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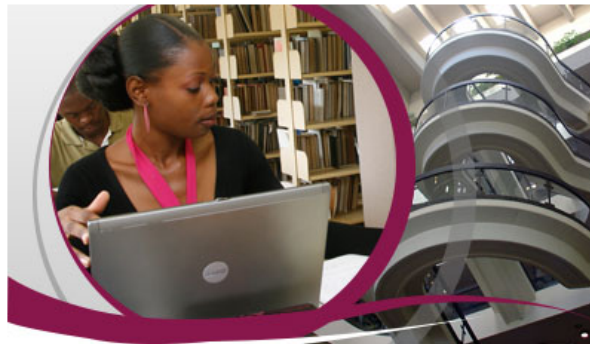
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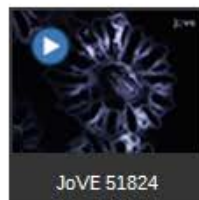
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Multimodal Optical Microscopy Methods Reveal Polyp Tissue Morphology and Structure in Caribbean Reef Building Corals

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Published **September 5, 2014**. Keywords: [Environmental Sciences](#), [Serial block face imaging](#), [two-photon fluorescence microscopy](#), [Montastraea annularis](#), [Montastraea faveolata](#), [3D coral tissue morphology and structure](#), [zooxanthellae](#), [chromatophore](#), [autofluorescence](#), [light harvesting optimization](#), [environmental change](#)

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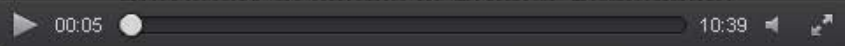
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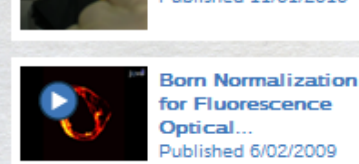
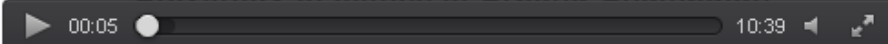
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Abstract

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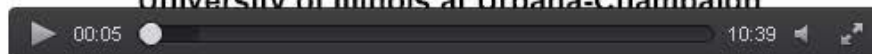


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Keywords: Environmental Sciences, Issue 91, Serial block face imaging, two-photon fluorescence microscopy, *Montastraea annularis*, *Montastraea faveolata*, 3D coral tissue morphology and structure, zooxanthellae, chromatophore, autofluorescence, light harvesting optimization, environmental change

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An integrated suite of imaging techniques has been applied to determine the three-dimensional (3D) morphology and cellular structure of polyp tissues comprising the Caribbean reef building corals *Montastraea annularis* and *M. faveolata*. These approaches include fluorescence microscopy (FM), serial block face imaging (SBFI), and two-photon confocal laser scanning microscopy (TPLSM). SBFI provides deep tissue imaging after physical sectioning; it details the tissue surface texture and 3D visualization to tissue depths of more than 2 mm. Complementary FM and TPLSM yield ultra-high resolution images of tissue cellular structure. Results have: (1) identified previously unreported lobate tissue morphologies on the outer wall of individual coral polyps and (2) created the first surface maps of the 3D distribution and tissue density of chromatophores and algae-like dinoflagellate zooxanthellae endosymbionts. Spectral absorption peaks of 500 nm and 675 nm, respectively, suggest that *M. annularis* and *M. faveolata* contain similar types of chlorophyll and chromatophores. However, *M. annularis* and *M. faveolata* exhibit significant differences in the tissue density and 3D distribution of these key cellular components. This study focusing on imaging methods indicates that SBFI is extremely useful for analysis of large mm-scale samples of decalcified coral tissues. Complementary FM and TPLSM reveal subtle submillimeter scale changes in cellular distribution and density in nondecalcified coral tissue samples. The TPLSM technique affords: (1) minimally invasive sample preparation, (2) superior optical sectioning ability, and (3) minimal light absorption and scattering, while still permitting deep tissue imaging.

Video Link

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Introduction

Global warming and accompanying environmental change are directly affecting the health and distribution of tropical marine corals¹⁻⁴. Multiple impacts are being observed, including coral bleaching and the emergence of infectious diseases^{5,6}. However, more accurate prediction of future coral response to these environmental threats will require that a histological "baseline" be established, which defines tissue morphology and cell composition and distribution for "apparently healthy" corals. In turn, "impacted" corals can then be quantitatively compared. Furthermore, this baseline should be established for apparently healthy corals under a variety of environmental conditions, so that "healthy response" can also be gauged across environmental gradients. As an initial step toward establishing this baseline, a high-resolution 3D study has been undertaken of how apparently healthy coral polyp tissue morphology and cellular composition responds to increases in water depth (WD) and accompanying decreases in sunlight irradiance. Results can then be used to establish a more comprehensive mechanistic understanding of coral adaptation, as well as to gain insight into coral-symbiont evolution and the enhancement of light harvesting.

Stony corals (Scleractinia) are colonial marine invertebrate animals that play host to a complex assemblage of other microorganisms, collectively referred to as the coral holobiont^{7,8}. The research undertaken in the present study seeks to use a suite of cutting-edge imaging technologies to simultaneously track changes with increasing water depth in the tissue pigments and symbiotic zooxanthellae of apparently healthy host corals. This will establish the required comparative tissue cell "baseline" across a bathymetric gradient for apparently healthy corals and act as indicators of coral health⁹. Coral pigments, called chromatophores, act to absorb, reflect, scatter, refract, diffract, or otherwise interfere with incident solar radiation¹⁰. The zooxanthellae-chromatophore endosymbiotic relationship has enabled the coevolution of strategically advantageous light-harvesting optimization and skeletal growth strategies, as well as trophic plasticity (shifting feeding strategies back-and-forth from autotrophy to heterotrophy) for the coral animal¹¹.

The southern Caribbean island nation of Curaçao (formerly part of the Netherlands Antilles) lies approximately 65 km north of Venezuela within the east-west trending Aruba-La Blanquilla archipelago (Figure 1A). The 70 km long southern coast of Curaçao contains a continuous modern and Miocene-Pliocene-Pleistocene-Holocene ancient fringing coral reef tract^{13,14}. Mean annual SST on Curaçao varies approximately 3 °C

annually, ranging from a minimum of 26 °C in late January to a maximum of 29 °C in early September, with a mean annual temperature of 27.5±0.5 °C (NOAA SST Data Sets, 2000-2010). The coral reef at Playa Kalki (12°22'31.63"N, 69°09'29.62"W), lying near the northwestern tip of Curaçao (Figure 1A), was chosen for sampling because it has been previously well-studied and the marine ecosystem at this location is bathed in fresh nonpolluted seawater¹⁵⁻¹⁹. Two closely related scleractinian coral species, *M. annularis* and *M. faveolata*, were chosen for experimentation and analysis in this study because each species: (1) exhibits distinctly different and nonoverlapping bathymetric distributions on the reef tract with respect to the shelf break and the associated carbonate sedimentary depositional environments (*M. annularis* range = 0-10 m WD; *M. faveolata* range = 10-20 m WD²⁰; Figures 1B, 2A, and 2B); (2) is a common coral reef framework builder throughout the Caribbean Sea²¹; and (3) has well-studied ecological, physiological, and evolutionary relationships²².

Field sampling for the present study was conducted using standard SCUBA diving techniques offshore of Playa Kalki on Curaçao. A shallow-to-deep water bathymetric transect was established that ran across the shelf, over the shelf break, and into the deep water fore reef environments. Apparently healthy coral heads were then identified for sampling along this bathymetric transect, including: (1) three individual ~ 1 m diameter coral heads of *M. annularis*, all of which were at 5 m water depth (WD); and (2) three individual ~ 1 m diameter coral heads of *M. faveolata*, all of which were at 12 m WD. Photosynthetically active radiation (PAR) was measured as 33-36% PAR at 5 m WD and 18-22% PAR at 10 m WD. Sampling was conducted in January when the SST was 26 °C at the water depths of both the 5 m and 12 m. Each of these six coral heads was sampled in triplicate at equivalent spatial positions (i.e., approximately 45° N latitude on each of the six hemispherical coral heads). Each individual sample consisted of a 2.5 cm diameter coral tissue-skeleton core biopsy that was collected with a cleaned arch punch. Three coral tissue-skeleton biopsies were sampled on standard SCUBA with gloved hands from each of the coral heads (3 from *M. annularis* colonies at 5 m WD and 9 from *M. faveolata* at 12 m WD). Immediately upon collection at depth, each biopsy core sample was placed in a sterile 50 ml polypropylene centrifuge tube, screw-top sealed, and returned to the surface. The seawater was decanted from each centrifuge tube and each core biopsy was then immersed, stored, and transported in 4% paraformaldehyde.

SBFI imaging has previously been performed on a wide range of biological samples, including whole-brain and whole-heart human tissues, intact mouse embryos, zebra fish embryos, and multiple types of animal samples with intact bones²³⁻²⁵. Most of these studies utilized optical/light microscopy with either fluorescence or bright field techniques. However, studies have been conducted at ultra-high magnifications using scanning electron serial block face imaging in the past²⁶. In the present study, a modified SBFI protocol has been developed for and applied to corals for the first time. Because *M. annularis* and *M. faveolata* coral polyps are 1-2 mm in thickness, none of the routine light microscopy techniques would be capable of penetrating the entire thickness of coral polyp tissue. Therefore, we have SBFI sample preparation protocol specifically designed for coral samples. In addition, we have custom designed a stereomicroscope holder, which is motorized to move in both x and y directions. This apparatus takes images of the block face of the sample rather than collecting the sections using a regular microtome in front of the microscope. We also introduced another nonlinear optical two-photon microscopic technique to image the same coral polyps across the entire thickness of the coral tissues. This overcomes the limitations imposed by SBFI in terms of decalcification and the possibility of changes in tissue morphology and volume (shrinking) that may be induced by sample preparation (dehydration) and processing protocols. Furthermore, the emission profiles from the corals were spectrally resolved to identify their peak emissions and variations between the chromatophores and the photosynthetic zooxanthellae. These results were evaluated in the context of the method used and their individual advantages regarding acquisition time, analysis time, and the ability to resolve fine structural details without compromising structural integrity of the coral tissue.

Protocol

NOTE: Reagents to be prepared for Serial Block Face Imaging of Coral Samples

1. Preinfiltration Wax

1. Melt 3.6 g of STEARIN flakes in a glass beaker. Mix well on a hot plate (60-70 °C).
2. Add 400 mg of Sudan IV (to minimize wax background fluorescence). Mix well and wait until a red translucent solution is achieved.
3. Add 96 ml not molten paraffin (100%) and mix well.

1.2) Embedding Wax

1. Melt 7.2 g of STEARIN flakes in a glass beaker and mix well on a hot plate (60-70 °C).
2. Add 0.8 g of Sudan IV. Mix well and wait until a red translucent solution is achieved.
3. Add paraffin granules (162 g) and mix until paraffin melts completely.
4. Add 30 g of white granular Vybar and melt completely in the same beaker, once melted, mix.
5. Loosely close the glass bottle with a lid. Place the glass bottle in a 60 °C convection oven to keep the ingredients in a liquid state. Carry out all infiltrations in this oven.
6. Split the total volume of the 200 ml red wax in to two glass bottles of 100 ml each. Use one aliquot for infiltration and the other for final embedding.

1.3) Embedding Coral Tissues for Serial Block Face Imaging

1. Wash the coral polyps collected in the field (SI Video 1) and stored (3-6 months at 4-5 °C in paraformaldehyde) in phosphate buffered saline (3x 5 min) and decalcify when ready to be imaged. Decalcify the polyps in EtCaI solution for 24 hr or until as the polyps are totally devoid of CaCO₃. Incubate several decalcified coral polyps as a single block in a 25, 50, 75, and 100% ethanol series, followed by 1x xylene substitute to dehydrate the samples.
2. Place the processed polyps in a preheated 65 °C oven containing 100% xylene substitute. Incubate for 30 min twice by changing to fresh solution. Orient the polyps in such a way that the top of the polyps faces down and the top surface is as flat as possible.
3. Make solutions of 2:1, 1:1, and 1:2 xylene substitute and preinfiltration wax (step 1.1) in 50 ml Falcon tubes.
4. Incubate coral polyps with these three increasing concentrations of preinfiltration wax, followed by 3x incubation in 100% preinfiltration wax for 30-60 min each time.

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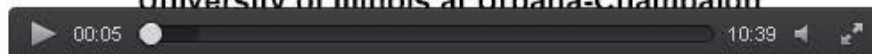
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Materials

Name	Company	Catalog Number	Comments
Coral Tissue Skeleton	None	None	2.5 cm Biopsy from natural habitat
Arch Punch Coring Device	C.S. Osborne and Company	No. 149	For Coral biopsy collection
Paraformaldehyde	Electron Microscopy Sciences	RT 15700	16% Pre-diluted
HistoClear/SafeClear II	Electron Microscopy Sciences	RT 64111-04	Non-Toxic alternate to Xylene, Dehydration and Deparaffinization
Xylene and Ethanol	Fisher Scientific	Fisher Scientific	Dehydration
Paraffin Wax	Richard Allen Scientific	Type H REF 8338	Infiltration solution
Vybar	The Candle Maker	None	Component of Red Wax
Stearin	The Candle Maker	None	Component of Red Wax
Sudan IV	Fisher Chemical	S667-25	Red Wax-Opaque background
Wheat Germ Agglutinin (WGA)	Life Technologies	W32466	For labeling Coral Mucus
Prolong Gold	Life Technologies	P36095	Anti-fade mounting media
Fluoro Dish	World Precision Instruments	FD-35-100	For two-photon imaging
XY Motor, Driver and Controller	Lin Engineering	211-13-01R0, R325, R256-RO	XY Translational Movement
Hot Plate	Corning	DC-220	Melting all wax
Convection Oven	Yamato	DX-600	Infiltration and Embedding
Tissue Processor	Leica	ASP 300	Dehydration, infiltration
Microtome	Leica	RM2055	Disposable knives
Stereo Microscope	Carl Zeiss	Stereolumar V 12	1.5x (30 mm WD) Objective
Fluorescence Microscope with ApoTome	Carl Zeiss	Axiolvert M 200, ApoTome I System	Imaging thin section of a polyp: Zooxanthellae
Axiocam camera	Carl Zeiss	MRm	Monochrome camera 1388x1040 pixels
Axiovision Software	Carl Zeiss	Version 4.8	Image acquisition program
Two-Photon Laser	Spectraphysios	Maitai eHP, pulsed laser (70 fs)	With DeepSee module
Laser Scanning Microscope	Carl Zeiss	LSM 710 with Spectral Detector	34 channel PMT detection
Zen Software	Carl Zeiss	2010 or above	for two-photon and spectral image acquisition
Imaris Suite Software	Bitplane, Inc.,	Version 7.0 or above	3D Volume, Iso-surface Rendering, Visualization