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Original Article

Safe Specimen Preparation for Electron Microscopy of Pathogenic Fungi by Freeze-substitution after Glutaraldehyde Fixation

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Abstract

A safe method is described for observing ultrastructure of highly infectious fungi by ultrathin sectioning electron microscopy. The fungal cells were first chemically fixed by glutaraldehyde to kill them. They were then rapidly frozen by propane slush in liquid nitrogen and freeze-substituted in acetone containing 2% osmium tetroxide. This method gave clear cell images with high resolution in a natural state, close to the image obtained by rapidly frozen freeze-substituted specimen of living cells. Although we have demonstrated the utility of this method using *Exophiala dermatitidis* and *Cryptococcus neoformans*, it could also be used for observing highly infectious fungi such as *Coccidioides immitis*.

Key words: pathogenic fungi, rapid freezing, freeze-substitution, chemical fixation, *Exophiala dermatitidis*, *Cryptococcus neoformans*

Introduction

Observation of fungal cell ultrastructure by ultrathin sectioning electron microscopy was difficult, since double fixation by glutaraldehyde and osmium tetroxide commonly used for animal cells gave no clear images. Fixation by potassium permanganate or glutaraldehyde fixation after cell wall digestion gave better cell images, although destruction of ribosomes or cell deformation was inevitable[1)]. Freeze-substitution fixation after rapid freezing of living cells has been effective in preserving ultrastructure of fungal cells[2-6)], since it fixes living materials in a vitrified state in milliseconds. This method would also be useful for observing pathogenic fungi. However, it may be hazardous to fix highly infectious living fungi by rapid freezing, because the method requires living cells in high density. During the study on dynamics of the spindle pole body in the cell cycle of the pathogenic yeasts[7-9)], we tried various fixation methods for better electron microscopy. We found a fixation method that may be safely used for observing highly infectious fungi by electron microscopy, and report this method here.

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Materials and Methods

Organisms and culture conditions

The strains used in this study were *Exophiala dermatitidis* IFM 4844, *Cryptococcus neoformans* IFM 5808, 5844 and 41464[10)]. Cells were cultured in 20 m*l* YPG medium (1% each of yeast extract, polypeptone, and glucose) at 30°C with shaking[11)].

Cell fixation and electron microscopy

For chemical fixation, cells were fixed with 3% glutaraldehyde- 1% paraformaldehyde at 4°C

overnight and 1% osmium tetroxide at 4°C for 1 hr, embedded in 2% agar, and dehydrated by a graded series of ethanol. For rapid freezing and freeze-substitution, living cells were sandwiched between two copper discs, rapidly frozen by plunging into propane slush in liquid nitrogen[12], and freeze-substituted in acetone containing 2% osmium tetroxide at −80° C for 2 days. For freeze-substitution after glutaraldehyde fixation, cells were fixed in a mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30-60 min or at 4°C overnight. They were collected by centrifugation, rapidly frozen by propane slush, and freeze-substituted in acetone containing 2% osmium tetroxide at −80°C for 2 days. These differently fixed and dehydrated samples were then embedded in epoxy resin and polymerized at 60°C for 24 hrs. Ultrathin sections were cut to a thickness of 70-90 nm with a diamond knife on an ultramicrotome (Leica Ultracut S) and mounted on copper grids. They were stained with uranyl acetate and lead citrate, coated with plasma-polymerized naphthalene support film[13], and observed in a JEM 1200EX transmission electron microscope (JEOL, Tokyo) at 80 kV.

Survival rate of cells after glutaraldehyde fixation

Cells were collected by centrifugation at exponential phase (OD_{660}= 1〜2) and stationary phase (OD_{660}= 22〜30), and fixed in a mixture of 3% glutaraldehyde and 1% paraformaldehyde for 30 min at room temperature or overnight at 4°C. They were washed 3 times with distilled water, inoculated on YPG agar plates at various cell quantities, and observed for the appearance of colonies at 24°C for 5 days.

Results and Discussion

By chemical fixation the cell became electron-dense. Although the nucleus, vacuoles, and mitochondria could be recognized, their images were not clear and their morphologies were irregular. Tri-lamellar structures of membrane systems were not clear either (Figs. 1A-1B).

Freeze-substitution after rapid freezing of living cells revealed the cell structure very well, and the cell wall, plasma membrane (Figs. 2A-2B, 2D, 3A and 3C), mitochondria (Figs. 2B and 3B), vacuoles, vacuolar membrane (Fig. 2C), nucleus, nucleolus, nuclear envelope, rough endoplasmic reticulum (Figs. 2A, 2E and 3A), glycogen granules (Fig. 3C), and ribosomes (Figs. 2A-2E and 3A-3C) were clearly visible in a natural state. Nucleus and vacuoles were spherical, and membranes appeared smooth (Figs. 2A-2E and 3A-3C). Tri-lamellar structures of the outer mitochondrial membrane, mitochondrial cristae, vacuolar membrane, plasma membrane, nuclear membrane, and rough ER were clearly visible (Figs. 2B-2E and 3B-3C).

Freeze-substitution after glutaraldehyde fixa-

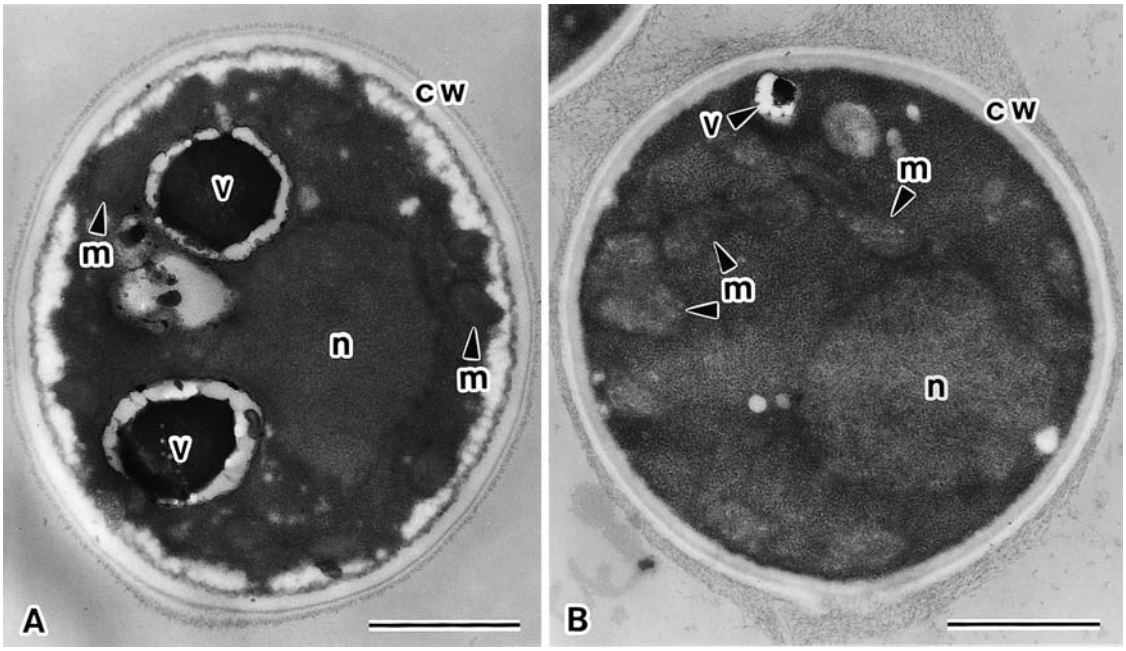

Fig. 1. Ultrathin sections of chemically fixed *Exophiala dermatitidis* (A) and *Cryptococcus neoformans* (B). Cells were fixed with 3% glutaraldehyde-1% paraformaldehyde and 1% osmium tetroxide. Cell structures are not clearly visible. cw, cell wall; m, mitochondrion; n, nucleus; v, vacuole. Scale = 1 μm.

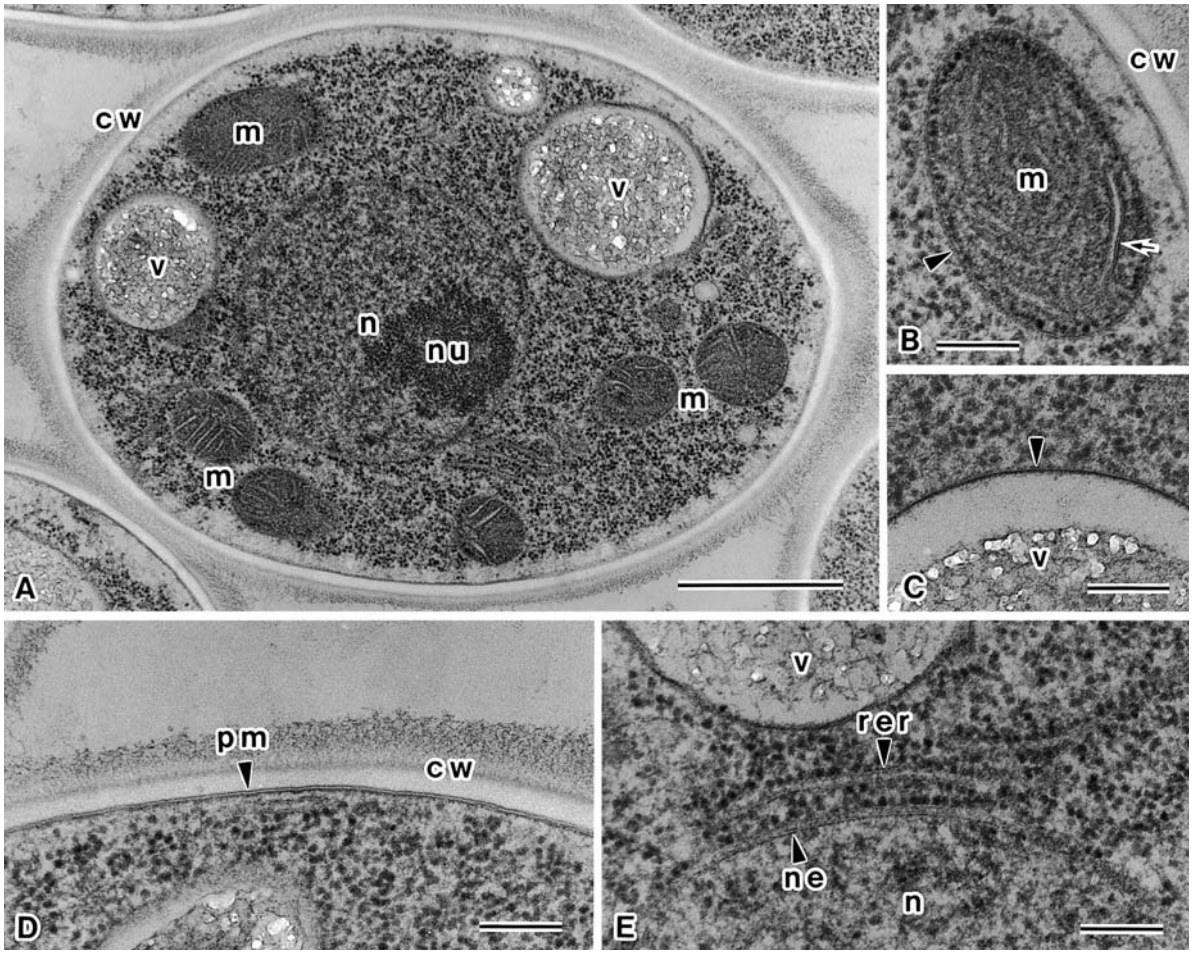

Fig. 2. Ultrathin sections of freeze-substituted *E. dermatitidis*. Cell structures are clearly visible. cw, cell wall; m, mitochondrion (arrow, cristae; arrowhead, outer membrane); n, nucleus; ne, nuclear envelope; nu, nucleolus, pm, plasma membrane; rer, rough endoplasmic reticulum; v, vacuole. A, scale = 1 μm. B-E, scale = 200 nm.

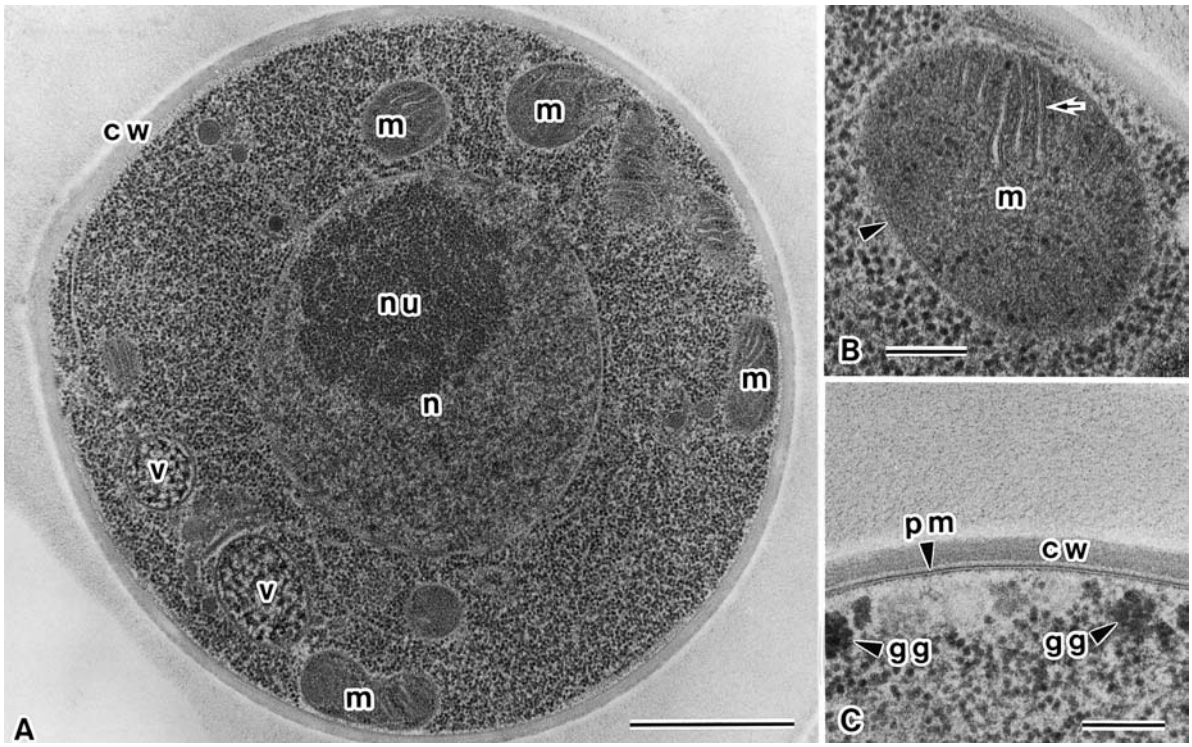

Fig. 3. Ultrathin sections of freeze-substituted *C. neoformans*. Cell structures are clearly visible. cw, cell wall; gg, glycogen granule; m, mitochondrion (arrow, cristae; arrowhead, outer membrane); n, nucleus; nu, nucleolus, pm, plasma membrane; v, vacuole. A, scale = 1 μm. B-C, scale = 200 nm.

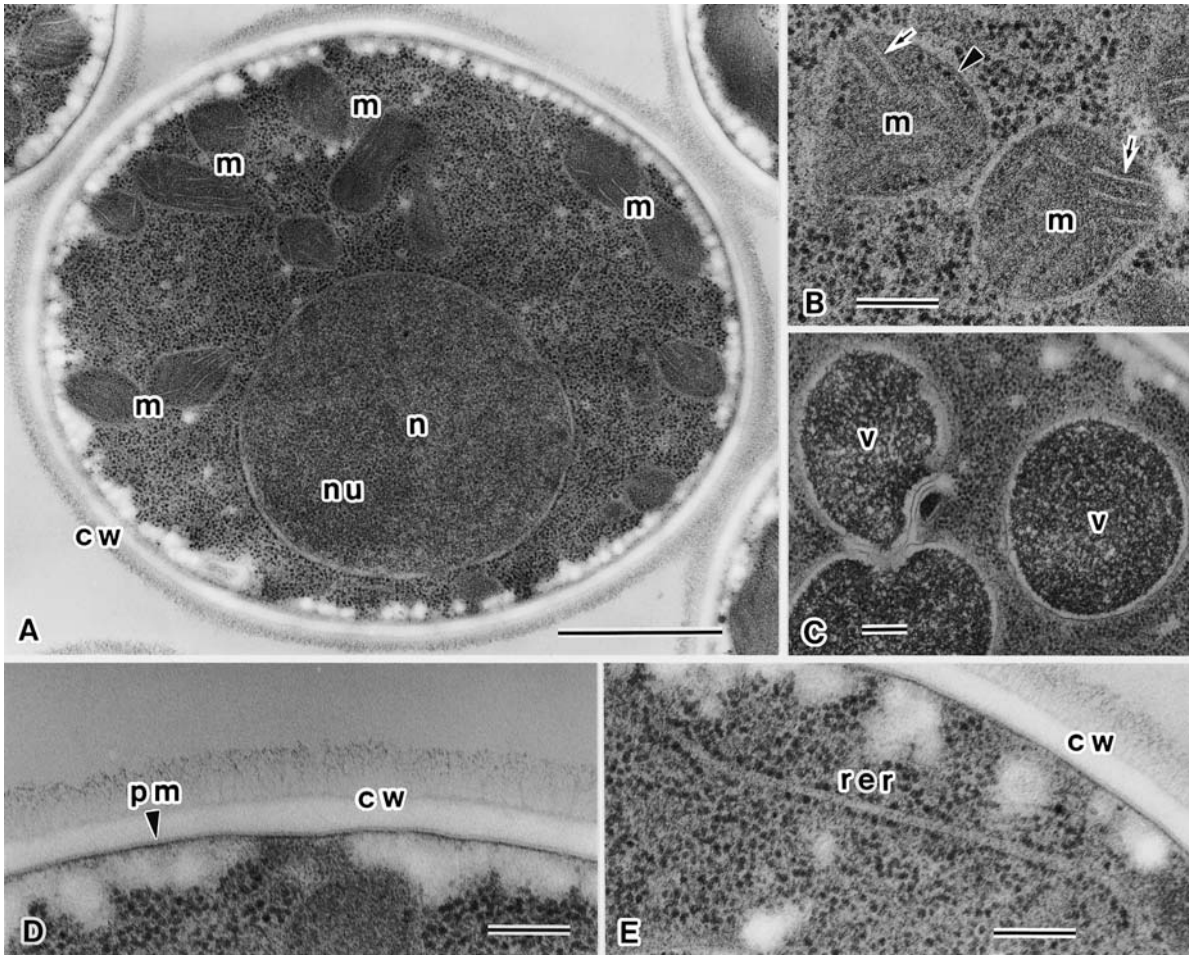

Fig. 4. Ultrathin sections of freeze-substituted *E. dermatitidis* after glutaraldehyde fixation. Cell structures are clearly visible. cw, cell wall; m, mitochondrion (arrow, cristae; arrowhead, outer membrane); n, nucleus; nu, nucleolus, pm, plasma membrane; rer, rough endoplasmic reticulum; v, vacuole. A, scale = 1 μm. B-E, scale = 200 nm.

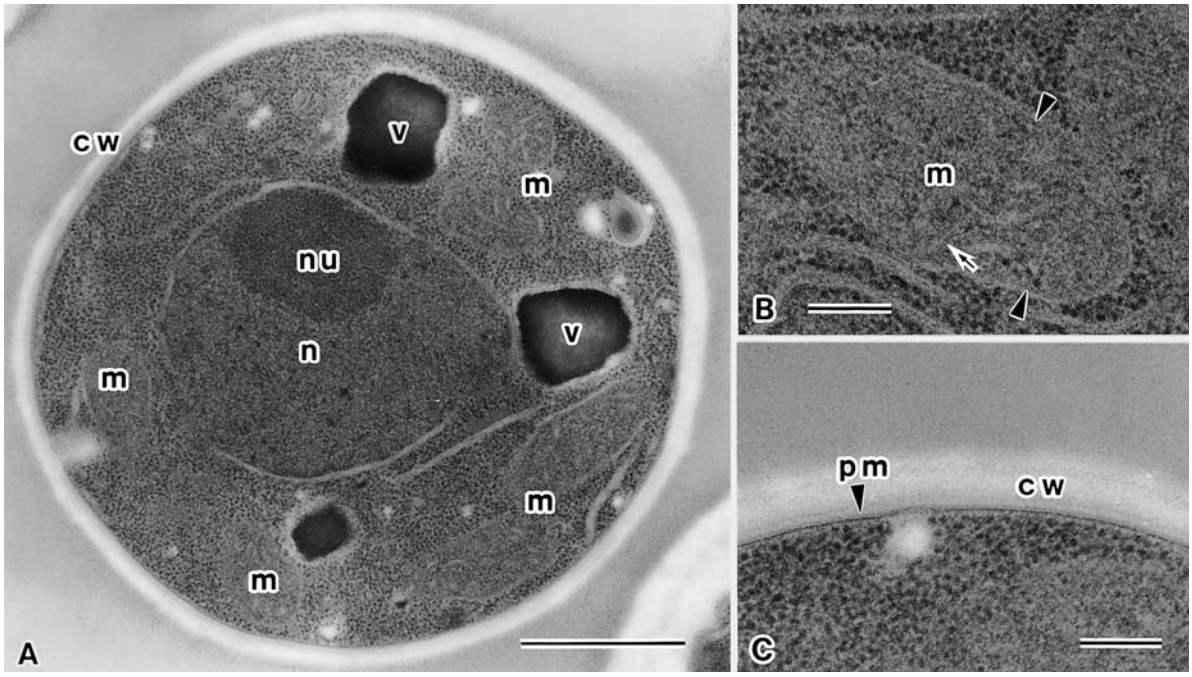

Fig. 5. Ultrathin sections of freeze-substituted *C. neoformans* after glutaraldehyde fixation. Cell structures are clearly visible. cw, cell wall; m, mitochondrion (arrow, cristae; arrowhead, outer membrane); n, nucleus; nu, nucleolus, pm, plasma membrane; v, vacuole. A, scale =1 μm. B-C, scale = 200 nm.

tion gave lighter cell images than chemical fixation, and revealed cell structures well (Figs. 4A-4E and 5A-5C). This method revealed ribosomes and nucleolus that could not be recognized by chemical fixation alone (compare Figs. 4A-4E and 5A-5C with Figs. 1A-1B). In *E. dermatitidis*, the nucleus and vacuoles were spherical and cell structures appeared natural (Figs. 4A-4E). The membranes appeared smooth, and tri-lamellar structures of the outer mitochondrial membrane, mitochondrial cristae, vacuolar membrane, and plasma membrane were visible (Figs. 4B-4D), although tri-lamellar structures of nuclear membrane and rough ER were not very clear compared with the images of freeze-substitution without glutaraldehyde fixation (compare Figs. 4A-4E with Figs. 2A-2E). In *C. neoformans*, cell structures appeared somewhat deformed. Tri-lamellar structures of the outer mitochondrial membrane, mitochondrial cristae, and plasma membrane were visible (Figs. 5B-5C), although tri-lamellar structures of vacuolar membrane, nuclear membrane and rough ER were not as clear as the images of freeze-substitution without glutaraldehyde fixation (compare Figs. 5A-5C with Figs. 3A-3C). Preservation of cell structures was better in *E. dermatitidis* than *C. neoformans*, though the reason for this is not clear at present.

All cells examined in both exponential and stationary phases of *E. dermatitidis* and *C. neoformans* were dead after glutaraldehyde fixation for 30 min or overnight (none were alive among $\sim 1.5 \times 10^6$ plated cells), confirming that rapid freezing after glutaraldehyde fixation is safe.

Generally, freeze-substitution of living fungi or yeasts after rapid freezing gives clear cell images and is recommended for electron microscopy for cell structure research. It is very dangerous, however, for handling highly infectious living fungi such as *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis* and *Penicillium marneffei*, especially because of the high concentration of cells needed for successful rapid freezing. Our results show that freeze-substitution after glutaraldehyde fixation reveals cell structures well. The images were close to those obtained by freeze-substitution after rapid freezing, and much better than the images obtained by chemical fixation alone, although the extent of preservation of the cell structures seems to vary from species to species. Glutaraldehyde fixation overnight did not give significantly different images from glutaraldehyde fixation 30-60 min before freeze-substitution.

There were several reports that used freeze-substitution after chemical fixation in various specimens[14–16], but none paid attention to the usefulness of the method for observing infectious fungi, nor compared the method with freeze-substitution of a rapidly frozen specimen of living cells. We suggest here using the method of freeze-substitution after glutaraldehyde fixation for safe observation of cell structure of dangerous pathogenic fungi at high resolution, and believe that this can offer a significant contribution to the structure research of pathogenic fungi.

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