Photoactivation in green to red converting EosFP and other fluorescent proteins from the GFP family

Jörg Wiedenmann[a*] & G. Ulrich Nienhaus[b,c**]

[a]Dept. of General Zoology and Endocrinology, University of Ulm, 89069 Ulm, Germany
[b]Dept. of Biophysics, University of Ulm, 89069 Ulm, Germany
[c]Dept. of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

ABSTRACT

The green fluorescent protein (GFP) from the hydromedusa *Aequorea victoria* and its derivatives have become indispensable imaging devices in cell biology. In previous years, a wide variety of GFP-like proteins were discovered in non-bioluminescent anthozoa. Some of them displayed exciting new properties, including photoactivated changes of the fluorescence emission intensity and wavelength. Photoactivatable proteins offer a high potential as tools for regional optical marking in live cells and tissues. This review aims to give an overview of photoactivatable marker proteins, focusing on the molecular basis of light-induced green to red photoconversion in EosFP.

Keywords: red fluorescent protein, green fluorescent protein, EosFP, GFP, dsRed, monomer, crystal structure, coral, photoconversion, photoactivation.

INTRODUCTION

The glaring history of fluorescent proteins (FPs) began in 1962 with Osamu Shimomura's discovery of a green fluorescent protein (GFP) in tissue extracts from the hydromedusa *Aequorea victoria*[1]. Further GFPs were subsequently found in other bioluminescent cnidarians such as *Renilla reniformis* or *Obelia genicula*[2]. Initially, GFPs were studied mainly in their functional roles as secondary emitters in the bioluminescence reaction. An important milestone was the determination of the primary sequence by Prasher and colleagues in 1992[3]. This accomplishment laid the basis for the second stage of GFP research, heralded by the recombinant expression of the protein in *Caenorhabditis elegans* by Chalfie et al. in 1994[4], which paved the way to the now widespread use of GFP as a genetically encoded fluorescent marker in living cells. Its strongly fluorescent chromophore, 4-(*p*-hydroxybenzylidene)-5-imidazolinone, forms autocatalytically from the tripeptide Ser-Tyr-Gly within a rigid, 11-stranded β-barrel protein fold[5-6], requiring nothing except molecular oxygen[7]. Detailed studies of GFP photophysics and photochemistry were greatly assisted by the availability of large amounts of recombinant protein from bacterial expression [e.g. 8-9], and further improvement by mutagenesis have rendered GFP a useful marker of gene expression and protein trafficking. Blue, cyan and yellow GFP variants were created by protein engineering[7]. These novel hues enabled multicolor applications, Förster resonance energy transfer (FRET) based studies of protein-protein interaction and the development of FRET based biosensors, e.g., for the measurement of intracellular Ca^{2+} levels[10-13].

Wild-type GFP displays two absorption bands of its chromophore, a large band at ~397 nm and a smaller one at ~475 nm that have been assigned to the neutral and anionic form of the chromophore, respectively[14-15]. Remarkably, excitation in either band leads to fluorescence emission in a single band at ~508 nm. This unusual feature has been explained by excited state proton transfer (ESPT)[16-17]: Absorption of light by the neutral chromophore results in efficient deprotonation of the phenolic oxygen of the chromophore and subsequent light emission by the anionic state. Intense irradiation of the neutral chromophore, however, leads to stable conversion

* joerg.wiedenmann@uni-ulm.de; phone ++49 731 50-22591; fax ++49 731 50-22581
** uli@uiuc.edu; phone ++49 731 502-3050; fax ++49 731 50-23059

Genetically Engineered Probes for Biomedical Applications, edited by Alexander P. Savitsky, Rebekka Wachter,
Proc. of SPIE Vol. 6098, 609801, (2006) · 1605-7422/06/$15 · doi: 10.1117/12.657565

to the anionic state due to decarboxylation of Glu222, causing a three-fold increase of fluorescence when exciting the protein bulk at ~488 nm[18]. The remarkable ability to control the fluorescence properties of GFP and other FPs by light irradiation has been denoted as photoactivation. The effect was further enhanced in the photoactivatable GFP variant (PA-GFP) by a single mutation, Thr203His[19]. For this variant, the protonation equilibrium of the chromophore is shifted almost completely to the neutral form. Intense exposure to ~400-nm light causes irreversible photoconversion to the anionic form, with excitation and emission maxima at 504 and 517 nm, respectively, leading to an increase in emission of the anionic form (for excitation at 488 nm) by a factor of ~100. The demonstration of interlysosomal protein exchange by PA-GFP-fusions proved the viability of this mutant for regional optical marking in live cells[19]. Already in 1997, Elowitz et al. made the observation that the fluorescence of "enhanced" GFP (EGFP) turned red upon illumination with blue light in a low oxygen environment and exploited this property to monitor protein diffusion in living *E. coli* cells[20].

In recent years, the range of fluorescence colors was greatly expanded by the discovery of GFP-like FPs in non-bioluminescent anthozoa[21-22]. Besides cyan, green and yellow emitters, these novel homologs included also the long-sought red fluorescent variants[22-25]. Moreover, strongly absorbing but essentially nonfluorescent GFP-like chromoproteins (CPs) were first found in sea anemones[23,26]. Photoactivation was observed for several of these proteins in their natural form or has been introduced by mutagenesis.

The purple chromoprotein asulCP, also known as asFP595, from *Anemonia sulcata* can be photoactivated by irradiation with green light[26]. Excitation in the major absorption peak at 572 nm gives rise to a red fluorescent state with an excitation maximum at 572 nm and an emission maximum at 595 nm. An engineered variant, the so-called "kindling" fluorescent protein (KFP1) shows a ~30 fold increase in red fluorescence upon irradiation with green light[27]. Interestingly, the red fluorescence can be reversibly "quenched" by blue light at ~450 nm. Beyond applications as optical highlighters, FPs featuring reversible switching between fluorescent and non-fluorescent states may play a key role for sub-diffraction optical microscopy based on reversible saturable optical fluorescence transitions[28]. In contrast to PA-GFP, where photoactivation derives from chromophore deprotonation induced by irreversible photochemistry, a photo-induced cis–trans isomerization of the chromophore is likely responsible for the reversible photowitching of the fluorescence in KFP and its variants[29].

A photoactivatable variant of the monomeric red fluorescent protein (mRFP1) was developed using KFP as lead structure[30-31]. The red fluorescence of this variant increases 70-fold upon stimulation with light around 380 nm.

Yet another type of photoactivatable protein was isolated from a *Pectiniidae* stony coral[32]; it was named Dronpa after the Japanese word Dron, a ninja term referring to instant disappearance of the body, and pa as an abbreviation for photo-activation. As with PA-GFP, irradiation of the neutral chromophore with near UV-light (~400 nm) causes a strong increase in excitability by blue light (~503 nm). Interestingly, the activated green fluorescence emission at 518 nm vanishes upon excitation. The reversibility of the reaction was demonstrated by on/off switching the protein for >100 times. Presumably, this process involves reversible protonation/deprotonation of the chromophore. Besides imaging of fast protein shuttling in living cells, this type of photoactivatable FP may also find application in data storage at the molecular level[32-33].

A photoswitching cyan fluorescent protein (PS-CFP) was engineered into aceGFP from another hydromedusa, *Aequorea coerulescens*[34]. Both its excitation and emission spectrum are shifted to the green spectral region upon activation by intense blue light around 400 nm. The monomeric nature of aceGFP, its variant PS-CFP and GFP from *A. victoria* reflect the close taxonomic relationship of the donor animals at the molecular level.

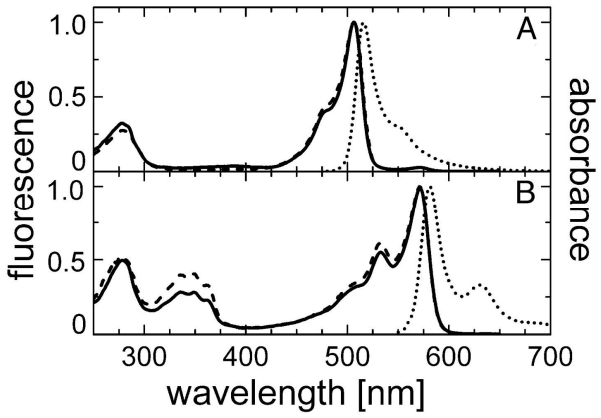

Fig. 1: Spectra of the green and red states of EosFP. Solid lines: absorbance; dashed lines: excitation; dotted lines: emission. (A) Green species at pH 7. Excitation (emission) spectra were measured with emission (excitation) set to 520 (490) nm. (B) Red species at pH 7. Excitation (emission) spectra were measured with emission (excitation) set to 590 (560) nm.

FPs that change their fluorescence from green to red upon irradiation with light around 400 nm form yet another class of optical highlighters. The first described example was Kaede (meaning maple leaf in Japanese) from the reef coral *Trachyphyllia geoffroyi*[35]. The molecular environment of the Kaede chromophore guided the engineering of a green to red convertible variant of the green fluorescent protein KikG from *Favia favus*[36]. The artificial photoconverting protein, named KikGR, is several fold brighter than its lead structure Kaede.
EosFP, cloned from the coral *Lobophyllia hemprichii*[37], is a protein with spectroscopic properties very similar to those of Kaede. Its name was inspired by the rosy-fingered goddess of dawn in Greek mythology. EosFP exists initially in a green fluorescent state with excitation and emission maxima at 506 and 516 nm, respectively, and can be switched by light around 400 nm into a red form, with excitation at 571 nm and emission at 581 nm (Fig. 1). EosFP is currently the best characterized representative of the class of green-to-red photoconverting FPs with respect to its structure and spectroscopy[37-40]. In this review, we shall focus on its structural and spectroscopic properties that make EosFP an extremely valuable tool in cellular imaging.

THE THREE DIMENSIONAL STRUCTURE OF EOSFP

X-ray structure analysis of both green and red forms of EosFP at resolutions of 1.85 Å and 2.0 Å, respectively[39-40], revealed the typical β-can fold first observed for GFP[5-6]. Its cylindrical shape is formed by 11 β-sheets, and a central helix runs in the interior along the axis of the cylinder (Fig. 2). It is interrupted by the chromophore that resides nearly in the geometrical center of the protein. In contrast to GFP, which crystallizes as dimers, all crystal structures of GFP-like proteins from anthozoan species examined so far display a tetrameric assembly, in which the subunits are arranged as dimers of dimers [36,39,41-49]. In EosFP, the two different interfaces (A/B and A/C) of the tetrameric assembly are slightly less extended than for the red fluorescent proteins DsRed and eqFP611[39]. Fortunately, only single point mutations in the A/B interface (Val123Thr) and in the A/C interface (Thr158His or Thr158Arg) were required to produce fully functional dimers. A combination of both mutations produced a variant, mEosFP, that is monomeric at concentrations relevant for cell biology applications (for the monomer-dimer equilibrium, K_d = 0.1 mM). Interfacial interactions appear to be crucial for the proper folding of anthozoan FPs[47]. For some FPs, especially modification of the A/C interface leads to a complete loss of functional protein[47]. Whereas dimeric EosFP expresses well in mammalian cell cultures at temperatures up to 37°C, expression of functional mEosFP is compromised above 30°C.

Fig. 2: Overall tetrameric structure of EosFP**.** The ribbon diagram of the EosFP tetramer shows the assembly as dimers of dimers typical of GFP-like proteins from anthozoa. The amino acids histidine, glycine and tyrosine forming the chromophore are represented by Van der Waals spheres.

THE GREEN FLUORESCENT CHROMOPHORE OF EosFP

The green emitting fluorophore forms autocatalytically from amino acids His62, Tyr63, and Gly64 and features the typical 4-(*p*-hydroxybenzylidene)-5-imidazolinone moiety of GFP (Fig. 3). Crystallographic refinement revealed an almost perfectly planar structure, rigidly held in place by multiple hydrogen bonds to charged or polar amino acids and structural water molecules in the immediate vicinity of the chromophore. The second and third chromophore-forming amino acids, Tyr63 and Gly64, are strictly conserved for all FPs known so far. Moreover, the two residues Arg91 and Glu212 (corresponding to Arg96 and Glu222 in GFP) have been suggested to play a pivotal role in chromophore formation[9]. Both observations suggest that the basic mechanism of chromophore formation is identical to the one in GFP from *Aequorea victoria*. The latter protein displays two different forms of the chromophore over a wide pH range, denoted as neutral (A) form, absorbing at ~400 nm, and anionic (B) form, absorbing at ~480 nm[14-15]. By contrast, most anthozoan GFPs show maximum absorption around 480 – 500 nm at neutral pH, indicating that their chromophores exist preferentially in the anionic B state. Exceptions are asFP499 from *A. sulcata*[23,50] and cgigGFP from *Condylactis gigantea*[51], which both show a dominant excitation band at ~400 nm.

The absorption of the anionic B form of the chromophore of EosFP is characterized by an extinction coefficient $\varepsilon(506\ \text{nm}) = 72{,}000\ M^{-1}\ cm^{-1}$; the fluorescence quantum yield was determined as 0.70 ± 0.02. Upon lowering the pH, the population of neutral chromophores absorbing at ~390 nm grows. The pH dependence can be described reasonably well by a Henderson-Hasselbalch relation, with $pK = 5.8 \pm 0.1$. Fluorescence of the anionic form upon absorption in the neutral A form is very weak, indicating that ESPT is inefficient, as indicated by the fact that the band at 390 nm is virtually absent in the excitation spectrum with detection of the emission at ~516 nm. Direct (blue) emission from the A band is also very weak (Fig. 1).

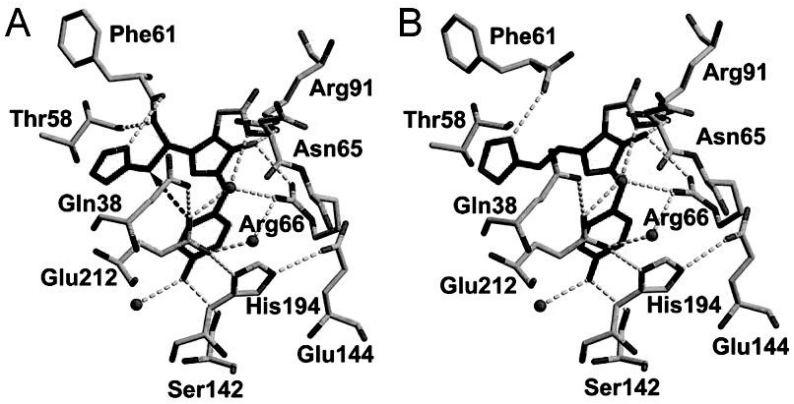

Fig. 3: Interactions between the green (A) and red (B) chromophores and amino acids of the surrounding protein scaffold. The stick model of the chromogenic triad His-Tyr-Gly is shown in black. Hydrogen bonds are represented by dashed lines. The arrow in (A) depicts the interaction between the Glu-212 carboxyl and His-62 C_β-H that is essential for cleavage of the His-62 N_α-C_α bond.

THE RED FLUORESCENT CHROMOPHORE OF EosFP

The absorption maximum of the red chromophore is located at 571 nm, with an extinction coefficient $\varepsilon(571\ \text{nm}) = 41{,}000\ M^{-1}\ cm^{-1}$. It has a strong vibronic sideband at 533 nm, and there are additional absorption bands between 300 and 400 nm that arise from excitations of higher electronic states (Fig. 1). The red fluorescence peaks at 581 nm, with a pronounced vibronic sideband at 629 nm. The quantum yield of the red-emitting form was determined as 0.55 ± 0.03.

Red fluorescence requires an extension of the conjugated π-electron system of the 4-(*p*-hydroxybenzylidene)-5-imidazolinone chromophore. In the red fluorescent proteins DsRed[22] and eqFP611[25], the extension of the GFP-type chromophore along the peptide backbone is realized by an C_α=N double bond (acylimine) within the first chromophore-forming amino acid, produced in a second oxidation step[41-43,45,52]. A further extension may even be possible by inclusion of the neighboring carbonyl group.

Although brightness and emission range of the red form of EosFP are comparable to those of FPs with a spontaneously forming red chromophore, a completely different mechanism is at work in this protein. We had earlier shown that the light-driven conversion from the green to the red chromophore is accompanied by cleavage of the peptide backbone[37]. Indeed, a break of the covalent N_α-C_α bond of His62 was confirmed by the x-ray structure of the red form[39]. Surprisingly, only minimal changes were observed in the spatial orientation of the red chromophore and the network of hydrogen bonds around the chromophore (Fig. 3). Cleavage at the corresponding site was also inferred for Kaede by mass spectroscopic analysis of tryptic peptides[53]. Concomitant with backbone cleavage, the π-conjugation of the chromophore is extended so that the His-62 imidazole ring system connects to the imidazolinone via an *all-trans* alkenylene bridge.

THE MECHANISM OF PHOTOCONVERSION

The photo-induced backbone cleavage likely occurs via an E2-type β-elimination mechanism, in which key roles are attributed to Glu212, His62 and the neutral phenolic sidechain of Tyr63 within the chromophore[39]. In this mechanism, Glu212 acts as a base abstracting a proton from His62-C_β (Fig. 4). Additional experimental support has been provided by introducing a glutamine in position 212[40]. Its sidechain is of similar shape as in glutamate but it cannot act as a nucleophile attacking one of the two His-62-C_β protons. In accord with our expectations, this mutant forms a functional green form but cannot be photoconverted.

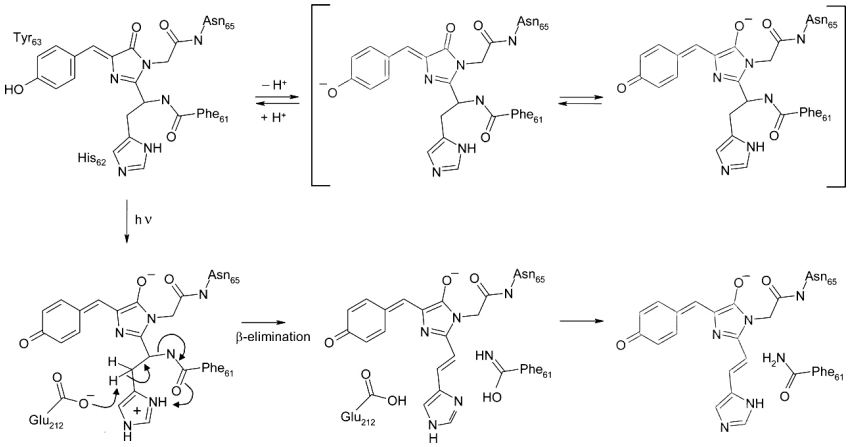

Fig. 4: Reaction mechanism depicting chromophore extension and backbone cleavage. Arrows illustrate electron displacements in the β-elimination reaction as discussed in the text.

Note that this β-elimination mechanism does not yet involve the requirement for photoexcitation. However, light is clearly indispensable for the conversion reaction to proceed, as purified EosFP solutions remain green when kept in the dark. The neutral chromophore acts as gateway structure in photoconversion, as shown by the following observations: (1) The action spectrum of photoconversion closely tracks the absorption spectrum of the neutral chromophore, peaking around ~390 nm. (2) The conversion rate increases with decreasing pH in proportion to the population of the neutral chromophore species. (3) Substitution of Pro141 by alanine yielded a mutant with an ~1.5 fold increase in the 390 nm absorption band and a concomitant increase in the photoconversion rate (Fig. 5). This mutation presumably causes a slight repositioning of Ser142, causing a less efficient hydrogen-bond stabilization of the anionic chromophore. Mizuno and coworkers[53] have suggested that electron redistribution in the excited state of the chromophore may be essential for the cleavage reaction to proceed. In addition, we have pointed to a crucial role of His62 because its replacement by any other amino acid results in a loss of photoconversion in Kaede[35] and EosFP[37]. Moreover, histidine is strictly conserved in the first position of the chromophore in all green to red photoconverting proteins identified so far, including mcavRFP, dendRFP and rfloRFP[54]. The His62 sidechain may become cationic by transient protonation transitions, for example after ESPT from the Tyr63 phenyl group.

The resulting charge displacement induced in the Phe61 carbonyl renders the peptide group between Phe61 and His62 an ideal carboximidic leaving group in the subsequent β-elimination reaction. The quantum yield of photoconversion is rather small even at low pH, where the neutral chromophore is significantly populated. As mentioned earlier, excitation at 390 nm produces only very weak green fluorescence, indicating that ESPT is a rare event in EosFP. Yet, the effect is consistent with the observed low photoconversion quantum yield.

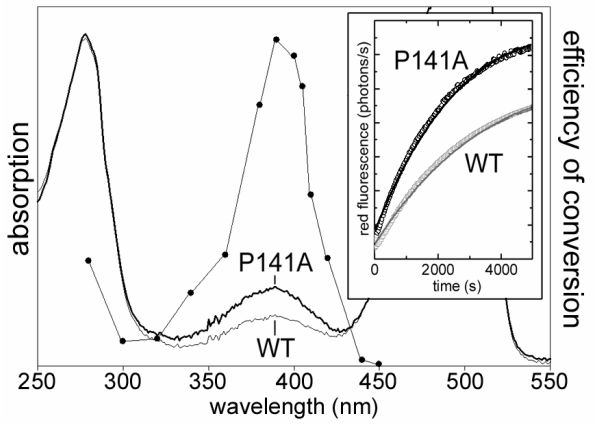

Fig. 5: Absorption spectra of EosFP (WT) and its variant Pro141Ala at pH 7.2. Spectra were normalized at the peak maximum at 506 nm. The action spectrum (dots) is also included. The faster conversion rate of the P141A mutant (inset) is correlated with the increased absorption at ~390 nm.

CONCLUSIONS

The x-ray structure analyses of both green and red forms of EosFP have yielded unprecedented insights into the structural determinants underlying its intriguing photoactivation mechanism. This detailed knowledge of the structure-function relationship of EosFP forms the basis for the development of advanced variants of green to red switchable marker proteins and, possibly, other photoactivatable proteins of the GFP family.

ACKNOWLEDGMENTS

The work was supported by the Deutsche Forschungsgemeinschaft (SFB 569 to GUN), the Fonds der Chemischen Industrie (to GUN), the Landesstiftung Baden-Württemberg (Elite-Postdoc-Förderung to JW) and Landesforschungsschwerpunkt Baden-Württemberg (to JW & GUN).

REFERENCES

1. Shimomura, O., Johnson, F.H., and Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* 59, 223-239.

2. Morin, J.G. (1974). Coelenterate bioluminescence. *In Coelenterate Biology. Reviews and new perspectives.* L. Muscatine, and H.M. Lenhoff, eds. (New York: Academic press), 397-348.

3. Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier , M.J. (1992). Primary structure of the Aeqourea victoria green fluorescent protein. *Gene* 111, 229-233.

4. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent proteins as a marker for gene expression. *Science* 263, 802-805.

5. Ormö, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. (1996). Crystal structure of the Aequorea victoria green fluorescent protein. *Science* 273,1392-1395.

6. Yang, F., Moss, L.G., and Phillips, G.N., Jr. (1996). The molecular structure of green fluorescent protein. *Nat. Biotechnol.* 14, 1246-1256.

7. Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* 67, 509-544.8.

8. van Thor, J.J., Pierik, A.J., Nugteren-Roodzant, I., Xie, A., and Hellingwerf, K.J. (1998). Characterization of the photoconversion of green fluorescent protein with FTIR spectroscopy. *Biochemistry.* 37, 16915-16921.

9. Sniegowski, J.A., Lappe, J.W., Patel, H.N., Huffman, H.A., and Wachter, R.M. (2005). Base catalysis of chromophore formation in Arg96 and Glu222 variants of green fluorescent protein. *J Biol Chem.* 280, 26248-26255.

10. Miyawaki, A., Llopis, J., Heim, R., McCaffe,ry J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* 388, 882-887

11. Pollok, B.A., and Heim, R. (1999). Using GFP in FRET-based applications. *Trends Cell Biol.* 9, 57-60.

12. Rizzo, M.A., Springer, G.H., Granada, B., and Piston D.W. (2004). An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 2, 445-449.

13. Nguyen, A.W., Daugherty, P.S. (2005). Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat. Biotechnol.* 23, 355-360.

14. Brejc, K., Sixma, T.K., Kitts, P.A., Kain, S.R., Tsien, R.Y., Ormö, M., and Remington, S.J. (1997). Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein. *Proc. Natl. Acad. Sci.* USA. 94, 2306-2311.

15. Palm, G.J., Zdanov, A., Gaitanaris, G.A., Stauber, R., Pavlakis, G.N., and Wlodawer, A. (1997). The structural basis for spectral variations in green fluorescent protein. *Nat. Struct. Biol*. 4, 361-365.

16. Chattoraj, M., King, B.A., Bublitz, G.U., and Boxer, S.G. (1996). Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc. Natl. Acad. Sci.* USA 93, 8362-8367.

17. Lossau, H., Kumer, A., Heinecke, R., Poellinger-Dammer, F., Kompa, C., Bieser, G., Jonsson, T., Silva, C.M., Yang, M.M., Youvan, D.C., and Michel-Beyerle, M.E. (1996). Time-resolved spectroscopy of wild type and mutant green fluorescent proteins reveals excited state deprotonation consistent with fluorophore-protein interactions. *Chem. Phys.* 213, 1-16.

18. Yokoe, H., and Meyer, T. (1996). Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat. Biotechnol*. 14, 1252-1256.

19. Patterson, G. H. and Lippincott-Schwartz, J. (2002). A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297, 1873-1877.

20. Elowitz, M.B., Surette, M.G., Wolf, P.-E., Stock, J., and Leibler, S. (1997). Photoactivation turns green fluorescent protein red. *Curr. Biol.* 7, 809-812.

21. Wiedenmann, J. (1997). Die Anwendung eines orange fluoreszierenden Proteins und weiterer farbiger Proteine und der zugehörenden Gene aus der Artengruppe Anemonia sp. (sulcata) Pennant, (Cnidaria, Anthozoa, Actinaria) in Gentechnologie und Molekularbiologie. Offenlegungsschrift DE 197 18 640 A1. *Deutsches Patent- und Markenamt*, 1-18.

22. Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. (1999). Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* 17, 969-973.

23. Wiedenmann, J., Elke, C., Spindler, K-D. & Funke W. (2000).Cracks in the β-can: fluorescent proteins from Anemonia sulcata. *Proc. Natl. Acad. Sci* USA. 97, 14091-14096.

24. Fradkov, A.F., Chen, Y., Ding, L., Barsova, E. V., Matz, M. V. , and Lukyanov, S. A. (2000). Novel fluorescent protein from Discosoma coral and its mutants possesses a unique far-red fluorescence. *FEBS Lett.* 479, 127-130.

25. Wiedenmann, J., Schenk, A., Röcker, C., Girod, A., Spindler, K.-D., and Nienhaus, G. U. (2002b). A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from Entacmaea quadricolor (Cnidaria, Anthozoa, Actinaria). *Proc. Natl. Acad. Sci.* USA. 99, 11646-11651.

26. Lukyanov, K.A., Fradkov, A.F., Gurskaya, N.G., Matz, M.V., Labas, Y.A., Savitsky, A.P., Markelov, M.L., Zaraisky, A.G., Zhao, X., Fang, Y., Tan, W., and Lukyanov, S.A. (2000). Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* 275, 25879-25882.

27. Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. (2003). Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* 21, 191-194.

28. Hofmann, M., Eggeling, C., Jakobs, S., and Hell, S.W. (2005). Breaking the diffraction barrier in fluorescence microscopy at low light intensities by using reversibly photoswitchable proteins. *Proc. Natl. Acad. Sci.* USA. 102, 17565-17569.

29. Andresen, M., Wahl, M.C., Stiel, A.C., Gräter, F., Schäfer, L.V., Trowitzsch, S., Weber, G., Eggeling, C., Grubmüller, H., Hell, S.W., and Jakobs, S. (2005). Structure and mechanism of the reversible photoswitch of a fluorescent protein. *Proc. Natl. Acad. Sci.* USA. 102, 13070-13074.

30. Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci*. USA. 99, 7877-7882.

31. Verkhusha, V.V., and Sorkin, A. (2005). Conversion of the monomeric red fluorescent protein into a photoactivatable probe. *Chem. Biol.* 12, 279-285.

32. Ando, R., Mizuno, H., and Miyawaki, A. (2004). Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* 306, 1370-1373.

33. Habuchi, S., Ando, R., Dedecker, P., Verheijen, W., Mizuno, H., Miyawaki, A., Hofkens, J. (2005). Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. *Proc. Natl. Acad. Sci*. USA. 102, 9511-95116.

34. Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E.A., Lukyanov, S., Lukyanov, K.A. (2004). Photoswitchable cyan fluorescent protein for protein tracking. *Nat. Biotechnol.* 22, 1435-1439.

35. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki A. (2002). An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci.* USA. 99, 12651-12656.

36. Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N., and Miyawaki, A. (2005). Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* 6, 1-6.

37. Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Röcker, C., Salih, A., Spindler, K.D., and Nienhaus, G.U. (2004). EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl. Acad. Sci.* USA., 101, 15905-15910.

38. Ivanchenko, S., Röcker, C., Oswald, F., Wiedenmann, J., and Nienhaus, G.U. (2005). Targeted Green-Red Photoconversion of EosFP, a Fluorescent Marker Protein. *J. Biol. Phys*. 31, 249-259.

39. Nienhaus, K., Nienhaus, G. U., Wiedenmann, J., and Nar, H. (2005). Structural Basis for Photo-Induced Protein Cleavage and Green-to-Red Conversion of Fluorescent Protein EosFP, *Proc. Natl. Acad. Sci.* USA 102, 9156-9159.

40. Nienhaus, G.U., Nienhaus, K., Hölzle, A., Ivanchenko, S., Röcker, C., Renzi, F., Oswald, F., Wolff, M., Schmitt, F., Vallone, B., Weidemann, W., Heilker, R., Nar, H., Wiedenmann, J. (2005/1). Photoconvertible Fluorescent Protein EosFP -.Biophysical Properties and Cell Biology Applications. *Photochem. Photobiol.*, doi: 10.1562/2005-05-19-RA-533

41. Wall, M.A., Socolich M., Ranganathan R. (2000). The structural basis for red fluorescence in the tetrameric GFP homolog DsRed. *Nat. Struct. Biol.* 12, 1133-1138.

42. Yarbrough, D., Wachter, R.M., Kallio, K., Matz, M.V., and Remington, S.J. (2001). Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution. *Proc. Natl. Acad. Sci.* USA., 98, 462-467.

43. Nienhaus, K., Vallone, B., Renzi, F., Wiedenmann, J., and Nienhaus, G.U. (2003). Crystallization and preliminary X-ray diffraction analysis of the red fluorescent protein eqFP611. *Acta Cryst.* D 59, 1253-1255.

44. Prescott, M., Ling, M., Beddoe, T., Oakley, A.J., Dove, S., Hoegh-Guldberg, O., Devenish, R.J., Rossjohn J., (2003). The 2.2 A crystal structure of a pocilloporin pigment reveals a nonplanar chromophore conformation. *Structure (Camb.)* 11, 275-284.

45. Petersen, J., Wilmann P.G., Beddoe T., Oakley A.J., Devenish R.J., Prescott M., and Rossjohn J., (2003). The 2.0-A crystal structure of eqFP611, a far red fluorescent protein from the sea anemone Entacmaea quadricolor. *J. Biol. Chem.* 278, 44626-44631.

46. Wilmann, P.G., Petersen, J., Devenish, R.J., Prescott, M., and Rossjohn, J. (2005). Variations on the GFP chromophore: A polypeptide fragmentation within the chromophore revealed in the 2.1-A crystal structure of a nonfluorescent chromoprotein from Anemonia sulcata. *J. Biol. Chem.* 280, 2401-2404.

47. Wiedenmann, J., Vallone, B., Renzi, F., Nienhaus, K., Ivanchenko, S., Röcker, C., and Nienhaus, G.U. (2005).The red fluorescent protein eqFP611 and its genetically engineered dimeric variants. *J. B. O.* 10, 014003 / 1-7.

48. Henderson, J.N., and Remington, S.J. (2005). Crystal structures and mutational analysis of amFP486, a cyan fluorescent protein from Anemonia majano. *Proc. Natl. Acad. Sci.* USA. 102, 12712-12717.

49. Remington S.J., Wachter R.M., Yarbrough D.K., Branchaud B., Anderson D.C., Kallio K., Lukyanov K.A. (2005). zFP538, a yellow-fluorescent protein from Zoanthus, contains a novel three-ring chromophore. *Biochemistry* 44, 202-212.

50. Nienhaus, K., Renzi, F., Vallone, B., Wiedenmann, J., and Nienhaus, G.U. (2006). Structural and Spectroscopic Analysis of Chromophore-Protein Interactions in the Anthozoan Green Fluorescent Protein asFP499. Submitted.

51. Labas, Y.A., Gurskaya, N.G., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Lukyanov, S.A., and Matz, M.V. (2002). Diversity and evolution of the green fluorescent protein family. *Proc. Natl. Acad. Sci*. USA. 99, 4256-4261.

52. Gross, L. A.,, Baird, G.S., Hoffman, R. C. Baldridge, K. K., and Tsien, R. Y. (2000). The structure of the chromophore within DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci.* USA. 97, 11990-11995.

53. Mizuno, H., Mal, T.K., Tong, K.I., Ando, R., Furuta, T., Ikura, M., and Miyawaki, A. (2003). Photo-induced peptide cleavage in the green-to-red conversion of a fluorescence protein. *Mol. Cell.* 12, 1051-1058.

54. Shagin, D.A., Barsova, E.V., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Labas, Y.A., Semenova, T.N., Ugalde, J.A., Meyers, A., Nunez, J.M., Widder, E.A., Lukyanov, S.A., and Matz, M.V. (2004). GFP-like proteins as ubiquitous metazoan superfamily: evolution of functional features and structural complexity. *Mol. Biol. Evol.* 5, 841-850