Scanning Microscopy

Volume 1994
Number 8 *The Science of Biological Microanalysis*

Article 11

4-19-1994

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Scanning Microscopy Supplement 8, 1994 (pages 129-138) 0892-953X/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

THE PREPARATION OF CULTURED CELLS FOR X-RAY MICROANALYSIS

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(Received for publication January 31, 1994, and in revised form April 19, 1994)

Abstract

X-ray microanalysis of cells in culture is being used increasingly for the study of relationships between element (ion) content and cell function. There is, however, no one single method which can be used for the preparation of all different cell types for study by microanalysis. Cells in suspension are usually concentrated by centrifugation, before cryofixation, cryosectioning, and freeze drying. On the other hand cells grown as monolayers are more often studied as whole cell mounts, which are washed to remove the external medium before cryofixation and freeze drying. The alternative approach, sectioning of cell monolayers is rarely used. Some of the difficulties encountered in preparing and monolayers of smooth muscle cells for X-ray microanalysis are discussed here.

Key Words: Cell culture, X-ray microanalysis, sodium, potassium, smooth muscle, cardiac myocyte

Introduction

The first major review of the application of electron probe X-ray microanalysis (EPXMA) to single and cultured cells was that of Wroblewski and Roomans (1984). Examination of this latter paper reveals that the preparation techniques used up to that date, especially for single cells, relied mainly on air drying, or on conventional (glutaraldehyde fixation plus embedding) techniques. In the intervening decade, there has been a considerable increase in the applications of EPXMA to the study of isolated cells and cells in culture, with the emphasis of preparation methods shifting towards the use of cryopreparation (Table 1). The purpose of the present paper is to review the techniques which are used most frequently for cryopreparation of isolated and cultured cells and to evaluate their application to the study of problems in biology.

Isolated cells and cells in culture can be divided into two types, those which are isolated or occur naturally in suspension, and those which are cultured as an adherent monolayer. Methods used for preparing the specimens differ for these two categories, in consequence, each will be considered separately here.

Cells Isolated or Cultured in Suspension

This category comprises cells which occur naturally in suspension, such as the components of the blood, cells grown in suspension, and cells, or cell aggregates, which are freshly isolated using various procedures. For these cells a straight forward preparation procedure which comprises concentration of the cells by centrifugation, cryofixation of the resulting pellet, cryosectioning, and freeze drying of the cryosections before analysis, is used. Cells which have been prepared in this way include: HeLa S_3 cells (Warley and Stephen, 1985); macrophages (Smith *et al.*, 1985); proximal tubule cells or aggregates (LeFurgey *et al.*, 1986; Zierold and Schäfer 1988); thymocytes (Warley, 1986); chromaffin cells (Ornberg *et al.*, 1988); liver cells (Zierold and

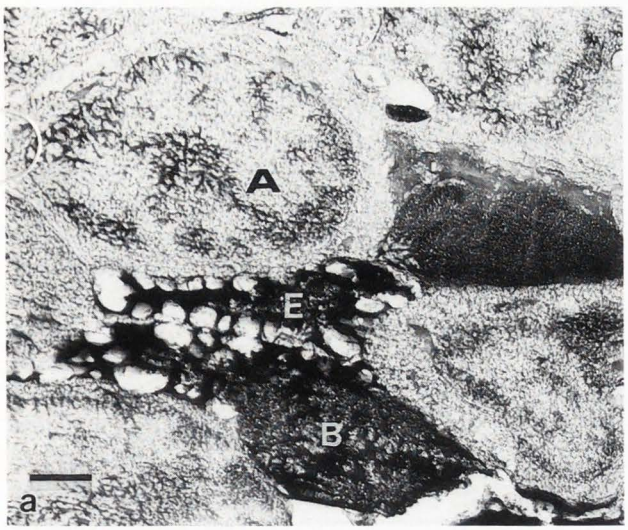
Table 1. Some applications of X-ray microanalysis to the study of isolated cells or cells in culture since 1983.

Cell type	Preparation method	Reference
Chondrocytes	Whole mounts/cryopreparation	Wroblewski *et al.* (1983)
Chromaffin	Cryosections	Ornberg *et al.* (1988)
Erythrocytes	Cryosections	Lew *et al.*, (1985)
Endothelial	Whole mounts/cryopreparation	Hall *et al.* (1992)
Epithelial	Whole mount/cryopreparation	von Euler *et al.* (1993)
Fibroblasts	Cryosections	Zierold *et al.* (1984)
	Whole mount/ cryopreparation	Abraham *et al.* (1985)
(CF patients)	Whole mount/ cryopreparation	von Euler and Roomans (1991)
HeLa S_3	Cryosections	Warley and Stephen (1985)
Liver	Cryosections	Zierold and Schäfer (1988)
Lymphocytes	Whole mounts/air dried	Hook *et al.* (1986)
Macrophages	Cryosections	Smith *et al.* (1985)
Muscle Cardiac	Cryosections	Wendt-Gallitelli and Isenberg (1989)
(Adult)	Cryosections	Ward and Warley (1990)
(Neonatal)	Cryosections	Buja *et al.* (1985)
	Cryosections	Murphy *et al.* (1986)
Muscle smooth	Whole mount/cryopreparation	Warley *et al.* (1993)
Myoblasts	Whole mount/cryporeparation	von Zglinicki and Wroblewski (1992)
Oil Palm	Cryosections	Warley *et al.* (1985)
Platelets	Whole mount/air dried	Boekestein *et al.* (1985)
Prostate	Cryosections	Tvedt *et al.* (1988)
Renal tubule	Whole mounts/cryopreparation	Larsson *et al.* (1986)
	Cryosections	LeFurgey *et al.* (1986)
	Cryosections	Zierold and Schäfer (1988)
	Whole mount/cryopreparation	Pavenstadt-Grupp *et al.* (1989)
Thymocytes	Cryosections	Warley (1986)

Table 2. Some substrates used for growing cells for study by X-ray microanalysis as whole mount preparations.

Substrate	Cells	Method of Analysis	Reference
Steel grids	Smooth muscle	STEM	James-Kracke *et al.* (1980)
Ti grids	Chondrocytes	STEM	Wroblewski *et al.* (1983)
	Fibroblasts/epithelia	STEM	von Euler *et al.* (1993)
Au grids	Glioma	STEM	Zierold (1981)
	Smooth muscle	STEM	Warley *et al.* (1993)
Carbon plate (plus formvar)	Chondrocytes	STEM	Wroblewski *et al.* (1983)
Graphite discs	Fibroblasts	EPMA	Abraham *et al.* (1985)
Silicon pieces	Renal tubules	EPMA	Larsson *et al.* (1986)
Thermanox	Fibroblasts	SEM	Zierold and Schäfer (1988)
Cytodex beads	Endothelium	SEM	Hall *et al.* (1992)
Collagen membranes	Fibroblasts	SEM	von Euler *et al.* (1993)

Note: Grids used for analysis in STEM are coated with a thin plastic film of either Formvar or Pioloform.



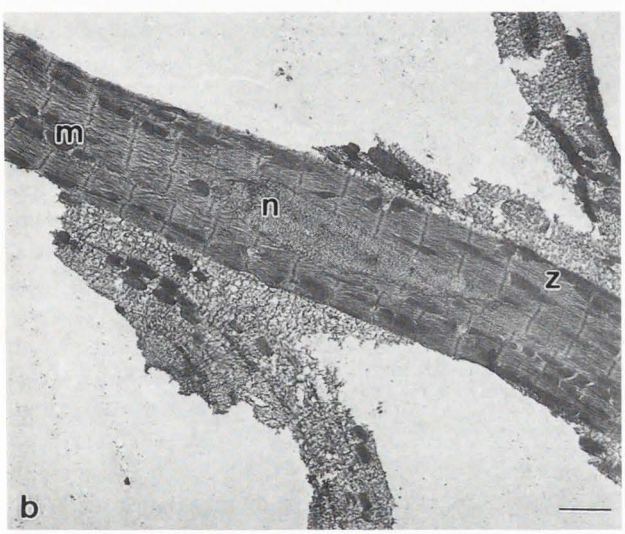


Fig. 1. Morphological detail in cells which have been pelleted and cryosectioned before X-ray microanalysis. a) Thymocytes isolated from diabetic rats. A: Thymocyte, B: pyknotic cell, E: Erythrocyte. Marker = 1 μm. b) Isolated cardiac myocyte, n: nucleus, z: z-line, m: mitochondria. Marker = 2 μm.

Schäfer, 1988) and cardiac myocytes (Ward and Warley, 1990).

The advantages of this preparation technique are (i) its relative simplicity, (ii) good morphological detail is obtained (see Fig. 1a, b) allowing analysis of element concentrations at the subcellular level, and (iii) there is no need for complete removal of the external medium, since medium which remains between the cells should not interfere with the subsequent analysis. There has been some argument, however, as to whether the steps involved, particularly centrifugation, might introduce

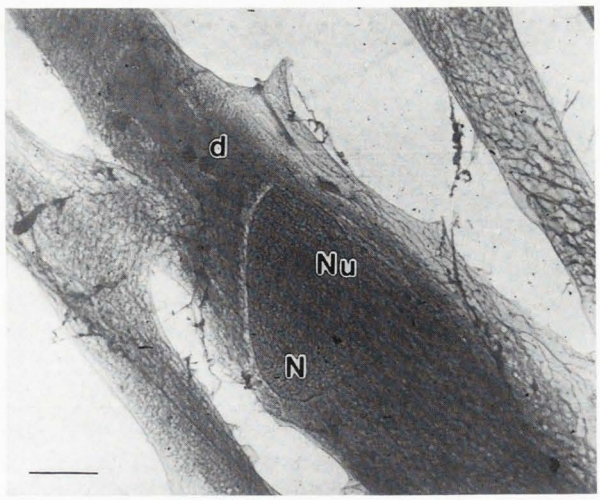


Fig. 2. Whole mount of ASM prepared for X-ray microanalysis by washing in distilled water. N = nucleus, Nu Nucleolus, d dense bodies. Marker = 5 μm.

artifacts. There is little evidence to support this contention. Many groups employ centrifugation in a microfuge (10,000 g) for short periods of time (maximum 3 min). The high K/Na ratios obtained in the ensuing analysis suggest that redistribution of intracellular elements has not occurred. Indeed, work on $HeLaS_3$ cells (Warley and Stephen, 1985) shows that redistribution of intracellular Na and K with that from the extracellular fluid only occurs if the cell membrane is already damaged before centrifugation is carried out, for example after infection of HeLa S_3 cells with virus. Although in a study on thymocytes (Warley, 1986) I was able to show some differences in element concentrations when the cells were prepared in the presence of different media, the differences were more likely to be caused by direct effects of the medium on the cells, rather than the centrifugation procedure. Interestingly, higher centrifugal forces may cause intracellular redistribution of ions. Cameron *et al.*, (1986) showed redistribution of K and Cl in *Euglena* which had been stratified by centrifugation at 100,000 g for one hour.

When freshly isolated cells are being studied, the isolation procedure itself often results in disturbance of intracellular elemental content, and it is important that freshly isolated cells are allowed a minimum time of incubation at 37°C in a compatible medium, to allow the intracellular element concentrations to recover. The necessity of the incubation step has been shown for thymocytes (Warley, 1986), rat liver cells and papillary collecting ducts (Zierold and Schäfer, 1988) and cardiac

myocytes (Ward and Warley, 1990).

Cells Grown as Monolayers

The majority of cells grown in tissue culture do not grow in suspension but form monolayers which adhere to the surface on which they are grown. For these cells the simple preparation procedures outlined above are not appropriate, the cells cannot be removed from their substrate before analysis since this is known to cause damage to the cell membrane and redistribution of ions (Szallasi *et al.*, 1988). The approach that is used to prepare such cells for microanalysis is, therefore, either to grow the cells on a substrate which is suitable for electron microscopy, and analyse the cells as whole cell mounts after washing away the growth medium, or to grow the cells on a substrate which can be sectioned, and to cryofix and cryosection the cells before analysis. This latter approach has been used in only a few applications to date.

Preparation as Whole Cell Mounts

This approach was introduced by Somlyo's group (James-Kracke *et al.*, 1980) and has proved popular, being used for studies using the scanning electron microscope (SEM), the electron probe microanalyser (EPMA) and the scanning transmission electron microscope (STEM). The substrate on which the cells are grown varies, the choice usually depends on the instrument which is to be used for the subsequent analysis (Table 2). The substrate should preferably not produce characteristic X-rays which would interfere with analysis especially when cells are being analysed in the SEM mode and penetration of the cell by the electron beam may occur. Problems which may occur in quantification of results from cells grown on a solid substrate and analysed in the SEM are discussed in von Euler *et al.* (1992) and Wroblewski and Wroblewski (1993).

Cells may be supported on the substrate by being allowed to attach after isolation and then used after a short period of time, this needs to be determined experimentally for each cell type. Cells can also be carried successfully through culture for a number of days. If cells are being supported, rather than grown on the substrate it is important, as outlined above, that some time is allowed to enable the cells to recover from the isolation period before analysis is undertaken, for example, von Euler *et al.* (1993) allowed a period of 4-6 hours. On the other hand, if cells are being grown on the substrate for prolonged periods, then it is important that the substrate does not interfere with the growth characteristics of the cells and experiments to determine increase of cell numbers on different substrates should be carried out (Abraham *et al.*, 1986; Warley *et al.*, 1994).

Cells which are prepared and analysed as whole mounts, need to be subject to a washing procedure to remove the external medium which interferes with the subsequent analysis. As emphasised by others (Wroblewski and Wroblewski, 1993; von Euler *et al.*, 1993), the ideal washing solution must be capable of removing the culture medium effectively, but should not cause damage to the cell membrane or leakage of ions. The solutions which are commonly used for this purpose are isotonic sucrose, isotonic ammonium acetate, or distilled water, all are usually used at a temperature of 0-4°C. The reactions of different cell types to these different washing solutions varies, and it is necessary to determine experimentally the medium of choice for the cell type under investigation. Airway smooth muscle (ASM) cells which have been grown on Pioloform covered gold grids, and washed in ice cold distilled water before freezing and freeze drying are shown in Fig. 2.

Undoubtedly, one of the difficulties with this approach is determining the effectiveness of the washing process, since often the intracellular element concentrations in the cell line of interest are not known, and there may not be sufficient cells available for conventional analysis to be performed. Various criteria which have been used to determine the effectiveness of washing include: comparison with unwashed controls; high intracellular [K] coupled with low intracellular [Na] leading to high K/Na ratios, and high K/P or K/S ratios, but each of these on its own cannot be considered entirely satisfactory. It might seem that comparison with unwashed controls should provide information about the removal of elements such as Mg and K, which are present at low concentrations in growth medium. However, it is sometimes found that these elements are lower in the unwashed cells than in the washed cells (see Table 3, and Wroblewski *et al.*, 1983; Pavenstadt-Grupp *et al.*, 1989); this is opposite to the expected result. The reason for this finding is not exactly clear but may, in part, be explained by absorption of characteristic X-rays by overlying medium in the unwashed cells, or by its contribution to the generation of continuum (Warley *et al.*, 1994). In consequence, comparison with the unwashed cells using results obtained by X-ray microanalysis may, on its own, not be an effective means of determining whether movement of elements has occurred. Comparison of results for K obtained from washed cells using X-ray microanalysis with those obtained from unwashed cells using another technique will, however, confirm the validity of the washing

Table 3. Element concentrations in rabbit airway smooth muscle cells after washing in different media.

	Animal	n	Not Washed	Sucrose	Distilled H_2O
Na	43	18	983 ± 106	169 ± 7	145 ± 10
	62	19	1026 ± 99	50 ± 5	80 ± 6
Mg	43	18	55 ± 22	38 ± 2	40 ± 2
	62	19	39 ± 2	38 ± 4	45 ± 2
P	43	18	235 ± 10	394 ± 15	417 ± 11
	62	19	398 ± 16	421 ± 17	484 ± 15
Cl	43	18	591 ± 80	183 ± 10	188 ± 7
	62	19	771 ± 72	113 ± 7	157 ± 7
K	43	18	291 ± 38	397 ± 16	439 ± 24
	62	19	512 ± 24	461 ± 25	594 ± 21

Data given in mmoles/kg dry weight, n = number of observations

technique (Abraham *et al.*, 1985).

High values for the ratio K/Na suggests that the washing procedure has been effective in removing Na, and thus the overlying medium from the cell, and as discussed in Zierold and Schäfer (1988) this is probably the best indicator of the health of the cells under study. High values for the ratios of K/P (Abraham *et al.*, 1985) and K/S (Roomans, 1991), have also been used to assess effectiveness of the washing procedure, but their use is based on the assumption that these elements represent adequately the dry mass content of the cell. In some cell types, however, one, or both, of the components of the ratio might vary in individual cells. For example, Zierold and Schäfer (1988) reported variations in cytoplasmic K and P in isolated rat liver cells, Hall *et al.* (1992) have described variation in K content in cultured endothelial cells as the cells approach confluence, we have shown variation in K concentrations in ASM cells in culture (Warley *et al.*, 1993), and von Euler *et al.*, (1993) have described variation in K content in cultured colon cancer cells. In such instances ratios of K to another element cannot be used reliably to assess the effects of different washing procedures.

Each method of assessing the effectiveness of a given washing technique may, on its own, have some problems associated with it. In the many studies which have now been carried out, however, it is generally found that the results from different methods of assessing effectiveness agree, and usually demonstration of the known effects of treatment with ouabain (see Fig. 3) is used to confirm the validity of the washing procedures.

Cryosections of Cells Grown as Monolayers

The ideal means for determining the validity of a given washing procedure should be comparison of the results from the washed cells with those from monolayers of cells which have been cryofixed and cryosectioned, that is, cells which have not been subject to removal of the growth medium. To date, however, few results have been obtained from such systems (see Table 4). The methods used by Buja *et al.* (1985) and Tvedt *et al.* (1988) where cells are grown on a support that can be sectioned seemed the best approach for sectioning monolayers of ASM which are extremely thin and difficult to section en-face. Our attempts to produce sections from ASM cells reliably, however, met with several difficulties. In our experience, the use of collagen as a substrate for cell culture should be approached carefully, since its presence may modify the behaviour of cells under study. For example, we have found that, whereas rabbit ASM cells did not grow on a collagen substrate, human ASM cells grew readily, but showed different behaviour (Hirst and Warley, unpublished results). Both rabbit and human ASM cells grew readily on laminin coated Pioloform which was supported over a Thermanox coverslip (details of this preparation are given in Warley *et al.*, 1994), but we experienced difficulties in supporting the preparation for cryosectioning. The preparation forms a thin wafer of cells plus Pioloform which is very delicate and tended to fracture easily when held in a vice type chuck as recommended in Buja *et al.* (1985) and Tvedt *et al.* (1988). We found that sections could be obtained on a more routine basis

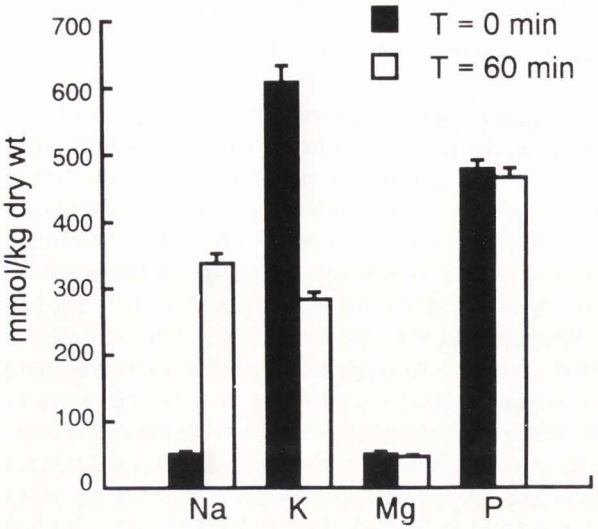


Fig. 3. Element concentrations in cultured rabbit ASM before (0 min) and after (60 min) treatment with ouabain (50 μM), details from Warley *et al.* (1993).

when the thin wafer of Pioloform plus cells was glued into the notch on a Reichert specimen pin (details in Warley *et al.*, 1994) using n-heptane as a cryoglue (Steinbrecht and Zierold, 1984).

When such a cryoglueing technique is used difficulties may be encountered with the freeze drying technique. Our routine drying procedure is to mount the grids bearing sections in a brass block which has been pre-cooled in liquid nitrogen, and to freeze dry these overnight in a conventional (tissue) freeze-dryer evacuated by a rotary pump (vacuum about 10^{-2} mbar). Sometimes the cryosections appeared rehydrated (Fig. 4a). Such rehydration never occurred with the whole mount preparations (Fig. 2) or with sections which had not been subject to cryoglueing (Fig. 1b). The appearance of the rehydration artefact was similar to that attributed by Hagler and Buja (1986) to melting of lipids, which suggested the possibility that, although all attempts were made to keep the surface of the specimen from contact with the n-heptane, sometimes contamination occurred, and that the n-heptane was melting during the uncontrolled freeze drying process which takes place at a relatively low vacuum and under which the temperature of the specimen is not controlled. As far as I am aware, such problems in using n-heptane as a cryoglue have not been reported previously. Steinbrecht and Zierold (1984) carried out freeze drying in the electron microscope at a much better vacuum (10^{-4} mbar) than we have used here. When freeze drying takes place under more controlled conditions of temperature and at higher vacuum (details in Table 5) using an Emitech K775

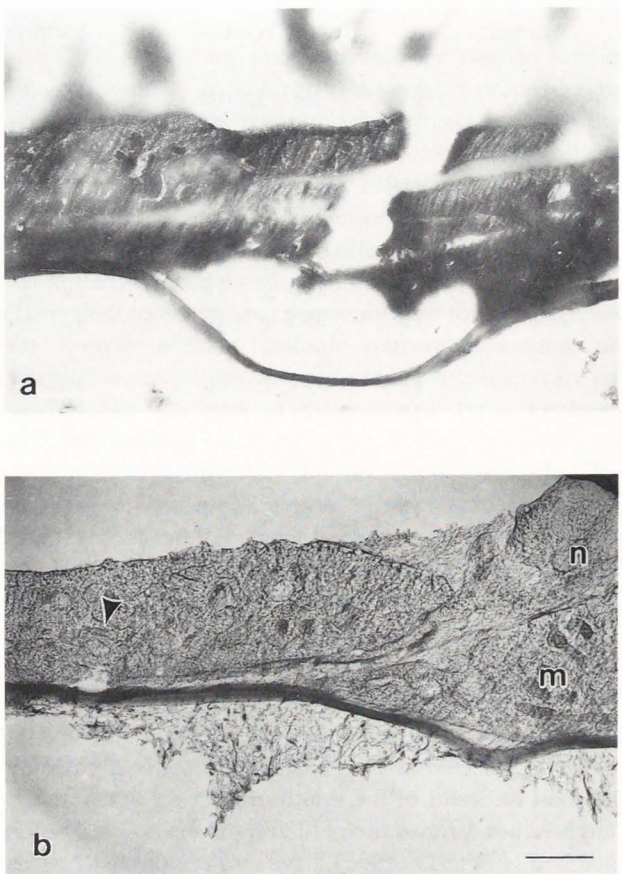


Fig. 4. a) Preparation of ASM which has been cryosectioned and freeze dried at low vacuum, note rehydrated appearance. b) Preparation of ASM which has been freeze dried under controlled conditions (for details see text), subcellular structures such as nucleus (n), mitochondria (m) and RER can be seen, Marker = 1 μm.

freeze dryer the rehydration artefact is not seen (Fig. 4b).

Comparison of Results from Washed Cells with Those from Sectioned Cells

To determine whether washing in distilled water caused gross disturbance of intracellular element concentrations in the ASM cells, we compared results obtained from whole cell mounts which had been prepared by washing in distilled water followed by cryofixation, with results from the analysis of sections of cultured cells derived from the same animal and passage number, and cultured under the same conditions (that is an identical source), which had been subject to cryofixation and cryosectioning. This comparison is shown in Fig. 5.

Table 4. Applications in which monolayers of cells have been cryosectioned and analysed by X-ray microanalysis

Cell type	Method of sectioning	Reference
Smooth muscle	Layer of cells + film gathered onto stub	James-Kracke *et al.* (1980)
Fibroblasts	en-face	Zierold *et al.* (1984)
Neonatal myocytes	Perpendicular to growth	Buja *et al.* (1985)
Prostate carcinoma	Perpendicular to growth	Tvedt *et al.* (1988)
Liver/renal tubule	en-face	Zierold and Schäfer (1988)

Table 5. Conditions used for freeze drying sections of ASM cells in a temperature controlled turbomolecular freeze drier (vacuum better than 10^{-2} mbar).

Temperature (°C)	Time (h)
-100	1
-100 to -70	1
-70	1
-70 to -50	1
-50	1
-50 to +25	1

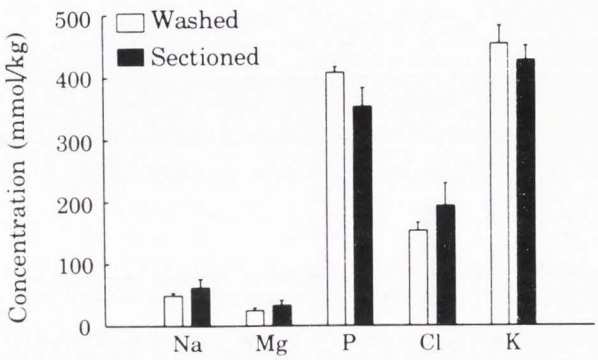


Fig. 5. Comparison of element concentrations in ASM from the same animal which have been prepared either as whole mounts washed in ice cold distilled water or cryosectioned. The only significant difference was in the concentration of P which was lower in the sectioned cells.

These results are mean values for element concentrations derived from analysis of 10 cells in each case. The only significant difference in elemental content between the two preparations was for [P] which was lower in the cryosectioned cells. We have now obtained results from a number of animals and they are essentially the same (Warley *et al.*, 1994). There are few other such comparisons which have been made. In their early work on cultured aortic smooth muscle cells James-Kracke *et al.*, (1980) compared cryosections of the cells with the unwashed whole mount preparations. They found that concentrations of Na and K were lower in their sectioned cells than in the whole cells. We find that our results for (washed) whole mount ASM cells compare favourably with those reported by James-Kracke *et al.*, (1980) for vascular smooth muscle. We do, however find lower concentrations of Na and Cl in our sectioned cells than those reported by the latter authors. The only other such comparison that we are aware of is that mentioned by Pavenstadt-Grupp *et al.* (1989) who compared the results from isolated whole mounts of papillary collecting duct cells, with the values published by Zierold and Schäfer (1988) for cryosections of cells from the same source. Good agreement was found between the two preparations.

Conclusions

The past decade has seen an increased use of cryotechniques coupled with X-ray microanalysis to study relationships between elemental content and function in a number of different cell types. There is no one method which can be used for preparing all cell types for X-ray microanalytical studies, and the method used depends very much on the type of cell and the purpose of the study. Cryosectioning of pelleted cells has proved to be a reliable method for study of suspended cells, whereas whole cell mounts are used more frequently for estimation of elemental content in monolayers of cells. Despite some uncertainties about the effects of the different washing procedures, this method has proved reliable for the measurement of fluxes of ions and has drawn attention to the variability of individual cells, which is not seen when conventional methods of analysis are used. This technique is very easy to use and the preparation requires a very small number of cells, and a short period of time, and no doubt, will be increasingly used with routinely cultured cells. Cryosectioning of monolayers still remains the most technically difficult of all the methods for preparing cultured cells for study by X-ray microanalysis, but the increasing realisation that spatial organisation of subcellular

elements may be important in control of cell function will add impetus to the continued development of this technique.

Acknowledgements

I would like to thank the Wellcome Trust for financial support, Dr S.J. Hirst for culturing the airway smooth muscle cells, and Mrs K.P.B. Cracknell for technical support.

This paper is dedicated to the memory of Dr B.L. Gupta in acknowledgement of the help and encouragement that he always gave so freely.

References

Abraham EH, Breslow JL, Epstein J, Chang-Sin P, Lechene C (1985) Preparation of individual human diploid fibroblasts and study of ion transport. Am J Physiol **248:** C154-164.

Boekestein A, Kuijpers GAJ, Stols ALH, Stadhouders AM (1985) Elemental analysis of individual rat blood platelets by electron probe X-ray microanalysis using a direct quantification method. Histochemistry **82**: 257-264.

Buja LM, Hagler HK, Parsons D, Chien K, Reynolds RC, Willerson JT (1985) Alterations of ultrastructure and elemental composition in cultured neonatal rat cardiac myocytes after metabolic inhibition with iodoacetic acid. Lab Invest **53:** 397-412.

Cameron IL, Hansen JT, Hunter KE, Padilla GM (1986) Elemental concentration gradients between subcellular compartments. J Cell Sci **81:** 283-297.

Hagler HK, Buja LM (1986) Effect of specimen preparation and section transfer techniques on the preservation of ultrastructure, lipids and elements in cryosections. J Microsc **141:** 311-318.

Hall SR, Sigee DC, Beesley JE (1992) Scanning X-ray microanalysis of microcarrier cultured endothelial cells: elemental changes during the transition to confluency and the effect of ionophore A23187. Scanning Microsc **6:** 753-763.

Hook GR, Elin RJ, Hosseini JM, Swyt C, Fiori CE (1986) A sample preparation for quantitative determination of magnesium in individual lymphocytes by electron probe X-ray microanalysis. J Microsc **141:** 69-78.

James-Kracke MR, Sloane BF, Shuman H, Karp R, Somlyo AP (1980) Electron probe analysis of cultured vascular smooth muscle. J Cell Physiol **103:** 313-322.

Larsson S, Aperia A, Lechene C (1986) Studies on final differentiation of rat renal proximal tubular cells in culture. Am J Physiol **251:**455-464.

LeFurgey A, Ingram P, Mandel LJ (1986) Heterogeneity of calcium compartmentation: electron probe analysis of renal tubules. J Memb Biol **94:** 191-196.

Lew VL, Hockaday A, Sepulveda MI, Somlyo AP, Somlyo AV, Ortiz OE, Bookchin RM (1985) Compartmentation of sickle-cell calcium in endocytotic inside out vesicles. Nature **315:** 586-589.

Murphy E, Wheeler DM, LeFurgey A, Jacob R, LoBaugh LA, Liebermann, M (1986) Coupled sodium-calcium transport in cultured chick heart cells. Am J Physiol **250:** C442-452.

Ornberg RL, Kuijpers GA, Leapman RD (1988) Electron probe microanalysis of the subcellular compartments of bovine adrenal chromaffin cells. J Biol Chem **263:** 1488-1493.

Pavenstadt-Grupp I, Grupp C, Kinne RH (1989) Measurement of elemental content in isolated papillary collecting duct cells by electron probe microanalysis. Pflugers Arch **413:** 378-384.

Roomans GM (1991) Cryopreparation of tissue for clinical applications of X-ray microanalysis. Scanning Microscopy Suppl **5**: S95-S107.

Smith NKR, Lewinski AJ, Mangos JA, Boyd RL (1985) Stimulus-permeability coupling in rat pulmonary macrophages challenged by *Pseudomonas aeruginosa*. An X-ray microanalysis study. Cell Tissue Res **240:** 461-465.

Steinbrecht RA, Zierold K (1984) A cryoembedding method for cutting ultrathin cryosections from small frozen specimens. J Microsc **136:**69-75.

Szallasi Z, Szallasi A, Bojan F, Zs-Nagy I (1988) Effect of enzymic (collagenase) harvesting on the intracellular Na^+/K^+ ratio of Swiss/3T3 cells as revealed by X-ray microanalysis. J Cell Science **90:** 99-104.

Tvedt KE, Halgunset J, Kopstad G, Haugen OA (1988) Quick sampling and perpendicular cryosectioning of cell monolayers for the X-ray microanalysis of diffusible elements. J Microsc **151:**49-59.

von Euler A, Roomans GM (1991) X-ray microanalysis of cAMP-induced ion transport in cystic fibrosis fibroblasts. Cell Biol Int Rep **15:** 891-898.

von Euler A, Wroblewski R, Roomans GM (1992) Correction for extraneous background in X-ray microanalysis of cell cultures. Scanning Microscopy **6:** 451-456.

von Euler A, Pålsgård E, Vult von Steyern C, Roomans GM (1993) X-ray microanalysis of epithelial and secretory cells in culture. Scanning Microsc **7:** 191-202.

Ward JPT, Warley A (1990) Subcellular elemental composition in isolated cardiac myocytes from rabbit following chronic potassium depletion and acute repletion, studied by X-ray microanalysis. Exp Physiol **75:**

855-858.

Warley A (1986) Element concentrations in isolated rat thymocytes prepared for cryofixation in different media. J Microsc **144:** 183-191.

Warley A, Stephen J (1985) X-ray microanalysis of synchronised $HeLaS_3$ cells infected with vaccinia virus. J Cell Sci **75:** 401-409.

Warley A, Ferdinando D, Hughes WA (1985) Application of X-ray microanalysis to cell suspensions of oil palm (*Elias guineensis* Jacq.). Planta **165:** 527-531.

Warley A, Hirst SJ, Twort CHC, Ward JPT (1993) Variable potassium content in rabbit cultured tracheal smooth muscle cells. Cell Biol Int Rep **17:** 689-690.

Warley A, Hirst SJ, Twort CHC, Ward JPT (1994) Preparation of airway smooth muscle cells for study of element concentrations by X-ray microanalysis. J. Microsc., in press.

Wendt-Gallitelli MF, Isenberg G (1989) X-ray microanalysis of single cardiac myocytes frozen under voltage-clamp conditions. Am J Physiol **256:** H574-583.

Wroblewski J, Roomans GM (1984) X-ray microanalysis of single and cultured cells. Scanning Electron Microsc 1984; IV, 1875-1882.

Wroblewski J, Roomans GM, Madsen K, Friberg U (1983) X-ray microanalysis of cultured chondrocytes. Scanning Electron Microsc 1983; II, 777-784.

Wroblewski J, Wroblewski R (1993) X-ray microanalysis of cultured mammalian cells. In: X-ray Microanalysis in Biology: Experimental Techniques and Applications. (Sigee DC, Morgan AJ, Sumner AT, Warley A, eds), Cambridge University Press, Cambridge, pp 317-329.

von Zglinicki T, Wroblewski R (1992) Examination of low-level cadmium effects by X-ray microanalysis. In: *Electron Microscopy 1992* (Proc Eur Cong Electron Microsc) (Rios A, Arias JM, Megias-Megias L, López-Galindo A, eds) vol 1, pp 371-372.

Zierold K (1981) X-ray microanalysis of tissue culture cells in SEM and STEM. Scanning Electron Microsc 1981; II, 409-418.

Zierold K (1993) Rapid freezing techniques for biological electron probe microanalysis. In: X-ray Microanalysis in Biology: Experimental Techniques and Applications (Sigee DC, Morgan AJ, Sumner AT, Warley A, eds), Cambridge University Press, Cambridge, pp 101-116.

Zierold K, Schäfer D (1988) Preparation of cultured and isolated cells for X-ray microanalysis. Scanning Microsc **2:** 1775-1790.

Zierold K, Schäfer D, Pietruschka F (1984) The element distribution in ultrathin cryosections of cultivated fibroblast cells. Histochemistry **80:** 333-337.

Discussion with Reviewers

J. Wroblewski: In Table 3 there is a large discrepancy in the Na concentration between the two animals used. What do you think has caused this difference?
Author: Data presented in Table 3 represents some of the earlier work done on this system. I have found that as more work has been carried out values for [Na] have become lower and more reproducible.

J. Wroblewski: X-ray microanalysis allows simultaneous analysis of several elements. In the majority of the published work, that you have referred to, data on the concentration of S and Ca were included. In the present work you have chosen to omit these elements. Could you please comment why, and why different elements are presented in Table 3 compared to Figs. 3 and 5?
Author: The exclusion of S and Ca from the current results reflects my own preferences. Because of the known susceptibility of S containing material to mass loss under the electron beam I am always a little wary of attaching meaning to values for this element, even though since the measurements were carried out under identical conditions mass loss could be assumed to be equivalent. Additionally since I am not sure about the exact nature of the S containing material in these particular cells it is difficult to attach significance to the results for this element. Ca was present at low levels in these cells (about 3 mmol/kg) , since analysis was carried out on whole cells and over a wide area these levels will represent both general cytosolic levels of Ca and Ca present in the intracellular stores. Since high levels of Ca which would be indicative of cell death were not found, the results for Ca were not included. The exclusion of values for Cl from Fig. 3 has no particular significance. Fig. 3 was originally published in Cell Biology International and the selection of elements was made to confirm with the column width of that journal. The values for [Cl] were 138 $\pm$ 19 and 179 $\pm$ 8 mmol/kg for T = 0 and T = 60 min respectively.

J. Wroblewski: The possible adverse effects of centrifugation are discussed but not the effects of isolation by enzymic digestion, especially the effects of different enzymes (trypsin versus collagenase etc.). From other cell biological studies it is known that mechanical stress (centrifugation) induces the expression of some proto-oncogenes (especially c-fos) and that enzymatic digestion is not harmless to these cells. Could you please comment on that.
Author: My emphasis on the possible adverse effects of centrifugation stems from the studies on thymocytes and lymphocytes which did not need enzymatic digestion for

removal from the tissue, and thus the cells were only subject to mechanical trauma. Naturally one would expect that trypsin and collagen would have different effects when used for isolating cells, since trypsin is a protease and thus might cause some membrane damage and could feasibly trigger cells. In our studies on cardiac myocytes the cells were isolated by exposure to collagen.

G.M. Roomans: Do you not think that you are being over critical of the use of K/P or K/S ratios to check the washing procedure? Of course, the washing procedure should be checked by exposing **the same culture** to different procedures. If K varies as a result of cell growth, this should not make any difference. Even in a heterogeneous system such as that described by von Euler *et al.* (1993) one could study the K/P or K/S ratios of the "high-K" population - of course one should not take the average of the whole culture.

Author: I probably am being over critical about the use of K/P and K/S ratios but this stems from my experiences with the smooth muscle cells where K/P ratios proved unreliable. I think that the use of K/P ratios can be used as you suggest in a heterogeneous system, but only if the heterogeneity is seen in the morphology of the cell.