

Red fluorescent proteins and their properties

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Abstract. The main groups of currently known red fluorescent proteins are characterized: their structure, folding and mechanisms of chromophore formation are discussed. The key applications of these proteins as markers and sensors in cell and molecular biology are demonstrated. The bibliography includes 118 references.

I. Introduction

It is usually believed that all colour diversity of living organisms is caused by chromoproteins or low-molecular-weight pigments. As a rule, chromoproteins contain a small molecule of non-protein origin or a metal ion that are responsible for the protein chromogenic properties. However, fluorescence of the green fluorescent protein (GFP) discovered in 1961 by Shimomura *et al.*¹ in the study of bioluminescence of the jellyfish *Aequorea victoria* of the class Hydrozoa is based on the interaction of the protein inner amino acid residues and requires no co-factors. For many years, GFP had been studied by a relatively small group of scientists as a protein that is a part of a bioluminescent system.² After cloning of the GFP gene, the interest in this gene increased jumpwise, and at present GFP and its mutants are used most widely among genetic markers in different biological applications.^{3–5} In 2008, American scientists O Shimomura, M Chalfie and R Tsien were honoured with Nobel prize in chemistry for the

discovery and development of the green fluorescent protein, GFP.⁶ Cloning genes of GFP-like proteins with yellow, orange, red and far-red fluorescence had the next impact on the development of this line of research in biotechnology.⁷ At present, fluorescent proteins of the whole range of colours (from blue to far-red) are known, which span the spectrum from 424 to 655 nm. The GFP-like red fluorescent proteins are still the least covered in the literature, and this review is dedicated to these proteins.

The red fluorescent proteins (RFPs) have maxima of fluorescence emission above 560 nm. All RFPs can be divided into two main groups: permanently red fluorescent proteins and photoactivatable red fluorescent proteins (PARFPs). In contrast to RFPs, the PARFPs require UV irradiation or visible blue light for red chromophore formation. Such GFP-like proteins as chromoproteins (CPs) should also be mentioned, they are able to absorb light efficiently, but however do not fluoresce. The red chromoproteins have usually one absorption maximum at 560–610 nm and the wavelength of this maximum defines the protein colour. The RFP maturation often involves blue or green fluorescent states. This property of RFPs was used to create the so-called fluorescent timers that fluoresce in the blue or green region of the spectrum at the early maturation stages and in the red region after complete maturation.

II. Physical and chemical properties of red fluorescent proteins

1. The sources of fluorescent proteins and chromoproteins

Labas was the first to suggest that non-bioluminescent organisms, such as corals, could contain GFP-like fluorescent proteins. In 1999, six new fluorescent proteins were isolated and cloned from non-bioluminescent Anthozoa species. One of the proteins named drFP583 differed from GFP dramatically in its spectral properties, demonstrating fluorescence in the red spectral region.⁷ The drFP583 protein gene was optimized for expression in mammalian cells, and the protein became the first commercially avail-

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Received 24 July 2009

Uspekhi Khimii 79 (3) 273–290 (2010); translated by K D Piatkevich

able RFP termed DsRed. This protein is also the first RFP obtained in the monomer state, it allowed further the development of a number of monomeric RFPs with improved properties.

Later, the majority of RFPs were isolated and cloned from Anthozoa species living in the Indo-Pacific region. A plenty of proteins with two-coloured fluorescence (red and green), as well as a plenty of proteins demonstrating the properties of fluorescent timers and non-fluorescent CPs, were found in corals of the Zoantharia subclass.^{8–12} It turned out that replacement of just one amino acid in CPs can lead to a remarkable increase in their fluorescence quantum yields.⁹

According to this strategy, several CPs isolated from *Heteractis crispa*, *Condylactis gigantea* and *Goniopora tenuidens* were converted into the FPs with far-red shifted fluorescence (from 615 to 640 nm).¹¹ Other far-red FPs

were obtained on the basis of CPs isolated from blue *Actinaria* corals.¹³

The eqFP611 protein is the most red-shifted natural RFP isolated from Anthozoa species found in the sea anemone *Entacmaea quadricolor*.¹⁴ The eqFP578 protein,¹⁵ a precursor of a whole series of the monomeric RFPs with improved properties,^{15, 16} was isolated from the same organism. The wild-type red fluorescent protein a Kusabira-Orange with the most blue-shifted fluorescence was cloned from the coral *Fungia concinna*.¹⁷

The red fluorescent proteins were also found in other classes of organisms. Thus the shape and position of the absorption spectrum of a GFP-like chromoprotein anm2CP (Ref. 18) from Anthomedusae (Hydrozoa class) are similar to those of the CPs and RFPs from the Anthozoa class. This similarity allowed purposeful introduction of such mutations that have led to the transformation of anm2CP to

Table 1. Properties of the red fluorescent protein.

Protein	Oligomeric state	Ex _{max} /nm	Em _{max} /nm	ϵ , /litre mol ⁻¹ cm ⁻¹	QY	pK _a	$\tau_{1/2}$ of maturation at 30 °C/h	Photo-stability/s	Brightness ^a	Ref.
mBanana	monomer	540	553	6 000	0.70	6.7	1.0	1.4	4	22
mKO	"	548	559	51 600	0.60	5.0	4.5	122	31	17
E2-Orange	tetramer	540	561	36 500	0.54	4.5	1.3	81	20	23
mOrange	monomer	548	562	71 000	0.69	6.5	2.5	9	49	22
mKOk	"	551	563	105 000	0.61	4.2	1.8	—	64	24
mOrange2	"	549	565	58 000	0.60	6.5	4.5	228	35	25
tdRFP2	dimer	552	579	120 000	0.68	4.8	—	—	82	26
dTomato	"	554	581	138 000	0.69	4.7	1	64	95	22
DsRed	tetramer	558	583	75 000	0.79	4.7	10	326	59	7
TagRFP	monomer	555	584	98 000	0.41	<4.0	1.7	37	40	15
TagRFP-T	"	555	584	81 000	0.41	4.6	1.7	337	33	25
mTangerine	"	568	585	38 000	0.30	5.7	—	5.1	11	22
E2-Red/Green	tetramer	560	585	53 800	0.67	4.5	1.2	93	36	23
DsRed-Express	"	554	586	30 100	0.42	—	0.70	71	13	27
DsRed2	"	561	587	43 800	0.55	—	6.5	—	24	27
DsRedT3	"	560	587	49 500	0.59	—	1.3	—	29	27
DsRed-Max	"	560	589	48 000	0.41	—	1.2	9	20	28
DsRed-Express2	"	554	591	35 600	0.42	—	0.7	64	15	28
DsRed-Monomer	monomer	557	592	27 300	0.14	—	1.3	15	4	29
mApple	"	568	592	75 000	0.49	6.5	0.5	4.8	37	25
mStrawberry	"	574	596	90 000	0.29	<4.5	0.8	11	26	22
mRFPmars	"	585	602	32 600	0.25	—	—	—	8	30
mRFPruby	"	585	603	32 300	0.25	—	—	—	8	31
mRuby	"	558	605	112 000	0.35	5	2.8	179	39	32
mRFP1	dimer	584	607	50 000	0.25	4.5	<1	8.7	13	26
mCherry	monomer	587	610	72 000	0.22	<4.5	0.25	135	16	22
KillerRed	"	585	610	45 000	0.25	—	—	—	11	20
JRed	"	584	610	44 000	0.20	5.0	slow	—	9	19
mKeima	"	440	620	14 400	0.24	6.5	—	—	3	33
mRaspberry	"	598	625	86 000	0.15	—	0.9	—	13	34
mKate2	"	588	633	62 500	0.40	5.4	<0.33	118	25	35
tdKatushka2	dimer	588	633	132 500	0.37	5.4	<0.33	144	50	35
Katushka	"	588	635	65 000	0.34	5.5	—	—	22	16
mKate	monomer	588	635	31 500	0.28	6.2	1.25	71	9	16
t-HcRed	dimer	590	637	130 000	0.04	—	—	—	5	36
HcRed	tetramer	592	645	70 000	0.05	—	—	—	4	11
mPlum	monomer	590	649	41 000	0.10	<4.5	1.7	77	4	34
AQ143	tetramer	595	655	90 000	0.04	—	—	—	4	13

Notes. Hereinafter, Ex_{max} is the excitation maximum, Em_{max} is the emission maximum, ϵ is the molar extinction coefficient, QY is the quantum yield. ^a Fluorescent protein brightness is determined as a product of a quantum yield and a molar extinction coefficient divided by 1000.

RFPs named JRed and KillerRed. Currently, these proteins are the only known genetically encoded photosensitizers that can be expressed directly in the target cells.^{19,20} However, an attempt to obtain RFP on the basis of enhanced GFP from *Aequorea victoria* failed.²¹

The wild-type red fluorescent proteins and some first-generation RFPs described above have a number of disadvantages limiting the range of their application greatly, *viz.*, the aggregation tendency, the tetramer formation, slow and incomplete chromophore maturation (Table 1).

2. Aggregation

Many GFP-like proteins from the Anthozoa class including DsRed tend to form aggregates both *in vivo* and *in vitro*.¹² Protein aggregation is undesirable for the *in vivo* use because of toxic effect of the aggregates on the cells.

Site-specific mutagenesis reckoning on a suggestion that the positively charged N-terminal residues of the protein play a crucial role in the aggregate formation was used to prevent aggregation of the DsRed protein. Non-aggregating mutants DsRed were supposed to be obtained upon replacement of the Arg and Lys residues by negatively charged or neutral amino acid residues. A mutant E57 with three mutations, V105A, I161T and S197A, was chosen for mutagenesis rather than the wild-type protein DsRed. This mutant possessed the fastest maturation rate, its red fluorescence appeared twice as fast as fluorescence of a wild type DsRed.³⁷ One of the results of this work was the preparation of a triple mutant E57-NA with mutations R2A, K5E and K9T that provided a minimum level of aggregation both *in vivo* and *in vitro*. Fluorescent characteristics of E57-NA (brightness, fluorescence excitation and emission maxima) appeared to be very similar to that of the initial E57 protein, which makes E57-NA mutant more attractive for further application.¹²

3. Oligomerization

In contrast to GFPs from Hydrozoa species which are dimers, RFPs obtained from the Anthozoa class, form stable tetrameric complexes in dilute solutions, which dissociate only in severe denaturing conditions.^{38,39} For example, tetramerization of DsRed is observed at both very low concentrations of the protein *in vitro* in solution (the oligomerization rate constant is estimated as 10^{-9} mol litre⁻¹)⁴⁰ and *in vivo*.³⁸ The oligomerization manifests itself in the dependence of the absorption spectrum profile on the protein concentration.

Structural data of the DsRed protein and amino acid residues in the regions of subunit interface turned out to be useful for creation of non-oligomeric mutants of RFPs. It was revealed that the loss of ability to form tetramers can lead to a substantial decrease in the fluorescence brightness and protein maturation rates.^{26, 41} The problem of oligomerization for DsRed variants was solved by site-directed mutagenesis.²⁶ A monomeric version of DsRed was developed, designated mRFP1, which contained 33 substitutions: three in the hydrophobic interface and ten in the hydrophilic interface, three in a short N-terminus, 13 in the internal regions of β -barrel and four surface substitutions, the exact influence of which on the protein functions and structure is unknown. Disadvantages of mRFP1 are the presence of a protein fraction, containing 'green' chromophore, and its tendency to dimerize, which limit the application of the protein in experiments using fluorescence resonance energy transfer (FRET) and in multicolour

microscopy. The mRFPmars (Ref. 30) and mRFPruby (Ref. 30) proteins were engineered on the basis of mRFP1 and their genes were optimized for expression in *Dictyostelium* and mammalian cells, respectively.

Enhanced monomeric versions of mRFP1 were derived using random mutagenesis in combination with high-throughput method of cell sorting on a flow cytometer (Fluorescence Activated Cell Sorting, FACS).^{22, 34} New monomeric RFPs, termed mFruits, cover a wide range of a fluorescence spectrum (from 562 to 649 nm) and have substantially enhanced characteristics as regards maturation, brightness and photostability in comparison with an initial mRFP1. Nowadays, the most popular protein among mFruits is mCherry as it has an optimum compromise of brightness, maturation time, photostability and fluorescence emission.

Other approaches were suggested to eliminate aggregation of hybrid proteins tagged with fluorescent proteins (FPs) from Anthozoa. One of the approaches is covalent 'head-to-tail' cross-linking of two identical FPs, which would result in an intramolecular dimer. This may be used as a non-oligomerizing tag for proteins. This approach was used successfully for HcRed (Ref. 36), for dimeric variants of DsRed (Refs 22 and 26) and for Keima (Ref. 42) and Katushka2 (Ref. 35). The best results in chromophore formation and fluorescence brightness were obtained for linkers with the length of 4 and 12 amino acid residues for HcRed and dimeric variants of DsRed, Keima and Katushka2 proteins, respectively. Tandem proteins usually have identical spectral characteristics as the original proteins but can dislocate greatly the target-protein they are connected with.

4. Maturation: protein folding and chromophore formation

The RFP maturation includes two consecutive steps, *viz.*, protein folding and chromophore formation. Generally, the external amino acid residues are responsible for the protein folding and internal ones are mostly responsible for the chromophore formation rate and effectiveness. Using site-directed mutagenesis, it was demonstrated that tetramer formation is very important step in the RFP maturation.

In contrast to GFP isolated from *Aequorea*, the majority of RFPs from Anthozoa species exhibit better maturation at 37 °C than at room temperature. Proteins eqFP578 and eqFP611 that mature at less than 30 °C are an exception.^{14, 15, 41} Such a difference can be explained by the temperature of the habitat of the organisms used for the isolation of the corresponding fluorescent proteins.⁴³

First attempts to improve DsRed maturation by random and site-directed mutagenesis were unsuccessful,³⁸ but mutations (K83M and Y120H) that lead to a dramatic shift of the fluorescence maximum toward longer wavelength (602 and 600 nm, respectively) were found. In turn, mutations V105A, S197T led to formation of a fluorescent timer E5, which changes its fluorescence from green to red over time.⁴⁴ Further mutagenesis of the DsRed gene resulted in the following mutants: E5up (V105A) and E57 (V105A, I161T, S197A) with complete fluorophore maturation and enhanced rate of maturation, respectively,³⁷ and a non-fluorescent chromoprotein DsRed-NF.⁴⁵

Seven rounds of random and site-directed mutagenesis yielded three DsRed mutants, called DsRedT1, DsRedT3 and DsRedT4, exhibiting faster maturation rates with half-times from 0.7 to 1.3 h.²⁷ It was shown that the N42Q substitution significantly improved chromophore maturation

tion rate with formation of a bond with the Q66 residue, which is a part of chromophore. The DsRedT1 protein became commercially available as DsRed-Express (Clontech, USA). Recently, enhanced versions termed DsRed-Express2, DsRed-Max,²⁸ E2-Orange and E2-Red/Green²³ have been developed on the basis of DsRed-Express; all of these proteins yield minimum cytotoxicity and high maturation rates, which makes them more attractive for expression in transgenic animals and stable cell lines. However, tetrameric state of these proteins does not allow using them in fusion proteins. Another drawback of these proteins is the existence of green fluorescence limiting their application for multicolour microscopy. DsRed-Monomer (or DsRed.M1), a monomeric version of the DsRedT4 protein, can be used as a marker for protein localization in cells.²⁹ However, DsRed.M1 is almost fourfold less bright than its precursor DsRedT4. DsRed.M1 and DsRedT4 differ from each other in 37 mutations whereof 31 are external.

Wild-type proteins eqFP578 and eqFP611 cannot be efficiently used for expression in mammalian cells due to weak folding at 37 °C. Random mutagenesis was applied to optimise maturation temperature of the proteins. In the case of eqFP611, two amino acid substitutions (I57V and F102I) significantly enhanced its maturation at 37 °C. In the case of the RFP630 protein, which contains an additional N143S mutation, other substitutions (S171F and V184D) improving protein maturation were found. Possibly, this can be explained by different chromophore conformations: eqFP611 contains *trans* chromophore, while RFP630 has chromophore in *cis* conformation (see Section V.2).⁴⁶ The amino acid substitutions R35G, L83F and S135P improved maturation rate and pH-stability of the eqFP578 protein dramatically.¹⁵

5. Photostability

All fluorescent proteins like low-molecular-weight fluorescent compounds undergo photobleaching upon extended irradiation by excitation light. Photostability plays a crucial role in the experiments that involve long-term visualization of a cell or all the observation field. The rate of photobleaching varies essentially for different fluorescent proteins. Even proteins with similar spectroscopic characteristics can differ in photobleaching time. Photostability of fluorescent proteins also depends strongly on oxygen access to the sample: photobleaching occurs much faster in the presence of oxygen.²⁵

The most unified method for the protein photobleaching rate measurements was proposed by Tsien and co-workers.⁴⁷ Protein photostability is measured in aqueous droplets formed under mineral oil. Droplets of a protein solution, comparable in size with typical mammalian cells, are photobleached with continuous irradiation with periodic detection of fluorescence intensity to generate a photobleaching curve. The obtained data are normalized to the extinction coefficient and quantum yield of the FP, the emission spectrum of the arc lamp used for excitation, and the transmission spectra of the filters. The result obtained in such a way shows the time each FP will take to lose 50% of the initial fluorescence emission rate (1000 photons s⁻¹). This analysis has a restriction to the intensity of irradiation used for photobleaching, which must be of about 10 W cm⁻²; more intense irradiation evokes non-linear effects that are difficult to predict during data normalization.

A comparison of half-bleaching times determined by that method revealed the proteins DsRed, TagRFP-T and mOrange2 to be the most photostable among RFPs (see Table 1). Obviously, when choosing a fluorescent protein for a particular experiment, it should be kept in mind that protein photostability strongly depends on the conditions of the protein biosynthesis and photobleaching.

III. Enhanced monomeric red fluorescent proteins

The first-generation monomeric RFPs such as mFruits,^{22,34} mKO,¹⁷ TagRFP,¹⁵ mKate¹⁶ can successfully be used as markers of protein localization, dynamics and interactions. However, limited brightness and low photostability of these proteins hampered their applications. Recently, a whole series of fluorescent proteins with enhanced brightness and photostability has been developed on the basis of these proteins. Monomeric fluorescent timers (FTs) were also created with different blue-to-red chromophore maturation rates⁴⁸ (Table 2). The mKeima protein should be particularly mentioned. This protein has the largest Stokes shift (180 nm) among all fluorescent proteins.³³ All RFPs can be divided into two groups according to the fluorescence emission maximum wavelength: red fluorescent proteins and far-red fluorescent proteins (see Table 1).

Table 2. Properties of monomeric fluorescent timers.⁴⁸

Protein	Ex _{max} /nm	Em _{max} /nm	ε (see ^a)	QY	pK _a	τ _{1/2} of maturation at 37 °C/h
Fast-FT	403	466	49 700	0.30	2.8	0.25
	583	606	75 300	0.09	4.1	7.1
Medium-FT	401	464	44 800	0.41	2.7	1.2
	579	600	73 100	0.08	4.7	3.9
Slow-FT	402	465	33 400	0.35	2.6	9.8
	583	604	84 200	0.05	4.6	28

^a In litre mole⁻¹ cm⁻¹.

1. Red fluorescent proteins

The mKO protein is an enhanced monomeric version of wild-type Kusabira-Orange protein¹⁷ cloned from the coral *Fungia concinna*, it has high brightness and photostability. These properties make mKO attractive for its utilization as a protein localization marker. The mKO protein can be also used as an acceptor in FRET-pairs with cyan fluorescent proteins. The main disadvantage of this protein is slow maturation at 37 °C: the maturation half-time (τ_{1/2}) is 4.5 h. The brightness and maturation time of mKO were improved by introduction of 7 mutations.⁴⁹ An enhanced version of mKO (mKOk) was successfully used as an FRET-acceptor.²⁴

The eqFP578 protein turned out to be rather prospective precursor of a series of monomeric RFPs. The key positions responsible for protein dimerization were determined on the basis of X-ray diffraction of its analogue eqFP611 (Refs 41 and 50) isolated from the same organism. Later, as a result of a few rounds of site-directed mutagenesis at these positions, the monomeric TagRFP protein was developed.¹⁵ In addition to high brightness and fast maturation, TagRFP possessed good pH-stability. However, the main disadvantage of TagRFP is its low photostability.

Tsien and co-workers²⁵ succeeded in improving photostability of the TagRFP and mOrange proteins significantly. Selection of the brightest bacterial colonies expressing TagRFP and mOrange mutants under long-term irradiation by intense green-yellow light allowed isolation of photostable clones. In the case of TagRFP, only one mutation, S158T, was sufficient to increase photostability almost ninefold; at the same time, spectral characteristics of the protein did not virtually change. However, three mutations (Q64H, F99Y and G196D) in mOrange leading to 25-fold increase of its photostability, reduced brightness and increased protein maturation time. Photostability of mOrange2 appeared to be almost insensitive to oxygen; in the case of TagRFP-T, photosensitivity to oxygen remained unchanged.²⁵

The monomerization of the RFP611 protein required only two amino acid substitutions (T122R and 194A).⁴¹ However, these mutations led to the total loss of fluorescence. During further rounds of random and site-directed mutagenesis, more 20 amino acid substitutions were introduced, and these mutations restored fluorescence through enhanced folding of the protein and chromophore maturation. Introduction of 10 amino acid residues to the C-terminus prevented its localization in organelles during expression in mammalian cells. As a result, the monomeric protein mRuby, which turned out to be an effective marker for visualization of peroxisome in mammalian cells, was obtained.³²

On the basis of a red chromoprotein cloned from the coral *Montipora sp.*, the fluorescent proteins with red fluorescence excitation maximum at 440 nm and large Stokes shift were developed.^{33,42} A combination of the fluorescent proteins with similar fluorescence excitation wavelength but different Stokes shifts was used in multicolour two-photon microscopy.⁵¹ FRET-sensors with complete separation of emission peaks can be designed on the basis of such proteins and these sensors could dramatically simplify the detection of the energy transfer effectiveness.

2. Far-red fluorescent proteins

The growing number of researchers are showing interest in far-red FPs due to their superiority over other RFPs for tissue and whole-body imaging,^{16,35} which can make them attractive for application in medicine. Far-red FPs can be used for multicolour microscopy of living cells.⁴⁷ Far-red FPs are the proteins of choice for tagging cellular proteins even in strongly autofluorescence conditions. Infra-red FPs obtained on the basis of phytochromes⁵² compete with GFP-like far-red FPs.

A whole family of bright far-red FPs has been developed on the basis of the eqFP578 protein. Random mutagenesis of eqFP578 gene yielded a dimeric far-red FP, named Katushka.¹⁶ A monomeric version of Katushka (mKate), can be applied for visualization of intracellular structures, but high pH-sensitivity and weak photoactivation of this protein complicate quantitation of the collected data.

The crystall structure analyses revealed⁵³ that mKate chromophore can exist in two configurations: *cis* and *trans*. While *cis* configuration of a chromophore is fluorescent, a protein with the *trans* chromophore is non-fluorescent. The *trans* chromophore is stabilized through a hydrogen bond formed by the hydroxy group of Ser165 residue, as in the

eqFP611 protein and chromoproteins. Replacement of Ser by hydrophobic Ala in position 165 led to almost twofold enhancement of brightness and pH-stability. Random mutagenesis of the S165A_mKate gene afforded three mutations (V48A, M151T and K238R), which substantially enhanced brightness and photostability; this new protein was named mKate2. Mutations V48A and K238R turned out to be responsible for a significant increase in the protein maturation rate and completeness. Thus the maturation half-time for mKate2 was < 20 min.³⁵

Probably, development of proteins with emission above 650 nm will require additional posttranslational modifications to further extend the conjugated π -electron system of the DsRed-like chromophore and to shift the protein absorbance to longer wavelengths. Alternatively, amino acid substitutions in the chromophore immediate environment can result in fluorescent proteins with larger Stokes shifts.

Some low-molecular-weight dyes change the fluorescence emission maximum as the solvent polarity increases due to the difference between dipole moments of the ground and excited chromophore states. Synthetic GFP-like chromophores demonstrate this solvatochromic effect shifting their fluorescence maximum in the response to solvent polarity changes. However, in contrast to the synthetic chromophores, the majority of GFP-like proteins exhibit small Stokes shifts due to the rigid chromophore environment necessary to prevent non-fluorescent relaxation to the ground state. Ultrafast time-resolved fluorescence spectroscopy revealed the evolution of the fluorescence maximum for mPlum from 625 to 650 nm in 500 ps.⁵⁴ These observations can be explained by fast rearrangement of amino acid residues that interact with the chromophore. From the protein structure it followed that chromophore environment in mPlum is more hydrophobic than, for example, in DsRed and the E16 residue plays the key role in the fluorescence shift to far-red region. The carboxylic group of the glutamic acid residue forms a hydrogen bond with an oxygen atom of the acylimine group, which can lead to the increase in the delocalization region of the chromophore p-electron density.

3. Fluorescent timers

The fluorescent timer (FT) DsRed-E5 changes the fluorescence colour from green to red over time. However, DsRed-E5 cannot be regarded as a FT, because green fluorescence intensity decreases due to the increase in FRET between 'green' and 'red' subunits of the tetrameric protein upon red chromophore maturation.

Recently, a series of monomeric fluorescent timers able to change fluorescence from blue to red at different rate have been derived from the mCherry protein.⁴⁸ For fast, medium and slow timers, blue fluorescence maxima at 37 °C are achieved after 0.25, 1.2 and 9.8 h, and half-maxima of red fluorescence are reached after 7.1, 3.9 and 28 h, respectively. With FT maturation, blue fluorescence intensity following attainment of maximum decreases almost to zero. In a wide range of temperatures (16–45 °C), the protein blue-to-red transition is complete, and maturation rate for each form of the fluorescent timer is temperature-dependent. Thus, the FT age can always be determined on the basis of red and blue fluorescence ratio. All forms of fluorescent timers are pH-stable (see Table 2).

IV. Photoactivatable and photoswitchable red fluorescent proteins

Recently, several so-called photoactivatable fluorescent proteins (PAFPs) have been developed. These proteins change fluorescence properties significantly under irradiation with intense light with a definite wavelength. Irradiation converts some PAFPs from the non-fluorescent state to the fluorescent state (photoactivation), while some other just change the fluorescence maximum (photoswitching). Photoactivatable red fluorescent proteins (PARFPs) created to date can be divided into four main groups according to the photoactivation mechanism.

The first group includes PARFPs that undergo irreversible photoconversion of the chromophore under UV-light. This group includes PAmRFP1 proteins⁵⁵ and newly developed protein PAmCherry1.⁵⁶ An irreversible UV light-induced photoactivation (340–380 nm, 25 min) was revealed for the PAmRFP1 protein constructed on the basis of mRFP1 with replacements in the positions 148, 165 and 203. The PAmRFP1-1 protein (the photoactivatable mutant of mRFP1 with the best characteristics) has initially weak blue fluorescence, and photoactivation leads to a 70-fold increase in red fluorescence intensity. However, the activated red fluorescent form is unstable: its lifetime ($\tau_{1/2}$) is 9 h at 37 °C. High phototoxicity, low brightness and the oligomeric state significantly limit PAmRFP1 applications. Besides, the stability of the activated form of PAmRFP1 is slightly lower than that of its precursor mRFP1.

All drawbacks inherent in the PAmRFP1 protein were overcome in a monomeric photoactivatable protein named PAmCherry1, which was absolutely non-fluorescent in the dark (non-activated) state, but easily activated by irradiation with the wavelength of 399 nm. The activated red form of the protein was stable, no reversion to the dark state was observed for at least 24 h and the photoactivation contrast (the ratio of fluorescence intensities before and after photoactivation) achieved 4000. Moreover, the PAmCherry1 protein had fast maturation, and the absence of extra peaks in the excitation and emission fluorescence spectra allowed using it in multicolour high-resolution microscopy.⁵⁶

Photoactivatable proteins from the second group undergo an irreversible green-to-red photoconversion under UV or blue light (illumination by light with longer wavelengths does not lead to any noticeable photoactivation). The effectiveness of photoactivation for all proteins from this group is strongly pH-dependent. All green-to-red photoactivatable proteins share the same chromogenic tripeptide HisTyrGly (H–Y–G). This class of proteins includes proteins from coral polyps: the protein Kaede originates from *Trachyphyllia geoffroyi*,⁵⁷ the proteins mCavRFP, rFluorFP (Ref. 7) and EosFP were isolated from *Lobophyllia hemprichii*, recombinant forms tdEosFP, mEosFP,⁵⁸ mEos2,⁵⁹ IrisFP,⁶⁰ and KikGR and mKikGR are the mutant forms of the KikG protein from *Favia favaus*.^{61, 62}

The wild-type proteins such as Kaede, mCavRFP and rFluorFP are easily photoactivated by 350–400 nm light, but mCavRFP and rFluorFP also exhibit the properties of fluorescent timers being converted to the red fluorescent state gradually without UV-light illumination.⁸ The Kaede protein does not possess such property, which allows it to be used as a photoactivatable fluorescent marker.⁵⁷ The photo-

activation contrast for Kaede is 2000. Effective maturation of Kaede at 37 °C allows its expression to be carried out in animal cells.

The wild-type EosFP, named after the goddess of dawn in Greek mythology, shares the 84% amino acid homology with the Kaede protein and undergoes irreversible green-to-red photoconversion upon irradiation at 390 nm (the emission maxima are at 516 and 581 nm, respectively).⁵⁸ With decreasing pH, the photoactivation rate of EosFP increases indicating that it is the protonated chromophore that undergoes photoconversion. The tandem-dimeric (tdEosFP) and monomeric (mEosFP) versions of EosFP with spectral properties similar to those of the precursor were developed. The mEosFP protein does not mature at 37 °C, which hampers significantly its expression in mammalian cells. An enhanced version of mEosFP (mEos2) developed recently matured effectively at 37 °C; this can be used as a marker for localization of different proteins.

The mEos2 protein is one of the lately obtained. Its chromophore can exist in two fluorescent states. Similarly to the parental protein, mEos2 possesses high brightness, photoactivation contrast and photostability.⁵⁹

It should be mentioned that a new protein IrisFP, derived from EosFP by introduction of only one mutation F173S combines the properties of photoactivatable and photoswitchable proteins. The IrisFP protein is irreversibly green-to-red photoactivated under UV light, both green and red fluorescent forms can be reversibly switched on and off by light with the wavelength of 440 and 561 nm, respectively.⁶⁰ The main disadvantage of IrisFP is still its tetrameric state.

Taking into account the structural data, rational mutagenesis of the green fluorescent protein KikG, which does not possess any photoconvertible properties, was performed to generate mutants that can be photoswitched under UV light. In the first step of rational mutagenesis, the H–Y–G chromophore was obtained by introduction of the 65H mutation. After 30 rounds of molecular evolution, a photoactivatable protein KikGR, which has 8 mutations in comparison to its precursor, was obtained. UV-illumination of the green KikGR species with the emission maximum at 517 nm drives to the red form with the fluorescence maximum at 593 nm.⁶¹ Further KikGR optimization resulted in its monomeric version mKikGR with the same spectral properties.⁶² The photoactivation effectiveness of the KikGR and mKikGR proteins strongly depends on the wavelength of the activation light (350–420 nm) and pH, the contrast of their photoactivation achieves the value of 560. The proteins can be activated by soft irradiation of an IR laser. The red form of KikGR is almost fourfold more photostable than the red form of mEosFP, though it has more complex kinetics of photo-bleaching.

The third group of PAFPs includes the dendGFP protein cloned from *Dendronephthya* sp. and the monomeric protein Dendra2 recently derived from dendGFP.⁶³ The proteins of this group can be irreversibly green-to-red photoconverted under both UV light and blue light. After complete photoconversion of the Dendra2 protein under 405 or 488 nm light, the red fluorescence intensity with a maximum at 575 nm increases 150–300-fold, and the level of green fluorescence with a maximum at 505 nm decreases 15–20-fold. The Dendra2 protein is the first PAFP, which is monomeric, folds effectively at 37 °C in bacterial and mammalian cells and can be photoactivated by 488 nm light

with low phototoxicity. However the high-intensity light is required for Dendra2 photoconversion, the low-intensity light with the wavelength in the range of 400–490 nm does not activate the protein even under long-term irradiation. This property allows excitation of green fluorescence without protein photoconversion and local photoactivation of the green form by choosing the light with proper intensity. The red species of Dendra2 are fourfold more photostable than DsRed, which significantly simplifies extended visualization of the protein.

The fourth group of PAFPs includes KFP1, a mutant version of the asFP595 chromoprotein cloned from the sea anemone *Anemonia sulcata*,^{64,65} rsCherry and rsCherryRev created on the basis of the mCherry protein.⁶⁶ The proteins of this group photoswitch from the non-fluorescent forms to the fluorescent ones and *vice versa* under light with definite wavelengths.

The wild-type protein asFP595 effectively absorbs light (absorption maximum at 568 nm), but it does not fluoresce. The protein becomes fluorescent ('kindling') with absorption and emission maxima at 575 and 595 nm, respectively, as a result of intense green irradiation.⁹ Upon cessation of green-light irradiation, the protein relaxes to the initial non-fluorescent state quickly ($\tau_{1/2} < 10$ s). On the other hand, fluorescence quenching of this protein can be performed through illumination by blue light (450 nm). Both 'kindling' and quenching are reversible.^{64,65} A short lifetime of the fluorescence state prevents the use of the wild-type asFP595 as a highlighter for proteins and organelles *in vivo*. Therefore, a mutant form of asFP595, named KFP1 (Kindling Fluorescent Protein, KFP), with longer lifetime of the fluorescent state ($\tau_{1/2} \approx 50$ s) was developed. KFP1 differs from asFP595 only in three amino acid substitutions: the

mutation A148G increases the lifetime of the fluorescent state and K9T and K10E prevent aggregation of the protein. Protein KFP1, similarly to asFP595, switches to the fluorescent state under intense green light illumination and quenches under blue light. The rate and the degree of reversible 'kindling' and quenching depend on the intensity and duration of the respective irradiation. Activated KFP1 exhibits absorption and emission maxima at 580 and 600 nm, respectively. Moreover, high intensity green light or extended illumination (or both factors simultaneously) lead to irreversible conversion of KFP1 to the fluorescent state, with the fluorescence intensity increasing at least 30-fold in comparison to the initial state. Fluorescence of irreversibly activated KFP1 is stable for at least a year.

Site-directed mutagenesis of chromoproteins cgigCP and hcriCP yielded a whole series of 'kindling' fluorescent proteins.⁶⁵ All of these proteins are capable of both reversible and irreversible photoconversion from the non-fluorescent state to the fluorescent state. Two mutants of hcriCP with single mutation N165A or N165G undergo photoconversion under blue light. An advantage of these variants in contrast to other KFPs is the lack of background photoconversion in the process of fluorescence detection during the 'kindling' of the proteins. 'Kindling' FPs in the 'kindled' state have fluorescence maxima in the red region of the spectrum (the excitation maximum is 580–590 nm, the emission maximum is 600–630 nm). However, all proteins derived from the chromoprotein asFP595 are tetramers.

rsCherry and rsCherryRev are the first monomeric photoswitchable fluorescent proteins, to be developed from mCherry.⁶⁶ The proteins rsCherry and rsCherryRev have an opposite modes of photoswitching, *i.e.*, yellow light (550 nm) activates red fluorescence of rsCherry, and blue

Table 3. Properties of photoactivatable and photoswitchable red fluorescent proteins.

Protein	Oligomeric state	$E_{x_{max}}$ /nm	$E_{m_{max}}$ /nm	ϵ /litre mol ⁻¹ cm ⁻¹	QY	pK _a	Photo-stability/s	Ref.
Dendra2	monomer	490	507	45 000	0.50	6.6	45	63
		553	573	35 000	0.55	6.9	378	63
IrisFP	tetramer	488	516	52 200	0.43	—	—	60
		551	580	35 400	0.47	—	—	60
EosFP	"	506	516	72 000	0.70	5.7	58	58
		571	581	41 000	0.55	—	489	58
tdEosFP	dimer	506	516	84 000	0.66	5.7	47	67
		569	581	33 000	0.60	—	380	67
mEosFP	monomer	505	516	67 200	0.64	—	—	58
		569	581	37 000	0.62	—	—	58
mEos2	"	506	519	56 000	0.84	5.6	42	59
		573	584	46 000	0.66	6.4	323	59
PA-mCherry1	"	404	—	6 500	—	—	18	56
		564	594	18 000	0.46	6.3	—	56
PAmRFP1-1	dimer	578	605	10 000	—	4.4	—	55
		Kaede	tetramer	508	518	98 800	0.88	5.6
mKikGR	monomer	572	580	60 400	0.33	5.6	386	57
		505	515	49 000	0.69	—	14	62
KikGR	tetramer	580	591	28 000	0.63	—	21	62
		507	517	28 200	0.7	4.2	—	61
KFP1	"	583	593	32 600	0.65	5.5	—	61
		570	600	123 000	<0.001	—	—	64
rsCherryRev	monomer	570	600	59 000	0.07	—	—	64
		572	608	85 000	0.0003	5.5	—	66
rsCherry	"	572	608	84 000	0.005	5.5	—	66
		572	610	81 000	0.009	6.0	—	66
		572	610	80 000	0.02	6.0	—	66

light (450 nm) quenches red fluorescence of rsCherry, while in the case of rsCherryRev both yellow and blue light have opposite action. Relaxation half-times ($\tau_{1/2}$) of the red forms for rsCherry and rsCherryRev to the equilibrium state are 40 and 13 s, respectively. More detailed study of properties of rsCherry and rsCherryRev⁵⁶ revealed a large subpopulation of the protein molecules, which can form the chromophore but remain non-fluorescent and cannot be photoactivated resulting, in general, in low brightness of activated assemblies of protein molecules. The other disadvantages of the proteins rsCherry and rsCherryRev are the low contrast of photoswitching and residual fluorescence in the 'off' state. These facts significantly limit the protein application in high-resolution microscopy. In all key parameters, the proteins are inferior to their competitors.

The main physical and chemical characteristics of the known photoactivatable and photoswitchable proteins are summarized in Table 3.

V. The structure of fluorescent proteins

1. Crystal structure of fluorescent proteins

Amino acid alignment of the first isolated RFPs with GFP demonstrated that, despite low amino acid homology (20%–30%), the tertiary structure of all known fluorescent

proteins is highly conserved and represents the so-called β -barrel. However, contrary to GFP, which is ring-shaped when observed from the above, the monomer RFPs has an elliptical shape, which influences directly the chromophore microenvironment and probably matters during the protein maturation. Inside of each β -barrel, there is an α -helix containing a chromophore. The chromophore is located in the centre of the β -barrel cavity, which was established by X-ray diffraction analysis of the first RFP.^{68,69} Short α -helical sites, which turned out to be remarkably conserved, form the 'bottom' and the 'lid' of each barrel; N- and C-termini of the protein are exposed out of the β -barrel, therefore they are available to be employed as linkers to fusion proteins. The monomeric subunit has the molecular mass of 25–30 kDa and consists of 220–240 amino acid residues, the monomer size is 4.2×2.4 nm.

In the tetramer, the monomeric subunits form two pairs of dimers where monomers are oriented antiparallel to each other; the monomer chromophores represent mirror images relative to each other. Each monomer in the tetramer interacts with two other monomers and forms two regions of intersubunit interaction or interfaces, *viz.*, A/B (C/D) and A/C (B/D) (Fig. 1). The interactions in these regions have different nature. Hydrophobic interactions of the A/B interface are based on a cluster of closely packed hydrophobic amino acids, and the A/C interface includes many electro-

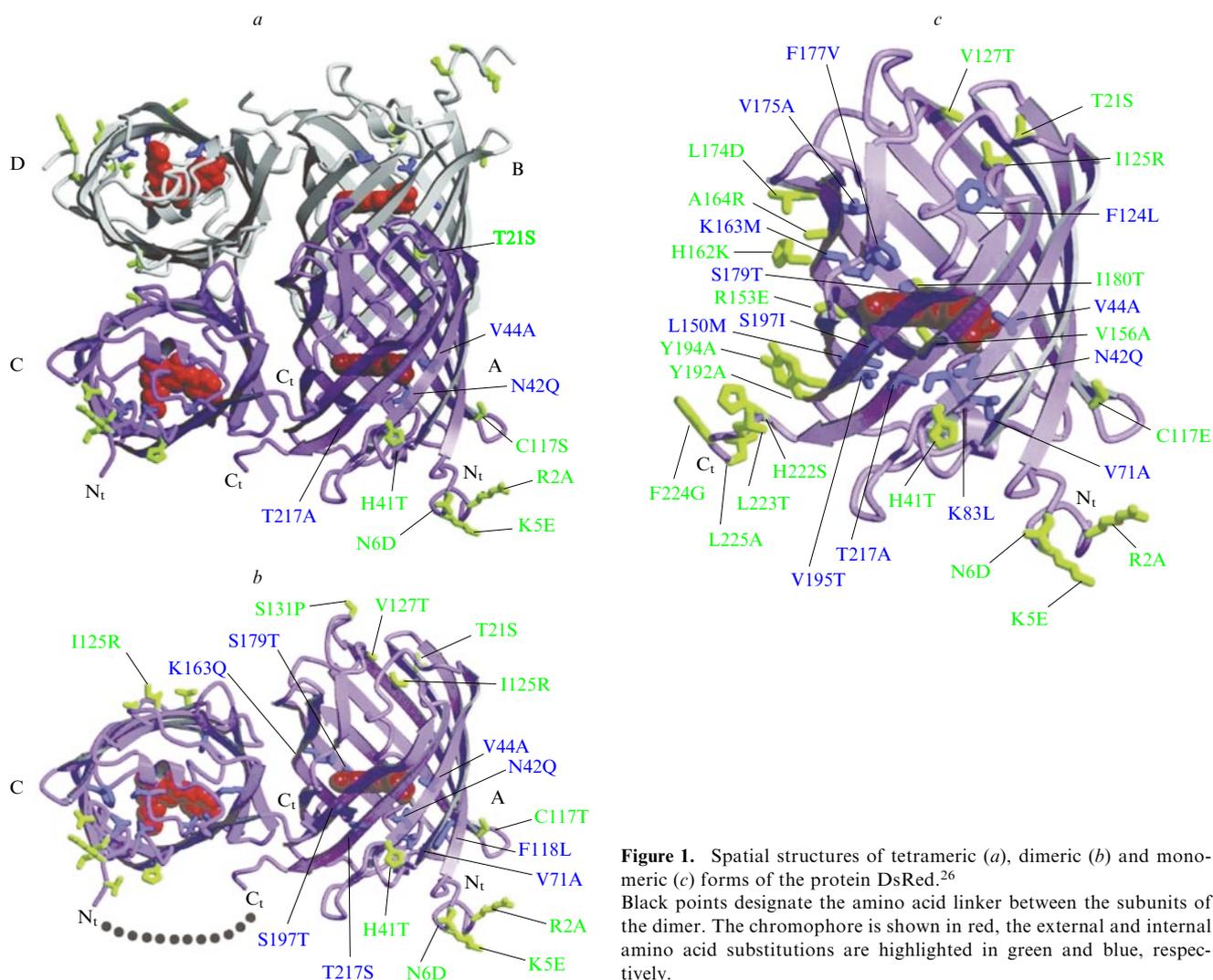


Figure 1. Spatial structures of tetrameric (a), dimeric (b) and monomeric (c) forms of the protein DsRed.²⁶ Black points designate the amino acid linker between the subunits of the dimer. The chromophore is shown in red, the external and internal amino acid substitutions are highlighted in green and blue, respectively.

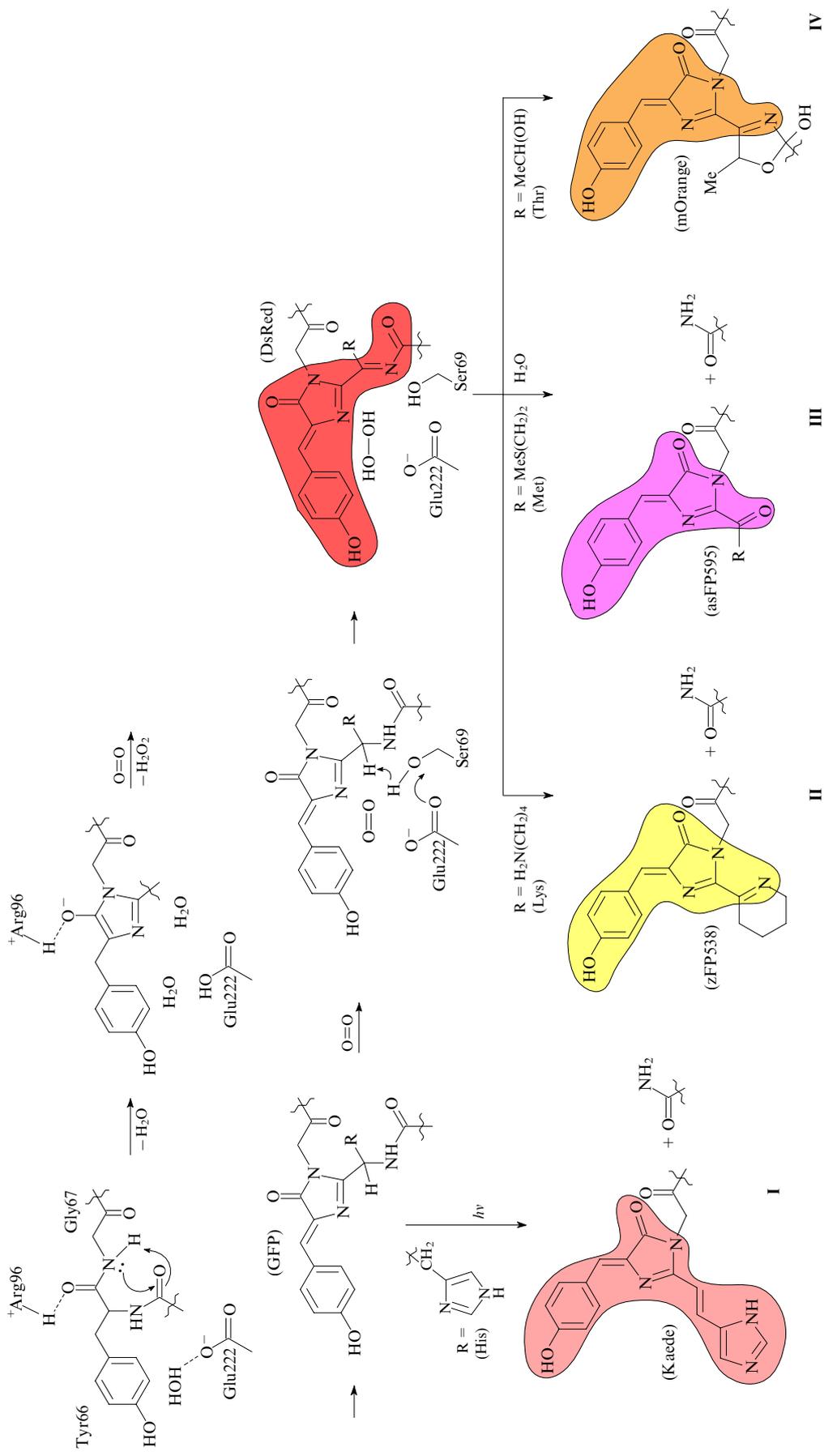


Figure 2. The mechanism of chromophore formation and the types of the red chromophore (see the text).

static interactions and hydrogen bonds between polar amino acids and water molecules, as well as a rare structure, 'clamp', formed by the C-terminus of each monomer.^{68, 69} Amino acid alignment of the interfaces in different Anthozoa FPs demonstrated low homology among the proteins.^{8, 50}

2. The structure and formation of the chromophore

The main peculiarity of GFP-like fluorescent proteins is that the formation of the chromophore responsible for protein fluorescence properties occurs without any co-factors or enzymes, but requires molecular oxygen. In particular, in the study of the protein DsRed chromophore it was hypothesized that the chromophores of RFPs are chemically different from those of GFP.⁷ Indeed, a significant red shift of spectrum can hardly be explained only by the difference in chromophore microenvironment. However, the formation of a red chromophore passes *via* green GFP-like chromophore. Moreover, the chemical synthesis of compounds similar in structure to the DsRed chromophore revealed that introduction of an additional double bond to the GFP-like chromophore brings about a remarkable red-shift of the absorption and emission spectra.⁷⁰

Red chromophores are divided into two types: DsRed-like chromophores⁷¹ and Kaede-like chromophores,⁷² these are named after the proteins where they were first found (Fig. 2). The fluorescent proteins with DsRed- or Kaede-like chromophores can easily be distinguished by the shape of the excitation and emission spectra. Thus the Kaede-like chromophore (I in Fig. 2) has narrow peaks with a smaller Stokes shift and a characteristic shoulder at 630 nm in the fluorescence spectrum. In addition, there is a distinct difference in the spectra of different groups of RFPs under denaturing conditions. The DsRed-like chromophores undergo hydrolysis with 1 M NaOH resulting in a green chromophore with an absorption maximum at 445 nm.⁷¹ In turn, the fluorescent proteins with the Kaede-like chromophores in 1 M NaOH absorb at 499 nm.⁷³

Purple-blue chromoproteins characterized by high molar extinction coefficients, have another chromophore (III in Fig. 2), which is an isomer of DsRed-like red chromophore.⁷⁴ Examples of protein fluorescence spectra are shown in Fig. 3.

Three derivatives of the DsRed-like chromophore are known, and each type is represented by at least one fluorescent protein.

The 'kindling' fluorescent protein KFP initially reported as a chromoprotein isolated from the actinia *Anemonia sulcata*⁹ has a fragmented DsRed-like chromophore⁷⁵ that is formed upon autocatalytic hydrolysis of acylimine.⁷⁶

The first amino acid residue of tripeptide chromophore (lysine) in the yellow fluorescent protein zFP538 cloned from *Zoanthus sp.*⁷ undergoes intramolecular cyclization with formation of tetrahydropyridine ring to afford a chromophore comprising three rings (II in Fig. 2).⁷⁷

The third type of DsRed-like chromophore (IV in Fig. 2) was found in the mOrange protein,⁷⁸ as well as in the wild-type KO and its monomeric variants.⁷⁹ In this group of proteins, the side chain of the amino acid residue (threonine in the case of mOrange or cysteine in the case of KO) that is a chromophore constituent, reacts with the amide group resulting in a dihydrooxazole ring.

So far, there is no definite answer to the question as to whether alterations in the amino acid sequence can lead to such a variety of chromophore structures.

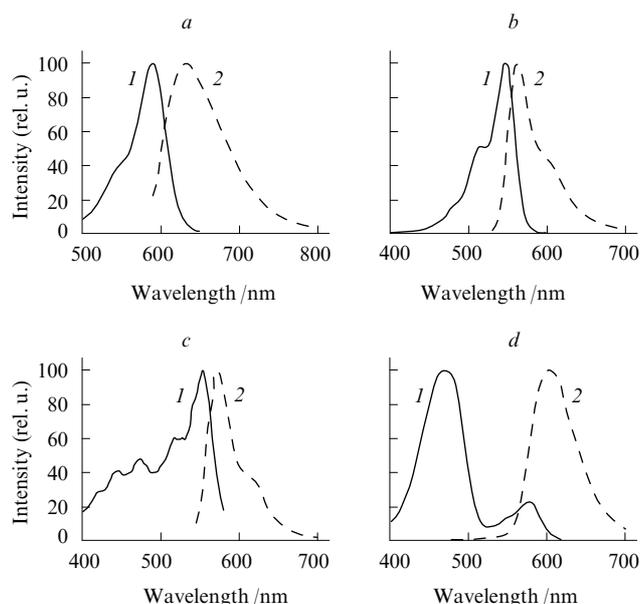


Figure 3. Excitation spectra (absorbance) (1) and fluorescence spectra (2) of some red fluorescent proteins: mKate (a), mOrange (b), Dendra2 in the activated state (c) and mKeima (d).

The red chromophore is generated as a result of four consecutive autocatalytic reactions with three amino acid residues that constitute the internal α -helix. The nucleophilic attack by the nitrogen atom of the peptide bond on the carbon atom of the carbonyl group occurs in the first step. Cyclization is followed by dehydrogenation of the intermediate product (removal of the hydrogen atoms occurs from the C_{α} - C_{β} bond of the Tyr66 residue) under the action of molecular oxygen resulting in a green chromophore [4-(*p*-hydroxybenzylidene)-2-imidazolin-5-one]. Subsequent autocatalytic oxidation by molecular oxygen leads to the extension of a conjugated π -system due to the formation of the acylimine bond. Though the same amino acid sequence is also found in other proteins, but no chromophore is formed in them, which suggests the key role of the protein spatial structure in the formation of the green chromophore.

Amino acid alignment of more than a hundred of known fluorescent proteins allows inferring that four amino acid residues among the proteins are absolutely conserved. These are Tyr66 and Gly67 of the chromogene tripeptide as well as Arg96 and Glu222 (hereinafter, the amino acid numbering according to sequence of the wild-type GFP is employed), which are in immediate chromophore environment. The Arg96 residue is located in close vicinity to the oxygen atom of imidazolinone and the Glu222 residue is closer to the chromophore in DsRed than in GFP. Obviously, the nitrogen atom of the Gly67 side chain is a nucleophile in the first step of chromophore cyclization, while Arg96 and Glu222 residues perform catalytic function. According to the structure of the R96A mutant with the pre-cyclized chromophore it was shown that during protein folding the chromogenic amino acid residues are situated in the most convenient position to form a ring.

Recently, the role of the R96 and E222 residues has been studied,^{80, 81} the results obtained revealed that R96 is an electrostatic catalyst that stabilizes the enolate intermediate in the first step of the chromophore formation. Substitution

of any amino acid (except for lysine) for this arginine residue results in retardation of protein maturation to several months. Interestingly, the lost function of maturation in the R96A mutant can be recovered by the Q183R substitution.⁸⁰ It is assumed that R96 stabilizes the glycine enolate form in the chromophore thereby facilitating nucleophilic attack on the carbon atom of the carbonyl group. This hypothesis is proved by crystallographic data of GFP and blue FP (BFP).⁸² The reduced GFP and BFP proteins obtained in the absence of molecular oxygen contain the enol form of imidazolinone. The role of the Glu222 residue is less clear but the experimental data suggest the side-chain anion serves as a base catalyst that deprotonating the amide group of Gly67.⁸¹

Practically nothing is known about initiation and control over the acylimine bond formation. The reaction usually proceeds slowly and its result is often unpredictable, so it does not always result in the formation of the expected products. It was also demonstrated that the acylimine bond formation is a limiting step in red chromophores maturation.⁴⁷ The evolutionary tree shows¹⁹ that the emergence of RFPs took place fairly recently through several independent pathways. Recently, a surprisingly successful experiment has been carried out resulting in the synthesis of a few genes of RFP precursors.⁸² Notably, the gene expression results in fluorescent proteins with green and red emission; their maxima coincide with those for GFPs and RFPs.⁸³

There are two essential facts that limit significantly the number of plausible mechanisms of acylimine bond formation. First, the appearance of intermediates containing protonated GFP-like chromophore with blue fluorescence in the RFP maturation suggests that the anionic form of the green chromophore cannot be involved in the acylimine bond formation.^{48,84} Second, the presence of an additional positively charged side chain turns out to be necessary but not sufficient for red fluorescence, since lysine or arginine are virtually always present in the chromogenic pentapeptide X–Y–GX–K/R in position 5. For example, in the DsRed protein the K70 residue can be replaced only by Arg,²¹ otherwise protein maturation ceases at the step of the green chromophore formation.

According to X-ray diffraction data obtained for DsRed,⁶⁸ it can be suggested that the E222 and S70 residues play a key role in the acylimine bond formation. Relative arrangement of E222 and Q66 is critical for the red chromophore formation. Thus the E222 residue is involved in both the green chromophore formation and its further conversion into the red chromophore.

In the DsRed molecule, the E222, Q65 and S68 residues interact with the N42 residue, which is responsible for their correct position. Probably, this is the reason for a significant increase in green fluorescence upon substitution of the N42 residue in DsRed.⁸⁵

In the case of z2FP574 (RFP from *Zoanthus sp. 2*), decarboxylation of D66 is required for the acylimine bond formation; D66 is the first amino acid residue in the chromogenic tripeptide DYG.^{86,87} Further oxidation results in the DsRed-like chromophore formation. The kinetics of maturation of z2FP574 and its mutants shows that, in contrast to other RFPs, the red chromophore formation in the z2FP574 protein proceeds *via* the anionic form of the green chromophore.⁸⁶

Usually, the chromophore has a planar structure, though deviation from planarity is possible. As a rule, the chromophore consists of the benzene ring of Y66 and a *cis*

or *trans* configured cross-conjugated five-membered heterocycle (imidazolinone). In the case of the Kaede proteins, the chromophore contains an additional conjugated system including the imidazole ring. The chromophore can have *cis* or *trans* configuration of the double bond $C_{\alpha}=C_{\beta}$ between the aromatic systems (Fig. 4).

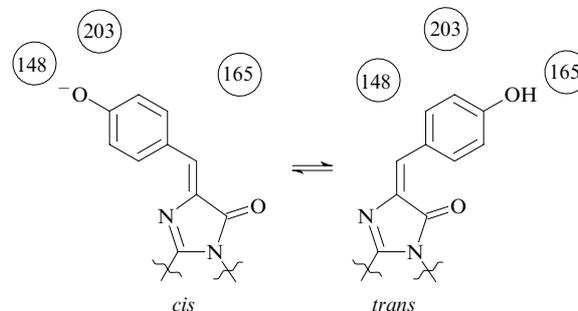


Figure 4. Scheme of chromophore *cis*–*trans*-isomerization under reversible transfer.

The positions of amino acid residues stabilizing the chromophore in *cis*- or *trans*-configuration are indicated.

The amino acid residue in position 148 is very important for the stabilization of the *cis* configuration. The amino acid residues able to form hydrogen bonds stabilize the anionic *cis* chromophore. The data from mutagenesis show that position 148 is crucial for the interconversion between the fluorescent proteins and chromoproteins.^{9,45} The amino acid residue in position 165 plays a key role in the stabilization of the *trans* configuration. Small hydrophilic amino acids in position 165 stabilize the *trans* chromophore by hydrogen bonding with the OH group of the chromophore tyrosine residue, whereas hydrophobic amino acids destabilize the *trans* chromophore.

For instance, the DsRed chromophore is planar and contains phenolic tyrosine fragment in the *cis* configuration. The results of X-ray diffraction analysis and high-resolution mass spectrometry demonstrate that the majority of RFPs contain the DsRed-like chromophore with the *cis* configuration.^{74,88–90} However, all the known chromoproteins (in contrast to DsRed) contain non-planar DsRed-like chromophore in the *trans* configuration. The protein eqFP611 also contains a DsRed-like chromophore in the *trans* configuration,⁵⁰ but unlike CPs it has a planar structure; however, the chromophore in the mutant RFP630 containing only one amino acid substitution N143S has the *cis* configuration.⁴⁶ The chromophore microenvironment in eqFP611 is favourable for both planar *cis* and *trans* configuration; thus, this protein is the first example of a GFP-like protein that is fluorescent with both *cis* and *trans* configuration of the chromophore.

Non-co-planarity of both *cis* and *trans* chromophore concerns the β -carbon atom (tilt, τ) and the C_{β} – C_{γ} bond (twist, φ) (Fig. 5). The X-ray diffraction data allowed establishment of an unambiguous correlation between the protein fluorescence quantum yield and its chromophore planarity:⁷⁸ the larger the tilt- and twist-angles the smaller the quantum yield.

Spectral characteristics of fluorescent proteins depend not only on the chromophore configuration but also on its charge. The red chromophore can exist in the neutral or in

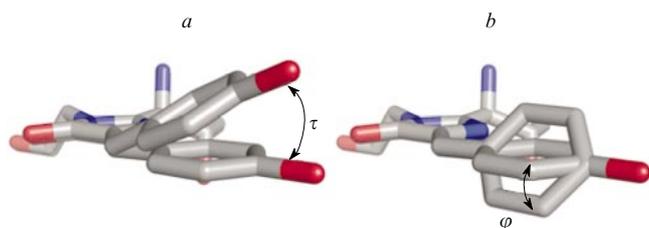


Figure 5. Chromophore planarity distortion: tilt (a) and twist (b) angles of the red chromophore.⁸⁶ In both cases, two different positions of the same ring are shown.

the anionic form. The anionic DsRed-like chromophore absorbs light at 570–590 nm and it fluoresces at 590–640 nm, whereas the neutral form absorbs light at 440–460 nm and fluoresces at 470–490 nm. Obviously, RFPs contain the chromophore in the anionic form. Apparently, the RFP mKeima with a large Stokes shift is an exception from the rule.³³ The excitation maximum for mKeima is at 440 nm, which corresponds to the neutral form of the red chromophore, but the emission maximum for mKeima is at 620 nm. This observations can be explained by excited-state proton transfer (ESPT), which is detailed for GFPs.⁹¹ For example, upon blue-light excitation the neutral chromophore of the protein Sapphire transfers to the excited state. The excited chromophore releases proton passing to the anionic form, which emits green light⁹² and afterwards returns to the neutral form. The equilibrium between the neutral and anionic forms of the chromophore is effected through a hydrogen bond network, and the side chain of glutamic acid or aspartic acid usually acts as a proton acceptor. The suggested ESPT pathway explains the large Stokes shift of mKeima, and the results of spectral and structural investigations confirm this fact.⁹³

VI. Photophysics and photochemistry of red fluorescent proteins

The photophysics and photochemistry of photoactivatable and photoswitchable fluorescent proteins refer to the most various and interesting issues.

The suggested photoactivation mechanism of the PAmRFP1 and PAmCherry proteins includes two consecutive steps. First, decarboxylation of the amino acid residue E222 occurs and initiates rearrangement of hydrogen bonds inside the β -barrel, and then irreversible photoconversion of the red chromophore from the neutral to the anionic form occurs under UV light.⁹⁴ Deprotonation of the residue Y66 can also be accompanied by the transition of the chromophore from the non-fluorescent *trans* configuration to the fluorescent *cis* configuration, which is stabilized by the amino acid residues in positions 148 and 167.⁵⁵

The exact mechanism of the PAmRFP1 photoactivation is still unknown, but from a number of characteristics of the activated and non-activated states of PAmRFP1 one can judge on possible alterations in the protein structure upon photoactivation. Thus the absorption spectra of the denatured mRFP1 and non-activated PAmRFP1 in 1 M acid are similar and characterized by a wide band with a maximum at 382 nm. However, the spectrum of PAmRFP1 denatured under alkaline conditions differs from the mRFP1 spectrum; in addition to the peak at 450 nm, it contains a band

at 330 nm, which probably corresponds to the photoactivatable protein molecules.

Other PAFPs undergo irreversible green-to-red photoconversion upon illumination with UV or blue light. The chromophore of this group of proteins is formed from the tripeptide H65–Y66–G67. The single bond between the nitrogen and C_{α} -carbon atoms of the H65 residue of the neutral chromophore is cleaved under UV light to form a double bond between C_{α} and C_{β} atoms of the H65 residue. Extension of the conjugated π -system leads to red shift of the fluorescence spectrum.⁹⁵ The H65 residue plays a crucial role in the β -elimination reaction by the E2 mechanism. It was demonstrated that replacement of H65 by any other amino acid residue blocks photoactivation of the fluorescent protein.^{58, 61}

The high-resolution crystal structures of green and red forms allowed specification of the mechanisms of photoconversion for Kaede,⁹⁶ EosFP⁶⁷ and IrisFP.⁶⁰ The mechanisms of photoactivation of all proteins of this group turned out to be similar. The E222 residue plays a role of a catalyst in photoactivation: its anion acts as a proton acceptor in the β -elimination reaction. The Q38 Residue is also critical for Kaede photoconversion; the side chain of Q38 forms a hydrogen bond with H65 through a water molecule. It was found that reversible photoswitching of green and red forms of IrisFP occurs through *cis*–*trans*-isomerization of the chromophore. Notably, the immediate environment of the chromophore is virtually unchanged upon photoactivation.

The chromophores of dendGFP and Dendra are built from three amino acids H62–Y63–G64. The histidine in position 62 is typical of all Kaede-like photoactivatable proteins. However, none of the Kaede-like PARFPs photoconvert under blue light. The reasons for Dendra sensitivity to blue light are still unknown. Amino acid alignment of the chromophore microenvironment for dendGFP/Dendra and the Kaede, EosFP and KikGR proteins showed⁶³ that position 116 in Dendra is occupied by glutamine, whereas in Kaede-like proteins it is asparagine.⁶³ The analysis of the EosFP crystal structure showed that the longer side chain of the Gln residue in position 116 can interact with the polypeptide chain near the chromophore-forming residue H62.⁶⁷ Probably, this interaction favours cleavage of the polypeptide chain and the formation of the red chromophore in response to blue-light irradiation. Elucidation of the mechanism of Dendra photoconversion requires further studies of this protein.

A model of reversible transition from the non-fluorescent to the fluorescent state related to *cis*–*trans*-isomerization of the chromophore was suggested for the photoswitchable proteins asFP595 and KFP1. In a series of the Anthozoa fluorescent proteins, the Ser148 residue is highly conserved and the A148S mutation in asFP595 leads to a significant increase in quantum yield.^{68, 69} It was thus suggested that configurations of the asFP595 chromophore in the fluorescent state and of Anthozoa FPs chromophores and GFP chromophore from *Aequorea* are similar. Therefore, if photoconversion is induced by *cis*–*trans*-isomerization of the chromophore, the Y66 residue of the asFP595 chromoprotein must interact with the S165 residue.

In the DsRed1 protein, the I165 residue creates steric hindrance to this configuration. It is suggested that S165 stabilizes the asFP595 chromophore in the non-fluorescent state, since serine is not found in this position of other FPs. At the same time, there is a probability of transition of the

excited chromophore into the fluorescent state. This model explains well the spectral characteristics of KFP1 and asFP595 and reversible switching from the non-fluorescent to the fluorescent state. This model was confirmed by crystal structures of asFP595 mutants in both fluorescent and non-fluorescent states.⁹⁷ The mutant protein asFP595-A148S exhibiting extended life-time of the fluorescent state was chosen to elucidate the protein structure in the activated state. Following irradiation with high-intensity light, the crystals were flash frozen, and then the electron density maps with different resolution were obtained. The experimental data showed that immediately after the intense irradiation the chromophore had *cis* configuration. If the irradiation was short, two chromophore configurations were observed. It should be noted that the *cis* chromophore is stabilized by a hydrogen bond between the tyrosine OH group of the chromophore and the S148 side chain. This explains the increased life-time of the activated state of asFP595-A148S as compared with KFP1 (A148G) where no stabilizing hydrogen bonds are formed. Structural analysis of KFP1 in the activated state were performed additionally. The electron density maps for the KFP1 crystal obtained upon irradiation by the high-intensity light with the wavelength 540 nm unexpectedly revealed the chromophore in the *trans*-configuration involved in stacking interaction with H203. Thus, the observed structures are similar to structures obtained for the non-activated state of the KFP1 protein⁹⁸ except for the chromophore planarity. The angles τ and ϕ for the non-fluorescent state are 8.8° and 21°, and for the activated state they are 1.2° and 13°, respectively. The further structural analysis of the KFP1 protein in the activated and non-activated states at temperatures from -173 to 25 °C showed that activation is the result of the H197 residue movement up to the position favourable for its stacking interaction with the chromophore and an increase in the chromophore planarity.⁹⁹ It is of note that in the case of the asFP595-A148S mutant the S148 residue blocks H203 in 'stacking' with the chromophore in such a way that the conformation of H203 is not changed upon photoswitching. This can imply that the mechanisms of photoswitching for the A148G and A148S mutants are different.

The structure of the permanently fluorescent mutant asFP595-S158V with resolution of 1.7 Å has been solved.⁹⁷ The structure of the asFP595-A148S/S165V mutant was obtained independently.⁹⁹ It was found that in both proteins the chromophore has *cis* configuration and is planar. In the case of asFP595-A148S/S165V, a hydrogen bond between the tyrosine OH group of the chromophore and S148 was observed as in asFP595-A148S.⁹⁷ However, a more detailed comparison of these structures demonstrated a big difference (up to 3 Å) in the chromophore location inside the β -barrel. It can be explained by the interaction of the V165 residue with the chromophore. But it does not explain the mechanism of irreversible photoactivation of KFP1, which is the matter of further study.

Photoinducible *cis-trans*-isomerization of chromophore is also typical of other RFPs.^{100, 101} Fluorescence activation, fast photobleaching or alternation of spectral characteristics under illumination can be connected with *cis-trans*-isomerization.^{16, 101} Thus, stabilization of the chromophore in one configuration may significantly improve protein photostability or prevent undesirable photoactivation.

The ability of many RFPs such as mPlum, HcRed1, mKO, mKO2, mKate, Katushka, mOrange, mOrange2 to undergo light-induced photoconversions has recently been revealed.^{102, 103} For example, Katushka, mKate and HcRed1 upon irradiation by light with wavelength 405, 561 or 750 nm convert into green fluorescent proteins with virtually complete loss of red fluorescence. The photoconversion contrast can reach a value of 82 in both solution and living cells that express the protein. It is worth mentioning that TagRFP and TagRFP-T derived from the same precursor as mKate and Katushka do not exhibit similar photoconversion.

Upon irradiation of the mOrange and mOrange2 proteins by 488 nm light, their emission maxima shifted towards the far-red region (up to 640 nm). In contrast to photoswitchable proteins, the effectiveness of RFP photoconversion does not virtually depend on pH. The mechanism of RFP photoconversion is still unknown.

VII. The main application areas of red fluorescent proteins

Fluorescent proteins are invaluable tools widely applied to study of different biological systems. The application of GFP-like proteins in biochemistry, biotechnology and cytology allowed not only imaging cell dynamics in a novel way, but also accelerated development of new techniques in microscopy. For more than a decade, the green fluorescent protein has been used as a marker for different fusion proteins for studying their intracellular localization and movements. Moreover, the green fluorescent protein has been utilized for labelling of living cells and whole organisms.¹⁰⁴ Wide-field or confocal microscope can be used for the fluorescence visualization, and high-intensity lamps and lasers can be used as sources of excitation light.^{105, 106} Recently, a new method of microscopy has been developed that allows collecting images with resolution up to ~10 nm, though only photoactivatable and photoswitchable fluorescent proteins are used.¹⁰⁷ The creation of RFPs broaden the palette of fluorescent proteins making possible multicolour microscopy. The GFP-like mutant, recombinant RFPs developed recently differ from the wild-type precursors in stability, quantum yield, the character of the absorption spectra, fluorescence excitation and emission.

An advantageous feature of the GFP-like proteins that can form chromophore without any co-factors other than molecular oxygen is their high stabilities. In addition, both N- and C-termini of fluorescent proteins are accessible for fusion, which allows coupling of a fluorescent protein with the target protein. It is of note that FPs rarely affect functional characteristics of the target protein. The GFP-like proteins are mostly non-toxic for cells. The main advantage of RFPs as compared to green proteins is that the level of autofluorescence is much lower in the red spectral range and lesser scattering of the longer wavelength light. The three main subjects of FP application are cell, organelle and protein labelling.^{95, 108}

1. Red fluorescent proteins as reporter markers

The fluorescent protein gene (or reporter gene) located under control of a certain gene promoter allows studying temporal and spatial expression of the gene by measuring the fluorescence signal in living cells and tissues. The oligomeric state of an expressed fluorescent protein does not matter for its application as a marker of gene expres-

sion. Slow chromophore posttranslational maturation and stability of the expressed FP are the main factors that limit the use of FPs in studies of fast activation of transcription. From this point of view, the rapidly maturing DsRed-Express2 protein and its improved versions E2-Orange and E2-Red/Green and a far-red protein Katushka2 are preferred for the use as reporter markers. It was shown that cytotoxicity of DsRed-Express2 and its improved version with respect to mammalian cells are comparable to that of EGFP (GFP with improved fluorescence), and their photostabilities are much higher than those of many improved RFPs.^{23, 28} Far-red fluorescence with a maximum at 633 nm is the main advantage of the Katushka2 protein as a reporter of gene expression providing whole-body imaging of its expression.³⁵

Fluorescent proteins are most often used as markers for protein labelling. Such fusion proteins enable the analysis of localization and dynamics of proteins, organelles and even cells in living organisms, studies of protein–protein interactions and determination of their biological function. Protein localization demands that a fluorescent protein, to be used as a marker, was present in the monomeric state and possessed high brightness. The tendency of the fluorescent protein to oligomerization affects strongly the possibility of localization of a protein under study, especially if it is an oligomer itself. The efficiency of fluorescent protein maturation is also of great importance since complete folding of fusion proteins often prevents inclusion bodies formation. Thus, TagRFP, mCherry, mKate, mKO, *etc.* are the proteins of choice for fusion. The oligomeric state of fluorescent proteins is not so important for labelling organelles and cells. However, the utility of RFPs for two-photon microscopy is limited by low efficiency of their excitation with a standard Ti-sapphire laser. In this case, the Keima protein and its mutants with excitation maxima at 440 nm can be used for multicolour two-photon microscopy.⁵¹ In turn, the development of new monomeric photoswitchable proteins enabled the use of super-resolution microscopy (~10 nm).⁹⁴

Photostable fluorescent proteins are highly important for long-term visualization of labelled proteins. Photostability and the absence of such photoinduced effects as photoactivation and photoswitching are substantial for quantitative analysis. For this purpose, the proteins mOrange2, TagRFP-T, mKate2 can successfully be employed. The use of PAFPs for constant imaging of fluorescently tagged subject simplifies the experiment.^{109, 110} Photoswitchable proteins allow elimination of problems related to photobleaching and phototoxicity of fluorescent proteins. Monomeric photoactivatable proteins enable a single protein molecule tracking in a cell.^{111, 112}

2. Methods based on fluorescence resonance energy transfer

FRET is one of the most powerful methods for protein–protein interaction studies both *in vivo* and *in vitro*. This method is based on non-radiative energy transfer between two fluorophores where the emission spectrum of one fluorophore (the energy donor) overlaps with the absorption spectrum of another (the energy acceptor). FRET efficiency depends on the mutual orientation of the donor and acceptor and on the distance between them.

The fluorescence of the donor and acceptor can be measured simultaneously and the efficiency of transfer can be judged from the ratio of fluorescence intensities at two wavelengths corresponding to emission of the donor and

acceptor or from the increase in fluorescence intensity upon acceptor photobleaching. The efficiency of FRET can be calculated from the intensities of the donor and acceptor signals before and after photobleaching. Another method of determination of FRET efficiency is based on the measurement of life-time of donor fluorescence. This method requires fewer measurements and is faster than the method described above but it requires more sophisticated equipment.

The most efficient FRET-pairs for cell microscopy are the following: GFP/RFPs that provide minimum overlap of emission spectra of the donor and acceptor fluorescence and possess high photostability and brightness.¹¹³ Pairs GFP/mRFP1 (Ref. 114) and GFP/mCherry (Ref. 115) demonstrated higher FRET efficiency than the widely used pair CFP/YFP (cyan/yellow FP). In the construction¹¹⁶ of FRET-pairs for studying protein configurational alterations or interaction of several proteins, such protein pairs as mKO and MiCy,¹⁷ mKOok and mUKG,²⁴ TagRFP and TagGFP,¹¹⁷ mKate2 and mCitrine¹¹⁸ were successfully used.

* * *

Protein engineering in combination with high-throughput methods of cell sorting significantly extended colour variety of fluorescent proteins and improved such physicochemical characteristics of the proteins as maturation, brightness, photostability, *etc.* The first attempts to improve wild-type RFPs resulted in monomerization since the oligomeric state limited their application greatly. Later on, optimization of physicochemical characteristics of monomeric FPs has been performed. Fluorescent proteins became indispensable markers for studies in biochemistry, biotechnology and cell biology. FPs enable visualization of dynamic processes in cells in a new way; moreover, they triggered development of new microscopy techniques. Development of photoactivatable and photoswitchable fluorescent proteins for super-resolution microscopy of cell structures and significantly simplified real-time visualization in living cells became an important step in the development of fluorescent protein technology.

References

1. O Shimomura, F H Johnson, Y Saiga *J. Cell. Comp. Physiol.* **59** 223 (1962)
2. H Morise, O Shimomura, F H Johnson, J Winant *Biochemistry* **13** 2656 (1974)
3. D C Prasher, V K Eckenrode, W W Ward, F G Prendergast, M J Cormier *Gene* **111** 229 (1992)
4. M Chalfie, Y Tu, G Euskirchen, W W Ward, D C Prasher *Science* **263** 802 (1994)
5. R N Day, M W Davidson *Chem. Soc. Rev.* **38** 2887 (2009)
6. A Miyawaki *Cell* **135** 987 (2008)
7. M V Matz, A F Fradkov, Y A Labas, A P Savitsky, A G Zaraisky, M L Markelov, S A Lukyanov *Nat. Biotechnol.* **17** 969 (1999)
8. Y A Labas, N G Gurskaya, Y G Yanushevich, A F Fradkov, K A Lukyanov, S A Lukyanov, M V Matz *Proc. Natl. Acad. Sci. USA* **99** 4256 (2002)
9. K A Lukyanov, A F Fradkov, N G Gurskaya, M V Matz, Y A Labas, A P Savitsky, M L Markelov, A G Zaraisky, X Zhao, Y Fang *J. Biol. Chem.* **275** 25879 (2000)
10. J Wiedenmann, C Elke, K D Spindler, W Funke *Proc. Natl. Acad. Sci. USA* **97** 14091 (2000)

11. N G Gurskaya, A F Fradkov, A Terskikh, M V Matz, Y A Labas, V I Martynov, Y G Yanushevich, K A Lukyanov, S A Lukyanov *FEBS Lett.* **507** 16 (2001)
12. Y G Yanushevich, D B Staroverov, A P Savitsky, A F Fradkov, N G Gurskaya, M E Bulina, K A Lukyanov, S A Lukyanov *FEBS Lett.* **511** 11 (2002)
13. M A Shkrob, Y G Yanushevich, D M Chudakov, N G Gurskaya, Y A Labas, S Y Poponov, N N Mudrik, S Lukyanov, K A Lukyanov *Biochem. J.* **392** 649 (2005)
14. J Wiedenmann, A Schenk, C Rlcker, A Girod, K D Spindler, G U Nienhaus *Proc. Natl. Acad. Sci. USA* **99** 11646 (2002)
15. E M Merzlyak, J Goedhart, D Shcherbo, M E Bulina, A S Shcheglov, A F Fradkov, A Gaintzeva, K A Lukyanov, S Lukyanov, T W J Gadella, D M Chudakov *Nat. Methods* **4** 555 (2007)
16. D Shcherbo, E M Merzlyak, T V Chepurnykh, A F Fradkov, G V Ermakova, E A Solovieva, K A Lukyanov, E A Bogdanova, A G Zaraisky, S Lukyanov, D M Chudakov *Nat. Methods* **4** 741 (2007)
17. S Karasawa, T Araki, T Nagai, H Mizuno, A Miyawaki *Biochem. J.* **381** 307 (2004)
18. Yu G Yanushevich, D A Shagin, A F Fradkov, K S Shekhabzov, E V Barsova, N G Gurskaya, Yu A Labas, M V Matts, K A Lukyanov, S A Lukyanov *Bioorg. Khim.* **31** 49 (2005)^a
19. D A Shagin, E V Barsova, Y G Yanushevich, A F Fradkov, K A Lukyanov, Y A Labas, T N Semenova, J A Ugalde, A Meyers, J M Nunez, E A Widder, S A Lukyanov, M V Matz *Mol. Biol. Evol.* **21** 841 (2004)
20. M E Bulina, D M Chudakov, O V Britanova, Y G Yanushevich, D B Staroverov, T V Chepurnykh, E M Merzlyak, M A Shkrob, S Lukyanov, K A Lukyanov *Nat. Biotechnol.* **24** 95 (2006)
21. A S Mishin, F V Subach, I V Yampolsky, W King, K A Lukyanov, V V Verkhusha *Biochemistry* **47** 4666 (2008)
22. N C Shaner, R E Campbell, P A Steinbach, B N Giepmans, A E Palmer, R Y Tsien *Nat. Biotechnol.* **22** 1567 (2004)
23. R L Strack, D Bhattacharyya, B S Glick, R J Keenan *BMC Biotechnol.* **9** 32 (2009)
24. H Tsutsui, S Karasawa, Y Okamura, A Miyawaki *Nat. Methods* **5** 683 (2008)
25. N C Shaner, M Z Lin, M R McKeown, P A Steinbach, K L Hazelwood, M W Davidson, R Y Tsien *Nat. Methods* **5** 545 (2008)
26. R E Campbell, O Tour, A E Palmer, P A Steinbach, G S Baird, D A Zacharias, R Y Tsien *Proc. Natl. Acad. Sci. USA* **99** 7877 (2002)
27. B J Bevis, B S Glick *Nat. Biotechnol.* **20** 83 (2002)
28. R L Strack, D E Strongin, D Bhattacharyya, W Tao, A Berman, H E Broxmeyer, R J Keenan, B S Glick *Nat. Methods* **5** 955 (2008)
29. D E Strongin, B Bevis, N Khuong, M E Downing, R L Strack, K Sundaram, B S Glick, R J Keenan *Protein Eng. Des. Sel.* **20** 525 (2007)
30. M Fischer, I Haase, E Simmeth, G Gerisch, A Müller-Taubenberger *FEBS Lett.* **577** 227 (2004)
31. M Fischer, I Haase, S Wiesner, A Müller-Taubenberger *FEBS Lett.* **580** 2495 (2006)
32. S Kredel, F Oswald, K Nienhaus, K Deuschle, C Röcker, M Wolff, R Heilker, G U Nienhaus, J Wiedenmann *PLoS ONE* **4** e4391 (2009)
33. T Kogure, S Karasawa, T Araki, K Saito, M Kinjo, A Miyawaki *Nat. Biotechnol.* **24** 577 (2006)
34. L Wang, W C Jackson, P A Steinbach, R Y Tsien *Proc. Natl. Acad. Sci. USA* **101** 16745 (2004)
35. D Shcherbo, C S Murphy, G V Ermakova, E A Solovieva, T V Chepurnykh, A S Shcheglov, V V Verkhusha, V Z Pletnev, K L Hazelwood, P M Roche, S Lukyanov, A G Zaraisky, M W Davidson, D M Chudakov *Biochem. J.* **418** 567 (2009)
36. A F Fradkov, V V Verkhusha, D B Staroverov, M E Bulina, Y G Yanushevich, V I Martynov, S Lukyanov, K A Lukyanov *Biochem. J.* **368** 17 (2002)
37. A V Terskikh, A F Fradkov, A G Zaraisky, A V Kajava, B Angres *J. Biol. Chem.* **277** 7633 (2002)
38. G S Baird, D A Zacharias, R Y Tsien *Proc. Natl. Acad. Sci. USA* **97** 11984 (2000)
39. P V Vrzheschch, N A Akovbian, S D Varfolomeyev, V V Verkhusha *FEBS Lett.* **487** 203 (2000)
40. A A Heikal, S T Hess, G S Baird, R Y Tsien, W W Webb *Proc. Natl. Acad. Sci. USA* **97** 11996 (2000)
41. J Wiedenmann, B Vallone, F Renzi, K Nienhaus, S Ivanchenko, C Rlcker, G U Nienhaus *J. Biomed. Opt.* **10** 14003 (2005)
42. T Kogure, H Kawano, Y Abe, A Miyawaki *Methods* **45** 223 (2008)
43. H Mizuno, A Sawano, P Eli, H Hama, A Miyawaki *Biochemistry* **40** 2502 (2001)
44. A Terskikh, A Fradkov, G Ermakova, A Zaraisky, P Tan, A V Kajava, X Zhao, S Lukyanov, M Matz, S Kim, I Weissman, P Siebert *Science* **290** 1585 (2000)
45. M E Bulina, D M Chudakov, N N Mudrik, K A Lukyanov *BMC Biochem.* **3** 7 (2002)
46. S Kredel, K Nienhaus, F Oswald, M Wolff, S Ivanchenko, F Cymer, A Jeromin, F J Michel, K D Spindler, R Heilker, G U Nienhaus, J Wiedenmann *Chem. Biol.* **15** 224 (2008)
47. N C Shaner, P A Steinbach, R Y Tsien *Nat. Methods* **2** 905 (2005)
48. F V Subach, O M Subach, I S Gundorov, K S Morozova, K D Piatkevich, A M Cuervo, V V Verkhusha *Nat. Chem. Biol.* **5** 118 (2009)
49. A Sakaue-Sawano, H Kurokawa, T Morimura, A Hanyu, H Hama, H Osawa, S Kashiwagi, K Fukami, T Miyata, H Miyoshi, T Imamura, M Ogawa, H Masai, A Miyawaki *Cell* **132** 487 (2008)
50. J Petersen, P G Wilmann, T Beddoe, A J Oakley, R J Devenish, M Prescott, J Rossjohn *J. Biol. Chem.* **278** 44626 (2003)
51. H Kawano, T Kogure, Y Abe, H Mizuno, A Miyawaki *Nat. Methods* **5** 373 (2008)
52. X Shu, A Royant, M Z Lin, T A Aguilera, V Lev-Ram, P A Steinbach, R Y Tsien *Science* **324** 804 (2009)
53. S Pletnev, D Shcherbo, D M Chudakov, N Pletneva, E M Merzlyak, A Wlodawer, Z Dauter, V Pletnev *J. Biol. Chem.* **283** 28980 (2008)
54. X Shu, L Wang, L Colip, K Kallio, S J Remington *Protein Sci.* **18** 460 (2009)
55. V V Verkhusha, A Sorkin *Chem. Biol.* **12** 279 (2005)
56. F V Subach, G H Patterson, S Manley, J M Gillette, J Lippincott-Schwartz, V V Verkhusha *Nat. Methods* **6** 153 (2009)
57. R Ando, H Hama, M Yamamoto-Hino, H Mizuno, A Miyawaki *Proc. Natl. Acad. Sci. USA* **99** 12651 (2002)
58. J Wiedenmann, S Ivanchenko, F Oswald, F Schmitt, C Röcker, A Salih, K D Spindler, G U Nienhaus *Proc. Natl. Acad. Sci. USA* **101** 15905 (2004)
59. S A McKinney, C S Murphy, K L Hazelwood, M W Davidson, L L Looger *Nat. Methods* **6** 131 (2009)
60. V Adam, M Lelimosin, S Boehme, G Desfonds, K Nienhaus, M J Field, J Wiedenmann, S McSweeney, G U Nienhaus, D Bourgeois *Proc. Natl. Acad. Sci. USA* **105** 18343 (2008)
61. H Tsutsui, S Karasawa, H Shimizu, N Nukina, A Miyawaki *EMBO Rep.* **6** 233 (2005)
62. S Habuchi, H Tsutsui, A B Kochaniak, A Miyawaki, A M van Oijen *PLoS ONE* **3** e3944 (2008)
63. N G Gurskaya, V V Verkhusha, A S Shcheglov, D B Staroverov, T V Chepurnykh, A F Fradkov, S Lukyanov, K A Lukyanov *Nat. Biotechnol.* **24** 461 (2006)
64. D M Chudakov, A V Feofanov, N N Mudrik, S Lukyanov, K A Lukyanov *J. Biol. Chem.* **278** 7215 (2003)
65. D M Chudakov, V V Belousov, A G Zaraisky, V V Novoselov, D B Staroverov, D B Zorov, S Lukyanov, K A Lukyanov *Nat. Biotechnol.* **21** 191 (2003)
66. A C Stiel, M Andresen, H Bock, M Hilbert, J Schilde, A Schlnle, C Eggeling, A Egner, S W Hell, S Jakobs *Biophys. J.* **95** 2989 (2008)
67. K Nienhaus, G U Nienhaus, J Wiedenmann, H Nar *Proc. Natl. Acad. Sci. USA* **102** 9156 (2005)

68. M A Wall, M Socolich, R Ranganathan *Nat. Struct. Biol.* **7** 1133 (2000)
69. D Yarbrough, R M Wachter, K Kallio, M V Matz, S J Remington *Proc. Natl. Acad. Sci. USA* **98** 462 (2001)
70. X He, A F Bell, P J Tonge *Org. Lett.* **4** 1523 (2002)
71. L A Gross, G S Baird, R C Hoffman, K K Baldrige, R Y Tsien *Proc. Natl. Acad. Sci. USA* **97** 11990 (2000)
72. H Mizuno, T K Mal, K I Tong, R Ando, T Furuta, M Ikura, A Miyawaki *Mol. Cell* **12** 1051 (2003)
73. F Oswald, F Schmitt, A Leutenegger, S Ivanchenko, C D'Angelo, A Salih, S Maslakova, M Bulina, R Schirmbeck, G U Nienhaus, M V Matz, J Wiedenmann *FEBS J.* **274** 1102 (2007)
74. M Prescott, M Ling, T Beddoe, A J Oakley, S Dove, O Hoegh-Guldberg, R J Devenish, J Rossjohn *Structure* **11** 275 (2003)
75. M L Quillin, D M Anstrom, X Shu, S O'Leary, K Kallio, D M Chudakov, S J Remington *Biochemistry* **44** 5774 (2005)
76. I V Yampolsky, S J Remington, V I Martynov, V K Potapov, S Lukyanov, K A Lukyanov *Biochemistry* **44** 5788 (2005)
77. S J Remington, R M Wachter, D K Yarbrough, B Branchaud, D C Anderson, K Kallio, K A Lukyanov *Biochemistry* **44** 202 (2005)
78. X Shu, N C Shaner, C A Yarbrough, R Y Tsien, S J Remington *Biochemistry* **45** 9639 (2006)
79. A Kikuchi, E Fukumura, S Karasawa, H Mizuno, A Miyawaki, Y Shiro *Biochemistry* **47** 11573 (2008)
80. T I Wood, D P Barondeau, C Hitomi, C J Kassmann, J A Tainer, E D Getzoff *Biochemistry* **44** 16211 (2005)
81. J A Sniogowski, J W Lappe, H N Patel, H A Huffman, R M Wachter *J. Biol. Chem.* **280** 26248 (2005)
82. D P Barondeau, J A Tainer, E D Getzoff *J. Am. Chem. Soc.* **128** 3166 (2006)
83. J A Ugalde, B S Chang, M V Matz *Science* **305** 1433 (2004)
84. V V Verkhusha, D M Chudakov, N G Gurskaya, S Lukyanov, K A Lukyanov *Chem. Biol.* **11** 845 (2004)
85. J Wiehler, J von Hummel, B Steipe *FEBS Lett.* **487** 384 (2001)
86. A A Pakhomov, V I Martynov *Biochemistry* **46** 11528 (2007)
87. N Pletneva, V Pletnev, T Tikhonova, A A Pakhomov, V Popov, V I Martynov, A Wlodawer, Z Dauter, S Pletnev *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **63** 1082 (2007)
88. M C Chan, S Karasawa, H Mizuno, I Bosanac, D Ho, G G Privig, A Miyawaki, M Ikura *J. Biol. Chem.* **281** 37813 (2006)
89. V I Martynov, B I Maksimov, N Y Martynova, A A Pakhomov, N G Gurskaya, S A Lukyanov *J. Biol. Chem.* **278** 46288 (2003)
90. A A Pakhomov, N V Pletneva, T A Balashova, V I Martynov *Biochemistry* **45** 7256 (2006)
91. X Shu, P Leiderman, R Gepshtein, N R Smith, K Kallio, D Huppert, S J Remington *Protein Sci.* **16** 2703 (2007)
92. K Brejc, T K Sixma, P A Kitts, S R Kain, R Y Tsien, M Ormö, S J Remington *Proc. Natl. Acad. Sci. USA* **94** 2306 (1997)
93. S Violot, P Carpentier, L Blanchoin, D Bourgeois *J. Am. Chem. Soc.* **131** 10356 (2009)
94. T J Gould, V V Verkhusha, S T Hess *Nat. Protoc.* **4** 291 (2009)
95. K A Lukyanov, D M Chudakov, S Lukyanov, V V Verkhusha *Nat. Rev. Mol. Cell Biol.* **6** 885 (2005)
96. I Hayashi, H Mizuno, K I Tong, T Furuta, F Tanaka, M Yoshimura, A Miyawaki, M Ikura *J. Mol. Biol.* **372** 918 (2007)
97. M Andresen, M C Wahl, A C Stiel, F Gräter, L V Schäfer, S Trowitzsch, G Weber, C Eggeling, H Grubmüller, S W Hell, S Jakobs *Proc. Natl. Acad. Sci. USA* **102** 13070 (2005)
98. P G Wilmann, J Petersen, R J Devenish, M Prescott, J Rossjohn *J. Biol. Chem.* **280** 2401 (2005)
99. J N Henderson, S J Remington *Proc. Natl. Acad. Sci. USA* **102** 12712 (2005)
100. D C Loos, S Habuchi, C Flors, J Hotta, J Wiedenmann, G U Nienhaus, J Hofkens *J. Am. Chem. Soc.* **128** 6270 (2006)
101. K Nienhaus, H Nar, R Heilker, J Wiedenmann, G U Nienhaus *J. Am. Chem. Soc.* **130** 12578 (2008)
102. G J Kremers, K L Hazelwood, C S Murphy, M W Davidson, D W Piston *Nat. Methods* **6** 355 (2009)
103. J Goedhart, J E M Vermeer, M J W Adjobo-Hermans, L van Weeren, T W J Gadella Jr *PLoS ONE* **2** e1011 (2007)
104. R M Hoffman *Methods Cell Biol.* **85** 485 (2008)
105. W R Zipfel, R M Williams, W W Webb *Nat. Biotechnol.* **21** 1369 (2003)
106. J M Girkin, S Poland, A J Wright *Curr. Opin. Biotechnol.* **20** 106 (2009)
107. T J Gould, M S Gunewardene, M V Gudheti, V V Verkhusha, S R Yin, J A Gosse, S T Hess *Nat. Methods* **5** 1027 (2008)
108. D M Chudakov, S Lukyanov, K A Lukyanov *Trends Biotechnol.* **23** 605 (2005)
109. D M Chudakov, K A Lukyanov *Biochemistry* **68** 952 (2003)
110. D Kedrin, B Gligorijevic, J Wyckoff, V V Verkhusha, J Condeelis, J E Segall, J van Rheenen *Nat. Methods* **5** 1019 (2008)
111. D M Chudakov, S Lukyanov, K A Lukyanov *Biotechniques* **42** 553 (2007)
112. D M Chudakov, S Lukyanov, K A Lukyanov *Nat. Protoc.* **2** 2024 (2007)
113. K I Anderson, J Sanderson, S Gerwig, J Peychl *Cytometry* **69** 920 (2006)
114. M Peter, S M Ameer-Beg, M K Hughes, M D Keppler, S Prag, M Marsh, B Vojnovic, T Ng *Biophys. J.* **88** 1224 (2005)
115. M Tramier, M Zahid, J C Mevel, M J Masse, M Coppey-Moisan *Microsc. Res. Tech.* **69** 933 (2006)
116. D W Piston, G J Kremers *Trends Biochem. Sci.* **32** 407 (2007)
117. D Shcherbo, E A Souslova, J Goedhart, T V Chepurnykh, A Gaintzeva, I I Shemyakina, T W Gadella, S Lukyanov, D M Chudakov *BMC Biotechnol.* **9** 24 (2009)
118. H Mutoh, A Perron, D Dimitrov, Y Iwamoto, W Akemann, D M Chudakov, T Knöpfel *PLoS ONE* **4** e4555 (2009)

^a — *Russ. J. Bioorg. Chem. (Engl. Transl.)*