Fluorescence color diversity of great barrier reef corals

Grigory Lapshin^{*,†,‡,§,¶}, Anya Salih[‡], Peter Kolosov[§], Maria Golovkina[†],
Yuri Zavorotnyi[¶], Tatyana Ivashina^{||}, Leonid Vinokurov^{**},
Victor Bagratashvili^{††} and Alexander Savitsky^{*,¶}

**Physical Biochemistry Lab, INBI RAS, Leninsky Prospekt
33, Build. 2 Moscow 119071, Russia*

*†Faculty of Bioengineering and Bioinformatics
Moscow State University*

Leninskiye Gory 1-73, MSU GSP-1 Moscow 119991, Russia

*‡Confocal Bio-Imaging Facility (CBIF), Division Deputy Vice-Chancellor
(Research & Development)
University of Western Sydney, Locked Bag 1797
Penrith South DC, NSW 1797, Australia*

*§Institute of Higher Nervous Activity and Neurophysiology
The Russian Academy of Sciences
5A, Butlerova St., Moscow 117485, Russia*

*¶Skobeltsyn Institute of Nuclear Physics, Moscow State University
1(2) Leninskie Gory GSP-1, Moscow 119991, Russia*

*||Molecular Microbiology Lab, Skryabin Institute of Biochemistry
and Physiology of Micro-organisms RAS
Prospect Nauki 5 Pushchino, 142290 Moscow Region, Russia*

***Group of Bioengineering of Reporter Proteins
Branch of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry
of the Russian Academy of Sciences
Prospect Nauki 6 5 Pushchino, 142290 Moscow Region, Russia*

*††Institute of Laser and Information Technologies RAS
2 Pionerskaya, Troitsk, Moscow 142190, Russia*

‡‡grigory.lapshin@gmail.com

¶¶apsavitsky@inbi.ras.ru

Received 25 September 2014

Accepted 6 January 2015

Published 11 February 2015

^{¶¶}Corresponding author.

This is an Open Access article published by World Scientific Publishing Company. It is distributed under the terms of the Creative Commons Attribution 3.0 (CC-BY) License. Further distribution of this work is permitted, provided the original work is properly cited.

A group of variously colored proteins belonging to the green fluorescent protein (GFP) family are responsible for coloring coral tissues. Corals of the Great Barrier Reef were studied with the custom-built fiber laser fluorescence spectrometers. Spectral analysis showed that most of the examined corals contained multiple fluorescent peaks ranging from 470 to 620 nm. This observation was attributed to the presence of multiple genes of GFP-like proteins in a single coral, as well as by the photo-induced post-translational modifications of certain GFP-like proteins. We isolated a novel photo-convertible fluorescent protein (FP) from one of the tested corals. We propose that two processes may explain the observed diversity of the fluorescent spectra in corals: (1) dark post-translational modification (maturation), and (2) color photo-conversion of certain matured proteins in response to sunlight.

Keywords: Coral fluorescence; GFP-like proteins; fluorophores; Kaede.

1. Introduction

The color diversity of the Great Barrier Reef ecosystem in general, and its fluorescence color diversity specifically, is surprising. At the same time the functional role of such diversity remains largely unknown.¹ The natural palette of coral colors is limited by coral host tissue pigments and by the natural brown color of photosynthetic pigments (chlorophyll a and c₂, peridinin) of the endosymbiotic zooxanthellae. Coral host-specific pigments fluoresce under visible light or UV excitation,² while red emissions at 680 nm arise from chlorophylls of zooxanthellae.

In the last two decades it has been established that the fluorescence color diversity of corals is caused by numerous homologs of the green fluorescent protein (GFP).^{3,4} GFP was originally discovered in the jellyfish *Aequorea victoria*⁵ as a part of its bioluminescence system, and being in a pair with the primary blue emitter aequorin, it defines the green color of the jellyfish bioluminescence. The unusual feature of GFP protein superfamily is that, in contrast to most other pigments, the GFP-like proteins do not require any cofactors or prosthetic groups for autocatalytic chromophore formation.^{6,7} Moreover, it is now well known that the GFP-like proteins are not only responsible for the fluorescent colors of corals, but also for their nonfluorescent blue, pink and purple hues.^{6,8}

The functional role of GFP-like proteins remains largely unknown. The main hypothesis centers on the protective and light modulating properties of GFP-like proteins in order to optimize the internal coral tissue light environment for the endosymbiotic dinoflagellates, protecting them from sunlight overexposure and by harvesting of additional sunlight.⁸ Coral reefs are subjected to nonregular sun exposure as light conditions change rapidly and dramatically

due to wave lensing, with depth and over a reef surface. Such light differences may vary greatly even in a single colony. The photoprotective properties of GFPs in high concentrations have been experimentally demonstrated.⁹ Other functions include visual signaling to other reef organisms,⁹ antioxidant activity and immune responses.^{11,12}

To date, fluorescent proteins (FP) of different colors belonging to the GFP family have been found in numerous corals and in many other organisms possessing bioluminescence systems.¹¹ The fluorescence color diversity of corals is based on the variation in the structure of the autocatalytically formed chromophores inside the protein. Seven classes of chromophores according to their spectral properties and structure have been revealed.¹³ Among them, four classes of chromophores (cyan-green fluorescent, yellow fluorescent, red fluorescent and nonfluorescent chromoproteins) were discovered earlier,^{3,14} while the other three classes of chromophores (Kaede type with a very narrow orange-red fluorescence,^{15,16} the kindling FP type with a broad deep red fluorescence,¹⁷ and the artificially generated mutant form called mOrange¹⁸) were described more recently.

The diversity of GFP-based colors varies between species as well as within a species, which can exist as several color morphs.⁹ Colonies of *Montastrea cavernosa* were shown to demonstrate color polymorphism with color morphs of three colors: green, blue or red. For some species it has been shown that color differentiation is caused by relative variation in gene expression of multiple paralog genes of GFP-like proteins of different colors.¹ *Lobophyllia hemprichii*, *Favites ahadita* and *M. cavernosa*, in particular, belong to the *Faviina* suborder of stony corals and share the same paralogous groups

of GFP-like proteins.^{10,13} The coloration of a coral's body is not homogenous, with GFP-type pigments concentrated at various body parts such as tentacle tips, oral cones and calyces. Light can also cause upregulation of certain GFP-type proteins in many coral species so that the outermost coral branches are usually more fluorescently pigmented compared to the lower shaded branches.^{9,19,20} On the whole, information about natural color polymorphism of corals is still limited due to the relatively low number of the species that had been studied.

The present study reveals the characteristics of the fluorescence spectra of several species and color morphs of corals from the Great Barrier Reef. In order to excite effectively fluorescence emissions from all multiple colors FPs present in coral tissues and to study their whole visible fluorescence range, our spectral studies were accomplished by using lasers with different wavelengths (380, 473, 532, 598 and 635 nm) for *in situ* excitation of live coral samples.

2. Materials and Methods

2.1. Sample collection

Screening of scleractinian corals for fluorescence was done *in situ*, underwater, using blue light torches (Night Sea Inc) as described previously¹² at Heron Island and One Tree Island located in the center of the Capricorn Group of the Great Barrier Reef and ~100 km off the Queensland coast, Australia. The following coral species were sampled from the lagoon and reef flat of Heron Island: *Acropora pulchra*, *Acropora* sp., *Acropora ditifera*, *Acropora hyacinthus*, *Acropora divaricata*, *Acropora chesterfieldensis*, *Montipora digitata*, *Porites murrayensis*, *Porites cylindrica*, *Pocillopora damicornis*, *Cyphastrea microphthalma*, *Favites russelli*, *Platygyra lamellina*, *Fungia* sp., *Favites complanata* and *Favites rotundata*. On one tree island (OTI) the following samples were collected: *Nephtheidae nephthea*, *Acropora abrotanoides*, *Acropora secale*, *Montipora turgescens*, *Montipora monasteriata*, *Montipora turtlensis*, *Hydnophora pilosa*, *Seriatopora hystrix*, *Stylophora pistillata*, *F. complanata*, *Goniastrea retiformis*, *Echinophyllia aspera*, *Oxyopora* sp., *Psammacora* sp., *L. hemprichii*, *Lobophyllia pachysepta*, *Stylocoeniella armata*, *E. aspera* and *Goniopora* sp.

2.2. Measurements of coral fluorescence

Following screening of corals for fluorescence using underwater blue light torches, we observed heterogeneity in fluorescence color distribution over coral colonies. For further characterization of coral fluorescence, three different fiber optical spectrofluorimeters, thereby allowing the sampling of fluorescence spectra from small spots with the diameter of less than a few millimeters, were used to investigate the observed multi-color fluorescence heterogeneity.

The coral samples from Heron Island were examined using fluorescence spectroscopy by Spectra-Cluster with excitation at 532 and 635 nm and Oceanoptics s2000 (Ocean Optics LLC) fiber-optic spectrometers with excitation at 380 nm.²¹

Corals from One Tree Island were examined with a custom-designed fibreoptic imager based on Spectra-Cluster spectrofluorimeter. Its fiber bundle consisted of seven optical fibers. One of the fibers was used for sample excitation and guided beam from one of three solid state lasers: 473 (15 mW), 532 (5 mW) and 598 nm (5 mW). Six other fibers guided fluorescence from a sample to the polychromator, with spectral resolution of 1 nm and 400–900 nm spectral range. The interference filters were used for elimination of excitation light scattering. The optical fiber tip was attached to a two-coordinate actuator with two stepping motors. Each sample was observed through a lens with a 75 mm focal distance and 60 mm aperture. This setup provided a 15 × 15 mm spatial range with 10 μm resolution. A digital camera (Canon S3IS) was used for the overall sample imaging. The scheme of our custom-designed two-dimensional (2D) spectral imager at given in Fig. 1. The custom-made software was used for 2D spectral imager operation.

The set of lasers used for the excitation of samples allowed us to detect all possible FPs in the visible range of spectra. However, for long-wavelength excitation, at 598 and 635 nm, distinctive chlorophyll spectra of endosymbionts were observed in 650–750 nm range. Chlorophylls peaked at 685 nm, with a shoulder at 745 nm, were also observed upon excitation at shorter wavelengths but the latter were excluded from the analysis.

To identify the diversity of spectral bands of corals, all the fluorescence spectra obtained from our collection of corals were fitted with multiple

Fig. 1. Scheme of custom designed 2D spectral imager. 1 – spectrometer part with plane actuators, 2 – lens, 3 – digital camera, 4 – semitransparent mirror, 5 – focal plane actuator and A – sample.

peaks by Gauss algorithm in OriginPro 9.0 program. Example of fitting is shown in Fig. 2.

2.2.1. cDNA cloning and Gene construction

One sample of coral *Stylocoeniella armata* was selected for FP genes isolation due to its deep red color. Coral genetic material was stabilized in RNA-later and transported to the biochemical laboratory. Total DNA was isolated with TRIzol Reagent (Ambion). Coral samples were ground in a mortar by pestle, suspended in 1 mL Trisol, transferred to an eppendorf tube and intensely mixed by pipetting. Subsequently 0.5 mL of chlorophorm was added, vortex mixed for 30 s and centrifuged at 10,000 rpm for 10 min at 4°C. The water fraction was mixed with 0.5 mL of isopropanol, incubated

Fig. 2. Fluorescence spectra of *A. digitifera* (A), *A. hyacinthus* (B) *E. aspera* (C) from two different parts of colonies. Spectra are shown with solid blue and red lines. Fluorescence curves were analyzed and their Gauss peaks shown (narrow solid lines colored as analyzed curves). A and B spectra obtained with Spectral-Cluster and C with custom made spectrometer. Excitation is 380 nm (A and B) and 473 nm (C).

for 5 min at room temperature and centrifuged at 10,000 rpm for 10 min at 4°C. The precipitate of RNA was washed by 0.7 mL of 80% ethanol and dissolved in 20 µL of water. The cDNA library was synthesized from 1 microgram total RNA by Mint-2 cDNA synthesis kit (Evrogen) (the same as SMARTer PCR cDNA Synthesis Kit (Takara, Clontech)) according to the manufacturer's protocol. Total RNA was first mixed with the primer oligo-dT, denatured for 2 min at 70°C, then cooled to 42°C (primer annealing), mixed with Mint revertase and incubated for 30 min at the same temperature for synthesis of the first strand of single stranded cDNA. The second strand was synthesized by adding plugOligo primer and incubated for 90 min at the same temperature. cDNA library was amplified by 19 cycles. Before cloning, cDNA library was normalized by Trimmer-2 kit (Evrogen) according to the manufacturer protocol. The normalized library was cloned into the vector pGEM-T easy and transformed into the strain *E. coli* JM109-T7pol. For obtaining 100,000 individual colonies 37 dishes (90 mm diameter) with LB-agarose medium containing antibiotic were used for 2000–2500 colonies per dish. Dishes were first incubated for 12 h at 37°C; the second incubation was at least 2 weeks at 4–10°C in the refrigerator for protein expression in colonies coding the FPs. The third incubation continued for several days in sunlight at room temperature for photo conversion of light sensitive FPs.

Colonies were screened for fluorescence with a Nikon TE2000 fluorescence microscope using a wide range of excitation and emission wavelengths. Single fluorescent colonies were picked and cultivated for protein isolation.

2.2.2. FP expression in *E. coli*

For expression in *E. coli* cells the full-length Open reading frame (ORF) of the selected sample named SAASoti (669 bp) was amplified from pGEM-T-SAP4f using Kod Hot start polymerase (Novagen) and a pair of primers, and inserted into NdeI/EcoRI sites of pET22b (Novagen, USA) to yield pET-SAASoti. The recombinant plasmid pET-SAASoti was transformed into *E. coli* BL21(DE3), cultured at 37°C in LB medium with 100 mg of ampicillin to an optical density of 0.5, then induced with 0.25 mM IPTG, and further incubated for 24 h at 20°C.

For protein isolation, *E. coli* was disrupted with French press, proteins were precipitated with sodium sulfate and the colored fraction was separated by centrifugation. The protein was further purified with MonoQ based ion-exchange chromatography and gel-filtration on a Superdex 200 10/300 GL column.

2.3. Homology with other FPs

Homologs of the extracted *Stylocoeniella armada* FP were found with NCBI Protein BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with default settings and with search through the Swissprot database.

2.4. Spectral characterization of purified protein

Spectral properties and photoconversion of purified FP were studied using a Cary Eclipse fluorimeter and a Cary 300 spectrophotometer. For photoconversion studies, the solid state laser (405 nm, 80 mW power) was used for excitation and a Spectral-cluster spectrometer was used for the fluorescence emission measurements.

2.5. Phylogenetic analysis

The coding SAASoti DNA was aligned with aligned set of previously reported FP genes.¹¹ GTR+G+I evolution model was used after previous works on FP phylogeny.¹¹ The phylogenetic analysis was performed with MrBayes 3.2.²² The MCMCMC chain was run for 1,500,000 iterations with a sample frequency of 200 resulting in 7500 trees, of which the first 6000 were discarded while summarizing the data. The analysis was run three times to ensure convergence.

3. Results and Discussion

3.1. Natural coral fluorescence

The use of the fiberoptic 2D spectral imager allowed us to compare fluorescence spectra from different spot locations of each coral studied. The majority of the corals studied, such as *Acropora digitifera*, *A. hyacinthus* and *E. aspera* (Fig. 2), showed a distinct spectral heterogeneity over their surface, with at least two different spectral FP variants present.

A vast variety of fluorescence peaks were observed in the 470–630 nm spectral range. From the summary of the spectral peaks presented in Table 1, it can be concluded that every studied coral contains multiple fluorophores with fluorescent emitters covering the whole of the above mentioned spectral range. The most common fluorescence found in the majority of samples was in cyan 480–500 nm spectral range, followed by green-yellow peaks at 500–560 nm, and yellow-orange peaks in 560–580 nm range. Our findings support published data¹⁰ of multiple copies of GFP like genes present in corals. FPs with longer emission wavelengths are very rare and, if present, are partly hidden by strong chlorophyll fluorescence.

Three major color bands were well correlated with the previous observations of faviid suborder members.¹⁰ It was shown, for example, that *M. cavernosa* has three color morphs: cyan, green and red and their color was defined by the differences in the expression of FP genes. Our study revealed that almost every coral from the Great Barrier Reef has these three colors as a part of their natural coloration (Table 1).

Color polymorphism of *M. cavernosa* was described as a whole colony color change with three

different color morphs found to occur at reef locations and morph color was attributed to the main FP present. Color differentiation over a colony could be explained by varying expressions of FP genes in tissues of a single colony. Such pattern of coloration was described for *Acropora millepora* adult corals,¹⁰ as well as its larvae with green fluorescent body and distinct red fluorescent coloration on aboral pole.²³ In our study, we found a mix of spectral bands in the colony fluorescence and the ratio of these bands corresponded to the final coloration of each coral at a micro or mm-scale level. Visually, spectral resolution of coral tissues frequently did not match their perceived color. This was mainly the case for cyan FPs that we found to be the most abundant emitters in the majority of tested corals, and yet the latter did not appear cyan fluorescent, but had a gray, whitish or beige coloration in sunlight. This can be explained by the higher sensitivity of human visual receptors to the longer, more red-shifted, compared to the shorter fluorescent wavelengths, so that green and red FPs are perceived by humans as the more colorful.^{10,24} Moreover, high concentrations of cyan FPs have been

Table 1. Major and minor fluorescence peaks in the studied coral colonies. Big crosses contribute to major peaks, small to minor. Color of crosses codes excitation wavelength: blue, yellow and red corresponds to 380, 532 and 473 nm excitation, respectively.

	380	532	473	480-500	500-520	520-540	540-560	560-580	580-600	600-620	620-640	640-660
Heron Island												
<i>Porites cylindrica</i>	x	x	x				x	x	x	x	x	
<i>Acropora pulchra</i>	x	x	x					x	x	x	x	x
<i>Acropora</i> sp.	x	x	x				x	x	x	x		
<i>Montipora digitata</i>								x				
<i>Porites murrayensis</i>						x		x		x		x
<i>Pocillopora damicornis</i>	x						x	x		x		x
<i>Cyphastrea microphthalma</i>								x				
<i>Acropora ditifera</i>	x		x				x	x	x	x	x	
<i>Acropora hyacinthus</i>	x		x					x	x	x		
<i>Acropora divaricata</i>		x						x				
<i>Favites ruselli</i>	x							x				
<i>Platygyra lamellina</i>	x					x		x				
<i>Fungia</i> sp.		x						x		x		
<i>Favites complanata</i>	x	xx					x	xx		x		
<i>Acropora chesterfieldensis</i>	x	xx					x	x		x		
<i>Favita rotundata</i>	x	x				x		x				
One Tree Island												
<i>Nephtheidae Nephthei</i>		x						x				
<i>Stylophora pistillata</i>	x					x				x		
<i>Montipora turgescens</i>	x	x										
<i>Hydnophora pilosa</i>		x				x						
<i>Seriatopora hystrix</i>	x	x					x					
<i>Stylophora pistillata</i>	x					x		x				
<i>Acropora abrotanoides</i>					x		x					
<i>Acropora secale</i>												
<i>Favites complanata</i>												
<i>Goniastrea retiformis</i>												
<i>Echinophyllia aspera</i>	x	x						x				x
<i>Oxyopora</i> sp.		x				x		x				x
<i>Psammacora</i> sp.							x		x			
<i>Lobophyllia hemprichii</i>		x					x					
<i>Montipora turtensis</i>	x	x								x		
<i>Stylocoeniella armata</i>							x			x		x
<i>Lobophyllia pachysepta</i>	x	x						x		x		
<i>Montipora cf. monasteriata</i>	x	x						x		x		
<i>Echinophyllia aspera</i>	x	x						x		x		
<i>Goniopora</i> sp.								x				

shown to be highly light scattering, giving cyan coral tissues metallic gray or even whitish coloration.⁹ Another likely reason for the visual to spectral mismatch can be attributed to energy transfer by FPs in coral tissues: cyan and green FPs can form donor–acceptor Förster resonance energy transfer (FRET) pairs in corals, so that the emissions from donor cyan FPs are quenched by the acceptor green FPs.^{25,26}

3.2. Photoconvertible protein from *S. armata*

3.2.1. Homology to other FP

Another possible explanation of color differentiation may involve photoconversion of coral FPs from one color to another following irradiation by specific wavelengths. Photoconvertible proteins were originally described as proteins having green to red light

activated fluorescence shift.¹⁵ Coral species with photoconvertible FPs may have three color varieties achieved with only a single green-to-red photoconverting FP, producing either green, red or yellow colored tissues or colony color morphs when the two forms appear as mixed populations.^{16,19}

We carried out a homology analysis of the gene sequence obtained from one of the collected samples, *S. armata*, from which a GFP-like gene was extracted and found the presence of photoconvertible protein with close homology to the green-to-red converting FPs.

We named the protein SAASoti as this coral was collected from One Tree Island (hence, OTI). The chromophore-forming amino acids (Tyr-66, Gly-67) and the amino acids essential for chromophore formation (Arg-96, Glu-222) were conservative. The protein showed 50.2% identity with the original photo-converting FP known as Kaede.¹⁵ Similar to

		*		20	*		40	
avGFP	:	MSKGEEL----	FTGVVPI	LVELDGD	VNGHKFS	VS	GE	EGDA : 37
SAASoti	:	-----	MALSKQYIP	DDMELIF	HMDGCVN	GHYFTT	VATG	KAKP : 37
Kaede	:	-----	MSL----	IKPEMKIK	LLMEGNV	NHGFVIE	GDGK	GHP : 33
		*		60	*		80	
avGFP	:	TY--	GKLT	LKFICTTG--	KLPVPWP	TLVTTFSY	GVQC	FSRYPD : 76
SAASoti	:	--	YEGK	QNLKATV	TKGAPLP	FTDILSTV	MHYGN	RCIVHYPP : 77
Kaede	:	--	FEGK	QSMDLV	VKEGAPLP	FAYDILT	TAFHYC	NRVFAKYPD : 73
		*		100	*		120	
avGFP	:	HM	QHDFF	KSAMPEGY	VQERTIFF	KDDGN	YKTRAE	VKFEGD : 117
SAASoti	:	GI--	LDYFK	QSFPEG	YSWERTFA	FEDGGF	CTASAD	IKLKDN : 116
Kaede	:	HI--	PDYFK	QSFPGF	SWERSLM	FEDGGV	CIATND	ITLKGD : 112
		*		140	*		160	
avGFP	:	TL	VNR	IELKGI	DFKEDGN	ILGHKLE	YNYN	SHNVYIMADKQK : 158
SAASoti	:	CF	IHTS	MFHGVN	FPADGPV	MQRKT--	IQWEK	SIEKMTV--SD : 154
Kaede	:	TF	FNKVR	FDGVNF	PPNGPVM	QKKT--	LKWEA	STEKMYL--RD : 150
		*		180	*		200	
avGFP	:	NG	IKVN	FKIRHN	IEDGSVQL	ADHYQONT	PIGDGP	VLLPDNH : 199
SAASoti	:	GIV	KDIT	MTMFL	LLLEGG	KYRCQF	HTSYKAK--	KV--VEMPQSH : 193
Kaede	:	GVL	TGDI	TMALL	LKGDVH	YRCDFR	TTYKSR--	QEGVKLPGYH : 190
		*		220	*		240	
avGFP	:	YL	STQS	ALS	KDPNEK	RDHMLLE	FVTAAG	ITHGMDELYK-- : 238
SAASoti	:	YVEH--	SIERT	NDDGTQ	FELNEHA	VAR--L	NEI-----	: 222
Kaede	:	FVD	HCIS	ILRH	DKDYN--	EVKLYE	HAVA--H--	SGLPDN--VK : 225

Fig. 3. Amino acid sequence (single-letter code) alignment of the novel protein SAASoti with Kaede and avGFP (*A. victoria* GFP).²⁷ The numbering is based on *A. victoria* GFP. In the sequence of avGFP sheet-forming regions are underlined. Residues whose side chains form the interior of the β -can³ are shaded. Residues responsible for chromophore synthesis are indicated by black shade.

Fig. 4. Absorption of SAASoti before and after sunlight exposure.

Kaede protein, SAASoti contained a histidine residue in the chromophore (Fig. 3).

Unfortunately, the full genome of *S. armata* was not available. However, NCBI GeneBlast contains two proteins from *Stylocoeniella* sp. with 65% and 50% identity with SAASoti protein sequence. Both proteins contain a chromophore that is different from the chromophore of SAASoti.

3.2.2. Spectral properties of SAASoti

The absorption spectrum of the purified protein in Tris buffer (pH = 7.5) (Fig. 4) had a major

Fig. 5. Emission and excitation spectra of the green form of SAASoti. Change of green form to red form fluorescence intensity ratio (R:G) in the course of 405 nm laser irradiation. Inset — Time-course of the green to red fluorescence conversion shown as a red to green ratio.

absorption peak at 510 nm with a shoulder at 480 nm. Both these bands were active in fluorescence excitation with 519 nm fluorescence emission peak (Fig. 5). The protein was found to be highly photoactive, so that direct sunlight exposure was sufficient for its photoconversion from green to red color.

For precise characterization of the conversion process we used the 405 nm laser since photoconversion of Kaede-type proteins is known to be induced by UV light. The protein was exposed to laser irradiation (power density — 300 mW/cm²) in a silica cell. In the course of laser exposure, the 519 nm peak decreased and the 589 nm peak appeared (Fig. 5). The maximal photoconversion rate was reached after 10 min. The ratio of red to green peak intensities increased from 1 to 13 (Fig. 5). With continuing exposure, the red form slightly bleached.

Since the majority of the described photoconverting FPs, as well as SAASoti, are sensitive to UV-A wavelengths, this property may be linked to the ambient light conditions of shallow tropical reefs (this paragraph was moved from conclusion).

The described spectral properties of SAASoti were found to be similar to Kaede. With absorption peak at 508 and 475 nm shoulder, Kaede has maximal emission of the green form at 518 nm. Similar to Kaede, SAASoti's chromophore showed a strong pH dependence both in the absorbance and the excitation and it lost almost all fluorescence at pH < 6, while attaining maximal fluorescence at pH > 8 (Fig. 6).

Fig. 6. Effect of pH on fluorescence and absorbance of green form of SAASoti protein. Absorbance at 508 nm and fluorescence at 500 nm. Measurements were performed in 20 mM Tris-HCl 100 mM NaCl 30 mM imidazole.

Fig. 7. Bayesian phylogenetic tree of coral sourced FPs. Genes of interest are contrasted with bold font. Clades and subclades coded by letters.

3.2.3. Phylogenetical analysis

We carried out a phylogenetical analysis of SAASoti gene based on the known GFP-like protein genes set from Ref. 11. Bayesian phylogenetic tree of coral-sourced FPs is presented in Fig. 7. Clades were labeled from B to D and it had been reported that each of these clades has a strong phylogenetic support.^{11,14} Clade B is comprised mostly of the purple-blue non-fluorescent chromoproteins, which have been cloned from families *Acroporidae*, *Pocilloporidae*, *Poritidae*, *Faviidae*, *Pectinidae*, *Oculinidae* and *Dendrophylliidae*.¹¹ Clade C contains GFP-like proteins from predominantly the order *Scleractinia*, several from *Zoanthidea* and a single cyan FP from *Anemonia majano* from the order Actiniaria.¹¹ Subclade C1 contains representatives from several coral families and may correspond to a grouping of these families into one of the Robusta subclades in accordance to the novel coral phylogeny.^{11,28} Subclade C2 contains green and cyan proteins from Archaeocoeniina suborder (families *Acroporidae* and *Pocilloporidae*) and a cyan protein from the sea anemone *A. majano* (amajCFP, original name amFP486).¹¹ The C3 subclade is again a mixture of coral suborders. Clade D is also a mixture of different coral families, mostly from the suborder *Faviina*.¹¹

Examining the GFP-like proteins from our studied coral species, two genes from *Stylocoeniella sp.* were previously reported and included in the phylogenetic analysis: stylGFP and stylCP.¹¹ Although these proteins and SAASoti are sourced from the same coral species, our analysis showed that they appeared in different clades. SAASoti gene nested in C3 clade, stylCP and stylGFP genes nested in clades B and C2, correspondingly. This indicates that the three genes evolved separately over a long time. It could be that some of them were adopted by the horizontal gene transfer from other species or genes have been duplicated over time. Such phylogenetic separation is not however an uncommon situation: different FP genes sourced from one species—*A. millepora*, *Acropora aculeus*, *Galaxea fascicularis* and several other corals were also nested in different clades; while GFP-type proteins with a similar chromophore were also sometimes grouped into different clades, as different clade as we found for SAASoti and Kaede. In contrast, Kaede²⁹ and another photoconvertible protein — EosFP,¹⁶ were grouped in the same subclade. This grouping points to a common

ancestor for the Kaede-like proteins. It is still unclear what the evolutionary relationships are between SAASoti protein and other photoconvertible FPs.

4. Conclusion

We analyzed several coral samples with fiber spectrofluorometers and discovered that every coral contained multiple fluorescent wavelength bands covering nearly the whole visible spectral range. Fluorescence profiles were heterogeneous over the coral surfaces and generally were due to mixtures of FPs from different spectral classes. We concluded that the differences in coral surface tissue color in some cases can be explained by the photoconversion of FPs. In summary, the variety of color bands in corals can be determined by fewer genes and corals may vary their color in post-translational manner, one of which may be the photo-conversion effect. This conclusion supports the earlier work on green-to-red converting FPs from *M. cavernosa* and *Lobophyllia* spp.¹⁹ Our study indicates that such photonvertable proteins may be much more common in corals than previously realized.

Acknowledgments

RAS presidium grant “Molecular cellular biology”, RFBR 06-02-02100, RFBR CCDFR 13-00-40303. Australian Research Council and FABLS grants to A. Salih. Corals collected under Great Barrier Reef Marine Park Authority permit to A. Salih.

References

1. I. V. Kelmanson, M. V. Matz, “Molecular basis and evolutionary origins of color diversity in great star coral *Montastraea cavernosa* (Scleractinia: Faviida),” *Mol. Biol. Evol.* **20**, 1125–1133 (2003).
2. C. Mazel, “Spectral measurements of fluorescence emission in Caribbean cnidarians,” *Mar. Ecol. Prog. Ser.* **120**, 185–191 (1995).
3. M. V. Matz, A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zaisky, M. L. Markelov, S. A. Lukyanov, “Fluorescent proteins from non-bioluminescent Anthozoa species,” *Nat. Biotechnol.* **17**, 969–973 (1999).
4. S. Dove, O. Hoegh-Guldberg, S. Ranganathan, “Major colour patterns of reef-building corals are due to a family of GFP-like proteins,” *Coral Reefs* **19**, 197–204 (2001).

5. O. Shimomura, F. H. Jonhson, Y. Saiga, "Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydro-medusan, *Aequorea*," *J. Cell. Comp. Physiol.* **59**, 223–239 (1962).
6. K. A. Lukyanov, A. F. Fradkov, N. G. Gurskaya, M. V. Matz, Y. A. Labas, A. P. Savitsky, M. L. Markelov, A. G. Zaraisky, X. Zhao, Y. Fang, W. Tan, S. A. Lukyanov, "Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog," *J. Biol. Chem.* **275**, 25879–25882 (2000).
7. C. W. Cody, D. C. Prasher, W. M. Westler, F. G. Prendergast, W. W. Ward, "Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein," *Biochemistry* **32**, 1212–1218 (1993).
8. E. G. Smith, C. D'Angelo, A. Salih, J. Wiedenmann, "Screening by coral green fluorescent protein (GFP)-like chromoproteins supports a role in photoprotection of zooxanthellae," *Coral Reefs* **32**, 463–474 (2013).
9. A. Salih, A. Larkum, G. Cox, M. Kühl, O. Hoegh-Guldberg, "Fluorescent pigments in corals are photoprotective," *Nature* **408**, 850–853 (2000).
10. M. V. Matz, N. J. Marshall, M. Vorobyev, "Are corals colorful?," *Photochem. Photobiol.* **82**, 345–350 (2006).
11. N. O. Alieva, K. A. Konzen, S. F. Field, E. A. Meleshkevitch, M. E. Hunt, V. Beltran-Ramirez, D. J. Miller, J. Wiedenmann, A. Salih, M. V. Matz, "Diversity and evolution of coral fluorescent proteins," *PLoS One* **3**, 12 (2008).
12. A. Salih, "Screening reef corals for novel GFP-type fluorescent proteins by confocal imaging," *Methods Mol. Biol.* **872**, 217–233 (2012).
13. S. F. Field, M. V. Matz, "Retracing evolution of red fluorescence in GFP-like proteins from *Faviina* corals," *Mol. Biol. Evol.* **27**, 225–233 (2010).
14. Y. A. Labas, N. G. Gurskaya, Y. G. Yanushevich, A. F. Fradkov, K. A. Lukyanov, S. A. Lukyanov, M. V. Matz, "Diversity and evolution of the green fluorescent," **99**, 4256–4261 (2002).
15. R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno, A. Miyawaki, "An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein," *Proc. Natl. Acad. Sci. USA* **99**, 12651–12656 (2002).
16. J. Wiedenmann, S. Ivanchenko, F. Oswald, F. Schmitt, C. Röcker, A. Salih, K.-D. Spindler, G. U. Nienhaus, "EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion," *Proc. Natl. Acad. Sci. USA* **101**, 15905–15910 (2004).
17. M. L. Quillin, D. M. Anstrom, X. Shu, S. O'Leary, K. Kallio, D. M. Chudakov, S. J. Remington, "Kindling fluorescent protein from *Anemonia sulcata*: Dark-state structure at 1.38 Å resolution," *Biochemistry* **44**, 5774–5787 (2005).
18. X. Shu, N. C. Shaner, C. A. Yarbrough, R. Y. Tsien, S. J. Remington, "Novel chromophores and buried charges control color in mFruits," *Biochemistry* **45**, 9639–9647 (2006).
19. F. Oswald, F. Schmitt, A. Leutenegger, S. Ivanchenko, C. D'Angelo, A. Salih, S. Maslakova, M. Bulina, R. Schirmbeck, G. U. Nienhaus, M. V. Matz, J. Wiedenmann, "Contributions of host and symbiont pigments to the coloration of reef corals," *FEBS J.* **274**, 1102–1109 (2007).
20. P. J. Ralph, U. Schreiber, R. Gademann, M. Kühl, A. W. D. Larkum, "Coral photobiology studied with a new imaging pulse amplitude modulated fluorometer1," *J. Phycol.* **41**, 335–342 (2005).
21. Y. F. Loginova, N. I. Kazachkina, V. V. Zherdeva, A. L. Rusanov, M. V. Shirmanova, E. V. Zagaynova, E. A. Sergeeva, S. V. Dezhurov, M. S. Wakstein, A. P. Savitsky, "Biodistribution of intact fluorescent CdSe/CdS/ZnS quantum dots coated by mercaptopropionic acid after intravenous injection into mice," *J. Biophotonics* **5**, 848–859 (2012).
22. J. P. Huelsenbeck, F. Ronquist, "MRBAYES: Bayesian inference of phylogenetic trees," *Bioinformatics* **17**, 754–755 (2001).
23. A. Salih, A. W. Larkum, T. W. Cronin, J. Wiedenmann, R. Szymczak, G. C. Cox, "Biological properties of coral GFP-type proteins provide clues for engineering novel optical probes and biosensors," A. P. Savitsky, L. Y. Brovko, D. J. Bornhop, R. Raghavachari, S. I. Achilefu, Eds., *SPIE Proceedings, Color Proteins: Photophysics and Biosensors III*, Vol. 5329, pp. 61–72 (2004).
24. C. H. Mazel, M. P. Lesser, M. Y. Gorbunov, T. M. Barry, J. H. Farrell, K. D. Wyman, P. G. Falkowski, "Green-fluorescent proteins in Caribbean corals," *Limnol. Oceanogr.* **48**, 402–411 (2003).
25. A. M. Gilmore, A. W. D. Larkum, A. Salih, S. Itoh, Y. Shibata, C. Bena, H. Yamasaki, M. Papina, R. Van Woesik, "Simultaneous time resolution of the emission spectra of fluorescent proteins and zooxanthellar chlorophyll in reef-building corals," *Photochem. Photobiol.* **77**, 515–523 (2003).
26. G. Cox, M. Matz, A. Salih, "Fluorescence lifetime imaging of coral fluorescent proteins," *Microsc. Res. Tech.* **70**, 243–251 (2007).
27. R. Y. Tsien, "The green fluorescent protein," *Annu. Rev. Biochem.* **67**, 509–544 (1998).
28. A. M. Kerr, "Molecular and morphological supertree of stony corals (Anthozoa: Scleractinia) using matrix representation parsimony," *Biol. Rev. Camb. Philos. Soc.* **80**, 543–558 (2005).
29. H. Mizuno, T. K. Mal, K. I. Tong, R. Ando, T. Furuta, M. Ikura, A. Miyawaki, "Photo-induced peptide cleavage in the green-to-red conversion of a fluorescent protein," *Mol. Cell* **12**, 1051–1058 (2003).