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Research paper

Three-dimensional observation of foraminiferal cytoplasmic morphology and internal structures using uranium–osmium staining and micro-X-ray computed tomography

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ABSTRACT

Benthic foraminifera are common protists that inhabit a broad environmental range of the seafloor. Because of their large biomass, these organisms play crucial roles in biogeochemical cycles, but our knowledge of their ecology and cell biology are still limited. One reason is that the calcareous or agglutinated tests (shells) of foraminifera hamper observing or measuring the cytoplasm from outside the organism. Here, we report the three-dimensional (3-D) observation of the cytoplasm and several ultrastructures of deep-sea benthic foraminifera using the combined techniques of micro-X-ray computed tomography (CT) and the osmium- and uranium-based cytoplasm-staining methods that are standard protocols for transmission electron microscope (TEM) observation. Osmium and uranium bind to organic membranes, proteins, and nucleic acids, rendering them visible by X-rays due to their high X-ray attenuation and enabling their reconstruction as 3-D images. This methodology revealed not only the external cellular morphology but also vacuoles and plugs inside the cell. Furthermore, volumetric calculation of cytoplasm and vacuoles is possible using the obtained CT data to evaluate their biomass and roles of vacuoles. Our method offers rapid (less than an hour in this study) evaluation of whole single or multiple foraminiferal cells and can be applied to investigate the biology and ecology of the foraminifera and other testate eukaryotes whose internal features are obscured by their external test structures.

1. Introduction

Benthic foraminifera are unicellular eukaryotes that are distributed abundantly throughout the ocean. Their tests (shells) are made of calcite or aragonite, agglutinated sediment particles, or organic material and are often used for age determination and paleoenvironmental reconstruction of sediment. Foraminifera dominate the benthic biomass, particularly in dysoxic to anoxic environments (Bernhard et al., 2000; Koho and Piña-Ochoa, 2012), implying that they are important components in the marine biogeochemical cycles through which vast quantities of organic matter are degraded.

Their cytoplasmic structure is key to understanding how benthic foraminifera adapt to various environments, such as the oxic-to-anoxic gradient in sediments. Foraminiferal cytoplasmic morphology, which is determined primarily by calcareous or agglutinated test structures, plays an important role in the interaction between ambient seawater and cytoplasm. Foraminifera can incorporate various ions and chemicals from the surrounding seawater or excrete them for calcification of the test (Ca^{2+} and CO_3^- incorporation and Mg^{2+} and CO_2 excretion; e.g., Kulie et al., 1989; Toyofuku et al., 2008), respiration (O_2 incorporation and CO_2 excretion; e.g., Hannah et al., 1994; Nomaki et al., 2007; Geslin et al., 2011), nitrate respiration, namely, denitrification (NO_3^- incorporation and N_2 excretion; e.g., Risgaard-Petersen et al., 2006; Høgslund et al., 2008), photosynthesis of symbiotic algae (CO_2 and nutrient incorporation and O_2 excretion; e.g., Rink et al., 1998; Hallock, 1999; Fujita et al., 2014), pH control (proton incorporation and excretion; de Nooijer et al., 2008, 2009), nutrition (dissolved organic matter incorporation and CO_2 and nutrient excretion; e.g., Lee et al., 1966; Nomaki et al., 2011). Increasing the surface area of the cytoplasm by forming pseudopodia may enhance these seawater–cytoplasm interactions. In addition, the presence of vacuoles in foraminifera may help to store various compositions of seawater for their subsequent use as nitrate pools for denitrification (Risgaard-Petersen et al., 2006; Piña-Ochoa et al., 2010); although the actual nitrate storage site in the cell is still unknown. For these reasons, elucidating the morphology of the cytoplasm and its internal structures is crucial to understanding how benthic foraminifera adapt to various environments through the exchange of chemicals between the cytoplasm and the ambient seawater or the seawater stored in their vacuoles.

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Foraminiferal cytoplasm exterior morphologies are often regarded as those of test interior morphologies. Except for that of soft-walled species, direct observation of the foraminiferal cytoplasm is difficult because of the masking effect of the calcareous or agglutinated test. Transmission electron microscope (TEM) observation using sequential ultra-thin sections, as is common for small protists (Yabuki et al., 2012, 2013), or so-called array tomography (Micheva and Smith, 2007) may yield insights into the ultrastructures of foraminiferal cells. However, these techniques are difficult to apply to foraminifera because of their large cell size, which would require hundreds to thousands of ultra-thin sections. Recent advances in focused ion beam scanning electron microscope (FIB-SEM; e.g., Heymann et al., 2006; Lucas et al., 2014) technology enable the reconstruction of three-dimensional (3-D) images of cytoplasm at a voxel size of 5 to 10 nm (Muller-Reichert and Verkade, 2012), with a maximal sample size of $50 \times 30 \times 30\ \mu m^3$ in volume. Again, however, foraminifera are too large for the application of such techniques. Furthermore, FIB-SEM operation sequentially scrapes away the surface of samples, which hampers their subsequent analysis.

X-ray computed tomography (CT) is a powerful tool for observing and reconstructing the internal structures of target objects. Because the absorbance of X-rays differs depending on a target's electron density, we can distinguish between materials with high or low electron densities. Key advantages of CT include its rapid (typically within 1 h) and non-destructive scanning of internal structures of target materials. X-ray CT has diverse medical and industrial applications and has recently been used for foraminiferal test biometry (Speijer et al., 2008; Briguglio and Hohenegger, 2014; Briguglio et al., 2011, 2013; Gorog et al., 2012; Hohenegger and Briguglio, 2012; Ferrández Cañadell et al., 2014) and also for calcite test dissolution as indicated by X-ray attenuation (Johnstone et al., 2010; Iwasaki et al., 2015). Micro-X-ray CT techniques can differentiate a foraminiferal test that is denser than air and return 3-D reconstructed images of it. In addition, the internal structure of tests, which defines the space available for storing cytoplasm, has been reconstructed as an index of cytoplasmic volume (Briguglio et al., 2013; Briguglio and Hohenegger, 2014). However, micro-X-ray CT has not yet been applied to exploring the foraminiferal cytoplasm itself.

Here, we report the 3-D cell observation of four deep-sea benthic foraminiferal species – *Uvigerina akitaensis* Asano, *Globobulimina affinis* (d'Orbigny), *Bolivina spissa* Cushman, and *Chilostomella ovoidea* Reuss – which we obtained using micro-X-ray CT. To make an electron density contrast among foraminiferal cells and keep their cytoplasmic structures for measurements, we followed a sample preparation protocol designed for the TEM. Specifically, we fixed the foraminiferal cytoplasm using glutaraldehyde and osmium tetraoxide, stained the membranes and cytoplasm with uranyl acetate in addition to osmium tetroxide, and embedded stained samples in resin block (Hayat, 2000). This protocol further allows us to use a single specimen for both micro-X-ray CT imaging and corresponding TEM or optical microscopic observations. We compared the resulting micro-CT images with TEM images of ultra-thin sections and optical microscope images of semi-thin sections that were sliced from the same specimens to assess which parts of the specimen exhibited high and low X-ray attenuation. The main purposes of this study were 1) to demonstrate the effectiveness of osmium tetroxide and uranyl acetate staining methods coupled with micro-X-ray CT imaging for revealing the 3-D cellular and cytoplasmic morphology of foraminiferal specimens and 2) to show how foraminiferal cytoplasmic morphology differs between species, thus demonstrating the importance of observing the morphology of the cytoplasm, not just that of the calcareous test.

2. Materials and procedures

2.1. Sediment sampling and foraminiferal isolation

Surface sediments containing benthic foraminifera were collected from central Sagami Bay, Japan (34°58.0′N 135°23.9′E; water depth, 1480 m), during cruise KS13-T02 of the R/V *Shinseimaru* in October 2013. Sediment samples collected with a multiple corer were immediately sliced onboard into 1-cm sections to a depth of 6 cm and were preserved at 4°C in separate plastic bags without additional seawater. In a laboratory, deep-sea bottom water was added to the sediment to remove fine particles by decantation, and foraminiferal specimens were collected immediately from the remaining sediments under a binocular stereomicroscope. Four deep-sea benthic foraminiferal species – *U. akitaensis* and *B. spissa*, collected from the 0- to 1-cm depth section, and *G. affinis* and *C. ovoidea*, collected from the 4- to 5-cm depth section – were examined with a TEM and by micro-X-ray CT. *U. akitaensis* and *B. spissa* mainly thrive in oxic sediment and *G. affinis* and *C. ovoidea* mainly thrive in dysoxic to anoxic sediment (Nomaki et al., 2005). Both *Uvigerina* and *Globobulimina* are abundant foraminiferal genera at the bathyal depths of continental slopes and have been characterized by the TEM (Goldstein and Corliss, 1994).

2.2. Sample fixation, staining, and embedding into resin

Sample fixation, staining with uranium and osmium, and subsequent embedding into resin followed the protocol for TEM observation (e.g., Nomaki et al., 2014, 2015; Tsuchiya et al., 2015) except that a different type of resin was used. Isolated specimens (10 specimens for *B. spissa* and *U. akitaensis*, 13 specimens for *C. ovoidea*, and 14 specimens for *G. affinis*) were fixed overnight in 2.5% glutaraldehyde in filtered seawater and stored at 4°C. Fixed samples were embedded in 1% aqueous agarose and then cut into approximately 1-mm cubes. Samples were decalcified with 0.2% EGTA in 0.81 mol L^{-1} aqueous sucrose solution (pH 7.0) for several days, rinsed with filtered seawater, and then postfixed with 2% osmium tetroxide in filtered seawater for 2 h at 4°C. Samples were rinsed with an 8% aqueous sucrose solution and stained *en bloc* with 1% aqueous uranyl acetate for 2 h at room temperature. Stained samples were rinsed with distilled water, dehydrated in a graded ethanol series, and embedded in Quetol 651 resin (Nisshin EM, Tokyo, Japan).

During the fixation and staining, osmium and uranium were used to stain the foraminiferal cytoplasm to yield high-contrast images during TEM and X-ray CT observation. Osmium tetroxide is a convenient tissue fixative that typically is used as a postfixation agent (Hayat, 2000). Osmium tetroxide preserves lipids, particularly the unsaturated fatty acids of phospholipids, thus generating a high-electron density at membranous regions of organelles; it also reacts with some amino acids, peptides, and proteins. In contrast, uranyl acetate stains nucleic acids and, to a lesser extent, proteins (Hayat, 2000). Together, both osmium and uranium, representing the high electron density part in the foraminiferal cell, were mainly bonding with the membranes of cell and organelles, nucleic acids, and proteins.

From the fixed and stained foraminiferal samples embedded in resin block, the following analyses were carried out (Supplementary Fig. 1):

1. Semi-thin section (500-nm-thick) observation using the optical microscope to assess the cytoplasm condition
2. Ultra-thin section (thickness, 60 nm) observation using TEM to examine organelle distribution and the condition of the specimen
3. Micro-X-ray CT using HMX225-ACTIS (*U. akitaensis*, *G. affinis*, *B. spissa*, and *C. ovoidea*) or SkyScan1272 (*U. akitaensis* and *G. affinis*)
4. Semi-thin section (500-nm-thick) observation of a single specimen of *U. akitaensis* using the optical microscope to compare cytoplasmic distribution observed by micro-X-ray CT and optical microscopy

2.3. TEM observation and EDS analysis

After confirming the cytoplasm condition using semi-thin sections (thickness, 500 nm) under a light microscope (BX51, Olympus), ultra-thin longitudinal sections (thickness, 60 nm) were cut from the selected foraminifera (3 specimens of *B. spissa*, 5 specimens of *U. akitaensis*, 4