

Cell organization and ultrastructure of a magnetotactic multicellular organism

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Abstract

Magnetotactic multicellular aggregates and many-celled magnetotactic prokaryotes have been described as spherical organisms composed of several Gram-negative bacteria capable to align themselves along magnetic fields and swim as a unit. Here we describe a similar organism collected in a large hypersaline lagoon in Brazil. Ultra-thin sections and freeze fracture replicas showed that the cells are arranged side by side and face both the external environment and an internal acellular compartment in the centre of the organism. This compartment contains a belt of filaments linking the cells, and numerous membrane vesicles. The shape of the cells approaches a pyramid, with the apex pointing to the internal compartment, and the basis facing the external environment. The contact region of two cells is flat and represents the pyramid faces, while the contacts of three or more cells contain cell projections and represent the edges. Freeze-fracture replicas showed a high concentration of intramembrane particles on the edges and also in the region of the outer membrane that faces the external environment. Dark field optical microscopy showed that the whole organism performs a coordinated movement with either straight or helicoidal trajectories. We conclude that the organisms described in this work are, in fact, highly organized multicellular organisms.

Keywords: magnetotactic bacteria, multicellularity, uncultured bacteria, freeze-fracture, magnetotactic multicellular aggregate, many-celled magnetotactic prokaryote

1. Introduction

In 1983, Farina *et al.* described magnetotactic multicellular aggregates (MMAs) as spherical organisms composed of several Gram-negative bacteria capable of aligning themselves along magnetic fields and swim as a unit. A many-celled magnetotactic prokaryote (MMP) similar in morphology and behaviour was later described and supposed to be a multicellular prokaryote (Rodgers *et al.*, 1990). As the unicellular magnetotactic bacteria, MMAs and MMPs have magnetic crystals inside the cytoplasm, which are responsible for aligning the whole organism along the magnetic fields (Farina *et al.*, 1983; Farina *et al.*, 1990; Rodgers *et al.*, 1990). In most magnetotactic bacteria, these crystals are composed of magnetite (Fe_3O_4) (Bazylinski and Moskowicz, 1997), whereas in MMAs (Farina *et al.*, 1990; Lins de Barros *et al.*, 1991) and MMPs (Mann *et al.*, 1990) iron sulphide crystals have been found. The iron sulphides of MMPs were found to be of three types: greigite (cubic Fe_3S_4), which is weakly magnetic, and the non-magnetic iron sulphides mackinawite (tetragonal FeS) and cubic FeS (Pósfai *et al.*, 1998). Phylogenetic analysis on the 16S rDNA sequences showed that MMPs belong to a large cluster of sulphate-reducing bacteria in the δ subdivision of the Proteobacteria (DeLong *et al.*, 1993).

The outermost layer of MMAs is a capsule disposed radially (Farina *et al.*, 1983). Rodgers *et al.* (1990) showed that MMPs present many flagella only in part of the cell, presumably at the part of the membrane that faces the exterior environment. They observed regions of constant distance between the outer membranes of MMP cells and proposed that this structure would be involved in cell-to-cell communication to coordinate swimming.

When the osmolarity of the environment is lowered by adding distilled water, MMAs and MMPs break up into many ovoid parts that remain immotile (Farina *et al.*, 1990; Lins de Barros *et al.*, 1990; Rodgers *et al.*, 1990; Lins de Barros *et al.*, 1991). Living organisms similar to MMA cells were never observed (Farina *et al.*, 1990). Lins and Farina (1999) and Lins *et al.* (2000) showed bridges between the cells of partially disrupted MMAs by scanning and transmission electron microscopy, respectively. Lins and Farina (1999) showed that the size of the organism is proportional to the size of the individual cells, and proposed that MMAs would grow by increasing the size of the individual cells and not by increasing the cell number, implying that MMAs would have a fixed number of cells. MMAs and MMPs have not been cultured until now, and so all studies were made using uncultured organisms recovered directly from environmental samples (Farina *et al.*, 1983; Farina *et al.*, 1990; Mann *et al.*, 1990; Rodgers *et al.*, 1990; DeLong *et al.*, 1993; Pósfai *et al.*, 1998; Lins and Farina, 1999; Lins *et al.*, 2000; Lins and Farina, 2001).

Although some studies have been published on MMA (Farina *et al.*, 1983; Lins and Farina, 1999; Lins *et al.*, 2000) and MMP (Rodgers *et al.*, 1990) ultrastructure, the tri-dimensional

arrangement of cells in these organisms remained largely unknown. In this work, we describe the organization of cells in a magnetotactic multicellular organism composed of Gram-negative bacteria, similar to MMAs and MMPs in behaviour, morphology and elemental composition of the magnetic crystals. This organism also performs the complex coordinated movement as described for MMAs and MMPs. We show that their cells are organized side by side around an acellular internal compartment and face both this compartment and the external environment. These new findings led us to conclude that this organism is, in fact, a prokaryotic multicellular organism.

2. Materials and Methods

2.1. Sampling and magnetic separation

Samples of water and sediment were collected in Araruama Lagoon, a large hypersaline coastal lagoon in Rio de Janeiro State, Brazil (22°50'21" S 42°13'44" W), and maintained in the lab for up to three weeks at room temperature. The sediments of this lagoon were dark and presented a strong H₂S smell. The water salinity was around 74.55 ‰, and the pH around 7.3.

The samples used in all preparations were magnetically separated as described by Lins *et al.* (in press). Briefly, water and sediment were transferred to a glass flask containing one large opening on the top through which we put the samples, and a lateral capillary end through which water containing large amounts of magnetotactic multicellular organisms were collected after exposition to a magnetic field generated by a coil in the appropriate polarity.

2.2. Optical microscopy

Drops of water containing magnetically separated organisms were put onto coverslips. Excavated slides were used to allow the drop to be suspended under the coverslip. A commercial magnet was used to direct the magnetotactic organisms to the border of the drop. Micrographs were taken at a Zeiss Axioplan in the Nomarski interference contrast mode. Trajectories of magnetotactic multicellular organisms were photographed from small drops of water between a common slide and a coverslip using the dark field mode for 4 seconds.

2.3. Scanning electron microscopy

The samples were put onto coverslips and fixed in 2.5% glutaraldehyde in cacodylate buffer 0.1M pH 7.3 prepared with lagoon water, overnight. After rinsed in the buffer, they were post-fixed in OsO₄ 1% in the buffer for 1h, rinsed again and dehydrated in acetone series. They were critical-point dried and sputtered with chromium (high resolution ion beam coater Gatan – Model 681). Observation was done in a field-emission scanning electron microscope (Jeol FESEM 6340F) operating at 5.0 kV.

2.4. Transmission electron microscopy and elemental analysis

Samples for conventional transmission electron microscopy were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.1M pH 7.3 prepared in lagoon water, overnight, rinsed in the buffer, post-fixed in OsO₄ 1% for 20 min, rinsed, dehydrated in acetone series and embedded in Polybed 812. To evidence polysaccharides, we made the same procedure, except that we used ruthenium red at 0.2% in all solutions used, prepared the buffer in distilled water and increased the

post-fixation time to 1h. Ultra-thin sections were obtained in an ultramicrotome and stained with uranyl acetate and lead citrate.

For observation of whole bacteria and elemental analysis, magnetically-isolated samples were placed onto Formvar-covered electron microscopy grids, gently rinsed with distilled water and dried in air. They were observed and analysed in a Jeol 1200 EX transmission electron microscope equipped with a Noran accessory for energy-dispersive X-ray analysis (EDXA). Analyses were done at 120kV for 400s (livetime) using a spot size of about 80nm in diameter.

2.6. Freeze-fracture

Magnetically isolated samples were fixed in either 1.25% or 2.5% glutaraldehyde in sodium cacodylate buffer 0.1mM pH 7.3 in lagoon water. After 2h, they were rinsed in lagoon water. The lagoon water was gradually substituted by distilled water. The samples were slowly infiltrated with 30% glycerol for cryoprotection, mounted in gold stubs and frozen in liquid Freon 22. Subsequently they were transferred to a Balzers freeze-fracture device, where they were fractured and sputter-coated with platinum at 45° and with carbon at 90°. After washing in either 70% sodium hypochlorite or 70% sulphuric acid, the replicas were washed in water and mounted onto nickel electron microscopy grids. They were observed in a Jeol 1200 EX transmission electron microscope.

3. Results

Most samples presented magnetotactic multicellular organisms as the only magnetotactic microorganisms (Figure 1a); a few samples presented also unicellular spirilla. Living unicellular magnetotactic bacteria similar to the cells of the multicellular organisms were never observed. The magnetotactic multicellular organisms from Araruama Lagoon present the same complex movements observed in MMAs (Lins de Barros *et al.*, 1991; Lins and Farina, 1999) and MMPs (Rodgers *et al.*, 1990). While swimming freely in the water under an applied magnetic field, the magnetotactic multicellular organisms from Araruama Lagoon move toward the south magnetic pole with either a straight or a helical trajectory aligned with the magnetic field lines (Figure 1b). When they reach the edge of the drop, they stay rotating around themselves. In another kind of movement, the microorganisms swim backward aligned with the magnetic field, returning to the edge in the free-swimming movement.

Transmission electron microscopy and energy-dispersive X-ray analysis showed that cells of magnetotactic multicellular organisms contain pleomorphic magnetic crystals composed of iron sulphides (fig. 2), very similar to those described in MMAs (Farina *et al.*, 1990; Lins de Barros *et al.*, 1991; Lins and Farina, 2001) and MMPs (Mann *et al.*, 1990; Pósfai *et al.*, 1998).

These organisms have the same general appearance of MMAs and MMPs when observed in optical, scanning or transmission electron microscopy (figs. 1, 3-4). They are formed by many Gram-negative bacterial cells containing large lipid and/or polyhydroxyalkanoate inclusions, their magnetosomes are found mainly near the periphery of the organisms, and they are covered externally by a capsule composed of radially-arranged filaments (fig. 4), similarly to those described by Farina *et al.* (1983). Samples processed with ruthenium red showed a highly stained capsule, suggesting that polysaccharides are its main component. Additionally, we observed a thin covering layer over the radial component, perpendicular to the radial fibres, observed only in ruthenium red stained samples, which is easily pulled apart during preparation (fig. 4). The whole capsule is 94 ± 18 nm thick in ruthenium red stained ultra-thin sections.

Cells in the magnetotactic multicellular organisms were arranged radially in a way that all cells face both the external environment and an acellular space present in the organism center, which we called internal compartment (Figs. 4 and 5). Ruthenium red stained ultra-thin sections showed a belt of filaments between the apices of the cells, in the periphery of the internal compartment (Fig. 4). Many membrane vesicles are present in this compartment, most of them near the cells (Fig. 6a). Occasionally, we observed small membrane vesicles at the exterior of the organism, mainly near the contact region of two adjacent cells (Fig. 6b).

In ultra-thin sections and freeze fracture replicas, we observed that both the cell and outer membranes of two adjacent cells were very close to each other. The peptidoglycan layer was very thin, being hardly seen. Ultra-thin sections showed a constant distance between the outer membranes of neighbor cells (Fig. 7). As the lateral membranes of adjacent cells are juxtaposed, the interface between two cells is flat, as shown in figures 5 and 8. Thus, the final shape of one cell results from the arrangement of its neighbors. Because the cells are arranged radially in a sphere, most of them have pyramidal shapes (fig. 8). Indeed, the lateral walls of the cells were trapezium-shaped and presented edges or projections of cytoplasm between two side walls (figs. 5 and 8). These edges were at the contact regions between three or more cells in both ultra-thin sections (not shown) and transversally fractured cells (fig. 8).

We observed that these projections were straight and oriented radially in relation to the whole organism (Fig. 5). The projections measured $1.3 \pm 0.3 \mu\text{m}$ in length ($n = 15$). For comparison, the cells measured $2.4 \pm 0.3 \mu\text{m}$ in length ($n=25$) in organisms cut or fractured in a plane containing the internal compartment. In the P-face of the outer membrane (POM), the intramembrane particles were concentrated on the projections, mainly toward the basal region of the cells (the base of the cell corresponds to the pyramid base and is the region that is in contact with the external environment). These particles formed rows until they reach the external end of the projection, where they are continuous with a region containing a high concentration of intramembrane particles (Fig. 9a). Thus, the POM is divided into two regions: an intramembrane particles rich region, which corresponds to the region of the membrane that is in contact with the external environment, and an intramembrane particles poor region, which is the region that is in contact with another cell or with the internal compartment. The E-face of the outer membrane (EOM) showed a similar pattern, but in this case we had grooves instead of projections and a gradient of intramembrane particles concentration growing toward the internal compartment (Fig. 9b). Because of their location, we suggest that the intramembrane particles of the basal region of the cells are mainly proteins involved in metabolic exchange, since they are placed in the part of the membrane that have direct contact with the environment.

In some E-face outer membrane fracture surfaces, we observed the outline of the underlying cell (fig. 10). This outline is clearer in the basal region than near the apex of the cell and further confirms the juxtaposition of the cells of magnetotactic multicellular organisms. Figure 10 also shows clearly the trapezium-shaped side walls resulting from the fitting of the cells to each other. At the larger base of the trapezium, the membranes bend (fig. 10b), further confirming that this is the region where the membrane stops the contact with another cell and faces the external environment.

4. Discussion

The cells of magnetotactic multicellular organisms are very close to each other, and the outer membranes of two adjacent cells present extensive regions of constant distance indicative of a specialized structure joining the two cells, as described previously (Rodgers *et al.*, 1990). Besides, both the peptidoglycan layer and the cell membrane were closely apposed to the outer membrane. Differently of the organisms described here, which present juxtaposed cells, the aggregates formed by other bacteria and archaea do not present this kind of structure, having an organic matrix between the cells (e. g. Holt and Canale-Parola, 1967; Robinson, 1986). However, no structures that could be responsible for maintaining the constant distance between the outer membranes of juxtaposed cells have been found in the outer membrane leaflets of freeze-fracture replicas.

No structures similar to plasmodesmata (Emons *et al.*, 1992), microplasmodesmata (Giddings Jr. and Staehelin, 1981) or gap junctions (Dunia *et al.*, 1998), which could be involved in the cell-to-cell communication necessary to co-ordinate swimming, were observed in outer membrane surfaces of magnetotactic multicellular organisms. Instead, cell communication could be performed through the internal compartment, since this is a closed environment faced by all cells. Small concentrations of soluble molecules would rapidly reach all cells of the organism in this closed space. In fact, cell-to-cell communication in bacteria is done mainly through soluble molecules (Kaiser and Losick, 1993; Gray, 1997). Membrane vesicles found in the internal compartment could also transmit chemical signals from one cell to the others. According to Beveridge (1999), membrane vesicles are blebs of the outer membrane containing periplasmic components. Being made of the same substance, they can adhere to the outer membrane of Gram-negative bacteria and fuse with it, releasing the luminal components into the periplasmic space of the receiver cell.

The organization of cells in the magnetotactic multicellular organisms, as shown in this work, is unique among the prokaryotes. All their cells face both the internal compartment and the external environment, forming a hollow sphere, similar to *Eudorina* species (which are eukaryotic algae). Besides, flagella are found only in the part of the cell that faces the external environment in both organisms (*Eudorina* and the organism described here). In addition, certain aspects of their cellular organization are analogous to that of epithelia: the cells are arranged side by side delimitating the internal compartment and are polarized as most epithelia.

The shape of one cell results from the arrangement of their neighbor cells. To fit in perfectly, the cells are pyramid-shaped, having trapezium-shaped membrane surfaces at the contact between two cells (the pyramid faces), and membrane projections at the contact between three or more cells (the pyramid edges). The pyramid base corresponds to the part of the cell that faces the external

environment. In the larger base of the trapezium-shaped lateral membrane, there is a bending angle, confirming that in this region the cell ends the contact with another cell and faces the external environment.

Because the concentration of dissolved solutes inside bacterial cells is higher than outside, there is a considerable turgor pressure that would cause cell rupture unless the peptidoglycan layer was present. Both the turgor pressure and the shape of the peptidoglycan layer give the shape of a bacterial cell (Koch, 1990). However, this can not explain the shape of the cells of magnetotactic multicellular organisms. These cells have a very thin peptidoglycan layer, which would not be as rigid as in most bacteria and would permit the deformation of the cells. The binding with the neighbour cells would be the main factor driving the deformation and fitting of the cells in the organism. The rows of particles observed on the cell projections are certainly involved in maintaining cells together, as they are placed in the regions where there is a higher pressure tending to separate the cells. Besides, the filament belt observed in the internal compartment could also be involved in maintaining cells together, as it is composed of thin filaments linked to cell apices which probably cross-link all cells in an organism.

Some aspects of three-dimensional cell organization are similar to the organisation of soap bubbles because they obey to the same surface forces (Thompson, 1966; Williams, 1979; Koch, 1990). The tri-dimensional arrangement of bubbles of equal volume happen in such a way that all bubbles face the exterior and the mass of bubbles has no empty spaces if their number is 14 or less. However, with 15 bubbles, one bubble stays inside the mass and the other 14 stay around it (Williams, 1979). In this way, if subjected only to the same kind of forces and pressures, the 15 to 30 cells (Lins de Barros *et al.*, 1991) that compose a magnetotactic multicellular organism would not fill all the space without having internal cells. In fact, the cells do not fill all the space available and the organism have no internal cells, which suggests that specific forces are acting in cell organisation. As the internal compartment fills the organism centre, the general organization obtained for 15 soap bubbles is maintained. Anyway, the similarities with the bubbles are striking, showing that physical forces have their place in shaping this organism: the interface between two cells is flat and the part of the cell that faces the external environment is rounded. Moreover, the shape of the organism approaches a sphere, the minimal surface-to-volume ratio geometry.

Recently, it has grown the discussion on the existence of multicellular prokaryotes because new findings on the molecular mechanisms of intercellular communication between prokaryotic cells challenged the traditional concept of bacteria as being unicellular and independent organisms (e. g. Kaiser, 1986; Shapiro, 1998; Shimkets, 1999; Kaiser, 2001). In fact, there is presently a clear tendency of considering microorganisms like filamentous nitrogen-fixing cyanobacteria and myxobacteria as multicellular organisms (Kaiser, 2001). This tendency generated new concepts of multicellularity, with

less defined boundaries, as exemplified by the proposal that multicellular organisms are those that have many cells in close contact that coordinate growth, movements and biochemical activities (Shapiro, 1998; Kaiser, 2001).

According to more conservative concepts, multicellular organisms present characteristic shape and cell organization and lack both cell autonomy and competition. Colonies are intermediate structures that present a fairly defined distribution of cells, significant intercellular interactions and co-operation, but also some competition. Populations of single-celled organisms are composed of randomly arranged cells interacting fortuitously and competing with themselves. In practice, the classification of individual organisms in this system can be difficult because the transitions between populations, colonies and multicellular organisms are diffuse and several organisms represent intermediate steps. According to this definition, highly organized prokaryotic organisms like filamentous cyanobacteria, actinomycetes and myxobacteria would be colonial, because their cells are organized in a characteristic way but are relatively autonomous (Carlile, 1980).

The results presented here show that the organisms described in this work are in fact multicellular organisms. They fulfil all the requirements proposed by Carlile (1980), Shapiro (1998) and Kaiser (2001) listed above: (i) the organism have a well defined cellular organization including specialized membrane structures involved in the cell-to-cell interactions; (ii) their cells are not autonomous, as free-moving magnetotactic bacteria similar to cells of magnetotactic multicellular organisms were never found in our samples and the cells remain immotile after disaggregation of the organism; (iii) do not seem to compete, as MMAs seem to have a fixed number of cells (Lins and Farina, 1999), which precludes the possibility of independent cell division; and (iv) the cells coordinate the highly complex swimming movements of the whole organism.

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FIGURE LEGENDS

Fig. 1: (a) Nomarski interference contrast light micrograph of magnetotactic multicellular organisms concentrated near the border of a drop of lagoon water. Organisms indicated by arrows show elongated cells surrounding an approximately spherical central region. Bar, 20 μm . **(b)** Two trajectories of magnetotactic multicellular organisms, one straight (small arrow) and the other helicoidal (large arrow), obtained with dark field optical microscopy. Bar, 50 μm .

Fig. 2: (a) Transmission electron micrograph of part of a magnetotactic multicellular organism placed over a Formvar film. Arrowheads point to magnetosomes. Bar, 200 nm. **(b)** Energy-dispersive X-ray analysis of a typical magnetosome.

Fig. 3: Scanning electron micrograph showing the external morphology and the outline of the cells of a magnetotactic multicellular organism. Bar, 1 μm .

Fig. 4: Ultra-thin section of a magnetotactic multicellular organism prepared with ruthenium red (to evidence polysaccharides) showing the general organisation of cells and the internal compartment (asterisk). Observe that all cells have a double membrane characteristic of Gram-negative bacteria (small arrow) and face both the external environment and the internal compartment. The internal compartment is acellular and is surrounded by a belt of filaments (large arrow) linked to the cell ends. Many filaments cover externally the organism forming a capsule (arrowheads) composed of a radial component and a covering transversal component. Lipid or polyhydroxyalkanoate inclusions are marked by stars. Bar, 1 μm .

Fig. 5: Freeze-fracture replica of a magnetotactic multicellular organism showing mainly outer membrane fracture surfaces. The P-face of the cell membrane (PCM) is seen in the apices of some cells. Observe the internal compartment (asterisk), the radial projections (black arrows) in the E-face of the outer membrane (EOM) and the corresponding grooves (white arrows) at the P-face of the outer membrane (POM). The radial projections and grooves delimit trapezoidal lateral membrane surfaces, where two adjacent cells are in close contact. Cytoplasmic fractures (C) are also shown. Arrowheads point to flagella. Dark regions correspond to magnetosomes that remained attached to the replica. Bar, 1 μm .

Fig. 6: Freeze-fracture replicas showing membrane vesicles. The E-face of the outer membrane (EOM), the P-face of the outer membrane (POM) and the cytoplasm (C) are shown. **(a)** High magnification of the internal compartment (asterisk) showing that it contains many membrane vesicles (arrowheads) mainly near the cells. Dark regions are magnetosomes attached to the replica. Bar, 200 nm. **(b)** Membrane vesicles external to the organism (arrowheads), near two adjacent cells. Bar, 100 nm.

Fig. 7: Ultra-thin section showing that the four membrane units of two adjacent cells are very close to each other and that their outer membranes maintain a constant distance over most of the contact interface (arrowheads). Bar, 100 nm.

Fig. 8: Freeze-fracture replica showing a peripheral fracture of a magnetotactic multicellular organism. We interpreted this micrograph as if we were looking from the center of the organism toward its periphery. Note the square outline of the central cell (star) and its pyramidal tri-dimensional shape, caused by the flat side membranes (asterisks) separated by cytoplasmic projections or edges (arrowheads). Bar, 0.5 μm .

Fig. 9: Freeze-fracture replicas showing the straight projections in the P-face of the outer membrane (POM) and corresponding grooves in the E-face of this membrane (EOM). **(a)** Two projections (small arrows) containing high concentration of intramembrane particles in the POM and a groove (large arrow) in the EOM. **(b)** A groove (arrow) in the EOM. Part of the P-face of the cell membrane (PCM) is also shown. **(a, b)** Both outer membrane leaflets have a higher concentration of intramembrane particles in the basal region of the cells (asterisks), whereas a lower concentration of particles in the POM and a particle concentration gradient growing toward the center of the organism in the EOM (stars). This difference in the intramembrane particles concentration divides the outer membrane into two regions (arrowheads point to the division region). In **(b)**, this division is continuous in the outer membranes of two adjacent cells (arrowheads). Bar, 0.5 μm .

Fig. 10: Freeze-fracture replicas showing the E-face of the outer membrane (EOM), where the outline of the underlying cells are seen (arrowheads). The P-face of the outer membrane (POM) of the underlying cell is also seen. The internal compartment is marked by asterisks. **(a)** Basal region of the outline. **(b)** Apical region of the outline. At the end of the contact with the underlying cell, the outer membrane has an angle (arrows), which further confirms that in this region the membrane of one cell stops the contact with the membrane of an adjacent cell and faces the external environment. These micrographs confirm that the side walls of the cells are trapezium-shaped. Bar, 0.5 μm .

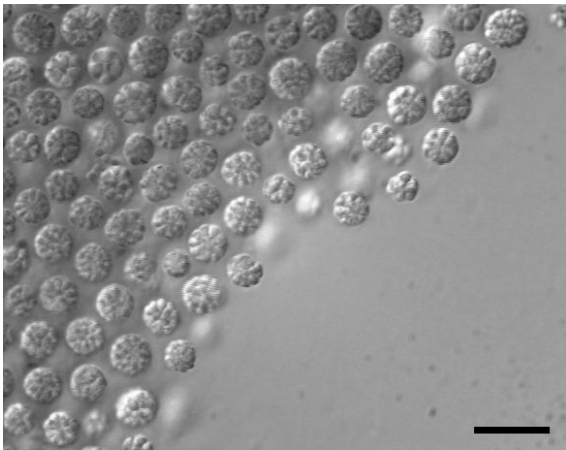


Figure 1a

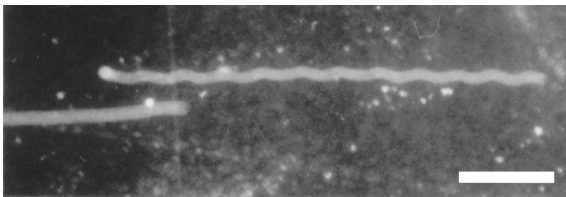


Figure 1b

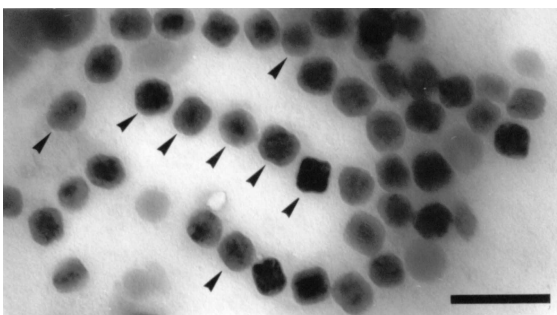


Figure 2a

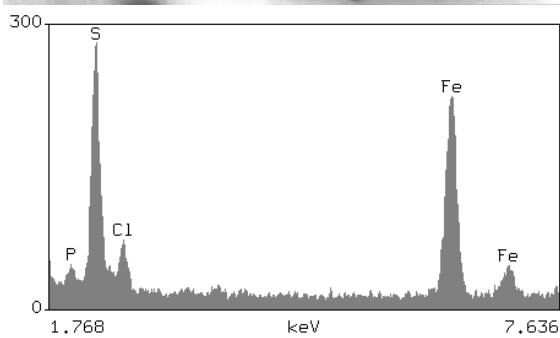


Figure 2a

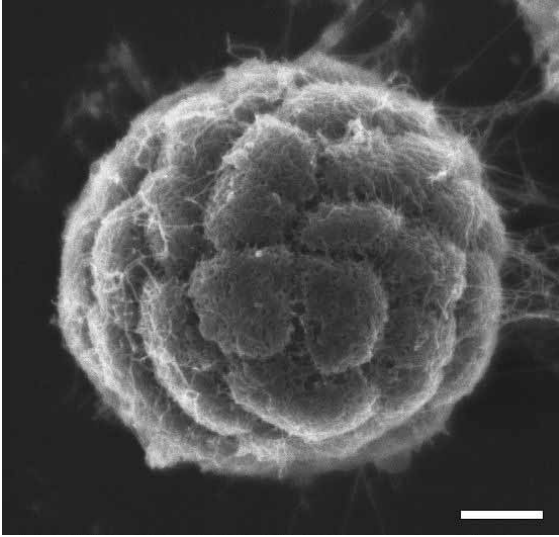


Figure 3

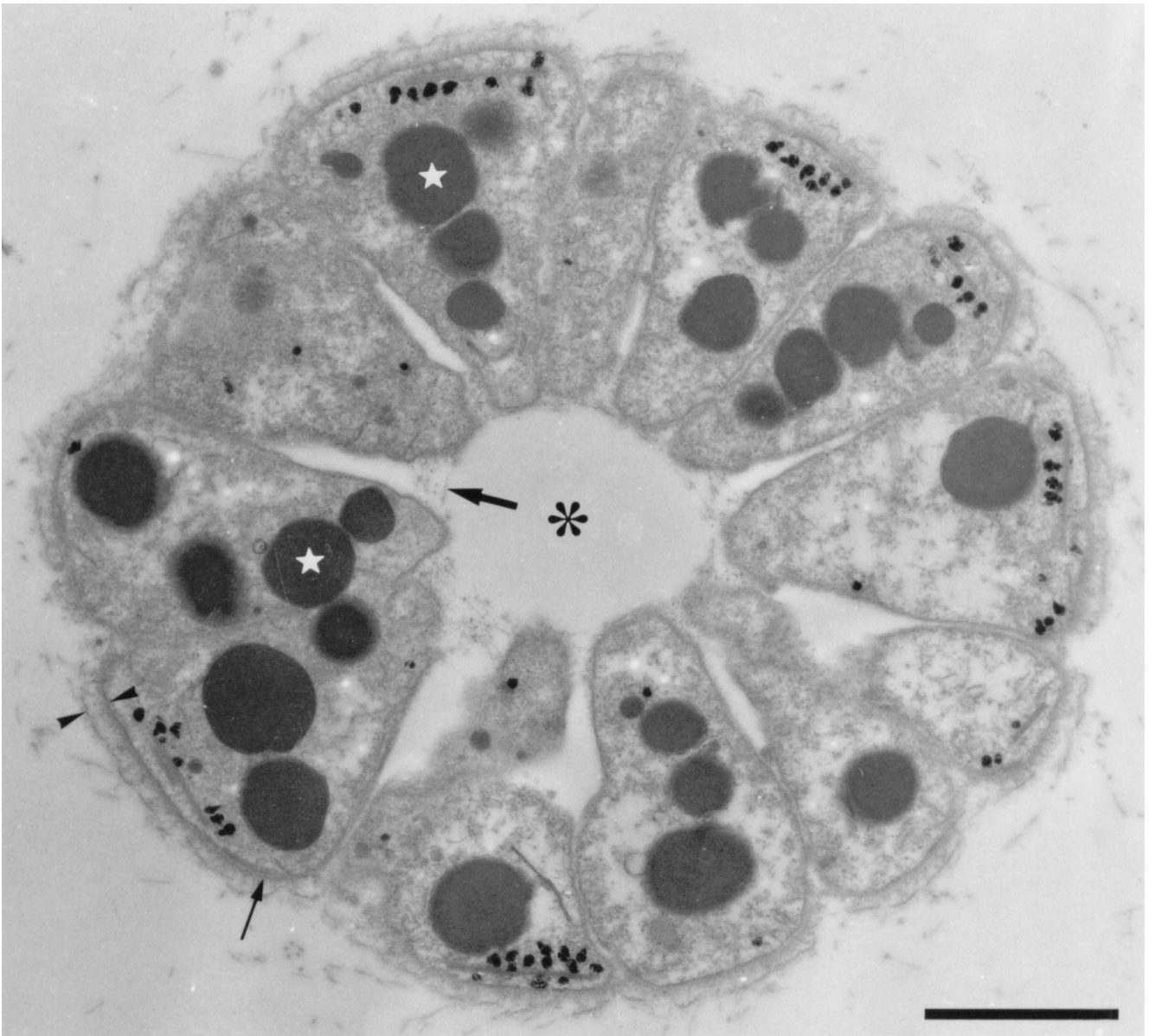


Figure 4

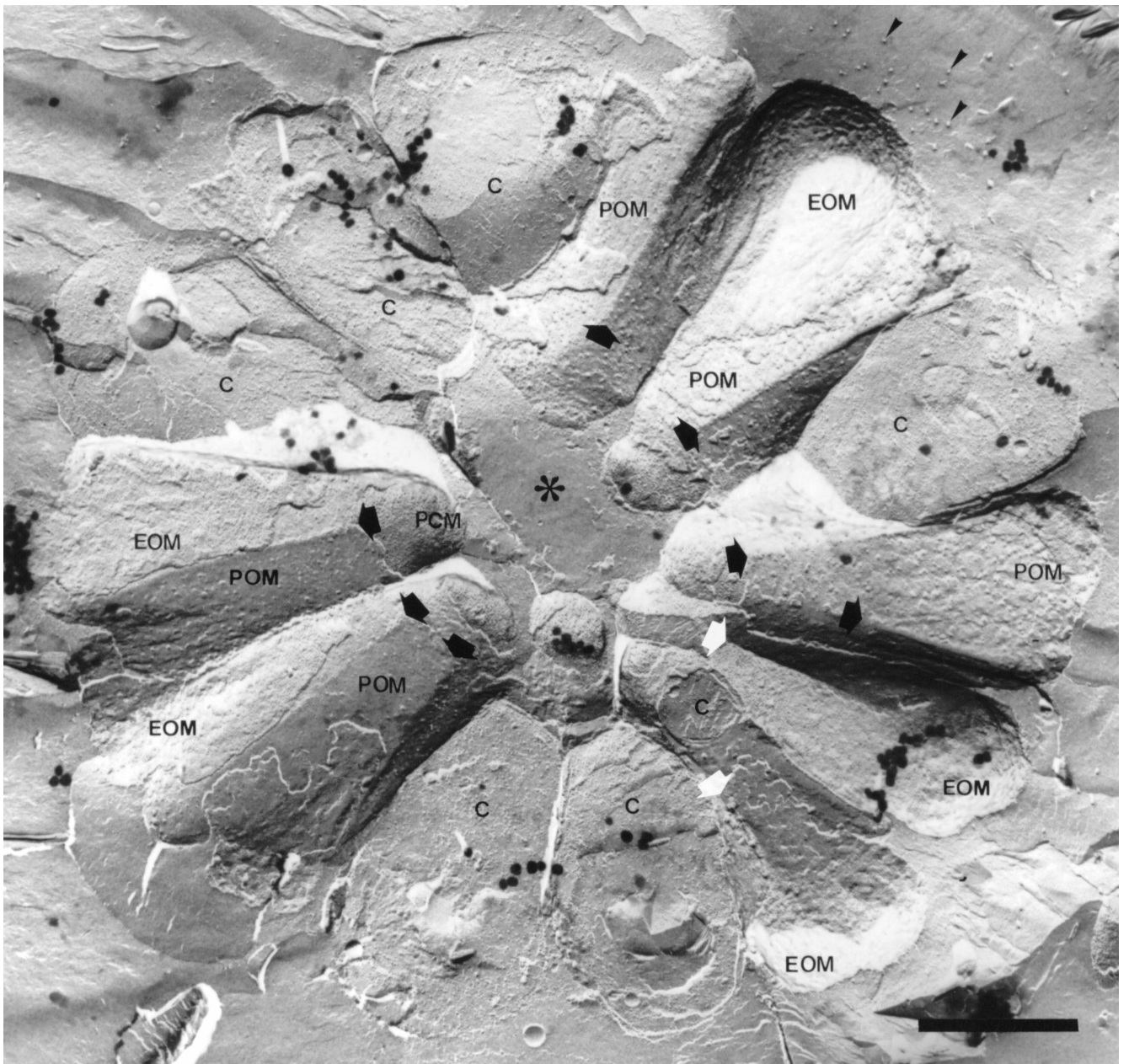


Figure 5

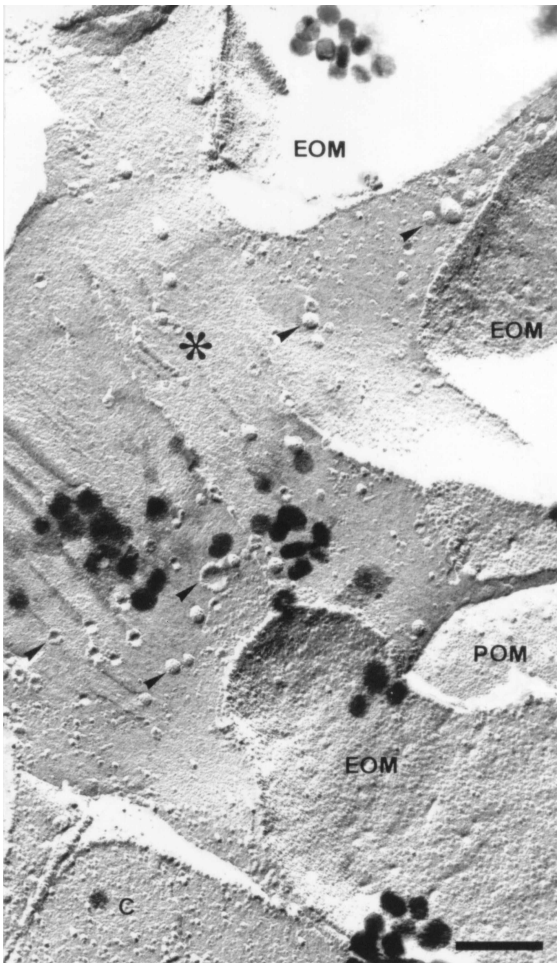


Figure 6a

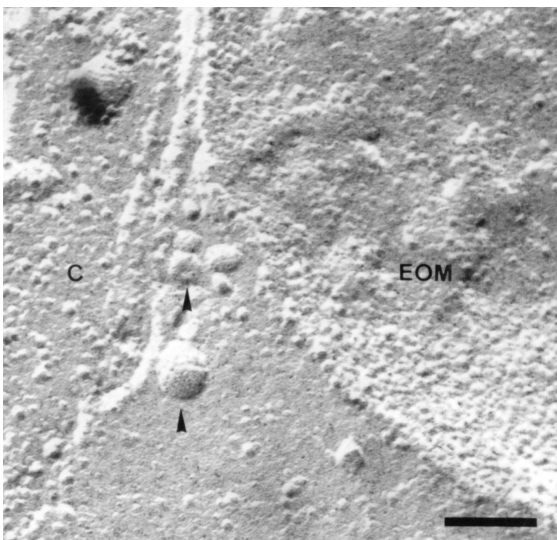


Figure 6b

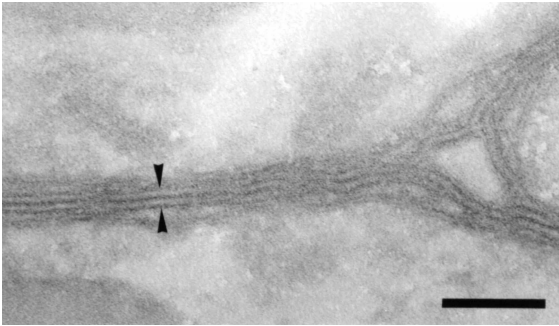


Figure 7

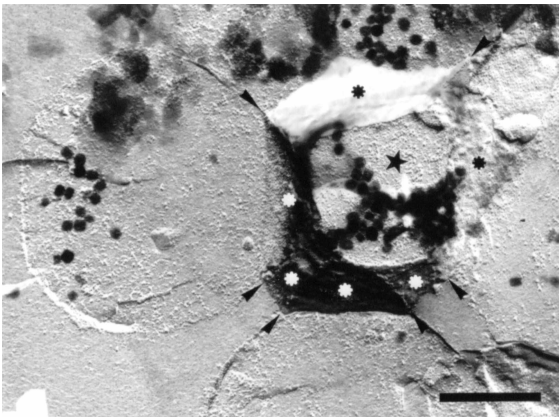


Figure 8

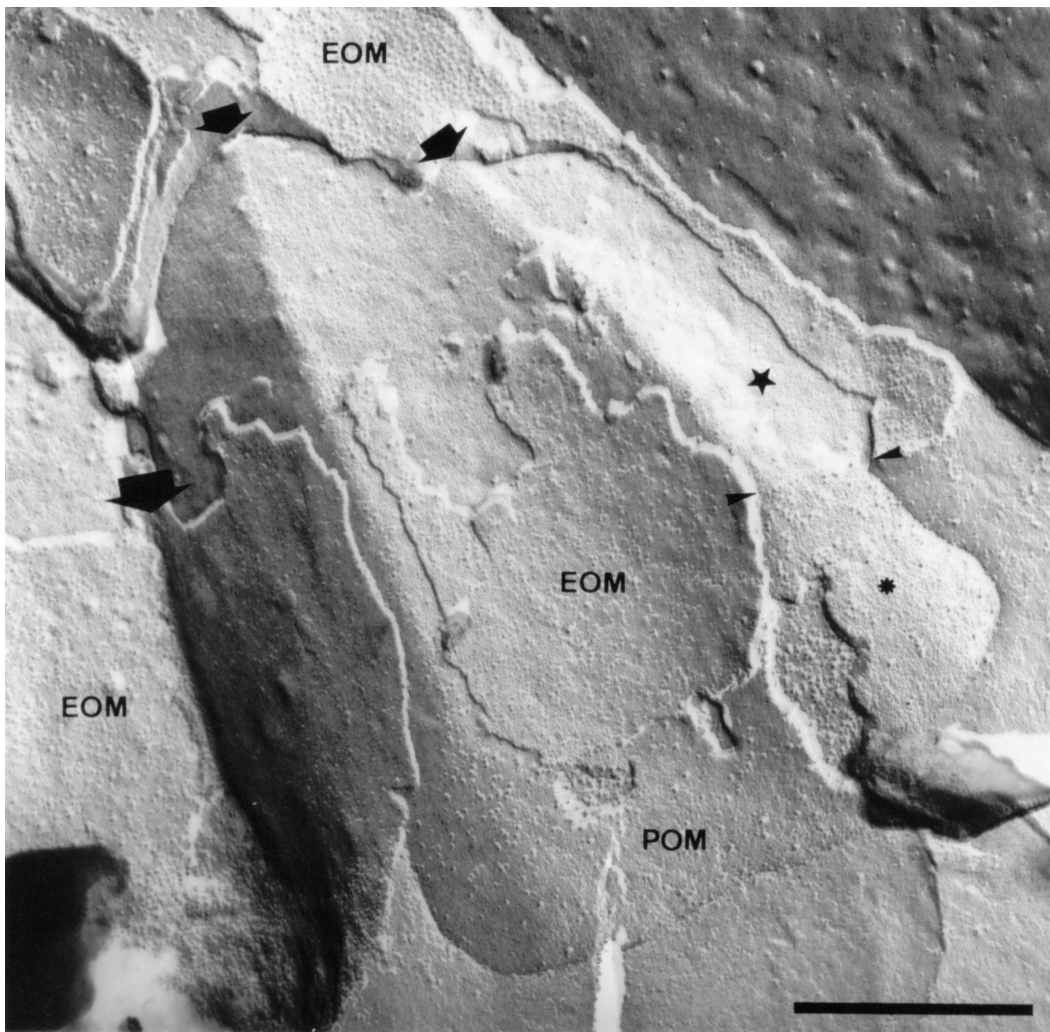


Figure 9a

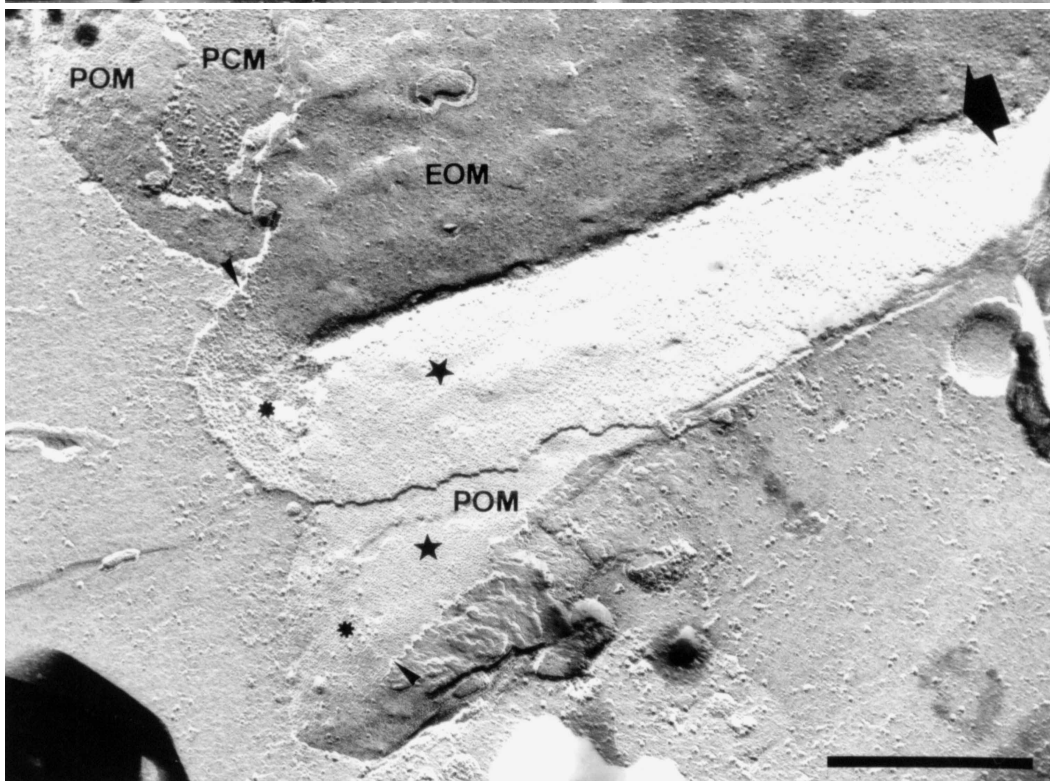


Figure 9b



Figure 10a

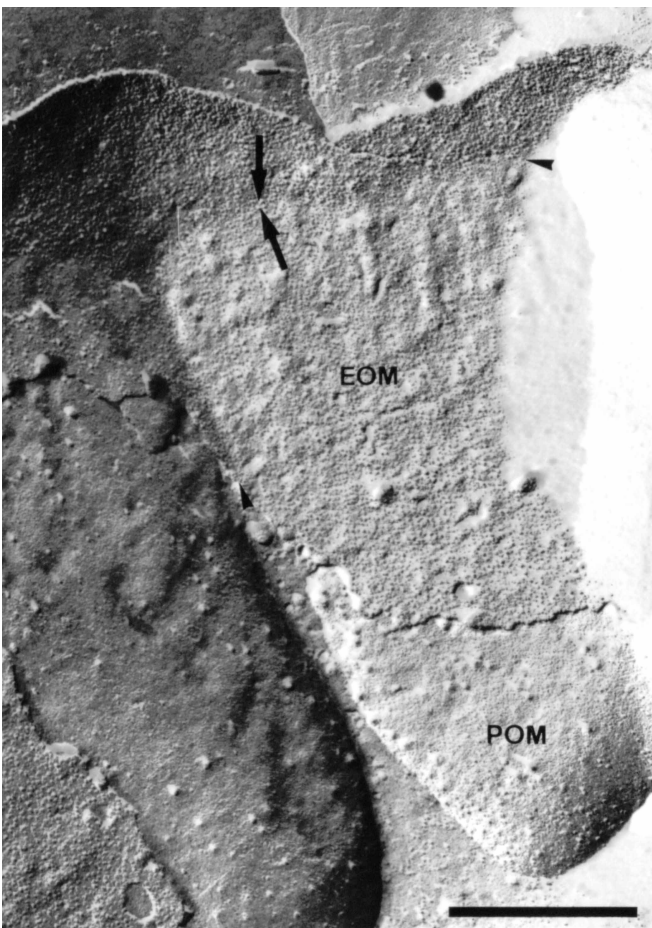


Figure 10b