METHODS IN MICROBIOLOGY

Volume 20
Electron Microscopy in Microbiology

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Edited by

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1988

ACADEMIC PRESS
Harcourt Brace Jovanovich, Publishers
London San Diego New York Berkeley Boston
Sydney Tokyo Toronto

ACADEMIC PRESS LIMITED
24–28 Oval Road
London NW1

United States Edition published by
ACADEMIC PRESS INC.
Orlando, Florida 32887

British Library Cataloguing in Publication Data
Methods in microbiology. — Vol. 20:
Electron microscopy in microbiology.
1. Microbiology. Laboratory techniques
I. Mayer, Frank
576'.028

ISBN 0-12-521520-6

Printed in Great Britain by Galliard (Printers) Ltd. Great Yarmouth

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PREFACE

Volume 20 of "Methods in Microbiology" is concerned with electron microscopic techniques applied for the elucidation of microbial structures and structure-function relationships at cellular, subcellular, and macromolecular levels. Many of the recent findings on the ultrastructural features of microorganisms have been obtained with newly developed methods; cryotechniques are of major importance in this respect. Nevertheless, classical approaches have not lost their validity. Both conventional and newer methods have therefore been incorporated into this volume. The topics dealt with are meaningful not only in bacterial cytology, but also in physiology, enzymology, biochemistry, and molecular biology, as well as aspects of medical and biotechnological application.

At the cellular level the topics covered include the analysis of bacterial surface structures (polysaccharides, S-layer), membranes, and special inclusions (R-bodies), and approaches for the qualitative and quantitative investigation of low and high molecular weight cellular components. At the subcellular level, the analysis of isolated membranes and of proteoliposomes is described. At the macromolecular level, nucleic acids and selected enzymes are treated. A more general chapter on cryopreparation of microorganisms for transmission and scanning electron microscopy provides an introduction to the field; a chapter on computerized image evaluation and reconstruction was put at the end of the volume because the quantitative extraction of structural data and their handling are prerequisites for the improved understanding of what is seen on electron micrographs. Some of the individual chapters may contain descriptions of procedures also presented in other chapters. In part, this was unavoidable; it was also desirable in that the reader may discover that specific procedures work in a number of variations. This appears to be a feature of the majority of electron microscopic approaches, which often demand a specific skill and patience, and which have developed in different directions after their basic features had become common knowledge.

It is hoped that this volume will encourage workers in a variety of fields of microbiology, and that it contributes to a wider application of modern preparation, imaging, and image evaluation techniques.

The editor wishes to express his gratitude to the authors for their co-operation, to G. Gottschalk for initiating this volume and for his advice, to the staff of Academic Press in London, and to the Chairman of the Advisory Board, John R. Norris.

Frank Mayer

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Cryopreparation of Microorganisms for Electron Microscopy

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I. Introduction

The aim of an electron microscopical study is to provide a structural basis for the correlation of structure and function. Structural information is therefore more valuable when the structures are preserved more completely; preparation techniques should aim to preserve the smallest significant details.

Electron microscopy is the only tool with the inherent power to observe structural details at macromolecular dimensions within the context of complex biological systems. It is therefore important to continue the development of this unique feature of electron microscopy to complement the biochemical and molecular biological techniques available. In practice, however, electron microscopy is used extensively in its "instamatic function", i.e. to illustrate subjective expectations derived from *a priori* information

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obtained by other techniques. With the exception of immunocytochemical methods, biological electron microscopy is rarely used as a source of primary information. This use of electron microscopy can be understood when conventional preparative procedures based on chemical fixation and dehydration are used; it is no longer justified, or at least to a much lesser extent, with the advent of appropriate cryofixation techniques.

The main problem encountered during preparation of biological specimens for electron microscopy arises from the necessity to transform the aqueous biological sample into a solid state in which it can resist the physical impact of the electron microscope (high vacuum, electron beam irradiation). The visualization of intracellular structures by transmission electron microscopy requires very thin sections. Biological samples embedded in plastic compounds, epoxy or methacrylate resins, yield the requisite thin sections. Dehydration of the samples in graded series of an organic solvent, usually acetone or ethanol, is required since these resins are not totally miscible with water. Some effects of the organic solvent, e.g. extraction of lipids (Weibull *et al.*, 1983), gross conformational changes, collapse phenomena, aggregation of proteins and other macromolecules due to complete dehydration (Kellenberger, 1987), are reduced by previous chemical fixation. Chemical fixatives react relatively slowly and cannot preserve all cellular components. Most of the diffusible ions are lost or redistributed during sample preparation. Fixation influences the diffusion properties of the membranes and therefore results in alterations of shape, volume, and content of the cell and its components. Dehydration of fixed samples results in further dimensional changes (shrinkage) (Lee, 1984), which are by no means isotropic. It becomes evident from this that the initial potential of electron microscopy cannot be approached by specimen preparation procedures based on chemical fixation and dehydration.

Ideally one would like specimen preparation procedures that simultaneously guarantee absolute preservation of the dimensions and of the spatial distribution of diffusible elements. Antigens, receptors, lectin-binding sites, etc., should become demonstrable through immunocytochemical techniques. A universal specimen preparation procedure will perhaps remain a dream. One must nevertheless attempt to realize it so that the integrating potential of electron microscopy can further develop into a complementary tool in modern biological research.

Dehydration is the most limiting step with respect to the preservation of the structural integrity. It affects the complex interactions of macromolecules, membranes, cellular water, bound and diffusible ions, in a way that cannot be controlled by means of chemical fixation. Cryofixation represents the alternative; it halts the physiological processes very rapidly. Chemically unpretreated biological material can be immobilized in its natural environ-

ment; only minor dimensional changes are introduced (when frozen the volume of water increases only by 9%: Ushiyama *et al.*, 1979).

II. Cryofixation

The structural integrity of the biological material is guaranteed only if cryofixation brings about solidification of water or solutes in a vitreous or microcrystalline state in the absence of any chemical treatment (aldehyde prefixation, for example, reduces possible effects of cryoprotectants: Rash and Hudson, 1979).

Freezing cellular water in the vitreous, amorphous state would be ideal, since the basic nature of the liquid would be preserved. Vitrification of cellular water, however, requires very high cooling rates, present only in very thin layers at the specimen surface. These layers are usually too thin ($< 1\,\mu m$) to represent the bulk of the sample or to be processed further for electron microscopy. Their thickness is, however, related to the composition of the cellular fluid (i.e. the concentration of components that act as natural cryoprotectants). Dubochet *et al.* (1987) claim vitrification of thicknesses up to 5–10 μm of non-cryoprotected tissue of liver or kidney. True vitrification of biological solutions has been demonstrated by low temperature electron diffraction (Dubochet *et al.*, 1982) in a transmission electron microscope equipped with a cold stage using the "bare grid" technique (Adrian *et al.*, 1984). By this technique thin ($\sim 100\,nm$) aqueous layers of suspensions are formed within the meshes of an electron microscope grid and vitrified by immersion into liquid ethane or propane at $\sim -180°C$. Thus true vitrification is currently limited to biological samples (suspensions of viruses, phages, liposomes, macromolecules, etc.) that can be prepared in this way.

Heat can be extracted only through the surface of the sample. Heat transfer from deeper within the specimen is limited by the low thermal conductivity of the water and the developing solid layer. Very high cooling rates can be achieved which may lead to the immobilization of a thin layer in the vitreous state at the surface of the sample. Insufficient cooling rates allow ice crystals to form deeper in the sample. More heat is produced by ice crystal formation with increasing depth than is transferred through the ice to the cooled surface. This progressively reduces the cooling rate and results in increasing ice crystal dimensions. Solutes are excluded from the crystal lattice and concentrated between neighbouring ice crystals to form a eutectic as ice crystals form. In electron micrographs of biological material cryofixed with insufficient cooling rates, the eutectic appears as a network of segregation compartments. In practice, the absence of a segregation pattern is a good indication of adequate cryoimmobilization with perhaps only very small ice

crystals, if any, and is referred to as *microcrystalline*. A correct analysis of the state of the cryoimmobilized cellular water is, however, only possible by electron diffraction of frozen hydrated cryosections (Dubochet *et al.*, 1983).

Under optimized freezing conditions (sample mass: Bachmann and Schmitt, 1971; Gulik-Krzywicki and Costello, 1978; optimized application of the cold: Müller *et al.*, 1980b; Costello and Corless, 1978; Dempsey and Bullivant, 1976a,b) and optimized specimen geometry the first detectable segregation patterns appear at a depth of 5–10 μm. This depth depends on the composition of the cellular fluid, as mentioned above. It cannot be significantly increased by any increase in cooling rates at the specimen surface.

Phase separation occurs whenever ice crystals form. Other physiological effects within the cells, such as increase in local solute concentration or change in pH, must therefore follow. These may affect smaller structural details even if no visible segregation compartments are present. It is important to be aware of such effects in order to avoid mis- or overinterpretation of the micrographs.

The above discussion of cryofixation is perfunctory at best. A few aspects relevant to practical application should be stressed:

(1) Cryoimmobilization (by very high cooling rates), in the microcrystalline state, of untreated aqueous samples is only possible in very thin (~10 μm) superficial layers.

(2) The term "microcrystalline" refers to the usable layer, in which no compartmentalization due to excessive ice crystal formation can be detected. Minor structural alterations due to effects of phase separation may only be excluded if true vitrification can be demonstrated.

(3) True vitrification of untreated biological samples is obtained only in extremely thin superficial layers, and at present is therefore, with the exception of the "bare grid technique" (Dubochet *et al.*, 1982), of limited practical value.

(4) The very high cooling rates (e.g. 10^4 K s^{-1}) required for adequate cryofixation bring about a rapid arrest of physiological processes and therefore allow the study of dynamic events with an estimated time resolution of 0.1 ms (Knoll *et al.*, 1987).

(5) During vitrification, water expands linearly by about 2% (Dubochet *et al.*, 1982). During the change of water from a liquid to a solid phase an increase in *volume* of about 9% has been reported (Ushiyama *et al.*, 1979). These very small changes indicate that cryoimmobilization in the microcrystalline state may well preserve the dimensional relations close to the living state, thus forming the basis of a technique to

measure significant structural details as a function of the physiological state, rather than of the applied preparative procedure (as may be the case in the conventional procedures based on chemical fixation and dehydration).

During the last few years our knowledge of the complex behaviour of cellular water during freezing has improved greatly. A comprehensive introduction to cryofixation is given by Robards and Sleytr (1985) and by Bachmann and Mayer (1987). These reviews deal with the fundamental aspects of freezing and are highly recommended to everybody already using, or planning to enter the field of cryofixation-based electron microscopy. In depth knowledge about the physics of water and ice is essential for correct interpretation of results, i.e. to what extent our results reflect the true living state.

Rapid cooling techniques guarantee optimal structural preservation in a thin ($\sim 10\,\mu m$) layer; the very high cooling rates immobilize the cellular water in the vitreous, or in the microcrystalline state, and concomitantly rapidly arrest physiological processes, i.e. dynamic events at membranes (Heuser *et al.*, 1979; Knoll *et al.*, 1987). Rapid cooling techniques are therefore only applicable to samples that can be prepared in a thin layer (e.g. suspensions of macromolecules, isolated cell organelles, viruses, microorganisms, fungal hyphae, etc.) or to natural or cut surfaces of biological tissue samples. Thicker, more complex systems (e.g. plant or animal tissue, fungus–host interactions, root nodules) can be studied by cryofixation-based electron microscopy only if the physical properties of the cellular water are influenced in such a way that cryoimmobilization in the vitreous or microcrystalline state is achieved with much slower cooling rates. The impregnation of larger samples with cryoprotectants, usually in combination with aldehyde prefixation, is frequently used (Skaer, 1982). Numerous artefacts, however, have been shown to be introduced by this procedure (Plattner and Bachmann, 1982), for example the loss of diffusible ions and the redistribution of intramembraneous particles.

The aim of cryofixation is solely to immobilize the specimen physically. A method of cryofixation based on the application of high hydrostatic pressure was developed by Moor and coworkers (Moor and Höchli, 1970; Riehle and Höchli, 1973; Moor *et al.*, 1980; Müller and Moor, 1984b). The effect of the high pressure can be elucidated by applying the principle of Le Chatelier: freezing increases the volume of water. This expansion, and consequently the crystallization, is hindered by high pressure. This effect is demonstrated by a lowering of the freezing point and by reduced rates of nucleation and ice crystal growth (Riehle, 1968). Consequently, less heat is produced by crystallization and has to be extracted per unit time by cooling. This means

that vitrification can be achieved with reduced cooling rates. As deduced from the phase diagram of water (Kanno *et al.*, 1975) the most profitable pressure zone is at 2045 bar, where the melting point of water is lowered to the minimum of 251 K. Samples with dimensions of up to 500 μm can be adequately cryoimmobilized by high pressure freezing. The optimal sample dimensions again depend on the composition of the cellular fluid, i.e. on the presence of components that bring about cryoprotective activity. The suitable specimen thickness for plant material is limited to about 300 μm, whereas animal tissue blocks up to 600 μm thick are frequently successfully cryofixed (Hunziker *et al.*, 1984; Moor *et al.*, 1980). High pressure freezing thus permits structural analysis of more complex systems, i.e. fungus–host interactions or, in the centre of a tissue sample, of cells that have not suffered from traumatic excision. These advantages are somewhat reduced by the relatively slow cooling rates (approx. 500 K s^{-1}) achieved in the centre of the sample. These rates may be too slow to catch dynamic events at membranes or to prevent structural alterations due to lipid phase transition and segregation phenomena. On the other hand, the transition temperature of membrane lipids is raised by about 20 K $kbar^{-1}$ (Macdonald, 1984). This means that by applying a pressure of more than 2 kbar (which is attained in ~15 ms) the membrane lipids may be immobilized very quickly, purely by the action of high pressure. Experimental data supporting this assumption as well as on other short-lived high pressure effects on biological material are not yet available. Possible reactions of biological specimens to high pressure have been discussed by Müller and Moor (1984b) and Moor (1987).

III. Follow-up procedures

Successfully cryoimmobilized samples have to be further processed for electron microscope analysis by various follow-up procedures, each of which yields different information and poses different technical problems. Subsequent processing has to be performed at sufficiently low temperatures such that devitrification and secondary ice crystal growth are avoided. The devitrification range for vitreous, amorphous water was found at ~140 K by means of low temperature electron diffraction (Dubochet *et al.*, 1983); vitreous water recrystallizes into cubic ice at higher temperatures (Dubochet *et al.*, 1983). In this state no effects of phase separation due to ice crystal formation are yet visible when employing the most frequently used follow-up procedures (freeze-fracturing and freeze-substitution). At higher temperatures, i.e. above 190 K, cubic ice may be transformed into hexagonal ice, in which modification ice crystals may rapidly grow and alter the specimen.

A. Physical procedures

Low temperature electron microscopy of vitrified thin aqueous layers and of cryosections (Stewart and Vigers, 1986), as well as freeze-fracturing, are considered to be direct, purely physical procedures. They provide reliable structural information, most closely related to the living state (Dubochet *et al.*, 1987). Cryosectioning of untreated cryofixed biological material at present is still a very demanding technique (Dubochet and McDowall, 1984) and the sections are generally too thick to provide structural identification at high resolution. On the other hand, cryosections observed in the microscope at low temperatures, either frozen hydrated or freeze-dried (Zierold, 1987), represent the best, if not the only, way towards qualitative and quantitative information of the spatial distribution of diffusible ions by X-ray microanalysis. None of these direct physical procedures is suitable for immuncytochemical work unless mild chemical fixation and cryoprotection precedes cryofixation [compare the cryosectioning technique of Tokuyasu (1984) for the labelling of intracellular antigens and the label-fracture techniques of Pinto da Silva and Kan (1984)].

Freeze-fracturing represents the simplest and best established physical technique for obtaining a "safe" representation of structural details (down to ~5 nm). Robards and Sleytr (1985), among others, have reviewed this technique in detail, and its application to microorganisms has been considered by Chapman and Staehlin (1986). Freeze-fracturing allows the description of specific structural aspects which depend on the fracturing behaviour of the sample and its components. It is especially suited to characterizing membranes, since, for energetic reasons, the fracture plane proceeds through the hydrophobic interior of the membranes, thus providing information about size and distribution of intramembraneous particles (IMP). The pattern formed by the IMPs is characteristic for each specific membrane fracture face. Alterations of these specific patterns may reflect dynamic processes at membranes (Knoll *et al.*, 1987) or, if the sample was cooled too slowly, the occurrence of phase transitions and segregation phenomena of the lipid phase. The nature of the IMPs is still under discussion. They may indicate the positions of transmembrane or intramembraneous proteins but, as pointed out by Verkleij (1984), may also be of lipidic nature.

B. Hybrid techniques

Freeze-fracturing is a straight-forward, easily handled technique which provides reliable structural information. Its major disadvantages are that it is rarely applicable to any purpose other than structural description and that the fracture plane proceeds at random. These problems can be partially overcome

by hybrid techniques which combine the advantages of cryofixation with those of the conventional plastic embedding and thin sectioning procedures. *Freeze-substitution* and *freeze-drying* are frequently used for these purposes (for review see Steinbrecht and Müller, 1987). Both procedures are essentially dehydration processes. Freeze-substitution dissolves the ice in a cryoimmobilized specimen by an organic solvent, and freeze-drying eliminates the frozen water by sublimation in a vacuum chamber. Freeze-drying and freeze-substitution must be performed well above the devitrification range of amorphous water ($\sim$ 140 K). Due to the low vapour pressure, freeze-drying at temperatures below about 170 K would lead to impractically long drying times. The temperature limits during freeze-substitution are set by the melting point of the solvent used and the amount of water the solvent can take up at low temperatures (Humbel and Müller, 1986). Generally, temperatures of 180–190 K are considered to be "safe" for cryofixed biological samples because of the rather high natural cryoprotective activity of many cellular components (see Steinbrecht, 1980). After completion of the dehydration process the samples are warmed to room temperature, infiltrated with the embedding resin, and heat polymerized. With respect to the preservation of the structural integrity, these hybrid techniques are much more obscure than the purely physical follow-up procedures such as cryosectioning or freeze-fracturing outlined above, since effects of the organic solvents (e.g. lipid extraction: Weibull *et al.*, 1984) and the embedding chemistry (Causton, 1986; Weibull and Christiansson, 1986) are not excluded. The structural description provided by the physical procedures, in which the water remains in the specimen, thus represents the standard by which all the other procedures have to be measured. Freeze-substitution and freeze-drying may allow an accurate control of the dehydration process, but, due to our incomplete knowledge of cellular water, they are still insufficiently understood.

Our present knowledge about the role of water in the cell and the effects of its removal are summarized by the following statements (for details see Bachmann and Mayer, 1987; Kellenberger *et al.*, 1986; Kellenberger, 1987; Steinbrecht and Müller, 1987):

(1) Water in the cell exhibits different physicochemical properties and is classified into two major groups, namely, bulk, or free water, in contrast to anomalous water referred to as "bound water", "non-freezable water", etc. This anomalous water is thought to be closely associated with surfaces of macromolecules, membranes and ions, and is sometimes also termed hydration shell, surface-modified water, or vicinal water.

(2) This surface-modified water is extremely important for metabolism (Clegg, 1979; Negendank, 1986) as well as for the maintenance of the

structural integrity of proteins and other cell constituents (Tanford, 1980).

(3) One may conclude that the bulk water is more easily removed and affects the preservation of the structural integrity less than the water of the hydration shells during the dehydration process in biological electron microscopy.

The above assumptions are supported by the non-linear shrinkage behaviour of cells and tissues during conventional dehydration by organic solvents at room temperature. The cells start to shrink when ~70% of the cellular water is replaced by the organic solvent. Fully dehydrated, they shrink up to 30–70% of their initial volume (Lee, 1984). This is a first indication that part of the cellular water can be removed which does not introduce gross dimensional changes. However, there is some water closely associated with the cellular structures. Removing this residual water may lead to conformational changes of cellular components (collapse) and aggregation (Kellenberger, 1987).

The temperatures above which different types of macromolecules collapse when exposed to dehydrating agents such as organic solvents and vacuum were determined by MacKenzie (1972). These temperatures range from 215 K to 263 K and seem to depend only on the temperature and the polarity of the dehydrating agent. Wildhaber *et al.* (1982) and Gross (1987) studied the freeze-drying of test specimens containing deuterium oxide D_2O instead of H_2O and followed its escape with a mass spectrometer. They observed a first peak of D_2O evaporating in the temperature range 180–190 K which approached zero after 2 h at 190 K. A second peak of D_2O was observed only after heating the specimen further and had a maximum between 220 K and 230 K. This suggests that the water in the different groups is held in the tissue by different forces, and it may be concluded that some of the specimen water is bound, and therefore needs a higher energy for evaporation than the free solvent. The temperature at which the second peak of D_2O was observed by Wildhaber *et al.* (1982) is in the temperature range of the collapse temperature of MacKenzie (1972), and it may be speculated that it corresponds to the release of the water of the hydration shell.

C. Freeze-drying and freeze-substitution

Ideally, freeze-drying and freeze-substitution could be used to control the residual water content, i.e. how much water has to be left so that the cells maintain their structural and functional integrity, and how much water has to be removed to allow successful plastic embedding. Experiments, however, have shown that the hydration shells can prevent an efficient copolymerization between biological material and resin (Humbel and Müller, 1986).