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PREPARATION OF BIOLOGICAL SAMPLES FOR ION MICROSCOPY

(Technical Report)

Prepared for publication by

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Preparation of biological samples for ion microscopy (Technical Report)

Abstract - Ion Microscopy is a powerful technique for intracellular elemental localization in biological specimens. The effective use of this technique depends on the reliability of sample preparation. An ideal sample preparation should preserve the chemical and structural integrity of a living cell, and it should also satisfy the instrumental requirements for analysis. This article critically reviews different methods of sample preparation for diffusible element analysis in soft biological tissues and cultured cells. Frozen freeze-dried tissue cryosections mounted on a smooth conducting substrate provide reliable specimens for ion microscopic analysis. For tissue culture cells, a cryo freeze-fracture freeze-dry method can be used for diffusible element localization and ion transport studies. Ion images of physiologically important diffusible elements such as Ca, K, and Na are included to show their intracellular distribution in tissue sections and cultured cells. Typical sample preparation artifacts are also discussed.

1. INTRODUCTION

Diffusible elements such as Ca, Na, K, and Mg play important roles in cell physiology, and their subcellular localization has become a major area of research. Ion microscopy, based on secondary ion mass spectrometry (SIMS) provides a powerful technique for these studies (ref. 1-7). The high sensitivity and isotopic discrimination capabilities of ion microscopy make the technique ideally suited for intracellular elemental localization studies in biological systems. Ion images provided by the CAMECA IMS-3f ion microscope reveal intracellular elemental gradients with a spatial resolution of about 0.5 μm. The true potential of this technique for biological research can only be realized if reliable sample preparation procedures are used. Physiologically important cations such as Ca^{2+}, K^{+}, and Na^{+} are highly diffusible in nature, and large gradients of these ions exist between the cellular environment and extracellular fluids. Even within the cell their distributions may be asymmetric. Live cells cannot be analyzed because of the high vacuum environment in the ion microscope. Therefore, cells must be preserved in their native state so that the analysis reveals the chemical composition of the live state. An ideal sample preparation should not alter the chemical and structural integrity of a living cell, and at the same time it should also satisfy the instrumental requirements for analysis. In this report we will critically review different methods of sample preparation for ion microscopic analysis. We recommend that fast freezing followed by freeze-drying provides a reliable sample preparation method for diffusible element analysis in biological specimens.

2. INSTRUMENTAL (ION MICROSCOPIC) REQUIREMENTS FOR SAMPLE PREPARATION

In ion microscopic analysis, the sample is held at 4500 volts in a high vacuum chamber (about 10^{-6} Pa) of the ion microscope (CAMECA IMS-3f). Therefore, the first requirement is that the sample be electrically conducting (or that neutralization of surface-charging can be effected). Second, the sample should withstand the high vacuum condition without showing any adverse effect on its chemical and structural integrity. The third and final requirement is planarity. Significant sample roughness can cause image distortion and degrade the quantitative information in secondary ion images. One should also be careful about the thickness of the specimen to be analyzed. Since biological samples normally are electrically nonconducting, a thick section (more than a few micrometers) may suffer from charging artifacts. On the other hand, thin sections as used for transmission electron microscopy are prone to quick destruction due to the surface sputtering nature of ion microscopy. In our experience 0.5-2.0 μm is a reasonable section thickness for SIMS analysis, and such sections can be analyzed without conductive coating.

3. SAMPLE PREPARATION METHODS

Sample preparation methods can be classified into three categories. First are the conventional methods which are typically used for transmission electron microscopy ultrastructural analysis (ref. 8,9). In brief, these methods involve chemical fixation of small pieces of tissues using fixatives such as glutaraldehyde, osmium tetraoxide, etc. in a buffered solution. The fixation is followed by dehydration using acetone or ethanol. Plastic resins are then impregnated into the tissue pieces. Curing of plastic impregnated material at elevated temperatures (about 60^0C) produces a solid plastic-embedded fixed tissue block. Agents such as tetrapotassium diantimonate or sodium oxalate have been used to immobilize Ca^{2+} along with chemical fixatives for in situ Ca^{2+} localization studies in conventional methods of sample preparation. Sections from conventionally fixed material can be cut on water and then deposited on conducting substrates such as silicon, gold, tantalum, etc. for ion microscopic analysis. These sections generally possess excellent details for morphological recognition but their chemical integrity is lost, especially for diffusible elements (ref. 10). These specimens produce excellent quality ion micrographs showing, however, absolutely meaningless diffusible elemental distributions.

The second category involves semi-cryo methods such as freeze substitution, and freeze drying followed by plastic impregnation. These methods have two common characteristics. In both methods, the specimen is first frozen to immobilize diffusibles, and then resins are impregnated after dehydration. The method of dehydration differs between these methods. In freeze substitution the sample is held frozen at low temperatures (about -80^0C) and water is removed by the use of organic solvents such as diethyl ether, acrylaldehyde, acetone, ethanol, etc. (ref. 11). In contrast, the freeze-drying and plastic impregnation method uses the conventional freeze-drying approach to remove water (ref. 12). Tissue prepared by either of these methods must be cut and mounted dry for SIMS analysis. Floating of sections on water may result in relocation and loss of diffusible elements (ref. 13). These methods are much better than conventional methods for diffusible elemental localization studies, but data obtained with these methods need confirmation with frozen-hydrated or frozen freeze-dried materials. Another problem that these methods face for SIMS analysis is alteration of the tissue matrix. Upon resin impregnation, tissue material becomes the minor part of the matrix. This is further complicated by the differing water density regions within the tissues (or cells). Ion microscopy matrix effects have been shown to be significant between the plastic impregnated tissue and the adjacent pure plastic regions (ref. 14,15). Unless reliable corrections for these effects are made, SIMS ion images from such specimens present qualitatively and quantitatively inaccurate distributions.

The third category of sample preparation methods involves cryo procedures without embedment, where samples are analyzed frozen-hydrated or freeze-dried. These methods are preferred since they preserve the specimen in its native state without the addition of a foreign substance such as plastic. In freeze-dried material, however, water is removed from a frozen specimen prior to analysis. Frozen-hydrated analysis is a recent innovation in ion microscopy (ref. 16,17), and a preliminary evaluation of cultured cells has shown a preferential water removal from the sample matrix upon primary ion beam bombardment (ref. 18). This complication needs to be thoroughly characterized before this procedure can be pursued further. At present, frozen freeze-dried sample preparation is the method of choice for biological ion microscopy of diffusible elements. We will discuss this method for both tissues and cultured cells.

4. FROZEN FREEZE-DRIED SAMPLE PREPARATION FOR SOFT BIOLOGICAL TISSUES

Methods for the preparation of soft biological tissue sections using cryotechniques have been driven by the need for samples of high elemental integrity in several areas of biological research. The most crucial step in cryogenic tissue preparation, both in terms of the elemental preservation and the preparation requirements for the subsequent sectioning, is the freezing of the specimen. The tissue may be dissected, but into small pieces (1 mm^3 to facilitate rapid heat transfer), and fast frozen in cryogenic fluids (ref. 19). Alternatively, *in vivo* slam-freezing methods have been developed and applied to certain tissue types to produce large volumes of well frozen specimens (ref. 20,20a). Once frozen, the tissue block may then be sectioned at low temperatures using a cryomicrotome. Typically, 0.5 to 2.0 μm thick sections are cut dry and pressed onto a planar, conducting substrate and transferred under liquid nitrogen to a freeze-drier where they are freeze-dried at or below the sectioning temperatures in preparation for ion microscopic analysis.

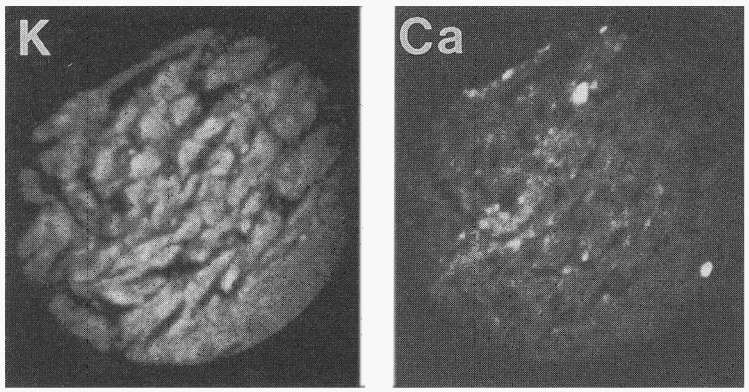

Fig. 1. Secondary ion images of $^{39}K^+$ and $^{40}Ca^+$ from cryogenically prepared freeze-dried rat liver cryosection. A 250 nA O_2^+ primary ion beam (spot size of about 60 μm) was directed onto a 250 μm^2 raster for these images. A 60 μm contrast aperture was employed to focus the secondary ion signals for these images. Image field of view is 150 μm in diameter. Ion image exposure times: $^{39}K^+$ = 1/8 s.; $^{40}Ca^+$ = 30 s.

The importance of using cryoprepared soft tissue sections for diffusible element localization in ion microscopy has been shown (ref. 10). Using improved cryomethods and indium as a specimen support, a routine method of cryosection preparation for ion microscopy has been demonstrated (ref. 21). An example of ion images obtained from a freeze-dried section of *in vivo* frozen rat liver tissue is shown in figure 1. The dark regions in the potassium image represent the sinusoids which separate the hepatic laminae. Within the hepatic cells, potassium seems to be nearly homogeneous, while calcium shows higher cytoplasmic intensities.

5. FROZEN FREEZE-DRIED SAMPLE PREPARATION FOR CULTURED CELLS

The adherence of cell growth medium to the plasma membrane of cultured cells complicates direct ion microscopic analysis of this sample type. Intracellular elemental signals can be obscured by strong elemental signals from the medium. Attempts to wash out the nutrient medium result in exposure of cells to an additional treatment and such treatments may also affect the cellular ion composition (ref. 22). This challenge has been overcome by using a sandwich freeze-fracture method developed in our laboratory (ref. 23). In brief, the sandwich freeze-fracture method involves growing cells on a conducting and nontoxic substrate like silicon. Polystyrene beads (8-12 μm) are then added 60,000-100,000 per 10 mL of the nutrient media) to the petri dish at the time of cell seeding or at the next media change. These beads act as spacers in sandwich fracture methodology. The cells now can be sampled at the desired confluency or after experimental treatments. For fracturing, the silicon piece containing the cells is removed from the nutrient medium and excessive medium is removed by tilting the silicon piece and gently touching its one edge to a filter paper. Another clean silicon piece is then used for sandwiching the cells between the two silicon surfaces. The sandwich is fast frozen in cryogenic fluids, and then fractured by prying apart the two halves under liquid nitrogen. The cells are subsequently freeze-dried at about -80 to -90^{0}C for 24 to 48 h. Such a fracture produces a few randomly scattered areas containing up to hundreds of cells grouped together where the fracture plane has passed through the apical side of the cell monolayer, leaving the half-membranes and the extracellular fluid on the nonsubstrate side of the sandwich. These apical side fractured cells allow the direct ion microscopic analysis of intracellular Ca, Na, K, etc. distributions within individual cells. The strength of this method lies in its ability to sample individual cells without perturbation of their environment.

This method has been successfully used for fracturing many established cell lines such as 3T3 mouse fibroblast, chinese hamster ovary (CHO), normal rat kidney (NRK), L6 and L5 rat myoblast cells, etc., and its reliability has been confirmed by successfully imaging Na^+/K^+ ion transport after the inhibition of Na^+, K^+-adenosine triphosphatase with ouabain (ref. 24). The spacer size may have to be adjusted according to the needs of a particular cell line. As a rule of

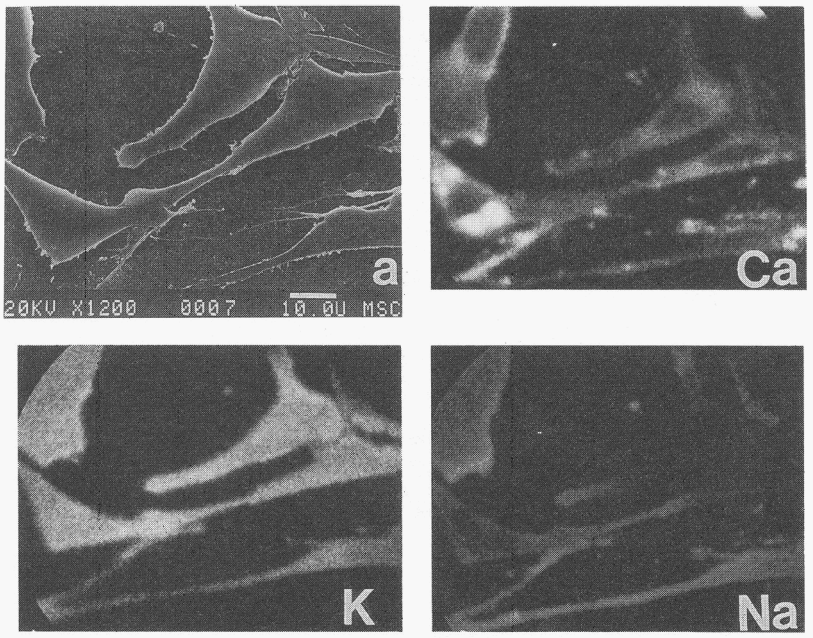

Fig. 2. Secondary electron microscopy image (SEM) of an area which contains several cryogenically prepared fractured freeze-dried dual transformed 3T3 cells (a). Intracellular distribution of Ca, K, and Na in these cells is shown in the respective secondary ion images. A 250 nA O_2^+ primary ion beam (spot size of about 60 μm) was directed onto a 250 μm^2 raster for these images. Image exposure times for potassium and sodium images were 1/8 s. The calcium image was integrated for 220 s.

thumb, the thicker the cells the bigger the bead size. In our experience 8-10 μM beads have shown good results for fracturing 3T3 cells, NRK's and CHO's, while about 12 μM beads are preferred for dual transformed 3T3's. The majority of our work has utilized cells cultured to confluency over a period of several days directly on the silicon. Samples can also be prepared by seeding a desired density of cells over the silicon and allowing the cells to attach over a few hours of incubation.

The cell confluency is not a requirement for sandwich-fracture method, an example of this is shown for dual transformed 3T3's in figure 2. Figure 2a shows a secondary electron micrograph of sandwich fractured freeze-dried cells. Intracellular distribution of Ca, K and Na in these cells is shown in the respective secondary ion images. The Na image was normalized to the K image by keeping the instrumental and photographic conditions identical between these images (ref. 23). These cells show high K, low Na intensities. While K is nearly homogeneous, the cell cytoplasm shows higher intensities of Ca.

Using the same sandwich-fracture methodology, we have additionally fractured mixed cultures for intracellular elemental studies. Figure 3a shows a SEM of a fractured area which contains mixed cultures of liver hepatocytes (rounder cell) and L6 rat muscle myoblasts. The intracellular distribution of K and Ca in these cells is shown in the respective secondary ion images. Such an analysis of mixed cultures would allow one to study the response of two different cell types to a stimulus by examining the same field of view. The mixed culture approach, however, is limited to cell lines which show clear differences in their morphological spread.

6. UNDERSTANDING SAMPLE PREPARATION ARTIFACTS

There are two main types of artifacts for ion microscopic analysis of biological samples. The first type includes sample preparation oriented artifacts, and the second category is analysis artifacts or SIMS matrix effects. The common artifacts observed in the first category are ion redistribution (spatial redistribution, gain or loss), and structural damage. These are not independent, since structural damage from poor freezing can result in ion redistribution. The second category

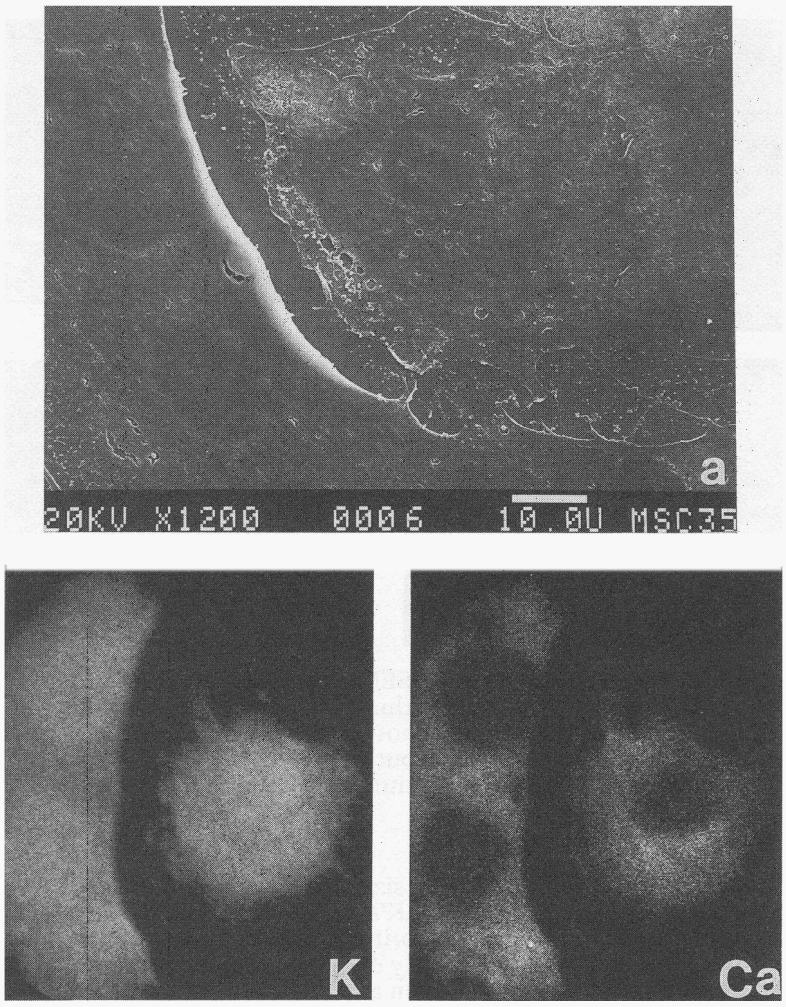

Fig. 3. Secondary electron microscopy image of an area which contains freeze-fractured freeze-dried mixed cultures of rat liver cells (rounder cell) and rat muscle myoblast cells (a binucleate myotube) (a). The intracellular distribution of K and Ca in these cells is shown in the respective secondary ion images. A 250 nA O_2^+ primary ion beam (spot size of about 60 μm was directed onto a 250 μm^2 raster for these images. The ion image exposure times for potassium and calcium images were 1/8 s. and 80 s., respectively.

includes preferential sputtering, mass interferences, and practical ion yield variations between the tissue/intracellular matrix. Obviously, there is no point studying SIMS matrix effects unless one has overcome the artifacts of the first category. SIMS matrix effects were found to be negligible between the cytoplasm and the nucleus of fractured freeze-dried cells (ref. 26,27). Similar observations have been made in frozen freeze-dried intestine and liver tissue sections (ref. 21).

An ideal sample preparation should preserve the structural and chemical integrity of a living cell. While structural damage can be precisely evaluated by electron microscopy, ionic redistribution, especially at a subcellular scale, is difficult to evaluate. However, certain criteria may be used as guidelines to evaluate the preservation of the chemical integrity of a cell. For example, healthy cells maintain a relatively high potassium/low sodium intracellular environment by the action of plasma membrane enzymes. The extracellular nutrient medium is low potassium/high sodium by comparison. Injury to the plasma membrane results in ion fluxes which elevate intracellular sodium and lower intracellular potassium. A massive accumulation

of Ca in mitochondria is an additional characteristic of an injured cell (ref. 25). A dead cell would typically show high sodium-low potassium signals, reflecting the composition of the nutrient medium. The extent of the injury may differ from cell-to-cell, and the reason of the injury may be the sample preparation or the treatment in question. A poor freezing of the specimen would not only damage the cellular structure but also result in redistribution of ions since damaged membranes can no longer serve as barriers to the ion gradients within the cell, and between the cell and extracellular fluid. We have previously shown alteration of ion composition in damaged cultured cells (ref. 23). The strength of ion microscopy lies in its ability to analyze individual cells and indicating damaged or dead cells based on their ion signals alone so that such cells can be discarded from any physiological explanations.

7. CONCLUSION

A reliable sample preparation is the first necessity for ion microscopic analysis of diffusible ions in soft biological matrices. At present, frozen freeze-dried unembedded material provides the best sample type. High sensitivity and isotopic detection capabilities of ion microscopy are well suited for many biomedical problems. With the understanding of SIMS matrix effects, ion image quantification has been achieved in fractured freeze-dried cultured cells (ref. 28). This would enhance the biomedical applicability of ion microscopy.

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