

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

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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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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 pennatulacean *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 *Corynactis 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 actinians under irradiation [46].

A chromatophore system containing fluorescent pigments was found in the endodermal 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,

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