Research Report

Confocal Imaging of In Situ Natural Microbial Communities and Their Extracellular Polymeric Secretions Using Nanoplast® Resin

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ABSTRACT

A novel method using excision and fixation in Nanoplast®, a hydrophilic embedding resin, allows confocal imaging of natural microbial communities and their extracellular polymeric secretions (EPS) while in situ. Prestaining with fluorescent probes permits the observation of specific cellular and extracellular components. Marine stromatolite sediments were examined using this method. Optical sectioning using confocal laser scanning microscopy (CLSM) permitted high-resolution imaging through sediments. Delicate arrangements of the EPS that are associated with sedimentary microbial biofilms were imaged using a fluorescein isothiocyanate (FITC)-labeled lectin (concanavalin-A) probe. Close microspatial associations of heterotrophic bacteria cells and autotrophic cyanobacteria cells were also observed. The nanoplast resin produces no detectable autofluorescence. Further coupling of multi-photon scanning laser microscopy (2P-LSM) with a conventional single photon CLSM allowed concurrent imaging of DAPI-labeled microbial cells, FITC-labeled EPS and autofluorescent carbonate sand grains. The multi-photon infrared laser permits deep (approximately 1 mm) penetration of samples and the excitation of DAPI, which normally requires UV-excitation with minimal disturbance to samples. The unique combination of Nanoplast with fluorescent probes, CLSM and 2P-LSM allows for the preservation and imaging of natural microbial communities in their in situ state, a method easily adapted for examinations of other microbial systems.

INTRODUCTION

Microbial processes are a global-scale regulatory mechanism that influence the biogeochemical cycling and remineralization of nutrients (2,3). However, the actual sites of microbial activities often occur within microenvironments that have spatial scales measured in micrometers (2,4,12). Such information is critical to understanding the inherent natural variability of microbial rate processes and the roles of diverse microbial microenvironments.

Attached bacterial cells in marine systems, whether they are associated with water-column particulates, sediments or other surfaces, are enclosed within a matrix of extracellular polymeric secretions (EPS). This combination is collectively referred to as a microbial biofilm (7). The EPS component of some microbial biofilms form a structural anchor for microbial cells and has definable architecture. The EPS further forms a structuring matrix that may enhance the development of sharp geochemical gradients and microenvironments. However, the close association of bacterial cells and their EPS matrix has generally not been acknowledged in marine systems because of the many logistical difficulties of observing these associations at their appropriate spatial scales.

A difficulty in imaging EPS is that it consists of a delicate matrix of highly hydrated polymeric molecules. These molecules are easily destroyed or altered when using conventional sample preparation methods (e.g., a series of sample dehydrations) for transmission electron microscopy (TEM), scanning electron microscopy (SEM) and even light microscopy (6,8,9,13,18,23). Aquatic sediments are particularly difficult systems in which to image bacteria in their intact state. Modern marine stromatolites found in the Bahamas have provided an aquatic sediment model for understanding how novel microbial interactions can influence their highly layered structure and how fossil stromatolite forms were generated. Stromatolites are long-lived geological features and represent the oldest macroscopic evidence of life on earth (1). These layered structures were formed by complex interactions of bacteria, sediments and the environment.

For the natural spatial arrangements of microbial cells to remain in an intact state, a sample must be preserved while it is hydrated. This will preserve the delicate hydrated EPS that often are in close association with cells. The introduction of a hydrophilic embedding resin, Nanoplast® (Ted Pella, Redding, CA, USA), successfully preserves the intact hydrated state of cells and EPS within natural samples in TEM studies and other colloidal materials in both marine and freshwater systems (16,17). Furthermore, the recent development of two-photon laser scanning microscopy (2P-LSM) (10,22) has allowed high-resolution imaging, fluorescence lifetime imaging and deeper optical sectioning of biological structures with negligible background interference. Using 2P-LSM, it is possible to image probes such as 4′ 6-diamidino-2-phenylindole:2 HCl (DAPI), which are normally excited by UV wavelengths.

Using a modern marine stromatolite as a model in this study, we report a

novel embedding method for confocal imaging to observe the microspatial relationships among microbial cells, their EPS and carbonate sand grains in situ.

MATERIALS AND METHODS

Collection of Samples

All stromatolite samples were collected from an intertidal marine environment at Highborne Cay in the Exumas chain of islands in the Bahamas. This site has been previously described in more detail (21) and is under current investigation through the Research Initiative on Bahamian Stromatolites (RIBS) project.

Nanoplast Embedding Preparations

Samples of stromatolite were prepared in several steps. First, freshly collected samples were sectioned using sterile razor blades. Immediately after collection, the sections (0.75 × 0.75 × 1 cm) of stromatolite were preserved in EM-grade (methanol-free) formaldehyde (Ted Pella) in seawater (3% final concnetration). A double-labeling experiment was performed on the sections to determine the spatial relationship of microbial cells and their EPS, using 100 ng/mL, DAPI (Sigma Chemical, St. Louis, MO, USA) for cellular DNA and FITC concanavalin A (FITC-Con A) (Molecular Probes, Eugene, OR, USA) for EPS. Concanavalin A specifically binds to D(+)glucose and D(+)mannose groups on EPS (11).

After incubations of approximately 15 min, excess unbound probes were washed 3× with 20 mL sterile-filtered sea water for 10 min. Excess water was gently removed using pipets. Sections were trimmed (0.5 × 1 cm × 5 mm) using a sterile razor, then placed in BEEM® embedding molds (supplied in the Nanoplast kit). Care was taken to minimize any minor perturbations of the stromatolite section surface by the razor during trimming. However, the effects of such minor perturbations are negligible because the outer millimeters of the stromatolite blocks will be removed during later thick-sectioning procedures. Nanoplast is a hydrophilic embedding resin originally designed for TEM (16). Resin and catalyst were thoroughly mixed on site and then added to molds containing the stromatolite samples. This mixture provided a suitable hardness for later thick sectioning. The molds were placed in a temperature-controlled heat block at 25°C for 48–60 h to allow slow penetration and complete mixing of the Nanoplast resin with the hydrated sample. The hydrated nature of this medium allows it to completely mix with sample, while it is still in a hydrated state. After penetration of the medium, the temperature is raised to 40°C for 48 h to dry and then to 60°C for 48 h to harden the medium into blocks. The resulting blocks were thick sectioned (approximately 2 mm thick), mounted on glass microscope slides using Epon 812™ (Shell Oil) and then observed using CLSM.

Single-Photon and 2P-CLSM

All LSM was conducted using an MRC 1024MP® system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with an Eclipse TE 300 compound-inverted microscope (Nikon, Tokyo, Japan). Resolution was 1280 × 1024 with a 3-detector assembly, 6-position emission wheel, 2-stage pre-amplifier non-descanned, and 2 photomul-

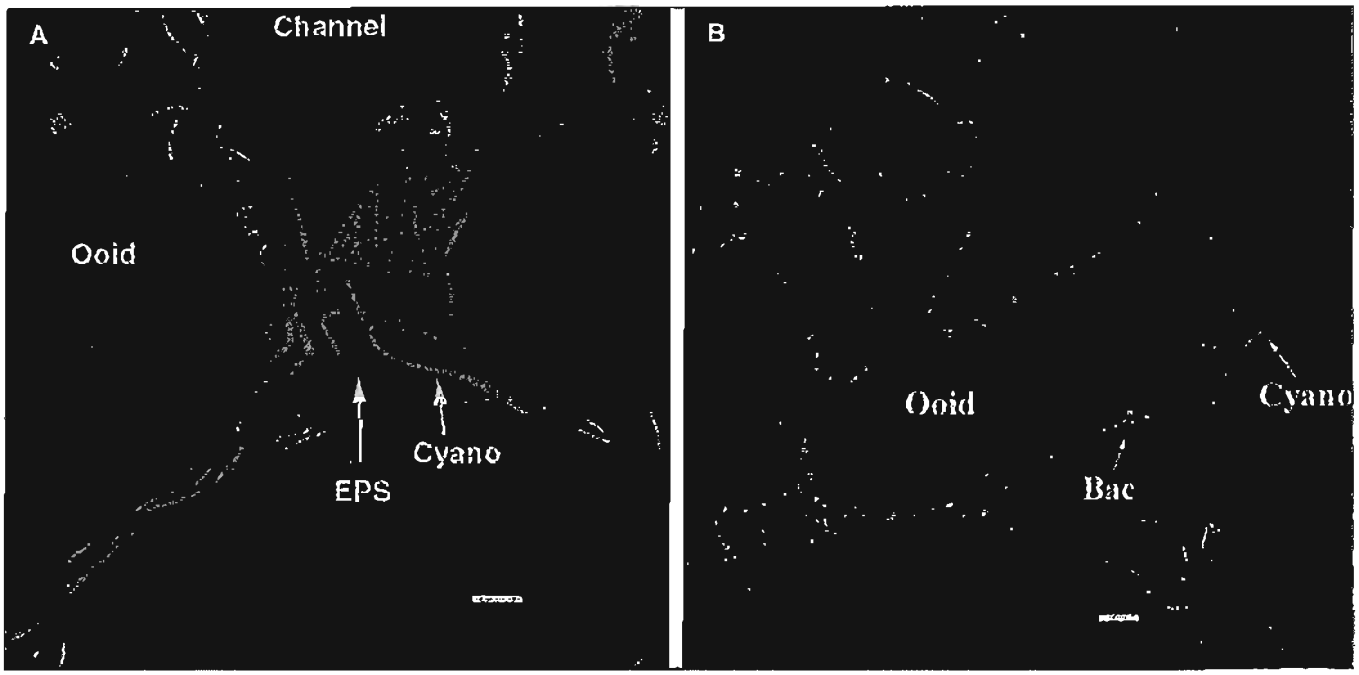

Figure 1. (A) Low-magnification CLSM micrograph of a stromatolite showing sand grain ooids and microbial extracellular polymers, cyanobacteria and porewater channels (channel) within an EPS matrix. (B) 2P-LSM micrograph showing DAPI-labeled bacteria cells (Bac) associated with sand grains (Ooid); scale bars are 10 μm.

tiplier tubes (PMT 1 and PMT 2) equipped with a variable confocal aperture. Sample slides were viewed using either Nikon Plan Apo 60×/1.40 oil or Nikon Plan Apo 100× /1.40 oil objective lenses.

Confocal Imaging of EPS and Carbonate Sand Grains

A single-photon (Krypton-Argon) laser 488 nm line was used for imaging FITC-Con A EPS (PMT 2; emission = 522 nm). Autofluorescence was used for imaging carbonate sand grains (ooids), the crusted layers of the stromatolite (micritic layers) and $CaCO_3$ precipitates (PMT 1; emission = open channel). Typically, laser intensity was set at 30%. Z-sections were collected at 5 µm intervals.

2P-LSM Imaging of Microbial Cells

A 2P Titanium-Sapphire laser (Coherent, Santa Clara, CA, USA) was used for the imaging of DAPI-labeled microbial cells. The 2P Ti-Sapphire source of the multi-photon system is an infrared wavelength laser (Hazard Classification = Class 3b laser) that produces photon pulses (approximately 1140 nm) at femtosecond intervals; it was set at 770 nm. The external detectors (DAPI/FITC filters combination, emission filter E625SP and emission filter cube B/G 808) were used to collect maximum emission from the sample instead of internal detectors.

Co-Localization of DAPI-Labeled FITC-Con A Microbial Cells, FITC-Con A-Labeled EPS and Autofluorescent Carbonate Sand Grains

To image microbial cells, EPS and carbonate sand grains in a single image, the two images were merged. The internal detectors were used for both CLSM and 2P-LSM to avoid the image shift. The emission filter was set to 460LP (460 nm–650 nm), and the iris was set wide open (at 8/8) to collect maximum emission. Gain-setting ranged from 1000–1300 to acquire the optimum image of DAPI-labeled microbial cells. By sequentially collecting images of carbonate sand grains and EPS by CLSM (as described above) and microbial cells by 2P-LSM, the three digital images were precisely merged using the LaserSharp Program (Bio-Rad Laboratories). Image analyses were conducted using a Model 400 MHZ XPS® computer system (Dell Computer, Round Rock, TX, USA) outfitted with 100 MB zip drives. Then, images were digitally converted, and final image processing was conducted using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA, USA).

RESULTS AND DISCUSSION

The technique developed here preserves the intact hydrated structure of microbial microenvironments and their delicate EPS. Using Nanoplast, the embedding of fixed samples and subsequent imaging through the use of single-photon CLSM and 2P-LSM permits a high-resolution and potentially quantitative tool for the examination of microbial cellular and extracellular components. When samples were preserved and embedded using this method, they were stable for more than two years (that is, the length of time up to submission of this manuscript).

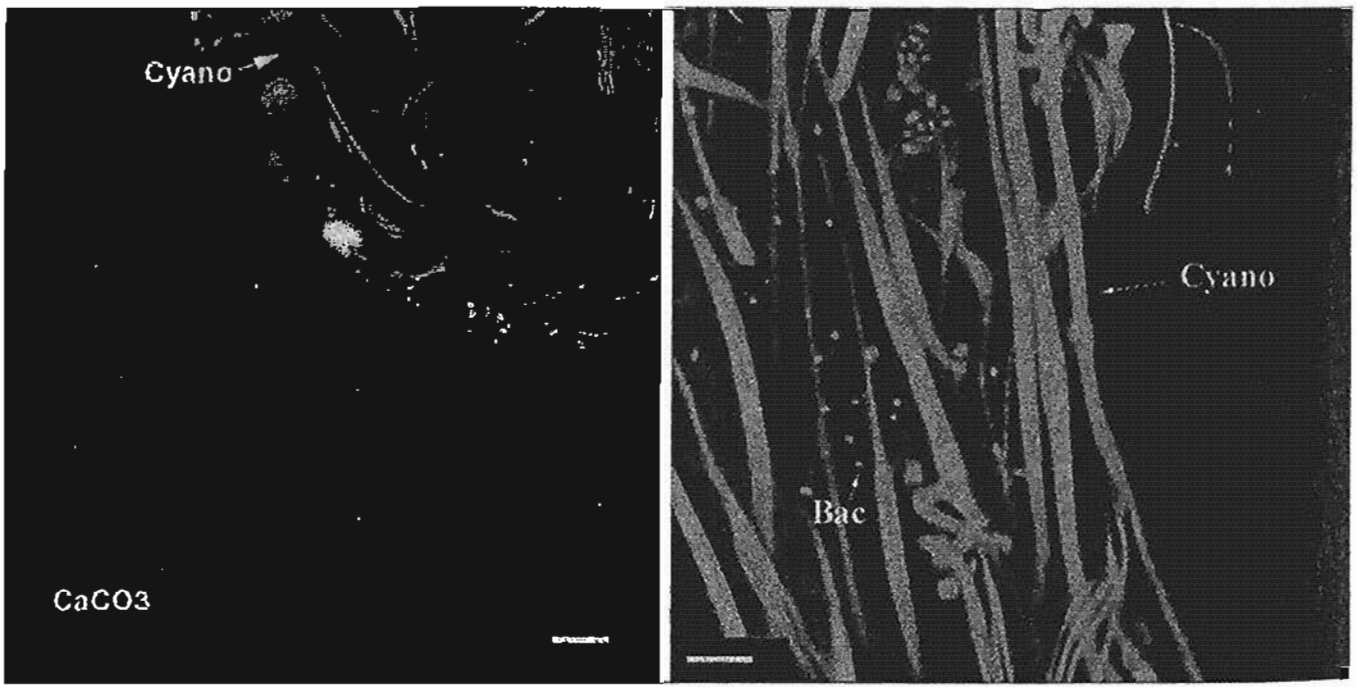

Figure 2. (A) Low-magnification confocal laser overlay micrograph showing microspatial partitioning of blue DAPI-stained photosynthetic cyanobacteria cells and sheaths taken by 2P-LSM and orange autofluorescence natural micritic layer composed of precipitated calcium carbonate ($CaCO_3$) taken by CLSM; scale bar is 10 µm. (B) High-magnification image using 2P-LSM showing close spatial association of DAPI-labeled photosynthetic cyanobacteria cells and heterotrophic bacteria cells. Z-axis thickness of optical section is 2.0 µm; scale bar is 5 µm.

Nanoplast Embedding Technique

Nanoplast was found to be an ideal embedding resin for CLSM and 2P-CLSM because there was no detectable autofluorescence at the major emission wavelengths of most major fluorophores. EM-grade formaldehyde should be used as the primary fixative agent because a lower-grade formaldehyde may contain varying amounts of other solvents, such as methanol, which may alter the delicate EPS. Another common fixative agent, glutaraldehyde, which is typically used for electron microscopy (EM), causes intense autofluorescence and degrades polysaccharides (11). Normally, a final fixative concentration of 1.5 % formaldehyde is used to preserve bacteria in aquatic samples. We used a slightly higher (3.0%) final concentration of formaldehyde to fix our stromatolite samples to ensure complete fixation of the dense matrix of stromatolite biofilm. The formaldehyde fixation most likely targets N-rich molecules, such as proteins in the natural EPS.

During initial preparations of stromatolite samples, it is important to physically stabilize the sample and prevent any movement of carbonate sand grains in the stromatolite. Minor alterations at the edges of the sample, due to the coring tube or sample device, may be trimmed away when the mounted block has hardened. It is preferable to minimize the initial sample block size as this will facilitate better diffusive dispersal of the fixative, fluorescent probes and later infiltration of the Nanoplast resin. In initial preparations using sample blocks as large as 2 cm^3 we did not obtain complete penetration of the sample by fluorescent probes. For complete penetration of Nanoplast, the sediment must be incubated longer than 24 h at 25°C. The length of the

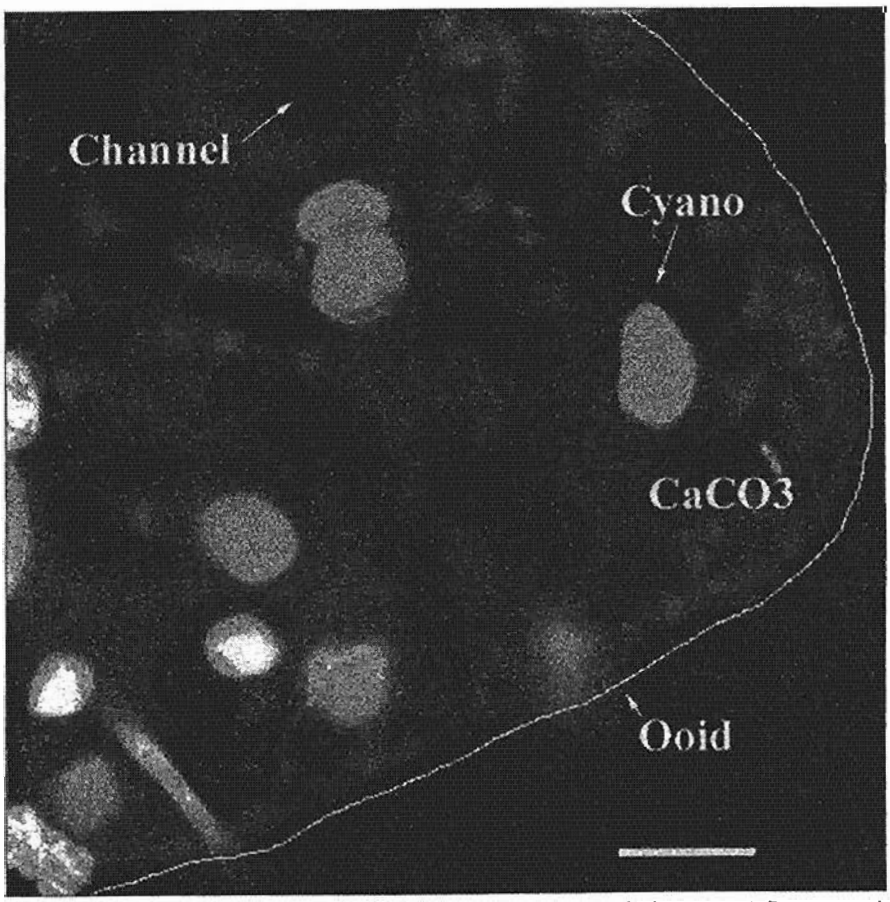

Figure 3. Confocal laser micrograph of calcium carbonate sand grain (orange autofluorescence) outlined in white, with FITC-labeled EPS surrounding Endolithic cyanobacterium. These cyanobacteria form tunnels or channels within the carbonate sand grain; scale bar is 10 µm.

incubation time will depend on the porosity of the sample. This important step facilitates a slow mixing and more complete penetration of Nanoplast with the naturally hydrated components of the sample. The type of incubation mounting tray used for sample resins can strongly influence the physical integrity of the hardening process. For example, silicon resin mounting trays that are often used in EM preparations produced bubbles within our sample during the hardening process, which was due to volatilization of the formaldehyde in the resin on being heated.

The amount of B-52 hardener catalyst can be adjusted to influence the final hardness of Nanoplast. Although 0.25 g of B-52 (per 10.0 g MME) provides the hardest resin for TEM, we found it was best to use 0.15 g for thick-section preparations for CLSM. This resulted in a more consistent retention of carbonate grains during thick sectioning. Ideally, our experimental design would have compared images of fixed, embedded stromatolites with non-fixed, non-embedded living stromatolites using CLSM. However, this was not possible because the distance and significant travel time from the sample collection site to our CLSM facility would have introduced potential artifacts in any effort to observe intact non-fixed stromatolites using CLSM.

Confocal Imaging of In Situ Natural Microbial Community and their EPS

Single-photon CLSM and 2P-LSM images can provide a high level of spatial resolution and acuity. For images to be viewed optimally, a number of parameters must be optimized; these are reviewed in more detail elsewhere (5,14,15). The confocal laser images provide a 3-D optical section with a Z-axis thickness that can be adjusted from 0.5 to 10 mm.

EPS were well imaged in the interstitial spaces between sand grains (Figure 1A) using the FITC-Con A probe. This result suggests that much of the EPS was composed of carbohydrates contain either D-glucose or D-mannose monomers. In most cases, the thickness (Z-axis) of the optical plane of imaging was just 2 μm and was sufficient for us to observe fluorescence of FITC-Con A-labeled EPS. The intensity of fluorescence was not homogeneous but showed patchy areas that ranged from dense fluorescence to barely detectable fluorescence. Wolfaardt et al. (25) found similar non-homogeneity of lectin probe binding to EPS. This finding suggests that the EPS consists of varying compositions and/or that distinct water channels in the EPS matrix occur within the interstitial spaces between carbonate sand grains.

Carbonate sand grains and precipitated $CaCO_3$ (Figure 1A) could be readily observed by the inherent autofluorescence (excitation = 488 nm; emission = open channel). Red color (mixer A) was assigned to image these structures. An orange color with many irregularities, as revealed by the autofluorescence intensity of individual carbonate sand grains using CLSM, suggests that an EPS matrix exists within some carbonate grains. Small precipitates, ranging in size from 1–5 μm, could also be observed within the EPS matrix. $CaCO_3$ precipitates as small as 1 μm in diameter could be easily imaged by autofluorescence. Being able to image the relative positions of such small precipitates provides important information on how major precipitation events such as different types of micritic layers occur within stromatolite layers.

The capability of the multi-photon laser to image fluorophores such as DAPI (which usually require UV-wavelength excitation) greatly enhances our ability to image microbial systems. DAPI probes, which are conventionally imaged using an excitation $\lambda = 365$ nm (20), were imaged best on our multi-photon system using an excitation wavelength at 770 nm. The 2P-LSM allowed clear imaging of bacteria using DAPI and a deep penetration of the laser through sediment grains. DAPI-labeled microbial cells were easily imaged while many layers of carbonate sand grains lay above and below the focal plane (Figures 1B, 2A and 2B). Optical sectioning made possible the imaging of "endolithic" cyanobacteria cells, *Solentia* sp., which were easily seen (Figure 3) in tunnels within carbonate sand grains. Images collected using the multi-photon LSM system ac-

curately and precisely complemented our observations after using a conventional single-photon CLSM. Wiggli et al. (24) examined cyanobacterial communities in the microbial mats of alpine bogs using CLSM and were able to relate their vertical distributions to average sunlight intensities reaching cells.

The two-photon laser allows deeper penetration with less damage to the sample than the Ar-Kr laser of a conventional single-photon CLSM system. In part, this is because the infrared photons converge in a narrow optical plane, and therefore image fluorescence is strictly confined to only one narrow plane of focus and sample damage due to photobleaching is thus minimized (19). No photobleaching of any fluorescence probes was observed in our study. The interaction of fluorescent probes with the Nanoplast resin appears to enhance the stability of the probes against degradation. Once embedded, the fluorophores are quite stable and appear highly resistant to potential photobleaching effects, even during relatively high-power laser scanning using either a single-photon (Krypton-Argon) or 2P (Ti-Sapphire) laser. After samples are preserved and mounted, fluorophores are stable for several years.

Post-imaging reconstruction can be used to merge successive Z-axis images and provide a 3-D depth-of-field effect. This is often helpful when viewing the entirety of elongated sheath-containing cyanobacteria cells (Figure 2B). Within certain layers of the stromatolite, the cells and sheaths were seen to have a predominantly vertical orientation (Figure 2B).

Future Potential Applications For the Technique

The method we developed, combining fluorescent probes with Nanoplast and following with CLSM and 2P-LSM imaging, allowed us to image in situ microspatial arrangements of microbial cells and their EPS within relatively opaque marine sediment systems (stromatolites). Sediment systems are sites for diverse microbial activities, and often contain dense aggregations of bacteria containing novel strains (and/or biochemical pathways). Sediments also represent a sink for the concentration of environmental contaminants. Unique biotransformations of contaminants by microbial processes make sediments an active site for exploration in biotechnology applications. Our methodology for imaging sediment systems represents an important step in understanding microbial microenvironments and an approach to a more quantitative assessment of microbial processes at small spatial scales. Such a tool could be easily adapted for use in water-column aggregates.

The future application of fluorescence lifetime imaging (22) in conjunction with 2P-LSM of such systems may provide for more detailed chemical characterizations of microbial microenvironments.

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