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Fluorescent pigments in corals are photoprotective.

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All reef-forming corals depend on the photosynthesis of their dinoflagellate algal symbiont and are therefore restricted to the photic zone. In this zone light intensity declines over several decades of intensity from high and damaging levels (accompanied by UV radiation) at the surface to extreme shade conditions at the lower limit[1]. The ability of corals to tolerate this range implies effective mechanisms for light acclimation and adaptation[2]. Here we show that the fluorescent pigments[3-9] (FPs) of corals provide a photobiological system for regulating the light environment of coral host tissue. Previous studies have suggested that under low light, FPs may enhance light availability[4,5]. We now present evidence that in excessive sunlight, FPs are photoprotective through dissipation of excess energy at wavelengths of low photosynthetic activity, as well as by reflection of visible and infra-red light by FP-containing chromatophores. We also show that FPs enhance the resistance to mass bleaching of corals during periods of heat stress, which has implications for the impact of environmental stress on the diversity of reef-building corals.

The bright colours of corals (Scleractinia) and other Anthozoa are due to pigments of animal-host origin[3], many of which are intensely fluorescent under UVA and blue light, with emission maxima at 420-620 nm[4-9] (Fig. 1). In many corals distinct morphs are found which differ greatly in the concentration of FPs. FPs are part of a group of coral pigments for which the generic term "pocilloporins" has been proposed[9,11]. It includes both brightly coloured, low fluorescence forms and highly fluorescent forms[9] described here. Both types of pigments are partially homologous to Green Fluorescent Protein (GFP)[8,9], first found in the luminescent jelly-fish *Aequorea*[10] and widely used in cell biology. While the function of GFP in a luminescent system is known, the role of similar FPs in non-luminescent anthozoans has hitherto been unclear[6,11,12].

We surveyed the distribution of fluorescent corals on the Great Barrier Reef (GBR) (see methods) and found that 124 spp of 56 genera in 16 sampled families contained fluorescent morphs, often found growing side by side with non-fluorescent morphs. Colour polymorphism is typical of corals[12,13], but many FPs are invisible in daylight, therefore this widespread abundance was not previously known. The highest numbers of fluorescent morphs were recorded at the shallowest sites; thus 97% of sampled reef flat corals at Heron Island, southern GBR, contained medium or high FP concentrations. Moreover, their relative concentration was significantly higher in sun-exposed as compared to their shaded colony parts ($P<0.001$, T-test). We therefore explored an early suggestion[3] that fluorescent pigments

in shallow water corals might function in photoprotection, by comparing fluorescent and non-fluorescent morphs.

We found that the emission maxima of FPs ranged from blue to green to red (Fig 1**),** which is consistent with studies done on the isolated FP proteins of corals[9]**.** The shorter wavelength FPs were more abundant**.** Microscopically we identified 2 broad groups: FPs bound within 0.2-8µm fluorescent pigment granules (FPGs) as reported previously[3,5,7] and inter- or intracellular FPs, not enclosed in granules (CFPs). Significantly, the majority of corals contained multiple FPG and CFP types. Correspondences between emission and excitation maxima of FPs which occur in close association (Fig 1, *e-f*) suggest that energy transformation to longer, non-photosynthetically active wavelengths might in some cases be a sequential process, with the fluorescence of one pigment exciting another, as expected from spectra of isolated proteins[8,9]. We demonstrated that this process could occur by comparing fluorescence of green FPGs (excitation max. 482.5 nm) alone and mixed with blue FPGs (excitation max. 382.5 nm). Only weak green fluorescence is seen under 330-380nm excitation; addition of blue FPGs emitting at 480nm enhanced green fluorescence intensity 4 to 7-fold. (The effect was strongly dependent on distance, with maximal enhancement when blue and green granules were less than 10µm apart.) The final energy spill would then (depending on the pigments involved) lie between the two major peaks of the coral photosynthetic action spectrum and hence be relatively inactive in photosynthesis (Fig 1*f*). This would be the inverse counterpart of the process of light transfer *to* photosynthesis which others have proposed in light limited habitats[4-5] (Fig 1 *g*)

High light causes photodamage and photoinhibition[14] in coral symbionts[15-19]. We hypothesized that FPs may reduce the susceptibility to photoinhibition of fluorescent corals by filtering out damaging UVA and excessive PAR. We compared the degree of day-time photoinhibition in a polymorphic intertidal species, *Acropora palifera*, by exposing replicate sub-colonies made from green fluorescent, brown medium fluorescent and beige non-fluorescent mother colonies to full sunlight and monitored photosynthesis by chlorophyll fluorescence analysis with a pulse amplitude modulation (PAM) fluorometer[19,20]. As expected[15,16], corals showed pronounced photoinhibition during periods of peak irradiance; non-fluorescent morphs, however, were significantly more photoinhibited and recovered to pre-inhibition rates slower than fluorescent morphs ($P<0.001$, ANOVA) (Fig. 2*a*). Similar measurements with other polymorphic species (*Acropora nobilis, Pocillopora damicornis, Goniastrea retiformis*) also indicated that FPs are correlated with reduced photoinhibition.

Since high solar radiation is a factor in the widely observed mass bleaching of corals[17,21,22], FPs might affect susceptibility to bleaching. Bleaching occurs as a consequence of damage to dinoflagellate photosynthesis caused by combined effects of thermal stress and sunlight[18,19,22]; consequently the dinoflagellates either degrade or are expelled from the host. During the severe 1998 GBR mass bleaching event, we sampled 21 common coral species affected by bleaching to varying degrees and found a significant correlation ($r^2 = 0.9471$; $P<0.0001$) between bleaching resistance (i.e., high tissue dinoflagellate biomass) and the concentration of FPs within the tissue (Fig. 2*b*).

Light scattering is an important factor linked to the sun screening function of FPs. We measured spectral reflective properties of coral tissues with fiber-optic microprobes[23,24] positioned over specific parts of single coral polyps. The highly reflective bare coral skeleton was used as a reflection standard (100%). FPs greatly modified the surface light environment not only by their emissions but also by light scattering and reflectance, which was higher in areas with high FPG concentrations (Fig. 3*a*). White pigmented regions of tissues, formed by dense layers of FP chromatophores, had 60-100% reflectivity (Fig. 3, *a*-*b*). The most pigmented, and most reflective, parts of colonies: (i) branch tips and colony edges and (ii) the oral disk/cone and tentacle tips, which on polyp retraction form a sun-screening polyp 'plug'[7] (Fig. 4*c*), correspond to known areas of highest cell division and areas immediately above reproductive organs, respectively. This distribution points to a photoprotective role of FPs in screening sensitive coral tissues as well as symbionts.

Our observations also indicate that corals actively vary the areal density of pigment chromatophores via polyp expansion/contraction. During expansion more light penetrates into the tissues through the gaps between FPGs. Under high light, polyp contraction leads to denser concentration of tissue FPGs and cytoplasmic FPs, forming a thicker and a quasi continuous FP layer, acting as an effective sunscreen (Fig. 3, *b*-*c*) by light scattering and by radiant fluorescence energy transfer from shorter to longer wavelengths. We also found that FP-containing polyps of shade-adapted and high light-adapted corals exhibited differences in spectral reflectivity. Shade-adapted polyps absorbed most of the incident light, in line with previous observations[25], while high light-adapted polyps were generally 20-100% more reflective (Fig. 3*b*).

What are the causes of such different tissue optical properties of shade- and high light-adapted corals? The 3-D cellular localization of FPs in corals showed a clear difference in the distribution of FPs relative to the layers of endosymbionts[7]. In high light-acclimated corals, FPs are localized above the endosymbionts (Fig. 4 *a*-*c*) and are in a position to screen them from excess sunlight. In shade-adapted corals from light-limited habitats, FPs are localized endodermally, among or below the layers of endosymbionts (Fig. 4*d*), consistent with their proposed function of light enhancement for photosynthesis via wavelength-transformation and back-scattering[4,5].

The results presented above all suggest that FPs reduce the photoinhibitory effect of high levels of solar radiation, which in conjunction with thermal stress leads to bleaching. These findings improve our understanding of the causes of observed inter- and intraspecific variability in bleaching[17,26] and may provide an insight into how changing global climatic conditions will influence the species diversity and rate of change of coral reef communities[22].

In conclusion, our study provides a new and more complete understanding of the role of coral FPs, which appear to be involved in regulation of the internal light microenvironment of coral tissues. The evidence presented here indicates that the role of FPs in photoprotection in shallow water, hitherto neglected, is at least as significant as the function

of light capture in deep water previously assigned to them. Dinoflagellate photosynthesis is vulnerable to both UV[27,28] and high levels of PAR[16-19]. While accessory pigments in dinoflagellates can dissipate excess PAR as heat[29], FPs can dissipate excess light energy via fluorescence and light scattering. FPs may also supplement UV-screening by mycosporin-like amino acids (MAAs)[30] since some FPs can transform absorbed UVA radiation to longer non-actinic wavelengths via fluorescence. By screening chlorophylls and peridinin from high levels of solar radiation and by absorbing UVA, FPs thereby decrease the likelihood of irreversible photoinhibition, photooxidation and subsequent coral bleaching. By changing their optical properties with the help of these GFP-like pigments, coral polyps are able to optimise the photosynthetic activity of their tissues for the better survival of the organism.

Methods

Survey sites, sampling and manipulations: Surveys of fluorescent corals were made at the inter-tidal lagoon, reef flat and inner and outer slope of Heron Island (23°26'S, 151°55'E) and One Tree Island (OTI) (23°30'S, 152°06'E), and at several GBR mid-shelf-reefs (1-20m depths). Corals were sampled by chiselling pieces from replicate colonies (n = 3-6). Each sample was broken in two, and, subsequently, one subsample was frozen and the other was chemically fixed as described previously[7] for microscopy. Polymorphic *Acropora palifera* colonies used in photoinhibition experiment were collected from the OTI lagoon from ~1m depth. Three colonies of each colour morph were broken into replicate sub-colonies and fixed in horizontal position in flowing seawater (27-28°C) with one side exposed to sunlight and the other shaded. Controls were kept at 50-80µmol photons $m^{-2}s^{-1}$. During the March, 1998 bleaching event samples were taken at Coats (17°28'S, 146°30'E) and Cayley (18°30'S, 147°E) mid-shelf reefs from 1-6m depths. Replicate samples (n=3-6) were taken from species selected by susceptibility to bleaching: 9 bleached (including non fluorescent *Acropora nobilis* morph;); 5 partially bleached (including fluorescent *A. nobilis)*; 7 unbleached.

Microscopy: Frozen, glutaradehyde-fixed and live coral samples were analysed by fluorescence widefield and confocal (CLSM) microscopy as described previously[7]. Fluorescence characteristics of FPs were not substantially affected by glutaraldehyde fixation. Confocal imaging used 488nm excitation, with detection at 520-550 nm (FPs) and >585nm (chlorophyll). 3-D reconstruction from optical sections was done with VoxelView Ultra 2.1.2 (Vital Images, USA). A fluorescence microscope fitted with cooled CCD camera (PCO Sensicam) was used to test energy transfer from blue to green FPGs, both extracted from *Plesiastrea versipora*. Intensity (in the green) was measured as the ratio of emission excited at 330-380nm to the emission excited at 450-490nm, thereby compensating for differences between granules. Relative FP concentrations in light- and shade-samples as well as in post-bleaching samples were measured, semi-quantitatively, by CLSM as the fluorescence intensity per μm^2 of imaged coral surface (replicate 3-6 colonies / specimen). Zooxanthellae were extracted from samples, their biomass cm^{-2} of coral surface was determined microscopically and correlated to the relative concentration of FPs in surface tissue as determined by CLSM.

Coral surface areas were measured as described previously[18].

Spectroscopy: Fluorescence excitation and emission spectra of FPGs isolated by homogenization and repeated centrifugation of coral tissues in phosphate buffer (0.1M, pH 7.2) were determined by a Perkin Elmer Luminescence Spectrometer S50B. All spectra were normalized to their peaks. Reflectance spectra were measured on live corals in seawater by a tapered (40µm tip) fibre-optic field radiance microprobe[23] positioned ~100µm above the coral surface. The microprobe was connected to a fibre-optic diode-array spectrometer (Hamamatsu PMA-11) with a 300-800nm spectral range. A micromanipulator was used to position the microprobe tip above specific single polyp regions, as viewed under a dissection microscope. Samples were illuminated by a UV-VIS metal halide light source via a 1 mm quartz fibre equipped with a collimator at the output end. Spectra of reflected light from the corals were normalized to the spectrum of reflected light from a reflectance standard in order to obtain reflectance spectra corrected for the spectral composition of incident light (normalized reflectance). Data was subsequently expressed as percentages of surface downwelling radiance reflected from cleaned coral skeleton (relative reflectance).

Active fluorescence measurements: Throughout the day (06:00, 09:00, 12:00, 14:00, 18:00) photoinhibition of light-exposed and shaded portions of sub-colonies (n=3 per morph), and shaded controls, was measured as decrease in the maximal potential quantum yield (*Fv/Fm*) of PSII by a pulse amplitude modulation fluorometer (DIVING-PAM)[19,20] after 30 min dark-adaption. Photosynthetically active radiation (400-700nm) during the experiment was measured by LI-190SA quantum sensor.

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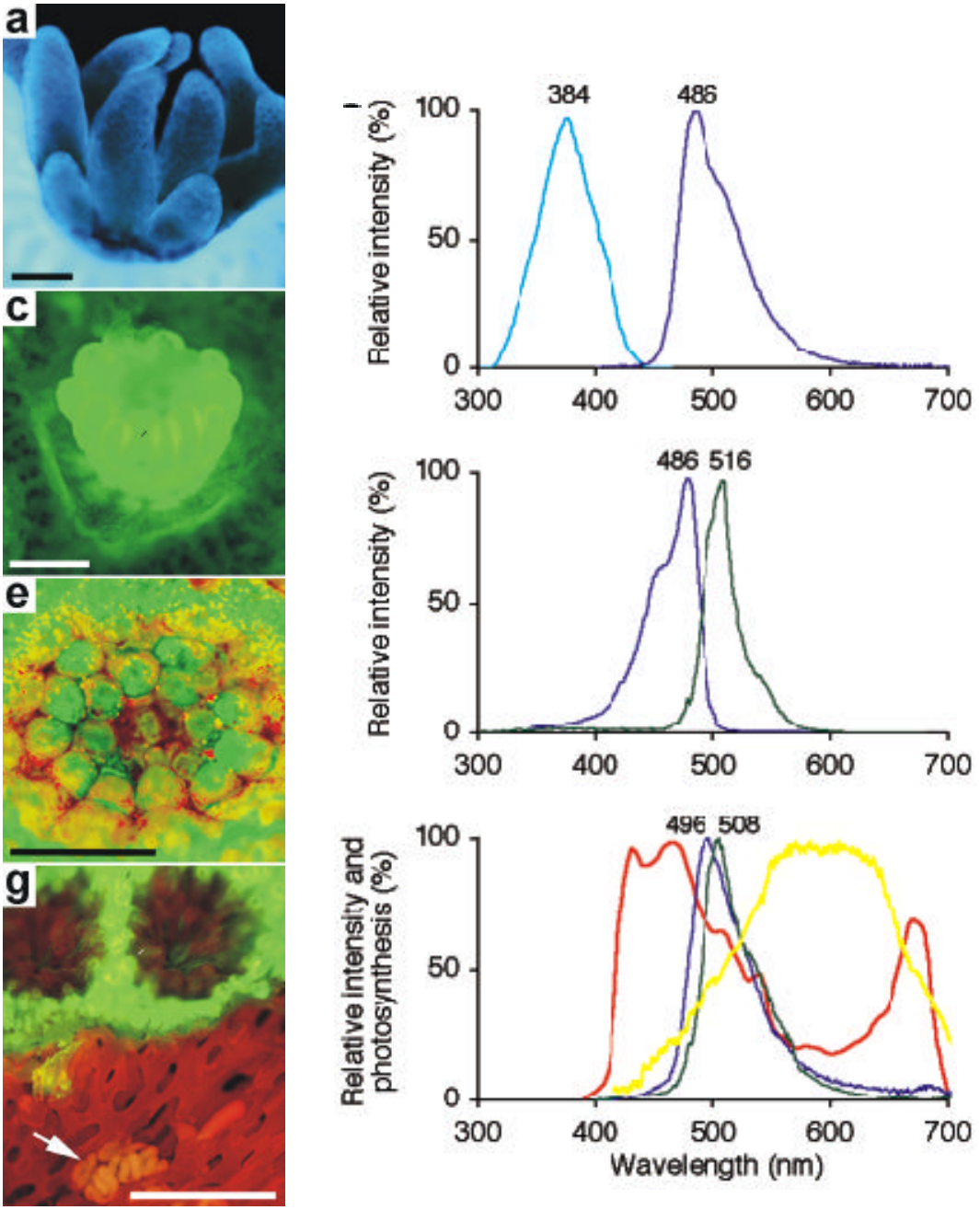

Figure 1 Main types of fluorescent pigments in coral polyps found in blue, green, yellow and red combinations with overlapping excitation and emission spectra. **a-b**, Mainly blue, in *Acropora nobilis*. **c-d**, Mainly green, in *Pocillopora damicornis*. **e-f**, Emissions of outer blue/green and underlying yellow FPs in 'sun' *Porites cylindrica*. Coral photosynthetic action spectrum[24] (red line) shows that much of the energy is emitted at wavelengths not usable in photosynthesis. **g**. Sub-surface red FPs in green *Montipora digitata*. Arrow, red FPs in mesenterial filaments. Scale bars 0.5mm

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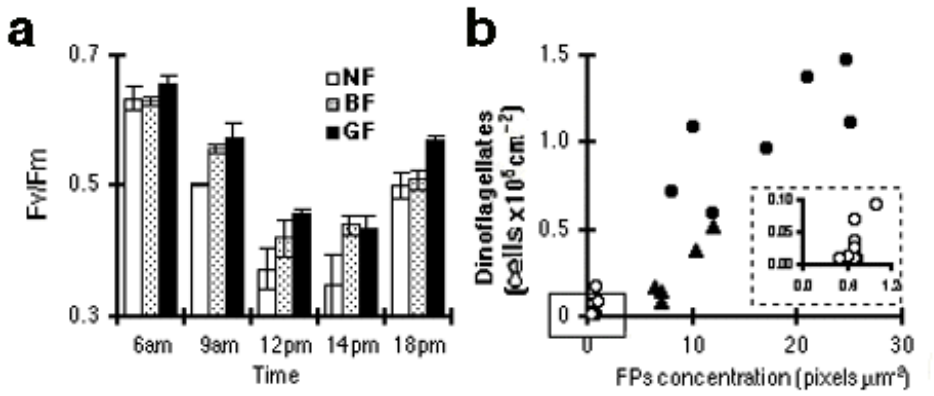

Figure 2 Photoinhibition and bleaching responses of corals. **a**, Maximal potential quantum yield (*Fv/Fm*) of dinoflagellates in green highly fluorescent (GF), brown medium fluorescent (BF) and non fluorescent (NF) *Acropora palifera*. Results are means ± S.E. for 3 sub-colonies x morph x 2 tanks x time interval. **b**, Dinoflagellates cm^{-2} and relative concentration of fluorescent pigments μm^{-2} of sampled corals: ○ - bleached, ▲ - part-bleached, ● - unbleached. Inset - enlarged section of graph marked in square.

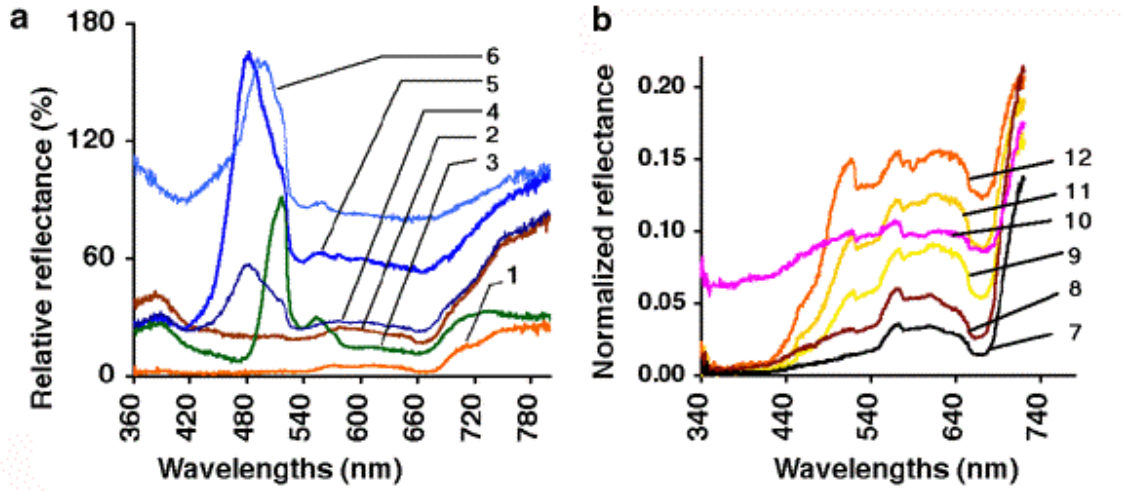

Figure 3 Apparent reflectance. **a,** *Plesiastrea versipora* - tissues lacking FPs (1); tissue with blue FPs overlying skeletal ridge (2); contracted tentacle with green FPGs (3); blue FPGs in expanded (4) and contracted (5) oral disc; dense blue FPGs in white oral disc of shallow-water *Platygyra daedalea* (6). **b**, Intertidal yellow *Porites cylindrica* - 'shade' expanded (7); contracted (8); and 'sun' expanded (11), contracted (12) tentacles; edge of polyp calyx (9); septal skeleton with thin tissue (10). Dips in spectra are due to absorption by photosynthetic pigments. Spectral peaks due to FPs emission and reflection.

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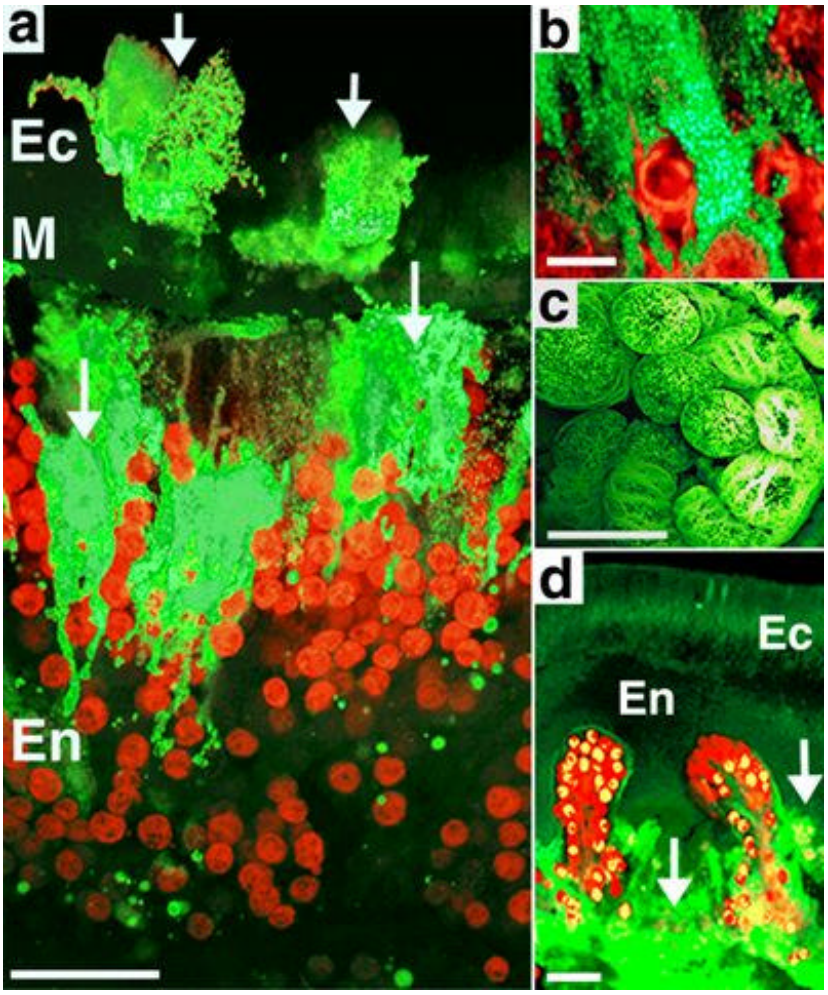

Figure 4 Reconstruction of serial confocal sections through tissues of 'sun' and 'shade' corals. **a**, FPs in chromatophores above endosymbionts in polyp tentacle of 'sun' intertidal *Goniopora tenuidens*. **b**, FPG-filled chromatophores with long extensions enveloping endosymbionts. Bars - 50µm and 10µm, respectively. **c**, Retracted tentacles with dense FPGs form a 'plug' over polyp. Bar - 1 mm. **d**, Endodermal FPs below dinoflagellates in 'shade' *Lobophyllia corymbosa*. Bar - 50µm. FPs shown in green/yellow, symbiotic dinoflagellates in red. Arrows - FP chromatophores. Letter captions: Ec - ectoderm (epidermis), M - mesogloea; En - endoderm (gastrodermis)