 \pm 0.014 \) ns; for H2A–mScarlet: \( \tau_\phi = 3.521 \pm 0.016 \) ns and \( \tau_M = 3.642 \pm 0.022 \) ns.

**Ratiometric FRET analysis**

The RFPs were fused to YPet, this latter sequence was derived from pPBbsr2-Raichu-2247x and was a kind gift of Miki Matsuda. To ensure equal linker length from the last amino acid from the beta-barrel of the RFP until the M1 of YPet the linker had a different length for mRuby2 (SGLRSRAQNSAVDGT, 15 aa) as for the other RFPs used (SGLRSRAQSASAVDGT, 17 aa). Cells were seeded as described in the general methods. The cells were co-transfected with 4.5 \( \mu \)g PEI, 450 ng carrier DNA (empty cloning vector) and 17.5 ng plasmid (RFP–YFP fusion). To measure direct excitation of the RFP at the donor
excitation wavelength and the shape of the acceptor and donor spectrum, cells were transfected with acceptor or donor only (4.5 µg PEI, 450 ng carrier DNA and 17.5 ng pRFP–C1 or pYPet–C1). The low plasmid concentration was used to prevent by-stander FRET (this was confirmed by fluorescence lifetime measurements of YPet in a similar P2A construct). The cells were imaged two days after transfection. The samples were mounted as described in the general methods.

The spectral imaging set-up was previously described43 and was equipped with a Plan Neofluar 20x air objective (Zeiss). First, cells were selected in an unbiased manner towards acceptor maturation by using donor microscope settings, hence 500/20 nm for excitation (HQ500/20, Chroma Technology Incorporation), a 528 dichroic mirror (525dcxr, Chroma Technology Incorporation), and a 542/27 nm emission filter (FF01-542/27, Semrock).

The sensitized spectrum was captured using 500/20 nm excitation filter, an 80/20 beam splitter (20/80bs, Chroma Technology Incorporation), and a 530 long pass emission filter (#46-059, Edmund optics worldwide). Immediately after, an acceptor-only image was recorded to correct for protein production, using a 577/20 nm excitation bandpass filter (D577/20, Chroma Technology Incorporation), a 596 nm dichroic mirror (600dcxr, Chroma Technology Incorporation), and a 630/60 nm emission filter (HQ630/60, Chroma Technology Incorporation). For each variant at least 37 cells were imaged and analyzed.

Briefly, two spectral images were acquired, one was acquired using donor excitation with the longpass filter, and subsequently one was acquired using acceptor excitation with the bandpass filter. Cells were selected semi-automatically using a custom made Matlab script and the spectrum obtained with donor excitation settings was normalized with the peak intensity of the spectrum as measured using acceptor excitation settings. Using these microscope settings, the donor was not excited with acceptor excitation light, however, the acceptor was directly excited when using the donor excitation settings. Spectra of the samples that contained both donor and acceptor were unmixed using linear regression with the spectra measured in cells expressing donor only and acceptor only, and all spectra from the samples were averaged over multiple cells. The amount of direct acceptor excitation was estimated from normalized spectra measured in cells expressing acceptor only. The wavelength axis and spectral sensitivity (tail of long pass filter and camera) were corrected in order to obtain calibrated spectra. Finally, for each mRFP variant the spectra were normalized with respect to the donor peak at 527 nm.

**Single chain FRET sensor for RhoA activity**

In the DORA-RhoA sensor–wt biosensor (a kind gift from Yi Wu27) the donor (mCerulean3) was replaced by SGFP2 using BamHI and Nhel restriction sites. The SGFP2 insert for this color swap was obtained by performing PCR on a Clontech-style pSGFP2-C1 (22881, Addgene) using RhoA primers set 1 (Supplementary Table 1), thereby introducing BamHI and Nhel restriction sites before and after the SGFP2, respectively. Next, the acceptor
(cpVenus6) was replaced by either mCherry or mScarlet-I in a two-step process. First, a BglII restriction site was introduced into the sensor plasmid, behind the acceptor FP sequence and simultaneously the original acceptor is swapped for mNeonGreen (a kind gift from Nathan Shaner44) via overlap-extension PCR45,46. The first PCRs were performed on the clontech-style C1 plasmid containing mNeonGreen using RhoA primers set 2 (Supplementary Table 1) and on the RhoA sensor plasmid using RhoA primers set 3 (Supplementary Table 1). The second PCR was performed on a mix of both PCR products using RhoA primers set 2 FW and RhoA primers set 3 RV. The resulting PCR product and the RhoA sensor are digested with AgeI and HindIII and the PCR product containing the BglII restriction site and mNeonGreen is ligated into the sensor. Second, mNeonGreen was replaced by mCherry or mScarlet-I using AgeI and BglII restriction sites. The mCherry and mScarlet-I inserts for this color swap were obtained by performing PCRs on a clontech-style pmCherry-C1 and pmScarlet-I–C1 using RhoA primers set 4 (Supplementary Table 1). To create the constitutive active forms of the GR-RhoA sensor (ca-GR-RhoA sensor) containing SGFP2 and mCherry/mScarlet-I, the part containing the constitutive activating Q63L mutation from the DORA-RhoA sensor-ca (a kind gift from Yi Wu27) was introduced using HindIII and NheI restriction sites.

Response curves
HeLa cells were seeded as described in the general methods. The cells were transfected with 500 ng plasmid encoding wtGR-RhoA sensor and 500 ng plasmid untagged H1R receptor (HRH0100000, cDNA). For bleedthrough controls cells were transfected with 500 ng pSGFP2–C1 (22881, Addgene), pmCherry–C1 or pmScarlet–I–C1. One day after transfection the samples were mounted as described in the general method section and were placed on a widefield fluorescence microscope described by van Unen et al. 27. A time series with 3 sequential images (donor excitation–donor emission (d), donor excitation–acceptor emission (S), acceptor excitation–acceptor emission (a)) was recorded using an oil-immersion objective (Plan-Neo-fluor 40×/1.30; Carl Zeiss GmbH) at 37 °C. The time interval in the time series was 4.4 seconds and the image size was 348×260 pixels with pixel size of 340 nm. The donor was excited at 480/10 nm. The excitation light passed through a 375-490 nm (e490sp, Chroma Technology Incorporation) excitation filter, was reflected by a 490 nm dichroic mirror (490dcxr, Chroma Technology Incorporation). The donor emission was filtered by a 500 nm long pass filter (HQ500LP, Chroma Technology Incorporation) and a 525/40 nm bandpass (BP525/40, Chroma Technology Incorporation). The acceptor emission was filtered by a 600/37 nm bandpass (FF01-600/37, Semrock). The acceptor was excited at 545/10 nm, the excitation light passed a 375-580 nm excitation filter (e580sp, Chroma Technology Incorporation) and was reflected on the sample by a 591 nm dichroic mirror (585dcxr, Chroma Technology Incorporation). The acceptor emission was filtered by a 593 nm longpass (E590lpv2, Chroma Technology Incorporation) in combination with a
600/37 nm bandpass (FF01-600/37, Semrock). Cells were stimulated with 100 µM histamine (Sigma Aldrich) and 10 µM mepyramine (Sigma Aldrich).

In order to extract the pure donor emission and sensitized emission, filter FRET coefficients were determined from cells expressing donor only and acceptor only; the same microscope settings were used for these measurements. The bleedthrough of the donor in the acceptor emission channel (β), the bleedthrough of the acceptor in the donor channel (δ) and the direct excitation of the acceptor with donor excitation light (γ) were determined\(^4\). The background intensity of each image in the time series was calculated using a hand drawn region of interest, and was subsequently subtracted. The background images were stored in an ImageJ tif hyperstack containing the three channels (D, S and A). Before applying the filter FRET calculations, the images with sensitized emission (S) were registered to the images with donor emission (d) using the Lucas-Kanade algorithm. The images with direct acceptor emission (a) were registered to the registered sensitized emission images (S). Filter FRET was applied and the resulting corrected images were stored in a separate ImageJ tif hyperstack, Regions of interest were drawn around cells using ImageJ and the time traces of the mean intensity for all channels were extracted. The ratio of the pure sensitized emission and pure donor emission was calculated and baseline subtracted. The latter retains the effect of the quantum yield on the amplitude of the FRET ratio change. Sharp spikes due to pipetting or other causes in the time traces were removed with the aid of a median filter algorithm. The 95% confidence interval of the mean response curve was calculated using statistical bootstrapping.

**FRET spectra**

HeLa cells were seeded as described in the general methods and transfected with 500 ng plasmid encoding wtGR-RhoA or caGR-RhoA sensors. For unmixing controls cells were transfected with 500 ng pSGFP2–C1 (22881, Addgene), pmCherry–C1 or pmScarlet–I–C1. One day after transfection the sample was mounted as described in the general methods section. The spectra were recorded on a widefield spectral imaging microscope as described in the section ratiometric FRET analysis in a similar manner. Only here, the donor was excited by using a 436/20 nm excitation filter (D436/20, Chroma Technology Incorporation) and the emission was filtered by a 458 nm longpass (BLP01-458R, Semrock). The same analysis was applied as described in the section Ratiometric FRET analysis.

**Multimeric ratiometric FRET biosensor**

An intermolecular ratiometric FRET biosensor was created that contains PH-PLCδ\(_1\)-RFP and SYFP2-PH-PLCδ\(_1\) produced in a three to one ratio due to an IRES sequence. The PH-PLCδ\(_1\) encoding sequence was derived from plasmid PH-PLCD1-GFP (51407, Addgene) and the IRES encoding sequence was obtained from Gβ-2A-epV-Gγ2-IRES-Gai3-mTq2 (69625, Addgene). The cells were cultured as described in the general methods. U-2 OS cells were transfected with 300 ng carrier-DNA (empty cloning vector), 100 ng untagged H1R receptor (HRH0100000, cDNA) and 100 ng of the biosensor plasmid.
**Acceptor comparison**

Two days after transfection, the sample was mounted as described in the general methods and was stimulated with histamine to a final concentration of 0.1 mM. A time series of images was acquired on an Olympus FV1000 confocal microscope equipped with a 488 nm laser (LDH-P-C-485, PicoQuant). The excitation light was directed via a 488 dichroic mirror (Di01-R405/488/561/635, Semrock) and focused by a 10× UPLSApo NA0.4 air objective (Olympus). The donor signal was collected with an open pinhole and filtered using a 525-555 nm bandpass filter (Chroma Technology Incorporation). The acceptor signal was filtered by a 610-710 nm bandpass filter (Chroma Technology Incorporation).

The images in both channels were background corrected and an upper threshold of 4000 counts was applied such that pixels associated with background and pixels associated with cells producing extremely high amounts of protein were excluded. For each time point the mean intensity was calculated for both the RFP and the YFP channel. Time traces from three different measurements were averaged and the baseline was subtracted (Fig. 2j).

**Ratiometric FRET movie with mScarlet-I as acceptor**

One day after transfection, the sample was mounted as described in the general methods and were stimulated with histamine to a final concentration of 0.1 mM. The sample was placed on an Eclipse Ti-E (Nikon) equipped with a 513 nm LED (SpectraX, Lumencor). The excitation light passed through a 510/24 bandpass and was directed via a triple band cube (blue, teal, yellow) (MXU74157, Nikon) and focused by a CFI Plan Apochromat K 60X NA 1.4 (Nikon) objective in the sample. The donor emission was filtered with a 550/49 nm bandpass (FF01-550/49, Semrock), the acceptor emission was filtered with a 593/46 nm bandpass (FF01-593/46, Semrock). The optical filters were moved by an optical filter changer (Lambda 10-B, Sutter instrument). The donor and acceptor emission were sequentially recorded by an ORCA-Flash4.0 V2 Digital CMOS camera (C11440-22CU, Hamamatsu Photonics). Images of 2048×2044 pixels, with a pixel size of 108 nm, were taken every 1.6 seconds. The cells were stimulated with histamine (0.1 mM) in order to activate PLC.

Each frame in both the RFP and YFP channel was background corrected and the channels were registered using the Lucas-Kanade algorithm to remove any spectral shift between the channels. Images were subsequently smoothed with a 5×5 box filter, and the ratio image of acceptor over donor intensity was calculated. A mask was applied to the ratio image in order to remove background. The mask threshold was based on a smoothed intensity image. The ratio was visualized using the Fire false color LUT and an overlay image was created combining the smoothed intensity image and the false color-coded image associated with the FRET ratio.

**Statistical methods**

Data were analyzed according to the methods described in the sections above. In case an analysis was based on individual cells, the data from any cell showing possible signs of dying
(e.g. rounding up) was excluded. The resulting parameters are presented including their 95% confidence intervals, except for the time-domain fluorescence lifetimes and cytotoxicity data that are presented with standard errors. In order to make sure the results were repeatable and consistent the experimental design for each type of experiment was systematically optimized. Analysis was performed on results obtained from optimal experiments and confidence intervals were calculated and evaluated, if necessary sample size was increased in order to obtain more accurate estimates. Error propagation was used in the case of composite parameters that were calculated based on two or more measured parameters. Confidence intervals were calculated using the inverse Student's t-distribution, which included the number of degrees of freedom. Statistical bootstrapping applied to average maturation curves was used to calculate the confidence intervals for the maturation delay times. Boxplots where generated using BoxPlotR (http://boxplot.tyerslab.com/)\textsuperscript{48}.

**Acknowledgments**

We thank Ronald Breedijk for technical assistance with advanced microscopy, Janina Pfeilschifter for her help with cloning fusion constructs and QY determination and dr. Joachim Goedhart (JG) and other lab members for discussions and useful suggestions. We thank dr. Renée van Amerongen for proofreading the manuscript. The ESRF is acknowledged for access to beam lines via its in-house research program. This work was supported by ‘Middelgroot’ investment grant 834.09.003 (MAH) and CW-Echo grants 711.01.01812 (MAH and TWJG) and 711.013.009 (JG & TWJG) and ALW-VIDI grant 864.09.015 (MP) from the Netherlands Organization for Scientific Research (NWO), by grant 12149 (TWJG) from the Foundation for Technological Sciences (STW) from the Netherlands, and the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 706443 (KEW).
Supplementary Figure 1 | The evolution from mRed7 to the mScarlet series depicted as amino acid alignment. The intermediates in the evolution of mRed7 to the mScarlet variants are given in order of occurrence. The chromophore is shown in grey boxes. Amino acid mutations highlighted in blue originate from quantum yield OmniChange mutagenesis, in green from OmniChange side mutagenesis, in magenta from random mutageneses. Amino acid residues highlighted in red, orange and brown are specific for mScarlet, mScarlet-I and mScarlet-H, respectively.
Supplementary Figure 2 | Ratiometric screen for brightness and maturation. (a) Systematic representation of the used screening construct for bacterial and mammalian expression. To correct for protein concentration, mTurquoise2 is fused to an RFP of interest. To minimize FRET in bacteria, the FPs are linked through a large spatial linker. The linker is followed by a P2A sequence to exclude FRET in mammalian cells. (b - f) Bacteria transformed with the vector described in (a) with three different RFP mutants, grown in a 3 × 3 square on a Petri dish. Three images are recorded sequentially with red settings (b), green setting (c), cyan settings (d). Ratios of red/cyan (e) and red/green (f) fluorescence intensities are calculated. An elevated ratio red/cyan (e *) shows a variant with an increased brightness. An elevated ratio red/green (f +) represents an RFP mutant that has decreased dead-end green component, thereby improved maturation. Ratio images are pseudo-colored using the rainbow LUT displayed.
Supplementary Figure 3 | Absorbance and fluorescence emission spectra of RFPs. (a) Normalized absorbance spectra of purified RFPs. (b) Fluorescence emission spectra of RFPs area normalized to the calculated brightness (ε·QY). (c) Fluorescence emission spectra of RFPs area normalized to the brightness observed in mammalian cells (Table 1). (a, b, c) mScarlet (solid red), mScarlet-I (dotted red), mScarlet-H (dashed red), mRuby2 (green), mKate2 (magenta), TagRFP-T (dark yellow), mApple (light blue), mCherry (dark blue) and dTomato (grey).
Supplementary Figure 4 | Fluorescence decay of the mScarlet variants. Time-resolved fluorescence decay curves of purified mScarlet (a), mScarlet-I (b) and mScarlet-H (c) in phosphate buffer. The measured decay curves (gray dots) were analyzed using a mono- and bi-exponential fit model (black line, Eq. 2) including the instrumental response function (blue line), the parameters are listed in Supplementary Table 2. The residuals of the fits are shown for a mono- (middle, red) and bi-exponential (bottom, black) fit model.
mScarlet: a novel bright monomeric red fluorescent protein for cellular imaging
Supplementary Figure 5 | RFP brightness in mammalian cells. Mammalian cells produce mTurquoise2 and an RFP variant of interest separately in a 1:1 ratio. Dot plots show the mean fluorescence intensity of individual cells in the RFP channel (y-axis) against the CFP channel (x-axis). A higher RFP/CFP ratio corresponds to an increased RFP brightness in cells. The expression vector mTurquoise2-P2A-RFP that was used for transfection with the different RFP variants: mScarlet (a), mScarlet-I (b), mScarlet-H (c), mRuby3 (d), mRuby2 (e), mKate2 (f), TagRFP-T (g), mApple (h), mCherry (i) or dTomato (j) is shown in Supplementary Figure 2. (k) A C- and N-terminus polypeptide can influence the brightness of FPs in cells, as is observed for mRuby2, but not for mScarlet. Red dots represent mScarlet (mTurquoise2-P2A-mScarlet, shown in panel a), dark red dots represent mScarlet with a C-terminus fused polypeptide (mScarlet-T2A-mTurquoise2), light green dots represent mRuby2 (mTurquoise2-P2A-mRuby2, shown in panel e), and dark green dots represent mRuby2 with a C-terminus fused polypeptide (mRuby2-T2A-mTurquoise2). (l) Photochromic behavior of RFPs interfering with brightness analysis in cells. Cells containing TagRFP-T are imaged multiple times in the red and cyan channels. Due to blue light induced photochromic behavior, the red fluorescence intensity increased while the mTurquoise2 fluorescence intensity remained constant. The consecutive measurements are displayed in the order: yellow, orange, brown, and black, respectively.
Supplementary Figure 6 | pH sensitivity of the mScarlet variants. The normalized spectral-integrated fluorescence intensity for mScarlet (a), mScarlet-I (b) and mScarlet-H (c) were plotted against pH and fitted using Equation 3 (red curve), the parameters are listed in Supplementary Table 2. Data points indicate measurements from four concentration series.
Supplementary Figure 7 | Apparent maturation delay time relative to mTurquoise2. Fluorescence intensity rise of recently divided mammalian cells separately producing mTurquoise2 and an RFP variant of interest in a 1:1 ratio. The delay between the cyan fluorescence intensity (cyan) and red fluorescence intensity (red) corresponds to the initial delay time of the synthesis of RFP compared to mTurquoise2. The expression vector mTq2-P2A-RFP that was used for transfection with the different RFP variants: (a) mScarlet, n=32; (b) mScarlet-I, n=26; (c) mScarlet-H, n=47; (d) mRuby3, n=27; (e) mRuby2, n=42; (f) mKate2, n=32; (g) TagRFP-T, n=45; (h) mApple, n=45; (i) mCherry, n=30; or (j) dTomato, n=32; is shown in Supplementary Figure 2a.
**Supplementary Figure 8** | Photostability of RFPs in living cells. Photon emission rate under continuous widefield (a) or confocal spinning disk (b) illumination: mScarlet (solid red), mScarlet-I (dotted red), mScarlet-H (dashed red), mRuby2 (green), mKate2 (magenta), TagRFP-T (dark yellow), mApple (light blue), mCherry (dark blue) and dTomato (grey). *All RFPs are bleached with the same illumination intensity, and the time axis is computationally normalized to an initial photon emission rate of 1000 photons s\(^{-1}\) molecule\(^{-1}\).
**Supplementary Figure 9** | Quantification of photostability under widefield and spinning disk imaging conditions in living cells. The time to reduce the emission rate from 1000 to 500 photons s\(^{-1}\) molecule\(^{-1}\) \((t_{1/2})\) was calculated per cell under continuous widefield (a) and confocal spinning disk (b) imaging conditions. Each dot represents one cell (total number of cells is indicated above the horizontal axis). Thick lines represent the median \(t_{1/2}\) value in seconds. *All RFPs are bleached with the same illumination intensity, and the time axis is computationally normalized to an initial emission rate of 1000 photons s\(^{-1}\) molecule\(^{-1}\). Only the variants that lack photochromic behavior are shown (Fig. 2h and Supplementary Fig. 17).
Supplementary Figure 10 | Assessment of oligomeric state of RFPs in living mammalian cells by OSER assay. U-2 OS cells were transfected with plasmids encoding fusions of CytERM to different FPs: (a) mScarlet, (b) mScarlet-I, (c) mScarlet-H, (d) dTomato, (e) mCherry, (f) mKate2, (g) mRuby2, thereby anchoring the FP to the membrane of the ER. To enhance visualization of cells with bright OSER structures, the gamma of all images was adjusted to 0.8. Scale bars are 10 µm. (h) Table displaying the results of the OSER assay. ‘Normal looking cells’ are cells with reticular shaped ER, without nuclear envelope (NE) thickenings, without whorl (OSER) structures, and without incorrect localization. * FPs that are monomeric according to definition given by Costantini et al.\textsuperscript{22}.
Supplementary Figure 11 | The median ratio OSER/NE of several FPs determined with the OSER assay. The intensity ratio of the whorls structure (OSER) over the mean intensity of the nuclear envelope (NE) was measured in cells expressing different CytERM–FP constructs. Each dot represents an OSER structure. Thick lines in each FP variant represent the median intensity ratio OSER/NE. The bar at 2.3±0.6 represents the monomeric threshold$^{22}$. 

Chapter 2
**Supplementary Figure 12** | mRuby2 and mRuby3 localize to the Golgi apparatus in the OSER assay. U-2 OS cells were co-transfected with plasmids encoding fusions CytERM-mScarlet and mTurquoise2–Giantin (a-c), CytERM-mRuby2 and mTurquoise2–Giantin (d-f), or CytERM-mRuby3 and mTurquoise2–Giantin (g-i). a, d, g: RFP channel showing localization of CytERM fusion, b, e, h: CFP channel showing localization of Golgi marker, c, f, i: Overlay of Red and Cyan channels. (a-i) boxed area is zoomed in the upper right corner. Scale bars are 10 µm.
Supplementary Figure 13 | mScarlet-I and mScarlet-H as fusion tag to visualize intracellular structures in living cells. U-2 OS cells (a-f, i, j) or HeLa cells (g, h) were transfected with plasmids encoding fusion constructs with mScarlet-I (a, c, e, g, i) or mScarlet-H (b, d, f, h, j). (a, b) LifeAct–7aa–mScarlet-I/H (F-Actin), (c, d) MTS1–4aa–mScarlet-I/H (mitochondria), (e, f) mScarlet-I/H–7aa–Giantin (Golgi apparatus), (g, h) mScarlet-I/H–7aa–α-tubulin (microtubules), and (i, j) mScarlet-I/H–SRL (direct fusion, peroxisomes). Scale bars are 10 µm. The fluorescence intensities were pseudo-colored according to the indicated Scarlet-LUT (bottom).
Supplementary Figure 14 | Cytotoxicity of mRFPs in HeLa cells. The percentage of positive (fluorescent) HeLa cells was calculated 2 and 6 days after transfection. The percentage of positive cells at day 6 relative to day 2 were normalized to EGFP. The lines represent the mean values of three independent experiments from transfections on three different days, the individual experiments are shown as dots.
Supplementary Figure 15 | Fluorescence lifetime-unmixing of mScarlet and mScarlet-H. U-2 OS cells were cotransfected with plasmids encoding mScarlet–H2A (Histon 2A, nuclei) and mScarlet-H–NES (nuclear export sequence, cytoplasm). Wide-field fluorescence lifetime imaging showing (a) steady-state fluorescence intensity image, (b) the phase fluorescence lifetime image ($\tau_p$), and (c) $\tau_p$ histogram. $\tau_p$ is pseudo-colored as indicated by the LUT in the $\tau_p$-histogram. The unmixed fluorescence intensity image of (d) mScarlet-H–NES and (e) mScarlet–H2A. The overlay of the unmixed fluorescence intensity images (f) of mScarlet-H–NES (green pseudo-color) and mScarlet–H2A (blue pseudo-color). Scale bar is 10 µm.
Supplementary Figure 16 | Unmixing of YFP–RFP FRET spectra. Fluorescence emission spectra were measured in U-2 OS cells expressing YFP–RFP fusions. Each spectrum (colored line, Fig. 2g) corresponds to the mean spectrum based on multiple cells. The FRET spectra (upper colored lines) were unmixed into the YFP donor component (black line) and the total RFP acceptor component (grey line) and subsequently normalized to donor emission maxima. The component arising from direct acceptor excitation in the FRET spectra (lower colored line) were measured in cells only containing the acceptor, and normalized to the average donor emission maximum. Hence, the grey areas represent the net sensitized emission from the RFP acceptors, the mean values are presented in Figure 2h and the values and statistics from individual cells are shown in Supplementary Figure 18a. The RFP spectra and their sample size are presented in the following order: (a) mScarlet, n=50; (b) mScarlet-I, n=47; (c) mScarlet-H, n=41; (d) mRuby2, n=37; (e) mKate2, n=45; (f) TagRFP-T, n=43; (g) mApple, n=56; and (h) mCherry, n=54.
Supplementary Figure 17 | Photochromic behavior of RFPs in mammalian cells. U-2 OS cells were transfected with the plasmid shown in Supplementary Figure 2a. The cells were widefield illuminated with alternating light of 556/20 nm and 448/20 nm light for multiple illumination cycles. Photochromic RFPs exhibit a fast decrease and subsequent recovery of the red fluorescence intensity upon alternating excitation with yellow and blue light, respectively. The photochromic amplitudes represented by the blue arrows were calculated using Equation 4. In this figure only one representative recording is shown for each RFP, amplitudes and statistics for all measurements are presented in Supplementary Figure 18b. RFPs are displayed in the following order: (a) mScarlet, (b) mScarlet-I, (c) mScarlet-H, (d) mRuby3, (e) mRuby2, (f) mKate2, (g) TagRFP-T, (h) mApple, (i) mCherry, and (j) dTomato.
**Supplementary Figure 18** | Quantification of net sensitized emission and photochromic amplitude of RFPs. (a) Boxplot of the net sensitized emission from each RFP calculated for individual cells (see grey area in Supplementary Figure 16) relative to mCherry (100%). The mean values of the net sensitized emission is presented in Figure 2h and listed in Supplementary Table 2. (b) Boxplot of all determined photochromic amplitudes (see blue arrows in Supplementary Figure 17) based on 2-4 recordings each with 3 illumination cycles. The mean values of the photochromic amplitudes is presented in Figure 2h and listed in Table 1.
Supplementary Figure 19 | Spectra of GR-RhoA sensors in resting state and in GTP-locked state. Fluorescence emission spectra measured in HeLa cells expressing GR-RhoA sensors constructs were recorded, each spectrum corresponds to the mean spectrum based on measurements from multiple cells. The FRET spectra (colored lines) are normalized to the intensity at the wavelength where the GFP donor spectrum (black lines) peaks. (a) FRET spectrum of the wtGR-RhoA sensor in unstimulated cells (blue dashed line, \( n = 26 \)) and constitutive active caGR-RhoA sensor in the GTP-locked state (blue solid line, \( n = 28 \)) with mCherry as acceptor; (b) FRET spectrum of wtGR-RhoA sensor in unstimulated cells (red dashed line, \( n = 28 \)) and caGR-RhoA sensor in the GTP-locked state (red solid line, \( n = 36 \)) with mScarlet-I as acceptor.
Supplementary Figure 20 | Crystal packing of mScarlet. (a) Visualization of the hydrophobic AB\textsuperscript{5} (green) and hydrophilic AC\textsuperscript{5} (orange) interaction interfaces in the crystal structure of mScarlet. (b) Orthogonal view (rotation around the horizontal axis of a).
## Supplementary Table 1 | Primer list

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<td>72 + 74</td>
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<td>145 + 146</td>
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FW = Forward, RV = Reverse. Bases indicated with * are phosphorostilated bases.
**Supplementary Table 2 | Characteristics of RFPs (extended)**

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<td>0.52 ± 0.19</td>
<td>4.8</td>
<td>57 ± 4</td>
<td>363 ± 4</td>
<td>225 ± 19</td>
<td>129 ± 12</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>mScarlet-H</td>
<td>551</td>
<td>597</td>
<td>0.20 ± 0.02</td>
<td>1.30 ± 0.02</td>
<td>2.01 ± 0.03</td>
<td>80 ± 1</td>
<td>1.06</td>
<td>1.28 ± 0.27</td>
<td>0.92 ± 0.27</td>
<td>4.6</td>
<td>15 ± 1</td>
<td>75 ± 1</td>
<td>574 ± 36</td>
<td>99 ± 10</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>mRuby3&lt;sup&gt;1&lt;/sup&gt;</td>
<td>588</td>
<td>592</td>
<td>0.45 ± 0.01</td>
<td>2.76 ± 0.01</td>
<td>1.53 ± 0.08</td>
<td>80 ± 2</td>
<td>1.21</td>
<td>2.51 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>4.8</td>
<td>57 ± 2</td>
<td>13.7 ± 0.4</td>
<td>*</td>
<td>19 ± 1</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>mRuby3&lt;sup&gt;2&lt;/sup&gt;</td>
<td>551</td>
<td>590</td>
<td>0.39 ± 0.01</td>
<td>2.47 ± 0.01</td>
<td>x</td>
<td>100 ± 1</td>
<td>1.21</td>
<td>2.47 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>4.8</td>
<td>57 ± 2</td>
<td>13.7 ± 0.4</td>
<td>*</td>
<td>19 ± 1</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>mApple&lt;sup&gt;2&lt;/sup&gt;</td>
<td>568</td>
<td>593</td>
<td>0.45 ± 0.01</td>
<td>2.90 ± 0.02</td>
<td>x</td>
<td>100 ± 0</td>
<td>1.02</td>
<td>2.90 ± 0.02</td>
<td>1.91 ± 0.01</td>
<td>4.8</td>
<td>57 ± 2</td>
<td>13.7 ± 0.4</td>
<td>*</td>
<td>19 ± 1</td>
<td>4.9 ± 0.4</td>
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<tr>
<td>mCherry&lt;sup&gt;3&lt;/sup&gt;</td>
<td>586</td>
<td>597</td>
<td>0.23 ± 0.01</td>
<td>1.28 ± 0.03</td>
<td>2.00 ± 0.07</td>
<td>71 ± 0</td>
<td>1.08</td>
<td>1.49 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>4.8</td>
<td>57 ± 2</td>
<td>13.7 ± 0.4</td>
<td>*</td>
<td>19 ± 1</td>
<td>4.9 ± 0.4</td>
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<tr>
<td>dTomato&lt;sup&gt;4&lt;/sup&gt;</td>
<td>555</td>
<td>581</td>
<td>0.49 ± 0.01</td>
<td>3.47 ± 0.01</td>
<td>1.21 ± 0.07</td>
<td>95 ± 0</td>
<td>1.36</td>
<td>3.36 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>4.8</td>
<td>57 ± 2</td>
<td>13.7 ± 0.4</td>
<td>*</td>
<td>19 ± 1</td>
<td>4.9 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>*Absorbance maximum. *Emission maximum. *Extinction coefficient at maximum absorbance. *Quantum yield relative to dTomato. *Fluorescence lifetime component 1. *Fluorescence lifetime component 2. *Amplitude of fluorescence lifetime component 1. *Chi-square fluorescence lifetime fit. Average fluorescence lifetime weighted by amplitude. *Apparent pKa value. *Hill coefficient. *pH at half maximal fluorescence intensity. *Calculated brightness, product of extinction coefficient and quantum yield. *Brightness in mammalian cells normalized to mCherry. *Time in seconds to reduce emission rate from 1000 to 500 photons s<sup>-1</sup> molecule<sup>-1</sup> under wide field conditions. *Time in seconds to reduce emission rate from 1000 to 500 photons s<sup>-1</sup> molecule<sup>-1</sup> under confocal spinning disk conditions. *Percentage that shows photobleaching behavior. *Accumulation in cells normalized to mCherry, brightness in cells divided by calculated brightness. *Apparent delay time of maturation relative to mTurquoise2 in mammalian cells. *Sensitized emission, corrected for direct excitation of donor and acceptor, normalized to mCherry. *Percentage of cells with correct smooth ER labeling. *OSER ratio. *Not applicable due to photobleaching behavior. *not determined. c d j k l m n q r s t v z confidence interval of 95%, e f g standard deviation. Values displayed in bold represent our data, for mRuby3 accumulation in cells the brightness in cells was divided by the published calculated brightness<sup>11</sup>. Values displayed in italic represent published data from the original publication.
Supplementary Table 3 | Data collection and structure refinement statistics

<table>
<thead>
<tr>
<th><strong>Data collection</strong></th>
<th>mScarlet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.978</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>pH</td>
<td>7.8</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>$a$, $b$, $c$ (Å)</td>
<td>84.2, 35.64, 86.01</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
<td>90, 109.7, 90</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>81.0 - 1.47 (1.52 - 1.47)*</td>
</tr>
<tr>
<td>Total reflections</td>
<td>136,140 (13,610)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>40,759 (4,026)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.3 (3.4)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.7 (98.2)</td>
</tr>
<tr>
<td>Mean I/sigma(I)</td>
<td>8.74 (1.94)</td>
</tr>
<tr>
<td>Wilson B-factor (Å²)</td>
<td>20.6</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>0.082 (0.539)</td>
</tr>
<tr>
<td>$CC_{1/2}$</td>
<td>0.996 (0.611)</td>
</tr>
</tbody>
</table>

**Structure Refinement**

| Resolution (Å) | 39.6 - 1.47 (1.52 - 1.47)* |
| $R_{work}$ / $R_{free}$ | 0.1368 (0.2495) / 0.1669 (0.3038) |
| N. of atoms     | 2,128 |
| Protein         | 1,842 |
| Chromophore     | 23 |
| Ligand          | 65 |
| Water           | 196 |
| B-factors (Å²)  | 17.5 |
| Protein         | 15.5 |
| Chromophore     | 14.6 |
| Ligand          | 33.3 |
| Water           | 31.8 |
| R.m.s deviations |        |
| Bond lengths (Å) | 0.011 |
| Bond angles (°)  | 1.54 |

* Values in parentheses are for the highest resolution shell.
Supplementary Video 1 | Microtubule dynamics visualized with mScarlet. Live cell imaging of HeLa cells transfected with mScarlet–7aa–α-tubulin. The Scarlet-LUT was used.

Supplementary Video 2 | Ratiometric FRET imaging with mScarlet-I. U-2 OS cells were co-transfected with the multimeric FRET biosensor with mScarlet-I as acceptor and unlabeled histamine1 receptor. The cells were stimulated with histamine in the same manner as for Figure 2j. The FRET ratio is shown (left) and pseudo-colored with the fire-LUT. The FRET ratio multiplied with the sum of the fluorescence intensity of both channels (right).
Supplementary Note 1. Motivation for mRed7

1. To start the development of a new monomeric RFP, a new synthetic template was designed based on the good maturating monomeric RFP mCherry, several other RFPs, chromoproteins and from the knowledge to break the AC and AB interfaces in the monomerization of tetrameric coral FPs. Also high quantum yield RFPs as DsRed and tdTomato were considered for internal amino acids.

2. In principle, the sequence for mCherry was leading with the following motivation for changes:

3. The shorter N-terminus sequence is copied from tdTomato, which maintains the Kozak sequence and the first 7 amino acids (MVSKGEE) of avGFP in front of two conserved hydrophobic amino acids (VI) that precede the first beta chain. The C-terminus is kept identical to mCherry, with the last 7 amino acids from GFP (GMDELYK) and preceding RHST sequence after the last beta-chain.

4. In the monomerization of mRFP1 and mCherry from tetrameric DsRed 3 mutations were done in the A-B interface: I126R, V128T and I180T. These 3 were conserved in mRed7.

5. To break the A-C interface, 10 amino acids were introduced in mRFP1 (R154E, H163K, A165R, L175D, Y193A, Y195K, H223S, L224T, F225G, and L226A). In mCherry, two substitutions A193N and A226G were introduced. In DsRed.M1 two more mild mutations R154Q, H163S were found and 165A was not changed, which was adopted in mRed. L175D was omitted, assuming that breaking the interaction between R154-E101 (by R154Q) the contribution of the leucine in the interface was marginal. Y193A and Y195N were adopted from mCherry to reduce the hydrophobic interface. The last 4 mutations that break the dimer interface were already introduced by adoption of the C-terminus from mCherry. Together, this yields 8 AC-interface changes as compared to DsRed of R154Q, H163S, Y193A, Y195N, H223S, L224T, F225G, and L226G. The amino acids 165A and 175L were kept. Later in the evolution towards mScarlet, the Q154E and S163K monomerizing mutations from mRFP1 were reintroduced into the new synthetic 7Q1S1BM template. That latter template also harbors the most beneficial amino acid changes found from random mutagenesis by ratiometric screening from the 7Q1S1 template (Supplementary Fig. 1).

6. In the turning loops between the beta sheets, amino acids from several naturally occurring RFPs and chromoproteins were compared. Here we tried to introduce consensus loops. For the first beta-turn (aa 24-27) NGHE, NGHY and NGHK are consensus. We introduced NGHE (also found in DsRed and mCherry). For the second beta-turn (aa 37-40) RPYE is consensus. EDGG is consensus (aa 101-104). EGDT is consensus for the third beta-turn (aa 115-118). We introduced EDGT to allow the stabilizing H-bond between 115E and 118T. It is of note that Q115E is also found in DsRed.T4SF, mTFP1 and eqFP611 and that E118T occurs in td-
mScarlet: a novel bright monomeric red fluorescent protein for cellular imaging

Tomato\(^7\) and DsRedM1\(^50\). PPDG is the consensus turn for aa 131-134, which was introduced into mRed7. Note that the S132P mutation is also found in tdTomato\(^7\), mTFP1\(^52\), mKeima\(^54\), gfasCP\(^49\) and ceriantRFP\(^55\). YPEDGV is the consensus loop of aa 152-157. Here we introduced YPEQGV in view of the monomerization-required 154Q. The longer loops of KLKDGGH (aa 167-173), KKPVQLPG (aa 185-192) and TSHNEDYTV (aa 203-211) were not changed in view of larger sequence diversity and possibly unpredictable results.

7. A number of external mutations not involved in the dimerization interfaces were introduced that were found to be beneficial in evolution of other RFPs, including R14Q (from DsRed.M1\(^50\)), V105A (from DsRed.T4SF\(^51\)), A146P (found in DsRedT3 and T4\(^56\), DsRedM1\(^50\), mKeima\(^54\) and several naturally occurring RFPs and chromoproteins\(^49\)), S148T (in DsRed.M1, tdTomato, mKeima, mTFP and several naturally occurring RFPs and chromoproteins), A157V (from DsRed, tdTomato, DsRedM1 and gfasFP\(^49\)), E178D (found in DsRedT4SF, DsRedM1, pporRFP, mTFP), K183M (from DsRed, tdTomato, DsRedT4 and DsRedT4SF), N197D (found in DsRed, DsRedT4, DsRedT4SF, DsRedM1, mApple, tdTomato, mKeima, mTFP, pporRFP, gfasFP, gfasCP\(^49\)) and I211V (DsRedM1). These mutations were supposed to be folding-enhancing mutations without interfering with oligomerization.

8. A couple of internal mutations were introduced: L84F, F119L, L125F, A176V, V178F, I198T and A218S. L84F is found in mApple, mKeima, mTFP1 and many natural RFPs and chromoproteins; F119L is found tdTomato, DsRedT4SF; L125F occurs in DsRedM1; A176V is found in DsRed, mTFP; V178F is found in DsRed, tdTomato, DsRedM1, mKeima; I198T occurs in tdTomato; and A218S occurs in tdTomato and gfasCP.

9. The chromophore forming residues 67-69 (MYG) were kept as in mCherry as well as the closely positioned 43Q that was identified to greatly accelerate folding in the tetrameric precursor of mCherry DsRedT4\(^56\). Also P64 found in mCherry and in DsRed was kept since it was assumed that this would introduce extra rigidity in the internal alpha helix.

The resulting template was dubbed mRed7 (Supplementary Fig. 1) and while designed to adopt the general fold of DsRed and mCherry (specifically concerning the conserved internal alpha helix), mRed7 harbors 30 amino acid changes as compared to mCherry and 44 as compared to DsRed.

**Supplementary Note 2. Evolution of mScarlet**

mRed7 – the template that we used for engineering a bright monomeric red fluorescent protein with a high quantum yield – showed red fluorescence upon yellow excitation, but displayed a low brightness, a low modulation fluorescence lifetime (\(\tau_M\)) of only 0.7 ns and a fluorescence quantum yield below 1%, meaning there was much room for improvement. To
increase the quantum yield of mRed7, we performed a targeted multiple site-directed mutagenesis on five amino acids (71R/K, 84F/I/K/L/N/Y, 150I/L/M, 198C/H/N/P/R/S/T/Y and 218A/G/R/S/T, for numbering see Supplementary Fig. 1) above the chromophore in an unbiased manner using the OmniChange method. Previous evolution of other bright RFPs has indicated that these 5 amino acids are involved in a complex H-bonding network above the chromophore that greatly influences the brightness and quantum yield of the FP. By their simultaneous mutagenesis we hoped to find the combination to establish the optimal H-bonding network above the chromophore.

The library of 1440 new RFP variants was screened (± 4500 colonies) for increased fluorescence lifetime as a measure for increased quantum yield. The efficiency was assessed by sequencing the DNA of 62 new variants (including several non-fluorescent) and showed an almost complete coverage of the possible amino acids on the five positions. The library yielded RFPs with strikingly enhanced fluorescence lifetimes of up to 3.0 ns. Sequence comparison of improved RFPs revealed that the amino acids at position 71 and 198 had the largest influence on the fluorescence lifetime. We selected one RFP variant with a fluorescence lifetime of 2.1 ns.

To better accommodate the amino acid on position 71, we performed a site directed mutagenesis saturated on position 72 (NNK) and A/F/I/L/M/S/T/V on position 74 both in the internal alpha helix.

Next, we performed a second OmniChange (145D/E, 146A/P, 162A/F/I/L/S/T/V, 164H/I/K/L/M/N/Q, 178F/I/L/M/V, 200F/I/L/M/V), selecting amino acids facing towards the side of the chromophore in an attempt to optimally accommodate the phenolate moiety of the chromophore. The library yielded 4900 new variants and was screened (± 12000 colonies) both on quantum yield (by fluorescence lifetime screening), and brightness and correct maturation of the RFP (by ratiometric screening). The brightness of RFPs expressed by bacteria is measured as a ratio of the fluorescence intensity of the RFP to mTurquoise2. Correct maturation to a red chromophore and not to a dead end green chromophore was simultaneously screened by taking the ratio of red over green fluorescence from the colonies (Supplementary Fig. 2). Colonies with a high fluorescence lifetime, and increased red/cyan or red/green ratio were sequenced. Sequencing results from a total of 146 variants showed that also in the second OmniChange almost all possible amino acids were incorporated in the FP variants.

The top variant mRed7Q1S1 had a seriously increased fluorescence lifetime of 3.3 ns, was reasonably bright but still showed relatively slow maturation. In order to improve the latter parameters, we performed random mutagenesis on four mRed7 variants, including mRed7Q1S1 (Supplementary Fig. 1). We wanted to find folding enhancing mutations and mutations that efficiently promote maturation of mRed7Q1S1 in the conformation that we optimized with the OmniChange mutageneses. In total 80 plates with ±150 mutants each were screened for improvement in fluorescence lifetime, brightness and/or maturation. Instead of selecting the brightest variant and continuing with further random mutagenesis
thereof, the seriously enhanced variants were sequenced. Mutations occurring multiple times and/or those connected to enhanced properties in the screens were pooled. Mutations causing possible dimerization (in the AB and AC interfaces) and neutral mutations were not adopted. In total 17 amino acid changes were combined (positions 7, 14, 23, 58, 73, 86, 112, 125, 146, 154, 161, 163, 167, 173, 183, 190, 199) into a new synthetic gene (7Q1S1BM, **Supplementary Fig. 1**). This RFP has a fluorescence lifetime of 3.5 ns and displayed strongly enhanced brightness and maturation. We reasoned that not all single beneficial mutations in the new template would be additive. Hence the new synthetic template was subjected to two new rounds of random mutagenesis, first yielding 176A, 199K as beneficial mutations. The last round of random mutagenesis yielded two enhanced variants, one with the additional mutation F84Y that we dubbed mScarlet and one with the mutations F84Y and T74I, which we dubbed mScarlet-I (**Supplementary Fig. 1**). Subsequent random and directed mutageneses did not yield further improvements in brightness, maturation or fluorescence lifetime. mScarlet-I and mScarlet displayed similar extreme brightness. mScarlet-I showed an increased maturation speed and efficiency, yet at the cost of a decreased fluorescence lifetime (and quantum yield).

Importantly, many of the initially introduced amino acids in the synthetic consensus template mRed7 that differ from mCherry, remained throughout the evolution towards mScarlet (i.e.: 8V, 84F/Y, 105A, 115E, 118T, 119L, 132P, 148T, 151L, 157V, 165A, 175D, 177E, 178F, 197D, 211I and the deletion of aa 9-12 of mCherry). This suggests that both the initial template and screening-approach are key ingredients for the enhanced properties of mScarlet.

To increase the photostability of mScarlet, both site-directed and random mutagenesis were performed. As the photostability screen in bacteria gave inconsistent results even within the same sample, the screening was performed in mammalian cells in 96-wells plates. The variant with the most improved photostability has a M164H substitution and is named mScarlet-H (**Supplementary Fig. 1**). While this variant displays a reasonable brightness and maturation in cells, the increased photostability was at the cost of a seriously decreased quantum yield and fluorescence lifetime. In the photostability screen, we found this anti-correlation between photostability and fluorescence lifetime or maturation in many more RFP variants, possibly indicating that photobleaching occurs mainly from the excited state.

**Supplementary Results**

a) *The mScarlet variants are true monomers*

Because it is essential that FPs behave as monomers if they are to be used in fusion constructs, we scrutinized the monomeric state of the mScarlet proteins by using the organized smooth endoplasmic reticulum (OSER) approach\textsuperscript{25}. In the OSER assay an FP is fused to an ER anchored transmembrane protein (CytERM), with the FP facing the cytosol. Upon dimerization of the FPs, the opposing ER membranes are brought together, thereby
disrupting the tubular network of the ER and forming intensely labeled whorls, which are large structures of coiled smooth endoplasmic reticulum (SER) membrane. Typical ER structures are shown in Supplementary Fig. 10a-g. To quantify the monomeric behavior, both the percentage of normal looking cells and the ratio of OSER to nuclear envelope (NE) fluorescence intensities were determined (Supplementary Fig. 10h). The ER of cells expressing the mScarlet variants looked normal in 76% to 82% of cells and the OSER/NE ratio values ranged from 1.4 ± 0.2 to 2.2 ± 0.2, which is below the threshold value of 2.3 ± 0.6 for monomeric FPs (Supplementary Fig. 11).

For comparison, the OSER assay was also applied for a set of reference FPs, including a known dimer (dTomato) and established monomers such as mCherry and SGFP2 carrying the monomerizing mutation A206K. As expected, cytERM–dTomato expression resulted in pronounced whorl structures with a low percentage of normal looking cells and a high OSER/NE ratio value, clearly above the 2.3 threshold. On the other hand, mCherry and SGFP2 behaved as monomers (Supplementary Fig. 10). Remarkably, several bright RFPs (mKate2, mRuby2 and mRuby3) did not pass the OSER test (Supplementary Fig. 10). Surprising was the excessive Golgi localization of both mRuby3 and its precursor mRuby2 in the OSER assay, which we scored as abnormal cells (Supplementary Fig. 10g).

To confirm the Golgi localization of mRuby2 and mRuby3 in the OSER assay, we performed a colocalization with a Giantin–mTurquoise2 Golgi marker for mRuby2 and mRuby3 with mScarlet as control (Supplementary Fig. 12), which clearly indicates that mRuby2 and mRuby3–CytERM fusions move onwards to the Golgi system whereas mScarlet–CytERM does not.

b) mScarlet variants perform well in fusion constructs

Because of its high brightness, monomeric behavior and high stability at low pH, we expected mScarlet to be a suitable probe for fusion constructs and organelle labeling. To confirm this, constructs encoding mScarlet fused to proteins that localize to several subcellular structures were expressed in mammalian cells (Supplementary Fig. 13). The F-actin labeling shows clear stress fibers. The structures of the mitochondria, Golgi apparatus, nuclei and peroxisomes all look authentic. Note that expression of mScarlet-tagged alpha tubulin resulted in bright dynamic microtubules with minor background fluorescence (Fig. 2e, Supplementary Movie 1), supporting the notion that mScarlet is monomeric in living cells. Importantly, the performance of mScarlet is equal with or without a polypeptide fused at the C- and N-terminus in terms of brightness and correct localization. In contrast, we detected a different performance for mRuby2 (Supplementary Fig. 5k).

c) mScarlet variants display low cytotoxicity

To evaluate cellular cytotoxicity of mScarlet and other red fluorescent proteins, we performed transient overexpression experiments in HeLa cells and scored the percentage of fluorescent cells over time using flow cytometry. We included cells expressing EGFP and FusionRed, an mRFP that has previously been demonstrated to exhibit low cytotoxicity.
as controls in the analysis. Initially, the percentage of positive (fluorescent) cells was measured 2 days after transfection. 6 days after transfection, the measurement was repeated and the proportion of cells that still remained fluorescent was calculated relative to day 2. Reasoning that EGFP is the non-cytotoxic gold standard, we set the percentage of residual fluorescent cells to 1 and compared the data of all RFPs to this value. The mScarlet series display low cytotoxicity and are comparable to FusionRed and clearly outperform mRuby2, mKate2 and mCherry (Supplementary Fig. 14).

d) Fluorescence lifetime-unmixing of mScarlet and mScarlet-H

Besides the excellent performance of the mScarlet variants in localization studies, these new mRFPs are also very useful in functional imaging, such as in fluorescence lifetime imaging microscopy (FLIM) and FLIM-based unmixing. The latter method can extract the quantitative spatial distribution of multiple fluorophores that each have a distinct fluorescence lifetime using only one spectral channel\textsuperscript{41,42}. Because mScarlet and mScarlet-H have nearly identical fluorescence emission spectra (Fig. 1a), but a large difference in fluorescence lifetime, they should be compatible for lifetime-unmixing experiments. To test this, we analyzed mammalian cells co-expressing nuclear-localized mScarlet (fused to Histon 2A) and cytoplasmic-localized mScarlet-H (fused to a nuclear export sequence) by FLIM and determined the steady-state fluorescence intensity image and the fluorescence lifetime image (Supplementary Fig. 15a-c). While the intensity image shows hardly any contrast within the cells, the fluorescence lifetime image reveals the low fluorescence lifetime of mScarlet-H in the cytoplasm and the high fluorescence lifetime of mScarlet in the nucleus. After lifetime-unmixing, their individual localization becomes visible (Supplementary Fig. 15d-f). We propose that the combination of mScarlet and mScarlet-H can be employed in multiparameter imaging and multiplexing hyperspectral FLIM applications\textsuperscript{64,65}.

Supplementary Discussion

The performance of newly engineered FPs can be analyzed after recombinant expression, isolation and purification of the proteins under well-defined artificial environments. However, their performance in living cells will be more decisive whether or not the new FPs will become widely adopted. Therefore, we extensively characterized the mScarlet variants and various popular published RFPs within mammalian cells. Rather than combining our numbers and published data in a single table, we used the same methods, both in vitro and in cells, to allow a thorough side-by-side comparison.

Surprisingly, we measured substantial differences between the in vitro and cellular brightness for several published mRFPs the (Table 1, Supplementary Table 2 and Supplementary Fig. 3). The in vitro brightness only considers fully matured mRFPs, and therefore can be regarded as maximum theoretical brightness. In cells however, the observed brightness is also determined by the protein synthesis rate, the total maturation efficiency and the stability of the proteins. Notwithstanding these additional factors, both the theoretical and cellular
brightness of mScarlet and mScarlet-I are much higher than for all other mRFPs. By dividing the cellular brightness with the *in vitro* brightness, the accumulation of properly matured mRFPs in cells can be derived. Here, the mScarlets, mCherry and mApple display roughly similar values between 89-129% relative to mCherry. The variants mKate2 and mRuby3 accumulate at roughly half of the efficiency of the mScarlets, whereas mRuby2 and TagRFP-T display even lower accumulation in cells. The overall cellular maturation speed was fastest for mScarlet-I, quickly followed by mCherry and mApple. These values should not be confused with published mRFP maturation times for which usually only the higher speed of chromophore oxidation of already synthesized and folded purified recombinant mRFP proteins is determined\textsuperscript{13,66}.

Previously, it was presented that the brightness of FPs is highest for YFPs and decreases with increasing wavelength\textsuperscript{1}, mainly caused by lower quantum yields of monomeric red and far-red FPs\textsuperscript{14,15}. Here, we obtained two mRFPs that break this trend with the calculated brightness of mScarlet and mScarlet-I being ~2-fold and 1.5-fold higher when compared to EGFP, respectively. Based on our results, we think that there is considerable room for improvement of FPs from the far-red spectral class.

Besides low brightness or incomplete maturation, residual dimerization can be a serious problem in FPs, as dimer formation can cause mis-localization of fusion-proteins or yield false positive FRET results while assessing protein-protein interactions. On the other hand, dimerization can be beneficial in the design of intramolecular (single chain) FRET sensors\textsuperscript{67,68}. To assess residual dimerization, we analyzed the RFPs using the OSER method\textsuperscript{25} since it is sensitive, quantitative and performed in living mammalian cells, rather than in artificial environments following ultracentrifugation, non-denaturing gels or size-exclusion chromatography. Our data and ordering of RFPs from monomer to oligomer are consistent with those obtained by Cranfill et al.\textsuperscript{17}, who also adopted the OSER assay. The aberrant behavior of TagRFP-T and mKate2 in the OSER assay is consistent with other studies\textsuperscript{17,25,61}.

In support of the monomeric behavior observed in cells, the mScarlet crystal structure shows that the side chains involved in the AB and AC interaction interfaces in naturally occurring RFP tetramers are not in contact with adjacent beta-barrels (Supplementary Fig. 19). Furthermore, the crystal contact areas observed in the mScarlet crystal structure are two to three times smaller than those in crystal structures of non-monomeric FPs such as EGFP or DsRed.

Another issue that can limit the practical use of FPs is cytotoxicity. It was argued that cytotoxicity of FPs is mainly caused by (residual) oligomerization or aggregation\textsuperscript{13,69}. Previously, mKate2 that showed residual aggregation was subjected to mutagenesis and screened for decreased cytotoxicity, resulting in the low cytotoxic mRFP FusionRed\textsuperscript{13}. Here we assessed cytotoxicity by comparing the percentage of RFP producing cells at two time points: 6 days after transfection and 2 days after transfection, normalized to the percentage of cells expressing non-cytotoxic EGFP (Supplementary Fig. 14). This experiment confirmed the earlier reported decreased cytotoxicity of FusionRed as compared to mKate2.
in HeLa cells. In line with their great performance in the OSER assay, the mScarlet series perform very well in this cytotoxicity experiment: they belong to the least cytotoxic mRFPs, as good as FusionRed and clearly better than mCherry, mKate2 and mRuby2. A prolonged high percentage of RFP-expressing cells is clearly a beneficial aspect for long term expression experiments.

In view of their high intrinsic brightness, monomeric behavior in cells and low cytotoxicity, the mScarlet series are the preferred mRFPs for tagging of proteins and long term expression experiments. In addition, they lack cysteine residues (unlike mRuby2, mRuby3, TagRFP-T, mKate2 and FusionRed) or N-glycosylation consensus sequences, which is relevant for experiments addressing the secretory pathway and/or oxidizing compartments.

In the comparative analysis, a surprising finding was that besides mApple, also TagRFP-T, mRuby2 and mRuby3 display strong fast photochromic behavior in cells upon alternating blue and green-yellow excitation. Importantly, fast photochromicity is negligible for the mScarlets, for mCherry, mKate2 and for dTomato. Especially in quantitative experiments, such as ratiometric FRET, but also in blue light-activated optogenetic systems, strong photochromic behavior is highly undesirable.

In the comparative cellular intramolecular FRET analysis, we deliberately used YFP-mRFP fusion proteins with equal spacing between the two beta-barrels. For the intermolecular ratiometric FRET comparison we used a sensor that does not depend on protein-protein interaction, but on protein-lipid interaction and surface concentration-induced bystander FRET. Therefore, we think that the observed differences reflect the general performance of mRFPs in ratiometric FRET due to differences in intrinsic brightness and maturation rather than mRFP-dependent conformation or -interaction effects. Based on the similar YFP-quenching that we observed (Fig. 2j), we expect that mCherry, mScarlet and mScarlet-I will perform equally well for donor-based FRET methods such as FLIM-FRET or acceptor photobleaching FRET. However, for ratiometric FRET, mScarlet and mScarlet-I are clearly the preferred mRFP acceptors in view of their much higher quantum yield and sensitized emission. This is demonstrated for a novel ratiometric GR-RhoA sensor, that shows a much higher sensitized emission change with mScarlet-I as acceptor than with mCherry as acceptor (Fig. 2i, Supplementary Fig. 18).
Author contributions
DSB, LH and LvW cloned the constructs, performed the mutagenesis & screening experiments, and expressed and purified the RFPs; TWJG designed the synthetic template and mutagenesis strategy; DSB, MAH and LH performed the in vitro spectroscopic characterization; MP, DSB, MAH and TWJG analyzed the spectroscopic data; DSB, LH and LvW performed the cellular localization, photobleaching, maturation FLIM and FRET experiments; DSB performed and analyzed the photochromicity experiments; DSB and KEW performed and analyzed the cytotoxicity experiments; LH & MM performed and analyzed the OSER experiments and the GR-RhoA FRET experiments; DSB, TWJG and MP designed the automated cellular ratiometric, maturation and photobleaching screens and performed data analysis; MP performed the statistical analyses and made the mScarlet LUT; SA crystallized mScarlet, SA, GG and AR performed the X-ray diffraction experiments and analyzed the structure; LH, DSB, AR and TWJG wrote the manuscript. All authors reviewed the manuscript.

References


