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mScarlet3: a brilliant and fast-maturing red fluorescent protein

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► **To cite this version:**

Theodorus Gadella, Laura van Weeren, Jente Stouthamer, Mark Hink, Anouk Wolters, et al.. mScarlet3: a brilliant and fast-maturing red fluorescent protein. *Nature Methods*, 2023, 20 (4), pp.541-545. 10.1038/s41592-023-01809-y . hal-04162894

HAL Id: hal-04162894

<https://hal.science/hal-04162894>

Submitted on 25 Nov 2023

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1 **1. Extended Data**

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3

Figure or Table # Please group Extended Data items by type, in sequential order. Total number of items (Figs. + Tables) must not exceed 10.	Figure/Table title One sentence only	Filename Whole original file name including extension. i.e.: Smith_ED_Fig1.jpg	Figure/Table Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Table. 1	Properties of red fluorescent proteins	Gadella_ED_Table1.pdf	^a Absorbance maximum. ^b Emission maximum. ^c Extinction coefficient at maximum absorbance. ^d Quantum yield. ^e Fluorescence lifetime component 1. ^f Fluorescence lifetime component 2. ^g Amplitude of fluorescence lifetime component 1. ^h Chi-square fluorescence lifetime fit. ⁱ Average fluorescence lifetime weighed by amplitude. ^j Modulation lifetime in cells. ^k Apparent pKa value. ^l Hill coefficient. ^m Calculated intrinsic brightness, product of extinction coefficient and quantum yield. ⁿ Brightness in mammalian cells normalized to mScarlet-I. ^o Time in seconds to half the emission rate under wide field conditions. ^p Time in seconds to half the emission rate under confocal spinning disk conditions. ^q Percentage of photochromic behavior. ^r Accumulation in cells normalized to mScarlet-I (brightness in cells divided by intrinsic brightness). ^s Apparent delay time of maturation relative to mTurquoise2 in mammalian cells. ^t Initial fluorescence at pH 13.5 relative to pH 7.4. ^u Time required to reduce fluorescence by 50% at pH 13.5. Sensitized emission by FRET in a RFP-SYFP2 fusion, corrected for direct excitation of donor and acceptor, normalized to mScarlet-I. ^w Percentage of cells with a correct ER labelling upon expressing an Cyterm-RFP fusion. ^x Percentage of cells visible OSER structures upon expressing an Cyterm-RFP fusion. ^y Number of analyzed cells. ^z Values from Semiakina et al..

			c,d,e,f,j,k,n,m,n,q,r,s ± standard deviation, or value cited from literature. Values in <i>Italics</i> from Bindels et al., 2016.
Extended Data Fig. 1	Brightness of RFPs in HeLa cells 24 h after transfection	Gadella_ED_fig1.pdf	For each RFP an mTurquoise2 construct was coexpressed in cells in a 1:1 molecular ratio. The average red fluorescence and cyan fluorescence was quantified for each cell (red data points). a mScarlet3 n=231, b mScarlet-I3 n=303, c mScarlet n=238, d mApple n=266, e mCherry n=272, f mScarlet-I n=303, g mKate2 n=294, h mRuby3 n=275, I FusionRed n=156, j TagRFP-T n=320, and k dTomato n=268. The red fluorescence values were corrected for spectral throughput of the microscope (correcting for instrument-dependent excitation and detection efficiencies of the different RFPs). The slopes of the linear regression line that is forced the origin was used for calculating the relative brightness of the RFPs (as compared to mScarlet) and indicated in Extended Data Table I and Fig. 1d. The slope was calculated as $\sum RC / \sum C^2$ in which R is the RFP intensity value of a single cell (vertical axes) and C is the CFP intensity of the same cell (horizontal axes). The standard error of the slope (indicated in Fig. 1d and Extended Data Table 1) was calculated as $\text{Sqrt}((\sum R^2 - (\sum RC)^2 / \sum C^2) / ((n-1)\sum C^2))$. In which n is the number of cells.
Extended Data Fig. 2	Alkaline denaturation speed of diverse purified RFPs at pH 13.5.	Gadella_ED_fig2.pdf	a: logarithmic time axis, b: normal time axis.
Extended Data Fig. 3	Photostability of RFP variants	Gadella_ED_fig3.pdf	a: Widefield bleaching of mScarlet variants without time-axis correction, b: same data as a) but y-axis scaled according to cellular brightness; c: confocal bleaching of

			<p>mScarlet variants; d: confocal bleaching of mScarlet variants and other RFPs. In order to fairly compare the photostability of different RFP variants under confocal imaging conditions, the time axis is corrected in c and d for spectral properties relative to mScarlet to mimic an identical overall emission rate (photons/s) for all RFPs at the start of the experiment. e: Blue light-induced photochromicity of RFP variants. The RFP variants were expressed in HeLa cells and continuously exposed with 550 nm excitation light. At t=15, 31, 46 and 52 s, the 550 nm excitation light interrupted by a brief excitation at 470 nm for 1 s (not recorded on time axis) after which the 550 nm excitation was continued. The sudden increase after blue light excitation is caused by photochromic behavior.</p>
Extended Data Fig. 4	<p>Evaluation of RFPs as fusion tag in HeLa cells</p>	Gadella_ED_fig4.pdf	<p>a-h: representative images of selected Cyterm-RFP fusions in HeLa cells 24h after transfection. a FusionRed, b mScarlet3; c TagRFP-T; d mCherry; e mScarlet-I3; f mScarlet-I3-NCwt; g mScarlet-I; h mScarlet. For a-h bar is 10 μm and the fluorescence intensities were pseudo-colored between minimum and maximum intensities according to the mScarlet lookup table as indicated in image d and subsequently gamma-corrected (value 0.5, including the lookup table) to better visualize the low intensity areas. For cell numbers imaged and evaluated see Extended data Table 1, images shown are representative of these. I-I: mScarlet3 as fusion tag for cellular localization studies. mScarlet3 was fused to: i alpha-tubulin (microtubule cytoskeleton); j EB3 (+ends of microtubules); k Giantin (Golgi apparatus); and l a 3x</p>

			<p>repeated nuclear localization signal (NLS) (nuclei) and expressed in HeLa cells. For i-l bar is 10 μm and the fluorescence intensity was pseudo-colored between minimum and maximum fluorescence according to the mScarlet lookup table indicated in l. Micrographs shown in i-l are representative of at least 4 images made from 2 independent transfections.</p>
<p>Extended Data Fig. 5</p>	<p>FRET in HeLa cells expressing SYFP2 alone or SYFP2-RFP fusion proteins</p>	<p>Gadella_ED_fig5.pdf</p>	<p>. a. Average FRET spectra from HeLa cells expressing SYFP2 alone or SYFP2-RFP fusion proteins. All spectra were normalized to the unquenched SYFP2 donor. b. Quantification of FRET spectra from a. E (blue bars) represent the energy transfer efficiency (average \pm sd), and Qs (red bars) depict the sensitized emission quantum yield (average \pm sd). Qs is defined as the number of sensitized emission quanta emitted by the RFP acceptor divided by the number of quanta absorbed by the SYFP2 donor and is calculated by $Q_s = E \cdot Q_a$, in which Q_a is the acceptor quantum yield of fluorescence. Qs is not dependent on the emission spectra and is proportional to the integrated acceptor sensitized emission signal. Each data point represents quantitative unmixing analysis of a single cell spectrum, 18-25 single cell spectra were recorded for each condition. c. SYFP2 fluorescence lifetime of HeLa cells expressing SYFP2 alone or SYFP2-RFP fusion proteins as determined by frequency-domain FLIM. Tau(phi) (red bars) is the average lifetime estimated from the phase shift (\pm sd), Tau(mod) (blue bars) is the average fluorescence lifetime estimated from the demodulation (\pm sd). For c each data point represents the average lifetime of an image with 4-</p>

			20 individual cells, 4-6 FLIM recordings were done for each condition.
Extended Data Fig. 6	Relative cell viability of RFP transfected cells	Gadella_ED_fig6.pdf	mEGFP and RFPs were transfected separately into HeLa cells at day 0. At day 2 each of the RFP transfected cells were split and mixed with mEGFP transfected cells. At days 4 and 6 the cell mixtures were resplit. At days 2, 4 and 6 after (4-5 h after splitting/mixing) the number of RFP and mEGFP expressing cells in the mixtures was scored. The ratio of RFP to mEGFP expressing cells was normalized to that obtained at day 2 to correct for bias in transfection efficiencies. For each RFP 8-12 technical replicates (depicted as individual data points) were measured. The average normalized ratio of red to green cell numbers \pm sd is indicated.
Extended Data Fig. 7	Brightness of RFPs in mammalian cells after fixation relative to mScarlet	Gadella_ED_fig7.pdf	Indicated is the average relative brightness (\pm sd), corrected for spectral throughput determined from single cell red to cyan ratios and linear regression (though the origin) like in Extended Data Fig. 1.
Extended Data Fig. 8	Illustration of the potential van der Waals interactions between 9 residues of mScarlet3 near the surface of the one face of the β-barrel	Gadella_ED_fig8.pdf	Residues belong to the central α -helix traversing the β -barrel (Thr74), to the α -helix capping one head of the β -barrel (Trp84), to the loop linking the latter helices (His76), to the 7 th strand (Leu151), to the 8 th strand (Leu158), to the loop bridging the 9 th and 10 th strands (Pro191), to the 10 th strand (Phe194, Ile196), to the 11 th strand (Ala220). Potential van der Waals interactions (dashed black lines) are highlighted by inter-carbon atom distances below 4.0 Å. a. View from the side of the β -barrel. The set of vdW interactions forms a crown-like structure. b. 90° view (from the barrel head) showing residues organized in three layers: the main one is

			formed of residues Thr74, Trp84, Leu158, Phe194 and Ala220, with residues His65 and Pro191 ahead of this layer, and residues Leu151 and Ile196 behind it.
Extended Data Fig. 9	Evolution path for mScarlet3 (seq-id #15) and mScarlet-I3 (#16) from mScarlet (#1) and mScarlet-I (#2), respectively	Gadella_ED_fig9.pdf	The numbers correspond with the sequence IDs in Extended Data Table 2. The horizontal axis depicts the (modulation) fluorescence lifetime as determined in live HeLa cells ~ 24 h after transfection and corresponds with intrinsic brightness (more to the right is better), and the vertical axis depicts the cellular brightness (higher is better) normalized to mScarlet-I. F. Vertical error bars are SD of the slope of the line fitted to the red to cyan cellular ratios (of 22-77 cells, e.g. see Extended Data Fig. 1) or the average modulation lifetime of the mScarlet variant in HeLa cells (from 3 individual FLIM recordings with > 5 cells each)

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5 **1. Supplementary Information:**6 **A. PDF Files**

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Item	Present?	Filename Whole original file name including extension. i.e.: Smith_SI.pdf. The extension must be .pdf	A brief, numerical description of file contents. i.e.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
Supplementary Information	YES	Gadella_SI.pdf	Supplementary notes, references, Figure, Tables and movie legends

Reporting Summary	Yes	NMETH-BC49688Breportingsu mm.pdf
Peer Review Information	No	<i>OFFICE USE ONLY</i>

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12
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B. Additional Supplementary Files

Type	Number Each type of file (Table, Video, etc.) should be numbered from 1 onwards. Multiple files of the same type should be listed in sequence, i.e.: Supplementary Video 1, Supplementary Video 2, etc.	Filename Whole original file name including extension. i.e.: <i>Smith_Supplementary_Video_1.mov</i>	Legend or Descriptive Caption Describe the contents of the file
Supplementary Video	Supplementary Video 1	Gadella_Supplementary_Video_1.avi	Confocal timelapse of mScarlet3-Cyterm imaged in U2Os cells 48 h after transfection. In addition to ER and nuclear envelope labelling also motile punctate endomembrane structures appear that are possibly involved in removal of mScarlet3-Cyterm from the ER. This is particularly apparent in the top right cell. Total Video duration 42 s at 0.32 s frame rate
Supplementary Video	Supplementary Video 2	Gadella_Supplementary_Video_2.avi	Prolonged confocal imaging of actin dynamics using Lifeact-mScarlet3. Actin dynamics was imaged in HeLa cells transfected with pLifeact-mScarlet3. 1000 frames were captured each with 2

			scans averaged. No noticeable bleaching is observed. Total Video duration is 16 h at 1 min frame rate.
Supplementary Video	Supplementary Video 3	Gadella_Supplementary_Video_3.avi	Microtubule polymerization visualized by EB3-mScarlet3 in HeLa cells. Total Video duration is 2 minutes in real time, bar is 10 μm .
Supplementary Video	Supplementary Video 4	Gadella_Supplementary_Video_4.avi	Dynamic microtubules visualized by expression of mScarlet3-alpha tubulin in HeLa cells. Total Video duration is 2 minutes in real time, bar is 10 μm .
Supplementary Video	Supplementary Video 5	Gadella_Supplementary_Video_5.avi	mScarlet3 applied for prolonged live-cell imaging of nuclei in pancreatic cells in zebrafish larvae. H2A-mScarlet3 was expressed under an elastase promotor and examined on a light sheet microscope. Nuclear localization is found only in the exocrine cells of the pancreas. Movement is caused by motion of the larva. Total Video duration of 12 h in real time at 15 min frame rate.
Supplementary Video	Supplementary Video 6	Gadella_Supplementary_Video_6.avi	Lightsheet microscopy of H2B-mScarlet3 labelled cell nuclei in a developing zebrafish larva – full larva. Total duration 355 min.
Supplementary Video	Supplementary Video 7	Gadella_Supplementary_Video_7.avi	Lightsheet microscopy of H2B-mScarlet3 labelled cell nuclei in a developing zebrafish larva - zoom on mitotic cells. Total duration 355 min (zoom). Note several dividing cells.

15 **3. Source Data**

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Parent Figure or Table	Filename Whole original file name including extension. i.e.: <i>Smith_SourceData_Fig1.xls</i> , or <i>Smith_Unmodified_Gels_Fig1.pdf</i>	Data description i.e.: Unprocessed western Blots and/or gels, Statistical Source Data, etc.
Source data Fig. 1	Gadella_source_data_Fig1.xlsx	Source data for Fig 1a-i
Source data Fig. 2	Gadella_source_data_Fig2.xlsx	Source data for Fig 2i
Source data ED Fig. 1	Gadella_source_data_ED_Fig1.xlsx	Source data for extended data Fig 1
Source data ED Fig. 2	Gadella_source_data_ED_Fig2.xlsx	Source data for extended data Fig 2a,b
Source data ED Fig. 3	Gadella_source_data_ED_Fig3.xlsx	Source data for extended data Fig 2a,b
Source data ED Fig. 5	Gadella_source_data_ED_Fig5.xlsx	Source data for extended data Fig 3a-e
Source data ED Fig. 6	Gadella_source_data_ED_Fig6.xlsx	Source data for extended data Fig 5a-c
Source data ED Fig. 7	Gadella_source_data_ED_Fig7.xlsx	Source data for extended data Fig 7
Source data ED Fig. 9	Gadella_source_data_ED_Fig9.xlsx	Source data for extended data Fig 9

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18 mScarlet3: a brilliant and fast maturing red fluorescent protein

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35 **We report the evolution of mScarlet3, a cysteine-free monomeric red fluorescent protein**
36 **with fast and complete maturation, record brightness, -quantum yield (75%) and -**
37 **fluorescence lifetime (4.0 ns). The mScarlet3 crystal structure reveals a barrel rigidified at**
38 **one of his heads by a large hydrophobic patch of internal residues. mScarlet3 behaves well**
39 **as fusion tag, displays no apparent cytotoxicity and it surpasses existing red fluorescent**
40 **proteins as Förster resonance energy transfer (FRET) acceptor and as reporter in transient**
41 **expression systems.**

42

43 Red fluorescent proteins (RFPs) found in Anthozoa species¹ are structurally related to GFP
44 found in *Aequoria victoria* and fold in highly similar yet tetrameric beta-barrel structures².

45 Much effort has been devoted to monomerize RFPs. After the first, relatively dim, mRFP1 was
46 described³, other brighter variants were created as mCherry⁴, TagRFP⁵, mRuby3⁶, mKate2⁷

47 and FusionRed⁸. However, irrespective of improvements, all of these second generation
48 mRFPs still suffer from relatively low quantum yield, photochromicity or residual dimerization
49 tendency. For this reason, we developed the mScarlet series of RFPs⁹. mScarlet displays the
50 highest intrinsic brightness (multiplication of extinction coefficient and quantum yield), no
51 photochromicity and is monomeric, but its maturation is relatively slow. mScarlet-I with lower
52 intrinsic brightness is brighter in cells owing to faster and more complete maturation.
53 Interestingly, in a recent study mCherry-XL with an equal fluorescence quantum yield (70%)
54 as mScarlet was evolved, but owing to reduced maturation, the brightness in cells was half of
55 mScarlet¹⁰. Obviously, a variant combining fast maturation and the highest possible intrinsic
56 brightness is highly desirable. For instance, for building FRET sensors with an RFP acceptor,
57 the extent of RFP maturation defines the extent of donor quenching, whereas its intrinsic
58 brightness determines the intensity of the sensitized emission, both contributing to high FRET
59 contrast.

60 Because random mutagenesis to create brighter mScarlet variants proved unsuccessful⁹,
61 we devised a targeted approach towards amino acids surrounding Threonine-74, since this is
62 the only mutated amino acid in mScarlet-I as compared to mScarlet. With multiparameter
63 screening¹¹, we evolved variants of mScarlet-I with a higher quantum yield and variants of
64 mScarlet with faster maturation (see Supplementary note 1).

65 After several rounds of targeted and random mutagenesis we generated two final
66 variants mScarlet-3 and mScarlet-I3 (for spectra see Fig. 1a and b). Spectroscopic evaluation
67 of purified mScarlet3 (Extended Data Table I) revealed that it has a quantum yield of 75%, and
68 extinction coefficient of $104,000 \text{ M}^{-1} \text{ cm}^{-1}$ and it displays a long monoexponential fluorescence
69 lifetime of 4.0 ns (Fig. 1c). Spectroscopic analysis of mScarlet-I3 revealed a seriously enhanced
70 quantum yield (65%) and an 21% increased intrinsic brightness as compared to mScarlet-I,
71 approaching that of mScarlet (95%) (Extended Data Table I). Intrinsic brightness of purified
72 FPs, however, does not always report on the actual brightness found in cells, since the latter
73 additionally depends on the extent and speed of chromophore maturation. Importantly, in
74 cells, mScarlet3 and mScarlet-I3 are 76% and 80% brighter as compared to mScarlet and > 5-
75 fold brighter than mCherry, owing to a substantially faster and more complete maturation
76 (Extended Data Table I, Fig. 1d, Extended Data Fig. 1). By directly comparing the intrinsic
77 brightness with the cellular brightness, one can infer the overall extent of maturation. It was
78 found that after 24 h mScarlet3 matures to an equal extent (96%) as the very efficiently

79 maturing mScarlet-I (Fig. 1e). mScarlet-I3 maturation is more complete (113%) than mScarlet-
80 I. mScarlet3 matures 4 times faster than mScarlet and equally fast as mCherry (Extended Data
81 Table I, Fig. 1f). mScarlet-I3 is the fastest maturing monomeric RFP tested. Surprisingly, in
82 alkaline denaturation experiments, we found a marked stability of mScarlet3: where mScarlet
83 and mScarlet-I denature instantaneously (< 1 s) at pH 13.5, mScarlet3 retained more than
84 half of its initial fluorescence for over 40 minutes (Extended Data Fig. 2). mScarlet3 ($pK_a=4.5$)
85 and mScarlet-I3 ($pK_a=4.2$) have better acid resistance as compared to mScarlet(-I) ($pK_a=5.4$),
86 which would be beneficial for applications of these RFPs in endomembrane studies (Fig. 1g,
87 Supplementary Video 1). At high excitation power, the photobleaching kinetics are slightly
88 faster as compared to the precursors, with mScarlet3 performing better in confocal bleaching
89 (see Extended Data Table I, Extended Data Fig. 3a-d). Notwithstanding, mScarlet3 is
90 photostable enough to permit 1000 confocal scans under live cell confocal timelapse imaging
91 conditions without noticeable photobleaching (Supplementary Video 2). We also tested
92 photochromicity in alternating blue and green excitation and we found that both mScarlet3
93 and mScarlet-I3 are non-photochromic (like mScarlet, mScarlet-I and mCherry) (Extended
94 Data Table I, Extended Data Fig. 3e), which is in great contrast to mApple, mRuby3, TagRFP-T
95 and FusionRed^{9,12}. mKate2 and TagRFP-T showed increased red fluorescence after several
96 photochromic cycles (Extended Data Fig 3d,e). Photochromic behavior can be highly
97 problematic in quantitative ratiometric FRET studies⁹. mScarlet3 and mScarlet-I3 display
98 monoexponential fluorescence decay with long lifetimes of 4.0 and 3.6 ns, respectively,
99 facilitating possible application as donors in FRET-FLIM.

100 Since both mScarlet3 and mScarlet-I3 contain new N and C termini and various mutations
101 on the exterior of the beta-barrel (see Supplementary note 1, Supplementary Fig. 1), we
102 performed an OSER (organized smooth ER) assay to address their monomeric behavior¹³.
103 Indeed, the OSER test revealed no enhanced oligomerization (Extended Data Table 1,
104 Extended Data Fig. 4a-h).

105 Several fusion constructs with mScarlet3 to label specific cellular organelles and
106 structures were made. In all instances bright labelling of the correct structures and
107 localizations was found (Fig. 2a-f, Extended Data Fig. 4 i-l, Supplementary Video 3). Also, the
108 direct fusion to alpha-tubulin, which is known to be very adversely affected by residual
109 dimerization tendency of FPs, revealed nice microtubular structures displaying dynamic
110 instability¹⁴(Supplementary Video 4).

111 The new mScarlets are superior FRET acceptors of a monomeric yellow fluorescent
112 protein SYFP2: in ratiometric spectral FRET, mScarlet3 quenches SYFP2 fluorescence intensity
113 as efficiently as mScarlet-I (both 53%) and better than mCherry (46%) or mScarlet (37%) (Fig.
114 1h & Extended Data Figs 5a,b). The yield in sensitized emission is substantially improved for
115 mScarlet3 (140% as compared to mScarlet-I, 154% as compared to mScarlet and 377 % as
116 compared to mCherry). The better quenching of SYFP2 is also seen by FLIM analysis of the τ_{ϕ}
117 fluorescence lifetime of SYFP2 that is more reduced with mScarlet3 (1.67 ns) than with
118 mScarlet (1.85 ns) or mCherry (1.77 ns) (Extended Data Fig. 5c). We found a small negative
119 effect of the new N and C termini on FRET levels, possibly due to another average orientation
120 despite our efforts in keeping the linker length identical in the comparison of the diverse
121 fusions (see Extended Data Table 1, Extended Data Fig. 5). Although mScarlet-I3 seems to be
122 a slightly better quencher of SYFP2 than mScarlet3, it produces less sensitized emission due
123 to its lower quantum yield. Hence, mScarlet3 is the best FRET acceptor of yellow fluorescent
124 proteins, providing equal YFP quenching but 40% more sensitized red emission as compared
125 to mScarlet-I (the previously best performing RFP).

126 Because mScarlet3 and mScarlet-I3 incorporate the new C and N termini originally
127 described for mScarlet-I2¹⁵ for which it was shown to reduce cytotoxicity in bacteria, we
128 tested for cytotoxicity in mammalian cells. Within 48 h after transfection, we found no
129 apparent cytotoxicity of mScarlet3 or mScarlet-I3 expression (Fig. 1i). By monitoring
130 fluorescently labeled cells at 4 and 6 days after transfection, we found slightly more cells
131 producing mScarlet-I3 or mScarlet3 as compared to other RFPs (Extended Data Fig. 6), also
132 suggesting no apparent cytotoxicity. We tested the resistance to paraformaldehyde fixation,
133 where mScarlet3 proved to be the brightest RFP variant after fixation (Extended Data Fig. 7).

134 To understand the structural basis for the improvements found in mScarlet3, we
135 determined its crystal structure at physiological pH at 1.33 Å resolution (see online Methods
136 and Supplementary Note 2). The conformations of the chromophore and its surrounding
137 residues (first coordination sphere) are perfectly superimposable on those observed in the
138 pH 7.8 crystal structure of mScarlet⁹. Yet, the key residue Glu216 adopts two alternate
139 conformations, like in the bright green FP EGFP (Fig. 2g). We focused our analysis on the
140 mutations within the bulk of the protein barrel: Y84W, Y194F, V196I, and G220A.
141 Coincidentally, these four mutations remove two polar atoms and add six non-polar atoms,
142 which results in the replacement of a small amphipathic cavity by a hydrophobic patch purely

143 involving methyl or methine carbon atoms (Fig. 2h). This hydrophobic patch is linking together
144 the tips of 4 of the 11 beta-strands of the protein and of the chromophore-bearing alpha-
145 helix, as well as a loop and a helix bridging pairs of strands (Extended Data Fig. 8). Thereby it
146 contributes to the increased rigidification of one head of the beta-barrel and the increased
147 fluorescence QY of mScarlet3.

148 To assess the performance of mScarlet3 for *in vivo* applications, we first compared
149 mScarlet and mScarlet3 for brightness in zebrafish larvae. 24 h after injection of plasmid, a 2-
150 fold increased red fluorescence was detected for mScarlet3 in larvae (Fig. 2i), in good
151 agreement with the values found in mammalian cells (Fig. 1d, Extended Data Figs. 1, 7).
152 Additionally, injections were made of H2B-mScarlet3 produced in exocrine cells of the
153 pancreas (Fig. 2j; Supplementary Video 5). Finally, we imaged H2B-mScarlet3 for 6 hours in 5
154 min intervals (z-stack of 309 frames) in all cells in zebrafish larvae following mRNA injection
155 and found many cells going through mitosis (Fig. 2k, Supplementary Video 6 and 7). Thus,
156 mScarlet3 is useful for *in vivo* examination of cells in zebrafish larvae.

157 In conclusion, we have evolved a brilliant new red fluorescent protein mScarlet3 that
158 uniquely combines the highest quantum yield of RFPs, monomeric behavior, no apparent
159 cytotoxicity and absence of photochromic behavior with fast and complete maturation
160 (Extended Data Fig. 9). In all tested fusions, mScarlet3 demonstrated excellent performance
161 as fusion-tag, as FRET acceptor and in live cell applications. While chromophore rigidity is
162 essential for high quantum yield, too much rigidity may hamper FP maturation that requires
163 a certain degree of internal flexibility to assist fast kinetics of chromophore formation and -
164 oxidation. We speculate that the engineered hydrophobic patch (Fig. 2h) exactly does that
165 job. We do not exclude that other existing bright FPs can be still improved to combine
166 maximal brightness and maturation by following the same recipe of multiparameter (at least
167 brightness and fluorescence lifetime) screening¹¹.

168

169 **Acknowledgments**

170 We are grateful to Ronald Breedijk (Amsterdam) for his assistance and the maintenance of
171 the LCAM microscopes and to Sylvain Engilberge (Grenoble) for his assistance in drawing
172 mScarlet3 structural figures. Part of the work has been performed in the UMCG Microscopy
173 and Imaging Center (UMIC). This work was supported by a grant from the French Agence
174 Nationale de la Recherche (ANR-17-CE11-0013 to J.D.).

175

176 Author contributions

177 TWJG designed and supervised the project. LvW & JS performed the mutagenesis
178 experiments. LvW prepared bacteria, purified proteins, cultured and transfected mammalian
179 cells. TWJG performed and analyzed the spectroscopic characterization and cellular imaging
180 experiments. MAH performed and analyzed the TCSPC experiments. AHGW and BNGG
181 performed and analyzed the zebrafish experiments. SA, JD and AR purified mScarlet3 for
182 crystallization, crystallized mScarlet3 and solved and analyzed the crystal structure. TWJG and
183 AR wrote the manuscript with input from all authors.

184

185 Competing interests

186 The University of Amsterdam has applied for a European Patent (EP 33108) and international
187 PCT Patent (WO 35192) (patents pending) on "Improved variants of monomeric scarlet red
188 fluorescent protein" with TWJ Gadella and L van Weeren as inventors. Otherwise, the authors
189 declare no competing interests.

190

191 Figure Legends

192 **Fig. 1. Characterization of mScarlet3 and mScarlet-I3.** Normalized absorbance, excitation,
193 and emission spectra of mScarlet3 (a) and mScarlet-I3 (b). For the fluorescence excitation
194 spectra, the emission was set at 620 nm, for the fluorescence emission spectrum excitation
195 was set at 540 nm. c. Fluorescence decay of mScarlet3 fitted with a single exponential
196 lifetime of 3.96 ns. The weighted residuals show a perfect fit ($\chi^2=1.18$). d. Relative
197 brightness (average \pm sd) of RFPs in HeLa cells 24 h after transfection, determined by
198 normalized, spectrally-corrected ratios of red to cyan fluorescence intensity found in cells
199 upon co-production of RFP and mTurquoise2 (See Extended Data Fig. 1). e. Maturation
200 extent of RFPs in HeLa cells relative to mScarlet-I (average \pm sd) 24 h after transfection
201 calculated by dividing the cellular brightness (Fig 1d) by the intrinsic brightness (Extended
202 Data Table 1). f. Maturation speed of RFPs in mammalian cells measured as delay (average \pm
203 sd in h) relative to co-produced mTurquoise2, n=7-10 cells. g. pH dependence of the
204 fluorescence intensity of mScarlet3, mScarlet-I3 and mScarlet. h. Spectral analysis of FRET
205 between SYFP2 and diverse RFPs in living HeLa cells. All spectra are corrected for detector
206 sensitivity and normalized to the unquenched SYFP2 signal. i. Check for cytotoxicity of

207 fluorescent protein expression in HeLa cells 48 h after transfection quantified by
208 NAD(P)H/live cell-dependent conversion of WST-8 into blue light absorbing formazan
209 (average absorbance at 460 nm \pm sd for each 6 independent transfections).

210

211 **Fig. 2. Characterization of the cellular and in vivo applications of mScarlet3 and of the**
212 **mScarlet crystal structure. a-f** usage of mScarlet3 as fusion tag in HeLa cells, bar=10 μ m, red
213 fluorescence intensity was scaled according to the mScarlet LUT (in a) micrographs are
214 representative of at least 4 images in 2 independent transfections. Cells were producing ER-
215 mScarlet3 (**a**, endoplasmic reticulum), LaminB-mScarlet3 (**b**, nuclear envelope); 4xmito-
216 mScarlet3 (**c**, mitochondria); lifeact-mScarlet3 (**d**, actin cytoskeleton); mScarlet3-peroxi (**e**,
217 peroxisomes); and LCK-mScarlet3 (**f**, plasma membrane). **g**. Alternate conformations of
218 Glu216 in mScarlet3. $2F_{obs} - F_{calc}$ electron density map contoured at a 1.5σ level and
219 superimposed on the chromophore and the surrounding residues Arg198 and Glu216.
220 Glu216 unambiguously exhibits two distinct conformations of its side chain, leading to the
221 positioning of one water molecule in alternate conformations. **h**. Comparison of a region
222 near one head of the beta-barrel between mScarlet (blue) and mScarlet3 (magenta),
223 showing how the concerted mutations of four residues lead to the formation of a
224 hydrophobic patch. Arrows indicate the additional non-polar atoms introduced in mScarlet3
225 contributing to both the volume decrease of a small amphipathic cavity and the increase in
226 the number of potential van der Waals interactions. **i**. Brightness of mScarlet (green
227 symbols) and mScarlet3 (red symbols) in fixed zebrafish larvae 24h post injection. Points
228 represent measurements of average fluorescence intensity found in confocal pictures of
229 individual cells relative to a coproduced mTurquoise2, lines represent linear regression
230 through the origin. **j**. mScarlet3 applied for live-cell imaging of pancreatic cells in zebrafish.
231 H2B-mScarlet3 was expressed under an elastase promotor and examined on a light sheet
232 microscope. Nuclear localization is found in the exocrine cells of the pancreas. Note the cell
233 division in the boxed area. Bar: 5 μ m. **k** Lightsheet microscopy of H2B-mScarlet3 labelled cell
234 nuclei in a developing zebrafish larva (see also corresponding Supplementary Video 6).

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- 276
- 277
- 278

279 **Online Methods:**280 General Methods

281 All red fluorescent proteins (RFPs) were cloned into a pDRESS plasmid (Addgene 130509)¹¹
282 or pDX vector (a modified TriEX vector, expressing a FP in both bacteria, under a rhamnose
283 promoter, and in mammalian cells, under a CMV promoter) using the AgeI and BsrGI
284 restriction sites. mRuby3⁶, TagRFP-T¹², mApple¹², mCherry⁴ mKate2⁷ and FusionRed⁸ were
285 obtained as described in⁹. The new vectors pDRESS_mTurquoise2_spatial-linker-
286 P2A_mScarlet3 (189752, Addgene), pDx_mScarlet3 (189754, Addgene),
287 pDRESS_mTurquoise2_spatial-linker-P2A_mScarlet-I3 (189755, Addgene), pDx_mScarlet-I3
288 (189757, Addgene) were deposited at Addgene. Mammalian cell imaging was done with U2OS
289 cells (HTB-96, ATCC) or HeLa cells (CCL-2 ATCC). Mammalian cells were grown in 24 well plates
290 with glass bottom (MatTek P24G-1.5-13-F) in DMEM (61965059, Thermo Fisher Scientific)
291 containing 10% fetal bovine serum (10270106, Thermo Fisher Scientific) or with colorless
292 DMEM 11594416 Thermo Fisher Scientific) supplemented with 1% of Glutamax (11574466
293 Thermo Fisher Scientific) under 7% humidified CO₂ atmosphere at 37 °C. For transfection
294 polyethylenimine (PEI) in ddH₂O (1 mg/ml, pH 7.3, 23966, Polysciences) was used. The
295 transfection mixture was prepared in Opti-MEM (31985047, Thermo Fisher Scientific) with 2
296 µl PEI solution and 50-200 ng plasmid. For some transfections, carrier DNA (empty plasmid)
297 was added to prevent overexpression. The transfection mixture was incubated for 20–45 min.
298 Cells were used for imaging 15-48 h after transfection.

299 Mutagenesis

300 mScarlet variants were obtained by site-directed and random mutagenesis using standard
301 protocols, see⁹. Evolution started from mScarlet and mScarlet-I by introducing G220A (Seq-
302 ID 3,4 (see Supplementary Fig 1)), followed by V196I (Seq-ID 5,6) targeted mutagenesis. With
303 random mutagenesis the combination of T107S, G156V and E219V was found to be beneficial.
304 A new template mScarlet-2A (Seq-ID 7) was ordered as geneblock carrying T107S, G156V,
305 V196I, E219V and G220A as mutations relative to mScarlet. T74I was introduced into
306 mScarlet-2A to yield mScarlet-2A-I (Seq-ID 8). Y84W was introduced in mScarlet-2A by
307 targeted mutagenesis, yielding mScarlet-2A-84W (Seq-ID 9). We also introduced T74I into
308 mScarlet-2A-84W to yield mScarlet-I-2A-84W (Seq-ID 10). These two new templates were

309 subjected to an additional round of random mutagenesis. From screening of several variants
310 of mScarlet-2A-84W were found with increased maturation, one with two mutations T109A,
311 K183R, one with the single mutation Y194F and one with the single mutation K48R with
312 increased brightness. We first combined T109A, K183R and Y194F, yielding mScarlet-2A-84W-
313 R8-194F (Seq-ID 11). Introducing K48R yielded a slight further brightness increase in
314 mScarlet3-NCwt (Seq-ID 13). With PCR new N and C termini¹⁵ were introduced (Δ 1, V2M, S3D,
315 K4S, G5T and M227S, D228G, E229G, S230S, Δ 231, Δ 232) yielding mScarlet3 (Seq-ID 15). In
316 the random mutagenesis of mScarlet-2A-I-84W a combination of 6 mutations originating from
317 different mutated variants were found to be beneficial. These mutations were introduced by
318 ordering a new template mScarlet-I-2A-84W-nt1 (Seq-ID 12) containing K93R, N99I, A105T,
319 T128G, K140R, Y194F relative to the mScarlet-2A-I-84W template. Subsequent scrutinizing
320 demonstrated that reversal of R93K by targeted mutagenesis was beneficial yielding
321 mScarlet-I3-NCwt (Seq-ID 14). With PCR new N and C termini were introduced (Δ 1, V2M, S3D,
322 K4S, G5T and M227S, D228G, E229G, S230S, Δ 231, Δ 232) yielding mScarlet-I3 (Seq-ID 16).

323

324 Protein purification

325 His-tagged recombinant RFPs were purified from E coli bacteria as described⁹ except for the
326 final affinity purification and dialysis steps. For affinity purification the crude E coli protein
327 extract was obtained from defrosted E coli pellets and incubation on ice with 5 ml ST buffer
328 (20mM Tris/HCl, 200mM NaCl, pH 8.0) supplemented with lysozyme (1 mg/ml, L7651, Sigma-
329 Aldrich), benzoase nuclease (5 unit/ml, Merck/Millipore, 71205-3) and 50 μ l 100x Halt
330 Protease Inhibitor Cocktail (Thermo Scientific 87785). After 2h of incubation, 100 μ l of 10%
331 (v/v) NP-40 (Thermo Fisher Scientific, 85124) was added to the lysate after which it was
332 centrifuged (30 min, 40,000g, 4°C). The supernatant was added to 2 ml of Co²⁺ loaded
333 HisPurTM Cobalt Superflow Agarose resin (Thermo Scientific, 25228) and incubated for at
334 least 1 hour at 4 °C. The resin was washed with 9-10 times with 2 ml wash buffer (ST-buffer
335 supplemented with 15 mM imidazole) until no detectable protein (as measured by OD 280)
336 was washed from the resin. His-tagged proteins were eluted with 2x 1 ml elution buffer (ST
337 buffer supplemented with 150 mM imidazole). The eluent was desalted and obtained in a 10
338 mM Tris-HCl pH 8.0 solution using Sephadex-G25 desalting columns (GE Healthcare 17-0852-

339 01). Proteins were short-term stored at 4 °C, or flash frozen and stored at -80 °C for long-term
340 storage.

341

342 Spectroscopy

343 *Extinction coefficient*

344 Purified proteins were diluted in PBS (50 mM Na₂HPO₄ – NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl,
345 pH 7.4). Absorbance spectra were acquired with a spectrophotometer (Libra S70, Biochrom).
346 The spectra were recorded in the wavelength range of 260-700 nm, with a step size of 1 nm.
347 PBS was used as a background reference. The samples were diluted such that the absorbance
348 of the red chromophore peaked between 0.15 and 0.5. To denature the RFPs 10-200 µl 10 M
349 NaOH was subsequently added to the samples, which was directly mixed by pipetting. Spectra
350 were acquired continuously after addition of the sodium hydroxide until the absorbance
351 spectra showed a complete loss of the absorbance peak associated with the red chromophore
352 and displayed only the peak associated with the green chromophore at 457 nm. This
353 absorbance spectrum was used for further analysis, and if necessary, the average absorbance
354 value in the wavelength range 670 - 680 nm was subtracted from the spectra, in order to
355 correct for a minor offset. The concentration of the denatured green chromophore was
356 calculated assuming an extinction coefficient of 44,000 M⁻¹cm⁻¹ at 457 nm for the green
357 chromophore in the denatured RFP^{16,17}. Based on the concentration of the red chromophore
358 the extinction coefficient for the red chromophore was determined at the maximum
359 absorbance wavelength. The above procedure was repeated at least three times per RFP
360 variant and the average extinction coefficient was calculated.

361

362 *Quantum yield*

363 Purified proteins were diluted in PBS (50 mM Na₂HPO₄ – NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl,
364 pH 7.4). Absorbance spectra were recorded with a spectrophotometer (Libra S70, Biochrom)
365 in the wavelength range 260 - 700 nm with a step size of 1 nm. PBS was used as a background
366 reference. Three dilutions of each RFP variant were prepared with an absorbance at 540 nm
367 (A_{540}) of $0.005 < A_{540} < 0.05$. Fluorescence emission spectra were taken from the same sample

368 cuvette with a fluorimeter (Model FP-8500, Jasco with a red extended PMT tube model R928-
 369 23, operated by Jasco Spectral Manager v 2.15). The excitation wavelength was set at 540 nm,
 370 the emission spectrum was recorded from 550 to 800 nm with a step size of 1 nm at a scan
 371 speed of 200 nm·min⁻¹. The excitation as well as the emission slits were set at 2.5 nm. To
 372 obtain more accurate A_{540} absorbance values of the low absorbing samples, their absorbance
 373 spectra were fitted to an absorbance spectrum of the same RFP at high concentration (OD
 374 0.1-0.3) and a variable (constant) offset value using a linear least squares fit. The A_{540}
 375 absorbance of the quantum yield samples was obtained from the fitted spectral component.
 376 Fluorescence spectra were background corrected by subtracting a spectrum measured with
 377 PBS. The emission spectra were corrected for spectral sensitivity using a calibrated white light
 378 source (ESC-842, Jasco) and the spectral area (I_{em}) was obtained by integrating from 550-800
 379 nm. The absorbance at 540 nm (A_{540}) was plotted versus the area under the emission
 380 spectrum, subsequently the slope of the line was determined using linear regression. The
 381 regression lines were constrained to go through the origin, hence $I_{em} = a \cdot A_{540}$.

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

383

$$384 \quad QY_s = QY_r \frac{a_s}{a_r} \quad \text{Eq. 1}$$

385

386 In **Equation 1** QY denotes the quantum yield (s and r denote sample and reference RFP,
 387 respectively) and a corresponds to the acquired slope. mScarlet was used as a reference with
 388 a quantum yield of 0.704⁹.

389

390 *Fluorescence lifetime*

391 Fluorescence lifetime measurements of purified RFPs diluted in PBS (50 mM Na₂HPO₄ –
 392 NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) were performed at an Olympus FV1000 confocal
 393 microscope equipped with a PicoHarp 300 TCSPC module (PicoQuant, Germany operated with
 394 SymPhoTime v 64.2.1) as described⁹.

395

396 *pH dependence of RFP fluorescence intensity*

397 A pH buffer series was created (pH 3-10) using a universal 50 mM citric acid, 50 mM
 398 phosphoric acid and 50 mM boric acid, 100 mM NaCl buffer. Buffers at the desired pH were
 399 made by titrating a 2-times concentrated stock solution with 1M NaOH (Merck 109137) and
 400 adjusting the volume to obtain a two-fold dilution. Citric acid and phosphoric acid (85%) were
 401 from Merck (art 818707 and 563, respectively), boric acid was from Sigma (B-0252). The final
 402 pH value was measured 24h after preparation at room temperature and yielded pH values of
 403 2.96, 3.90, 4.85, 5.94, 6.89, 8.16, 9.21 and 10.75. Fluorescence emission and absorbance
 404 Purified RFPs (section protein purification) were diluted in a black μ -clear 96 wells plate
 405 (655090, Greiner). For each pH triplicate samples for one RFP were made ranging from pH 3.0
 406 to 10.8. With a Biotek FL-600 fluorescence plate reader using the KC4 v3.0 software, the
 407 fluorescence intensity was measured using a 555/25 excitation filter and a 620/40 emission
 408 filter. The curves of pH versus the fluorescence of the samples, $F(\text{pH})$, was fitted using the Hill-
 409 function, **Equation 2** to obtain the apparent pK_a of the RFP and the Hill-coefficient n :

410

$$411 \quad F(\text{pH}) = \frac{F_{max}}{1+10^{n(pK_a-\text{pH})}} \quad \text{Eq. 2}$$

412

413 For Fig. 1g data from 3 technical replicates were pooled into one data set for mScarlet3 and
 414 mScarlet-I3.

415

416 *Stability at pH 13.5*

417 Fluorescence intensity was monitored as a function of time in a fluorimeter (Model FP-8500,
 418 Jasco with a red extended PMT tube model R928-23) using 560 nm excitation and 610 nm
 419 emission (slits at 5 nm). Measurements were started with blank PBS solution (50 mM Na_2HPO_4
 420 – NaH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4) to which 10 μl of purified RFP was added. After
 421 1 min 100 μl of 2 M NaOH was added and the mixture was quickly mixed by pipetting up and
 422 down in the cuvette. To determine the stability of RFP fluorescence at alkaline pH, the blank
 423 intensity was subtracted and the signal was normalized to the blank-corrected RFP
 424 fluorescence signal at normal pH. The signal after addition of NaOH was corrected for dilution.
 425 After NaOH addition the final unbuffered NaOH concentration was 0.33 M, yielding a pH of
 426 13.5. Stability measurements were performed in duplo yielding essentially similar results.

427

428 Cellular microscopy-based methods429 *Brightness mammalian cells*

430 The brightness of the diverse RFPs was determined in U2Os and HeLa cells as described in ¹¹.
431 Briefly, 50 ng of pDress vector encoding a 1:1 expression of mTurquoise2 cyan fluorescent
432 protein and the respective RFP was mixed with 150 ng carrier DNA and transfected in
433 mammalian cells in glass bottomed 24 well plates as described under general methods. 24 h
434 after transfection cyan, yellow and red fluorescence images were recorded on a Nikon wide
435 field microscope. The widefield microscope consisted of an Eclipse Ti-E (Nikon) equipped with
436 440, 508 and 555 nm LEDs (SpectraX, Lumencor). The excitation light from these LEDs was
437 passed through a 440/20, 510/24 or 550/15 nm bandpasses, respectively. For 440 nm and
438 508 nm excitation, a triple-band cube (MXU74157, Nikon) was used, for 555 nm excitation a
439 quad band cube (MXU 71640, Nikon) was used. Emission was additionally filtered with a
440 479/40, 550/49 nm or 593/46 nm bandpass (all from Semrock) placed in an optical filter
441 changer (Lambda 10-B, Sutter instrument). For the three detected channels cyan, yellow and
442 red, the effective excitation and emission bands were cyan: 430-450 nm excitation, 459-490
443 nm emission, yellow: 498-523 nm excitation, 526-555 nm emission, red: 543-558 nm
444 excitation and 570-616 nm emission. A 10x CFI Plan Apochromat NA 0.45 (Nikon MRD00105)
445 objective was used. Images were acquired on an ORCA-Flash4.0 V2 Digital CMOS camera
446 (C11440-22CU, Hamamatsu Photonics) using Nikon NIS Elements AR v 4.51.01 64 bit. For each
447 well a 5x5 tile of Images of 512 x 511 pixels each was acquired using a central ROI of
448 1024x1022 pixels with 2x2 binning and automated image stitching with 10% overlap, resulting
449 in a final image size of 2253x2253 pixels corresponding to a 2.91x2.91 mm imaged area in
450 each well. LED power was 10 %, 20% and 10% for 440, 508 and 555 nm LEDs for the cyan,
451 yellow and red images, respectively. Integration time per image was 60 ms, 200 ms and 60
452 ms for the cyan, yellow and red images, respectively. The ratio of red to cyan fluorescence
453 was calculated using the ratio_96-wells_macro_v7 as described ¹¹. The brightness in
454 mammalian cells was obtained by correcting these ratios for spectral throughput of the
455 imaging device. To this end, spectra of the corresponding purified RFPs were used and the
456 average excitation in the 543-558 nm excitation bandpass was calculated from the normalized
457 excitation spectrum and the integrated fluorescence in the 570-616 bandpass was divided by

458 the total integrated emission (550-800 nm). These corrections were normalized to the
459 throughput for mScarlet. Consequently, the detected Red/Cyan ratios were multiplied by 1.0
460 for mScarlet, 1.015 for mScarlet-I, 0.985 for mScarlet3, 0.964 for mScarlet-I3, 1.522 for
461 mCherry, 0.919 for mApple, 0.667 for TagRFP-T, 0.696 for mRuby3, 1.245 for FusionRed and
462 3.066 for mKate2 to obtain the relative cellular brightness values corrected for spectral
463 throughput. Cellular brightness experiments experiments were repeated at least 5 times in
464 different months and in different cell types and yielded essentially similar results.

465

466 *Brightness in mammalian cells after fixation.*

467 The brightness of the diverse RFPs following was also determined after paraformaldehyde
468 fixation in HeLa cells. Transfection, imaging and quantification was performed as described
469 above for living cells, but prior to determining the red to cyan fluorescence ratio, cells were
470 washed once with PBS and subsequently fixed for 20 minutes at room temperature with
471 freshly prepared 4% paraformaldehyde in PBS. After fixation, cells were washed once with
472 PBS and taken up in microscopy medium (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂,
473 10 mM glucose, and 20 mM HEPES pH 7.4) and imaged on the same day.

474

475 *Maturation*

476 The extent of maturation was calculated by dividing the corrected cellular brightness by the
477 intrinsic brightness (see Extended Data Table I) for the respective RFP and normalizing to
478 mScarlet-I.

479 For measuring the maturation speed, the same cells, transfection and setup as described for
480 the brightness in mammalian cells was used. For image acquisition the yellow image was
481 omitted and cyan and red single image using the full sensor ROI at 2x2 binning was used.
482 Starting 5 hours after transfection, for each well in the 24 well plate a red and cyan image was
483 recorded every 15 minutes for 24 h. ROIs were manually drawn around 6-12 individual cells
484 in each timelapse that remained viable for prolonged time and showed steady FP
485 accumulation over time, but that started with no visible FP accumulation. The background
486 corrected average intensity in the ROI was calculated for both the cyan and red channels.

487 Subsequently, only traces that first showed a nonlinear increasing accumulation of both RFP
488 and CFP fluorescence, followed by a linear increasing accumulation and ending with a slowing
489 down of the increase were selected. Cells with discontinuous time traces (due to cell division
490 or apoptosis) were discarded. The RFP and CFP trace were normalized to maximal intensity
491 and a straight line was fitted to the curve at the point a maximal slope of normalized
492 fluorescence increase with time was calculated. The intercept of this line with the time axis
493 was calculated and the delay between the intercept found for the red and cyan curves were
494 calculated for 6-15 individual cell traces and averaged.

495

496 *FRET-FLIM*

497 For all RFPs, direct fusion constructs with SYFP2 were generated using PCR- and restriction
498 enzyme-based cloning. To enable a good comparison, the linker between the SYFP2 and RFP
499 was chosen such that a constant number of amino acids separated the two beta barrels in the
500 fusion construct (i.e. correcting for differences in the length of N- and C- termini). In
501 Supplementary Table 1, the linker sequence between the different RFPs and SYFP2 is
502 described for the constructs used for the FRET studies depicted in Fig. 1h and Extended Data
503 Figs. 5.

504 50 ng of the SYFP2-RFP fusion constructs, SYFP2 or RFP single FP constructs, together with
505 150 ng of carrier DNA were transfected in HeLa cells growing in glass-bottomed 24-well plates
506 as described above. 46 h after transfection, the samples were analyzed with FRET-FLIM with
507 a frequency-domain widefield FLIM setup as described ¹¹. Briefly, the setup consisted of a
508 Nikon TiE inverted fluorescence microscope, with a Proscan-III automated stage, excitation
509 filter wheel and a Lambert Instruments FLIM Attachment (LIFA) system including a Multi-LED
510 light source and a LI2CAM detector (Lambert Instruments) operated with LiFLIM v 1.2.23 . For
511 measuring the SYFP2 lifetime a 506 nm LED modulated at 40 MHz was used (Lambert
512 Instruments), and the excitation light was additionally filtered with a 500/24-nm excitation
513 filter (BrightLine single-band bandpass filter; Semrock, cat. no. FF01-500/24). A filter cube
514 with a 523-nm dichroic mirror (Semrock, cat. no. Di02-R514), and a 542/27-nm emission filter
515 (BrightLine single-band bandpass filter; Semrock, cat. no. FF01-542/27-25) was used to
516 separate excitation from the SYFP2 fluorescence emission. A 40× CFI Plan Apochromat NA

517 0.95 air objective (Nikon) was used. For determining the phase and modulation of the
518 modulated excitation light at the sample position, a diluted (OD < 0.05) Alexa488 (Thermo
519 Fisher Scientific) solution in PBS was used, for which a monoexponential fluorescence lifetime
520 of 4.03 ns was assumed. FLIM measurements were done in culture medium at 37 °C under 5-
521 7% CO₂ atmosphere. For each SYFP-RFP sample 3-4 FLIM stacks were acquired with a different
522 field of view of approximately 20 cells each. The mean lifetime of all cells in each FLIM stack
523 was determined and averaged for all stacks acquired from the same sample. Repeated
524 experiments with independent transfections yield essentially similar results.

525

526 *FRET-SPIM*

527 48h after transfection, the medium of the same samples as described under FRET-FLIM was
528 replaced with microscopy medium (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10
529 mM glucose, and 20 mM HEPES pH 7.4) and spectral images were acquired at room
530 temperature using a home-built spectral imaging setup as described^{9,18}. Briefly the setup
531 consisted of a Zeiss Axiovert 200M fluorescence microscope, equipped with an HBO 100
532 Mercury lamp for excitation and an imaging spectrograph (ImSpector V7, Specim, Finland)
533 coupled to a CCD camera (ORCA ER, Hamamatsu, Japan) operated using MatLab 6.1 with the
534 DCAMAPI interface. A 10x plan Neofluar NA 0.3 objective was used. Two spectral images were
535 recorded: one with a 500/20 nm excitation filter (Chroma Technology Incorporation), a 80/20
536 dichroic mirror (20/80bs, Chroma Technology Incorporation) and a LP530 nm emission filter
537 (#46-059, Edmund optics worldwide) to record the full spectra and one with a 577/20 nm
538 excitation filter, (D577/20, Chroma Technology Incorporation), a 596 nm dichroic mirror
539 (600dcxr, Chroma Technology Incorporation), and a 630/60 nm emission filter (HQ630/60,
540 Chroma Technology Incorporation) to record the direct excited RFP acceptor signal. The
541 spectra were extracted from the spectral images and analyzed as described⁹. Briefly, the
542 direct excited acceptor spectrum was subtracted from the FRET spectrum by analyzing the
543 spectra from single RFP transfected cells. Subsequently, the remaining donor and sensitized
544 emission acceptor signals were obtained by spectral unmixing with linear least squares
545 method using the unfused SYFP2 donor spectrum and unfused RFP acceptor spectrum as
546 reference. After spectral correction for detector sensitivity of the unmixed spectra, the
547 amount of donor quenching (δ_{loss}) was calculated from the net sensitized emission (α) and

548 the ratio of quantum yields of donor (Q_d) and acceptor (Q_a) according to $\delta_{loss} = \alpha Q_d/Q_a$.
549 The spectral components δ_{loss} and α are expressed as number of photons, obtained by
550 dividing the corrected unmixed sensitized emission spectrum by the unit area full emission
551 acceptor reference spectrum. From the donor quenching (δ_{loss}) and the remaining detected
552 donor (δ obtained by dividing the corrected unmixed remaining donor emission spectrum by
553 the unit area full emission donor reference spectrum) the energy transfer efficiency E is
554 calculated according to $E = \delta_{loss}/(\delta + \delta_{loss})$. Typically, 10-30 single cell spectra were
555 analyzed per SYFP2-RFP fusion. The averaged spectra were normalized to the unquenched
556 donor signal.

557

558 *Localization*

559 The Giantin DNA coding sequence of FRB–ECFP(W66A)–Giantin (67903, Addgene) was cloned
560 in to pmScarlet3–C1, using the restriction enzymes BsrGI and BamHI. The following constructs
561 pLifeAct–mTurquoise2 (36201, Addgene), pmTurquoise2–alphaTubulin (36202, Addgene),
562 pmTurquoise2–Peroxi (36203, Addgene), EB3–mTurquoise2 (98825, Addgene), LCK–
563 mTurquoise2 (98822, Addgene) and ER–mTurquoise2 (36204, Addgene) were digested with
564 AgeI and BsrGI to exchange mTurquoise2 for mScarlet3 yielding pLifeact–mScarlet3_N1
565 (189767, Addgene), pmScarlet3–alphaTubulin_C1 (189768, Addgene), pmScarlet3–Peroxi_C1
566 (189769), pEB3–mScarlet3_N1 (189770, Addgene), pLCK–mScarlet3_C1 (189771, Addgene)
567 and pER–mScarlet3_N1 (189772, Addgene), respectively. 4xmts–mScarlet3 and 3xnls–
568 mScarlet3 were created by digesting 4xmito–mNeongreen (Addgene 98875) and 3xnls–
569 mNeongreen (Addgene 98875) with AgeI and BsrGI to exchange mNeongreen for mScarlet3,
570 yielding p4xmts–mScarlet3_N1 (189774, Addgene) and p3xnls–mScarlet3_N1 (189775,
571 Addgene). LaminB–mTurquoise2 (99830, Addgene) was digested with AgeI and BglII to
572 exchange mTurquoise2 for mScarlet3 yielding pmScarlet3–LaminB_C1 (189776, Addgene).

573 HeLa cells (CCL-2, ATCC) were seeded in uncoated glass-bottomed 24 well plates (MatTek
574 P24G-1.5-13-F) and transfected with 50 ng plasmid, 150 ng carrier DNA and 2 μ g PEI. 24h after
575 transfection, the cells were imaged in culture medium at 37 °C and 5% CO₂ atmosphere using
576 a spinning disk setup as described⁹. Briefly this microscope system consisted of a Nikon
577 Eclipse Ti-E microscope equipped a 561 nm laser and a Yokogawa CSU X-1 spinning disk unit

578 (operating at 5,000 r.p.m.). The excitation light was directed to the sample via a custom-made
579 dichroic mirror 405/488/561/640 through a 40× CFI Plan Apochromat NA 0.95 air objective
580 (Nikon). The red fluorescence was filtered with a 585-675 nm bandpass (FF01-512/630-25 m,
581 Semrock). Images were recorded with an iXon 897 EMCCD camera (Andor) controlled by
582 Nikon NIS Elements AR v4.51.01 64-bit. Images shown are representative of 3-4 images taken
583 from at least 2 technical replicates.

584

585 *Photostability*

586 For widefield photobleaching experiments transfection, mounting conditions and the
587 microscope used were identical as described under brightness mammalian cells. A 20x Plan
588 Fluor NA 0.5 air objective was used and the 555 nm LED was used set at 100% power. The
589 quad band cube (MXU 71640, Nikon) and 593/46 nm bandpass emission filter was used for
590 detection. Cells were focused under weak CFP illumination conditions in order to prevent any
591 RFP bleaching prior to the actual photostability measurement. A time-lapse recording was
592 made under perfect focusing at continuous 555 nm excitation with 25 steps of 5 s each. A
593 1024x1024 pixel central ROI of the camera was used at 1x1 binning and 10 ms integration
594 time. The power at the object plane was 3.96 W/cm².

595 For confocal photobleaching, the transfection and mounting conditions were identical as
596 described under brightness in mammalian cells. The microscope, the microscope objective
597 and filter settings were identical as described under localization. The 561 nm laser was
598 operated at 100% power, resulting in an illumination power at the object plane of 6.14
599 W/cm². Cells were focused under weak CFP illumination conditions in order to prevent any
600 RFP bleaching prior to the confocal bleaching. Under perfect focusing at continuous 561
601 excitation 10 images separated by 100 ms were recorded, followed by 10 recordings
602 separated by 400 ms, 10 recordings separated by 2 s, 10 recordings separated by 5s, 10
603 recordings separated by 10s and 10 recordings separated by 20 s, respectively, resulting in a
604 final stack of 60 images each with an integration time of 50 ms.

605 Wide-field photostability was analyzed using the Bleach_96wells_macro_v9 macro as
606 described¹¹. On average 5-15 cells per image were used for this automated analysis. For
607 analysis of the confocal photostability, the average fluorescence of 3-6 individual cells in each

608 recording was averaged and corrected for background fluorescence at each time point. The
609 intensities were normalized to the intensity at t_0 . For both confocal and widefield
610 photostability measurements, the time axis was adjusted relative to (the uncorrected time
611 axis of) mScarlet to account for differences in excitation efficacy and RFP quantum yield to
612 mimic a situation where all RFPs start with emitting an equal number of photons/s per
613 molecule at t_0 as compared to mScarlet¹². To estimate the time at which the FPs were
614 bleached by 50%, a linear interpolation method was used between the two most adjacent
615 time-points. Repeated experiments (3-4 times) yield essentially similar results.

616

617 *Prolonged confocal imaging*

618 For evaluating photostability under live cell confocal imaging conditions, pLifeact-
619 mScarlet3_N1 (189767, Addgene) was transfected in HeLa cells glass bottomed 24-well plates
620 that were imaged 24 h later using a commercial Leica SP8 confocal microscope with 561 nm
621 excitation (power at object plane 3 μ W) using a 63x NA 1.4 HC PL APO CS2 oil immersion
622 objective operated with Leica LAS-X v 3.5.7.23325 64 bit. Fluorescence was recorded with a
623 HyD detector using a detection bandpass of 575-650 nm. 1000 frames were recorded with 2
624 scans averaged per frame over a period of 16 h with a frame rate of 1 frame/min at a (single
625 scan) pixel dwell time of 0.4 μ s and pixel size of 0.18x0.18 μ m.

626

627 *Photochromicity*

628 For photochromicity experiments, the transfection, mounting and microscope were identical
629 is described for the wide-field photostability measurements. RFPs were switched on with
630 100% 440 nm for 0.5 s (3.3 W/cm² at the focal plane) and subsequently illuminated at 20%
631 continuous 550 nm LED power (0.8 W/cm² at the focal plane) for 15 s during which images
632 were recorded every 300 ms. Five consecutive cycles of 440 nm on switching and 15 s of
633 continuous 550 illumination were executed were executed of which the 550 nm excited
634 recordings were concatenated in a stack of 255 images in total for each RFP. Data were
635 analyzed using the Bleach_96wells_macro_v9 macro¹¹ to extract the average time-traces.
636 Each trace represents the average of 6-13 cells per RFP variant. Photochromicity (P) was

637 calculated as described⁹ from the RFP intensities just after 440 nm on-switching (I_a) and just
638 before 440 nm on-switching (I_b) as $P=(I_a-I_b)/I_a$. The 4 values found for the 5 consecutive
639 photochromicity cycles from the analysis of the average time traces per RFP were averaged
640 and indicated \pm sd in Extended Data Table 1.

641

642 *Oligomerization analysis with OSER method*

643 mScarlet-CytERM-N17, mScarlet-I-CytERM-N17, TagRFP-T-CytERM-N17 and mCherry-
644 CytERM-N17 were cloned as described in⁹. FusionRed-CytERM-N17, mScarlet3-CytERM-N17
645 (189778, Addgene), mScarlet-I3-CytERM-N17 (189777, Addgene) and mScarlet-I3-NCwt-
646 CytERM-N17 (189779, Addgene) were cloned by digesting mCherry-CytERM-N17 with
647 enzymes Agel and BsrGI to cut out mCherry and replace it with the other RFPs. Cells were
648 analyzed between 22h and 24 h after transfection. Imaging was performed with the same
649 microscope setup and transfection conditions as described under localization. To obtain an
650 overview of many cells, 16x16 tile scans were acquired with 10 % overlap and automated
651 image stitching in NIS elements software leading to 7040x7040 pixel images corresponding to
652 1.4x1.4 mm imaged areas in each well. Cells were analyzed as described in¹³. ROIs of
653 representative cells were assembled for Extended Data Fig. 4. Two days after transfection fast
654 moving punctate structures become visible in addition to the reticulate ER structure, likely
655 reflecting a degradation pathway involving the endolysosomal machinery. Using the same
656 spinning disk microscope, time-lapse imaging was performed at a frame interval of 0.32 s for
657 Supplementary Video 1. Repeated experiments (at least n=2) with independent transfections
658 yield essentially similar results.

659

660 *Cytotoxicity*

661 Cytotoxicity caused by expression of red fluorescent proteins in cells was tested with a
662 standard cell counting kit (96992, Sigma). This kit assesses living cells that produce NAD(P)H
663 to convert WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H
664 tetrazolium, monosodium salt) into formazan that can be detected by its blue light
665 absorbance¹⁹. We used standard conditions for inducing expression of fluorescent proteins
666 by PEI mediated transient transfection of HeLa cells in 96 wells format^{11,20}. On day 0, 3000

667 HeLa cells were seeded per well in 100 μ l DMEM in a Greiner (Cat. No. 655180) 96 well plate,
668 which was subsequently placed in a 37 °C humidified incubator with 5% (v/v) CO₂. On day 1,
669 300 ng of mEGFP-pDx, pmScarlet-pDx, pmScarlet3-pDx (189754, Addgene), pmScarlet-I3-pDx
670 (189756, Addgene), pmCherry-pDx or pmFusionRed-pDx was transfected with 1 μ l 1 % (w/v)
671 PEI/ 19 μ l optimum mix that was added per well in the 96 wells plate. As control, 300 ng empty
672 pDx vector (carrier DNA) without an open reading frame was transfected. As blank we used
673 wells without HeLa cells, that contained only 100 μ l DMEM medium. After transfection, the
674 96 wells plate was returned to the humidified cell incubator. Under these conditions about
675 50-70% of the cells are transfected. On day 3, 48 h after transfection, 50 μ l from a mix of 5.5
676 ml (containing 1 ml WST-8 cell counting kit, (96992, Sigma) and 4.5 ml DMEM), was added to
677 each well followed by 2 h incubation at 37 °C and 5% CO₂. Then the absorbance at 460 nm
678 was recorded using a Biotek FL-600 fluorescence/absorbance plate reader equipped with a
679 460/40 absorbance filter operated with BioTek KC4 v3.0. The average absorbance (\pm sd) of 6
680 wells that were transfected with the same plasmid was determined and corrected for blank
681 to quantify possible cytotoxicity. The experiment shown in Fig. 1i was replicated 3 times and
682 once in U2Os cells with similar results.

683

684 *Cell viability*

685 On day 0, 2.5 μ g of mEGFP-pDx, pmScarlet-pDx, pmScarlet3-pDx (189754, Addgene),
686 pmScarlet-I3-pDx (189756, Addgene), pmCherry-pDx or pmFusionRed-pDx was transfected
687 into HeLa cells cultured in T25 flasks. After approximately 4h, the medium was refreshed. Cells
688 were cultured as described under general methods. Cells were passaged at day 2. At day 2
689 trypsinized control mEGFP cells were mixed with each of the RFP transfected cells. From the
690 5 resulting cell mixtures, 4-5 biological replicates were subsequently grown in T25 flasks and
691 passaged on day 4 and on day 6. During passaging on day 2, 4 and 6, two aliquots of (mixed)
692 trypsinized cells were transferred to glass-bottomed 24-well plates supplemented with 0.5 ml
693 culture medium. After 4-5 h of incubation at at 37 °C under 7% humidified CO₂ atmosphere,
694 the 24-well plates were analyzed for the number of cells displaying green and red
695 fluorescence by fluorescence microscopy, amounting to 8-10 red/green cell number ratios
696 per time point per RFP. For microscopy, the same setup was used as described for the
697 brightness in mammalian cells. For GFP detection a 470 nm LED and for RFP detection a 555

698 nm LED (SpectraX, Lumencor) was used. The excitation light from these LEDs was passed
699 through 470/24 or 550/15 nm bandpasses, respectively. A quad band cube (MXU 71640,
700 Nikon) was used. Emission was additionally filtered with a 527/70 or 593/46 nm bandpass
701 (both from Semrock) placed in an optical filter changer (Lambda 10-B, Sutter instrument). For
702 the green and red detected channels, the effective excitation and emission bands were green:
703 458-483 nm excitation, 492-541nm emission, and red: 543-558 nm excitation and 570-616
704 nm emission. A 10x CFI Plan Apochromat NA 0.45 (Nikon MRD00105) objective was used.
705 Images were acquired on an ORCA-Flash4.0 V2 Digital CMOS camera (C11440-22CU,
706 Hamamatsu Photonics). For each well a 12x12 tile of Images of 512 x 511 pixels each were
707 acquired using a central ROI of 1024x1022 pixels with 2x2 binning and automated image
708 stitching with 10% overlap, resulting in a final image size of 2 channels of 5300x5300 pixels
709 corresponding to a 6.84x6.84 mm imaged area in each well. LED power was 50 % and 20% for
710 470 nm and 555 nm LEDs for the green and red images, respectively. Integration time per
711 image was 100 ms, for each exposure. Between 100 and 3000 fluorescent cells were counted
712 per well for each color using an automated ImageJ macro. Briefly this macro first reduced
713 noise, performed a background correction and applied a threshold intensity after which
714 individual cells were automatically counted using the analyze particles ImageJ command. The
715 threshold intensity for the green channel was identical for all analyzed samples, the threshold
716 intensity for the red channel was different for each RFP to account for differences in cellular
717 brightness, but identical for samples from different days. The effective cellular brightness of
718 the different RFPs was determined as described under brightness mammalian cells by
719 analyzing HeLa cells 48 h after transfection with pDress constructs driving expression of
720 mTurquoise2 and the respective RFPs in a 1:1 ratio. The relative brightness and RFP thresholds
721 were 1.307 for mScarlet, 1.899 for mScarlet3, 1.872 for mScarlet-I3, 0.331 for mCherry and
722 0.298 for FusionRed. This resulted in final thresholds of 24 counts for cells expressing GFP and
723 100 times the above relative brightness values for the RFP channel thresholds. For each
724 sample (RFP mixture, time point and replicate) the ratio of red fluorescent cells over green
725 fluorescent cells was calculated, normalized to the ratio obtained at day 2 (to correct for
726 possible differing transfection efficiencies) and displayed.

727

728 Structure determination

729 A batch of mScarlet3 protein sample was specifically expressed and purified for crystallization
730 purposes.

731

732 *Protein expression.*

733 Bacterial transformation of the pDX vector was achieved in E. coli BL21(DE3) (Invitrogen) and
734 incubated overnight at 30 °C on Kanamycin (30 µg/mL) selective LB-agar plates. A starting
735 culture of 100 mL of LB supplemented with Kanamycin (30 µg/mL) was grown overnight at
736 37 °C under agitation (165 rpm). 1 L of LB supplemented with Kanamycin (30 µg/mL) was
737 inoculated with 10 mL of starting culture, then grew at 37 °C, 145 rpm until the D.O. (600 nm)
738 reached 0.6. Protein expression was then induced with L-rhamnose at a final concentration
739 of 0.2 %, and grew overnight at 20 °C, 145 rpm.

740

741 *Purification.*

742 Cultures were centrifugated at 4000 g for 30 min at 4 °C. The pellets were then resuspended
743 in 25 mL of lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole, 20 mM MgSO₄,
744 1 tablet of the EDTA-free protease inhibitor cocktail complete (Roche, Basel, Switzerland),
745 400 µg/mL DNase) per liter of culture, flash-frozen and stored at -80 °C overnight. Thawed
746 pellets were sonicated and cell debris were centrifuged at 18,000 g for 45 min at 4 °C. The
747 clarified lysate was then loaded on nickel affinity HisTrap HP 1 mL (Cytiva) column for protein
748 purification. Proteins were eluted against an imidazole buffer (20 mM Tris pH 8.0, 200 mM
749 NaCl, 500 mM imidazole) with isocratic elution (5 to 500 mM imidazole in 10 % increments).
750 The final purification step consisted in a size exclusion chromatography using a HiPrep 16/60
751 Sephacryl 100 S HR column (Cytiva) against a 20 mM Tris pH 8.0 buffer. Purified mScarlet-3
752 proteins were concentrated to 15 mg/mL using an Amicon 15 (10 kDa cutoff, Merck Millipore)
753 for further crystallization assays.

754

755 *Protein crystallization and crystal harvesting.*

756 Crystallization conditions were set up in 96-well CrystalDirect plates using commercial screens
757 available at the HTX lab crystallization platform (<https://htxlab.embl.fr/>, EMBL Grenoble).
758 These plates are suitable for automated crystal harvesting based on photoablation of the
759 cover film ²¹. A condition consisting of 0.1 M NaCl, 0.1 M HEPES pH 7.5 and 1.6 M ammonium
760 sulphate in a mother liquor/protein ratio of 1:2 yielded a crystal of 800 x 400 x 200 μm^3 . The
761 position of the crystal was marked using the Crystal Harvesting Interface provided within the
762 plate viewing function of the CRIMS (Crystallographic Information Management System)
763 system and used for automated harvesting and flash-cooling of the sample. Cryoprotection
764 was achieved by removing excess of mother liquor around the crystal by aspiration prior to
765 crystal harvesting without addition of any cryoprotectant molecule.

766

767 *Data collection, processing and structure refinement.*

768 The data collection was performed at 100 K at beamline ID30B of the ESRF ²² using an X-ray
769 detector Pilatus3 6M (Dectris). Data were integrated using the automated processing pipeline
770 ²² accessible in the extended Laboratory Information Management System ISPyB
771 (<https://exi.esrf.fr/>) ²³. The best data integration was obtained with the XDSAPP pipeline and
772 yielded structure factors in the $P2_12_12_1$ space group he at 1.33 Å resolution using a $CC_{1/2}$
773 resolution cut-off of 0.5. Data collection statistics can be found in Supplementary Table 2. The
774 structure was solved by the molecular replacement method with PHASER v2.8.3 ²⁴ using the
775 previously-determined structure mScarlet structure (PDB ID: 5LK4) ⁹. The asymmetric unit is
776 composed of two molecules of mScarlet3. Cycles of structure refinement and model
777 rebuilding upon electron density map inspection were performed using REFMAC v5.8.0267 ²⁵
778 and COOT ²⁶, respectively. In the Ramachandran plot, 98.3% of residues are in the favoured
779 region, 1.7% are in the allowed region and 0.0% are outliers. Only 0.5% of side chain rotamers
780 are outliers. Structure refinement statistics can be found in Supplementary Table 2. The
781 structure of mScarlet3 at pH 7.5 has been deposited in the Protein Data Bank under PDB ID:
782 7ZCT.

783

784 *Zebrafish larvae experiments*

785 Wild-type zebrafish (AB) larvae were maintained at 28 °C in E3 medium. Injections were
786 performed on fertilized zebrafish eggs at the one cell stage.

787 Episomal expression was assessed by injecting pDRESS_mTurquoise2_spatial-linker-
788 P2A_mScarlet3 (189752, Addgene) and pDRESS_mTurquoise2_spatial-linker-P2A_mScarlet3
789 (130509, Addgene)¹¹ at a concentration of 250 ng/μl. After 24 hours, the larvae were fixed in
790 4% paraformaldehyde and subsequently analyzed on a confocal microscope with a 40x PLAN
791 Apochromat 1.3 NA objective (SP8X, Leica Microsystems). A white light laser set at 405 nm
792 (7.5%) and 569 nm (25%) was used to excite mTurquoise2 (Em:450-525) and mScarlet or
793 mScarlet3 (Em 580-655nm), respectively. For quantification, the relative fluorescence
794 intensity of the mScarlet(3) channel compared with the mTurquoise2 channel was calculated
795 for 35 regions of interest per construct.

796 Expression of mScarlet3 in the pancreas was allowed by placing mScarlet3 under the control
797 of the ElastaseA promoter in a Tol2 backbone and co-injecting this with Tol2 transposase
798 mRNA, both at 250ng/μl²⁷⁻²⁹. The Tol2 plasmid was a kind gift of Dr. Kawakami, Japan^{28,30}.
799 After 24 hours, pigment formation was inhibited by adding 1-phenyl 2-thiourea (PTU) to a
800 final concentration of 0.2 mM. Time-lapse imaging was performed on a single-plane
801 illumination microscope with a 10x PLAN Apochromat 0.5 NA detection objective (LS7, Zeiss)
802 at 15-min intervals for 12 hours with a Z-stack of 79 frames taken at each timepoint with 1.73
803 μm intervals using Zeiss Zen Black v3.1. Here, the zebrafish larva was illuminated through the
804 ventral and dorsal sides with a 561 laser and detection was done through a 575-615 nm band
805 pass filter with a camera set at 120 ms exposure time. For the illustration (Fig. 2j,
806 Supplementary Video 5), only the images originating from ventral sided illumination were
807 used. A maximum intensity projection of 21 frames in Z, surrounding a dividing cell, was
808 generated and time alignment with linear interpolation and subsequent cropping was
809 performed to account for drift over time (Zen blue v3.0, Zeiss). The image represents a single
810 experiment.

811 For the analysis of H2B-mScarlet3 in zebrafish larvae, mScarlet3 cDNA was cloned in frame
812 with histone 2B and subsequently cloned in the pCS2+ backbone containing the SP6
813 promoter. Capped RNA was *in vitro* transcribed using the mMACHINE SP6
814 Transcription Kit (Invitrogen, AM1340) and subsequently cleaned up using MEGAclean
815 Transcription Clean-Up Kit (Invitrogen, AM1908). Zebrafish embryos were micro-injected with
816 the capped RNA in the 1-cell stage and incubated in E3 medium containing 0.2 mM N-

817 phenylthiourea (PTU) (Sigma Aldrich, P7629) to inhibit pigment formation. At 30 hours post-
818 fertilization (hpf) the zebrafish embryos were sedated using 0.2 g/L ethyl 3-aminobenzoate
819 (Tricaine, MS-222) (Sigma Aldrich, E10521). For Fig. 2k and Supplementary Videos 6 and 7
820 imaging was performed on a single-plane illumination microscope using a 10X Plan
821 Apochromat 0.5 NA detection objective (LS7, Zeiss). Z-stack images were recorded with 1 μ m
822 intervals over 309 frames with 5 min intervals for 6 hours. Here, the zebrafish embryo was
823 excited from the ventral and dorsal sides with a 561nm laser and detection of the saggital
824 plane was done using a 585nm long pass filter with the camera set at 100ms exposure time
825 (this is a first experiment that was immediately successful).

826

827 **Reporting Summary**

828 Further information on research design is available in the Nature Research Reporting
829 Summary linked to this article.

830

831 **Data availability**

832 The data supporting this study are available within the article and the Supplementary
833 Information including the digital data underlying the figures. Other (raw) data supporting this
834 study will be available from the corresponding author upon reasonable request. Plasmids
835 constructed in this study, their maps, and sequences were deposited to the non-profit
836 organization Addgene ID: 189752-189779. The mScarlet3 crystal structure was deposited at
837 the Protein Data Bank as PDB ID: 7ZCT.

838

839 **Code availability**

840 Image analysis was performed in ImageJ/Fiji using macros described in ¹¹ and the code is
841 available on GitHub (see <https://github.com/molcyto/MC-FLIM-Petri-dish>,
842 <https://github.com/molcyto/MC-Ratio-Petri-dish>, [https://github.com/molcyto/MC-FLIM-96-](https://github.com/molcyto/MC-FLIM-96-wells)
843 [wells](https://github.com/molcyto/MC-Ratio-96-wells), <https://github.com/molcyto/MC-Ratio-96-wells>, [Bleach-96-wells](https://github.com/molcyto/MC-
844 <a href=)).

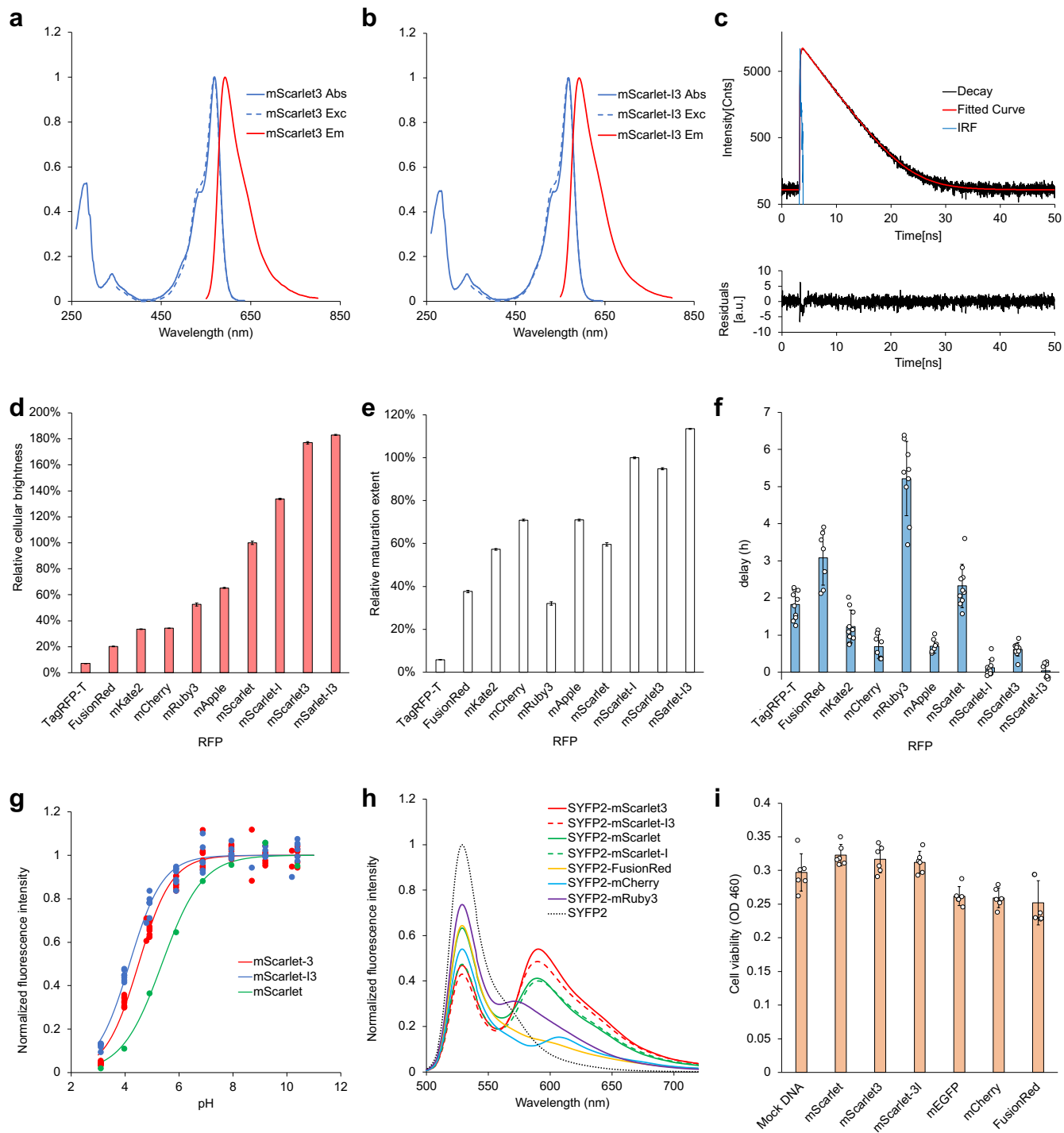
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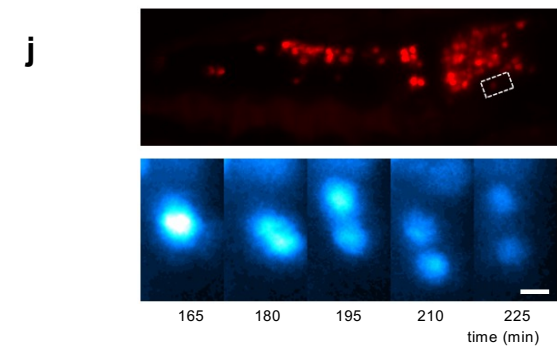
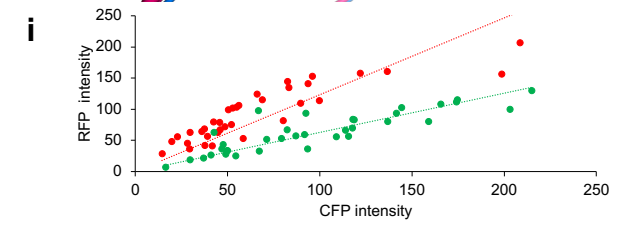
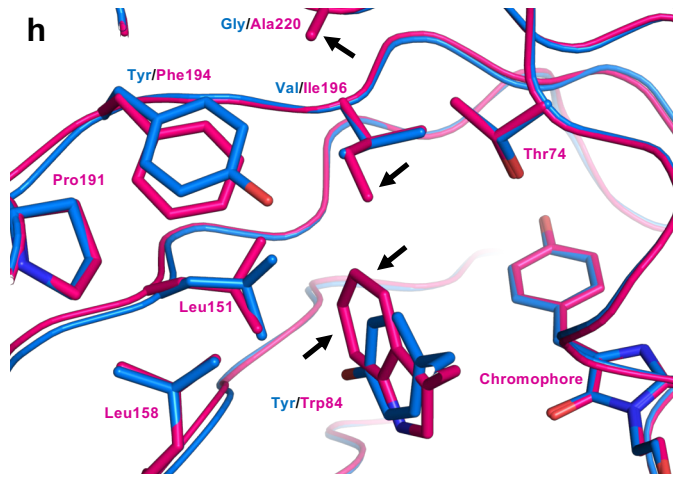
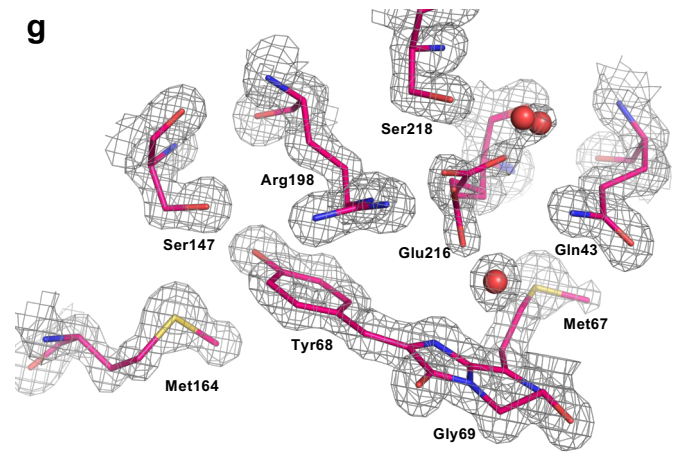
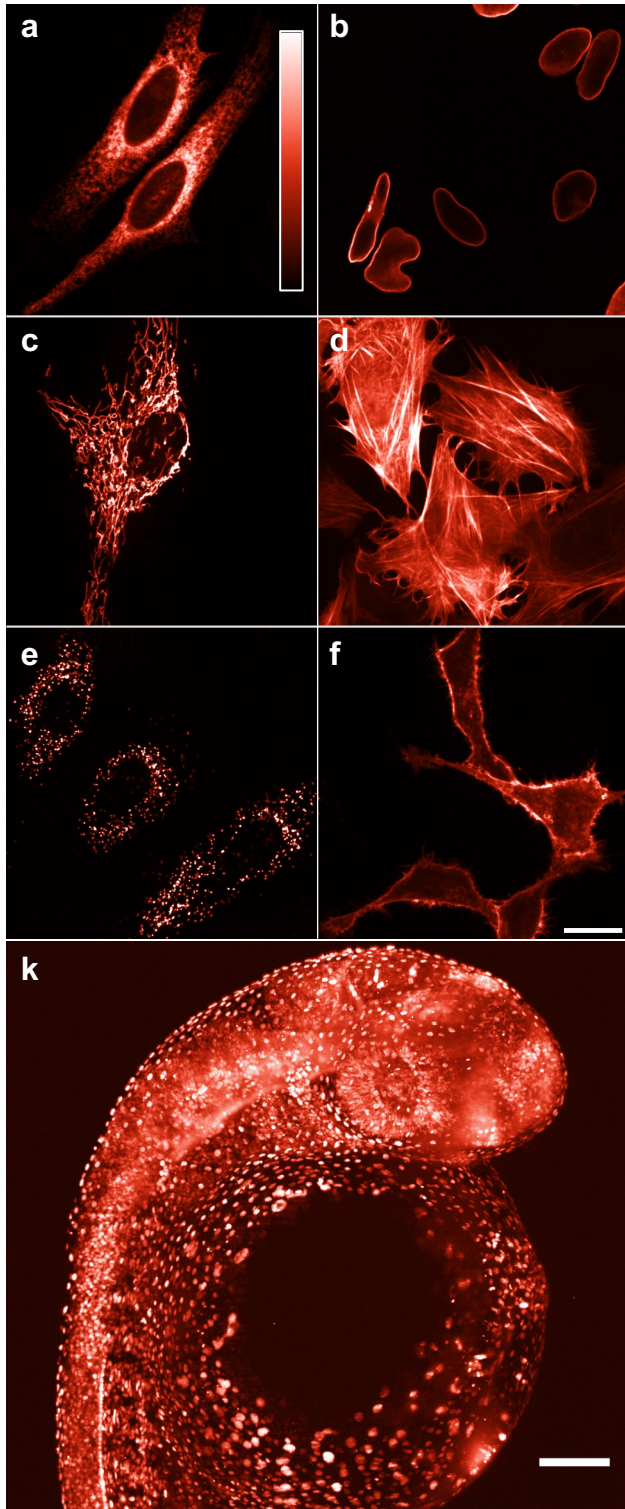
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886





	Spectroscopic Characteristics												Brightness		Photostability			Maturation		Stability pH13.5		Ratio FRET	Monomeric Assay		
	Abs max ^a (nm)	Em max ^b (nm)	ϵ^c 10 ³ M ⁻¹ cm ⁻¹	QY ^d (%)	τ_1^e (ns)	τ_2^f (ns)	α_1^g (%)	χ^{2h}	τ_{avg}^i (ns)	$\tau_M(\text{cells})^j$ (ns)	pK _a ^k	n ^l	EC*QY ^m	Cells ⁿ % 24h	WF t _{1/2} ^o (s)	Confocal t _{1/2} ^p (s)	PC ^q %	Accum ^r (% mSc-l)	delay ^s (h)	I ₀ ^t (%)	t _{1/2} ^u (min)	Sens em ^v (%)	Correct ER ^w (%)	# OSER ^x (%)	cells ^y n
mScarlet3	569	592	104±1	75.1±1	3.96±0.01	x	100	1.15	3.96	3.67±0.00	4.5±0.1	0.70±0.04	78±1	177±1	52	27	<1%	94.9±0.5	0.6±0.2	86	45.6	140±7	85	7	139
mScarlet-I3	568	592	105±2	64.5±1.1	3.64±0.01	x	100	1	3.64	3.35±0.00	4.2±0.1	0.70±0.04	69±2	183±1	47	16	1.1±0.3%	113.5±0.3	0.0±0.2	63	2.24	129±5	87	5	204
mScarlet-I3-NCwt	568	592	102±0.5	65.3±0.8	3.63±0.01	x	100	1.25	3.63	3.33±0.03	4.2±0.1	0.76±0.16	68±1	177±1	46	15		115±1	-	70	2	136±9	87	0	61
mScarlet	569	593	100±3	70.4±0.8	3.86±0.01	x	100	1.12	3.86	3.53±0.01	5.3±0.2	0.79±0.22	71±2	100±1	62	29	<1%	60±1	2.3±0.6	<0.5	x	92±23	92	8	39
mScarlet-I	569	594	104±3	54±4	3.52±0.02	1.67±0.04	76±1	1.11	3.08	3.01±0.04	5.4±0.4	0.52±0.19	57±4	134±1	55	21	<1%	100.0±0.4	0.1±0.2	<0.5	x	100±6	84	8	80
mRuby3	557	591	127±0.3	54±1	2.80±0.01	x	100	1.04	2.80	2.49±0.02	4.8		69±1	53±1	*	*	42±1%	32±1	5.2±1.0	50	0.53	50±24	0	24	17
mKate2	588	631	63±3	39±1	2.47±0.01	x	100	1.21	2.47	2.53±0.01	5.4		25±1	33.5±0.3	96	32	<1%	57.3±0.3	1.2±0.4	62	0.58		56	12	68
Tag-RFP-T	556	585	110±7	48±1	2.66±0.02	1.44±0.06	72±3	1.12	2.32	2.44±0.02	4.6		53±2	7.1±0.1	*	*	27±3%	5.6±0.1	1.8±0.4	<0.5	x		31	66	182
mCherry	586	610	88±1	23±1	1.28±0.03	2.00±0.07	71±0	1.08	1.49	1.56±0.01	<4.5		20±1	34.2±0.2	55	32	<1%	70.9±0.4	0.7±0.3	<0.5	x	37±3	82	18	87
FusionRed	575	605	93	24±1	1.58±0.03	2.72±0.15	84±4	1.01	1.76	1.71±0.03	4.6 ^e		22	20.3±0.3	*	*	26±0.2%	37.6±0.6	3.1±0.7	40	0.48	31±5	67	4	99
dTomato	555	583	90±1	69±1.3	3.47±0.01	1.21±0.07	95±0	1.36	3.36	3.28±0.02	4.7		62±1	116±1	154	14	<1%	112±2	1.6±0.4	<0.5	x		17	82	266

