

AN INTRODUCTION TO CRYO-PRESERVATION IN EM

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INTRODUCTION: The conditions in an electron microscope (high vacuum, high radiation dose) require specific steps to prepare aqueous biological material for analysis. The immobilisation of biological material kept under optimally controlled conditions is the first and most critical step when attempting to preserve the complex interactions of organelles, macromolecules, ions and water in close relationship to the living state. Immobilization must be sufficiently rapid; trapping dynamic events at membranes, (e.g. membrane fusion and exocytosis which occur on a millisecond time scale) and preventing the lateral displacement of lipids and proteins within the membranes) as well as the displacement of ions which, depending on their interaction with macromolecules and water molecules can move very fast.

With respect to time resolution, traditional techniques based on chemical immobilisation (aldehyde fixation) seem to approach their limits. 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 results therefore in alterations of shape, volume, and content of the cell and its components. It becomes evident that the initial potential of electron microscopy to serve as an integrating source of primary information on the structural complexity at cellular to macromolecular dimensions can hardly be approached by techniques based on chemical fixation. Cryoimmobilization represents a farther reaching alternative. Very high cooling rates (10^4 Ksec⁻¹ to 10^6 Ksec⁻¹) are required to prevent the formation and growth of ice crystals by which the structural integrity would be affected. The high cooling rates at the same time bring along a rapid arrest of the physiological events, i.e. a very high time resolution for dynamic processes in the cell and consequently structural immobilisation closely related to the living state.

METHODS: 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 at the surface of the sample. This may lead to the immobilization of a thin surface layer in the vitreous state. 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.

True vitrification of biological solutions has been demonstrated by low temperature electron diffraction using the "**bare grid**" technique (for review see Dubochet et al. 1988). Thin aqueous layers (< 100 nm) of suspensions of viruses, phages, liposomes and macromolecules are formed by this technique within the meshes of an electron microscope grid, either bare or covered with a perforated carbon foil, vitrified by immersion into liquid ethane, and observed in the microscope at low temperatures using a cold stage. Several "**rapid freezing techniques**" (review see Sitte *et al.* 1987), are available to adequately cryoimmobilize aqueous layers of suspensions of cells and microorganisms, organelles, tissue cultures up to a thickness of approx. 10 to 20 µm. The freezing rate achievable in such layers may be too low for vitrification but high enough to prevent growth of damaging ice crystals. Thicker, more complex systems can be studied by cryofixation-based electron microscopy only if the physical properties of the cellular water are manipulated in a way that adequate cryoimmobilization is achieved with much slower cooling rates. "**High pressure freezing**" (for review see Moor, 1987) is at present the only practical way of cryofixing larger untreated samples up to a thickness of 200 µm. It permits structural analysis of more complex systems, i.e., fungus-host interactions, tissue culture cells grown on established substrates (e.g. glass coverslips) or, in the centre of a tissue sample, of cells that have less or not suffered from traumatic excision. Compilation of high pressure literature is at <http://www.em.biol.ethz.ch/>. Subsequent processing has to be performed at sufficiently low temperatures at which devitrification and secondary ice crystal growth are avoided.

REFERENCES: Moor H (1987) pages 175-191 and: Sitte H, Edelmann L, Neumann K (1987) pages 87-113. In: Steinbrecht RA, Zierold K (eds) *Cryotechniques in biological EM*. Berlin, Springer. Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, McDowell AW, Schultz P (1988) In: *Quarterly Review of Biophysics* 21/2: 129-228.