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REVIEW

The molecular properties and applications of Anthozoa fluorescent proteins and chromoproteins

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The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its fluorescent homologs from Anthozoa corals have become invaluable tools for *in vivo* imaging of cells and tissues. Despite spectral and chromophore diversity, about 100 cloned members of the GFP-like protein family possess common structural, biochemical and photophysical features. Anthozoa GFP-like proteins are available in colors and properties unlike those of *A. victoria* GFP variants and thus provide powerful new fluorophores for molecular labeling and intracellular detection. Although Anthozoa GFP-like proteins provide some advantages over GFP, they also have certain drawbacks, such as obligate oligomerization and slow or incomplete fluorescence maturation. In the past few years, effective approaches for eliminating some of these limitations have been described. In addition, several Anthozoa GFP-like proteins have been developed into novel imaging agents, such as monomeric red and dimeric far-red fluorescent proteins, fluorescent timers and photoconvertible fluorescent labels. Future studies on the structure of this diverse set of proteins will further enhance their use in animal tissues and as intracellular biosensors.

The cloning of GFP from the jellyfish *A. victoria* (class Hydrozoa)[1], and the subsequent creation of wavelength-shifted and enhanced mutants, such as enhanced blue (EBFP), cyan (ECFP), green (EGFP) and yellow fluorescent protein (EYFP)[2], have had an enormous impact on biological research. Further breakthroughs have come with the recent cloning of novel GFP-like green, yellow and red fluorescent proteins (FPs)[3–6] and nonfluorescent chromoproteins (CPs)[6–8] from Anthozoa animals. In nature, these GFP-like proteins are thought to provide a photobiological system for regulating the light environment of host tissues: under low light they may enhance light availability[9] whereas in excessive sunlight, FPs and CPs have photoprotective functions[10].

GFP-like proteins are a family of homologous 25–30 kDa polypeptides that, together with *A. victoria* GFP mutants, cover the emission range from 442–645 nm. In comparison to other natural pigments[11], GFP-like proteins are unusual in that they can form internal chromophores without requiring accessory cofactors, external enzymatic catalysis or substrates other than molecular oxygen[12]. This property gives GFP-like proteins many advantages over low-molecular weight probes and other FPs and CPs. These advantages include the ability to form chromophores in live organisms, tissues or cells while maintaining their integrity[13], as well as molecular, organelle or tissue targeting and specificity[14]. Because of their β-can structures, GFP-like proteins are also useful for studying folding pathways and the thermodynamics of intermediates of predominantly β-folded proteins[15]. Finally, GFP-like proteins are increasingly used as quantitative, genetically encoded reporters for second messengers, intracellular chemical environments, protein-protein interactions and protein and cell tracking[16,17], and are widely available (**Box 1**).

In this review, we concentrate on the structural properties and potential uses of the Anthozoa members of the GFP-like protein family. For a recent review on *A. victoria* GFP, the reader is referred to ref. 18.

Spectral diversity

The most interesting feature of the coral GFP-like proteins is their color variety (**Fig. 1**), which can be classified into four main groups: GFPs, yellow FPs (YFPs), red FPs (RFPs) and nonfluorescent CPs of different hues, from orange to blue. Among known FPs, GFPs are the most abundant, as green fluorescence can be detected in the majority of Anthozoa species. GFPs are characterized by emission spectra that peak at 480–520 nm. Their excitation curves can possess either a single peak at 440–510 nm or two peaks at approximately 400 and 470–490 nm. Thus far, a single YFP has been isolated, zoanYFP (zFP538)[3] from *Zoanthus* sp., which shows excitation-emission maxima at 528 nm and 538 nm, respectively.

RFPs possess emission maxima at wavelengths greater than 570 nm. Often, these proteins go through a green fluorescence–emitting stage during their maturation. RFPs can be subdivided into two subgroups. The first subgroup is represented by drFP5833 (commercial name DsRed), the most popular and well-studied RFP to date. These RFPs are characterized by a broad (spectral width about 50–60 nm) emission spectrum that peaks at 570–610 nm. The second RFP subgroup is characterized by its need for UV or violet light irradiation for red chromophore formation[19]. In the dark, these proteins mature to a GFP, whereas UV-violet irradiation causes their fast transformation into RFP. The resulting red emission spectra are rather narrow (spectral width about 25 nm) and have a pronounced shoulder at about 630 nm.

Coral also possess GFP-like proteins that are CPs, which effectively absorb but practically do not emit light[5,7,8,20]. Known CPs possess single absorption maxima at 560–590 nm. The wavelength of the absorption maximum determines the particular CP color; one can see soft

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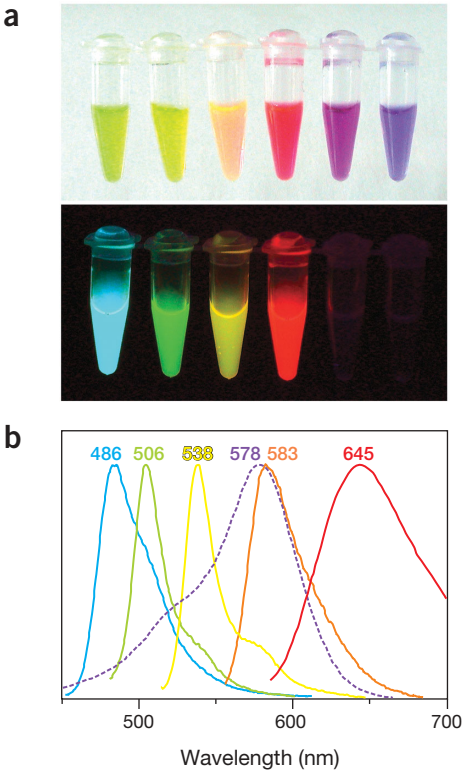

Figure 1 Spectral diversity of Anthozoa GFP-like proteins. (**a**) Purified protein samples representing different color groups visualized in daylight (upper photo) or UV-light (lower photo). From left to right: amajGFP (cyan FP), zoanGFP (green FP), zoanYFP (yellow FP), DsRed (red FP), asulCP and hcriCP (CPs). (**b**) Emission spectra for amajGFP (blue line), zoanGFP (green line), zoanYFP (yellow line), DsRed (orange line), HcRed (red line) and absorption spectrum for hcriCP (dashed purple line).

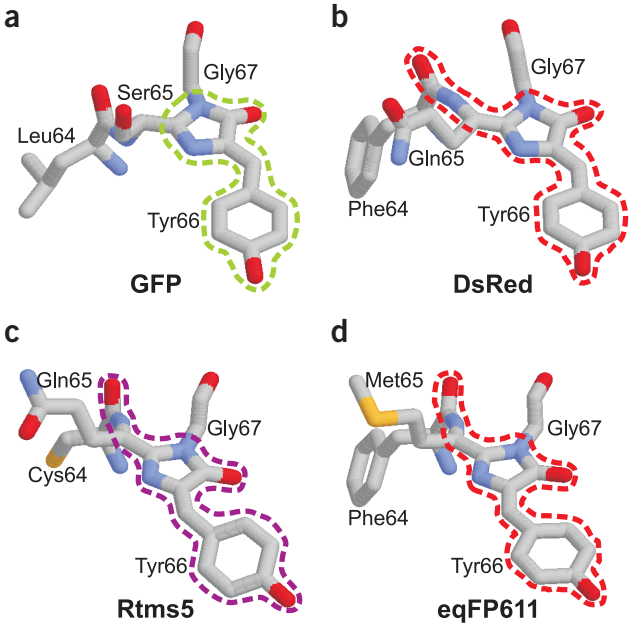

Figure 2 Variety of chromophores within GFP-like proteins. (**a**–**d**) Amino acids at positions 64–67 for green FP: 'sticks' representation of *Aequorea* GFP (**a**); red FP, *Discosoma* DsRed (**b**); nonfluorescent CP, *Montipora* Rtms5 (**c**); and far-red FP, *Entacmaea* eqFP611 (**d**). Carbon, nitrogen and oxygen atoms are gray, blue and red, respectively. Dashed lines of the corresponding colors schematically mark the chromophores.

hues of purple, crimson, lilac and almost-blue colors. In some CPs, extremely weak (quantum yield <0.001) red and far-red fluorescence can also be detected.

Two explanations for the color differences among GFP-like proteins can be suggested. First, different colors could arise from distinct noncovalent interactions of the chromophore with its microenvironment. Second, chemically distinct chromophores can determine drastic spectral shifts. The first explanation has been well documented for several *A. victoria* GFP mutants[21].

In Anthozoa proteins, an unexpected diversity of chromophore structures has been found (**Fig. 2**), suggesting the second possibility. DsRed, for example, derives its spectral quality from an additional autocatalytic dehydrogenation of the αC-N bond of Gln65 (to simplify comparison of numerous proteins, we use here and below the residue numbering to accord with that of *A. victoria* GFP), which extends the GFP-like chromophore by two strongly electron-withdrawing double bonds[22–24] (**Fig. 2b**). This enlarged system of conjugated double bonds leads to substantially red-shifted spectra.

The first crystal structure for a nonfluorescent blue CP, Rtms5, from the reef-building coral *Montipora efflorescens*, has been resolved[25]. In Rtms5, the DsRed-like chromophore was found to be in an unusual *trans*-conformation (in contrast to the chromophore *cis*-conformation in *A. victoria* GFP and DsRed) and to be nonplanar (**Fig. 2c**).

An additional conformational variant of the DsRed-like chromophore has been found in far-red FP eqFP611 from sea anemone *Entacmaea quadricolor*[26]. The chromophore of this protein is in *trans*-conformation, but in contrast to Rtms5, it is coplanar (**Fig. 2d**). These data suggest that a coplanar chromophore structure ensures high fluorescence quantum yield whereas *trans* nonplanar conformation may characterize nonfluorescent CPs[26,27].

Recently, a chromophore structure within UV-sensitive RFP Kaede from *Trachyphyllia geoffroyi*[19] has been determined[28]. A red-emitting chromophore within Kaede is formed as a result of an unusual cleavage of the protein backbone between the amide nitrogen and the αC carbon of His65 and subsequent formation of a double bond between αC and βC of His65.

The precise nature of chromophores within other Anthozoa GFP-like proteins is still unknown; however, measurements of absorption spectra for acid- and alkali-denatured proteins suggest that the YFP zoanYFP[3] and the purple CP asulCP from *Anemonia sulcata*[7] may carry novel types of chromophores. Thus, together with the GFP-like chromophore, several other distinct chromophore structures are embodied in coral's FPs and CPs.

Color transitions

To identify amino acid positions important for color transitions, site-directed and random mutagenesis have been used. Several dual-color and green mutants have been characterized for DsRed[29–33]. Work in one of our laboratories (K.A.L) has also demonstrated the interconversion of GFPs and YFP, as well as the appearance of an additional red peak in GFPs[34]. Far-red fluorescent mutants of nonfluorescent CPs have been generated[8], as well as the converse, and a DsRed FP has been mutated to a nonfluorescent chromoprotein[35]. On the basis of available three-dimensional structures for GFP and DsRed, it can be concluded that the majority of positions responsible for color changes in the mutants are those closest to the chromophore, specifically positions 65, 68, 148, 165, 167 and 203.

For practical use, two types of mutants with altered color properties look promising. First, a DsRed mutant, E5, which changes color from green to red with time, represents a novel tool, a so-called fluorescent timer, with which one can visualize the up- and downregulation of target promoters, the relative age of target organelles or follow cell differentiation[30,36–38] (**Fig. 3**). These color changes are based on a fluorescence resonance energy transfer (FRET) between the early-maturating green (5–6 h after induction of the expression) and late-maturating red (more than 9 h) monomers within a DsRed-E5 tetramer (see also below). Second, conversion of CPs into FPs has opened up a novel source of far-red FPs, such as HcRed, which has the longest reported emission peak at 645 nm[8]. The possibility arises that even more red-shifted proteins can be created if chromoproteins with red-shifted absorption spectra are found.

Oligomerization

DsRed was found to be an obligate tetramer, even at nanomolar concentrations[29,31,32,39,40], and crystallographic studies[22,23] have revealed the structural basis for tetramer formation (**Fig. 4**). Each DsRed monomer contacts the two adjacent protein molecules by two chemically distinct interfaces (**Table 1**). The hydrophobic interface includes a central cluster of closely packed hydrophobic residues surrounded by a set of polar side chains. The hydrophilic interface contains many salt bridges and hydrogen bonds between polar residues and buried water molecules, and also includes an unusual 'clasp' formed by several C-terminal residues of each monomer. Comparisons of the interface-forming residues in other Anthozoa FPs (according to their sequence alignment with DsRed) show that the tetrameric structure among other GFP-like proteins is highly conserved. At the same time, some specific features can be found in almost every protein. For instance, rfloRFP probably does not contain salt bridges around the hydrophobic interface because it carries Val19, Pro26 and His124 rather than the interacting Glu19, Lys124 and Glu26 found in DsRed. Also, rfloRFP lacks Arg157, which would interact with Glu101 in a hydrophilic interface. In contrast, mcavRFP possibly contains a salt bridge within the hydrophobic interface between Arg109 and Asp126. Interestingly, all three closely related *Zoanthus* FPs (zoanGFP, zoanYFP and zoanRFP) possess clearly distinct interfaces, with differences at positions 109, 124, 126, 128 and 157. It may be that each protein prefers to form homo- rather than hetero-tetramers, and the structural divergence observed has evolved to ensure that mixed FP tetramers will not occur in nature.

Box 1 Commercial availability

Since 2000, DsRed-encoding vectors have become available from Clontech Laboratories (now BD Biosciences). The company offers improved versions of this protein, DsRed2 and DsRed-Express, and a far-red dimeric HcRed1. Currently, BD Biosciences offers a whole color pallet of Anthozoa FPs, which contains DsReds and HcRed1 as well as mutants of amajGFP (commercial name AmCyan1), zoanGFP (ZsGreen1), zoanYFP (ZsYellow1) and asulCP (AsRed2). In 2003, three other companies entered the market of Anthozoa FPs: Promega offers a 'Monster Green,' the mutant of the GFP from Great Star Coral *Montastrea cavernosa*; Evrogen JSC has launched a Kindling Red (photoactivatable mutant of asulCP) and the tandem variant of HcRed1; and MBL International markets a green-to-red photoconvertible protein Kaede and tetrameric and monomeric versions of GFP Azami.

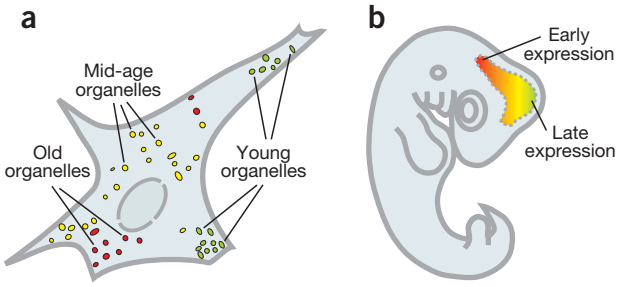

Figure 3 Monitoring temporal and spatial patterns of target events using fluorescent timer. (**a**) Fluorescence maturation of DsRed-E5 colors the tagged intracellular organelles (*e.g.*, vesicles) according to their age. Newly formed vesicles contain mostly green DsRed-E5 at early stages of their maturation; older vesicles are yellow or completely red due to the presence of DsRed-E5 mature (red) form. (**b**) Activation and downregulation of target promoter can be visualized on the whole organism scale. Green fluorescent areas indicate recent promoter activation (late expression), yellow regions correspond to continuous promoter activity, and red fluorescence denote areas in which promoter activity is currently stopped (early expression).

Wild-type Anthozoa tetramers are nondissociative and are only broken down by irreversible denaturation of polypeptides[29,32,39]. For DsRed and zoanYFP, it is not possible to dissociate tetramers into intact monomers by adding low concentrations of polar or nonpolar compounds, detergents, chaotropic agents, reducing agents, polyethylene glycol, or by changing the ionic strength, anion or cation concentrations[31,32,41]. However, the fast-maturating RFP, eqFP611, recently cloned from sea anemone, can be dissociated into intact monomers at mild concentrations of detergent[42]. DsRed fluorescence is stable within the pH range 5.0–12.0, which contrasts with the sensitivity of the majority of *A. victoria* GFP mutants to mildly acidic conditions, and pK_a for ionogenic groups in red monomers was estimated to be below 4.0–5.0 (refs. 29,31,39).

All reported GFP-like proteins from nonbioluminescent Anthozoa are tetrameric, suggesting that this state may be important for their functioning in coral polyps as photoprotective shields[10]. Of the two distinct interfaces of the β-cans of GFP-like proteins, one is usually involved in the formation of homodimers, whereas the other has diverged in function during evolution. In bioluminescent species, such as *A. victoria* and *Renilla reniformis*, the homodimer binds aequorin or luciferase[43], whereas in nonbioluminescent organisms, it binds a homodimer of the GFP-like protein. The distinct second interface might evolve to maximize photostability and thermotolerance of Anthozoa FPs living under intense tropical sunlight. It has been shown that tetrameric DsRed is four- to fivefold more resistant to photobleaching than *A. victoria* GFP and tenfold more resistant than its monomeric mutant mRFP1 (ref. 44), although differences in chromophore structures and internal environments should also be taken into account.

The oligomeric structure could confer Anthozoa FPs with greater stability than monomeric *A. victoria* GFPs at elevated temperatures and under other physical factors *in vitro*[45,46], and result in a longer intracellular lifespan *in vivo*[3,45]. The photostability and thermotolerance hypothesis correlates with the observation that Anthozoa FP folds optimally at higher temperatures—for example, DsRed folds at 32–38 °C (refs. 32,47)— and with the presence of a 330- to 340-nm absorption peak in RFPs, which may accept emission from UV-absorbing tryptophans and transfer it to the chromophores. In contrast, GFPs from *R. reniformis* and *A. victoria*, which live in regions

Table 1 Interface-forming residues in DsRed tetramer and their conservation and variability in other Anthozoa GFP-like proteins

	Interface residues[a]	DsRed (mRFP)[b]	zoan GFP	zoan YFP	zoan RFP	rflo RFP	mcav RFP	asul CP	hcri CP
Hydrophobic interface	97	V	S	S	S	T	S	T	T
	105	V	V	V	V	V	I	F	I
	107	T	I	I	I	T	T	T	T
	109	T	N	N	S	T	R	H	H
	126	I (R)	Y	N	Y	M	D	L	L
	128	V (T)	V	M	V	A	V	N	T
	184	I (T)	V	V	I	I	T	T	S
	19	E	E	E	E	V	E	E	E
	124	K	K	I	T	H	R	K	K
	26	E	K	K	K	P	N	Y	Y
Hydrophilic interface	101	E	E	E	E	E	E	E	E
	157	R (E)	K	K	S	C	R	V	E
	166	H (K)	S	S	S	A	N	L	V
	180	E	Q	Q	Q	D	D	H	H
	153	Y	I	M	I	F	Y	Y	Y
	176	H	R	R	R	H	H	H	R
	178	L (D)	R	R	R	R	R	T	I
	151	R	K	K	K	I	K	I	V
	168	A (R)	Y	Y	Y	S	A	A	A
	146	E	E	E	E	E	E	E	E
	198	Y (A)	W	W	W	Y	Y	F	F
	200	Y (K)	F	F	F	F	F	F	F
	147	A	P	A	P	P	P	P	P
	149	T	C	C	C	T	T	T	T
	229	H (S)	S	S	S	S	G	C	S
	230	L (T)	A	A	A	P	L	D	D
	231	F (G)	L	L	L	L	P	A	L
	232	L (A)	P	A	P	Q	R	A	P
	223	R	H	H	H	G	H	A	A
	225	E	I	I	I	V	E	V	V
	204	K	K	K	K	C	S	R	R

[a]Interactions between residues of the adjacent monomers are shown by arrows. The numbering is based on *A. victoria* GFP. [b]Amino acid substitutions within the hydrophobic and hydrophilic interfaces of DsRed, which have been introduced into its monomeric mutant, mRFP1, are shaded.

farther to the north than Anthozoa, do not have far-violet absorption and fold better at lower temperatures[48].

Improvements by mutagenesis

Great effort has been devoted to overcoming the drawbacks of using Anthozoa FPs as fluorescent labels. In some mutants, slow folding rate, an especially troublesome problem for DsRed, has been overcome. Random mutagenesis has generated a faster maturing mutant, E57 (ref. 33) as well as very fast DsRed (DsRed-Express) variants with a maturation half-time of 0.7–1.3 h (ref. 49).

Many Anthozoa GFP-like proteins also form nonspecific, high molecular weight aggregates both *in vitro* and *in vivo* that are toxic to cells[32,50]. These aggregates retain bright fluorescence, and therefore contain properly folded protein molecules. 'Sticky' hydrophobic patches on the molecule surface might cause aggregation. However, as DsRed does not contain pronounced hydrophobic areas, aggregation may also be due to electrostatic interactions. Computer-assisted calculations of wild-type DsRed tetramer electrostatic potentials have shown that its surface is negatively charged, except for a short N-terminal section of each monomer, which contains a group of positively charged basic residues. Mutating these residues to negative or neutral in DsRed variants and several other Anthozoa proteins prevents nonspecific aggregation and improves solubility[49,50] by eliminating the possibility of ionic-bond formation through positively charged residues.

The most serious problem with Anthozoa FPs is their tendency to form oligomers. Oligomerization does not limit the use of FPs simply as reporters of gene expression, but is a problem in applications in which FPs are fused to a target protein, especially one that is itself an oligomer. Although some examples of successful uses of DsRed in protein fusions have been reported, in many cases DsRed chimeras form intracellular aggregates[32,44,51].

Knowing the DsRed tetramer structure has provided a basis for creating mutants of interface-forming residues to generate nonoligomerizing FPs (**Fig. 5**). Mutagenic studies have shown that tetramerization is important for FP maturation. Single amino acid substitutions of interface-forming residues in DsRed1 resulted in a sharp drop in fluorescence intensity and maturation speed[44]. However, different Anthozoa proteins appeared to have different sensitivity to tetramer disruption. A fluorescent mutant of hcriCP was easily converted to a dimer by a single L126H substitution within the hydrophobic interface, without a substantial decrease in brightness[8]. However, the DsRed mutation at the same position (Ile126→Arg) resulted in a nearly nonfluorescent dimer[44]. Subsequent mutagenesis rescued fluorescence in the dimeric DsRed. A monomeric mutant, mRFP1, has been created that contains three amino acid substitutions within the hydrophobic interface, and ten within the hydrophilic (**Table 1**, shaded)[44]. Also, a monomeric variant of GFP from stony coral *Galaxea fascicularis* has been generated by introducing three amino acid substitutions within the tetramer interfaces[52].

Several other approaches have been used to overcome aggregation of Anthozoa RFP fusion proteins (**Fig. 5**). Covalently linked copies of identical RFPs forming head-to-tail tandem dimers may serve as a nonoligomerizing tag. This approach was successful for DsRed, HcRed and their dimeric mutants[44,53]. Tandem constructs displayed the same spectral characteristics as the parent proteins. Simultaneous coexpression of Anthozoa RFP-tagged proteins with an excess of either a fusion partner alone[51] or a nonfluorescent mutant of the respective RFP without the fusion partner may rescue targeting and function of fusion constructs[54,55]. In the last case, the resulting FP heterotetramers contain only a single target polypeptide, and therefore can be considered pseudo-monomeric.

Besides applications as a fluorescent tag, heterotetramers may provide a useful model for biophysical characterization of Anthozoa FPs. The tetrameric nature of FPs complicates data interpretation and hides the true behavior of monomers because of their close interactions within tetramers[56,57]. Thus, heterotetramers consisting of one

fluorescent monomer and three 'transparent' mutant monomers should provide unique information about properties of tetrameric FPs in pseudo-monomeric state in single-molecule and time-resolved studies.

Passive reporters

Anthozoa FPs have been successfully used in heterologous expression studies in a variety of cultured cells and diverse organisms, such as bacteria[7], yeast[49], plants[58], worms[30], insects[47] and vertebrates[30]. The red fluorescence of these proteins has been exploited in the following applications: counterstaining and multicolor complementation for *A. victoria* GFPs[47,59]; reporters for gene activation and bacteria-based biosensors[60]; markers to study cell lineage during development[30,47]; tags for localization of proteins, organelles or virus particles in living cells[51–55]; population markers in symbiotic studies[61]; and reporters of bacterial phagocytosis and pest control[61].

Promoters of various strengths are suitable to drive expression of both constitutive and inducible Anthozoa FPs, such as cytomegalovirus (commercial plasmids), *c-fos*[60] and heat shock–dependent[30] promoters. Anthozoa FPs have also been expressed in systems that use retroviruses[59], tetracycline transactivator (*Tet-On* and *Tet-Off*)[30], and *UAS-GAL4* (upstream activation sequence for transcription factor GAL4) constructs[47]. The advantages of Anthozoa RFPs as fusion tags for intracellular proteins include their higher tissue translucency and lower autofluorescence in the red wavelength range, as well as the spectral separation of their emission from *A. victoria* GFP and its mutants, enabling multicolor cell labeling.

FRET acceptors

Excited-state energy transfer, such as FRET and bioluminescence resonance energy transfer (BRET), in which a fluorescent or luminescent donor molecule transduces energy via a nonradiative dipole-dipole interaction to an acceptor molecule, have become important tools for studies of protein-protein interactions and in biosensors. FRET is detectable by sensitized emission from the acceptor, decreased excited-state life-time of the donor, or increased resistance to donor bleaching. Although *A. victoria* FRET pairs, such as EBFP/EGFP and ECFP/EYFP, have proven effective, they have several drawbacks, such as low quantum yield of the EBFP/ECFP donors, poor photostability of the EBFP donor, simultaneous excitation of the EYFP acceptor at the ECFP absorption range, and the parasitic donors' emission at the acceptor fluorescence wavelengths[62,63]. Anthozoa YFPs and RFPs represent attractive alternatives as FRET and BRET acceptors that would result in increased sensitivity of the analysis.

The calculated Forster radii (the distances at which FRET efficiency falls by a factor of two) for various *A. victoria* mutants and for DsRed are highest between EGFP, Sapphire or EYFP, and DsRed with values of 4.73, 4.90 and 4.94 nm, respectively[64]. These values are close to the Forster radius of 4.92 nm for the best ECFP/EYFP pair, and together with the long-wavelength tail of DsRed mutants, should facilitate detection of sensitized emission from these and other green donors. Indeed, when red monomers in tetrameric DsRed were photobleached *in vivo*, fluorescence from its green monomers increased by 2.7- to 5.6-fold in different cells, corresponding to FRET efficiencies of 68–83%[29]. These values are equal to or greater than the highest efficiencies seen for *A. victoria* GFP mutants, such as 68% for ECFP and EYFP linked by a Zn^{2+}-saturated zinc finger domain[63].

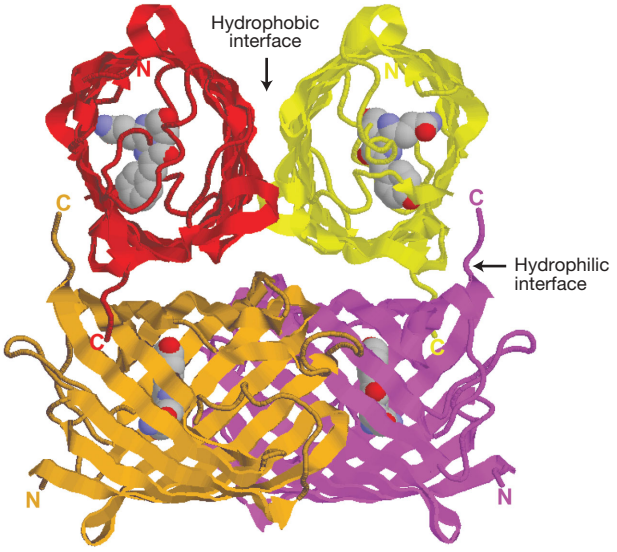

Figure 4 Overall DsRed tetramer structure. Monomers are shown in different colors. N and C termini for each monomer are designated in respective colors. Chromophores are represented as a 'spacefill' model. Carbon, nitrogen and oxygen atoms are gray, blue and red, respectively.

However, Anthozoa RFPs have several limitations in FRET applications, including tight tetramerization and a broad absorption spectra, which causes cross-excitation of GFP donors and RFP acceptors. Currently, oligomerization can be overcome using monomeric mRFP1, dimeric DsRed[44] or HcRed1 tandem[53] constructs as acceptors. The use of specific donors with greater differences between the excitation and emission maxima (Stokes shift) remedies cross-excitation. Sapphire as well as EGFP and EYFP exhibit excitation peaks and shoulders, respectively, in the 380- to 460-nm range, thus providing enough Stokes shift for emission and eliminating cross-excitation with Anthozoa RFPs. The superiority of Sapphire as the donor for DsRed and its tolerance to tetramerization has been demonstrated for genetically encoded FRET calcium indicators, so-called chameleons[65]. In mammalian cells, differ-

Table 2 Main properties of photoactivatable GFP-like proteins

Protein	Activating light	Quenching light	Reversibility of photoactivation	Fluorescence changes	Contrast, -fold	Oligomeric state	Reference
DsRed	3-photon, Infrared	No	Irreversible	Red to green	15	Tetramer	73
Kaede	UV-violet	No	Irreversible	Green to red	2,000	Tetramer	19
mcavRFP	UV-violet	No	Irreversible	Green to red	n.d.	Tetramer	6
rfloRFP	UV-violet	No	Irreversible	Green to red	n.d.	Tetramer	6
PA-GFP	UV-blue	No	Irreversible	None to green	100	Monomer	74
KFPs	Green or blue	Blue or no	Reversible and irreversible	None to red	70	Tetramer	27,76

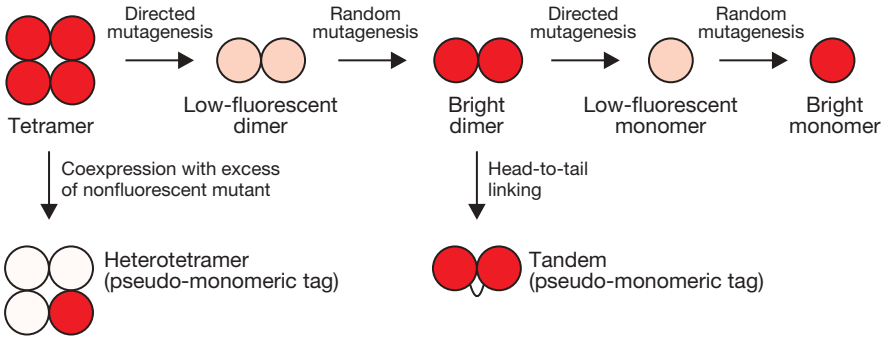

Figure 5 Ways to overcome FP tetramerization. The best solution to this problem is the creation of a monomeric FP. Site-directed mutagenesis of tetramer interface-forming residues can be used to generate dimeric and monomeric FP variants. Tetramer disruption frequently causes fluorescence loss, therefore, intensive random mutagenesis should be applied to restore the fluorescence. A second solution is coexpression of a tetrameric FP with excess of its chromophore-free mutant that results in the formation of the heterotetramers (so-called pseudo-monomeric tag). The third solution is to link a dimeric FP in tandem, which can be considered as a monomeric tag of the doubled molecular weight.

ent chameleon constructs with the DsRed acceptor have shown maximal changes of about 1.10-, 1.25- and 1.28-fold in the acceptor/donor emission ratio for ECFP, EYFP and Sapphire donors, respectively[32]. EGFP has been shown to be an acceptable donor for Anthozoa RFPs in a FRET pair consisting of phytochrome B/EGFP-tagged and cryptochrome 2/DsRed-tagged protein in *Arabidopsis* plant cells[58].

Work in one of our laboratories (K.A.L.) has demonstrated the potential use of Anthozoa RFP-tandem tags as acceptors for FRET using changes in the emission ratio of the EYFP and HcRed1-tandem FRET pair in a protease assay[53]. The ratio of donor/acceptor fluorescence changed by 2.5-fold after separation of the FRET pair, comparable to the FRET changes observed among *A. victoria* GFP mutants[62,65]. Because of the rather small quantum yield of HcRed1, the changes in donor/acceptor ratio in this case is attributed to the increase of the EYFP fluorescence (80%) rather than to the decrease of the acceptor emission (30%). This is distinct from the ECFP-EYFP pair, where the changes of the donor and the acceptor emissions are almost equal[62]. Monomeric or tandems of dimeric Anthozoa CPs might be useful in intracellular applications as effective fluorescence quenchers. EGFP and DsRed1 have also been used in dual-color fluorescence correlation spectroscopy (FCS) studies, demonstrating the advantages of cross-correlation (detection of concomitant signal fluctuations in two spectrally distinct emission channels; coincident signal fluctuations in both channels indicate the presence of tight linkage between the FPs) versus FRET analysis for large interchromophore distances[66].

Other than GFP-like proteins, Anthozoa RFPs have been paired with such donors as organic dyes and luciferases, which are suitable for intracellular FRET and BRET, respectively. Among the dyes, membrane-permeant, green-emitting ligands for the tetracysteine motifs in the biarsenical-tetracysteine system would be useful[67]. These dyes, such as biarsenylated fluorescein, specifically bind the tetracysteine motif in the amino acid sequence CC-XX-CC (where X is any amino acid except cysteine) when added to exogenously expressed protein, and become fluorescent only when bound[68]. Several applications of BRET using blue-emitting *R. reniformis* luciferase as the donor for *A. victoria* GFP mutants have already been reported[69,70]. We anticipate that insect luciferases[11], including the firefly *Luc* gene product with emission maxima in the 550- to 575-nm range, will also result in higher efficiency of BRET with Anthozoa RFPs, owing to substantial overlap of their excitation spectra.

Fluorescent photoconvertible labels

Similar to *A. victoria* GFP, the majority of Anthozoa GFP-like proteins can be irreversibly photobleached by intensive irradiation at their absorption maxima. This spatially restricted, photodynamic damage to chromophores allows one to carry out 'fluorescence-recovery-after-photobleaching' (FRAP) and 'fluorescence-loss-in-photobleaching' (FLIP) studies of the dynamics of GFP-fused proteins or tagged organelles[71,72]. The direct tracking of intracellular objects becomes possible with the introduction of photoactivatable fluorescent labels. Considerable progress in developing GFP-like proteins, which can be activated by light, has been achieved over the past few years (**Table 2**). The photolabeling technique based on the 'greening' of DsRed protein was first suggested in 2001 (ref. 73). This technique uses an intense three-photon laser to bleach red-emitting monomers within the DsRed tetramer, thereby enhancing emission from the green units by reducing the intra-tetrameric FRET. This results in a change of the red-to-green intensity ratio (*i.e.*, contrast) by about 15-fold.

Approaches allowing direct induction of the fluorescent signal in GFP-like proteins have also appeared. For example, green fluorescence of stony coral FP Kaede irreversibly converts to red by 300- to 400-nm light irradiation[19]. The resulting contrast between ground and pho-

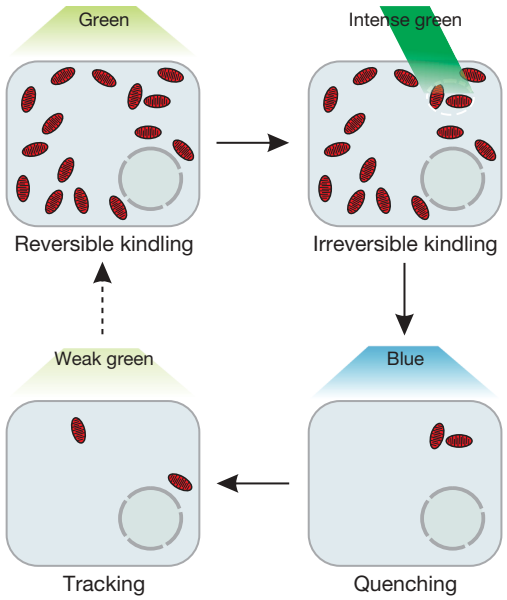

Figure 6 Tracking intracellular organelles tagged with kindling fluorescent protein. All KFP-containing organelles can be reversibly photoactivated by green light irradiation so they can be visualized. Intense green light causes irreversible kindling within the selected area. Then, reversibly activated KFP can be quenched by blue light (or it turns itself off with time), whereas irreversibly marked organelles remain constantly fluorescent. Weak green irradiation allows tracking of the irreversibly kindled organelles. Reversible kindling can be repeated at any time through the observation.

toactivated Kaede states reaches about 2,000-fold (considering both the decrease in the green peak and the increase in the red peak). Similar far UV-activated red FPs have been found in *Montastraea cavernosa* (mcavRFP) and *Ricordea florida* (rfloRFP)[6]. These color-changing properties provide a powerful technique for cell and organelle optical labeling, although disadvantages still exist, such as their tetrameric nature, the simultaneous presence of both green and red colors and damaging irradiation required for the photoconversion. Patterson & Lippincott-Schwartz[74] have described an *A. victoria* GFP mutant (PA-GFP) that is activated by UV and blue light. Photoactivation of PA-GFP is based on the photoinduced chromophore transition from the neutral to anionic state, similar to that described in an earlier report[75], but provides a substantially improved contrast reaching 100-fold. Because PA-GFP is monomeric, it provides a unique photolabeling method for tagged intracellular proteins.

One of the chromoproteins, asulCP, possesses a unique type of photoconversion[7]. Initially nonfluorescent, asulCP becomes red fluorescent (*i.e.*, 'kindles') with excitation-emission maxima at 575 and 595 nm, respectively, in response to intense green light irradiation. The protein then relaxes back to its initial nonfluorescent state or can be quenched instantly by blue light irradiation. Both kindling in green light and quenching in blue light are reversible processes for the wild-type protein. Through intensive mutagenesis of asulCP, cgigCP and hcriCP, one of our groups (K.A.L.)[27,76] has created a new group of kindling fluorescent proteins (KFPs), which can be reversibly photoconverted from the CP to FP state. However, more intense or more prolonged irradiation under the same conditions causes irreversible kindling of KFPs[76]. Kindled KFPs are fluorescent in the red range of spectrum (excitation at about 580 nm, emission at 600–630 nm for different KFPs) and contrast between initial dark and kindled KFP states reaches up to 70-fold for the best variants. The drawback of KFPs is the tetrameric nature of these proteins that hampers their use as protein tags. However, they can be successfully used for photolabeling and tracking of cells in tissues and cellular organelles (**Fig. 6**). The advantages of KFPs include their fluorescence in red and far-red regions and the absence of other fluorescent colors in their emission, which makes them useful for multicolor labeling simultaneously with GFP variants.

Reversible kindling also allows visualization of a whole KFP fluorescent pattern of the labeled cell before the precise irreversible subcellular photolabeling. Finally, dehydrated KFPs preserve the ability to be reversibly photoconverted (K.A.L., unpublished data) and therefore could be used in photoluminescent films, photochemical memories and in some nanotechnology applications.

Perspectives

In the past several years, research on Anthozoa GFP-like proteins has enhanced the advantages of these proteins and overcome some of the disadvantages. However, enormous challenges and opportunities still lie ahead. More structural studies on existing GFP-like proteins, together with the discovery of new CPs, could provide the basis for residue substitutions for creating different mutants and for moving the emission spectra further to far-red and infra-red regions (650–900 nm), where animal tissues have highest transparency. In addition, such information could be used to create new photoconvertible mutants and red-fluorescent intracellular biosensors. The new generation of laser scanning microscopes that split emission onto multichannel detectors will overcome the limits of existing multicolor labeling and FRET detection methods. We also anticipate future use of Anthozoa FPs in intact tissues and whole organisms where methods of cDNA introduction, such as 'gene gun' and homologous recombination, are well established. The combination of spatio-restricted and deeper multiphoton laser excitation with high-output fiber endoscopy will permit *in situ* FP-based analysis of numerous biological processes in transgenic animals.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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