

## Isolation of symbiosomes and the symbiosome membrane complex from the zoanthid *Zoanthus robustus*

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The zoanthid *Zoanthus robustus* was used as a model organism to develop procedures for isolating pure symbiosomes and symbiosome membranes. The symbiosome is comprised of a zooxanthella (*Symbiodinium* sp.) cell that divides rarely and is separated from the host gastrodermal cytoplasm by a symbiosome multimembrane complex. Devising a method to isolate membranes at the interface between the symbiotic partners is a critical first step in characterising the molecular components involved in the metabolic trafficking necessary to sustain an effective symbiosis. After zoanthid gastrodermal cells were extracted, symbiosomes were released by mechanical disruption, recovered by centrifugation, and then purified using discontinuous sucrose gradient centrifugation. The material forming the membrane complex around symbiosomes proved highly resistant to disruption. Methods used to dissociate this interface from symbionts included (1) Triton X-100 detergent solubilisation, (2) osmotic shock with mechanical disruption, and (3) vigorous mechanical disruptions, where powerful shearing forces were used, combined with a series of sucrose density gradient centrifugation steps. The lipophilic styryl fluorochrome FM 1-43, at a concentration of 30  $\mu$ M, selectively labelled the symbiosome membrane complex, both for isolated symbiosomes and those *in hospite*. Other cell membranes, including plasma membranes, endoplasmic reticulum, tonoplast, and organelle membranes, were not visibly labelled at this concentration. The selective labelling of the symbiosome membrane complex remained stable even after long exposure times (3 h). At 30  $\mu$ M concentration, FM 1-43 also labelled symbiosome membrane fragments isolated using methods (1), (2) and (3). Method (3) proved to be the most effective in producing a fraction enriched in FM-143-labelled membrane material, which we call a symbiosome membrane complex. Transmission electron microscopy, together with confocal and conventional epifluorescence microscopy of the FM 1-43-stained preparations, was used to validate the purity of symbiosome preparations and to infer the complexity of the symbiosome membrane complex. This membrane complex has regions where the membranes contributed by the alga are appressed, and punctate regions whose function remains unclear.

KEY WORDS: Symbiosome, *Zoanthus robustus*, *Symbiodinium*, Dinoflagellate, Symbiosome membrane

### INTRODUCTION

The concept of the “symbiosome” in cnidarian symbioses was first put forward by Neckelmann and Muscatine (1983) for the *Chlorella–Hydra* symbiosis. It has since gained currency in describing the structure of the symbiosis of *Symbiodinium* spp. in corals and other cnidarians where free-living *Symbiodinium* cells are ingested, probably via the host enteron, phagocytosed into gastrodermal cells of the host, and invested with a host-derived membrane. The host cell contains one, or more rarely, several zooxanthella cells. The internalised zooxanthella lacks flagella, and its cell wall structure is modified somewhat in comparison with free-living *Symbiodinium* (Wakefield *et al.* 2000).

The cnidarian symbiosome has been defined as the host-derived outer membrane plus the *Symbiodinium* algal cell and the space between these two, including multilayered membranes derived from the algal symbiont (e.g. Wakefield

*et al.* 2000, Wakefield & Kempf 2001); thus the physical barrier between the host and endosymbiont may be more accurately described as a membrane complex. The outer host-derived symbiosome membrane is the “definitive symbiosome membrane”, as determined by its immunological response. In an interesting hypothesis, Wakefield *et al.* (2000) suggest that the multilayered endosymbiont-derived membrane system originates when zooxanthellae *in hospite* periodically enter a form of delayed ecdysis, preparing new thecal plates that never eventuate. Instead, the cell wall breaks down and the plasma membrane and thecal vesicles that are shed form a membrane layer (Wakefield *et al.* 2000). Each terminated ecdysis adds another algal membrane layer, thus forming the variable multilayer membrane structure (Wakefield *et al.* 2000).

The symbiosome concept has been applied to nitrogen-fixing root nodules, where nitrogen-fixing bacteria (such as *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, etc.) are surrounded by a plant-derived vesicle membrane (Roth & Stacey 1989). A great deal of

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research has been carried out on nitrogen-fixing symbiosomes (e.g. Udvardi & Day 1997; Roberts & Tyermann 2002; Vincill *et al.* 2005), and the symbiotic interface of nitrogen-fixing symbioses is much better characterised than the one under examination here. The parallels between the two types of symbiosomes, one surrounded by an animal membrane and the other by a plant-derived membrane, have yet to be drawn in any detail and may be limited in that the endosymbiont in the former association is another eukaryote with a multimembrane symbiotic interface and the latter is a prokaryote with a relatively simple symbiotic interface comprising only a single membrane.

With regard to eukaryotic–eukaryotic associations, more is known about the process in some parasitic infections (Hackstadt 2000), especially in members of the phylum Apicomplexa. These obligate intracellular parasites (Morrisette & Sibley 2002) are closely related to dinoflagellates (Saldarriaga *et al.* 2004), making comparisons with the marine association valid. The best-studied Apicomplexan parasites are those of medical or veterinary importance, including *Toxoplasma* spp., *Cryptosporidium* spp. and *Plasmodium* spp. Here, the invasion process is actomyosin dependent, first involving attachment to the host cell, subsequent invagination of regions of the host cell plasma membrane that exclude integral membrane proteins, and, crucially, formation of a specialised parasitophorous vacuole (Morrisette & Sibley 2002). The parasite contributes lipids and proteins (Sinai & Joiner 2001) to this “enigmatic organelle”, which is so well integrated with host endoplasmic reticulum and mitochondria that it has resisted extensive attempts at biochemical, molecular and cell biological isolation, purification and characterisation (Martin *et al.* 2007).

Both the parasitophorous vacuole and the symbiosome membrane resist fusion with the host endolysosomal system, and presumably a cellular recognition process halts the normal endocytotic process. In the *Aiptasia–Symbiodinium* association, presence of an infecting endosymbiont interferes with Rab protein expression on the phagosome membrane, a crucial step in differentiation of the phagosome into the symbiosome (Chen *et al.* 2003).

The interfacial symbiosome membrane complex between the host gastrodermal cell cytoplasm and the endosymbiotic alga must play a key role in mediating communication between host and endosymbiont, including transport of metabolites and nutrients, and recognition of signals. The interface is likely to be the specific site for breakdown of the symbiosis and release of the endosymbiont during periods of stress, including mass coral bleaching [associated with a small increase in summer sea surface temperature (Hoegh-Guldberg 1999)] and overcrowding within the host cell [where expulsion of dividing cells has been proposed as a means of controlling endosymbiont population size (Baghdasarian & Muscatine 2000)].

Using monoclonal antibodies to distinguish between animal and algal membranes, Wakefield & Kempf (2001) identified the “definitive symbiosome membrane” as the outer single host-derived membrane, whereas the inner multiple membranes were considered to derive from the symbiont. Despite their prevailing immunological identities, it is not known whether these interfacial membranes remain

completely distinct as host or algal membranes, or whether, as occurs in Apicomplexan parasitophorous vacuoles (Martin *et al.* 2007), they exchange some materials and are thereby melded into a hyperstructure that transcends the individual nature of either host or algal membranes alone.

In this paper, we report methods for isolating large quantities of pure symbiosomes from the zoanthid *Zoanthus robustus* Carlgren, and from these symbiosomes, we purified membranes at the symbiotic interface. The approach we have taken is similar to the one taken by Trautman *et al.* (2002), but as the objective was to isolate membranes in large quantities for further analysis, it was necessary to make considerable modifications. It was also necessary to prove that we had isolated pure symbiosomes before proceeding. Confocal and electron microscopy were used to study the interface between the zooxanthellae and the host cell in detail. We are currently profiling the protein composition of the symbiosome membrane complex to better understand the functionality of symbiosome multi-membrane complex.

## MATERIAL AND METHODS

### Animal material

The invertebrate animals used in this study, *Z. robustus* Carlgren, were collected from –10 m at Fairlight Beach, Sydney Harbour (33°50'S, 151°22'E) and at Bare Island, Botany Bay (34°01'S, 151°19'E), Sydney, New South Wales, Australia. The animals were kept in an aquarium filled with fresh filtered seawater under aeration for up to two months.

Gastrodermal cells containing endosymbiotic *Symbiodinium* were gently extracted from healthy zoanthids using the negative pressure produced by the suction of a 5-ml syringe fitted with a 20-gauge needle, introduced through the mouth of the animal.

### Fluorescence-labelling the symbiosome–membrane complex

Symbiosomes, both isolated (see below) and *in hospite*, were fluorescence-labelled with FM 1-43 (Molecular Probes, USA) ( $\lambda_{\text{ex}}$  479 nm,  $\lambda_{\text{em}}$  598 nm). FM 1-43 is a vitally staining fluorochrome in the styryl family, used for detecting vesicle trafficking in eukaryotic cells (Bolte *et al.* 2004). We selected FM 1-43 for labelling symbiosome membranes because it reportedly identifies internalised or endocytosed membranes derived from the plasma membrane (Bolte *et al.* 2004). Stock solutions of FM 1-43 were made up in Milli-Q water at a concentration of 1 mM, and were kept at –20°C in the dark.

A range of final FM 1-43 concentrations (10 to 80  $\mu\text{M}$ ) was made up in a 1:1 volumetric ratio of phosphate-buffered saline (PBS) at physiological pH (7.4) and 50% filtered, sterilised seawater (PBS-50% FSSW). The FM 1-43 solutions were tested on symbiosome preparations to determine optimal conditions for labelling. Of these concentrations, 30  $\mu\text{M}$  FM 1-43 labelled the symbiosome membrane without labelling other cell membranes; whereas,

higher FM 1-43 concentrations (50  $\mu\text{M}$  and higher) faintly labelled gastrodermal cell membranes and cytoplasm. Thus, all samples were stained at the optimal 30  $\mu\text{M}$  concentration. The fluorescent nuclear stain 4', 6-diamidino-2-phenylindole (DAPI) ( $\lambda_{\text{ex}}$  350 nm,  $\lambda_{\text{em}}$  460 nm) was used to visualise nuclei, primarily to assure isolation of the symbiosomes. If the gastrodermal cell remained, its nucleus could be detected external to the symbiosome by labelling with DAPI at a final concentration of 1  $\mu\text{g ml}^{-1}$ .

FM 1-43 fluorescence was observed using a Zeiss Axiophot fluorescence microscope with the filter combination: excitation filter 450–490 nm, beam splitter 510 nm, and barrier filter 520 nm. Preparations were photographed using a Leica DC 300F camera and IM 100 software. Confocal fluorescence microscopy was performed using a Bio-Rad Radianc Plus 0.1 microscope with LaserSharp 2000 software and 488 nm krypton–argon and 520-nm lasers. The laser intensity, iris diameter, gain and background were optimised using LUTSET and were not changed during image acquisition.

Unstained symbiosome preparations were examined with epifluorescence microscopy using the same filter combinations to detect native autofluorescence at this wavelength.

Cultured *Symbiodinium* (CSIRO strain CS-73, Heron Island, Great Barrier Reef, Queensland, Australia; isolation date: 01 January 1978) was used for comparisons with symbiotic *Symbiodinium*. Cells were grown in f/2 (Guillard & Ryther 1962) at 25°C and 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Cells from 50 ml of cultured cell suspension were harvested by centrifugation (4000  $\times g$  for 10 min) at 4°C and resuspended in an equal volume of the same extraction buffer [without dithiothreitol (DTT)] used for symbiosome isolation. Cells were stained with FM 1-43 and examined under epifluorescence microscopy. For more details about the strain used here refer to [http://www.marine.csiro.au/microalgae/strainlist/CMAR\\_Strain\\_sep04.pdf](http://www.marine.csiro.au/microalgae/strainlist/CMAR_Strain_sep04.pdf).

### Viability of gastrodermal cells

Gastrodermal cell viability was determined by exposing crude gastrodermal tissue extracts containing symbionts to 10  $\mu\text{M}$  6-carboxyfluorescein diacetate (6-CFDA). 6CFDA is membrane-permeant and nonfluorescent. After uptake by living cells it is hydrolysed by plasma membrane esterases into nonpermeant and highly fluorescent 6-carboxyfluorescein (6CF). A 1 mM stock solution of 6CFDA (Molecular Probes, USA) was made up in methanol. This was diluted in PBS-50% FSSW to make a 10  $\mu\text{M}$  working solution. The fluorescence of 6CF was observed using the same excitation and barrier filters used with FM 1-43-labelled preparations.

### Separating symbiosomes from host gastrodermal cells

Gastrodermal cells of approximately eight healthy zoanthids were extracted and immediately mixed with an equal volume of PBS-50% FSSW containing 1 mM EDTA, 0.5% DTT, 0.5  $\text{mg l}^{-1}$  DNAase and the protease/proteinase inhibitors 0.5 mM phenylmethylsulphonylfluoride, 0.5 mM  $\alpha$ -caproic acid, and 0.5 mM benzamidine. EDTA and DTT were added to prevent the gastrodermal tissue

extract from aggregating. Aggregation was also reduced by having 50% seawater in the medium, and by temperatures above 15°C. The procedure produced 15 to 50 ml of gastrodermal tissue suspension depending on the size of the animals. Extracts were examined using epifluorescence, bright-field and transmission electron microscopy (TEM).

### Isolating symbiosomes from gastrodermal tissue suspension

The gastrodermal tissue suspension was homogenized (two cycles) in a loose glass homogenizer. The gastrodermal cells were then mechanically disrupted to release symbiosomes as follows. Extracts were either subdivided into 5-ml aliquots in 5-ml syringes, each of which was passed vigorously seven times through a 23-gauge needle (0.33 mm inner diameter) into a Falcon tube, or the entire 15–20-ml extract in a 20-ml barrel syringe was passed seven times through a 23-gauge needle into a Falcon tube. A different pattern of bands was obtained on discontinuous sucrose density gradients (see below) according to the vigour with which gastrodermal cells were disrupted.

The deaggregated and mechanically disrupted cell suspensions were examined using epifluorescence, bright-field and TEM.

### Separation of symbiosomes from animal cells and cell debris

Symbiosomes were separated from gastrodermal cell debris, cnidoblasts and other animal cells by sucrose gradient density centrifugation. The deaggregated and broken gastrodermal homogenate was loaded onto a sucrose gradient consisting of 30% (5 ml), 40% (10 ml), 50% (10 ml) and 50% (5 ml) sucrose made up in 50% FSSW. The gradients were centrifuged for 90 min at 26,500  $\times g$ , with slow acceleration/deceleration rates, at 12–15°C.

Gently disrupted material resolved into three bands of symbiosomes on the sucrose gradients: top, above 40% sucrose, middle, above 50% sucrose, and bottom, above 60% sucrose. Vigorously disrupted material resolved into a single large band above 40% sucrose. All symbiosome bands were assessed for purity using fluorescence and bright-field microscopy. The bottom band was contaminated with some animal cells, and was discarded. Remaining bands from both mechanical treatments were used in further work.

The symbiosome bands were collected, diluted threefold with extraction buffer (lacking DTT), and symbiosomes were recovered by centrifuging at 4000  $\times g$  for 10 min at 12–15°C. The symbiosome pellet was resuspended in an equal volume of PBS-50% FSSW (lacking DTT) and kept on ice.

Examination with phase-contrast optics revealed that a golden layer in the load zone of sucrose gradients was enriched in vesicles, and this layer was also harvested.

### Methods for enriching the symbiosome membrane complex fraction and associated proteins

Three different methods were used to obtain fractions enriched in symbiosome membranes or their associated proteins (or both); all methods used the same initial extraction buffer. The isolated symbiosomes (or cultured

*Symbiodinium* as control) were (1) treated with detergent to chemically solubilise membrane proteins at the surface of whole symbiosomes, (2) subjected to hypotonic treatments combined with mechanical shearing stress, and (3) subjected to imposed mechanical shearing forces and two sucrose density gradients.

(1) A maximum concentration of 0.3% Triton X-100 (10% Triton X-100 stock solution diluted with 50% FSSW) was used per 1 ml of symbiosomes (or cultured cell suspensions). Samples were exposed to detergent for: 1, 5, 15, 30, 45 or 60 min, and slowly stirred to facilitate solubilisation of the symbiotic interface. Samples were then transferred onto 30% sucrose (in 50% FSSW) and centrifuged ( $16,000 \times g$  for 45 s). Both the supernatant (membrane protein solution) and the pellet (algal pellet) were retained. Triton X-100 was removed from the supernatant using microconcentrators (10K Microsep™ devices PALL, Life Science). Proteins were concentrated by centrifugation (18 h, JA-17 rotor at  $3500 \times g$ ). The algal pellet was examined microscopically to assess the degree of detergent solubilisation.

(2) Combined osmotic and mechanical shearing methods were used in an effort to produce a fraction enriched in symbiosome complex membranes. One milliliter of resuspended symbiosomes was injected into 10 ml of hypotonic solution (~10-fold dilution in PBS) and rapidly stirred on a magnetic stirrer to produce shearing stress. The treatment was applied for ~30 min. In preliminary experiments we found that symbiosomes remained structurally stable in this hypotonic solution for up to 30 min. The procedure was carried out at two different temperatures, the first ~4°C in an ice bath and the second ~13.5°C. The first temperature was selected to avoid, and the second to approach, the lipid phase transition temperature at ~14–15°C (Drost-Hansen 2001).

In some experiments a range of different concentrations of Triton X-100 detergent (0.3% to 0.16% to 0.1%) was added to the hypotonic solution. In other experiments, the symbiosomes were subjected to 30 min of hypotonic treatment, and subsequently ground for ~15 min in PBS-50% FSSW in an Eppendorf tube with a micropestle, on ice, using glass beads (500 µm, Sigma).

After these treatments, the symbiosome solution was centrifuged at  $4000 \times g$  for 10 min at 12–15°C to recover whole symbiosomes, algal cells and debris. The supernatant was diluted in a 1:1 ratio with 20 mM ammonium acetate buffer (pH 6.8) and centrifuged at  $16,000 \times g$  for 40 min.

(3) The following optimised method involved reloading symbiosomes obtained from a sucrose density gradient onto a second such gradient. We aimed to minimise contamination from host cell membranes, and to produce a fraction enriched with membranes of the symbiotic interface. The gastrodermal cell extract was gently disrupted and three symbiosome bands were resolved on a sucrose gradient. The top and middle bands were pooled, diluted in PBS-50% FSSW, pelleted ( $1000 \times g$  15 min), and resuspended in PBS-50% FSSW containing the inhibitor cocktail. The resuspended symbiosomes were vigorously disrupted, by strong manual pressure on, and seven repeat passes through, the 23-gauge needle described above, loaded on to a second sucrose gradient, and centrifuged as described previously. The symbiosome membrane fraction was recovered as a golden layer of vesicles suspended in the load zone.

Membranes were concentrated using a modification of a classic method for erythrocyte membrane protein isolation (Mazia & Ruby 1968). The fraction was mixed in a 1:1 ratio with 20 mM ammonium acetate buffer (pH 6.8) and centrifuged at  $16,000 \times g$  for 90 min at 4°C. The resulting white pellet was collected, and the supernatant then ultracentrifuged at  $63,907 \times g$  (average) for 40 min at 4°C. The pellet was collected, and the supernatant, which contained a white flocculate, was centrifuged at  $113,613 \times g$  (average) for 40 min at 4°C (Beckman Ti60 rotor), producing a further white pellet.

The golden-layer material derived from both first and second sucrose gradients and the white pellets were stained with FM 1-43 and examined with epifluorescence microscopy. To assist with interpretation of the protocol, we offer a schematic (Fig. 1) that maps the optimised method with the structure components of the symbiosome membrane complex.

## TEM

TEM was carried out on preparations of (1) intact gastrodermal cells, (2) symbiosomes released from host cells, (3) symbiosomes purified using a sucrose density gradient and (4) symbiosomes treated with Triton X-100 0.3%. Samples were fixed using 2.5% glutaraldehyde in 0.1 M of phosphate buffer (pH 6.8 to 7.1), with 15% sucrose added as osmoticum (Larkum *et al.* 1987). Samples were fixed at room temperature for 1 h, rinsed twice with 0.1 M PBS containing progressively decreasing sucrose concentrations (15%, 10%, 5% and 0%), and then postfixed in 2% OsO<sub>4</sub> for 1 h. Samples were dehydrated through an ethanol series, embedded in Spurr's resin, ultrasectioned (Reichert Ultracut E ultramicrotome), and poststained with uranyl acetate and Reynold's lead citrate. Sections were examined using a Zeiss EM902A electron microscope incorporating Digital Micrograph 3.3.1 image-capture software.

## RESULTS

### Selective fluorescence-labelling of the symbiosome membrane complex by FM 1-43 and viability of host gastrodermal cells

A concentration of 30 µM FM 1-43 rapidly labelled (i.e. within a few milliseconds) the region around the symbiosome both *in hospite* (Fig. 2) and after isolation of whole symbiosomes from host gastrodermal cells. At 30 µM, FM 1-43 did not visibly label the plasma membranes of host gastrodermal cells, cnidoblasts, hyaline mesoglea or other animal cells present in the crude host cell extracts, nor did it label any other membranes such as the endoplasmic reticulum, tonoplast, or organelle membranes of either the host or the endosymbiont cells. [A bright-field image (Fig. 3) is provided for comparison.] Chlorophyll within chloroplasts of the endosymbionts produced red autofluorescence visible in Fig. 4. The selective labelling of the symbiosome membrane complex remained stable for at least 3 h under the conditions we describe (see Material and Methods).

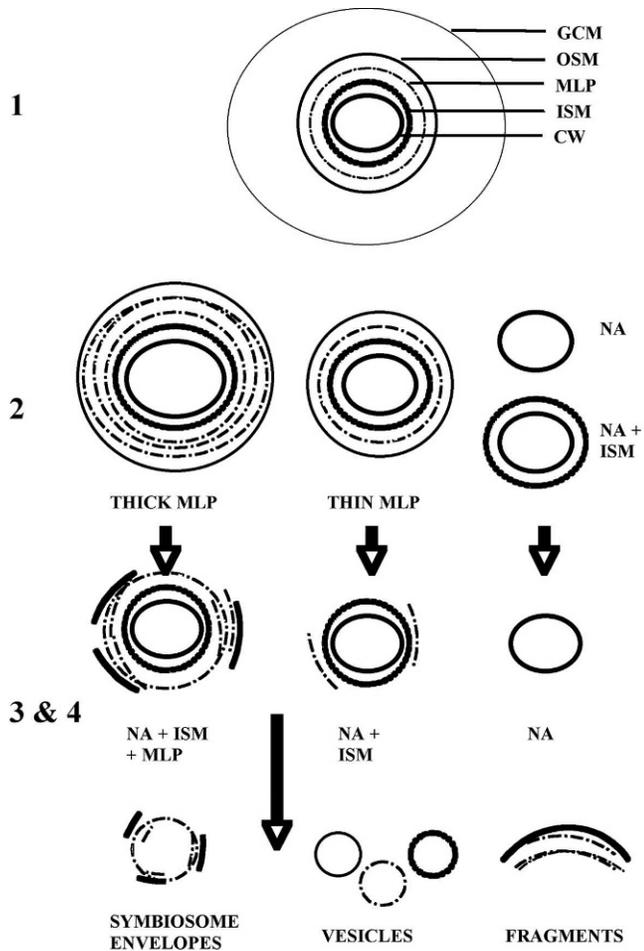


Fig. 1. Schematic diagram of the optimized protocol for isolating symbiosomes and symbiosome membrane complex from *Zoanthus robustus*. GCM = gastrodermal cell membrane; OSM = outer symbiosome membrane; MLP = multilayered membrane process; ISM = inner symbiosome membrane; CW = cell wall of the algal symbiont. **1-1.** The crude zoanthid extract contains gastrodermal cells with *Symbiodinium*. **1-2.** The sucrose density isolation resolves whole symbiosomes. We generate symbiosomes with different densities obtained from the gradients, and we infer from FM 1-43 staining that these populations include some symbiosomes with thicker multilayer processes (thick MLP), some symbiosomes with thinner multilayer processes (thin MLP), some with few or no multilayer processes (NA+ISM) and a proportion of naked algal symbionts (NA). We assume that the shear forces produced by syringe disruption and centrifugation are responsible for our observations. **1-3, 1-4.** The fraction containing stripped (naked) algae and a proportion of those retaining only a thin membrane (ISM) external to the cell wall are discarded. The remaining symbiosomes (with thick and thin MLP) are passed through a second sucrose density gradient procedure. Symbiosomes with initially the thickest MLP are disrupted to produce a punctuated OSM, plus MLP and ISM (NA+ISM+MLP); those with initially thinner MLP produce symbiosomes with remnants of MLP plus ISM (NA+ISM); a proportion of the algae has been stripped entirely (NA). The material above the load zone of the final sucrose gradient is highly enriched in FM 1-43-labelled membranes, including whole symbiosome envelopes, a population of vesicles of different sizes, and fragments of the complex of multilayer processes.

The blue light used to excite FM 1-43 fluorescence induced autofluorescence of aggregation bodies within the majority of symbiosomes, both isolated and *in hospite* (Fig. 4), and inclusion body autofluorescence has been

demonstrated previously by Muscatine *et al.* (1994). The emission spectrum of autofluorescent aggregation bodies was similar (visibly yellow) to that of FM 1-43; aggregation body autofluorescence of the symbiont, or of cultured *Symbiodinium* for that matter, cannot be taken as evidence of FM 1-43 internalisation via membrane trafficking.

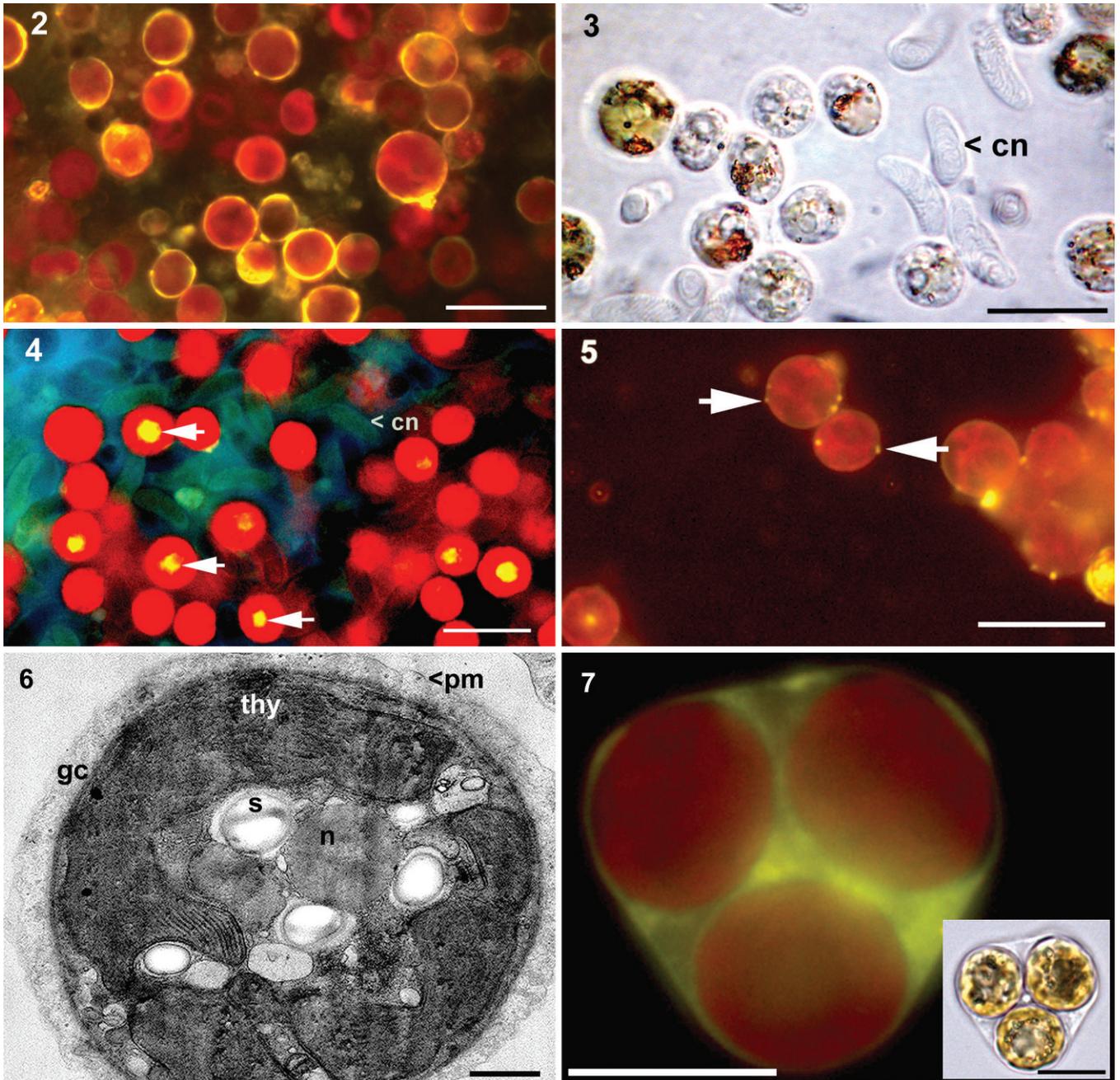
In contrast to their symbiotic counterparts, cultured *Symbiodinium* exposed to 30  $\mu\text{M}$  FM 1-43 did not give rise to the distinctive FM 1-43 halo of labelled membranes; this halo is a feature of symbiosomes alone. When the concentration of FM 1-43 was increased to 50  $\mu\text{M}$  (Fig. 5), we were able to detect some labelling that was restricted to extracellular punctate regions.

The gastrodermal cells contain from one to three endosymbionts *in hospite*. Fig. 6 shows a transmission electron micrograph of a gastrodermal cell containing a single *Symbiodinium* cell. The symbiont occupies most of the volume of the gastrodermal cell, whose cytoplasm forms a thin periphery bounded by a plasma membrane. Most of the volume of the symbiont is occupied by its large, multilobed chloroplast and associated starchlike granules, with a central nucleus. The symbiont is enclosed by a multilayered symbiosome membrane complex. This is separated from the animal cytoplasm by a discrete outer membrane, the symbiosome membrane.

We used 6CFDA to determine host cell's viability postisolation; the cytoplasm of the majority of symbiont-containing gastrodermal cells developed 6CF fluorescence. Fig. 7 shows the fluorescing cytoplasm of a gastrodermal cell hosting three *Symbiodinium* endosymbionts. The algal symbionts did not develop 6CF fluorescence, and neither did cultured *Symbiodinium*.

Symbiosomes purified using gentle mechanical disruption and a single sucrose density gradient centrifugation step (30–60% step gradient) resolved three distinct bands of symbiosomes. The bands were at the interface of 30–40% sucrose (top), 40–50% layer (middle) and at 50–60% layer (bottom) and a pellet (Fig. 8). The load zone and each zooxanthellal layer were examined under epifluorescence with FM 1-43 label and bright-field microscopy. The golden layer (at the interface of the load volume and 30% sucrose layers) contained some broken zooxanthellae but was rich with vesicles, visible under phase-contrast microscopy. Many of these vesicles were strongly FM 1-43 positive, suggesting that some symbiosome membrane material had been mechanically dislodged during centrifugation. However, the golden layer also contained numerous membrane vesicles that were not FM 1-43 positive (possibly remnants of gastrodermal cell plasma membranes), unidentified animal cells, cells of hyaline mesoglea and some whole symbiosomes.

The symbiosome layers (top, middle, and bottom) consisted of mainly intact symbiosomes (Figs 9–11) visualized by red autofluorescence from chlorophyll *a* (Chl *a*) and with the yellow fluorescent halo of the symbiosome membrane complex, labelled with FM 1-43. The precipitate contained animal cells (including cnidoblasts), zooxanthellae and hyaline mesoglea. Almost no small particles of mesoglea and sparse cnidoblasts were found in the top (Fig. 9) and middle (Fig. 10) layers; whereas, a few were present in the bottom layer (Fig. 11). In addition to isolated symbiosomes, the most aggregated, bottom, layer con-



**Fig. 2.** Specificity of FM 1-43 for the symbiotic interface between host cell and resident algae. *Symbiodinium* in the crude gastrodermal cell extract from *Zoanthus robustus* stained with FM 1-43 (30  $\mu$ M) using epifluorescence microscopy. Scale bar = 20  $\mu$ m.

**Fig. 3.** *Symbiodinium* in the crude gastrodermal cell extract from *Zoanthus robustus* examined under bright-field microscopy. cn = cnidoblast. Scale bar = 20  $\mu$ m.

**Fig. 4.** Autofluorescent inclusion bodies in *Symbiodinium*. *Symbiodinium* in the crude gastrodermal cell extract from *Zoanthus robustus* were examined using epifluorescence microscopy in the absence of FM 1-43. Inclusion bodies (arrowed) are evident only with extended exposure time. cn = cnidoblast. Scale bar = 20  $\mu$ m.

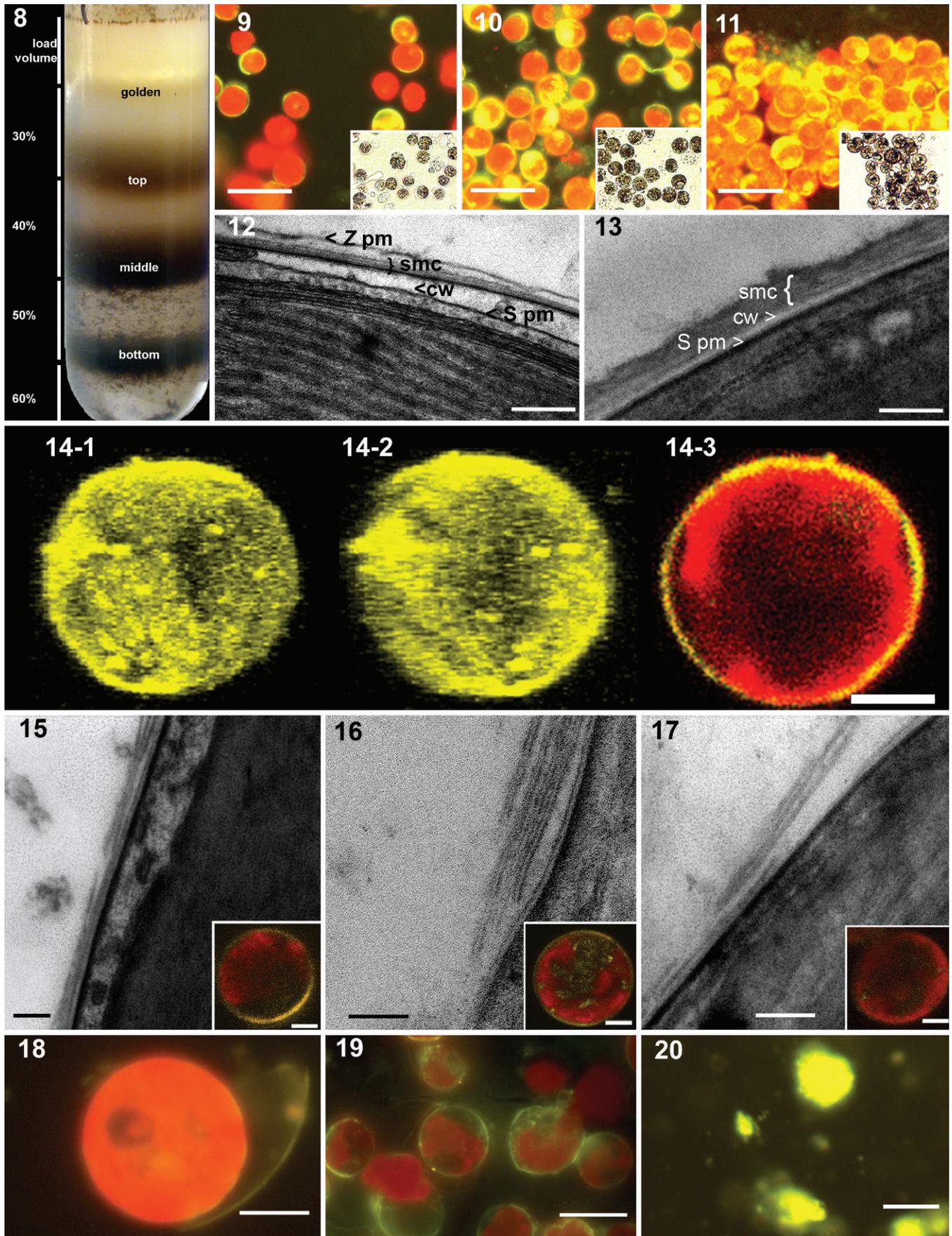
**Fig. 5.** Cultured *Symbiodinium* stained with high concentration of FM 1-43 (50  $\mu$ M). The degree of staining to the exterior of the alga is much reduced in comparison with their symbiotic counterparts, with the exception of small punctate regions (arrowed). Scale bar = 20  $\mu$ m.

**Fig. 6.** Isolated *Zoanthus robustus* gastrodermal cells examined under TEM: thy = thylakoid membranes, s = starch grain, gc = gastrodermal cell cytoplasm, n = nucleus, pm = host cell plasma membrane. Scale bar 1  $\mu$ m.

**Fig. 7.** Gastrodermal cell containing three endosymbionts stained with 6CFDA examined under epifluorescent microscopy (insert: bright-field of same field of view). Scale bars = 10  $\mu$ m.

tained mesogleal fragments, cnidoblasts, and some intact gastrodermal cells that were still motile. Such contamination was negligible in the top and middle layers. The results of experiments using DAPI staining and ester loading of

6CFDA to determine the extent of symbiosome isolation indicated that the majority (> 90%) of symbiosomes in top and middle layers had been stripped of host cell material (data not shown).



The difference in density of symbiosomes was coupled with visible differences in the extent of FM 1-43 fluorescence (Figs 9–11). The least dense symbiosomes (top layer, 30–40% sucrose interface) showed visibly less fluorescence (Fig. 9), having apparently thinner or even absent symbiosome membrane complexes. The denser symbiosomes (middle 40–50% and bottom 50–60% layers, Figs 10 and 11, respectively) invariably had FM 1-43-labelled symbiosome membranes, and these were, on the whole, visibly thicker. Although a quantitative image analysis of the fluorescence intensity was not possible because of fluorescent flaring, differences in density are nonetheless visibly coupled with differences in thickness of FM 1-43-labelled symbiosome membranes.

The periphery of a host gastrodermal cell containing an algal symbiont (Fig. 12) and the periphery of an isolated symbiosome (Fig. 13) were revealed by TEM. *In hospite* (Fig. 12), the outer symbiosome membrane encloses a multilayered symbiosome membrane with four visible layers, within which is situated the cell wall of the symbiont and the symbiont plasma membrane. Interior to this, in the symbiont cytoplasm, are faint vesicular structures that may represent the remains of thecal vesicles described by Wakefield *et al.* (2000). In contrast, the transmission electron micrograph of the isolated symbiosome (Fig. 13) shows the multilayered symbiosome membrane structure, possibly including the outer host membrane, as well as the cell wall and symbiont plasma membrane. The symbiosome membrane complex appears intact, whereas the host cytoplasm and cell membranes have been removed.

Fig. 14-1 and -2 shows confocal fluorescence micrographs of the FM 1-43-labelled surface of isolated symbiosomes. Three-dimensional reconstructions of the

symbiosome surface show that the symbiosome membrane complex is structurally heterogeneous, with variable thickness, numerous granular or swollen regions, and visible striations or furrows. The optical section through the centre of the symbiosome further demonstrates the heterogeneity of the membrane, with thinner and thicker regions, and a projected fluorescing membranous granule (Fig. 14-3).

### Isolating the symbiosome membrane complex

(1) DETERGENT EXTRACTION OF SYMBIOSOME MEMBRANE PROTEINS: Triton X-100 was used to solubilise the symbiosome membrane complex. Detergent-treated symbiosomes were examined under TEM (Figs 15–17) and with confocal microscopy (inserts Figs 15–17) to assess membrane solubilisation. The confocal images show both the autofluorescence from Chl *a* and the fluorescence of FM 1-43-labelled symbiosome membranes, indicating the extent of solubilisation over time. TEM images show that, after treating with 0.3% Triton for 1 min, the symbiosome interface was largely intact (Fig. 15), confirmed by FM 1-43 labelling (yellow fluorescent halo) symbiosome membranes (insert Fig. 15). After 15 min of Triton solubilisation the symbiosome membrane had been disrupted (Fig. 16), and at 30 min the symbiosome interface had become detached to some degree (Fig. 17). The electron-dense plasma membrane of the zooxanthella within the symbiosome complex membrane remained intact at both 15 min and 30 min (Figs 16 and 17, respectively), but, interestingly, the symbiosome membrane complex had clearly dissolved, although in a nonuniform way, with gaps appearing in the multilayered structure, and single or multiple leaflets separating away from those beneath. Different layers of the

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**Fig. 8.** Symbiosomes from *Zoanthus robustus* were purified using a discontinuous sucrose density gradient (30–60%). All bands were examined under bright-field and and epifluorescence (Figs 10–12).

**Fig. 9.** Symbiosomes isolated from the top (< 40%) band of the discontinuous sucrose density gradient were stained with FM 1-43 (30 μM) and examined under epifluorescence microscopy (insert: bright-field micrograph of the same field of view). Scale bar = 20 μm.

**Fig. 10.** Symbiosomes isolated from the middle (< 50%) band of the discontinuous sucrose density gradient were stained with FM 1-43 (30 μM) and examined under epifluorescence microscopy (insert: bright-field micrograph of the same field of view). Scale bar = 20 μm.

**Fig. 11.** Symbiosomes isolated from the bottom (< 60%) band of the discontinuous sucrose density gradient were stained with FM 1-43 (30 μM) and examined under epifluorescence microscopy (insert: bright-field micrograph of the same field of view). Scale bar = 20 μm.

**Fig. 12.** Verification of *Zoanthus robustus* symbiosome isolation protocol. TEM of periphery of isolated gastrodermal cell. Z pm = zoanthid gastrodermal plasma membrane, smc = symbiosome multilayered membrane complex, cw = algal cell wall, S pm = *Symbiodinium* plasma membrane. Scale bar = 0.2 μm.

**Fig. 13.** TEM of periphery of a symbiosome freed from the host cell plasma membrane using gentle mechanical disruption and recovered from the middle band of the 30–60% sucrose density gradient. Z pm = zoanthid gastrodermal plasma membrane, smc = symbiosome multilayered membrane complex, cw = algal cell wall, S pm = *Symbiodinium* plasma membrane. Scale bar = 0.2 μm.

**Fig. 14.** Confocal microscopy of symbiosome from the middle band labelled with 30 μM FM 1-43; 14-1 and 14-2 are composites of several images and demonstrate a degree of heterogeneity of the symbiosome membrane; note particularly the striations. 14-3 is a slice through the symbiosome confirming the specificity of this stain for the exterior components of the symbiosome membrane complex. Scale bar = 2 μm.

**Fig. 15.** Isolation of the symbiosome membrane complex using detergent solubilisation. TEM of Triton X solubilisation of *Zoanthus robustus* symbiosome membrane complex treated with detergent for 1 min. Scale bar = 10 nm. (Insert: confocal image of FM1-43-stained symbiosomes treated in the same way. Scale bar = 2 μm).

**Fig. 16.** Isolation of the symbiosome membrane complex using detergent solubilisation. TEM of Triton X solubilisation of *Zoanthus robustus* symbiosome membrane complex treated with detergent for 15 min. Scale bar = 10 nm. (Insert: confocal image of FM 1-43-stained symbiosomes treated in the same way. Scale bar = 2 μm).

**Fig. 17.** Isolation of the symbiosome membrane complex using detergent solubilisation. TEM of Triton X solubilisation of *Zoanthus robustus* symbiosome membrane complex treated with detergent for 30 min. Scale bar = 10 nm. (Insert: confocal image of FM 1-43-stained symbiosomes treated in the same way. Scale bar = 2 μm).

**Fig. 18.** FM 1-43-stained symbiosome that had been osmotically shocked examined under high-power epifluorescent microscopy. Scale bar = 5 μm.

**Fig. 19.** FM 1-43-stained symbiosomes that have been osmotically shocked examined under epifluorescent microscopy. Scale bar = 10 μm.

**Fig. 20.** FM 1-43-stained symbiosome membrane complex fragments isolated using the optimised method and examined under epifluorescent microscopy. Scale bar = 10 μm.

multilayered structure appear to have dissolved as separate “skins” and the supernatant, which we recovered, contained a mixture of solubilised layers that were originally either closer to or further from the surface of the symbiont. After both the 45-min and 60-min detergent treatments, the yellow fluorescence due to the FM 1-43 intercalating with the symbiosome membrane complex (i.e. the yellow halo) was no longer detected, the membrane complex having been completely solubilised (inserts Figs 16 and 17, respectively).

(2) OSMOTIC METHODS FOR DETACHING THE SYMBIOSOME MEMBRANE COMPLEX: Combining osmotic shock with vigorous stirring in hypotonic solution at 13.5°C, close to a lipid phase transition temperature (Drost-Hansen 2001), produced a supernatant enriched with membrane fragments and vesicles that labelled with FM 1-43 (data not shown). However, in addition to FM 1-43-positive vesicles, this fraction contained whole flattened symbiosomes and algal fragments; whereas, brown colouration of the pellet indicated that algal pigments (probably peridinin) had been released. This method, although effective in producing FM 1-43-positive membrane material, also disrupted the algal symbionts.

Very little FM 1-43-labelled material was obtained when the procedure was carried out at 4°C. When combined with Triton X-100 concentrations above 0.16% in the hypotonic solution, the method produced a dark brown pellet enriched in brown algal pigments, indicating that the endosymbiont had been ruptured. Similarly, osmotic shock combined with grinding produced a pigment-enriched pellet. This may be an excellent method for obtaining peridinin pigment and associated proteins, if not the symbiosome membrane complexes.

Fluorescence microscopic examination of the symbiosomes, pelleted after osmotic shock and mechanical agitation at 13.5°C treatment, showed that more than half had been completely stripped of their symbiosome membrane complexes, whereas, interestingly, some symbionts were partially stripped (Fig. 18). This again shows the remarkable structural integrity of the membrane complex. In some cases the entire membrane complex had slipped from an algal cell as a unit.

We determined the effect of hypotonic treatment on the FM 1-43-labelled symbiosome membrane complexes. After 10–30 min in hypotonic solution, the symbiosome interior visibly swelled, but the symbiosome membrane complex retained its integrity. After 50-min hypotonic shock (Fig. 19), many of the symbiosomes ruptured, ejecting chloroplasts and cellular material. Remarkably, however, the FM 1-43-labelled symbiosome membrane complex retained its form and structure and did not collapse. Some FM 1-43-labelled interior vesiculation was observed close to the cell wall of ruptured symbiosomes. This may represent internal layers of the symbiosome membranes vesiculating after rupture of symbiosomes and exposure to hypotonic solution. However, FM 1-43 labelled no other membranes, even those of ejected chloroplasts and cell contents, including endoplasmic reticulum.

These results suggest that the FM 1-43-positive membrane material can exist (1) in the form of whole membrane complexes, (2) in whole large half-moon-shaped fragments that are quite robust and do not reform vesicles and (3) in numerous vesicles of various sizes.

(3) OPTIMISED PROTOCOL FOR ISOLATING MEMBRANES AT THE SYMBIOTIC INTERFACE: The golden layer obtained from the load zone in sucrose density gradients was enriched with vesicles and fragments that were strongly FM 1-43 positive, suggesting that some symbiosome membrane material had been mechanically dislodged during centrifugation. However, this layer also contained contaminants, including vesicles that were not labelled with FM 1-43.

We enriched this material by subjecting the symbiosomes, collected from an initial sucrose gradient, to vigorous mechanical treatment (see “Material and Methods”) and passing them through a second such gradient. The symbiosome membrane fraction was recovered as a golden layer of vesicles suspended in the load zone of the second sucrose gradient. Since the symbiosomes had already been purified in the first gradient, we confidently attribute this material to the disrupted symbiosome membrane complex. The golden layer was replete with small vesicles, which stained strongly with FM 1-43 (Fig. 20). It contained only a rare whole symbiosome or unstained vesicle; whole animal cells were not detected.

## DISCUSSION

We report here the first well-supported isolation of intact symbiosomes. We have built upon the work of Tytler and Spencer Davies (1983) that used density gradient centrifugation to isolate viable zooxanthellae from *Anemonia sulcata* and may have actually isolated symbiosomes. Research on *Symbiodinium* has historically been focused on corals and anemones, which each has drawbacks as symbiosome model systems. Corals have a complex anatomy of thin tissue overlying a calcium carbonate skeleton (Veron 2000) that makes isolation of the gastrodermal cells extremely difficult. Anemones have the advantage that aposymbiotic varieties can be generated; however, their small size, in general, has deterred attempts to confirm that symbiosomes had indeed been isolated from the symbiotic varieties.

Zoanthids were chosen as a model organism, since they are abundant in many subtropical to temperate coastal regions of the world, and a large number of gastrodermal cells can be obtained by a simple procedure of sucking gently in the enteron cavity. These cells can be gently broken to release the intact symbiosomes when suitable precautions are taken to maintain *in vivo* osmolality and to avoid coagulation. We have previously found that certain ions, e.g. calcium, promote coagulation, and others, such as phosphate, do not. We have not investigated this phenomenon in detail; however, it seems to be related to coagulants on the symbiosome membrane complex. We were not able to use Percoll gradients to purify the symbiosomes, as is the standard technique for chloroplast isolation (Keegstra & Yousif 1986) because cell homogenates containing symbiosomes coagulated totally in media containing Percoll. We attempted to overcome this problem using a variety of surface charge reagents: EDTA, quarternary nitrogen compounds such as glycyl betain, and hydrophilic substances such as polyethylene glycol. None of these was

successful, so we adopted our standard medium of iso-osmotic solutions of sucrose made up with seawater plus PBS to physiological pH.

Like the work of Trautman *et al.* (2002), we used a fluorescent marker. The stain FM 1-43 was used here; this amphiphilic member of the styryl family intercalates into the outer, but not the inner, leaflet of the plasma membrane. When the orientation of the plasma membrane is reversed and the dye is orientated to the inner leaflet, it markedly increases its fluorescence (reviewed by Bolte *et al.* 2004). Thus it has been useful previously for tracking endocytosed membranes and other internalised membranes such as those of the symbiosome membrane complex. The brilliant staining we present here is a feature specifically of the symbiosome membrane complex associated with the symbiosis.

Importantly, we find that the styryl dye FM 1-43, extensively used for tracing endo- and exocytosis of synaptic vesicles (Rizzoli *et al.* 2003) specifically labels the symbiosome membrane complex, when used at a critical concentration of 30  $\mu\text{M}$  or below (Fig. 2). At this concentration, we confirm that neither the plasma membranes of gastrodermal cells, cnidoblasts, or hyaline mesogleal cells are labelled, nor are endoplasmic reticulum of either the animal or algal partners of the symbiosis. In cultured free-swimming *Symbiodinium*, FM 1-43 labelling is only faint at 30  $\mu\text{M}$  and, when the concentration was increased, was restricted to punctate extracellular regions, granules, which bear a strong resemblance to exocytotic vesicles of FM 1-43-labelled rat pituitary lactotrophs (Angleton *et al.* 1999). Autofluorescence of the aggregation bodies within symbionts is of a similar visible wavelength (yellow) to FM 1-43 under blue light excitation and would mask FM 1-43 staining of these aggregation bodies if vesicle trafficking was occurring.

Since FM 1-43 is impermeant, intercalating with the outer plasma membrane leaflet, how does it reach the symbiosome membrane complex *in hospite*? The possibility that FM 1-43 reaches the symbiosome because the gastrodermal membrane is damaged (or no longer intact) is untenable, because uptake of 6CFDA, and subsequent 6CF fluorescence in the host cell cytoplasm (Fig. 3), indicates that the extracted gastrodermal cells are viable, with intact plasma membranes. If, however, the symbiosome membrane complex is labelled by endocytosis of plasma membrane material, this process must be extremely rapid, since it appears to occur instantaneously. Rizzoli *et al.* (2003) report that FM 1-43 labelling occurs within milliseconds during extremely rapid endocytotic synaptic vesicle cycling. This rapidity is consistent with our observations. It occurs on a comparable timescale to the brain's neurophysiological events, which take place over milliseconds to hundreds of milliseconds (Hagan *et al.* 2002). Thus, it is likely that FM 1-43 reaches the symbiosome membrane system by vesicle transport so rapidly that to a human observer it appears instantaneous.

Why then does the plasma membrane of gastrodermal cells appear nonfluorescent whilst the symbiosome periphery is so brilliant and vividly apparent? At higher concentrations of FM 1-43 the plasma membrane of the cultured *Symbiodinium*, the gastrodermal and other animal

cells does indeed become fluorescent, as, faintly, does the cytoplasm of the animal cells. The TEM and confocal fluorescence images (Figs 12–14) show the complexity and relative thickness of the symbiosome membrane complex. Like Wakefield *et al.* (2000), we identified distinct components within the symbiosome membrane complex, including the host-derived outer membrane enclosing an inner and possibly symbiont-derived multilamellar layer, with varying numbers of layers. From the freeze-substituted material of Wakefield *et al.* (2000) and from our chemically fixed material we estimate that the multiple sandwiched inner layers are each approximately 3–5 nm apart. Together they form a complex that is at least an order of magnitude thicker than the plasma membrane (~3 nm) of the gastrodermal and other animal cells. We proposed that the fluorescence is due to FM 1-43 associating with the host-derived symbiosome membrane and the membrane layers that have accumulated beneath it. The 10-fold greater thickness of the symbiosome membrane complex, in comparison with the plasma membrane, is one explanation for its brilliant fluorescence. Furthermore, if the hypothesis of Wakefield *et al.* (2000) is accepted, and the multiple membrane layers originate as plasma membrane/thecal vesicles laid down after terminated ecdyses. We may expect these membrane leaflets to take up large amounts of FM 1-43 stain due to their high membrane content.

The confocal microscopy of FM 1-43-stained isolated symbiosomes shows that the symbiosome membrane complex is not homogeneous, consisting of thicker and thinner patches, with some regions barely stained or unstained, suggesting that some symbiosomes may have a pore or opening in the complex, as does the parasite *Toxoplasma gondii* when *in situ* (Martin *et al.* 2007). A thicker aggregation of the multilayered membrane system on one side of the symbiont was also noted by Wakefield *et al.* (2000). Furthermore, we found considerable variation amongst symbiosomes themselves, with some enclosed by a thicker FM 1-43-stained membrane complex than others (Figs 2, 9–11).

On the basis of these results, we suggest that symbiosomes exist as a population in which there are different thicknesses of the symbiosome membrane complex, resulting from different numbers of layers present in the multilamellar system. This is supported by the hypothesis that the multiple lamellae accrete as a result of multiple delayed ecdyses (Wakefield *et al.* 2000). We regard these multiple lamellae as being progressively accreted, akin to layers of an onion, as each attempted ecdysis occurs. At the same time it is probable that the isolation process damages and strips off some lamellae. We suggest that these layers can be stripped off mechanically as separate layers, with the outer layer stripping most easily. Successive interior layers may then strip off, forming vesicles if stripped as single layers, or lamellar fragments if entire coherent fragments of multilayers are removed (Figs 15–18). These vesicles and fragments then produce the rich population of materials that label with FM 1-43 and occupy the load zone of sucrose gradients, being the least dense constituents of the preparation. It is possible that the damaged or stripped cells swell, thereby causing changes in buoyancy. The result would be that the most affected cells would become more

buoyant, i.e. they would occur more in the top sucrose band, rather than the middle or bottom bands of developed gradients. This hypothesis is consistent with the observation that vigorous disruption (see Material and Methods) in the initial isolation of symbiosomes led to a single (top) band on developed sucrose gradients.

We have shown that symbiosome membranes can be harvested to near purity by mechanical treatment of intact symbiosomes from the combined top and middle bands from discontinuous sucrose gradients, following initial gentle breaking of the gastrodermal cells. Symbiosome membranes were tentatively identified at the same position in the first method: in both methods (a single 30–60% discontinuous sucrose density gradient step or two sucrose density gradient steps in series) small vesicles can be seen at the interface between the load zone and the 30% sucrose layer that stain strongly with FM 1-43. The first sucrose gradient material contains membranes that are not associated with the symbiosomes, but the top band of the second sucrose gradient is unequivocally derived in the main from the symbiosomes isolated in the first gradient.

We suggest that the FM 1-43-labelled vesicles that remain in the load zone, the golden band of the second sucrose gradient, are indeed derived almost solely from the disrupted symbiosome membrane complex. There are two reasons: the first experimental, the second theoretical. First, although it is possible that nonsymbiosome membranes could form vesicles presenting outer plasma membrane leaflet structure, the experimental method using two sucrose gradients makes it extremely unlikely that the rich suspension of light giant vesicles is from this source. The raw material is centrifuged, and under microscopic examination this removes many if not all of the gastrodermal cell membranes. This preparation is then passed through a sucrose gradient and separated into three bands of different density, and microscopic examination shows that these consist, almost entirely, of symbiosomes isolated from gastrodermal membranes. These symbiosomes are then subjected to vigorous mechanical disruption and passed through a second sucrose gradient. Since the resultant golden layer of low-density giant vesicles is so densely populated, it is extremely unlikely that the FM-positive vesicles of which it consists have any other major source than the symbiosomes.

Second, on a theoretical basis, it has become clear over the past decade that lipid bilayers of different organelle systems are distinct and many contain structural domains or rafts with specific functions controlled by membrane proteins. In particular, the lipid constitution of plasma membranes, late Golgi, and endosomes places constraints on “flip-flop” or lipid movements between inner and outer leaflets (Pomorski *et al.* 2004). Such flip-flop may be adenosine triphosphate dependent. The leaflets retain their orientation. Since the extremely hydrophobic FM 1-43 associates with the outer plasma membrane leaflet quite selectively (Bolte *et al.* 2004) where it does not fluoresce, and presumably to the inner leaflet of the symbiosome membrane complex where it does fluoresce, we reason that a sample so brilliantly stained with FM 1-43, in circumstances where the presence of other membranes has demonstrably been reduced, is enriched in membrane vesicles originating from the symbiosome mem-

brane complex, which is stripped by the various, mainly mechanical, methods in layers of different thickness (hence the analogy to layers like an onion), the thinnest of which form vesicles, the thickest of which remain as whole fragments.

The multilayers are each about 3–5 nm apart. This critical distance is equivalent to that over which strong repulsive hydration forces operate, forces that are considered a property of the solvent, and enable water to be drawn laterally inward, even against high applied pressures, in multilamellate materials including clays (Watterson 1989, 1991; reviewed in Shepherd 2006). At the same time, electrically neutral phospholipid membranes, attracted to each other by van der Waals forces, will spontaneously form stacked multilayers, and the lamellae find an equilibrium distance apart (of ~3 nm) when attractive and repulsive hydration forces are balanced (Parsegian and Rau 1984). The powerful hydration forces that enable clay to swell and crack concrete, or make it impossible to pull two wet microscope slides apart, explain why it is so difficult to separate the stacked array of symbiosome membranes (i.e. strip them off) by any means except mechanical shearing.

A final question remains: does the multilayered structure of the symbiosome membrane complex serve a particular function, or is it simply the consequence of repeated delayed ecdyses, as suggested by Wakefield *et al.* (2000)? A thick multilamellate membrane system seems an unlikely candidate as the interface across which host and symbiont must communicate. By increasing the distance over which dissolved gases and other materials must diffuse, and creating large unstirred layers, multiple membranes would presumably both slow the rate of transport and increase its energy demand. Alternatively, host and symbiont may communicate via a pore, as is the case with Apicomplexan associations (Martin *et al.* 2007). We found that FM-143-labelled symbiosome membranes *in situ* frequently showed a gap or pore, but further biochemical analyses are needed. Multilamellate membranes may have other functions. Ionic activity can alter the balance between attraction and repulsion between membrane layers, leading to swelling or shrinking, and ionic activity at membrane surfaces is a much-neglected regulatory mechanism for such fundamental and relevant processes as exocytosis and endocytosis (Parsegian and Rau 1984; Petrache *et al.* 2006).

Our work has concentrated on the isolation of symbiosome membranes from intact symbiosomes. In future, such symbiosome preparations will undoubtedly prove useful in physiological investigations of the movement of several biologically important substances across the symbiosome interface, as has occurred after the isolation of symbiosomes from root nodules (e.g. Roberts and Tyerman 2002; Krusell *et al.* 2005).

We found that the isolated symbiosomes could not be readily broken by osmotic shock techniques, under a variety of conditions. However, a symbiosome membrane fraction could be obtained by subjecting the osmotically shocked symbiosomes to mild treatment with Triton X-100 or by grinding the osmotically shocked cells with glass beads. The fact that detergent treatment or grinding was necessary indicates that in some places the symbiosome membrane is bound to the outer wall components of a

zooxanthella cell, and this is an intriguing possibility. We propose that the symbiosomes from the top band of the developed sucrose gradient are largely formed of zooxanthellae, where the symbiosome membrane complex is not intact, although it may appear so. Additionally, some of these symbiosomes may be at an early developmental stage. From TEM sections we have observed that the symbiosome membrane remains attached to the outer wall of zooxanthellae at more or less regular intervals. This suggests that there are strong physical attachments between the symbiosome membrane and the zooxanthellar wall, and that these occur at regular intervals. Little is known about such bonding. Lin *et al.* (2000) identified the participation of glycoproteins on the cell wall of zooxanthellae, which they claimed were necessary for the establishment of a symbiotic relationship in the sea anemone *Aiptasia pulchella*. Glycoproteins are a well-known component of the cell recognition apparatus. Furthermore, when freshly isolated zooxanthellae were treated with trypsin,  $\alpha$ -amylase, *N*-glycosidase F or *O*-glycosidase, reinfection of aposymbiotic anemones was inhibited. The most abundant glycoproteins had a molecular mass of 64 kDa, and the most abundant terminal sugar was mannose. In addition, Schwartz and Weis (2003) identified a Sym32 protein (32 kDa), which occurred on the symbiosome membrane of the anemone *Anthopleura elegantissima* under symbiotic conditions. These proteins belong to a class of cell adhesion proteins called fasciclin domain proteins, which function as adhesive domains in cell–cell and cell–extracellular matrix interactions. Characterising symbiosome membrane proteins using a range of tools such as proteomics and biochemistry will allow the physiological functions of the symbiosome membrane to be elucidated. By being able to generate preparations that are enriched for the symbiosome membrane we have taken a critically important step; we are now poised to characterise the protein profile, which will improve our understanding of the molecular basis of how marine algal symbioses work.

The symbiosome membrane complex is extremely robust, as demonstrated by the rather drastic methods we have needed to use to disrupt and isolate it. The parasitophorous vacuole of *T. gondii* is similarly robust, and little is known of the proteins responsible for trafficking and signalling there because of inherent difficulties in purifying this particular interface (Martin *et al.* 2007). Not only is the parasitophorous vacuole intimately associated with host cell organelles, but both host and parasite may contribute proteins to it through secretory activities (Martin *et al.* 2007). It is likely that both parasitophorous vacuole and the symbiosome membrane complex constitute structures that are greater than the sum of the parts contributed by either host or infecting organism.

The use of the dye FM 1-43, in combination with fluorescence or confocal microscopy, has been crucial to the success of this project. We chose FM 1-43 on the basis of the use of this dye to visualize, by fluorescence microscopy, vesicle membranes (reviewed by Bolte *et al.* 2004). Previously the dye MDY64 was used to visualise symbiosomes in *Z. robustus* (Trautman *et al.* 2002), but these symbiosomes were not purified to the degree described here, nor was the symbiosome membrane complex isolated.

As shown in the results, FM 1-43 was specific for the symbiosome membrane and did not resolve other host cell vesicles, as judged from an exhaustive search of cellular homogenates. The reason for this specificity is not readily apparent from the present work, although it may have something to do with the specific binding of the host-derived symbiosome membrane with membranous components on the zooxanthellar wall. The use of FM 1-43 makes the isolation of symbiosomes a feasible operation and will be of use in future for the isolation of symbiosomes from scleractinian corals and anemones.

The use of TEM in the present work allowed us to show that we can observe similar details and make similar conclusions for zooxanthellae and symbiosomes of *Z. robustus* as have been made for other cnidarian symbioses (e.g. Wakefield *et al.* 2000; Wakefield and Kempf 2001). Our TEM results say nothing about the origin of the symbiosome membrane. Previous work indicated that it was host-derived (Wakefield and Kempf 2001) and proteomic analysis in the future will undoubtedly provide much clearer evidence as to its origin. We hypothesise that there are adhesion sites between the host-derived and algal-derived components of the symbiosome membrane complex. When we applied mild detergent treatment, we observed portions of the membrane complex remaining attached to the multilayer structure at putative adhesion sites, which explains, in part, the difficulties we have had in separating the symbiosome membrane complex from the symbiont cells.

Because the symbiosome membrane and the multilayer structure are intimately associated and are physically interconnected at regular intervals, it seems preferable to regard both these structures as a functional unit. For this reason we prefer to use the term symbiosome membrane complex, which is defined as the host-derived symbiosome membrane plus the zooxanthellar-derived multilayer process.

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