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INVITED REVIEW

Cryopreparation of biological specimens for immunoelectron microscopy

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Summary

Cryopreparation methods for transmission electron microscopy comprise a variety of different low temperature approaches. For any particular specimen a careful comparison of preparation methods should be made with respect to the preservation of structure and antigenicity. Here an overview of cryomethods is provided especially suitable for immunoelectron microscopy. Replacing chemical fixation by cryofixation, which immobilizes a sample physically by rapid freezing, is the basis of several technical lines of ultrastructure research. Cryofixed samples can be cryosectioned and directly observed by cryo-electron microscopy in their most native, fully hydrated state. For the purpose of immunoelectron microscopy, cryofixed specimens are freeze substituted and embedded for room temperature sectioning or freeze fractured for replica cytochemistry. Since the invention of high-pressure-freezing (HPF) cryofixation is routinely applicable for larger specimens of up to 200 μm thickness. Cryosectioning of chemically fixed and cryoprotected samples according to Tokuyasu results in sections which are ideal substrates for immunogold labelling. Recently, rehydration protocols have been developed to combine cryofixation with routine Tokuyasu cryosectioning. Progressive lowering of temperature (PLT) protocols are also applied to chemically fixed samples. Here dehydration and embedding are carried out accompanied by a stepwise lowering of the temperature to minimize negative effects of dehydration on the distribution and antigenicity of proteins. Conclusions drawn from ultrastructural data should always be based on the best possible technique of sample preparation. Cryofixation is the state-of-the-art method for ultrastructural preservation, but for unstable and anoxia sensitive tissues, a combination of chemical prefixation with HPF and freeze substitution might be a valuable compromise.

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Introduction

After a period of decreasing interest, electron microscopy has returned to the repertoire of cell biologists, in part because the limitations of light microscopy have been realized; i.e. the relatively low resolution in the *x* and *y*, but also in the *z*-axis, and the missing structural reference space in relation to the fluorescent signal. In addition, parallel to improvements in resolution and digital imaging in light microscopy, technical innovations in specimen preparation and the development of three dimensional imaging have also greatly increased the potential of electron microscopy. In spite of all recent developments in light microscopy, only the electron microscope demonstrates the fine structure of a cell which is directly linked to the function of its components. However, since this requires sample preparation methods such as fixation, dehydration and embedding in a plastic resin, the induction of preparation artefacts is a persistent concern.

Indeed, getting the sample into the electron beam is a long and complex process, and every step has its potential to induce artefacts. The only method, which seems to be as close to nature as electron microscopy can get so far, is cryo-electron microscopy of vitreous sections or "CEMOVIS" (Al-Amoudi et al., 2004a, b; Dubochet et al., 2007). Here, living cells or tissues are vitrified by rapid freezing and only subjected to cryosectioning below −160 °C before being viewed fully hydrated at temperatures below −180 °C in a cryo-electron microscope. Cryo-electron microscopy is used routinely for 3D reconstruction of small specimens like proteins, viruses or even small cells vitrified in a thin layer of water. CEMOVIS makes it possible to apply the same kind of preservation and "nativity" to larger biological samples, which can be analyzed in 3D by electron tomography (Lučić et al., 2005; McIntosh et al., 2005; Hsieh et al., 2006). The remarkable difference to all other sample preparation methods lies in the fact that the contrast is formed by the biological material itself instead of adsorbed heavy metals like in conventionally stained specimens. Now the idea that electron microscopy could visualize the native structure of molecules directly inside the cellular environment comes closer to reality than ever (Studer et al., 2008). However, for most of the electron microscopists cryo-electron microscopy and CEMOVIS are out of reach and immunolabelling is impossible in frozen hydrated sections. Therefore, this review is focussed on cryopreparation techniques suitable for conventional room temperature electron microscopy and immunocytochemistry.

Immunoelectron microscopy combines important information: the localization of a defined protein or lipid antigen and fine structural details. This requires well-characterized antibodies and preparation methods that are compatible with antigen recognition and ultrastructure preservation. Ideally, during tissue processing for immunoelectron microscopy the antigens of interest should maintain their structure, location and immunoreactivity within the cell. The same antigen should be equally accessible in different well-preserved subcellular structures, and simultaneous labelling of different antigens should be possible without interfering with each other. Confronted with immunoelectron microscopy in practice, as a rule, not all of these requirements can be met at the same time with one technique. Whenever possible, different methods should be applied to find a suitable compromise and to gain insight into the kind of information which can be provided by a technique. Additionally, attention should be paid to the identification of specific artefacts associated with the various protocols for the respective sample.

In this review, I will discuss cryopreparation methods such as high-pressure freezing and freeze substitution as the most important cryofixation and embedding methods, as well as the cryosectioning method according to Tokuyasu, which can be