

Dialysis Membrane Technique for Ultrastructural Studies of Microbial Interactions

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Received 17 August 1983/Accepted 3 October 1983

A dialysis membrane technique was developed that enabled ultrastructural investigations of the interaction of nematode-trapping fungi and their nematode prey. It allowed the sectioning of individual traps that had been selected by light microscopy and was used in kinetic studies on trap formation, nematode capture, and subsequent nematode digestion. The method can also be used for enzyme cytochemical experiments.

Recently, a dialysis membrane technique for studies of microbial interactions was described (2). The technique involved the growth of organisms on a dialysis membrane placed on an agar support. It allowed direct observation of the interacting organisms by light microscopy and, after appropriate specimen preparation, by scanning electron microscopy; the technique was successfully used in studies on fungus-nematode and fungus-fungus interactions (2, 3; B. Nordbring-Hertz, in D. H. Jennings and A. D. M. Rayner ed., *The Ecology and Physiology of the Fungal Mycelium, British Mycological Society Symposium*, in press). Previous studies showed that *Arthrobotrys oligospora* Fres., a predatory nematophagous fungus, formed traps consisting of a three-dimensional adhesive network in response to certain environmental conditions (1, 2). At the subcellular level, the individual cells of these traps were characterized by the presence of a large number of dark bodies, whose nature and physiological function remained obscure. In an attempt to elucidate the functional role of these trap organelles, we decided to perform kinetic studies on trap development and nematode capture and digestion by electron microscopic techniques. Since such studies require methods which enable the selection of individual traps and captured nematodes for subcellular observations, the dialysis membrane technique, described previously (2), was extended for application in routine transmission electron microscopy and enzyme cytochemistry.

A. oligospora Fres. (ATCC 24927) was grown on dialysis membranes on the surface of a low-nutrient medium supplemented with a trap-inducing peptide as previously described (1). Young traps at different stages of development and mature, fully developed traps of different ages were fixed and prepared for transmission electron microscopy.

Fixation involved removal of the dialysis membrane carrying the fungal material from the agar surface. This was then placed in an empty petri dish and treated with the fixative, which was added dropwise. For routine sectioning, fixation was performed in 1.5% (wt/vol) KMnO₄ for 20 min at room temperature. After fixation, the KMnO₄ solution was carefully removed with a Pasteur pipette; subsequently, the material was washed in distilled water and stained with 1% (wt/vol) uranyl acetate for 4 to 8 h by the same flooding technique.

For kinetic studies of the interaction between fungal traps and nematodes, pieces of dialysis membrane (1 to 2 cm²)

containing plenty of traps (ca. 50 to 100 traps per cm²) were transferred to empty petri dishes, and a drop of water was added to the surface of each membrane. Subsequently, 50 to 100 nematodes were added to each membrane by applying 1 to 5 drops of a nematode suspension. Excess water was carefully removed with a Pasteur pipette, and the capture of nematodes was followed with a dissecting microscope. Capture started within 1 to 2 min, and individually captured nematodes could easily be identified. Usually ca. 5 nematodes (range, 2 to 10) were captured within 1 min after they were placed on the membrane surface. The nematodes that remained free after this time were then rapidly removed with a jet of water, and pieces of dialysis membrane with captured nematodes were fixed after different times of interaction. Preliminary results revealed that in the nematode-based experiments, a prefixation in formaldehyde-glutaraldehyde was required before the KMnO₄ fixation. This was due to the fact that the nematodes generally were able to withstand prolonged periods of incubation in glutaraldehyde or KMnO₄ solutions. However, in the presence of formaldehyde the nematodes were rapidly killed, and therefore routine fixation was performed in a mixture of 1.5% (vol/vol) formaldehyde and 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature. After the material was washed with distilled water, it was postfixed in KMnO₄ and poststained in uranyl acetate as described above.

In addition, cytochemical staining experiments were performed on formaldehyde-glutaraldehyde-fixed samples for the localization of hydrolytic and microbody-matrix enzymes. Acid phosphatase and glucose 6-phosphatase were demonstrated after incubations with Ce³⁺ ions and the enzyme-specific substrates, β-glycerophosphate and glucose 6-phosphate, by the methods described previously (6). Catalase activity was localized with diaminobenzidine and H₂O₂ (4, 5), whereas D-amino acid oxidase activity was demonstrated after incubations with CeCl₃ and D-alanine as the substrate (5, 7). In addition, after cytochemical staining procedures, the cells generally were washed with distilled water and postfixed in KMnO₄. After the material was dehydrated in a graded ethanol series, it was embedded in Epon 812. Generally, the pieces of dialysis membrane were embedded flat in a thin layer of Epon 812 in small petri dishes or aluminum foil cups.

As was reported earlier (2), the entire procedure may be performed on the dialysis membranes without further protection; when the fungal cells and the captured nematodes are handled carefully, they generally stick to these mem-

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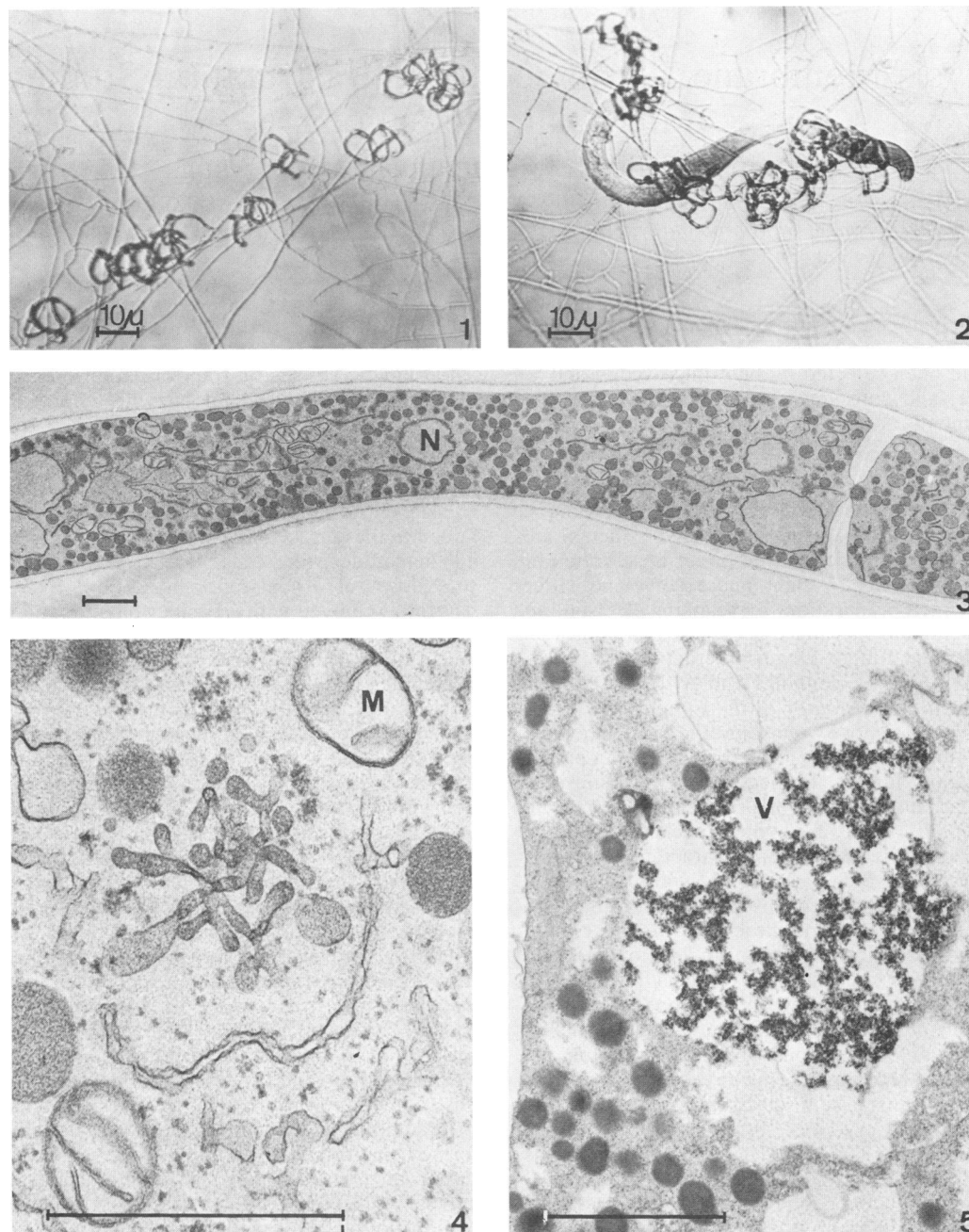


FIG. 1. and 2. Light micrographs of dialysis membrane-grown *A. oligospora* embedded in a thin layer of Epon 812. Traps (Fig. 1) and captured nematodes (Fig. 2) are easily recognized and prepared for electron microscopic observations (Fig. 1, KMnO_4 fixation; Fig. 2, formaldehyde-glutaraldehyde- KMnO_4 fixation).

FIG. 3. Survey of a trap cell showing the presence of numerous dark bodies (KMnO_4 fixation; N, nucleus). Bar, $1\ \mu\text{m}$.

FIG. 4. Detail of a young trap cell illustrating the development of dark bodies from specialized regions of the endoplasmic reticulum by budding (KMnO_4 fixation; M, mitochondrion). Bar, $1\ \mu\text{m}$.

FIG. 5. Cytochemical demonstration of glucose 6-phosphatase activity in the vacuole (V) of a mature trap; dark bodies are unstained (glutaraldehyde and KMnO_4 fixation; incubation was with CeCl_3 and glucose 6-phosphate). Bar, $1\ \mu\text{m}$.

branes. However, if necessary, the membranes can be embedded in agar or gelatin before dehydration and embedding.

After polymerization, the Epon blocks were removed from the petri dishes or aluminum foil cups. Single traps or a captured nematode could be easily detected with a dissect-

ing microscope and consequently could be cut or sawed out of the Epon block and mounted in a ultramicrotome holder for thin sectioning (Fig. 1 and 2). In addition to accurate selection of the desired specimen, another advantage of the above method is that the material to be sectioned can be oriented in any desired direction.

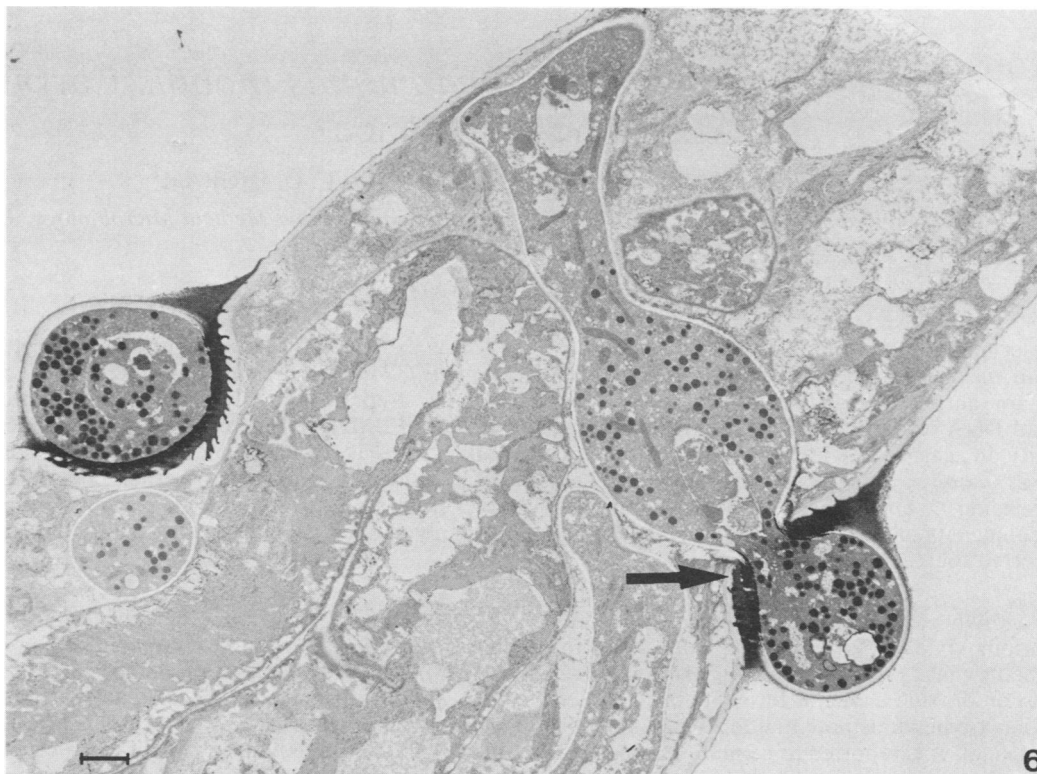


FIG. 6. Survey of area of penetration of a hypha of *A. oligospora* into a nematode. At the location of penetration the cell membrane, cell wall, and part of the adhesive coating are heavily stained for acid phosphatase activity (arrow). (Formaldehyde-glutaraldehyde-KMnO₄ fixation; incubation was with CeCl₃ and β -glycerophosphate). Bar, 1 μ m.

The method has been used to study the biogenesis and fate of the trap organelles and the significance of these organelles in trapping and subsequently digesting nematodes. Figures 3 through 5 show examples of electron micrographs of trap cells at different stages of development, and Fig. 6 illustrates the location of hyphal penetration into a captured nematode after 6 h of interaction. Numerous dark bodies are present in mature trap cells (Fig. 3), whereas the development of the dark bodies from specialized regions of the endoplasmic reticulum by budding in young trap cells is illustrated in Fig. 4. Furthermore, the method is also suitable for cytochemical experiments and was successfully used for the demonstration of hydrolytic enzymes (Fig. 5 and 6), catalase, and a peroxisomal oxidase (data not shown). The detailed results of these studies will be described elsewhere (M. Veenhuis, B. Nordbring-Hertz, and W. Harder, manuscript in preparation).

The present and earlier observations (2, 3; Nordbring-Hertz, in press) make clear that the dialysis membrane technique provides a powerful tool in studies on fungus-fungus and fungus-nematode interactions, on both the sub-structural and ultrastructural levels.

This work was performed during a research visit by B.N.-H. to the Department of Microbiology, University of Groningen, which was made possible by a grant from the Swedish National Science Research Council.

We thank J. Zagers for skillful assistance in preparing the electron micrographs.

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