Nature Methods

Molecular evolution approaches to design advanced red fluorescent proteins

Fedor V Subach, Kiryl D Piatkevich & Vladislav V Verkhusha

Supplementary Figure 1. Alignment of amino acid sequences of derivatives of RFPs with different phenotype.

Supplementary Table 1. Overview of suboptimal properties of current monomeric red fluorescent proteins.

Supplementary Table 2. Current red fluorescent proteins exhibiting the best specific property or maximally optimized for the specific imaging application.

Supplementary Table 3. Properties of the major chromophore structures observed in fluorescent proteins.

Supplementary Table 4. Amino acid residues in the supporting positions responsible for different phenotypes and properties of red fluorescent proteins.

Supplementary Note

Nature Methods: doi:10.1038/nmeth.1776

Supplementary Figure 1. Alignment of amino acid sequences of derivatives of RFPs with different phenotype.

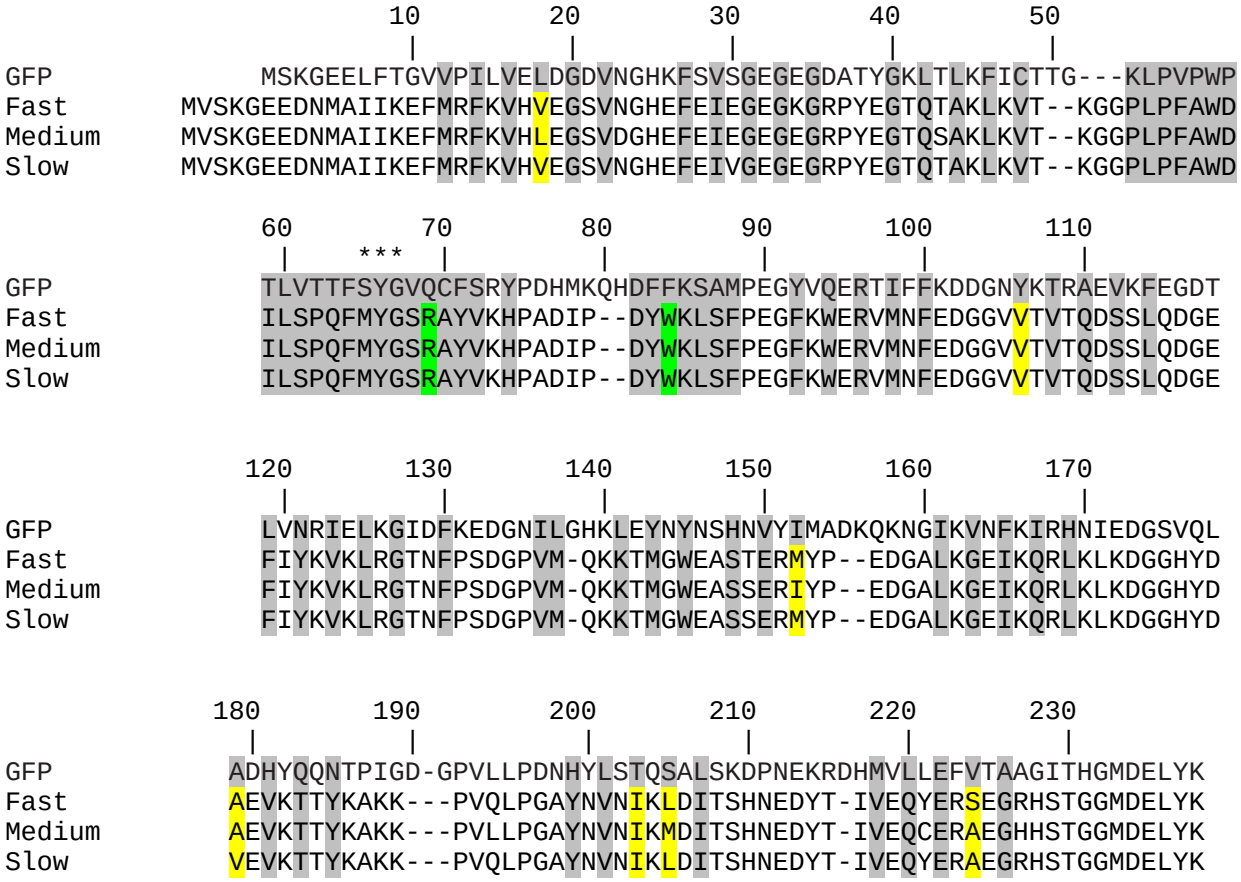
(**A**) TagBFP-like phenotype, (**B**) Fluorescent timer-like phenotype, (**C**) Photoactivatable-like phenotype, (**D**) Photoswitchable-like phenotype, (**E**) Far-red-shifted-like phenotype, (**F**) Large Stokes shift-like phenotype; and properties: (**G**) High quantum yield, (**H**) High photostability, (**I**) High pH-stability, (**J**) Fast maturation, (**K**) Monomeric state. Residues in key positions are selected with red, green, or blue colors to denote which of them are responsible for the respective phenotype independently, in concerted manner, or either of these two ways, respectively. Amino acid residues in the supporting positions are highlighted with yellow color.

(A) TagBFP-like phenotype

```
                        10        20        30        40        50
                         |         |         |         |         |
GFP               MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTG---KLPVPWP
Blue102     MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVT--KGGPLPFAWD
Blue124     MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVT--KGGPLPFAWD
mTagBFP           MSEELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVV--EGGPLPFAFD

                   60        70        80        90       100       110
                    |    ***  |         |         |         |         |
GFP                TLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDT
Blue102            ILSPQFLYGSRAYVKHPADIP--DYWKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGE
Blue124            ILSPQFMYGSRAYVKHPADIP--DYWKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGE
mTagBFP            ILATSFLYGSKTFINHTQGIP—-DFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGC

                  120       130       140       150       160       170
                    |         |         |         |         |         |
GFP                LVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQL
Blue102            FIYKVKLRGTNFPSDGPVM-QKKTMGWEAISERMYP--EDGALKGEIKQRLKLKDGGHYD
Blue124            FIYKVKLRGTNFPSDGPVM-QKKTMGWEAISERMYP--EDGALKGEIKQRLKLKDGGHYD
mTagBFP            LIYNVKIRGVNFTSNGPVM-QKKTLGWEAFTETLYP--ADGGLEGRNDMALKLVGGSHLI

                 180       190       200       210       220       230
                   |         |         |         |         |         |
GFP              ADHYQQNTPIGD-GPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK
Blue102          AEVKTTYKAKK---PVQLPGAYNVNIKLDITSHNEDYT-IVEQYERAEGRHSTGGMDELYK
Blue124          AEVKTTYKAKK---PVQLPGAYNVNIKLDITSHNEDYT-IVEQYERAEGRHSTGGMDELYK
mTagBFP          ANIKTTYRSKKPAKNLKMPGVYYVDYRLERIKE-ANNETYVEQHEVAVARYCDLPSKLGHKLN
```

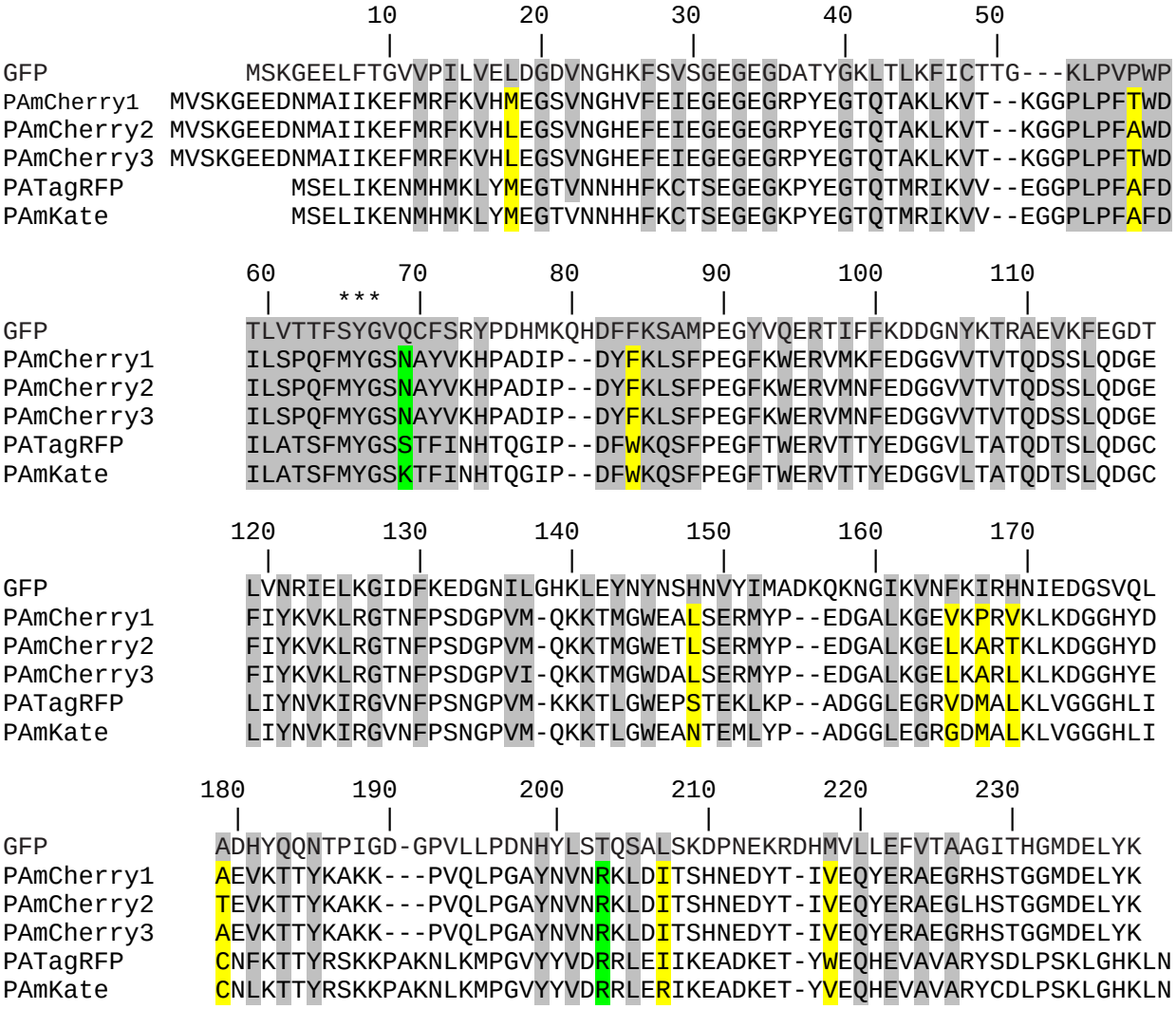
Nature Methods: doi:10.1038/nmeth.1776

(B) Fluorescent timer-like phenotype

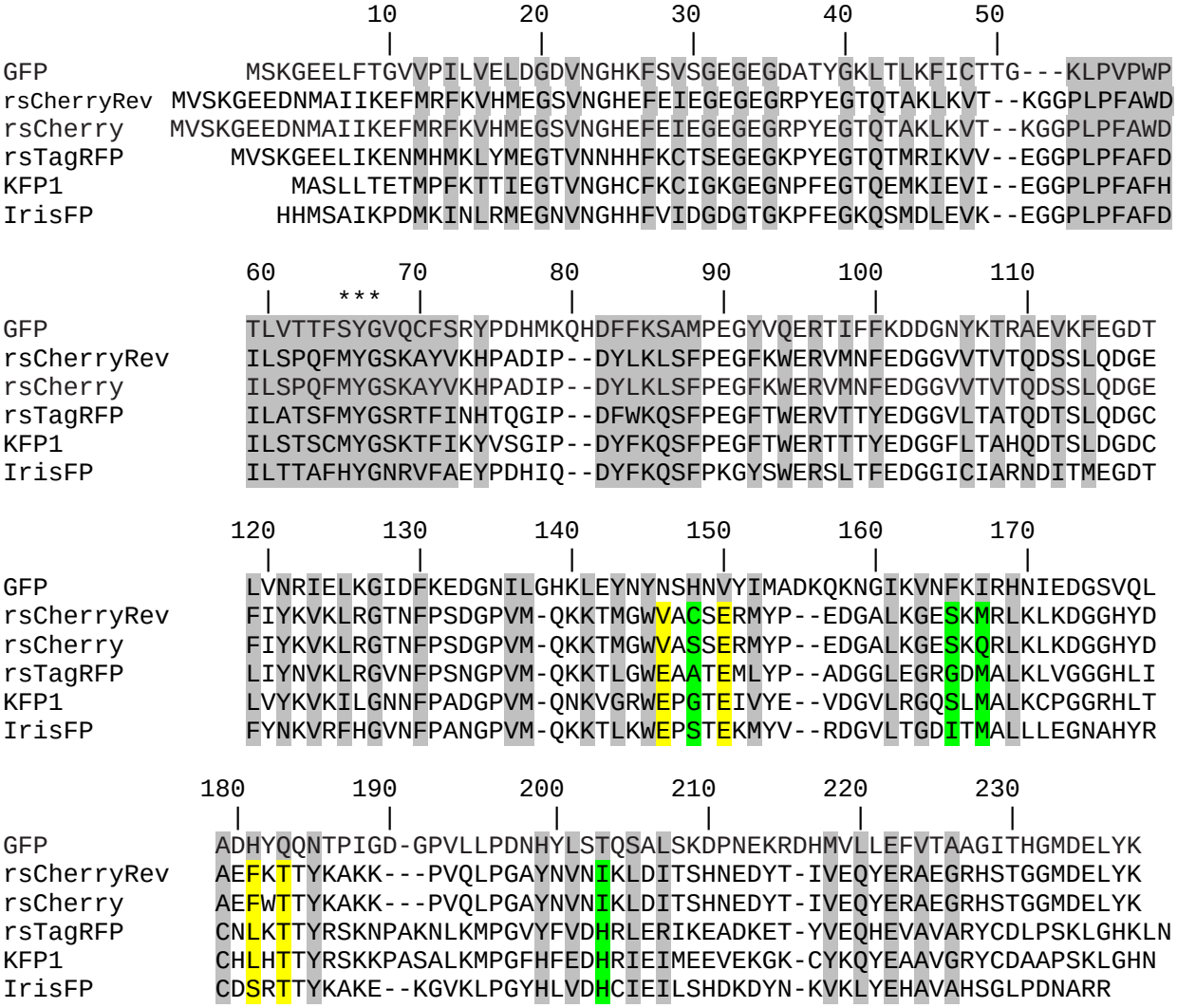


Nature Methods: doi:10.1038/nmeth.1776

(C) Photoactivatable-like phenotype

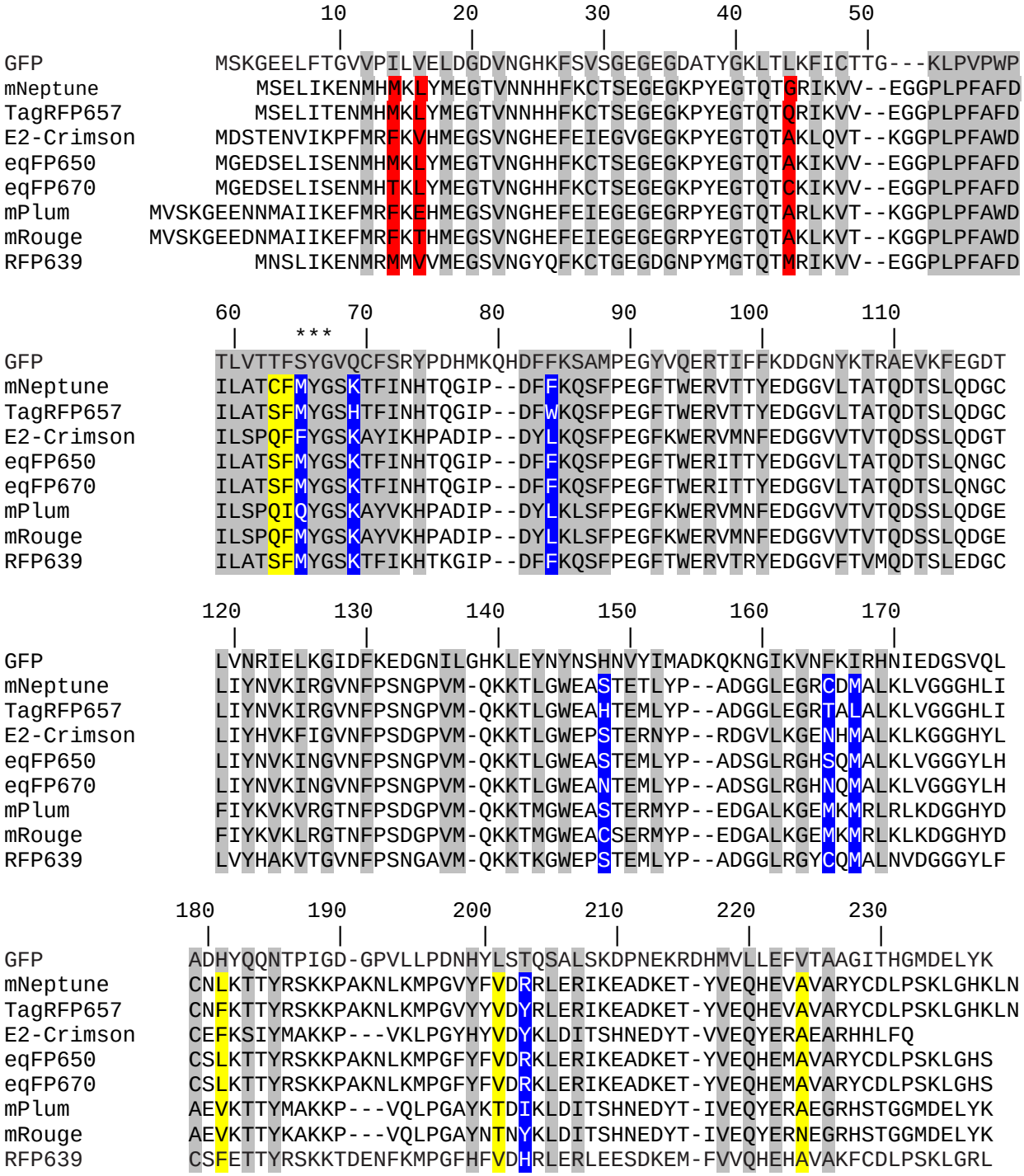


(D) Photoswitchable-like phenotype



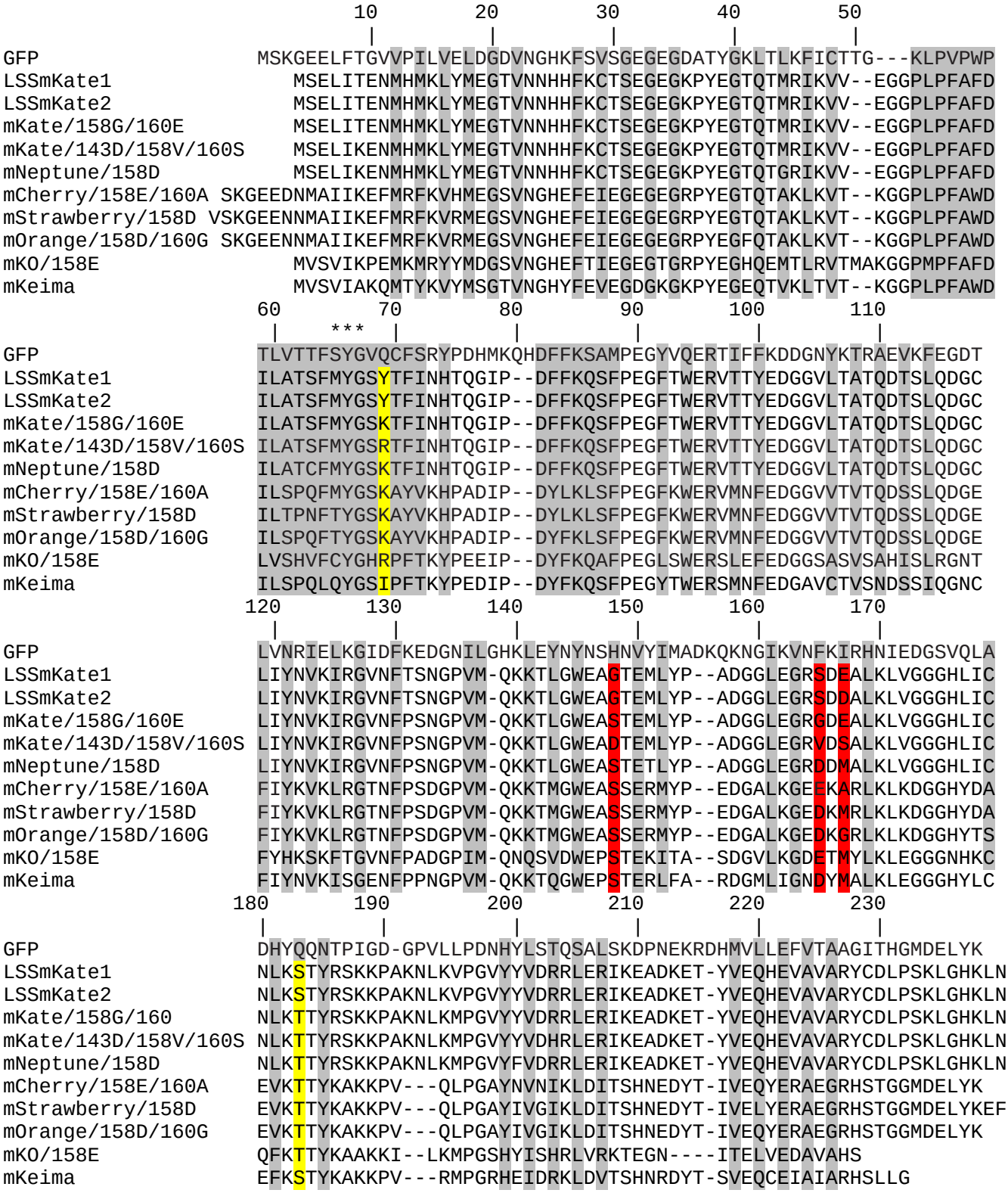
Nature Methods: doi:10.1038/nmeth.1776

(E) Far-red-shifted-like phenotype



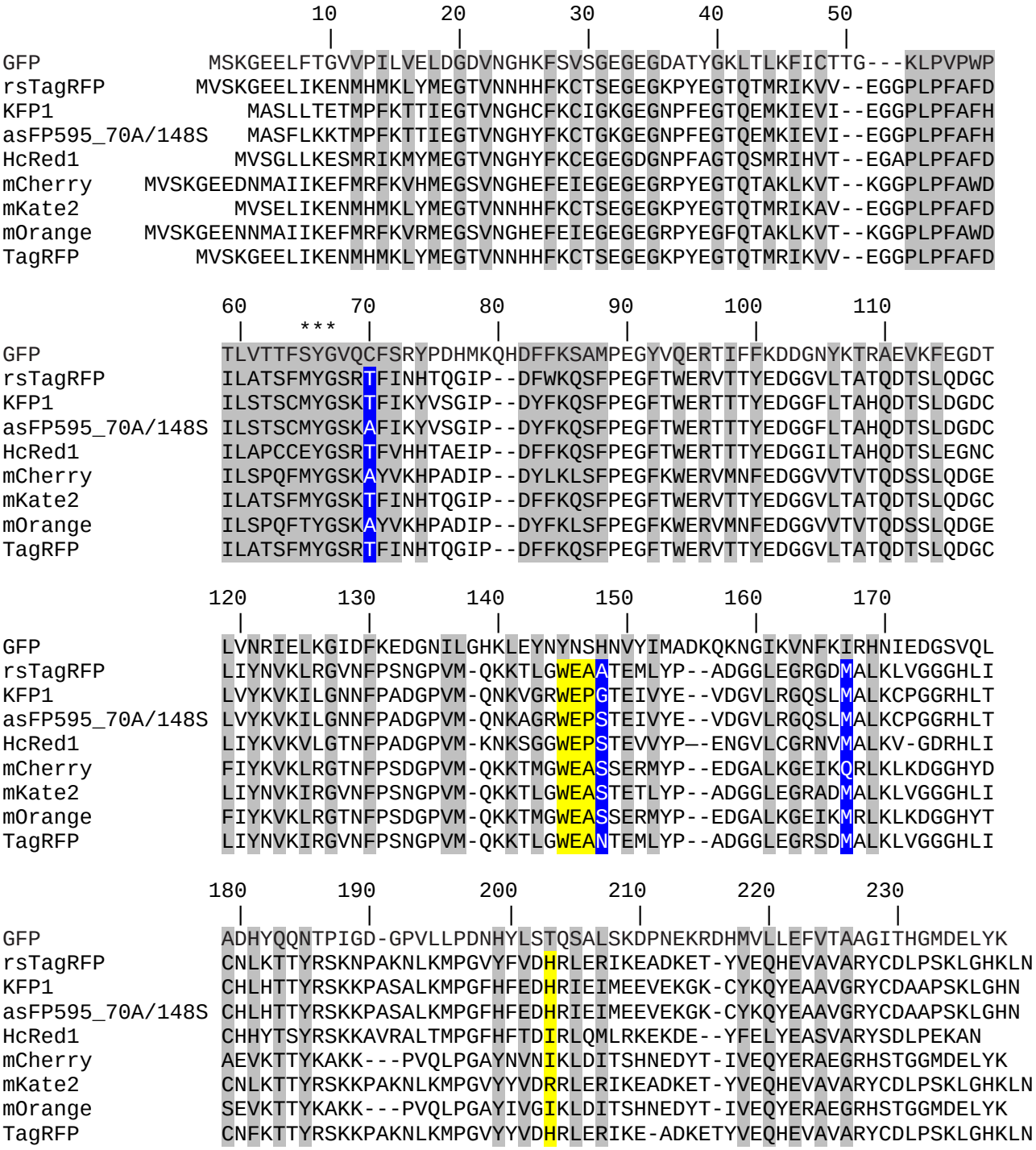
Nature Methods: doi:10.1038/nmeth.1776

(F) Large Stokes shift-like phenotype

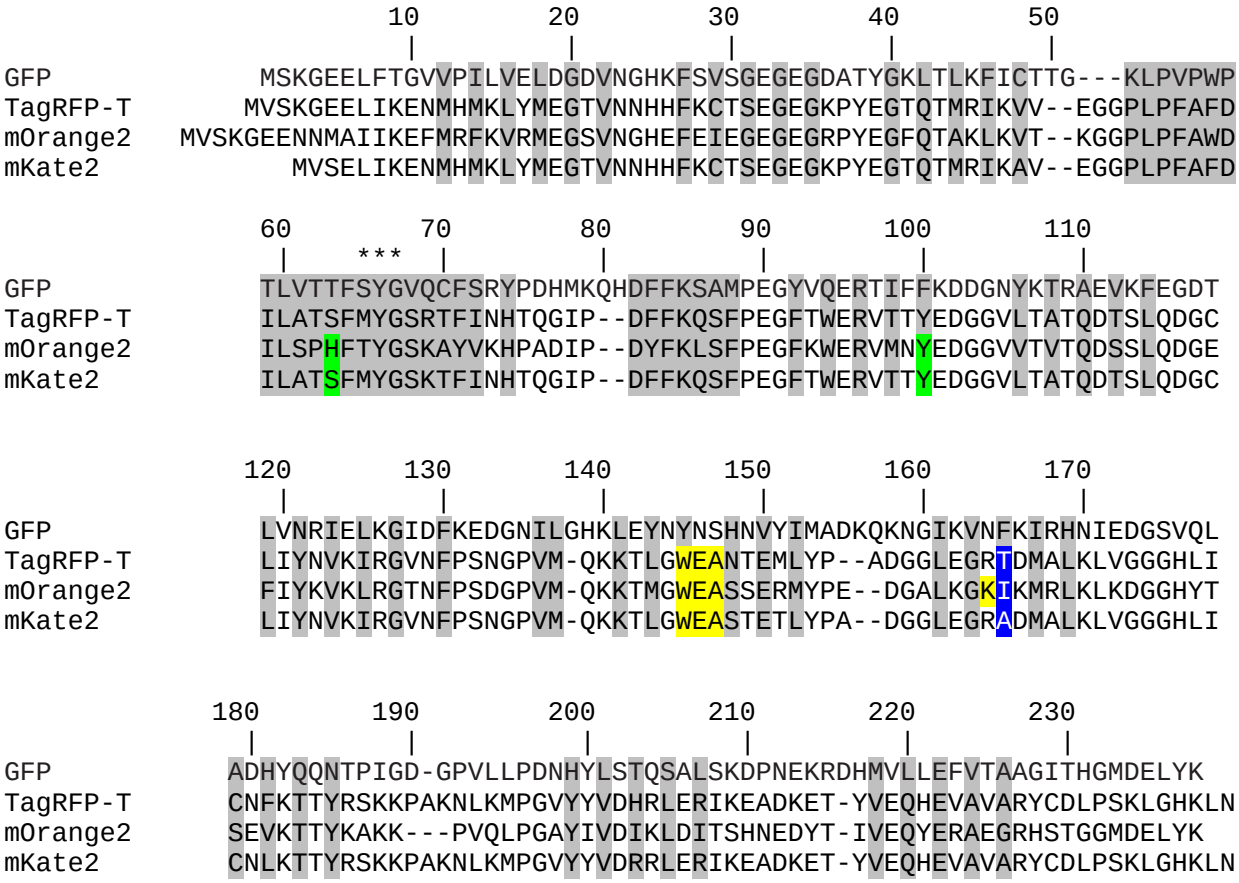


Nature Methods: doi:10.1038/nmeth.1776

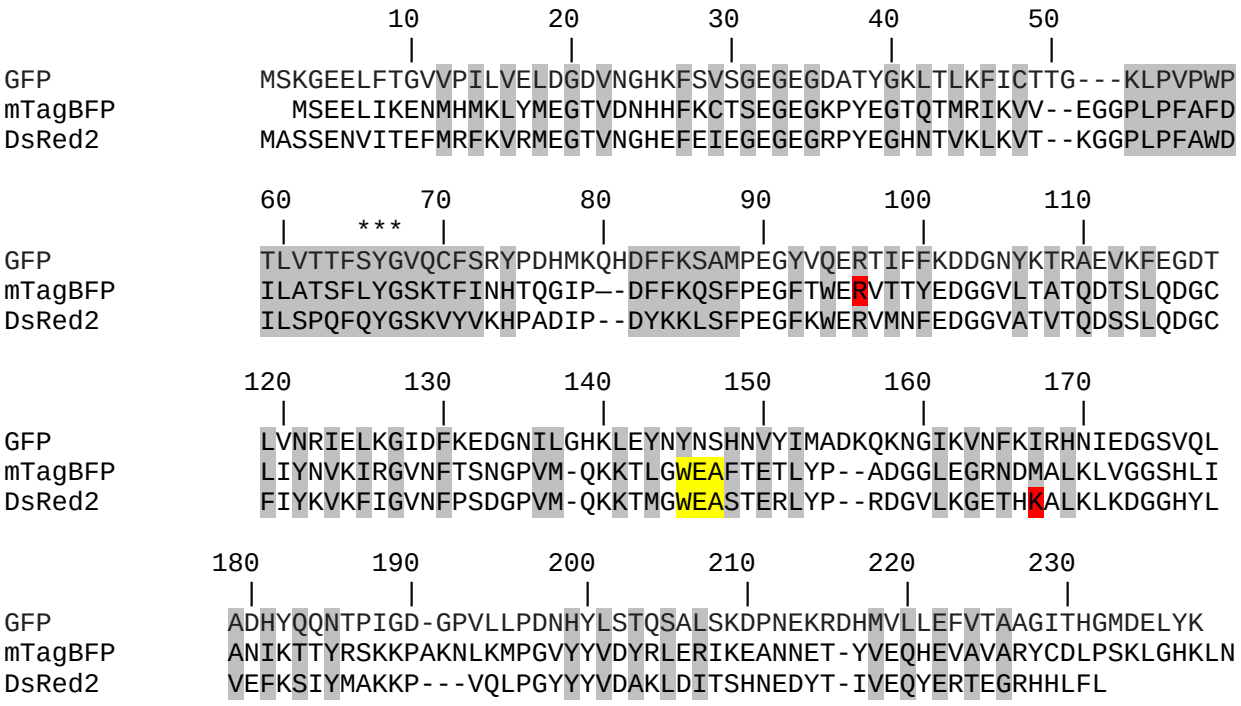
(G) High quantum yield



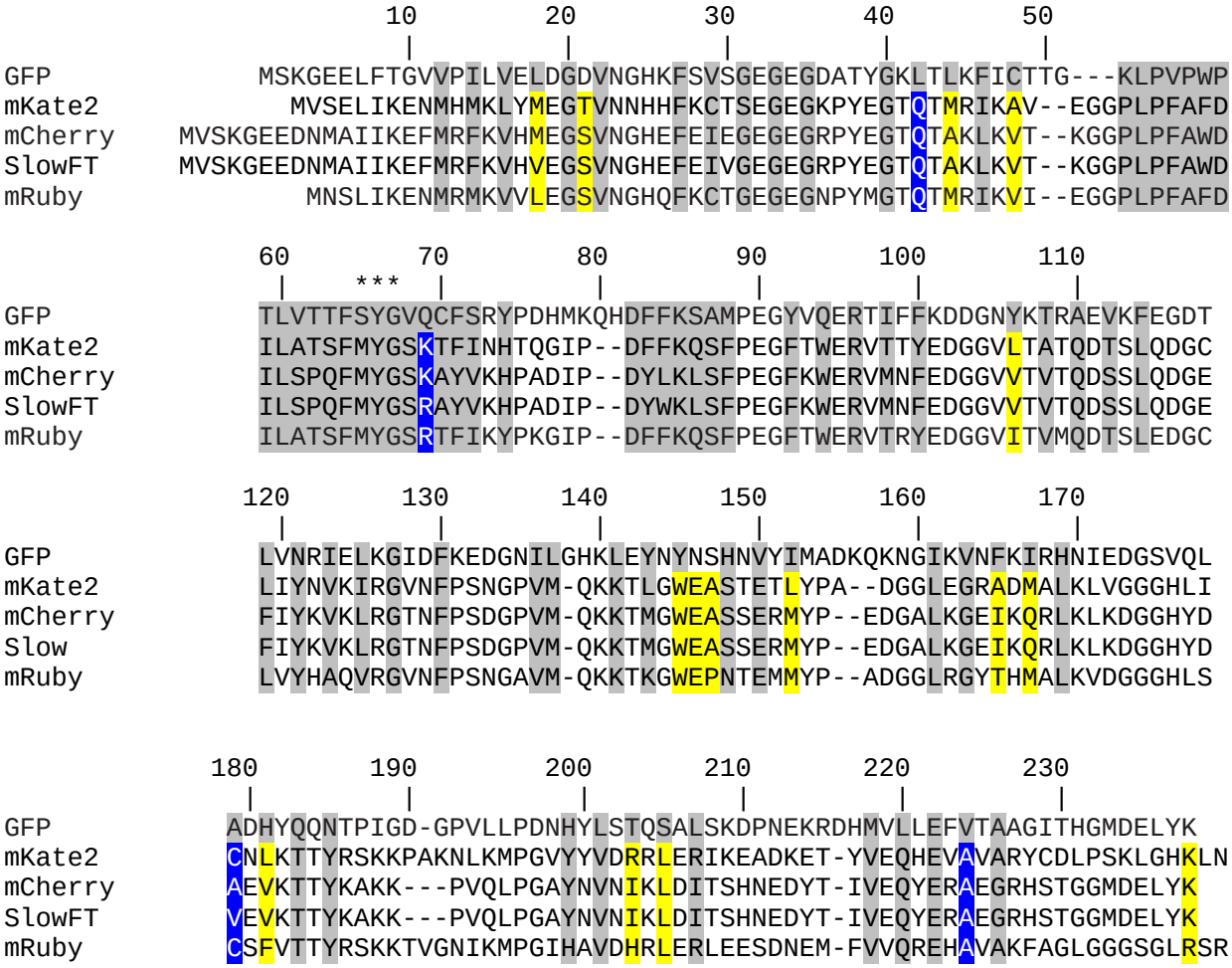
(H) High photostability



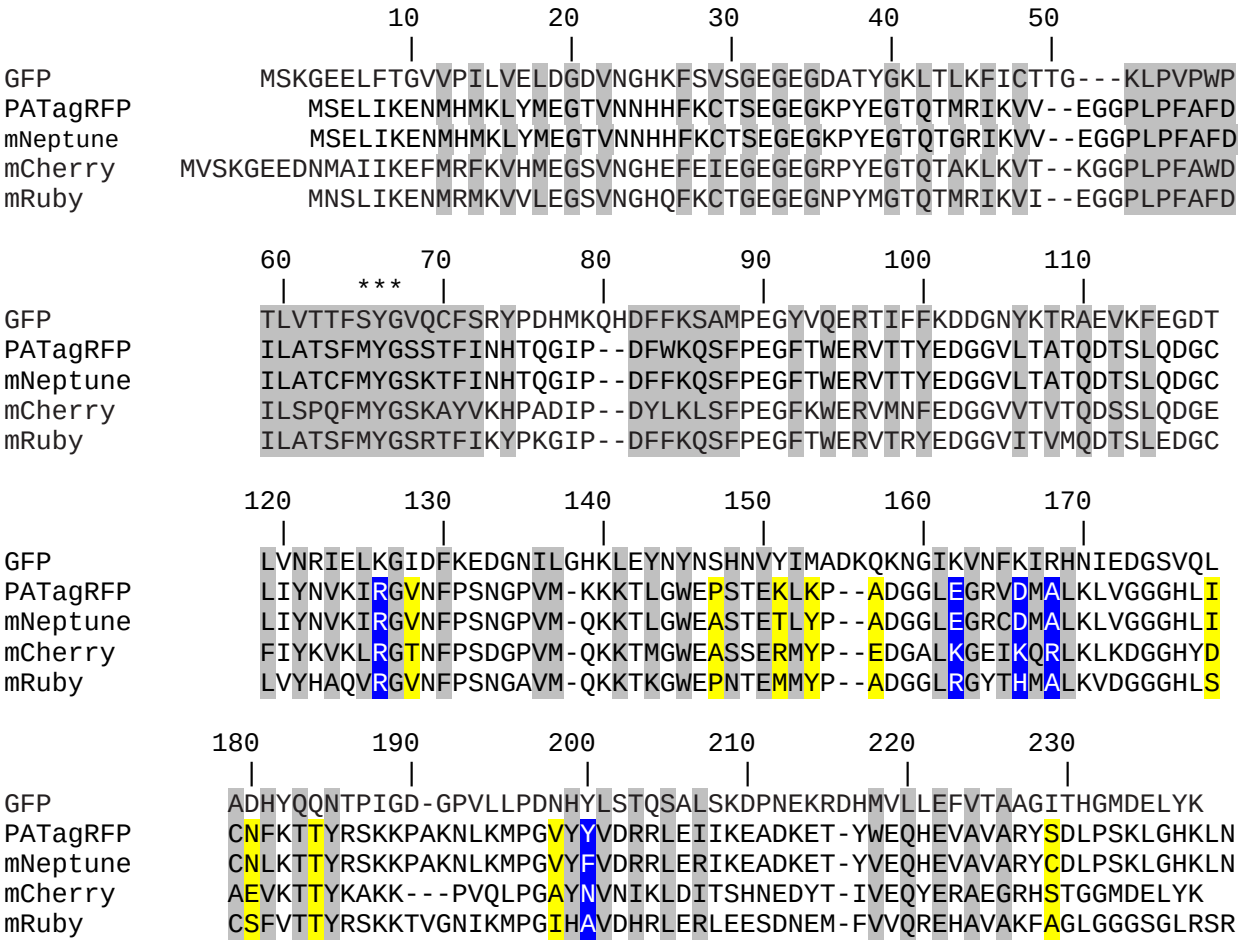
(I) High pH-stability



(J) Fast maturation



(**K**) Monomeric state



Nature Methods: doi:10.1038/nmeth.1776

Supplementary Table 1. Overview of suboptimal properties of current monomeric red fluorescent proteins.

Phenotype of RFP	Fluorescent protein	Form and its Excitation/Emission maxima, nm	Suboptimal properties	Ref.
Fluorescent	mKO	548/559	maturation, photoconversion to red spices	1
	mKO2	551/565	photostability	2
	mOrange	548/562	photostability, pH-stability, maturation, photoconversion to far-red species	3
	mOrange2	549/565	pH-stability, maturation, photoconversion to far-red species	4
	TagRFP	555/584	maturation, photostability, monomeric state	5
	TagRFP-T	555/584	maturation, monomeric state	4
	mRuby	558/605	maturation, photostability	6
	mStrawberry	574/596	quantum yield, photostability	3
	mRaspberry	574/596	maturation, quantum yield, photostability	7
	mCherry	587/610	quantum yield	3
	mKate2	588/633	pH-stability	8
	mPlum	590/649	maturation, brightness, photostability	7
	eqFP650	592/650	quantum yield, photostability	9
	mNeptune	600/650	quantum yield, maturation, monomeric state, photostability	10
	eqFP670	605/670	quantum yield	9
Large Stokes shift	mKeima	440/620	brightness, pH-stability, monomeric state, maturation, residual red fluorescence with yellow excitation light	11
	LSS-mKate1	463/624	brightness, maturation	12
	LSS-mKate2	460/605	brightness, maturation	
Fluorescent Timers	FastFT	Blue, 403/466	photostability	13
		Red, 583/606	quantum yield	
	MediumFT	Blue, 401/464	photostability	
		Red, 579/600	quantum yield	
	SlowFT	Blue, 402/583	photostability	

Nature Methods: doi:10.1038/nmeth.1776

		Red, 465/604	quantum yield	
Photoactivatable	PAmCherry1	Dark, 404/466	N/A	14
		Red, 564/594	extinction coefficient, pH-stability	
	PATagRFP	Dark, 351/none	N/A	15
		Red, 562/595	maturation	
Photoconvertable	mEos2	Green, 506/519	photostability, maturation, oligomeric state	16
		Red, 573/584	pH stability	
	mKikGR	Green, 505/515	photostability, pH-stability	17
		Red, 580/591	photostability	
Reversibly photoswitchable	KFP1	Dark, 580/600	N/A	18
		Red, 580/600	maturation, quantum yield	
	rsTagRFP	Dark, 440/none	extinction coefficient	19
		Red, 567/585	brightness, pH stability, maturation, contrast	
	rsCherry	Dark, 572/610	contrast	14,20
		Red, 572/610	brightness, maturation, complex photobehavior during switching,	
	rsCherryRev	Dark, 572/608	contrast	
		Red, 572/608	brightness, maturation, photoactivation during switching,	

N/A – not applicable.

Supplementary Table 2. Current red fluorescent proteins exhibiting the best specific property or maximally optimized for the specific imaging application.

RFP property	Conventional RFPs	Photoactivatable RFPs	RFP application	Conventional RFPs	Photoactivatable RFPs
Excitation beyond 610 nm	TagRFP657 [21]	not available	*STED*	TagRFP657	not applicable
Emission beyond 670 nm	eqFP670 [9]	not available	*Deep tissue imaging*	mNeptune and E2-Crimson	not available
One photon brightness	mKOκ [22] and tdTomato [3]	mEos2 [16]	*RESOLFT*	not applicable	rsTagRFP
Two-photon brightness	DsRed2 and dTomato [23]	unknown	*PALM*	not applicable	PATagRFP and tdEosFP
Photon counts per molecule	Unknown	tdEosFP [24]	*Multicolor PALM*	not applicable	green PA-GFP
pH stability	LSS-mKate2 [12] and TagRFP [5]	PATagRFP [15] and KikGR [25]			red PATagRFP
Maturation rate	mCherry [3] and mKate2 [8]	rsTagRFP [19]	*Multicolor imaging with a single excitation wavelength*	blue TagBFP	not available
Intracellular half-life and thermodynamic stability	mRFP1 [26]	Dendra2 [27]		green T-Sapphire	
Cytotoxicity	DsRed-Express2 [28] and E2-Crimson [29]	unknown		red LSS-mKate2	
Monomeric state and performance in fusions	mCherry [3]	PATagRFP [15], Dendra2 [30] and mEos2 [16]	*FRET-based biosensors*	Venus-mCherry [31]	EYFP-rsTagRFP [19]

Supplementary Table 3. Properties of the major chromophore structures observed in fluorescent proteins.

Chemical structure of chromophore	Chromophore name	Number of double bonds in conjugation	Typical absorbance/ emission max, nm	Examples of proteins sharing this chromophore
1	non-cyclized tripeptide X65-Tyr66-Gly67	none	none	majority of FPs
3	**aromatic α-enolate**	2	350-360/ undetectable	PATagRFP (dark state)
4	**TagBFP-like**	4	400-420/ 456-466	TagBFP, FTs (blue state), PAmCherry (dark state), mCherry-Blue102
5a, 6a, 8	**DsRed-like asFP595-like** (*anionic*)	7, 8	555-610/ 583-650	DsRed, mCherry, PAmCherry (*trans*), TagRFP (*trans*), mKate, PATagRFP, asFP595
5b, 6b	**DsRed-like** (*neutral*)	8	440-463/535, 605-624	mKeima, LSS-mKate1, LSS-mKate2, GmKate
9, 10	**mOrange-like**, **zFP538-like**, **mKO-like** (*anionic*)	7	540-548/ 561-565	mOrange, mKO, E2-Orange, zFP538
7	**DsRed-like a) H-bonding b) hydrophobic packing c) π-π stacking**	8	>610/ >650	TagRFP657, E2-Crimson, mNeptune, mRouge
11	**PSmOrange-like**	8	>610/ >650	PSmOrange
12	**GFP-like** (*anionic*)	6	484-493/ 502-510	EGFP, TagGFP2, Emerald
13	**GFP-like** (*neutral*)	6	399/ 511	wtGFP, Sapphire, Ametrine
14	**Kaede-like**	9	550-580/ 580-595	Kaede, mEos2, mKikGR, Dendra, mIrisFP

See Figure 2 for chemical structures of the chromophores. Additional characteristics of the listed proteins are summarized in the review papers[32-34].

Nature Methods: doi:10.1038/nmeth.1776

Supplementary Table 4. Amino acid residues in the supporting positions of red fluorescent proteins.

Phenotype/property (*structure/transition*)		Supporting positions										Ref.
TagBFP-like (*4*)		65 H,L	69 K,R	165 A,I,N	179 A	181 A,I						13,35
Fluorescent timer-like (*4→5a,6a*)		18 V,L,M		106 A	152 M,I	179 V,A	203 S,I	205 M,L	224 S,A			13,36
Photoactivatable-like (*4→5a,6a*)		18 L,M	58 T,A	84 F,W	148 L,S,N,H,T,F	165 V,L,S,A,G,Q	167 P,A,M,G	169 V,T,L	179 T,A,C	207 I,R	218 W,V	14,15,37,38
Photoswitchable-like (*5a↔6a*)		146 V,E	150 E	181 F,L,S	183 T							19,20,39,40
Far-red-shifted-like	H-bonding to N-acylimine oxygen (*7a*)	63 C,F	64 I,F	165 C								9,10,21,41-45
	Hydrophobic packing (*7c*)	181 F,L,V		201 T,V		224 C,N,A						
	π-stacking (*7b*)	181 F,L,V		201 A,T	224 C,N,A							
Large Stokes shift-like (*5b,6b*)		69 R,K,Y		148 G,S,N	165 G,A,V,N		167 S,M,A,G,L,N		183 T,S			11,12,46-48
High quantum yield		145 W	146 E	147 P,A	203 H,I,R							3,5,8,41,49-52
High photostability		145 W	146 E	147 P,A	163 K							4,8,52
High pH-stability		145 W	146 E	147 P,A								52-54
Fast maturation		18 V,L,M	21 T,S	44 M,A	48 A,V	106 I,V,L	152 M,I,L	165 A,I,T	167 M,Q	181 L,V,F	203 S,I,R,H	205 M,L	238 R	8,13,45,55-57
Monomeric state		128 V,T	151 K,T,R,M	153 K,Y	157 A,E		178 I,D,S	180 N,E,S	184 T	198 V,A,I	200 Y,F,N,A		229 S,C,A	6,10,15,56-58

The supporting residues correspond to those on Figure S1 highlighted with yellow color. Residues' numbering follows that for GFP.

Supplementary Note

Major steps of molecular evolution

A typical process of the development of fluorescent proteins with required spectral and biochemical properties includes a rational design and several steps of directed molecular evolution (**Fig. 1**). The starting points are design of new spectral phenotype of fluorescent protein based on the known mechanisms of chromophore transformations and choosing of an appropriate template gene.

Next step involves the rational design based on the knowledge of relationship between immediate chromophore environment and postranslational chemistry of fluorescent proteins and photochemical properties of the chromophore. At this step, the selected templates are subjected to multiple site-specific amino acid substitutions at the key and supporting positions, in order to generate a library of site-specific mutants. After screening this library typically in a low-throughput format, several primary clones, possessing the required spectral phenotype, are selected.

Several rounds of directed molecular evolution are then applied to improve the properties of imperfect primary variants. Major challenges to get a successful directed evolution include selecting an optimal biological system for expression, obtaining a sufficient number of clones in a library, and achieving reliable screening conditions. Each round of the directed molecular evolution begins with a generation of a large mutant library, using various stratagies of mutagenesis and recombination[59-62]. A high-throughput screening (HTS) of the library and subsequent evaluation of the selected clones in a low-throughput format then follows. The clones exhibiting prominent characteristics of the required phenotype are then applied to the next round of molecular evolution. After several rounds, the screened properties usually reach a plateau, suggesting that either a natural limit is achieved or some bias, caused by the mutagenesis stratagy used, is the culprit. To avoid the latter case, a saturated multi-site mutagenesis is typically applied to the selected mutants at amino acid positions found during the rounds of molecular evolution. Finally, testing of advanced variants with optimized characteristics by creating fusion proteins and their imaging in live cells comes before wide usage in the life sciences.

Historical aspects of the molecular evolution strategy in the development of fluorescent proteins

In the past, fluorescent proteins of new colors such as mFruits have been mainly developed using random mutagenesis on the basis of wild-type protein templates of required weak phenotype[3]. Over time the more structure-to-function information has become available for fluorescent proteins and it resulted into appearance of rational component in the creation of fluorescent proteins. The PAmCherrys[14], PATagRFP[15], rsCherrys[20], rsTagRFP[19], KikGR[25], mRouge[41] and series of TagBFP-[35] and LSS-like[46] fluorescent proteins have different colors and photochemical behavior and all of them were developed using rational mutagenesis at amino acids around chromophore to find weak phenotype followed by random mutagenesis for the further improvement of mainly brightness, maturation and photostability.

Correlation between residues at key positions and fluorescent protein phenotype

The residues at key positions 84, 148 and 203 for TagBFP-like phenotype mainly sterically hinder Tyr-ring of the chromophore occupies position necessary for the oxidation of

Nature Methods: doi:10.1038/nmeth.1776

Cα2-Cβ2 double bond between Tyr-ring and imidazolinone and improve brightness of blue chromophore 4.

The residues at key positions 69 and 84 for FT-like phenotype slow down 4-to-5a,6a transition and increase brightness of blue chromophore 4. The residues at crucial positions 69 and 203 for photoactivatable-like phenotype block 4-to-5a,6a transition. The residues at main positions 148, 165, 167 and 203 for photoswitchable-like phenotype provide space around Tyr-ring of the chromophore and ensure flexibility of the chromophore for *cis-trans* isomerization of the chromophore to occur.

The far-red shift in RFPs can be attributed by the formation of hydrogen bond between residues at positions 14, 16 and 44 and N-acylimine oxygen, by hydrophobic parking around chromophore provided by hydrophobic residues at positions 84, 148, 165 and 167 and by stacking interactions between aromatic residue at positions 65, 69, 148 and 203 and Tyr-ring of the chromophore.

Large Stokes shift-like phenotype relies on the Glu and Asp acidic amino acids at positions 148, 165 and 167 which stabilize neutral chromophore 5b,6b and support proton transfer in excited state.

High photostability of RFPs mainly depends on amino acids at positions 64, 99 and 165 which provide tight packing around chromophore and block photochemical reactions.

Fast maturation of RFPs is primarily affected by residues at positions 42, 69, 179, and 224 which facilitate 2-to-5a,6a transition.

The A-B and A-C dimerizing interfaces in RFPs are mainly disrupted by charged amino acid residues at positions 126, 162, 166 and 168.

High quantum yield in RFPs depends on the residues at positions 70, 148 and 167 which provide planar configuration and rigidity of the chromophore.

High pH-stability of blue and red chromophores 4 and 5a,6a, respectively, is provided by the residues at positions 96 and 167 which stabilize negative charge on CO-group of imidazolinone and Tyr-ring, respectively.

Maturation rate, pH-stability, quantum yield and photostability may be affected by the width of the water-filled pore which regulated by the residues at positions 145-147.

There are many examples in the world of fluorescent proteins where a particular feature (e.g., a red color in DsRed) is a synergistic effect of a large number of residue substitutions, including many residues that are remote from the active site. How these positions control fluorescence via coupling to each other is poorly understood. This is why the directed evolution approach will be useful for final turning of the residues remote from chromophore with found spectral phenotype.

Nature Methods: doi:10.1038/nmeth.1776

Supplementary references

1. Karasawa, S., Araki, T., Nagai, T., Mizuno, H. & Miyawaki, A. Cyan-emitting and orange-emitting fluorescent proteins as a donor/acceptor pair for fluorescence resonance energy transfer. *Biochem. J.* **381**, 307-312 (2004).
2. Sakaue-Sawano, A. et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* **132**, 487-498 (2008).
3. Shaner, N.C. et al. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567-1572 (2004).
4. Shaner, N.C. et al. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat. Methods* **5**, 545-551 (2008).
5. Merzlyak, E.M. et al. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat. Methods* **4**, 555-557 (2007).
6. Kredel, S. et al. mRuby, a bright monomeric red fluorescent protein for labeling of subcellular structures. *PLoS One* **4**, e4391 (2009).
7. Wang, L., Jackson, W.C., Steinbach, P.A. & Tsien, R.Y. Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc. Natl. Acad. Sci. USA* **101**, 16745-16749 (2004).
8. Shcherbo, D. et al. Far-red fluorescent tags for protein imaging in living tissues. *Biochem. J.* **418**, 567-574 (2009).
9. Shcherbo, D. et al. Near-infrared fluorescent proteins. *Nat. Methods* **7**, 827-829 (2010).
10. Lin, M.Z. et al. Autofluorescent proteins with excitation in the optical window for intravital imaging in mammals. *Chem. Biol.* **16**, 1169-1179 (2009).
11. Kogure, T. et al. A fluorescent variant of a protein from the stony coral Montipora facilitates dual-color single-laser fluorescence cross-correlation spectroscopy. *Nat. Biotechnol.* **24**, 577-581 (2006).
12. Piatkevich, K.D. et al. Monomeric red fluorescent proteins with a large Stokes shift. *Proc. Natl. Acad. Sci. USA* **107**, 5369-5374 (2010).
13. Subach, F.V. et al. Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. *Nat. Chem. Biol.* **5**, 118-126 (2009).
14. Subach, F.V. et al. Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. *Nat. Methods* **6**, 153-159 (2009).
15. Subach, F.V., Patterson, G.H., Renz, M., Lippincott-Schwartz, J. & Verkhusha, V.V. Bright monomeric photoactivatable red fluorescent protein for two-color super-resolution sptPALM of live cells. *J. Am. Chem. Soc.* **132**, 6481-6491 (2010).
16. McKinney, S.A., Murphy, C.S., Hazelwood, K.L., Davidson, M.W. & Looger, L.L. A bright and photostable photoconvertible fluorescent protein. *Nat. Methods* **6**, 131-133 (2009).
17. Habuchi, S., Tsutsui, H., Kochaniak, A.B., Miyawaki, A. & van Oijen, A.M. mKikGR, a monomeric photoswitchable fluorescent protein. *PLoS One* **3**, e3944 (2008).
18. Chudakov, D.M. et al. Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* **21**, 191-194 (2003).
19. Subach, F.V. et al. Red fluorescent protein with reversibly photoswitchable absorbance for photochromic FRET. *Chem. Biol.* **17**, 745-755 (2010).

20. Stiel, A.C. et al. Generation of monomeric reversibly switchable red fluorescent proteins for far-field fluorescence nanoscopy. *Biophys. J.* **95**, 2989-2997 (2008).
21. Morozova, K.S. et al. Far-red fluorescent protein excitable with red lasers for flow cytometry and superresolution STED nanoscopy. *Biophys. J.* **99**, L13-15 (2010).
22. Tsutsui, H., Karasawa, S., Okamura, Y. & Miyawaki, A. Improving membrane voltage measurements using FRET with new fluorescent proteins. *Nat. Methods* **5**, 683-685 (2008).
23. Drobizhev, M., Tillo, S., Makarov, N.S., Hughes, T.E. & Rebane, A. Absolute two-photon absorption spectra and two-photon brightness of orange and red fluorescent proteins. *J. Phys. Chem. B* **113**, 855-859 (2009).
24. Nienhaus, K., Nienhaus, G.U., Wiedenmann, J. & Nar, H. Structural basis for photo-induced protein cleavage and green-to-red conversion of fluorescent protein EosFP. *Proc Natl Acad Sci USA* **102**, 9156-9159 (2005).
25. Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N. & Miyawaki, A. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* **6**, 233-238 (2005).
26. Stepanenko, O.V. et al. Comparative studies on the structure and stability of fluorescent proteins EGFP, zFP506, mRFP1, "dimer2", and DsRed1. *Biochemistry* **43**, 14913-14923 (2004).
27. Kedrin, D. et al. Intravital imaging of metastatic behavior through a mammary imaging window. *Nat. Methods* **5**, 1019-10121 (2008).
28. Strack, R.L. et al. A noncytotoxic DsRed variant for whole-cell labeling. *Nat. Methods* **5**, 955-957 (2008).
29. Strack, R.L. et al. A Rapidly Maturing Far-Red Derivative of DsRed-Expres2 for Whole-Cell Labeling. *Biochemistry* **48**, 8279-8281 (2009).
30. Baker, S.M., Buckheit, R.W., 3rd & Falk, M.M. Green-to-red photoconvertible fluorescent proteins: tracking cell and protein dynamics on standard wide-field mercury arc-based microscopes. *BMC Cell Biol.* **11**, 15 (2010).
31. Hazelwood, K.L. et al. Searching the fluorescent protein color palette for new FRET pairs. *Proc. SPIE* **6868**, 68681-686812 (2008).
32. Davidson, M.W. & Campbell, R.E. Engineered fluorescent proteins: innovations and applications. *Nat. Methods* **6**, 713-717 (2009).
33. Chudakov, D.M., Matz, M.V., Lukyanov, S. & Lukyanov, K.A. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* **90**, 1103-1163 (2010).
34. Piatkevich, K.D., Efremenko, E.N., Verkhusha, V.V. & Varfolomeev, S.D. Red fluorescent proteins and their properties. *Russ. Chem. Rev.* **79**, 243-258 (**2010**).
35. Subach, O.M. et al. Conversion of red fluorescent protein into a bright blue probe. *Chem. Biol.* **15**, 1116-1124 (2008).
36. Terskikh, A. et al. "Fluorescent timer": protein that changes color with time. *Science* **290**, 1585-1588 (2000).
37. Subach, F.V. et al. Photoactivation mechanism of PAmCherry based on crystal structures of the protein in the dark and fluorescent states. *Proc. Natl. Acad. Sci. USA* **106**, 21097-21102 (2009).
38. Gunewardene, M.S. et al. Superresolution Imaging of Multiple Fluorescent Proteins with Highly Overlapping Emission Spectra in Living Cells. *Biophys. J.* **101**, doi:10.1016/j.bpj.2011.1007.1049 (2011).

39. Chudakov, D.M., Feofanov, A.V., Mudrik, N.N., Lukyanov, S. & Lukyanov, K.A. Chromophore environment provides clue to "kindling fluorescent protein" riddle. *J. Biol. Chem.* **278**, 7215-7219 (2003).
40. Wiedenmann, J. et al. From EosFP to mIrisFP: structure-based development of advanced photoactivatable marker proteins of the GFP-family. *J. Biophotonics* **4**, 377-390 (2011).
41. Chica, R.A., Moore, M.M., Allen, B.D. & Mayo, S.L. Generation of longer emission wavelength red fluorescent proteins using computationally designed libraries. *Proc. Natl. Acad. Sci. USA* **107**, 20257-20262 (2010).
42. Shu, X., Shaner, N.C., Yarbrough, C.A., Tsien, R.Y. & Remington, S.J. Novel chromophores and buried charges control color in mFruits. *Biochemistry* **45**, 9639-9647 (2006).
43. Shu, X., Wang, L., Colip, L., Kallio, K. & Remington, S.J. Unique interactions between the chromophore and glutamate 16 lead to far-red emission in a red fluorescent protein. *Protein Sci.* **18**, 460-466 (2009).
44. Strack, R.L. et al. A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. *Biochemistry* **48**, 8279-8281 (2009).
45. Kredel, S. et al. Optimized and far-red-emitting variants of fluorescent protein eqFP611. *Chem. Biol.* **15**, 224-233 (2008).
46. Piatkevich, K.D., Malashkevich, V.N., Almo, S.C. & Verkhusha, V.V. Engineering ESPT pathways based on structural analysis of LSSmKate red fluorescent proteins with large Stokes shift. *J. Am. Chem. Soc.* **132**, 10762-10770 (2010).
47. Henderson, J.N. et al. Excited state proton transfer in the red fluorescent protein mKeima. *J. Am. Chem. Soc.* **131**, 13212-13213 (2009).
48. Kogure, T., Kawano, H., Abe, Y. & Miyawaki, A. Fluorescence imaging using a fluorescent protein with a large Stokes shift. *Methods* **45**, 223-226 (2008).
49. Lukyanov, K.A. et al. Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* **275**, 25879-25882 (2000).
50. Pletnev, S., Subach, F.V., Dauter, Z., Wlodawer, A. & Verkhusha, V.V. Structural basis for reversible photoswitching of absorbance spectra in red fluorescent protein rsTagRFP. *Submitted*.
51. Gurskaya, N.G. et al. GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett.* **507**, 16-20 (2001).
52. Stiel, A.C. et al. 1.8 A bright-state structure of the reversibly switchable fluorescent protein Dronpa guides the generation of fast switching variants. *Biochem. J.* **402**, 35-42 (2007).
53. Wilmann, P.G. et al. The 2.1A crystal structure of copGFP, a representative member of the copepod clade within the green fluorescent protein superfamily. *J. Mol. Biol.* **359**, 890-900 (2006).
54. Bravaya, K.B., Korovina, N., Subach, O.M., Verkhusha, V.V. & Krylov, A.I. Puzzling over the mechanism of red chromophore maturation in the DsRed-like proteins: The nature of the blue intermediate revealed. *Submitted*.
55. Pletnev, S., Subach, F.V., Dauter, Z., Wlodawer, A. & Verkhusha, V.V. Understanding blue-to-red conversion in monomeric fluorescent timers and hydrolytic degradation of their chromophores. *J. Am. Chem. Soc.* **132**, 2243-2253 (2010).
56. Campbell, R.E. et al. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882 (2002).

57. Wiedenmann, J. et al. A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from Entacmaea quadricolor (Anthozoa, Actinaria). *Proc. Natl. Acad. Sci. USA* **99**, 11646-11651 (2002).
58. Nienhaus, K., Nar, H., Heilker, R., Wiedenmann, J. & Nienhaus, G.U. Trans-cis isomerization is responsible for the red-shifted fluorescence in variants of the red fluorescent protein eqFP611. *J. Am. Chem. Soc.* **130**, 12578-12579 (2008).
59. Wang, T.W. et al. Mutant library construction in directed molecular evolution: casting a wider net. *Mol. Biotechnol.* **34**, 55-68 (2006).
60. Farinas, E.T., Bulter, T. & Arnold, F.H. Directed enzyme evolution. *Curr. Opin. Biotechnol.* **12**, 545-551 (2001).
61. Yuan, L., Kurek, I., English, J. & Keenan, R. Laboratory-directed protein evolution. *Microbiol. Mol. Biol. Rev.* **69**, 373-392 (2005).
62. Giver, L. & Arnold, F.H. Combinatorial protein design by in vitro recombination. *Curr. Opin. Chem. Biol.* **2**, 335-338 (1998).