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Three methods for isolating viable anthozoan endoderm cells with their intracellular symbiotic dinoflagellates

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Abstract. Three maceration methods are described for the isolation of single endoderm cells from marine cnidarians. Two are enzymatic treatments suitable for fleshy anthozoans such as sea anemones and zoanthids. The third employs calcium free sea water and is suitable for stony corals. The viability and morphology of the endoderm cells is described using fluorogenic dyes and scanning and transmission electron microscopy.

Introduction

Anthozoans containing intracellular symbiotic dinoflagellates (zooxanthellae) have been investigated extensively at the ecological and organismal level (Muscatine 1973; Trench 1987). Given the abundance and distribution of these dynamic associations in the marine environment, it is remarkable how little is known of their cell biology. One obstacle has been the difficulty of obtaining individual living host cells containing symbiotic zooxanthellae by tissue maceration.

PAnthozoans are often fragile, contractile and secrete copious amounts of mucus. Some (e.g. corals) secrete proteinaceous and calcium carbonate skeletons which present additional technical problems. To date, methods for isolating individual cells have employed maceration following fixation of whole tissue (Hertwig and Hertwig 1879; David 1983). While useful information has been obtained from the static images resulting from these methods, it is often desirable to analyze the dynamic processes of living cells over time. The use of living tissues and single viable cell suspensions or cultures to study the cell biology of freshwater cnidarians and other phyla is advanced and well documented (Maurer et al. 1989; Ohtani 1988; O'Dell and Christensen 1989). We present three maceration methods for obtaining single viable anthozoan endoderm cells with their complement of symbiotic zooxanthellae. The methods circumvent the need for fixation, and the isolated cells are viable for up to five hours following maceration. The endoderm cells obtained using these methods are described using fluorogenic dyes and scanning and transmission electron microscopy.

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Methods

Specimens of the tropical sea anemone *Aiptasia pulchella* were relaxed in calcium-free artificial sea water (Harvey 1957; minus calcium chloride and trace elements) for 30 min to 1 h. Tentacles were removed with a scalpel, sliced into 1 mm long cylindrical sections and transferred to deep cavity slides. Mucus associated with the tissue was removed by washing at least 10 times with calcium-free sea water.

The tissues were macerated by replacing the final wash with one of two enzymes solutions: the first, 0.05% collagenase (Type I, Sigma Chemical Co.) in 0.1 M sodium phosphate buffer containing 3% sodium chloride and 0.004% calcium chloride (required to activate the collagenase), pH 7.4; and the second, 0.05% trypsin (Type III, Sigma Chemical Co.) in calcium-free sea water. The tissues were gently agitated for 30–60 min at 25° C. Motile endoderm cells containing zooxanthellae swam away from the tissue and were easily observed with a dissecting microscope. Both enzymatic treatments produced viable endoderm cells, however, trypsin appeared to macerate anemone tissues more rapidly than collagenase.

For reef corals with small polyps, a calcium-free maceration method was employed. Small branches were removed from colonies of *Pocillopora damicornis* and placed in calcium-free sea water for 3–4 h. Each of several polyps on a given branch was mechanically disrupted with a scalpel blade. Motile endoderm cells containing zooxanthellae moved out of the damaged tissue and collected in the bottom of the Petri dish.

Due to the fragile nature of the endoderm host cell plasma membrane, the isolated cells were collected by suction with a fine bore mouth pipette. They were expelled onto poly-L-lysine (0.1% in distilled water, mol. wt. >70000, Sigma Chemical Co.) coated slides for light microscopy and onto coated coverslips for scanning electron microscopy. The cells were allowed to settle and attach to the poly-L-lysine for at least 10 min prior to processing.

The viability of macerated cells was assessed by immersing the slides in the fluorogenic dye fluorescein diacetate (FDA, Sigma Chemical Co., stock solution 15 mg/ml in acetone; working solution

0.04 ml of stock in 9.96 ml 0.1 M sodium phosphate, 3% sodium chloride, 0.004% calcium chloride, pH 7.4) for 10 min. Non-specific esterases in viable cells hydrolyse non-polar FDA to polar molecular fluorescein which fluoresces at a wavelength of 450–490 nm (Schupp and Erlandsen 1987). The slides were rinsed twice in buffer (0.1 M sodium phosphate, 3% sodium chloride and 0.004% calcium chloride, pH 7.4) covered with a Vaseline supported coverslip and viewed under brightfield, phase contrast and epifluorescence with an Olympus BH-2 microscope at 40×. The endoderm cells from a single animal were placed in buffer (0.1 M sodium phosphate, 3% sodium chloride and 0.004% calcium chloride, pH 7.4) and incubated at 25° C for 5 h following isolation. A sample of cells was removed every hour and stained with FDA. The number of fluorescing cells was expressed as a percentage of the total number of cells counted for a particular sampling interval.

To visualize the host cell nucleus, endoderm cells were stained with the DNA specific dye Hoechst 33258 (Reynolds et al. 1986; Sigma Chemical Co. stock solution 5 mg/ml distilled water, working solution 0.04 ml of stock in 9.96 ml of 0.1 M sodium phosphate, 3% sodium chloride and 0.004% calcium chloride, pH 7.4) for 30 min. Following a brief wash in buffer (0.1 M sodium phosphate, 3% sodium chloride and 0.004% calcium chloride, pH 7.4) the cells were viewed using epifluorescence at a wavelength of 450–490 nm.

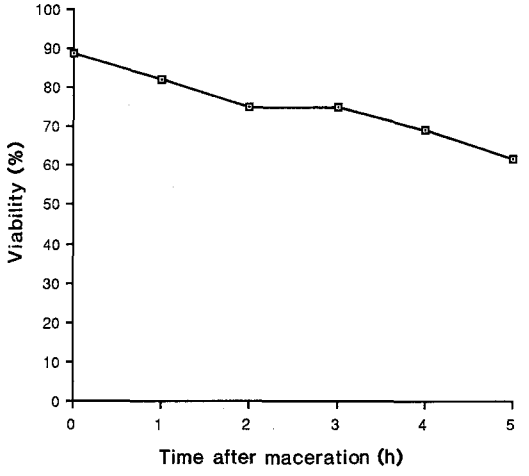

Fig. 1. The viability of *A. pulchella* endoderm cells as a function of time after isolation from the tentacle tissue

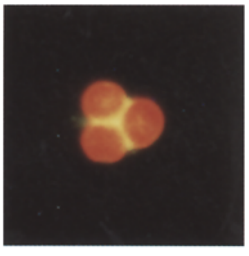

Fig. 2. An isolated *A. pulchella* endoderm cell containing three zooxanthellae and stained for viability with fluorescein diacetate (×4000)

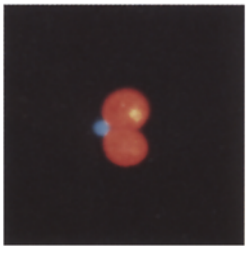

Fig. 3. An isolated *P. damicornis* endoderm cell containing two zooxanthellae (×4000) showing the position of the host cell nucleus, stained with Hoechst 33258

For scanning electron microscopy, endoderm cells attached to coverslips were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate for 1 h. Following two rinses in 0.1 M sodium cacodylate the cells were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 30 min. The coverslips were rinsed briefly in distilled water and dehydrated in 30%, 50%, 70%, 90%, 95% and 100% (×3) ethanol for 30 min each. The samples were immersed in hexamethyldisilazane (Applied Sciences, Inc.) for 5 min (Nation 1983) and air dried for 20 min. The coverslips were mounted on aluminium stubs, gold coated and viewed on a Cambridge 360 Scanning Electron microscope.

For transmission electron microscopy, endoderm cells were transferred to a microfuge tube (Gilson, 1.5 ml) and centrifuged (Eppendorf model 5414) for 30 seconds. The resulting pellet of cells was fixed in the microfuge tubes as described for scanning electron microscopy. After dehydration for 30 min each in 30%, 50%, and 70% ethanol, the ethanol was drained from the tubes and immediately replaced with 2% agar (in distilled water). The agar was allowed to set and the microfuge tube was then cut away from the agar plug containing the cells. The agar was trimmed and immersed in 70% ethanol. Dehydration was completed through 90%, 95%, and 100% (×3) ethanol. After transfer to propylene oxide the agar plug was embedded in Spurr's epoxy resin. Thin sections were cut using a Sorvall 6000 ultramicrotome, mounted on copper grids, stained with lead acetate and viewed on a JEOL transmission electron microscope.

Results and Discussion

Of the cell population released by maceration, 60% stained with FDA five hours following isolation (Fig. 1). Fig. 2 shows that in a stained endoderm cell, a large proportion of the intracellular compartment is taken up by the symbiotic zooxanthellae. Fluorescein is therefore restricted to the narrow host cell cytoplasmic compartment surrounding the red autofluorescent zooxanthellae. En-

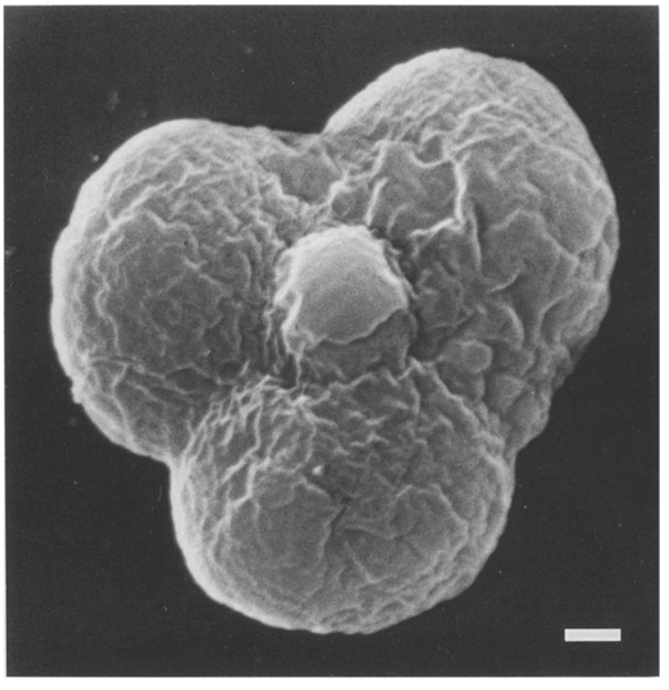

Fig. 4. A scanning electron micrograph of an isolated *A. pulchella* endoderm cells containing three zooxanthellae. Scale bar = 1 μm

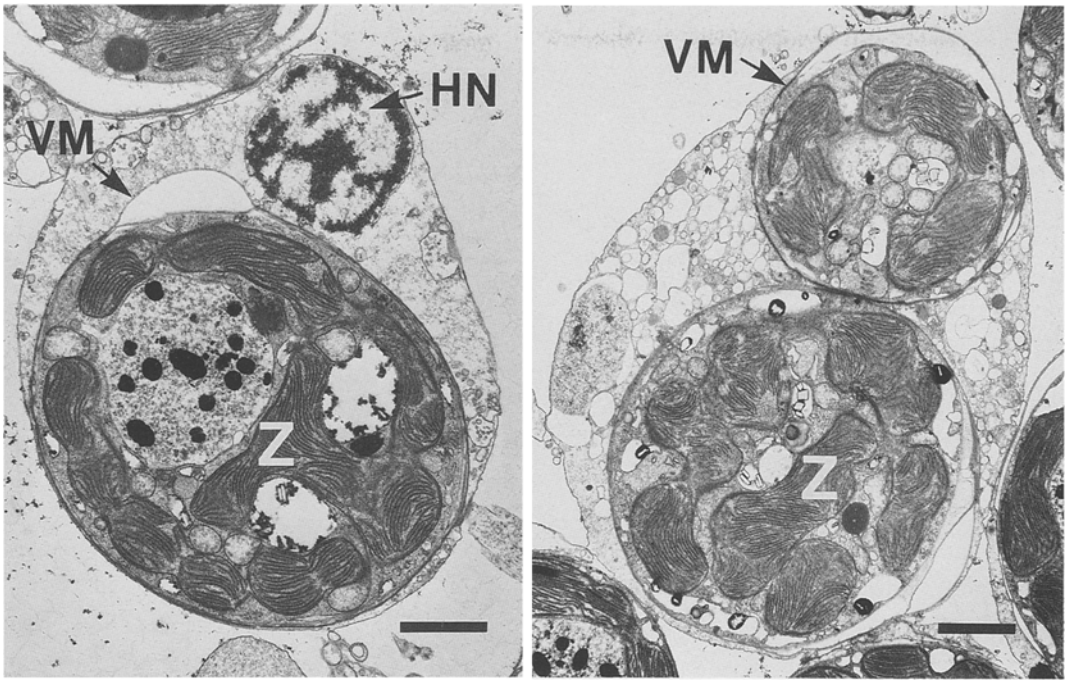

Fig. 5. Transmission electron micrographs of isolated *P. damicornis* endoderm cells containing one (left) and two (right) zooxanthellae. Scale bar = 2 μm. HN = host nucleus; VM = vacuolar membrane

doderm cells containing one zooxanthella are round (10 μm in diameter) while those with two zooxanthellae are elongate (20 μm in diameter). As the number of zooxanthellae per cell increases, the shape of the host cell tends to conform to the way the symbionts cluster. The host cell nucleus appears peripherally as a "bleb" in endoderm cells containing one or two zooxanthellae and centrally in cells containing three or more zooxanthellae (Fig. 3).

Scanning electron microscopy reveals the host cell nucleus as a small bulge beneath the plasma membrane sheath (Fig. 4). Transmission electron microscopy shows other host cell constituents including the host cell nucleus and the vacuolar membrane surrounding the zooxanthellae (Fig. 5). The plasma and vacuolar membranes are closely associated at various points around the cell. These membranes are fragile and frequently break during handling. This may explain why maceration often releases isolated zooxanthellae.

We have used these maceration methods to isolate endoderm cells from other anthozoans including *Zoanthus sociatus* (Order Zoanthidea), *Fungia scutaria* (Order Scleractinia) and *Boloceroides* sp (Order Actiniaria) collected from Checker Reef, Kaneohe Bay, Hawaii. The collagenase method also works on *Cassiopeia xamachana* scyphistomae (R. K. Trench, personal communication). We are currently using these techniques to obtain endoderm cells for cell culture.

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