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The Integrating Power of Cryofixation-Based Electron Microscopy in Biology

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ABSTRACT

The unique ability of electron microscopy in biology is its power to describe and integrate structural details down to molecular dimensions within the context of a complex living system.

The information density provided from an EM investigation is determined by the hierarchy of the steps involved in specimen preparation and imaging. The structural complexity of a living biological system can only be preserved as a whole. It can not be investigated in the linear analytical way characteristic of biochemistry or electron microscopy of isolated samples, because these techniques can only physiologically control simplified systems. Structural information closely related to the living state may therefore become an indispensable tool to integrate the analytical findings. It can be achieved by cryoimmobilisation techniques that vitrify the cellular water and at the same time rapidly arrest all physiological processes.

Immobilisation of the cellular water in the amorphous state (vitrification) for specimens up to 200 µm thick can be achieved by high-pressure freezing. Cell suspensions and monolayer tissue cultures can thus be vitrified in a physiologically controlled state. The information presently preserved in a vitrified sample of plant or animal tissue is, however, limited by unknown structural alterations that must occur during sampling (i.e. excision of small tissue pieces and preparation for high-pressure freezing may require up to 3 min.). Cryoelectronmicroscopy of cryosections in the frozen-hydrated state permits the analysis of the immoblised cellular water by electron diffraction and provides reference information by which structural alterations introduced by further processing may be judged.

THE HIERARCHY OF BIOLOGICAL SPECIMEN PREPARATION

The major task of high resolution biological electron microscopy is to provide the structural information with which to correlate structure and function; it is the only methodology with the inherent power to elucidate structures, down to molecular dimensions, within the context of complex biological systems. Specimen preparation and imaging techniques should therefore be directed towards preservation and imaging of the smallest significant details in order to fully exploit this unique, integrating feature of biological electron microscopy thereby complementing the progress of the techniques used in cell biology, biochemistry and molecular biology. The term "high resolution", in this context, means "high information density". The primary question is what are the smallest significant structural details that can be correlated with the physiological state of the visualised biological system.

It is not sufficient to simply list the ultrastructural components of a biological system, e. g. what organelles are present or where is a protein of interest located. We must additionally provide information about the exact size of the structures and their location at a particular time under given physiological conditions. Biological electron microscopy thus evolves beyond its "instamatic function" (an image is selected to illustrate subjective expectations derived from a priori information obtained by other techniques) into a source of primary information. Electron microscopy can effectively interact with the more linear analytical procedures and provide substantial conceptual contributions to the solution of relevant complex biological problems (e. g. virus infection, replication and virus release, muscle contraction, cell motility, intracellular signal transduction, cell-cell-interaction, etc.) at this level alone.

In order to achieve the highest possible information density for a given biological

question, the hierarchy of the steps involved in an electron microscopical investigation must stand as follows: 1. Sampling (excision of tissue samples, harvesting of cells from tissue cultures, centrifugation, filtration, etc.), 2. Immobilisation (chemical fixation, cryofixation, adsorption to a surface). 3. Follow-up procedures (pure physical procedures: cryosectioning, freeze-fracturing, freeze-drying; hybrid techniques: freeze-substitution; chemical techniques: dehydratation by organic solvents, critical point drying, embedding in resins, sugars or metal salts). 4. Contrast enhancement (metal coating for TEM and SEM, replication, metal staining with heavy metal salts, immunolabelling), 5. Information retrieval (signal generation and transfer function, analytical techniques, radiation damage). 6. Image interpretation and analysis (morphometry, averaging and filter techniques, 3D-reconstruction).

All of these steps must be developed interactively and the hierarchy has to be strictly observed if one is to achieve the goal of biological electron microscopy outlined above. The final result of this chain of procedures can never be better than the result of the first step, and, at best, each step may only preserve the information density of the previous step (e. g. even the best microscopes and the most sophisticated image analysis algorithms are unable to provide high density information from samples distorted during the sampling step; the morphometric analysis of a specimen that has suffered from unknown osmotic effects during chemical fixation is of limited use).

All the above steps are, however, associated with a myriad of specific and demanding instrumental, theoretical, and methodological developments that often create a momentum of their own. Abstractions have to be made in order to solve problems within a particular step or a combination of steps. These abstractions e. g. choosing the most accessible specimen and preparing in the most facile way to serve a particular purpose) are frequently selected to the disadvantage of the steps higher up in the hierarchy which, from the point of view of the biological question, have increased complexity. Theoretical, methodological and instrumental results extrapolated on the basis of a particular abstracted specimen may not be applicable to the general case and may therefore be of reduced use in terms of the integrating potential of biological electron microscopy. For further progress one can suffer from a strong tendency to look for similar other objects that can be treated in a similar way instead of attempting the transfer of the acquired knowledge to a level of higher complexity. The following example illustrates this point. Isolated periodic structures help to partially overcome the limiting effects of radiation damage by permitting spatial averaging. Structural resolution almost comparable to that of X-ray diffraction has been obtained with samples adsorbed to a support film and either negatively stained or embedded in glucose or vitrified ice. Ingenious computer algorithms for signal averaging have been developed, as well as important instrumentation for "minimum dose exposure". Periodic structures adsorbed to a carbon foil, rapidly frozen and freeze dried have also been used to establish the resolution limits of metal coating procedures at 1 to 1.5 NM using very thin, non-continuous ("optimal granularity") metal layers together with computer averaging techniques [5] Periodic structures characterised at this level of resolution now allow one to investigate the resolution limits of thicker, continuos coating layers required for the direct visualisation of non-periodic molecular objects in the TEM or the high resolution SEM. This experimentation furthers methodological and instrumental progress of general importance, but the information density of the results is limited by the fact that structural alterations due to isolation of the periodic structures from the intact biological system as well as the effects of specimen preparation (adsorption onto charged surfaces, effects of surface tension, interactions with negative stain and embedding solution, freezing, drying) are largely unknown. The problem is therefore the same as for an intact biological system with one important exception. The difference is that, prior to the preparation step, the conditions affecting isolated periodic structures may be more easily controlled by biochemical means and that structural analysis by independent techniques e. g. X-ray diffraction may also be possible.

Electron microscopy can thus be used as an efficient biochemical method provided that the biochemically controlled state of the sample can be preserved during sample preparation. This is also true for isolated, non-periodic, but biochemically defined systems (protein-protein,

protein-lipid, protein-nucleic acid interactions, etc). Structural information obtained from electron microscopy when used as a biochemical tool can only help to reach the aforementioned goal of biological electron microscopy as an integrating source of primary structural information if the biochemical controls exactly match these in the corresponding living system. Unfortunately this is impossible because the linear, analytical methods of biochemistry generally are not suitable for dealing with systems that exceed a certain level of complexity. Analysis of biochemically defined systems may, however, be an indispensable aid for correct identification and interpretation of structural details displayed in EM-micrographs of complex biological systems that have been immoblised closely related to the living state. One must therefore always start at the beginning of the hierarchy listed above. Fully exploiting the unique, integrating potential of electron microscopy in biology demands techniques that immobilise physiologically controlled specimens in-situ with a time resolution that produces the instantaneous arrest of all dynamic cellular processes. Immobilisation is the most critical step when attempting to preserve the complex interactions of organelles, macromelecules, ions and water in close relationship to the living state. Immobilisation must be suffieciently rapid to trap dynamic events at membranes, (e. g. membrane fusion and exocytosis that occur on a millisecond time scale) and to prevent the displacement of diffusible ions, as well as the movement of lipids and proteins within membranes. Techniques based on chemical immobilisation (aldehyde fixation) have reached their limits in this respect. Chemical fixatives react relatively slowly (on a second to minute time scale) and, in any case, 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.

Cryoimmoblisation represents a farther reaching alternative [13]. 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 (micro-to milliseconds) for dynamic processes in the cell and consequently structural immobilisation closely related to the living state [6].

Cryoimmobilisation procedures that lead to vitrification (immobilisation of the specimen water in the amorphous state) are the sole methods of preserving the interactions of the cell constituents, because the liquid character of the specimen water is retained (reviewed by Dubochet 1988) [3]. No conclusive information is available about the complexity of the above interactions at the molecualr level, because the analytical techniques presently available can only be applied to isolated, reduced systems. Nonetheless, of all techniques presently available, vitrification is still likely to preserve the highest information density.

Vitrification at ambient pressure requires very high cooling rates which are achievable only in a thin superficial layer (100-1000 nm) at the specimen surface. It can be accomplished by the "bare grid" approach [3] to freezing thin aqueous layers (100 nm) of suspensions containing isolated macro-molecules, liposomes, viruses, etc. and also by some rapid-freezing techniques (reviewed by Sitte, 1988) at the surface of suspension droplets or at the surface of natural or cut tissue samples.

High-pressure freezing [11, 12] is at present the only practical way of cryofixing larger non pretreated samples up to a thickness of 500 µm. A very high yield of adequately frozen (i. e. no detectable effects of ice crystal damage are visible after freeze substitution) specimens was demonstrated in TEM using suspensions of microorganisms as well as plant and animal tissue [14]. Recent progress in cryosectioning [9, 10] now permits the routine production of cryosections of high-pressure frozen biological material (Figure 1). In addition to providing a detailed structural description cryosections permit the identification of the state of the frozen water by electron diffraction (Figure 1, insert). High-pressure freezing can vitrify biological samples up to a thickness of 200 µm as deduced from such experiments. The actual thickness that can be vitrified depends on the specimen, particularly on the presence of substances that exhibit cryoprotectant activity. The possibility of vitrifying samples 200 µm thick allows one to

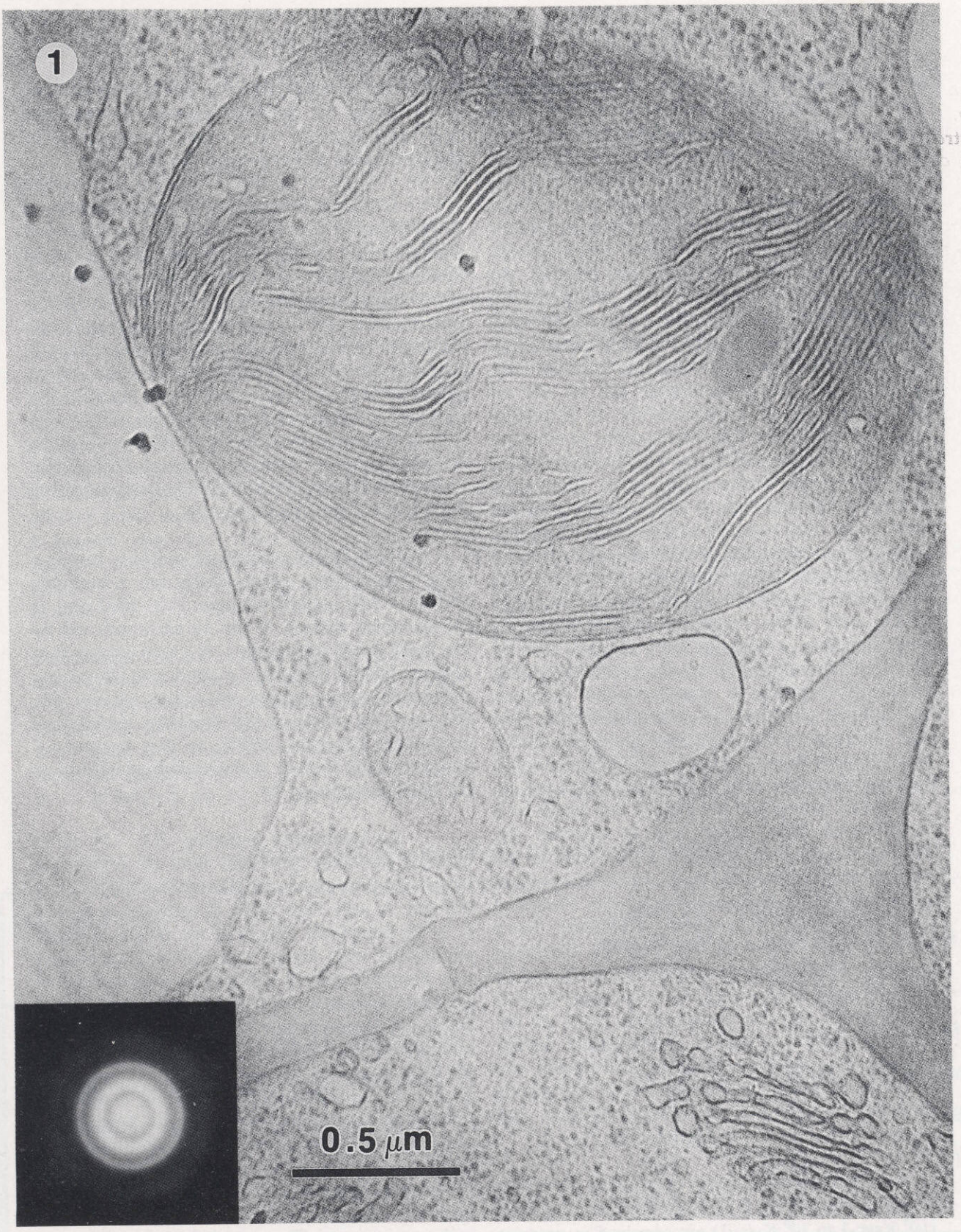

Figure 1: Frozen-hydrated cryosection of high-pressure frozen "Golden Delicious" apple leaf. The inserted electron diffraction pattern indicates the presence of vitreous ice. (Micrograph by M. Michel).

study more complex biological samples (e. g. plant or animal tissues, fungus/host interactions, root nodules) as well as larger volumes of suspensions and monolayer tissue cultures that are adherent to their "natural" substrate. Consequently, a high density of structural information may be preserved. Whereas the physiological control of cells in suspension or as a monolayer culture (medium, temperature, etc.) can be maintained until vitrification, the problems of sampling tissues remains far from being solved. Excision of small tissue samples and following preparation steps for high-pressure vitrification requires time (e. g. 1-3 min.) during which structural alterations may occur (e. g. interruption of blood supply and enervation may lead to changes in oxygen concentration, to local alterations of the pH, and to autolysis). The information density of such a sample will therefore be reduced, despite vitrification.

It is preferable to process samples that have been successfully cryoimmobilised by physical protocols for microscopic observation, e. g. cryo-sectioning, or freeze fracturing. Once the structures and the vitrified state of the sample are established by cryosectioning and electron diffraction, they can provide reference information against which the effects of additional preparation procedures can be judged. The vitrified cryosection can now be freeze dried (Figure 2) for ion measurements or subjected to a freeze substitution regime, that renders particular structures resistant against rehydration. In this state e. g. immunocytochemical experiments may be performed. The loss in information density produced by each step can thus be assessed by comparison with the frozen hydrated cryosection.

MATERIALS AND METHOD

High-pressure freezing of apple leaves:

Apple grafts of cv. "Golden Delicious" on scion M25, were plated in pots with a potting soil mixture and placed in a greenhouse with a controlled temperature regime (25° C during the day, 20° C at night) at a constant humidity of 70% and additional illumination, providing 16 hours of daylight. Discs of apple leaves (2 mm in diameter) were punched with a hole punch, and transferred into 1-hexadecene (Fluka, Buchs, Switzerland). The samples were then kept under a mild vacuum to replace intercellular gases, which are compressible under high-pressure. 1-Hexadecene optimises the transfer of pressure and cold to the specimen. No osmotic effects are expected because of its hydrophobicity [14].

The leaf discs were placed in the cavity (diameter 2 mm; depth 0.2 mm) of a cylindrical aluminum platelet (diameter 3 mm, thickness 0.5 mm) and sandwiched with a second platelet either with no cavity or with a cavity, that matched the specimen thickness as closely as possible [12]. The space between the sample and the platelets was filled with 1-hexadecene [14]. The specimen sandwich was loaded into the sandwich-holder and frozen under high pressure. The sandwiches were then transferred to liquid nitrogen for storage.

Cryoultramicrotomy:

High-pressure frozen "Golden Delicious" apple leaves were cryosectioned at low temperature with diamond knives (Diatome AG, Biel Switzerland), using a Reichert FCS microtome (Leica) equipped with the FCS cryoattachment [9, 10]. Good cryosections were obtained by optimising the cutting-parameters i. e. sectioning temperature, mechanical stability of the sample, and sectioning-velocity. Cutting-artefacts were minimised by reducing the electrostatic interactions between the knife surface and the cryosection. This was accomplished by sectioning the sample in the presence of an ionisation electrode. The ionisation device (Static Line, Diatome AG, Biel, Switzerland) with a primary voltage of 7-8 kV, produces positively and negatively charged nitrogen ions that neutralise the surface-charges of the knife and the section. This minimises the friction on the knife surface and results in ultra thin sections without crevasses or knife marks.

Improved contrast of the frozen-hydrated sections was obtained with the Zeiss EM 902 energy-filter microscope operated in the zero-loss mode.

Freeze-drying:

Cryosections were picked up on a carbon coated copper grid. Vitrification was assured by electron diffraction at ca. 100 K in a Zeiss EM 902 microscope before freeze-drying. The electron beam was then switched off and the specimen was dried by slowly warming to 173 K (readout of the cold stage)during 4 h by stepwise manual adjustment of the temperature control. The

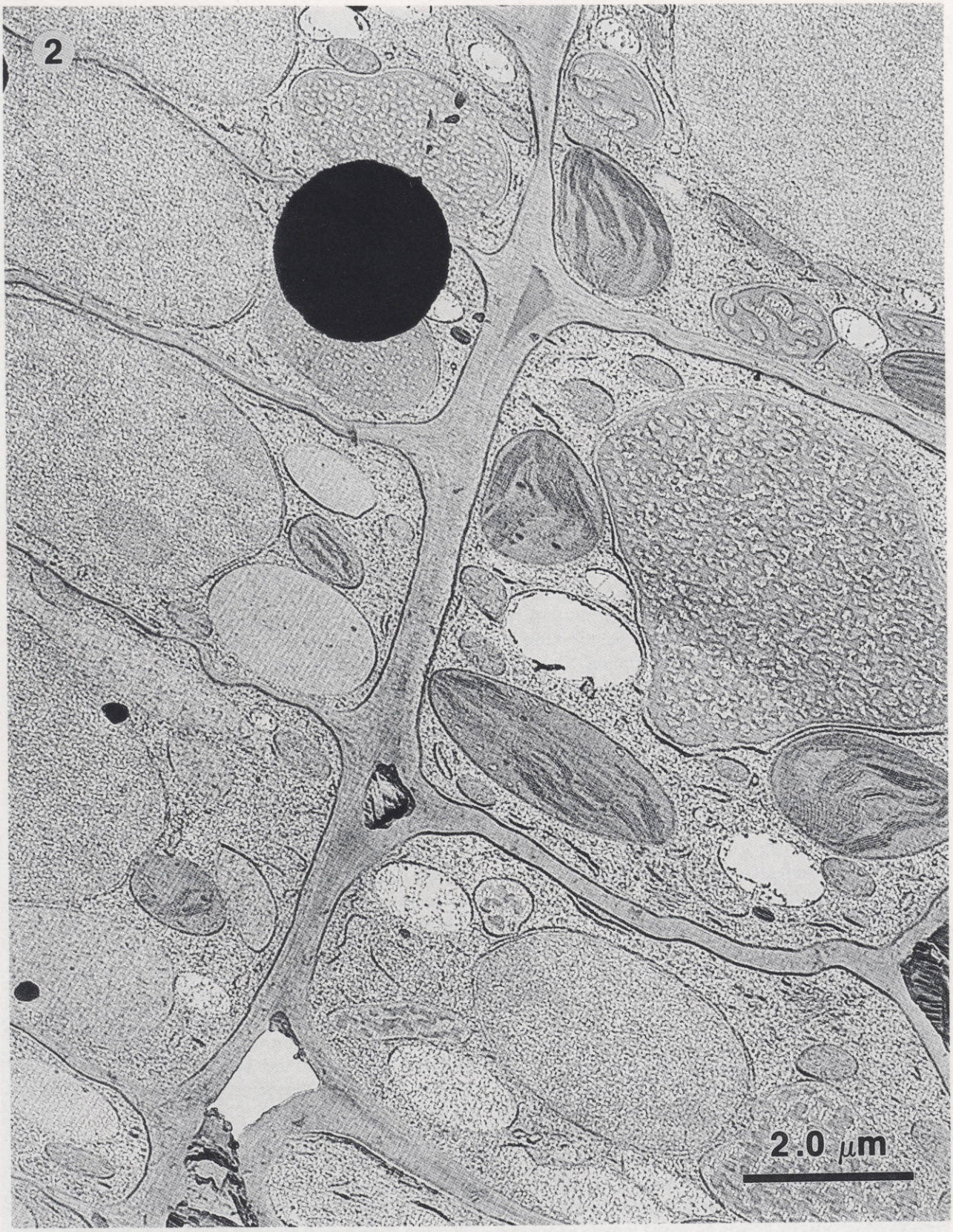

Figure 2: Freeze-dried cryosection of high-pressure vitrified "Golden Delicious" apple leaf. (Micrograph by M. Michel).

vacuum readout of the microscope was $<2x10^{-7}$ mbar. The sample temperature was then immediately returned to 100 K for photography.

RESULTS AND DISCUSSION

Figures 1 and 2 show cryosections of a high-pressure vitrified apple leaf in the frozen-hydrated (figure 1) and a partially freeze-dried (figure 2) state. They can, at present, be regarded as typical results of apple leaves, but by far, not as typical results of established procedures; many object specific aspects of cryosectioning such as local variation of hardness and density in the frozen specimen still demand optimisation for each individual sample. The process and the effects of freeze-drying are insuffieciently understood. Complete removal of the specimen water always results in shrinkage [1] but this is unfortunately essential for quantitative micronalytical studies since the neccessary high electron beam currents induce radiolysis of residual specimen water leading to rapid etching of the irradiated specimen area [15]. Shrinkage seems to be less pronounced if very slow drying regimes are applied [4, 8], whereas the standard procedure of freeze-drying at approx. 170 K followed by warming to room temperature may result in dramatic dimensional alterations. (e. g. the length of a sarcomere of initially 2.5 µm shrinks to approx. 1.6 µm, [7]. Conclusive, quantitative ion localisation studies therefore depend on compensation for shrinkage. Accurate data for compensation may be obtained from the corresponding cryosection observed in the frozen hydrated state. Figures 1 and 2 attempt to illustrate the reference function of frozen hydrated sections of vitrified samples. They may stand for a set of as yet unestablishedspecimen preparation procedures hierarchically ordered towards a quantitative biological electron microscopy as outlined above.

CONCLUSIONS

Biological, as well as biochemical, electron microscopy achieve their significance only by constantly referring to the living or biochemically controlled state. Immobilisation under physiological or analytical control by cryotechniques that produce vitrification, at present, have the best potential even though artefacts of vitrification, especially in the case of high-pressure freezing, cannot be excluded [2]. New and known specimen preparation procedures and instrumental solutions have to be interactively established along a hierarchy dictated by the complexity of the steps of specimen preparation. Frozen hydrated observation of vitrified cryosections may serve as a reference to judge the impacts of further preparative steps necessary to solve specific problems.

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