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Biological properties of coral GFP-type proteins provide clues for engineering novel optical probes and biosensors.

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ABSTRACT

In recent years, a variety of Green Fluorescent Protein (GFP)-like pigments have been discovered from corals and other marine organisms. They are widely used to expand the range of available GFP-type proteins in imaging applications, such as *in vivo* markers for gene expression and protein localization studies, FRET-based (Förster resonance energy transfer) multicolor imaging and biosensors. They have known diverse optical and biochemical properties but their *in vivo* spectral properties and biological function in marine organisms is only beginning to be understood. We have investigated their spectral diversity, optical properties and cellular microstructure in corals of the Great Barrier Reef with the aim of elucidating their photo-biological function/s as well as to identify novel proteins suitable for GFP-based technologies. We found numerous spectral variants, with emissions covering almost the full range of the visible spectrum. Many of these GFP-like proteins, especially in corals from the more extreme habitats, such as sun-exposed shallows or in deep water, showed a range of light-related spectral characteristics: high photostability, spectral tuning for energy transfer and dynamic photo-induced transformation properties. Intra-cellularly they were organized into spectral donor-acceptor pairs or even arrays, tuned for FRET. Coral color proteins thus offer an exciting potential to expand the use of the available GFPs in bio-imaging applications and as a basis for improved protein engineering.

Keywords: fluorescent proteins, GFP, FRET, fluorescence imaging, photoconversion, coral pigments, sunscreening

1. INTRODUCTION

The prototypical GFP from the bioluminescent jellyfish *Aequorea victoria* and its derivatives, has become one of the most important imaging tools in molecular and biological sciences, used as cell and protein labels, markers of gene expression and as cellular physiological reporters[1]. These proteins have also become important due to their potential use as a single-molecule optical storage device for computational and photonic applications[2]. GFP and derivatives are unique because their fluorophore forms in an autocatalytic reaction and requires no additional cofactors for color expression[1,3]. Recently, it has been discovered that the majority of the bright colors of Anthozoa – of reef corals, anemones and other related organisms, that have both the fluorescent blue, green, yellow and red and the low-fluorescent, purple-blue hues, are determined by proteins homologous to GFP[4-7]. The discovery of GFP-like proteins in non bioluminescent organisms has consequently greatly expanded multi-color labeling, FRET and other applications. A range of fluorescent proteins (FPs) from cyan to red colors from reef corals (RCFPs) are now commercially available and novel varieties are being constantly discovered, some with exciting optical properties, such as the red-to-green converting Kaede protein[8], that make them highly usable in cell biology and biotechnology. There is still considerable requirement to extend the available color palette of GFP-like proteins for multi-color labeling, especially in the far-red wavelengths[4,5,6,9] and for applications as donor-acceptor pairs suitable for FRET. Moreover, an improvement of certain molecular characteristics of GFP-like proteins, such as increased quantum yield and photostability, reduced aggregation, improved expression at elevated temperatures and discovery of proteins with novel optical properties are highly desirable. Some of these characteristics are attainable by genetic engineering[10] but further screening of GFP-like proteins from Anthozoa and other marine organisms offers exciting opportunities.

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Our research focused on investigating the diversity and photobiology of GFP-like proteins in corals of the Great Barrier Reef in order to elucidate their function in these organisms. Corals have formed a symbiotic relationship with dinoflagellate microalgae (zooxanthellae) that live within their endodermal cells. Consequently, corals are highly dependent on sunlight for the photosynthesis of the zooxanthellae from which they derive much of their own energetic requirements. However, corals live in the clear-water tropical seas where solar radiation penetrates to considerable depths and consequently, they have developed highly efficient strategies for photoprotection[33]. The negative consequences of 'life under the sun' can be seen when corals' sunscreening defenses are overwhelmed during stressful environmental perturbations, such as during periods of excessive solar radiation or while photosynthesizing under conditions of elevated seawater temperature. In addition, many environmental stressful conditions interfere with the ability of zooxanthellae to effectively process light via photosynthesis, leading to photo-oxidative damage that can cause coral bleaching[11,12,29]. Damage to photosynthetic apparatus results, causing a build-up of photooxidative stress[11] and a dramatic phenomenon termed bleaching – a process during which the symbiotic association breaks down due to photooxidation of zooxanthellar chlorophylls and symbiont expulsion, leaving behind whitened corals that often suffer wide-scale mortalities, reviewed by Coles & Brown (2003)[12].

By focusing on spectral, microstructural and eco-physiological studies of coral FPs *in vivo*, we recently proposed that they function in light optimization of coral tissues for photosynthetic requirements of their intracellular microalgal symbionts[13]. In shallow habitats, exposed to high solar radiation, GFP-like proteins act as sunscreens, as was originally suggested by Kawaguti[14]. We also suggested that multiple FPs common to corals function in photoprotection by transforming high energy, damaging (UV, blue) wavelengths into lower energy, less damaging (green to red) wavelengths via radiant energy transfer and via FRET among FPs with overlapping excitation and emission spectra; channel light into wavebands away from photosynthetic absorption; and screen zooxanthellae and coral tissues via light scattering[13,15-18]. We confirmed that FPs do afford photoprotection of zooxanthellae in corals by showing reduced light-induced photoihibition in fluorescent compared to non-fluorescent color morphs[13,19]. Moreover, our research has shown that GFP-like color proteins play an important role in increasing the ability of corals to survive climatic perturbations[13]. During the last decade unprecedented mass coral mortality on reefs worldwide has been recorded, linked largely to increasing seawater temperatures, possibly due to global climatic changes, which cause corals to bleach, weaken, become susceptible to diseases and die[12, 29]. Our recent discovery of the higher bleaching-resistance of fluorescent morphs compared to non-pigmented con-specifics[13], due to the inherent photoprotective properties of GFP-like proteins, has very important implications for reef management and conservation.

In the present study, we investigated the spectral diversity and the localization of GFP-like proteins in corals of the Great Barrier Reef. We also explored the cellular distribution of GFP-like color variants that might make them suitable for efficient FRET-type energy channeling of light within cells of live corals. FRET, is the exchange of energy between fluorescent donor and acceptor molecule pairs and in accordance with Förster equation occurs at high emission-excitation donor-acceptor spectral overlap; at intermolecular distances; and at strong molecular dipole-dipole alignment[20]. Recently, FRET between cyan and green GFP-like proteins was confirmed for the first time in several corals (*Plesiastrea versipora* and acroporiid spp)[17], expanding the potential for FRET previously shown within green and red tetrameres of anthozoan GFP-like protein, DsRed[21]. Moreover, since GFP-like proteins are known to possess a number of unique phototransformation properties and given our interest in investigating the role of GFPs and, consequently, the possibility of such processes in live coral cells and ultimately the biological function of such processes, we tested for a range of photoactivatable phenomena in our samples. The different mechanisms of phototransformation of GFP-like proteins have been addressed by a number of studies and due to the uniqueness of these processes among proteins and the biotechnological potential for their applications, the search for GFP-like proteins with similar or novel photoproperties is a focus of great scientific interest. Photoactivation behaviour was first reported in a wild-type GFP, which upon intense illumination with UVA light increased in fluorescence three-fold upon excitation at 488 nm as a result of the choromophore population undergoing photoconversion via a shift from neutral phenols to anionic phenolates[21,22]. As a result of a rapid and intense enhancement of fluorescence upon irradiation PA-GFP (photoactivatable GFP), that had been genetically modified for this purpose, has been shown to be highly suitable for analyzing protein kinetics within cells[23]. Another phototransforming process, the "greening" of GFP-like proteins - a red to green color shift following irradiation, was first described for GFP[24] and subsequently for DsRed, in the latter resulting from the selective bleaching of the red species of the tetramere by light and causing the fluorescence of the green species, that is normally quenched by FRET, to be enhanced[25, 26]. The "greening" property of DsRed is therefore

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used as a powerful technique for regional optical marking of cells and proteins. An even more exciting phototransformation ability of a GFP-like coral protein also utilized as an optical marker, was discovered in a coral protein named Kaede, that undergoes stable green to red conversion on irradiation by UV or violet light and is imaged at excitation by green light[8]. Another optical behaviour of anthozoan GFP-like proteins, the “kindling” of purple-blue chromoprotein, was recently discovered in *Anemonia sulcata*[7] in a protein named asCP that absorbs light at 568 nm causing purple coloration of anemone tentacle tips. Upon irradiation with intense green light, asCP becomes fluorescent with emission at 595 nm and can be quenched instantly by blue light. A model for kindling mechanisms was proposed[27] in which kindling was related to the cis-trans isomerization of the excited chromophore, from a non-fluorescent to a fluorescent state, similar to the switch from a fluorescent (cis) to the long-lived “dark” (trans) state first discovered in the wild-type GFP[28]. In this study, we explored a range of the above mentioned phototransformation properties of GFP-like proteins in selected corals.

2. MATERIALS AND METHODS

2.1 Sample collection

Scleractinian coral samples were collected at the inter-tidal lagoon, reef flat and inner and outer slope of Heron Island (23°26’S, 151°55’E), One Tree Island (OTI) (23°30’S, 152°06’E) and Osprey Reef, Coral Sea, Australia (from 0.5 to 40 m depths). Blue and green color-morphs of the faviid coral, *Plesiastrea versipora*, which occurs throughout much of the mainland coastline of Australia, were collected from 4 – 8 m depths in Port Jackson (NSW, Australia). Living samples were brought back in seawater and maintained in aquaria at the Electron Microscope Unit, University of Sydney. Samples were also fixed in glutaraldehyde, using a fixation protocol that was found to preserve cell structural integrity as well as the fluorescence characteristics of GFP-like proteins[13,15].

2.2 Fluorescence spectrophotometry

In vivo whole coral excitation and emission spectra of the major FPs in coral samples were obtained using fluorescence spectrophotometer (Varian Cary Eclipse). By using this instrument with a fiber-optic coupler in combination with remote read probe, it was possible to collect excitation/emission spectral scans of live coral samples immersed in seawater, without the need for protein extraction. Fluorescence spectra were collected at 5 nm slit width resolution. For each coral, a full complement of excitation and emission spectra was determined for as many FPs as was possible to resolve by this method.

Spectral characteristics of FPs *in vivo*, within individual live cells, were analyzed by epifluorescence microscopy (Olympus BH2-RFL or Zeiss Axioplan 2) connected to a radiometrically calibrated spectrometers (Ocean Optics S2000 or J&M Zeis, 5 or 3.5 nm slit-widths, respectively), coupled to the microscope's photographic head with a 2 m x 1000 µm fibre fibre-optic cable. Pieces of fluorescently pigmented tissues were dissected and mounted on glass slides in a drop of seawater under cover-slips. Excitation of FPs was by Hg arc lamp in combination with the microscope’s UVA filter sets (Ex 330-360 nm, FT 395, LP 420nm). An oil immersion objective x100 was used for all the measurements. By limiting the field of view with an iris diaphragm, spot measurements of spectral fluorescence at a resolution of single chromatophores or granules were made. Data were collected using a BiLink PC-type portable computer. Individual emission scans required about 200 to 1000 ms to collect, and typically averaged 10 scans for each stored spectrum. Spectral scans were taken of each FP type (blue, green, yellow, etc) from several different FCs or FPGs. Raw spectral data were post-processed to remove the offset due to the instrumental dark current.

2.3 Epifluorescence, single- and multi-photon laser scanning microscopy and spectrophotometric imaging.

Tissues dissected from live coral polyps were imaged using a Nikon Eclipse E800 microscope and standard epifluorescence system with a 100 W mercury arc lamp, using filters to excite blue FP (Ex 330-380 nm; DM 400 nm; BA 420 nm), green FPs (Ex 450–490 nm; DM 505 nm; BA 520 nm) and red FPs (Ex 510-540 nm) and fitted with a cooled CCD camera (PCO Sensicam). Photoactivation and photoconversion properties of FPs were tested by epifluorescent UVA, blue or green light by irradiating GFP-pigmented tissue samples or isolated pigment-containing cells. Red to green photoconversion of FPs in a red color morph of *Goniopora tenuidens* was examined by irradiating live pigmented tissues with blue epifluorescent light and Sensicam imaging. Changes in emission intensity in the blue, green and red were analysed by subsequent color separation into blue, green and red RGB channels of captured images.

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FPs in excised live polyp pieces were also imaged by Leica TCS spectrophotometer confocal inverted epifluorescence microscope (Leica Lasertechnik, Heidelberg, Germany) at excitation with Ar (458, 488, 514 nm lines) and green HeNe lasers (543 nm line). In each case, serial optical sections were made at increasing depths at 0.2 µm increments and were reconstructed into 3D images by Leica confocal software. Images showing varying emission in 3 PMTs were superimposed for combined visualization. For two-photon imaging a mode-locked Ti-Sapphire Laser (Coherent Mira 900) pumped by an 8 W argon-ion laser (Coherent Innova 300) was used, tuned to selected infrared wavelengths. All fluorescence emission spectra from cells were obtained by directing the emissions into a PMT1 at 8 bit resolution (256 greyscale levels) and measuring the entire spectral data (400-700nm) at µm and nm spacial resolution. Spectral scans were made at 100-200 steps to increase the number of points per peak. Spectral characteristics of individual chromatophores, granules or parts of granules were determined by selecting 6-12 areas of interest. The same set-up was used to test photoactivation and photoconversion properties of FPs by either irradiating GFP-pigmented tissue samples or isolated pigment-containing cells by epifluorescent light and by confocally imaging the emissions at 458, 488 nm or 415 nm laser lines or by continuous irradiation at single- or 2-photon excitation and imaging at 458, 488 or 415 nm.

3. RESULTS AND DISCUSSION

3.1 *In vivo* macro-spectral characterisation of GFP-like pigments

To spectrally characterise the diversity of GFP-like proteins of corals we measured the *in vivo* excitation and emission spectra in tissues of live specimens immersed in seawater. All corals had at least 1 major FP, present in low to high concentrations and most contained additional minor FP color variants. Of the 52 corals examined, only 9 contained a single FP and most contained FP mixtures. In total, we found over 40 spectral variants, with emissions covering almost the full range of the visible spectrum, although these may not necessarily represent distinct GFP-like proteins. In all cases, the successive overlapping spectral emission-excitation profiles of differently colored FPs indicated that they were suited for sequential energy coupling, as shown in **Fig 1 (a, b)** for 2 major FPs of blue and green *Plesiastrea versipora* color-morphs. Red FPs were also found to be very abundant in the sampled corals, although they were rarely the major pigments.

3.2 Distribution, cellular localisation and microstructure of GFP-like pigment cells.

To explore the diversity of GFP-like proteins from corals adapted to distinct and frequently stressful environments, such as those from shallow water sites exposed to excessive solar radiation or from deeper water, where light is limiting for growth, we analysed the fluorescence characteristics and cellular localization of FPs in coral tissues in samples collected from 0.5-1m, 5, 10, 20 and 40 m depths using confocal microscopic imaging. Cellular localization of GFP-like proteins in coral tissues were predominantly of two types: (i) within numerous fluorescent pigment granules (FPGs) (0.4-2.5µm diameter) packed into specialised cells, the fluorochromatophores (FCs), within the ectodermal (outer) and endodermal (inner) tissue layers[13,15,16] (**Fig. 2d, 3a**); or (ii) localized in non-FC specialized cells, such as within ectodermal and endodermal columnar and interstitial cells (**Fig. 2a-c**)[15]. FPs were mainly cytoplasmic but each cell also contained 1-3 brightly fluorescent granules. The low fluorescent, brightly colored (pink, purple and blue) GFP-like proteins, ie. chromophoric (CPs)[7], and other similar GFP-like CPs[30], were found localized within the cytoplasm and vacuoles of the columnar and interstitial cells of the ectoderm and did not form granules. All types of CPs and FPs, from blue to red, and all three combinations of the above cellular micro-structuring were recorded in corals at all sampled depths. The distribution and abundance of purple-blue CPs declined with depth; blue and cyan fluorescent emitters (470-499 nm) were more abundant at 40 m when compared to 1 m depths; greens (500 – 520 nm) were the most abundant FPs, but decreased at 40 m depth; while the reds (570-620 nm), the least abundant FPs, were highest at the shallowest (0.5-1 m) and also the deepest (40 m) sites and were rare at other depths. There were no significant differences in FP color varieties at intermediate depths (5 to 20 m). Equally, the only differences in pigment localization were found to occur in corals collected from the shallowest and the deepest sites. In the former samples, FPs, while present in both ectoderm and endoderm, were predominantly localized above the layer of zooxanthellae, while in the latter, FPs were localized endodermally among and below zooxanthellae, with the outer ectoderm being either unpigmented or containing the non-granular cytoplasmic FPs that allowed more light to be transmitted through tissues (**Fig. 2a**) than in corals in which FPs were in granules within FCs (**Fig. 2d, 3a**). Many corals in which FPs were localized within FPGs, such as *Goniopora tenuidens*, showed a modification of their granule microstructure in deeper water, forming rod-like or needle-like organelles within FCs or even within coral host-cells enclosing the zooxanthellae, that may channel light energy into chloroplasts of zooxanthellae. Such depth-related and consequently, light-related pigment organization, implies light-modulating adaptive function of GFP-like proteins.

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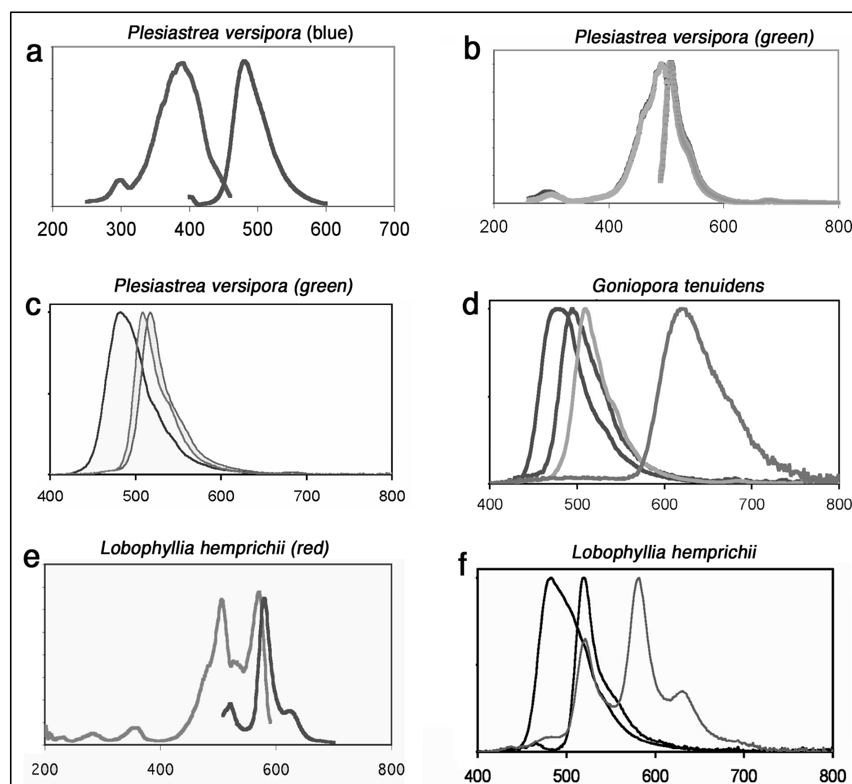

Figure 1. *In vivo* spectral characterization of fluorescent pigments (FPs) in coral tissues. **(a, b)** *Pleasiastrea versipora* color morphs – 1st spectral lines are excitation peaks, monitored at 485 nm for blue and at 510 nm for green morphs' emissions and 2nd emission spectral lines are emission peaks, monitored at 390 nm for blue and 495 nm for green excitation, using Varian Cary Eclipse fluorescence spectrophotometer. Note strong spectral overlap of excitation of green (acceptor) and emission of blue (donor) FP pairs. (**c**) Micro-spectrofluorometry of *P. versipora* single fluorochromatophores (FCs) and granules further resolved spectral diversity – here showing emissions of 3 of the 12 progressively red-shifted FP series recorded (485, 508, 516 nm). **(d)** Emission spectra of *Goniopora tenuidens* granules (480, 494, 509, 582 nm). (e) Whole coral excitation (peaks at 454, 510, 572 nm) and emission (peaks at 515, 580 nm) spectra and **(f)** emission microspectra of single FCs of red *Lobophyllia hemprichii* (483, 519, 581 nm).

3.3 *In vivo* diversity and microspectral characterisation of GFP-like proteins.

We next performed single-cell epifluorescence microspectrometric analysis of individual live FCs, FPGs and other types of non-specialized FP-containing cells dissected from coral tissues. This technique resolved an even higher spectral diversity than previous macro-spectrophotometric FP analyses. In *P. versipora*, a total of 12 spectrally distinct FPs were recorded in the 2 color morphs of *P. versipora* with emissions: in blue color morphs at 472 ± 1.8, 477 ± 2.1, 483 ± 1.4, 488 ± 0.3, 492 ± 1.2, 497 ± 1.2 & 501 ± 1.4 nm and in green color morphs at 497 ± 1.4, 503 ± 1.2, 508 ± 0.7, 511 ± 0.7, 515 ± 0.2 & 518 ± 0.4 nm (selected spectra shown in **Fig. 1c**). Granules packing the FCs were rarely of single color and typically were of 2-3 colors: granules in green FCs were mixtures of blue and green (e.g., 497 & 503 nm) or several greens (503, 509 & 512 nm); granules of blue FCs were blues or blue-greens mixtures (483 & 488 nm or 497 & 500 nm). Mixed color-morphs had any number of the 12 FP combinations and consequently, the FP diversity in different colonies varied considerably. Similarly, microspectrometry of individual FCs or FPGs of 46 other coral species of 11 families, also revealed multiple FP spectral types, from 2 to over 10 per specimen (e.g., *Lobophyllia hemprichii and Goniopora tenuidens* **Fig. 1 d, f**) and only 12% of sampled corals contained a single FP. Notably, different emitters within neighboring FCs or FPGs occurred in step wise, progressively red-shifted light emission series: blue-green, blue-green-yellow, green-yellow-red, etc.

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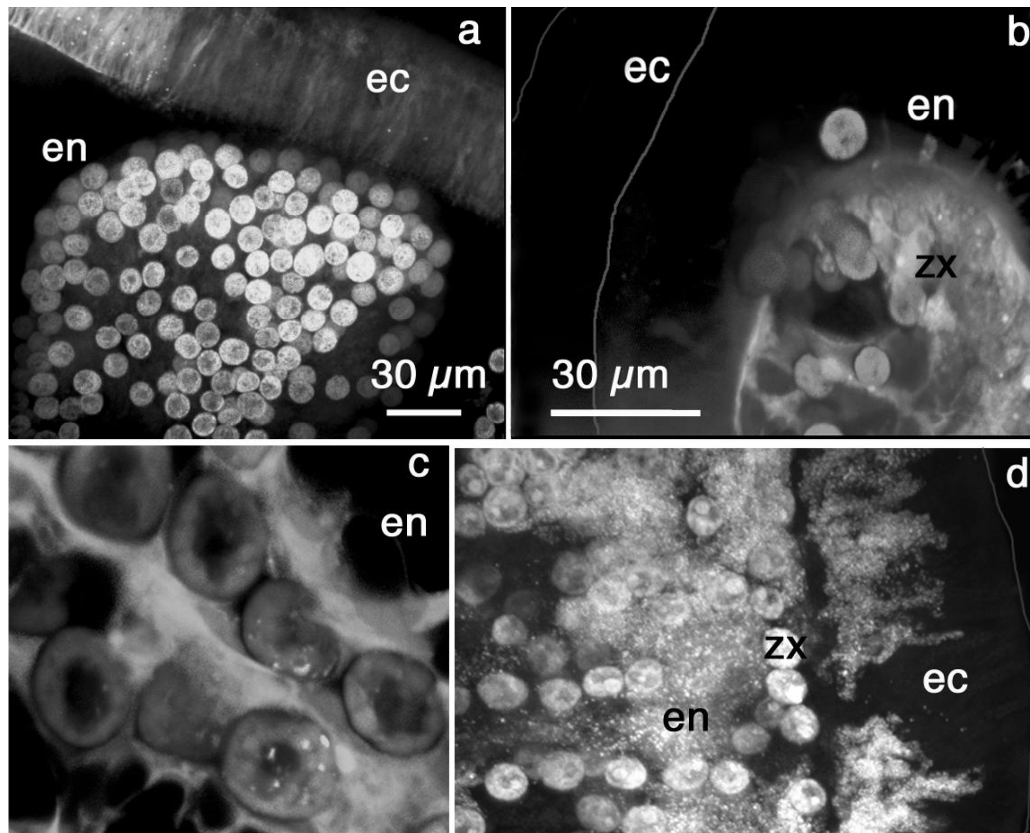

Figure 2. Light regulatory function of GFP-like FPs inferred from the analysis of their localization in cells of light- and shade-acclimated corals. **(a)** Endodermal layer with zooxanthellae devoid of FPs, which are concentrated in outer columnar cells of the ectodermal layer in light-acclimated *Lobophyllia corymbosa*. **(b, c)** Outer ectodermal layer devoid of FPs, which are concentrated in the inner endodermal layer, surrounding zooxanthellae, in shade-acclimated *L. corymbosa*. **(d)** FPs in fluorescent pigment granules (FPGs) within ectodermal and endodermal FCs in contracted polyp of *Goniopora tenuidens* from shallow water. In contracted tissues, FPs can effectively block much of the incident light from reaching the zooxanthellae. 3D projections from confocal data sets obtained at excitation by 488 nm laser line of green (GFPs) and red (chlorophylls) emissions imaged in 2 channels. ec = ectoderm, en = endoderm, zx = zooxanthellae.

3.4 Single- and multi-photon laser scanning microscopy and spectrophotometric imaging of GFP-like proteins.
We suspected that the diversity of FPs was even higher because epifluorescence microspectrophotometric detection allowed FP emissions from areas outside of the focus plane to be recorded by the detector and the strong signal from the major, highly concentrated FPs, often swamped emissions of less abundant ones[16]. To further resolve the diversity and cellular spatial localization of FPs in respect to each other, we used confocal laser scanning microscopy (LSM) imaging of small live tissue samples excised from coral polyps, combined with simultaneous spectroscopic characterisation of FPs by performing λ-scans across 400 -700 nm. LSM enables relatively thick tissues to be analysed by means of optical serial sectioning and the pinhole reduces fluorescence that originated from areas above or below the focal plane, thereby limiting FP spectroscopic detection to the plane of focus. First, we performed serial optical sectioning and visualization of FPs in tissues of green (n=5) and blue (n=4) morphs of *P. versipora,* capturing emissions of FPs at selected blue, green and yellow-red band-widths. Several different laser lines (458, 476, 488, 543 nm) were trialled to excite FPs; with excitation by 488 nm proving optimal for greens, 458 nm for blues and 547 nm for yellow-reds (data not shown). Strong green and blue, and weaker yellow-red FP emissions, were clearly imaged from individual FCs and for FPGs of green color-morphs, while blue color-morphs contained mainly blue, blue-green and green emitters. Excitation by 488 or 458 nm laser lines and spectrophotometric imaging, produced typical GFP-type emission spectra, with 2-4 minor peaks, indicating the presence of other minor FPs per color-morph. In support of the radiant energy transfer concept[13,16-18], we found that in many cases the higher energy blue FCs, excited by UVA wavelengths, were more abundant towards the

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outer tissue parts, while progressively longer wavelength green FCs, excitable by blue emissions, were localised immediately underneath.

When spectra were collected for individual FPGs, it was found difficult to resolve the full number of FPs present. Nevertheless, the analyses suggested that blue, green and red FPs within FPGs, with donor/acceptor properties, were situated close enough for FRET to occur. To examine cellular arrangement of FPs at even smaller scales, we used LSM at two-photon excitation at 780 nm, equivalent to single-photon wavelength of 390 nm, which is closest to optimal excitation of blue FPs, and emissions from whole FCs, single FPGs and from parts of single granules (10,000-40,000 nm^2) were spectroscopically analysed. The technique resolved the presence of 2-4 FPs within single FCs, seen as major and minor peaks on spectral curves, in either blue or green color-morphs and 2-6 spectral variants for individual FPGs per FC. Analyses at even smaller scale, of selected areas inside granules, made for several granules with different overall spectral peaks, revealed an astonishing diversity of FPs at such nano-scale distances, all arranged in strictly down-hill emission gradients. Green FPGs contained various combinations of 1-3 major blue-green and green FPs from the following series: 501-503, 506-508, 510-512 & 515-518 nm; with small peaks and shoulders due to 2-4 minor FPs, (blues at 477-479, 484, 488, 492-495, 497-499 nm & red-shifted FPs at ~520, 530 and 580 nm). Similarly, blue FPGs had 1-3 major and several minor blues (470-472, 477-479, 482-484, 486-488, 492-495 and 496-498 nm) as well as several minor greens (501-503, 506-508, 510-512 and 516-518nm) and occasionally, several yellow-orange emitters (> 530 nm). Given that the minor peaks recorded in blue color-morphs appeared as major peaks in greens and *vice versa* (**Fig. 3 e**), it seems probable that at least some of these are in fact distinct FP spectral variants, despite the difficulty of distinguishing them from the background noise, although admittedly many of these are unlikely to be different GFP-types. Nevertheless, the full complement of all spectral FP types in both color-morphs is clearly present and there may be over 6-14 FPs per single-color morph. Recently, comparative spectroscopic and molecular analysis of GFP-like proteins in a coral, *Montastraea cavernosa*, confirmed that a full complement of all color variants, from cyan to red, was present even in single colored morphs and that the color difference between the colonies was due to the relative expression levels of their genes[31].

The discovered typical arrangement of GFP-like proteins into series of donor-acceptor pairs, which at macroscopic level were shown to be localized in tissues in such a way that the shortest wavelength pigments were nearest to the outer, light-exposed tissue layers of the coral and the progressively longer wavelength pigments were in deeper tissue layers[13,16], suggests that they function in channelling of light energy. In high light conditions wavelength conversion by absorption and re-emission of fluorescence may occur and blue fluorescence may be converted to green, yellow and red (which is inactive for photosynthesis) in deeper cell layers. The absorption and emission peaks of many of these GFP-like proteins make them highly suited for such a process. We propose, consistent with our previous suggestions[13,15-18], that one function of the discovered intra-cellular GFP-like protein assemblies is also in photoprotection. By absorbing short wavelengths radiation and transforming it via radiative energy transfer and via FRET into longer wavelengths, GFP-type proteins dissipate excess sunlight excitation before it reaches and causes damage to zooxanthellae or to the vital organs of corals. Such photoprotective mechanisms would be very important for shallow tropical waters, where even UV wavelengths penetrate to depths over 20 m[34] and where blue light, known to be damaging to corals[32] has the highest proportion of incident irradiance and greatest depth penetration.

3.5 Photoactivation and photoconversion of GFP-like proteins.

We have identified a range of photoactivatable phenomena involving GFP-like proteins in live corals (Salih *et al. submit.*). These included four main types of light induced transformations of GFP-like proteins: (1) photoactivation – the rapid intensification of fluorescence upon irradiation by certain wavelengths, most commonly by blue wavelengths, but also in some cases by ultra-violet A (UVA), green light and multiphoton excitation and similar to that described for wild-type GFP[22] and its variant, PA-GFP[23]; (2) “greening” - red to green spectral transition following irradiation of samples by blue light or multiphoton wavelengths, as described for DsRed GFP-like protein[25,26]; (3) “reddening” - green to red color conversion upon irradiation by UVA and multi-photon wavelengths, equivalent to UVA single-photon range, as described for coral GFP-like protein, Kaede[8]; and (4) protein “kindling”, during which low fluorescent, blue-purple chromophoric proteins attained red fluorescence following irradiation by green light[7]. *In vivo* fluorescence activation and green to red photoconversion were recorded for pigmented tissues of several corals collected from shallow water sites (Salih *et al. submitted*). Upon irradiation with UVA, blue or green light, and in some cases at 2-photon excitation, fluorescence emission intensity increased rapidly during the first 1-5 minutes of irradiation, then more gradually for up to 30-60 minutes by 30% and to over 100% when subsequently viewed by epifluorescence microscopy or imaged by single-

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and multi-photon scanning. Green to red photoconversion was found to be a less common phenomenon but occurred in 7 of the examined corals when their live tissues were exposed to epifluorescent UVA illumination and imaged at green laser line excitation, e.g., green tissues of *Lobophyllia hemprichii* (Salih *et al. submit.,* Wiedenmann *et al. submit.*). "Greening" or red to green photoconversion of FPs was more commonly found in GFP-like proteins from deep water (40 m) then shallow corals – upon irradiation with blue light, their red emissions diminished, while green emissions intensified, e.g., in red color morph of *G. tenuidens* (**Fig. 4**). The kinetics of this behaviour were quite complex, with blue, green and red fluorescence intensifying during the first 1 to 2 minutes of irradiation, exhibiting photoactivation, followed by rapid reduction of red and an increase of green emissions. These stabilised and subsequently decreased upon more prolonged irradiation by blue light over 20-30 min (**Fig. 4**). In some cases, unusually, blue emissions were found to continue to increase for up to 1 hour. The diversity of photoactivatable GFP-like proteins that we discovered in Great Barrier Reef corals has promising potential for biotechnological needs.

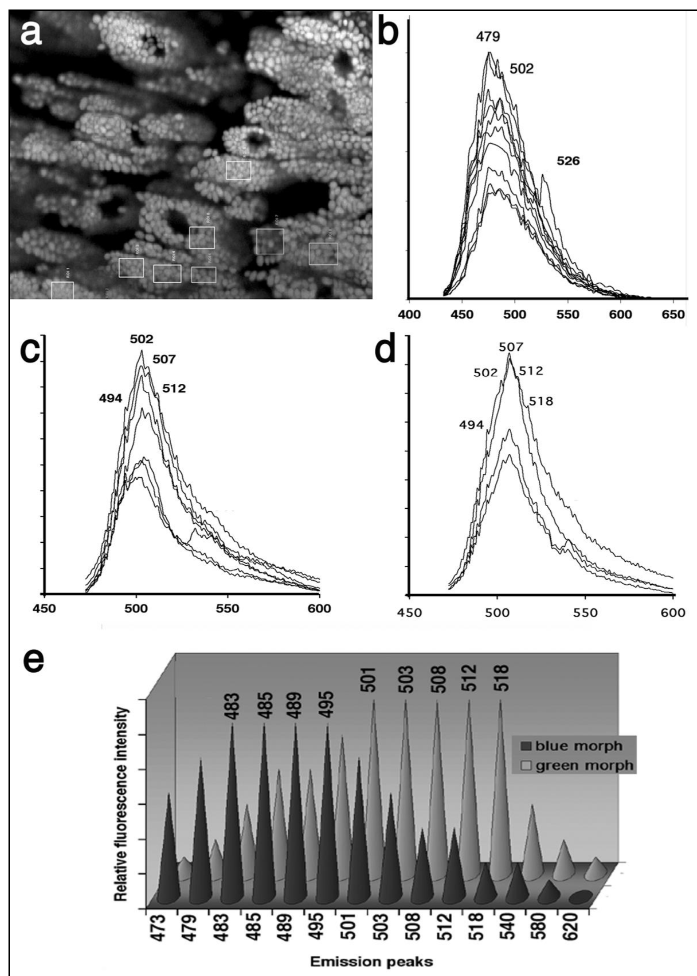

Figure 3. Spectral FP diversity resolved by microspectrophotometric imaging of *P. versipora* live cells at 2-photon excitation (760 nm). Spectrally sampled areas on images obtained by scanning at 1.75nm steps across 450-700nm spectrum using 5 nm detector slit of Leica SP at the level of FCs and fluorescent pigment granules (FPGs) within FCs. (**a**) Imaged FCs packed with FPGs of a green morph at excitation by 780 nm, showing some of selected areas of interest (AOI) used for spectral characterization. **(b-d)** Emissions with peaks due to major and minor peaks due to minor FPs of blue and green FCs and FPGs (note matching peaks of major and minor FPs within the series, confirming the presence of 2-4 major and several minor FPs. **(e)** Summary of FP emissions for all *P. versipora* color-morphs - blue major FPs (483-495nm) predominate in blue morph and match minor peaks in green morphs; green major FPs (501-518nm) in green morphs correspond to poorly pronounced minor peaks of blue morphs, indicating that almost all FPs can be found in both morphs. x-axes – wavelengths (nm); y-axis – relative fluorescence intensity.

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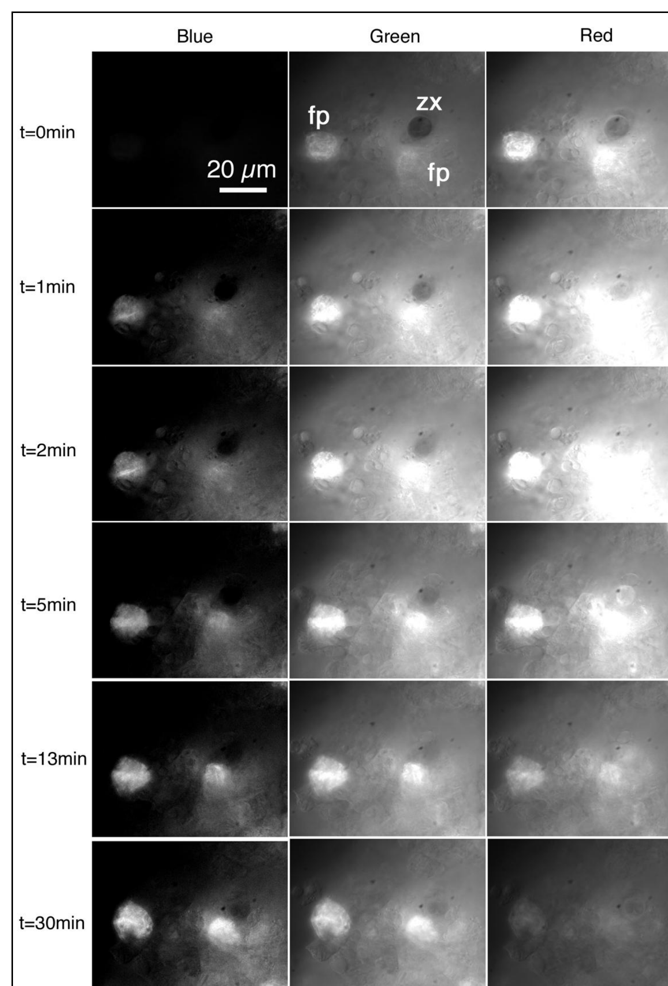

Figure 4. Phototransformation of orange-red GFP-like proteins (peak emission at 582 nm) within endodermal FCs in live tissues of *Goniopora tenuidens* on irradiation by blue epifluorescent light and imaged by Sensicam camera at selected time intervals. At 1-2 min, blue, green and red fluorescence intensity rapidly increased; at 5 min, blue and green emissions continued to increase, while red fluorescence decreased; at 13 & 30 min, only blue fluorescence continued to increase, red bleached, resulting in bright blue-green FC coloration.

The biological role of these complex light-induced changes in GFP-like proteins in corals and related organisms is under investigation by our group. Fluorescence photoactivation of GFP-like proteins in coral tissues by intense sunlight can function in photoprotection by increasing the dissipation of higher energy light, especially of the highly damaging blue wavelengths, to lower wavelengths, via fluorescent emissions. Similarly, green to red photoconversion can further enhance photoprotection by increasing the dumping of higher energy into lower wavelengths by red emitters. In the case of *G. tenuidens,* for example, the distribution of its color morphs at different depths supports this hypothesis. Green and cyan color morph are common in shallow water, while red color morphs, although being relatively rare at all depths, are more abundant at 20 to 40 m and have never been recorded by us in shallow water (0.5 to 15 m). It may be hypothesized that the intense sunlight of shallow water would preclude the occurrence of red pigmentation by rapid red to green conversion of tissue proteins. The kindling of purple-blue chromophoric GFP-like proteins may also function in photoprotection by increasing the dissipation of excessive solar radiation by absorbing incident green light and re-emitting it as low-energy red fluorescence. The predominance of CP-pigmented corals in shallow water supports this hypothesis. These phototransformation processes would be enhanced by the presence of multi-color FRET protein assemblies. Coral species span a wide depth range and show physiological adjustments analogous to the light/shade

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adaptations of higher plants[33], since attenuation of light with depth is accompanied by marked alteration in spectral composition so that deeper water lacks red wavebands and blues predominate[34]. In such situations, blue, green and red GFP-like proteins may function in light enhancement for the photosynthesis of zooxanthellae in which chlorophylls absorb blue and red wavelengths, while their accessory peridinins absorb blue and green light. Our depth- and cellular-distribution analyses of these proteins in corals support this hypothesis. There are of course other potential functions that can be attributed to GFP-like proteins. Such functions may be linked to their dynamic light activation properties and be involved in photoreception, circadian rhythms and possibly, in reducing tissue oxidation effects, that in symbiotic cnidarians are exacerbated by the photosynthetic activity of intra-cellular zooxanthellae. Alternately, the proteins may play important roles unrelated to their photo-properties. The presence of GFP-like proteins unrelated to bioluminescent systems have been reported in a wide variety of organisms, some of them present at depths where sunlight is dim (Wiedenmann *et al*. in press[35]; Shagin *et al*. in press[36]; Haddock & Schnitzler, *pers. com.)* and whilst certain organisms may utilize GFPs for color signalling[37,38], such function in corals has not been demonstrated and appears doubtful.

4. CONCLUSIONS

The research of the diversity, novel optical properties and of the biological roles of GFP-like proteins in corals and other marine organisms is a tremendously exciting area of science. The widespread distribution of these proteins in corals and other organisms, their tuning for energy transfer and the photo-inducible spectral properties, strongly suggest a functional role for GFP-like proteins and indicate that they form part of a complex adaptive mechanism for environmental adjustment. Our findings greatly strengthen the hypothesis that coloration in corals and other anthozoans is related to light modulation, although other functions are likely to be present. The study of the photoprotective mechanisms of corals has particularly important implications in the present times, since reefs are under threat both from global warming and enhanced ultraviolet radiation. The novel discovery of the radiative energy transferring and non radiative FRET-type pigment assemblies, which may have functions other than sunscreening, are under intense investigations by our group. Moreover, the identification of natural donor-acceptor novel GFP-like proteins for FRET applications is of great interest to cell biologists and biotechnologists since FRET microscopy has become a highly advanced technology. The identification of novel GFP-like proteins has already, and will continue to, expand the scope of the available bio-imaging techniques for studying cellular, organelle and protein dynamics, protein trafficking, in developing novel biosensors and in other biotechnological applications.

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