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Fluorescent Proteins II

Application of Fluorescent Protein Technology

Volume Editor:
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Aims and Scope

Fluorescence spectroscopy, fluorescence imaging and fluorescent probes are indispensible tools in numerous fields of modern medicine and science, including molecular biology, biophysics, biochemistry, clinical diagnosis and analytical and environmental chemistry. Applications stretch from spectroscopy and sensor technology to microscopy and imaging, to single molecule detection, to the development of novel fluorescent probes, and to proteomics and genomics. The *Springer Series on Fluorescence* aims at publishing state-of-the-art articles that can serve as invaluable tools for both practitioners and researchers being active in this highly interdisciplinary field. The carefully edited collection of papers in each volume will give continuous inspiration for new research and will point to exciting new trends.

Preface

A plethora of reviews, popular science books, and scientific textbooks have been written on the significance of fluorescent proteins in the life sciences. More than 30,000 references can be found in bibliographic databases which refer to at least one among the members of this protein family (see Fig. 1). Most of these narrate on how fluorescent proteins may be used to label gene products, how they may be visualized in cellular compartments by fluorescence microscopy, or how they may be expressed in individual cells, thus provoking novel findings in ontogenesis. In most of the experiments described, fluorescent proteins are being exploited as miniaturized light bulbs, the length scale is that of microns, and the time scale is that of seconds or longer. There is no doubt that fluorescent protein technology has revolutionized life sciences in that proteins have become universal and standard tools in molecular biology laboratories.

A minor fraction of roughly 5% of all publications deals with the *nanoscopic* properties of fluorescent proteins (FPs) acting as light bulbs. Early achievements include the crystallographic analysis of their molecular structure [1, 2], the discovery of excited-state proton transfer in the naturally occurring FP [3, 4], and the erratic light emission of individual members of FPs [5, 6]. Especially the last experiments, along with low temperature studies [7, 8], have revealed that FPs exhibit a tremendous heterogeneity in terms of structure and dynamics.

It is therefore not astonishing that FPs have had a large impact on other areas of biophysical research, e.g., in studies on protein folding [9–11]. However, the irregular emission of light by FPs also has impacted experiments in the life sciences: most operators of fluorescent protein technology, whom I was talking to, were concerned about weird experimental features like rapid initial fading in time-lapse microscopy, sometimes with sudden fluorescence recovery, or changing FRET-ratios upon continuous illumination. Such annoying findings can be traced back to the wealth of light-driven processes in the proteins, and I am quite sure that more surprises of that kind have been experienced by others. It should be emphasized here that such “strange” photodynamics have initiated seminal studies on protein diffusion and high-resolution microscopy [12–14].

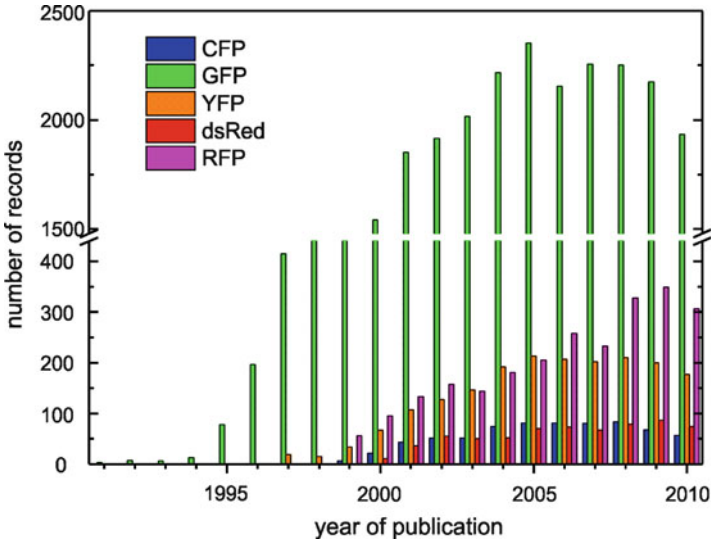

Fig. 1 Number of references related to fluorescent proteins (database: web-of-science). The number of articles dealing with Green Fluorescent Protein has reached saturation now at a level of typically 2,000 articles per year for almost a decade. Those on the Red Fluorescent Proteins are still increasing

Volumes 11 and 12 of the *Springer Series on Fluorescence* deal with various aspects of fluorescent proteins. The first volume (*Fluorescent Proteins I*) is devoted to the molecular, i.e., mainly optical, properties of fluorescent proteins. In the first part, the primary processes leading to fluorescence are discussed: excitation, relaxation, and other processes in the excited state and in emission. Fluorescence proteins are treated as "ordinary" fluorophores, and one article is highlighting our opportunities to circumvent the synthetic limitations given by nature. The second part focuses on the mechanisms that make the difference to conventional fluorophores: isomerization, protonation, as well as reversible and irreversible photochemical reactions. The knowledge on how these processes are affected by the surrounding of the FP allows for tailoring it with respect to spectacular applications, applications that are not conceivable with "ordinary" fluorophores.

In the second volume (*Fluorescent Proteins II*), the key aspect is on applications. Its first part is giving an overview on how many unconventional photophysical properties latently exist in naturally occurring and how double-resonance experiments enable the information to be extracted from microscopy data in an unprecedented way. More on high-resolution microscopy will be found in forthcoming volumes of this series. Quantitation, a central objective of analysis, is the comprehensive caption of the articles in the next part. We may state, justifiably, that researchers have reliable tools at hand to quantify some of the most abundant ions after more than a decade of development. Other physiological parameters of

overwhelming importance like the transmembrane potential still need to experience this development. The last part reports on three examples of utmost biological relevance and how ultrasensitivity in bioanalysis, i.e., single-molecule technology, is merged with FP technology. This combination has resulted in an understanding of processes on a molecular level and in detection limits that were not even thought of some 15 years ago.

A preface is also always the occasion to deeply acknowledge the support by others. First of all, I have to thank my family who tolerated my commitment to this experience. I also would like to express my thanks to my coworkers, to my colleagues, and to the representatives of Saarland University for their understanding. In times of growing competitiveness in many academic areas, it is not self-evident to dedicate a substantial amount of time to such a book project. For the same reason, I especially appreciate the immense work of all authors of these two volumes who are all passionate, but busy scientists and who (more or less) voluntarily spared no pains to complete their manuscripts in a wonderful and highly professional way. By now, it also may be appropriate to apologize for my e-mail bombardments!

Saarbrücken, Germany Gregor Jung

References

1. Ormö M et al (1996) Science 273:1392–1395
2. Yang F et al (1996) Nat Biotechnol 14:1246–1251
3. Chattoraj M et al (1996) Proc Natl Acad Sci USA 93:8362–8267
4. Lossau H et al (1996) Chem Phys 213:1–16
5. Dickson R et al (1997) Nature 388:355–358
6. Pierce D et al (1997) Nature 388:338
7. Creemers T et al (1999) Nat Struct Biol 6:557–560
8. Seebacher C et al (1999) J Phys Chem B 103:7728–7732
9. Craggs T (2009) Chem Soc Rev 38:2865–2875
10. Hsu S et al (2009) Chem Soc Rev 38:2951–2965
11. Mickler M et al (2007) Proc Natl Acad Sci USA 104:20268–20273
12. Yokoe E, Meyer T (1996) Nat Biotech 14:1252–1256
13. Patterson G, Lippincott-Schwartz J (2002) Science 297:1873–1877
14. Betzig E et al (2006) Science 313:1642–1645

Contents

Part I
Fluorescence Microscopy Beyond Imaging

Fluorescent Proteins: Nature's Colorful Gifts for Live Cell Imaging

Jörg Wiedenmann, Cecilia D'Angelo, and G. Ulrich Nienhaus

Abstract Fluorescence of marine organisms has fascinated researchers since the early twentieth century. The successful application of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in 1994 as genetically encoded marker resulted in a massive increase in interest for naturally fluorescent proteins. Methods are now established that allow the fast isolation of new genes encoding GFP-like proteins from marine creatures, resulting in an impressive array of glowing proteins with different biochemical and optical properties. Protein engineering has been applied to render natural variants into advanced optical tools for live cell imaging, promoting studies of protein localization and movement, gene activity, sensing of intra- and extracellular condition, and tracking of whole cells and organisms. Finally, photoactivatable proteins were discovered that enable pulse-chase experiments and live cell imaging of proteins with a resolution beyond the diffraction barrier of optical microscopy. Phylogenetic sequence analyses revealed interesting details about the molecular evolution of these proteins including the convergent evolution of colors. Marine organisms, especially corals, still harbor a huge number of GFP-like pigments, the majority of which are yet to be studied. Consequently, further important discoveries of useful marker proteins can be expected in the future.

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Contents

1 Introduction

The functional expression of the green fluorescent protein (GFP) in a nematode worm launched the era of live cell imaging and opened up new horizons for biomedical research [1]. Initially discovered during studies of jellyfish bioluminescence by Osamu Shimomura, the unusual biochemical properties soon made GFP from *Aequorea victoria* (avGFP), an indispensable tool for cell biology [2]. The protein can be expressed in its functional form in virtually any type of cell, facilitating the use of GFP as a genetically encoded marker of gene activity or for tracking of proteins in living cells [2]. The outstanding impact of GFP technology on life sciences research was recognized by the award of the Nobel Prize in Chemistry 2008 to Osamu Shimomura, Martin Chalfie, and Roger Tsien for the "discovery and development of the green fluorescent protein, GFP". Multicolor labeling was enabled by the generation of blue and yellow variants, and GFP-based sensor systems were developed that report changes in both intracellular and extracellular conditions. The discovery of GFP-like proteins in non-bioluminescent sea anemones and related organisms gave access to a great variety of homologous proteins with novel optical properties [3–6]. The gene hunt in the oceans resulted in novel variants including red fluorescent and photoactivatable proteins [7–9]. The evolution of the diverse spectroscopic properties of GFP-like proteins was analyzed using molecular biology and bioinformatics tools [10]. The phylogenetic tree of GFP-like proteins shows a clustering of optical features in certain taxonomic groups. This knowledge can be exploited in targeted searches for novel lead structures. Here, we outline the history of the development of the fluorescent marker protein technology, introducing marine organisms as source of novel marker proteins that enable fascinating live cell imaging applications.

2 Natural Sources of Fluorescent Proteins

2.1 History of Fluorescent Protein Research

The striking phenomenon of cnidarian bioluminescence in the marine realm was first described by Pliny the Elder (first century AD) and by Claudius Aeliani (second century AD) [11]. About eighteen centuries passed until the GFP of the hydromedusae *A. victoria* (avGFP) emanated as a "by-product" from studies of bioluminescence [12].

In 1925, Harvey observed the appearance of bluish fluorescence in previously nonfluorescent, light-emitting tissue upon stimulation of luminescence of the ctenophore *Mnemiopsis* [13]. The fluorescence was probably emitted by a substance similar to the blue fluorescent protein that appears as an intermediate in the luminescence reaction of the photoprotein aequorin isolated from *A. victoria* [12, 14, 15]. A yellow-green fluorescence was observed in the luminescent tissue of *Aequorea* and *Halistaura* [16]. During isolation and characterization of the photoprotein aequorin, Shimomura et al. identified the green fluorescent pigment of *A. victoria* as a protein [12].

FPs were also described for the hydroid *Obelia*, the hydromedusae *Aequorea*, and the pennatullacean *Renilla* [17–19]. In vitro, the bioluminescence reaction of these species produces bluish light with broad emission spectra, with maxima between 460 and 486 nm [18, 19]. However, the in vivo luminescence showed narrow peaks with maximal emission at 508 nm, matching the fluorescence emission spectrum of GFPs. The absence of the blue emission during the in vivo luminescence reaction indicates a non-radiative energy transfer between the light-generating proteins (luciferases, aequorin) and GFP [18]. GFPs or tissue fluorescence peaking around 508 nm was identified in numerous bioluminescent hydromedusae, hydropolyps, and pennatularians [11, 18–26]. GFPs with shorter emission wavelengths were found in *Halistaura* (497 nm) and *Phialidium* (498 nm) [26, 27]. In these bioluminescent cnidarians, GFPs were exclusively found in the photogenic cells [11, 24, 28].

The imidazolone structure of GFP was proposed by Shimomura in 1979 and later confirmed by Cody et al. [29]. During these early years of GFP research, details about the biochemical and optical properties also became available [27].

Finally, Prasher and coworkers determined the amino acid sequence of avGFP in 1992 [30]. The application potential of avGFP was fully realized when Chalfie and coworkers achieved the functional expression in the nematode worm *Caenorhabditis elegans* [1]. Their utility as genetically encoded marker is enabled by the autocatalytic formation of the chromophore in the presence of molecular oxygen [31–33].

The possibility to produce avGFP in unlimited quantities in recombinant systems stimulated further research on the biochemical and photophysical properties [2]. The molecular structure of avGFP was resolved by X-ray crystallography [34, 35], which enabled rational approaches to molecular engineering of the protein. Finally,

the possibility to alter the amino acid sequence by mutagenesis techniques opened the opportunity to customize GFP for imaging applications. These studies yielded, for instance, blue- and yellow-shifted emitters useful for multicolor labeling [2].

Today, it is well established that marine cnidarians host a variety of GFP-like protein pigments. However, already before the recent systematic studies, these pigments attracted sporadic interest from researchers. In 1927, UV-induced green fluorescence was demonstrated for a sea anemone from a rock pool in Great Britain [36]. Kawaguti noted in 1944 that green pigments of scleractinian corals in Palao exhibited green fluorescence [37].

Red fluorescence from a sea anemone was first observed by Marden [38] during a dive in the Red Sea. At a depth of 20 m, where the red components of the downwelling light are readily attenuated from the spectrum, a sea anemone appeared in bright red. He explained the phenomenon by the presence of red fluorescent pigments excited by blue-green light. Wobber [39] documented the red fluorescence of *Corynactics californicus* by photographing the animals under natural light at a depth of 40 m. A note on the fluorescence of the corals *Montastrea cavernosa* and *Mussa angulosa* was published by Read [40]. Orange and red fluorescence could be induced by exciting the corallimorpharian *C. californicus*, the coral *Balanophyllia elegans*, and a tube anemone *Cerianthus* sp. with ultraviolet, blue or green light [41]. Using UV light for excitation, Catala described the fluorescence of numerous corals [42–45]. Species belonging to 16 genera displayed fluorescence. For example, representatives of the genus *Flabellum* collected at a depth of 35–40 m showed intensive green fluorescence in the fleshy parts, whereas *Trachyphyllia* emitted orange fluorescence. In some specimens, he also observed a change of fluorescence color from green to pink upon prolonged or frequent irradiation with UV light [42]. UV-induced fluorescence was reported also for various corals, corallimorpharians, and actiniarians under irradiation [46].

A chromatophore system containing fluorescent pigments was found in the entodermal layer of the coral *Leptoseris fragilis* [47–50].

Mazel [51–55] provided photographic documentation and spectral characterization of fluorescent pigments of corals, corallimorpharians, and sea anemones from the Caribbean Sea. The pigments could be arranged in four major classes, with emission maxima around 486, 515, 575, and 685 nm. The red emission peaking at 685 nm could be attributed to chlorophyll of the symbiotic algae [54, 55]. Salih et al. [56] found fluorescent morphs among 124 species of 56 genera of Great Barrier Reef corals.

Despite the urgent need for red fluorescent marker proteins, the red-shifted emitters found in non-bioluminescent cnidarians were not considered as potential candidates for two reasons. (1) It was assumed that these pigments represent flavin-like compounds or phycobiliproteins. Both pigment types are products of complex biosynthesis pathways and therefore not suitable as genetically encoded markers. (2) In those days, GFPs were found only as secondary emitters in bioluminescent organisms and, consequently, their existence in non-bioluminescent cnidarians was ruled out.

The GFP-like protein nature of green and red fluorescent and the nonfluorescent pink pigments was realized by Wiedenmann in 1997 [4] and confirmed by the

cloning of several FPs with emission colors from cyan to red by Matz et al. [3] and Wiedenmann et al. [5, 6].

The following years yielded numerous natural FPs with novel spectral properties [57–73]. Protein engineering rendered them in even more useful tools. Milestones of the discovery and engineering of fluorescent proteins are outlined in Fig. 1.

2.2 *Marine Organisms as Sources of GFP-Like Properties*

2.2.1 Distribution Among Animal Phyla

As yet, GFP-like proteins have only been isolated from marine organisms (Fig. 2). Most of them belong to the phylum cnidaria [10]. However, green fluorescent homologs were also isolated from the taxa crustacea [65], ctenophora [74], and chordata [75]. The wide distribution suggests that, in principle, any metazoan organism can harbor GFP-like proteins. However, the taxon anthozoa proved to be the most rewarding source for innovative fluorescent marker proteins such as red fluorescent and photoactivatable proteins [7, 9].

2.2.2 Color Morphs

The existence of several morphs with striking color differences is common among many species of reef corals and sea anemones [76–79]. Already in the nineteenth century, numerous color morphs of *Anemonia sulcata* (=viridis) were described [80–82]. Five distinct color morphs of this species can be distinguished based on the presence of four GFP-like proteins in the tentacles [77] (Fig. 3). Also the morphs of the reef coral *M. cavernosa* owe their colors to differing tissue concentrations of cyan, green, and red fluorescent proteins [61, 79]. Interestingly, color morphs of both *A. sulcata* and *M. cavernosa* express the whole collection of pigments characteristic for each species. The color differences result from transcript levels that differ relative to each other among the morphs [61, 83]. This implies that novel GFP-like proteins can be discovered that display colors different from the color of the animal under study. Our studies of the sea anemone *Calliactis parasitica* revealed that red fluorescent proteins can even be cloned from animals that appear to be nonfluorescent ([67]; Gamber and Wiedenmann, unpublished).

2.2.3 Distribution in the Organism

GFP-like proteins can contribute up to 14% to the total soluble cellular proteins in the expressing tissue of some corals [79, 83]. In contrast to bioluminescent cnidarians where the expression of GFPs seems to be restricted to the photogenic tissue,

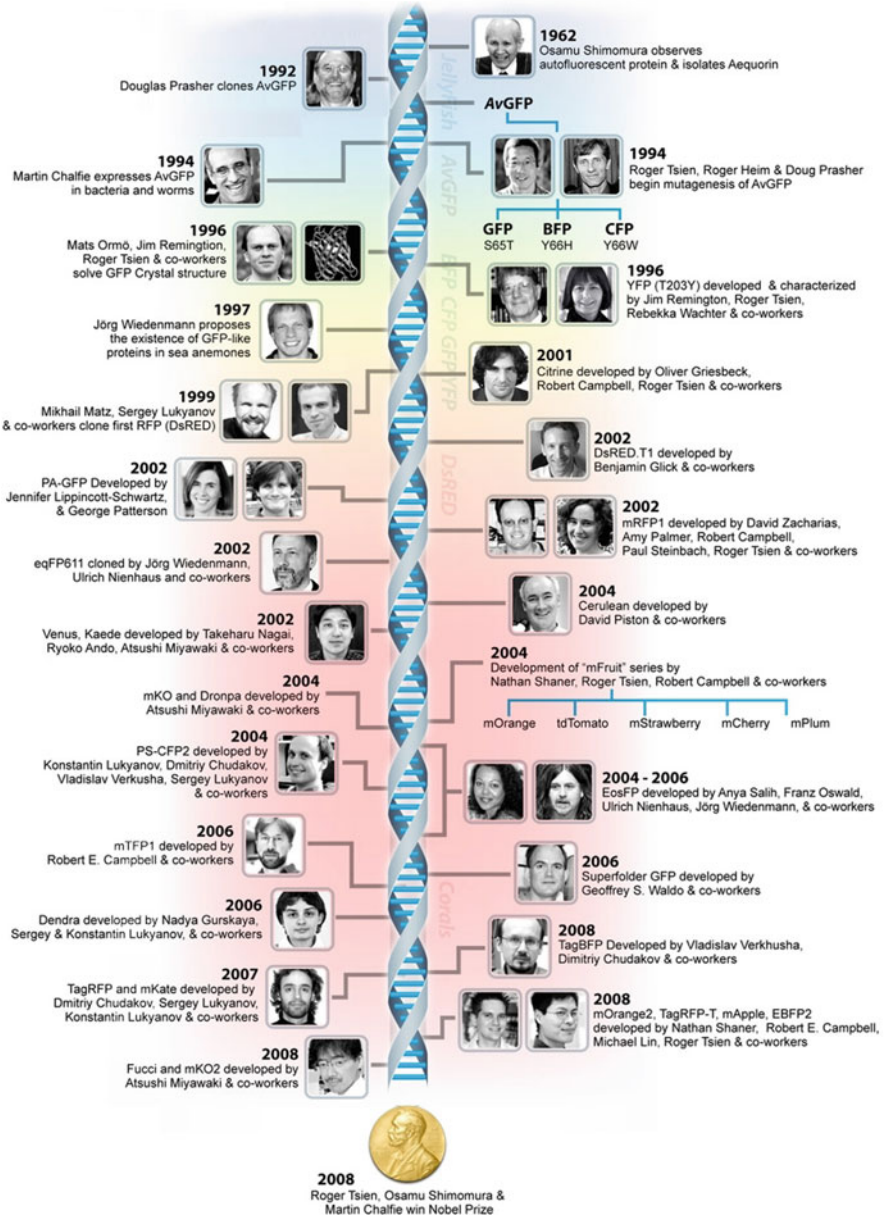

Fig. 1 Timeline of discovery and engineering of fluorescent proteins from cnidarians. This modified version of an image from reference [8] is a kind gift of Michael W. Davidson, Florida State University

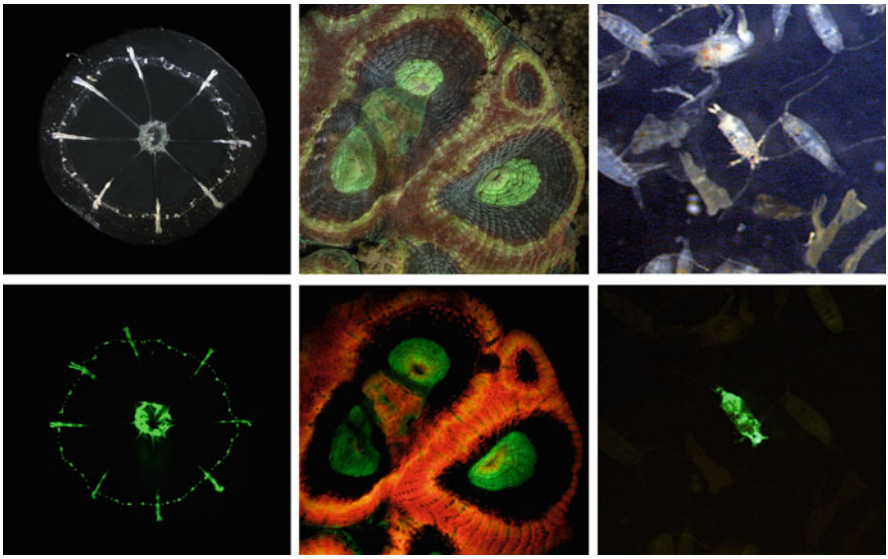

Fig. 2 Marine organisms as sources of fluorescent proteins. The *upper row* shows daylight photographies of a hydrozoan jellyfish (*left*), a faviid coral (*center*) from the Great Barrier Reef and plankton sample (mainly copepods) from the English Channel (*right*). Fluorescence images show the distribution of green and red fluorescent proteins. Only the calanoid copepod in the center of the image contains GFPs

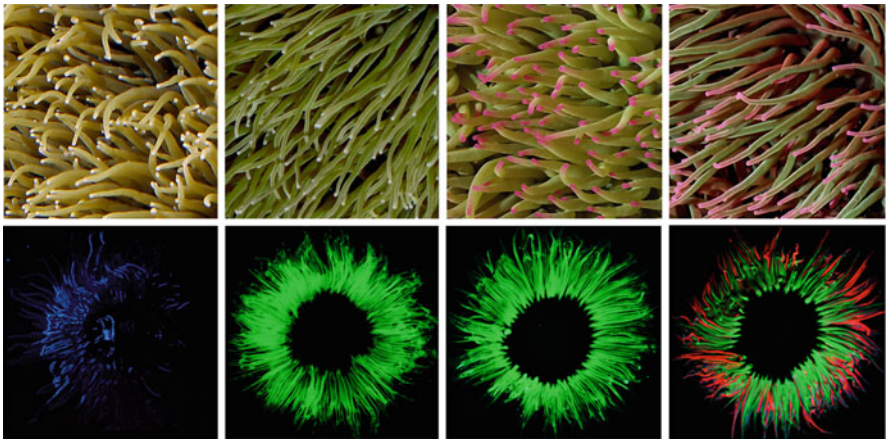

Fig. 3 Color morphs of *Anemonia sulcata* (=*viridis*) are defined by the tentacle content of green fluorescent proteins (asF499; asFP522), a red fluorescent protein (asFP595), and nonfluorescent, pink chromoproteins (asCP562). From *left* to *right*: var. rustica (no detectable content); var. viridis (GFPs); var. smaragdina (GFPs, asCP562); var. rufescence (GFPs, asFP595, asCP562) (not shown: var. vulgaris with asCP562). Animals were photographed under daylight (*upper row*) and UV (366 nm) light (*lower row*)

no uniform picture of the distribution of GFP-like proteins exists in non-bioluminescent anthozoans [11, 24, 28, 84].

Sea anemones and reef corals, in particular, show differing expression patterns of cyan, green, and red fluorescent proteins and nonfluorescent chromoproteins [77, 78, 83, 84]. Nevertheless, a few notable tendencies do exist. In many corals and sea anemones, the GFP-like proteins are found in highest abundance in the light-exposed part of the animals, such as the upper side of a colony or in the tentacles of polyps [77, 85]. Specifically, nonfluorescent chromoproteins are often localized in the tips of tentacles or branches and growth margins of coral colonies [77, 85]. Large differences in the distribution of GFP-like proteins in the tissue of scleractinian corals have also been documented at the microscopic scale.

Peloux [86] demonstrated that fluorescence was emitted from pigment granules localized in the entoderm in *Goniopora lobata*. In contrast, in *Euphyllia picteti*, the pigment granules were found in elongated cells of the ectoderm [86]. The orange-red fluorescent pigment of the solitary coral *Cynarina lacrymalis* appeared to be distributed in a diffuse, nongranular manner in the ectoderm. Kawaguti observed a membrane surrounding the pigment granules of *Lobophyllia robusta* as well as a close proximity of smaller granules with the endoplasmatic reticulum [87]. In the ectodermal cells of *Isophyllia sinuosa*, pigment granules are often surrounded by two membranes [88].

Salih and coworkers found that fluorescent granules were localized mainly above the symbiotic algae in corals from high light habitats, whereas in specimens from low light habitats, they were dispersed among or under the zooxanthellae layer [56]. Both, in *Lobophyllia hemprichii* and *M. cavernosa*, fluorescent pigments can engulf the vacuoles containing the symbiotic algae [79] (Fig. 4).

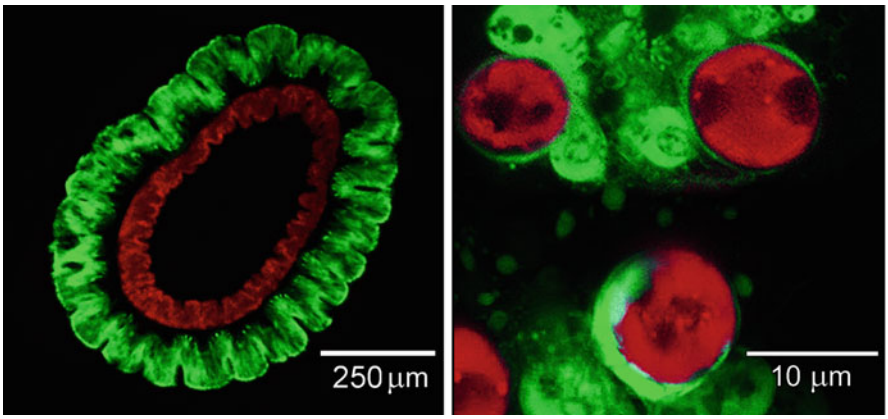

Fig. 4 Microscopic images of the distribution of GFPs in the tissue of anthozoans. The tentacle cross-section of the sea anemone *Anemonia sulcata* (var. smaragdina) (*left*) shows a dominant localization of GFPs in the ectodermal cells. The red fluorescence of the gastrodermal cells is caused by the chlorophyll of the zooxanthellae. In the reef coral *Montastrea cavernosa* (*right*), GFPs are also found in gastrodermal cells surrounding the red fluorescent zooxanthellae. Images modified from references [79, 89]. Copyright Elsevier 2007 and 2007 John Wiley & Sons Ltd

The green and red fluorescent pigments of the sea anemone *A. sulcata* are evenly distributed in the ectodermal cells of the tentacles [89] (Fig. 4).

2.2.4 Regulation of GFP-Like Protein Expression in Cnidarians

In many reef building corals, both fluorescent and nonfluorescent GFP-like proteins are most abundant in light-exposed parts of the colonies. For numerous species, this can be explained by a strong response of FP-encoding genes to the intensity of light experienced by the corals [85]. The blue region of the spectrum proved to be most effective in stimulating the expression of FPs on the transcriptional level. Interestingly, different groups can be distinguished based on their response to changes in the light climate (Fig. 5). Members of the low threshold group are upregulated already at low light intensities; however, under higher light levels, the tissue concentration of these proteins reaches a plateau or even decreases. In contrast, the amount of FPs belonging to the high threshold groups is negligible under low light, but increases nearly proportional to the amount of incident photons [85]. Representatives of a third group express FPs at high levels independent of the light intensity in the habitat [79, 83]. A striking example of light-independent expression of GFPs are tube anemones from the deep sea that are brightly fluorescent in the virtual absence of sunlight [90] (Fig. 5).

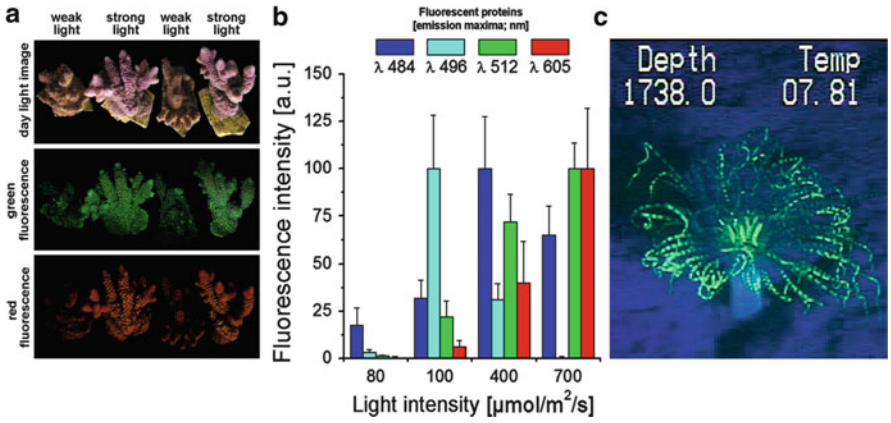

Fig. 5 Differential expression of fluorescent proteins in *Acropora millepora* in response to the light intensity (**a**). Cyan fluorescent proteins (amFP584, amFP496) are downregulated at high light intensities. Tissue concentrations of green and red fluorescent proteins (amFP512; amFP605) increase nearly proportional with the amount of incident light (**b**). The graph shows averages of five replicate measurements. Error bars represent standard deviation. (**c**) Fluorescence image of a tube anemone (ceriantharia) acquired in 530 m depth in the Gulf of Mexico. The depth (ft) and temperature (Temp, °C) are displayed. Panel (**c**) modified from [90] with permission

2.2.5 Spectral Properties of Natural GFP-Like Proteins

Most of the GFPs isolated from bioluminescent hydrozoa and anthozoa, such as *Renilla* or *Aequorea*, show a narrow emission spectrum peaking at ~508 nm and act as secondary emitters in the chemiluminescence reaction [11, 27]. Exceptions are found in the fluorescent proteins from *Phialidium* sp., where both blue-shifted (498 nm) and red-shifted (537 nm) emission maxima were observed and in *Halistaura* GFP which has also a blue-shifted (497 nm) emission maximum [26, 27, 65]. Regarding the position of excitation maxima, considerable differences were detected among GFPs from bioluminescent cnidaria. They were localized at 465 nm (Halistaura GFP), 485 nm (Phialidium GFP), 498 nm (Renilla GFP), and 525 nm (Phialidium YFP) [26, 27, 65]. The excitation spectrum of avGFP is characterized by a major maximum at 398 nm and a side maximum at 475 nm.

The set of fluorescent proteins from non-bioluminescent anthozoa FPs can be grouped in cyan, green, yellow, and red fluorescent proteins (Fig. 6). Cyan fluorescent proteins (CFPs) show excitation maxima of 400–460 nm and emission maxima of 480–486 nm. GFPs usually have excitation maxima between 480 and 518 nm and show emission spectra peaking between 490 and 522 nm. The excitation of some GFPs such as asFP499 show a second, more or less pronounced, maximum at ~400 nm [5]. This band corresponds to the absorption of the neutral chromophore and contributes to the excitation spectrum via excited state proton transfer [91, 92] (Fig. 6).

A GFP (Dronpa) cloned from a pectinidae coral shows an interesting photoswitching behavior [93]; green fluorescence peaking at 518 nm is emitted when Dronpa is irradiated with light around the excitation maximum at 503 nm. However, the protein undergoes photoconversion to a nonfluorescent state with an absorption maximum at 390 nm when being excited with such wavelengths. Conversely, upon excitation of the nonfluorescent form at 390 nm, the green fluorescent state is almost completely restored. Photoswitchable GFPs were also isolated from a deep sea cerianthid and a ctenophore [74, 90].

So far, only one truly yellow fluorescent protein (YFP) has been cloned from non-bioluminescent anthozoans (*Zoanthus* sp.) showing excitation/emission maxima at 528/538 nm [3]. Finally, the group of naturally red fluorescent proteins have emission maxima ranging from 583 to 611 nm. In this group, excitation spectra are characterized by maxima between 558 and 574 nm. The maturation of both yellow and red fluorescent proteins can produce minor amounts of green fluorescent side products [94]. These green fluorescent states have excitation and emission maxima at 475–500 nm and 500–520 nm, respectively [6, 95]. Their contribution to the total fluorescence depends on the protein. In eqFP611, the residual green fluorescence is less than 1% of the red emission [6], while in dsRed the contribution is considerably higher [94–96]. Fluorescence resonance energy transfer (FRET) can be observed if green and red emitting states of GFP-like proteins are present within a single tetramer [64, 95].

A special case is represented by photoconverting proteins such as Kaede or EosFP [59, 64, 65, 79]. Upon expression, these proteins form a green fluorescent

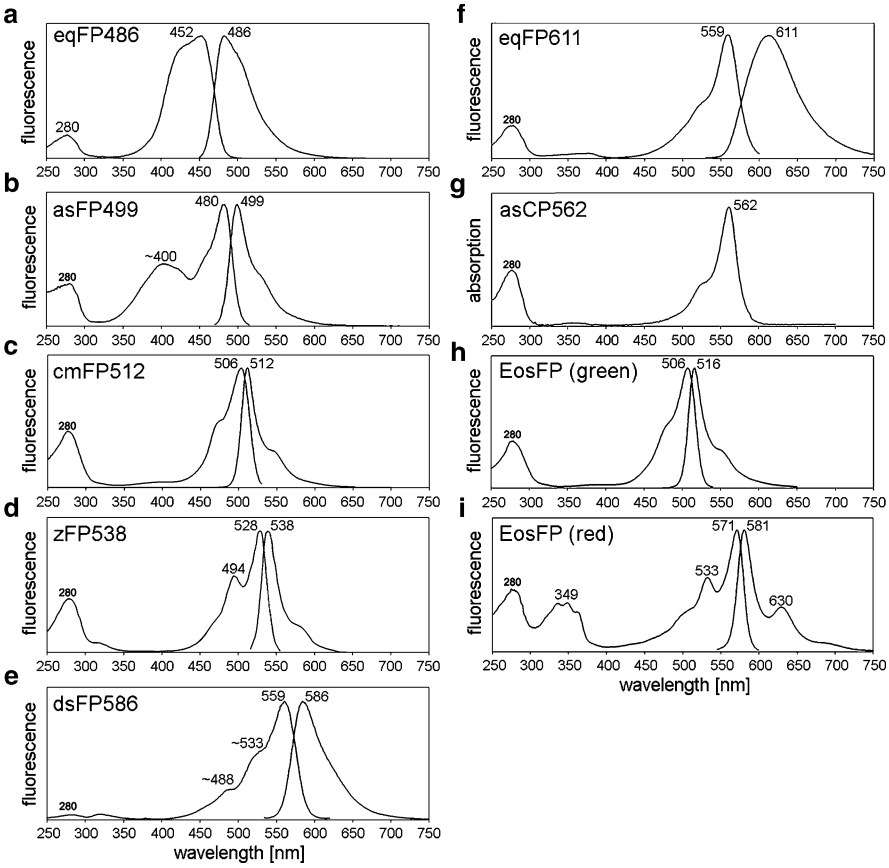

Fig. 6 Spectral properties of major color classes of GFP-like proteins. Panels (**a**)–(**i**) show the excitation and emission spectra of fluorescent proteins or the absorption spectrum in the case of the nonfluorescent chromoprotein asCP562. (**a**) CFP (eqFP486) from *Entacmaea quadricolor* (Wiedenmann, unpublished). (**b**) GFP (asFP499) from *Anemonia sulcata* [5]. (**c**) GFP (cmFP512) from *Cerianthus membranaceus* [67]. (**d**) YFP (zFP538) from *Zoanthus* [3] sp. (**e**) RFP (dsFP586) from *Discosoma* sp. (Wiedenmann, unpublished). (**f**) RFP (eqFP611) from *Entacmaea quadricolor* [6]. (**g**) Pink chromoprotein asCP562 from *Anemonia sulcata* [5]. (**h**) Green-to-red photoconverting protein EosFP from *Lobophyllia hemprichii* before, and (**i**) after photoconversion [64]. zFP538 spectra are a courtesy of M. Matz

state with excitation and emission maxima around 506 and 516 nm, respectively. Irradiation with ~390 nm light irreversibly changes the proteins to red emitters (λ_{max} ~ 581 nm) with an excitation maximum at ~572 nm [64].

Aside from the brightly fluorescent members, the GFP family also comprises a large number of homologous proteins that show intense color but no visible fluorescence [10, 68, 77, 78, 85]. Some of these nonfluorescent chromoproteins can, however, exhibit very weak red fluorescence detectable in a spectrophotometer [67, 68]. For asulCP, a quantum yield of <0.001 was determined. In contrast, with

a quantum yield of <0.0001, the blue chromoprotein Rtms5 from *Montipora efflorescens* is de facto nonfluorescent [97].

The chromoproteins asulCP and asCP562 undergo conversion into a red fluorescent state upon excitation with green light ([68]; Wiedenmann and Girod, unpublished). The process can be reversed by irradiation of the red fluorescent protein with blue light [68]. Photoswitching is associated with a *trans–cis* isomerization of the chromophore [98].

2.2.6 Structural Properties

In solution, avGFP exists as monomer at concentrations below 1 mg/ml [27]. At higher concentrations, dimerization occurs with a K_d of 0.1 mM [99]. In contrast, GFPs from *Renilla* sp., *Phialidium gregarium*, and *Halistaura* (*Mitrocoma*) *cellularia* form stable non-dissociatable dimers [27].

Throughout the different color classes of GFP-like proteins, the β-can fold is almost perfectly conserved (Fig. 7). However, most GFP-like proteins from anthozoans exist as homo-tetramers [6, 59, 60, 64, 67]. In some cases, the oligomerization tendency is reduced [6]. Among others, the cyan FP MiCy from a scleractinian coral forms a dimeric association [63].

The rigid β-can fold of coral FPs can help to explain the long half-lives of up to 3 weeks determined in vivo for the corals *L. hemprichii* and *M. cavernosa* [83] (Fig. 7).

The 4-(p-hydroxybenzylidene)-5-imidazolinone structure of the avGFP chromophore appears to be universally involved in the development of chromogenic

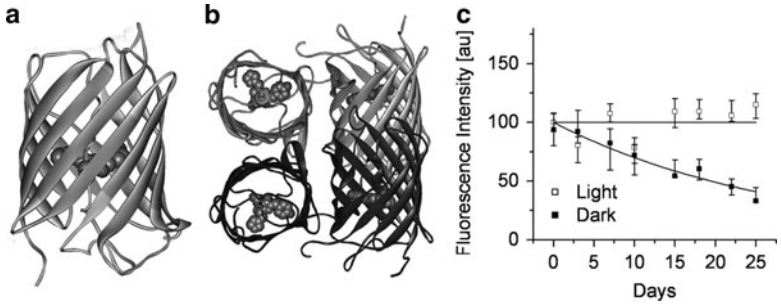

Fig. 7 Structural properties of GFP-like proteins (**a**) The β-can fold of EosFP from the reef coral *Lobophyllia hemprichii*. (**b**) Tetrameric assembly of EosFP. (**c**) Protein turnover measured in vivo and in situ in *L. hemprichii*. The diagram shows the decay of the tissue fluorescence at 581 nm in animals kept in the dark. Under these conditions, green-to-red photoconversion of newly synthesized protein is prevented, and the decrease of red tissue fluorescence can be used as indicator of the decay of the red fluorescent form of EosFP that was present at the beginning of the experiment. No changes in tissue fluorescence can be detected in illuminated corals. The diagrams show the medians; error bars display the first and third quartiles. Data from the dark-treated animals were fitted with an exponential decay. Panel (**c**) reprinted with permission from [83] (Copyright 2007 John Wiley & Sons Ltd)

properties in both fluorescent and nonfluorescent GFP homologs [7]. The conservation of the second and third chromophore-forming amino acids, tyrosine and glycine, and catalytic residues such as arginine in homologous positions to Arg96 in avGFP indicate that the basic mechanisms of chromophore formation are similar. Pronounced differences exist in regard to the modification of the GFP-type chromophore that yields the structures responsible for cyan, yellow, and red fluorescence or nonfluorescent pink to blue colors [7].

Structural features are discussed in greater detail in the chapter "Structure-Function Relationships in Fluorescent Marker Proteins of the GFP Family".

2.2.7 Molecular Evolution of GFP-Like Proteins

At the moment, more than 100 sequences coding for GFP-like proteins are available from databases, many of them awaiting further characterization of the coded protein. The length of the amino acid sequence of these FPs range from 225 (e.g., dsRed) to 238 (avGFP). One exception is the protein cFP484 from *Clavularia* sp. [3] showing a length of 266 amino acids. The length and composition of both the N- and the C-terminus vary considerably among different FPs (Fig. 8). The core protein is characterized by alternating conserved and variable regions. One striking feature is the strict conservation of the tyrosine–glycine motif within the chromophore. Moreover, the amino acids arginine and glutamine in positions homologous to residues Arg96 and Glu222 in avGFP are strictly conserved. These amino acids are thought to be involved in the autocatalytic formation of the GFP chromophore

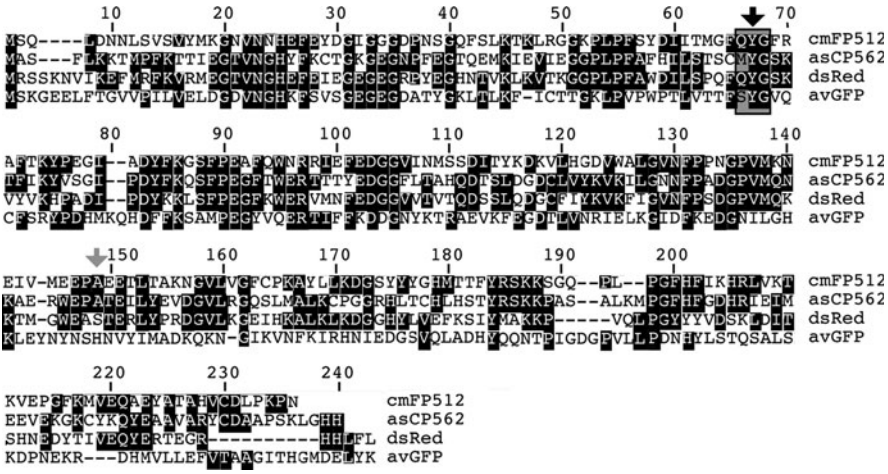

Fig. 8 Multiple sequence alignment of avGFP, a GFP (cmFP512 from *Cerianthus membranaceus* [67]), a nonfluorescent chromoprotein (asCP562 from *Anemonia sulcata* [5]), a red fluorescent protein (dsRed from *Discosoma* sp. [3]). Conserved residues are shaded in *black*. The chromophore-forming amino acids are shaded in *gray* and marked by a *black arrow*. The position corresponding to histidine 148 in avGFP is highlighted by a *gray arrow*

[32, 35, 100–102] (Fig. 6). In contrast, the first residue of the chromophore appears to be rather variable among differently colored GFP-like proteins. Consequently, prediction of the spectral properties based on this position is not reliable. Glutamine can serve as the first chromophore residue in both green and red fluorescent proteins [67] (Fig. 8). An exception is histidine in the first position of the chromophore. In all FPs examined so far, this type of chromophore underwent photoconversion from a green to a red fluorescent state upon irradiation with UV light [59, 64, 65, 103, 104].

In addition, the position corresponding to His148 in avGFP can be indicative of the spectral properties of GFP-like proteins. In highly fluorescent proteins, this residue is a serine, while in nonfluorescent GFP-like proteins this position is occupied by, for instance, alanine, cysteine, or aspartate [3, 73]. However, the proteins eqFP611 [6] and cmFP512 [67] provide exceptions to the rule. These proteins possess amino acids different from serine in the homologous position, aspargine and alanine, respectively, but exhibit a strong red (eqFP611) or green (cmFP512) fluorescence [6, 67, 105].

Molecular phylogeny approaches performed on amino acid sequences of FPs showed that the proteins group often according to the classical taxonomy of the host animals rather than to their spectral properties [5, 10, 58, 65, 106] (Fig. 9). The phylogenetic trees of GFP-like proteins suggest a multiple convergent evolution of red fluorescent proteins and nonfluorescent chromoproteins [10, 65].

The red fluorescent proteins dsRed, Kaede, and EosFP, for instance, show quite similar spectral properties: dsRed has an emission maximum at 583 nm, whereas the emission spectrum of red-converted Kaede and EosFP peaks around 581 nm. Nevertheless, the chromophore structure and underlying formation mechanisms are clearly different and suggest a convergent evolution of these red emitters [106, 107].

Based on the computational reconstruction of ancestral genes, a GFP was proposed to be the common ancestor of differently colored FPs in scleractinian corals [10, 108]. These results show that bioinformatics can help to localize key residues responsible for both biochemical and photophysical properties of FPs.

2.2.8 Biological Function(s) of GFP-Like Proteins

GFPs in bioluminescent cnidarians, as for instance avGFP, are exclusively found in the photogenic tissue of the animals [11, 16, 28]. They can form tight molecular interactions with photoproteins, as for example with luciferase in the case of *Renilla* [109]. The fact that they ubiquitously act as secondary emitters of light generated in the chemiluminescent reaction [11] suggests that their function might be associated with the bioluminescence function. Most cnidarians emit light upon mechanical stimulation [11, 110], which may be interpreted as a deterring response to the action of predators. The sudden flash might dazzle or confuse a nocturnal raider of pelagic jellyfish, allowing the prey to escape into the dark. As most cnidaria are quite well protected by their cnidae charged with potent toxins, they have rather few enemies specialized in feeding on them. A large number of predators of sessile cnidaria,

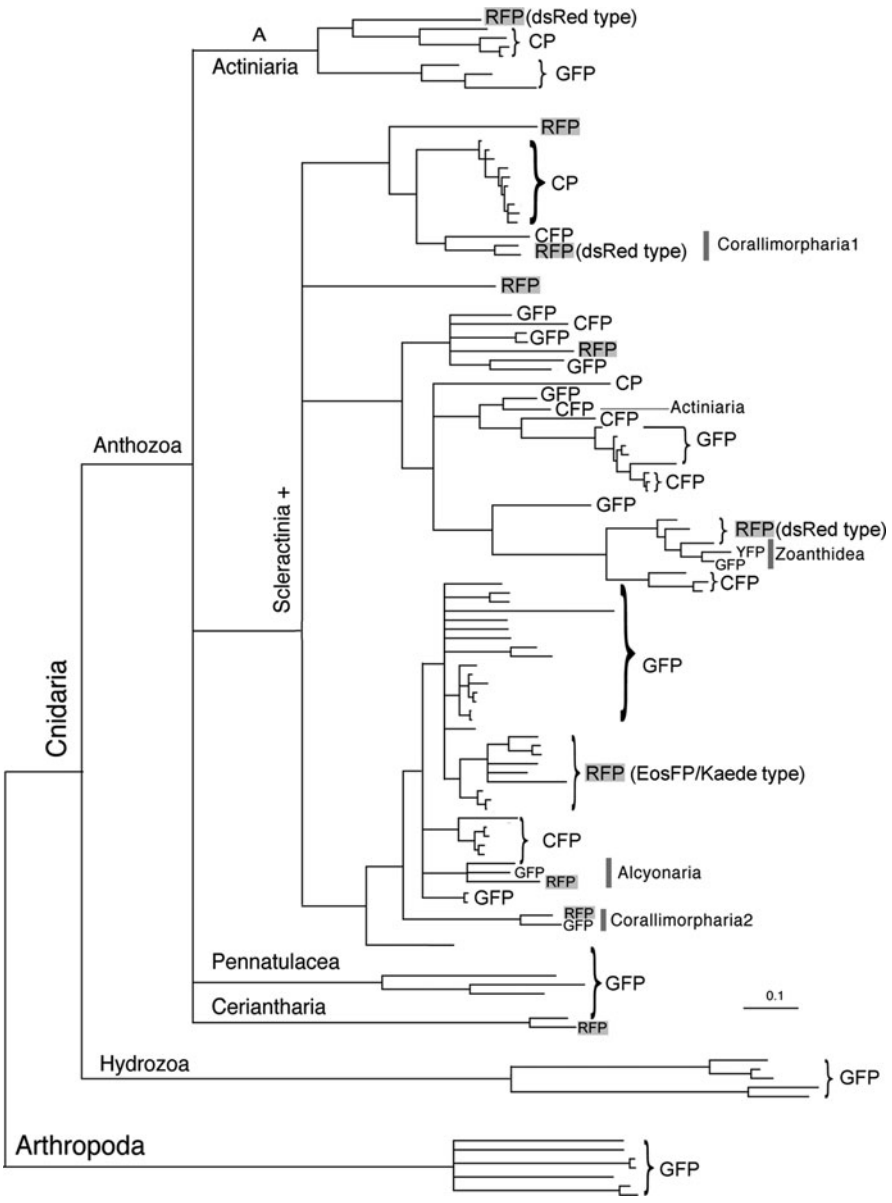

Fig. 9 Bayesian phylogenetic tree of GFP-like proteins from cnidarians showing Arthropoda FPs as outgroup. The emission color of the cyan, green, yellow, and red fluorescent GFP-like proteins are indicated by abbreviations (CFP, GFP, YFP, RFP). Red fluorescent proteins are highlighted by *gray shades*. Nonfluorescent chromoproteins are labeled with CP. The taxonomic origin of the animals from which the proteins were cloned is indicated on the branches. If a protein groups with representatives of a different taxonomic background, the deviating origin is given next to the branch ends. RFPs with different chromorphore types (dsRed type vs EosFP/Kaede type) represent a case of convergent evolution of red fluorescence. Many other cases are likely. Figure modified from [10] with permission