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Calcification process dynamics in coral primary polyps as observed using a calcein incubation method

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ABSTRACT

Calcification processes are largely unknown in scleractinian corals. In this study, live confocal imaging was used to elucidate the spatiotemporal dynamics of the calcification process in aposymbiotic primary polyps of the coral species *Acropora digitifera*. The fluorophore calcein was used as a calcium deposition marker and a visible indicator of extracellular fluid distribution at the tissue-skeleton interface (subcalicoblastic medium, SCM) in primary polyp tissues. Under continuous incubation in calcein-containing seawater, initial crystallization and skeletal growth were visualized among the calicoblastic cells in live primary polyp tissues. Additionally, the distribution of calcein-stained SCM and contraction movements of the pockets of SCM were captured at intervals of a few minutes. Our experimental system provided several new insights into coral calcification, particularly as a first step in monitoring the relationship between cellular dynamics and calcification *in vivo*. Our study suggests that coral calcification initiates at intercellular spaces, a finding that may contribute to the general understanding of coral calcification processes.

1. Introduction

Coral skeletons contribute to maintain a high level of biodiversity in ecosystems associated with coral reef formations. Although numerous studies have investigated the physiological and molecular aspects of coral calcification mechanisms, the actual calcification mechanism, particularly the initial nucleation and subsequent calcium carbonate ($CaCO_3$) crystal deposition, has been debated for more than a century [1]. Mechanistic aspects of this process, particularly those concerning calcium transport at the level of the calcifying cells (i.e., calicoblastic cells), remain unclear.

One of the major approaches for visualizing calcification is the microscopic observation of calcifying sites. For example, electron microscopic technique-based studies clarified the fine morphological features of the calcifying interface and described various nano-crystals, granular skeletal structures, and other components [2,3]. Isotope analyses, which include calcifying fluid pH measurement using a boron isotope [4] and calcium transmembrane transport evaluation using a calcium isotope [5,6], have also been used to investigate calcification mechanisms in corals. These techniques have provided additional details about coral calcification, but are restricted to static conditions for coral calcification.

Fluorescence live imaging technique with X-ray microanalysis was applied to the study of calcium transport and the storage from the seawater around the calcifying sites in coral tissues at early life stages (planula larvae and settled primary polyps) [7]. Recently, confocal live imaging has recently been used to investigate calcification mechanisms in live coral tissue; this technique enables us to observe dynamic coral calcification processes. For example, live imaging using fluorescent dyes has been used to visualize the pH of the subcalicoblastic medium (SCM) and clarify the biological responses of live coral tissues to ocean acidification [8–10]. These techniques have provided new insights into several important aspects of calcification, such as pH elevation in SCM and responses of the pH_{SCM} to seawater acidification.

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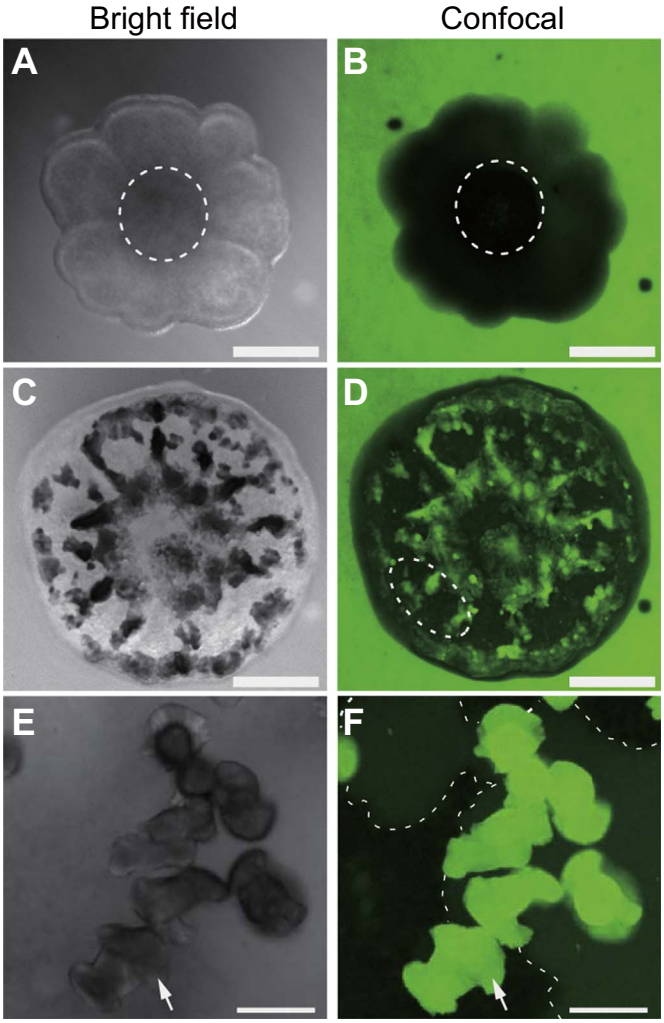

Fig. 1. Confirmation of coral skeletons and tissue staining patterns with or without calcein. Specimens were incubated in calcein-containing seawater during the experiment. **(A)** Bright-field image of a primary polyp 12 h after incubation. Scale bar: 200 μm. **(B)** Confocal image of the same position in **(A)**. **(C)** Bright-field image of the primary polyp in **(A)** at 56 h after incubation. The black area indicates the coral skeleton. Scale bar: 200 μm. **(D)** Confocal image of the same position in **(C)**. Dotted lines indicate the area approximately quarter to half from the periphery of the primary polyp. The coral skeleton was stained using calcein (green). **(E)** High-magnification bright-field image of coral skeletons from the polyps in **(A–D)** at 24 h after incubation. The white arrow indicates a dumbbell-shaped crystal. Scale bar: 20 μm. **(F)** Confocal image of the same position in **(E)**. White arrow indicates a crystal on the surface of the glass-based dish. Dotted lines indicate the periphery of the subcalicoblastic medium (SCM). The black area indicates the bottom of the coral tissue.

Calcein (fluorescein-3,3′-bismethylimino-discetic acid), which is a fluorescent calcium indicator that binds to calcium ion, is incorporated into precipitating calcium carbonate crystals. Calcein is a water-soluble molecule that cannot penetrate the cell membrane. These characteristics have led to the use of calcein for hard tissue staining in a wide variety of marine organisms (e.g., fish otolith [11]; foraminiferan shells [12]; coral skeletons [13]). In particular, the calcein has been used to image coral crystal growth [8,9,13,14] and SCM areas in corals on glass substrates [8]. Furthermore it has been used to image the intercellular spaces in corals [13].

Previous studies have demonstrated that short-term incubation with calcein did not appear to affect coral growth [15]; accordingly, calcein has been recommended instead of alizarin and Sr for tracing skeletal growth in some shellfish species [16,17]. The effect of calcein on the incorporation of Sr and Mg into calcite has been investigated in foraminifera, and these studies found that calcein did not affect the incorporation of these elements [18]. Additionally, calcein has few adverse effects on benthic foraminifera even during long-term exposure (4–5 weeks)[19]. Thereby, we hypothesize that continuous incubation of coral cultures in calcein is possible, and fluorescent imaging of corals subjected to long-term calcein exposure will enable a real-time visual observation of coral calcification, particularly the initiation of skeletal growth.

In the present study, we used a confocal imaging system to directly observe coral calcification processes *in vivo*. Based on the above-described merits, calcein was selected as a fluorescent marker for continuous skeletal growth monitoring. We also used an experimental system using coral primary polyps of *Acropora* species obtained at mass coral spawning events, which allowed us to observe initial calcification after the settlement of coral planulae [20]. *Acropora* planulae initiate calcification shortly after settlement by forming calcium carbonate ($CaCO_3$) structures at the interface between the larval tissues and substrate [21]. The simple morphology and the lack of symbiotic algae of these polyps render the calcification process easily visible. The present study proposes a detailed observational method of coral calcification in live tissues of early life stages which enables us to deeply understand the physiological aspects of coral calcification.

2. Materials and methods

2.1. Sample preparation

The scleractinian coral *Acropora digitifera*, which is one of the most common species in the Ryukyu Islands of Japan [22], was used in this study. Gravid colonies of *A. digitifera* were collected from a fringing reef at Sesoko Island, Motobu-cho in Okinawa, Japan. In addition, several colonies of a cryptic *A. digitifera* species (*Acropora* sp.1) [23] were also collected at Bise, Motobu-cho in Okinawa, Japan. The colonies were kept in a running seawater tank under natural light conditions at Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. Coral spawning occurred at night around the time of the full moon in the spring and summer seasons of 2013–2015. Gametes were collected after spawning as described by Morita et al. (2006) [24]. Primary polyps were prepared by inducing settlement of the planula larvae (3–30 days old) using the coral metamorphosis inducer peptide Hym-248 [25]. Hym-248 induces the synchronous metamorphosis and settlement of *Acropora* planulae, and is a useful tool for studies of *Acropora* larval metamorphosis [26]. Approximately 5–10 larvae were placed in a glass-based dish (No. 1S, thickness: 0.15–0.18 mm; IWAKI Glass, Tokyo, Japan) with 40 μL droplet of filtered seawater (FSW: pore size 0.22 μm). About 4–6 droplets were made on the surface of the glass-based dish. Next, a 10-μL aliquot of 2×10−4 M Hym-248 in FSW was added in each droplets and the larvae were incubated for 2 h to induce metamorphosis. Finally, approximately 10–20 larvae were settled on a glass-based. Larvae that settled on the seawater surface and the side of the glass-based dish were removed.

2.2. Calcein

Calcein was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution containing 2 gL^{-1} calcein was prepared in distilled water and buffered to pH 6 using sodium bicarbonate to enhance the solubility of calcein [13]. This solution was then diluted in FSW buffered to pH 8.1 (total pH scale) with NaOH to obtain a final concentration of 100 μM (FSW-calcein: salinity of approximately 35). After 2-h incubation with Hym-248, the solution was made up to 2000 μL with FSW-calcein. The pH was measured using a portable pH meter (D-71; Horiba, Ltd., Kyoto, Japan) as a total scale with a precision of ± 0.01 pH units. The detail effects of long-term calcein incubation on coral polyp were shown in Supplementary Fig. 1 and Supplementary Fig. 2.