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FREEZE-SUBSTITUTION: ITS APPLICATION AND SOME BASIC PRINCIPLES

Bruno M. HUMBEL [1]

ABSTRACT

An electron micrograph contains more information than any other imaging technique at high resolution. Therefore the goal of electron microscopist to image these details as realistic, as life-like as possible. During this quest many preparation-induced changes were discovered and openly discussed, which led to the negative reputation that electron microscopy only images artefacts. On the other hand it should be pointed-out that in the last decades, thanks to cryo-applications, the preparation methods have progressed so much that preparation artefacts are minimised and well understood. In this overview I sketch the present state of preparation methods for biological applications with attention to immunolabelling and electron tomography.

Keywords: Cryo-fixation, Freeze-substitution, Membrane contrast, Biology, Preparation methods.

DONDURARAK –YER DEĞİŞTİRME UYGULAMALARI VE TEMEL İLKELERİ

ÖZ

Elektron Mikroskobik görüntüler diğer yüksek çözünürlüklü görüntüleme tekniklerine göre daha çok bilgi vermektedir. Elektron mikroskobistlerin amacı bu detayları canlı haline en yakın görüntüliyebilmektir. Pek çok preperasyon teknikleri keşfedilmiştir ve bu tekniklerle hala tartışmaya açık olan görüntülerdeki artefaktlardır. Diğer yandan son yüzyılda kryo uygulamalarına teşekkür etmeliyiz, bu preperasyon metodu ile artefaktlar çok azaltılmış ve iyi anlaşılabilmiştir. Burada biyolojik uygulamalarda halen kullanılan immün işaretleme ve elektron tomografi teknikleri ele alınmıştır.

Anahtar Kelimeler : Kryo-sabitleme, Donma-yer değiştirme, Zar kontrastı, Biyoloji, Hazırlama metotları.

1. INTRODUCTION

Cryo-fixation is up to now the best method to preserve the cellular organisation at ultrastructural level. The most straightforward way is to image the sample in its frozen state in a cryo-electron microscope (Dubochet et al., 1988). For cellular systems only the thin border layer of cells can be imaged (Medalia et al., 2002) or the sample needs to be sectioned in the frozen state (Al-Amoudi et al., 2004; Leis et al., 2006). For most of the relevant biological samples, e.g., tissue, and for immunolocalisation studies, however, hybrid methods such as freeze-substitution (Humbel and Schwarz, 1989) or freeze-drying (Edelmann, 2002) are inevitable.

CRYO-FIXATION

Already in 1890 Altmann suggested that the method of cryo-fixation, freezing, is better than any chemical method to preserve the cellular structure as visible by light microscopy (Altmann, 1890). In the sixties of the last cen-

[1] Electron Microscopy & Structure Analysis, Molecular Architecture & Dynamics, Department of Biology, Faculty of Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

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tury the method was introduced for electron microscopy (Fernández-Morán, 1960; Van Harreveld and Crowell, 1964; Riehle, 1968) and in the years thereafter further developed (Bachmann and Schmitt, 1971; Heuser et al., 1976; Costello et al., 1983; Müller and Moor, 1984). The main important point is that freezing takes place a very high speed that the thermodynamically favoured hexagonal ice crystals cannot be formed. They would destroy the cellular organisation by pushing the components to the crystal boundaries. Today, there is a large variety of machinery to achieve cryo-fixation of which plunge-freezing and high-pressure freezing have become the most widely used. Plunge-freezing is successfully applied to embed small molecules and molecular complexes for single particle analysis or electron crystallography and high-pressure freezing for cellular electron tomography. With high-pressure freezing samples of up to 200 – 300 nm thick and 2 mm in diameter can successfully be vitrified. The most straight forward method is to image the vitrified samples directly by cryo-electron microscopy (Fernández-Morán, 1960; Henderson and Unwin, 1977; Dubochet et al., 1988) or after cryo-sectioning CEMOVIS (Al-Amoudi et al., 2004; Leis et al., 2006). This technique is best suited to gain high-resolution data but it has some drawbacks when larger cellular structures have to be investigated. In addition, except under very special circumstances (Frangakis and Förster, 2004) it cannot yet be applied for the cellular localisation of macromolecules. In these cases we have to rely and the so-called hybrid methods freeze-substitution (Humbel and Schwarz, 1989) and freeze-drying (Edelmann, 2002) followed by resin embedding.

FREEZE-SUBSTITUTION

Again freeze-substitution was introduced for light microscopy application (Feder and Sidman, 1958) and only thereafter its potential for electron microscopy was discovered (Fernández-Morán, 1959; Bullivant, 1960; Van Harreveld and Crowell, 1964). The so-called freeze-substitution (or cryo-substitution) method includes 3 steps: dehydration, chemical fixation and resin embedding.

Dehydration takes place at the lowest possible temperature that is around -90 ^{o}C to -95 ^{o}C, dictated by the freezing point of the solvents. Ideally the temperature should be lower than -140 ^{o}C, the recrystallisation/devitrification point of vitrified water (Fernández-Morán, 1960; Dubochet et al., 1988). At the first step at -90 ^{o}C the ice is dissolved by the organic solvent, mostly acetone. When chemical fixatives are added they are distributed homogeneously through the biological sample. The dehydration step is usually done for 8 hours until a few days depending on the protocols and experiences of the respective lab. It has been shown that the dehydration time is dependent on the solvent used the more polar the solvent the faster the dehydration at -90 ^{o}C (Humbel et al., 1983). On the other hand it has been suggested that the slower dehydration time of acetone is beneficial for the high resolution preservation of the ultrastructure (Studer et al., 1996).

In the second step the temperature is raised gradually or stepwise to the final, embedding temperature. At around -70 ^{o}C osmium tetroxide starts to react (White et al., 1976). Here osmium seems to react in the way as depicted in the text books of electron microscopy. The sample does not turn black as is typical for osmium tetroxide fixation. In addition these samples can be low-temperature embedded under UV polymerisation and can be successfully immunolabelled. At higher temperature -40 ^{o}C to -30 ^{o}C glutaraldhyde starts to cross-link (Humbel et al., 1983; Humbel and Schwarz, 1989). It has to be noted that the action of glutaraldehyde is not general but seems to be dependent on the proteins to be fixed, e.g., histones cannot be fixed with glutaraldehyde during freeze-substitution (Monaghan and Robertson, 1990).

At the last step the decision has to be taken in what resin the sample will be embedded and the resin determines the temperature of the final step, Lowicryl K4M, -30 ^{o}C; -50 ^{o}C HM20, -60^{o}C K11M, -80 ^{o}C HM23, LR gold, -20 ^{o}C. For epoxy or LR white embedding the first infiltration steps can be done already at -30 ^{o}C then temperature is raised with increasing concentration of resin (Matsko and Müller, 2005). Polymerisation is done the final embedding temperature for the Lowicryls and LR gold by UV light or at 60 ^{o}C for epoxy resins and LR white. There are exceptions to these protocols but they will not be discussed.

FREEZE-SUBSTITUTION AND REHYDRATION

For immunolabelling studies the cryo-sectioning method as described by Tokuyasu (Tokuyasu, 1973; Tokuyasu, 1980) and brought to perfection by the group of Slot (Liou et al., 1996; Raposo et al., 1997) proved to be the most sensitive in immunodetection of proteins. The method preserves the hydrated form of the proteins and due to the weak chemical fixation provides better three-dimensional access. For resin embedding the samples need to be dehydrated

that might have a crucial influence of the tertiary structure of the proteins. The surface of resin sections is rather smooth and only the epitops on the surface may be recognised by the antibodies. Since a long time the idea to combine the benefits of cryo-fixation with those of Tokuyasu sections lingered in the heads of several investigators but only recently two groups independently made this method available (Ripper et al., 2007; Van Donselaar et al., 2007). After cryo-fixation the samples are dehydrated and chemically fixed by freeze-substitution as described above. After freeze-substitution the samples are brought to 0^{o}C, on ice, and it is rehydrated stepwise first with water solvent mixtures and after 50 % rehydration, buffer solvent mixtures. After complete rehydration the samples are further processed for Tokuyasu cryo-sectioning according to routine protocols. This method indeed fulfils the expectations: the high label efficiency of the Tokuyasu technique paired with better morphology (Van Donselaar et al., 2007). The main impact of this technique is that it allows applying the Tokuyasu method to samples, which cannot be well-prepared with conventional chemical fixation such as e.g., plants, fungi, nematodes and insects (Ripper et al., 2007).

MEMBRANE CONTRAST

One of the main features of the freeze-substitution technique is the fact that the better the morphology the less intracellular membranes are visible. Visible means in the traditional way as either positively or negatively stained rail tracks. The lipid part of the membranes is devoid of any stain and ER can only be recognised by the ribosomes dotting the line. Also the membranes of mitochondria are barely visible. Many investigations, however, are dealing with compartments of the cells especially of the endocytic pathway (Murk et al., 2003), the Golgi system (Marsh et al., 2001; Trucco et al., 2004) or virus-cell interactions (Wild et al., 2001). Therefore a lot of attempts have been done to contrast the membranes in the substitution process. Obviously, the presence of osmium tetroxide seems to be important (Wild et al., 2001) but not always sufficient. The most promising additive is water (Walther and Ziegler, 2002). Depending on the material 1-5% water added to the acetone based substitution medium is sufficient to reveal the well-known bilayer appearance of intracellular membranes (Walther and Ziegler, 2002; Van Donselaar et al., 2007). The mechanism is not yet understood and investigations are on-going to get reproducible and well-defined membrane contrast.

ELECTRON TOMOGRAPHY

A thin-section contains three-dimensional information of a cell, even if they are very thin, e.g., 50 nm. The three-dimensional data are projected into a two-dimensional image. These images have given insight and understanding on the ultrastructure of cells and how the different tissues are organised. In general this information is sufficient to analyse the relationship of proteins and organelles within cells. For more detailed information on small continuities the chance that they are missed in physical thin sections is rather high. Here we get help with electron tomography (Crowther et al., 1970; Frank, 1992; Koster et al., 1992; Baumeister et al., 1999). With the three-dimensional impressions electron tomography provides, connections within the Golgi system could be demonstrated (Marsh et al., 2001; Trucco et al., 2004) and a new picture of neogenesis of peroxisomes was established: they can bud off from the ER system (Geuze et al., 2003). For electron tomography of a thick (300-400 nm) section a large number of images, tilted from -65^{o} to $+65^{o}$, are recorded and computed into a tomogram. In the computer this volume can be cut in slices as thin as 4 nm. By hand (Geerts et al., 2006) or more and more by automated processes the contours of interest are modelled (Frangakis and Hegerl, 2007; Lebbink et al., 2007). The model in turn can be rotated on the computer screen and observed from all angles, easing the interpretation of the results and making electron microscopy data also accessible to non-electron microscopists.

CONCLUSION

Cryo-fixation is the most reliable method to stabilise biological material in its native state. Freeze-substitution can translate the benefits of cryo-fixation to resin embedding with its advantages of immunolabelling and cellular electron tomography.

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Wild,P.,Schraner,EM.,Adler,H.Humbel BM. 2001. Enhanced resolution of membranes in cultured cells by cryoimmobilization and freeze-substitution. *Microsc Res Tech* 3, 3-321.

Bruno M. HUMBEL, I graduated in Biochemistry at the Federal Institute of Technology (ETH), Zürich, Switzerland and did my Ph.D. in Natural Sciences in the group of Dr. Martin Müller and Prof. Dr. Hans Moor at the Institute of Cell Biology, ETH. After a postdoc period of 4 years at the Max-Planck-Institute for Biochemistry in Munich I joined the lab for Electron Microscopy and Structure Analysis at Utrecht University. My main research interests are developing preparation methods for microscopy, which not only allow a glimpse into life at high resolution but also enable identification and localisation of the machinery of life within cells.