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X-RAY MICROANALYSIS OF CULTURED CELLS IN THE SCANNING ELECTRON MICROSCOPE AND IN THE SCANNING TRANSMISSION ELECTRON MICROSCOPE: A COMPARISON

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Abstract

X-ray microanalysis of cultured cells as "whole mounts" (i.e., not sectioned) is used frequently e.g., to study mechanisms of ion transport. Cells can be cultured either on solid substrates or on thin plastic films on grids. Cells cultured on solid substrates are analyzed in the scanning electron microscope at relatively low accelerating voltage, cells cultured on thin films can be analyzed in the (scanning) transmission electron microscope at high accelerating voltage. The main advantage of culturing the cells on a solid substrate is that the specimen is less easily damaged during preparation. Analysis of cells on thin films is more sensitive, and subcellular resolution is possible. The main problem here is the correction for extraneous background that has to be applied in quantitative analysis. The following test systems were used in this study: HT29 human colon carcinoma cells, primary cultures of human sweat gland coil cells, and normal bronchial human epithelial (NHBE) cells. The spread in the data is similar for cells grown and analyzed on a solid substrate compared to cells grown and analyzed on a thin substrate. The spread is much larger than expected on the basis of counting statistics, and is not reduced by using elemental ratios rather than net peak intensities. This means that the spread is not mainly caused by overpenetration or extraneous background, but that likely biological variation between the cells is responsible.

Key Words: X-ray microanalysis, cultured cells, scanning electron microscopy, scanning transmission electron microscopy, substrate, extraneous background.

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Introduction

X-ray microanalysis of cultured cells can be a useful technique to elucidate (patho)physiological mechanisms of ion transport. The use of cell cultures has several well-known advantages over the use of *in vivo* systems: the cell culture system is simpler, since it usually consists of only one cell type, systemic effects can be avoided, and especially in the case of human cells, *in vivo* experiments are often not possible because of practical or ethical constrictions. Preparation of cell cultures by freezing is often simpler than preparation of tissue, because dissection artefacts can be avoided. Cultured cells can be analyzed both as whole mounts at the cell level and after sectioning at the subcellular level (Warley, 1994).

Both cell lines and primary cell cultures have been used (reviewed, e.g., by Wroblewski and Roomans, 1984; von Euler et al., 1993; Warley, 1994; Hongpaisan *et al*., 1994; Wroblewski and Wroblewski, 1994; Roomans *et al*., 1996). Examples of systems studied include fibroblasts (Abraham *et al*., 1985; von Euler and Roomans, 1991), sweat gland cell lines (Mörk *et al*., 1995) and cell lines from colon cancer (von Euler and Roomans, 1992; W. Zhang and Roomans, 1998), primary cultures from sweat glands (Hongpaisan and Roomans, 1998), respiratory epithelium (Sagström *et al*., 1992), tracheal glands (A.L. Zhang and Roomans, 1997), and uterine epithelium (Jin and Roomans, 1998), airway smooth muscle cells (Warley *et al*., 1993, 1994) and uterine smooth muscle cells (Hongpaisan and Roomans, 1996).

There are, of course, alternative techniques to investigate ion transport in cultured cells, e.g., the Ussing chamber technique, radioisotope labeling, and techniques using fluorescent dyes. The advantage of X-ray microanalysis over the Ussing chamber technique and radioisotope labeling is that X-ray microanalysis gives information at the single cell level. This can be important if the cell culture is not homogeneous with regard to its elemental content, as we have observed in colon cancer cell lines (von Euler and Roomans, 1992) or if the cells are not homogeneous with regard to their response to a physiological stimulus (Mörk *et al*., 1995). The technique also needs very little material. With regard to fluorescent techniques, the

fura-2 technique to measure Ca^{2+} ions (Grynkiewicz *et al*., 1985) is not really comparable, because it measures free Ca^{2+} ions, whereas X-ray microanalysis measures total elemental Ca. Fluorescent techniques to measure intracellular chloride (Verkman, 1990) are more closely comparable with X-ray microanalysis, since they allow measurement at the single cell level, and the difference between the chloride activity measured by the fluorescent technique and the Cl concentration measured by X-ray microanalysis is not supposed to be very significant. However, it does not appear that a direct comparison of the two techniques on the same cell system has been carried out yet.

Many of the X-ray microanalytical studies on cultured cells were carried out by having the cells grow on a solid substrate (e.g., Abraham *et al*., 1985; von Euler and Roomans, 1991, Hall *et al*., 1992); these cells were then analyzed in the scanning electron microscope (SEM) at relatively low accelerating voltage. However, in other studies, grids covered with a thin plastic (Formvar, Pioloform) film were used to grow the cells on and the specimen was analyzed in the scanning transmission electron microscope (STEM) at relatively high accelerating voltage (James-Kracke *et al*., 1980; von Euler and Roomans, 1992; Warley *et al*., 1993, 1994). Since our group has experience with both ways of culturing and analyzing cells, a comparison of the advantages and disadvantages of these different methods might be of interest. The human colon adenocarcinoma cell line HT29 was grown on a solid substrate and analyzed in the SEM, whereas sweat gland coil cells and normal human bronchial epithelial (NHBE) cells were grown on grids and analyzed in the STEM. It should be emphasized that in the following, only analysis of cells as "whole mounts" is discussed, and not analysis of (cryo)sections of cell cultures, which is in principle not different from analysis of (cryo)sections of tissue.

Materials and Methods

HT29 colon adenocarcinoma cells analyzed in the SEM

HT29 human colon adenocarcinoma cells were grown in Dulbecco´s modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in an incubator. Subconfluent cells were harvested with 1 mM EDTA in Hank's balanced salts medium without Ca and Mg, and seeded directly on the cellulose nitrate filters which are compatible with the cell culture conditions and with X-ray microanalysis. After the cells had attached to the filters, 3-4 ml complete culture medium were added and the cells were allowed to grow for 2-3 days before the experiments.

To test the response of the cells to various physiological stimuli, the cells on the filters were incubated in standard Krebs Ringer's buffer (KRB) (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid] (HEPES) and 5 mM glucose, at pH 7.4, to which the agent studied was added. Prior to the incubation, the cells were quickly rinsed with KRB corresponding to the different incubation solutions to remove the culture medium. The incubation was terminated by washing the filters respectively with one of the following washing fluids: (a) distilled water, (b) 150 mM ammonium acetate or (c) 300 mM mannitol for 5 sec only to remove the NaCl-rich experimental solution. Comparison of the results showed that rinsing with distilled water or ammonium acetate gave adequate results (W. Zhang and Roomans, 1998). After blotting excess fluid with a filter paper, the cells were frozen immediately in liquid propane cooled by liquid nitrogen and freeze-dried overnight at -30°C. The dried filters were coated with a conductive carbon layer to avoid charging in the electron microscope.

The cells on the filter were analyzed in a Philips 525 scanning electron microscope (SEM) (Philips Electron Optics, Eindhoven, The Netherlands) with a Link AN 10000 energy-dispersive X-ray microanalysis system (Oxford Instruments, Oxford, UK) at 20 kV. Quantitative analysis was performed by determining the ratio (P/B) of the characteristic intensity (peak, P) to the background intensity (B) in the same energy range as the peak and comparing this P/B ratio with that obtained by analysis of a standard, which consisted of known concentrations of mineral salts in a 20% gelatin and 5% glycerol matrix, frozen, cryosectioned and freeze dried to resemble the specimen in its physical and chemical properties (Roomans and Sevéus, 1977; Roomans, 1988a). Each spectrum was acquired for 100 seconds. Only one spectrum was acquired from each cell. No correction for extraneous contributions to the spectrum was applied

Sweat gland coil cells and NHBE cells analyzed in the STEM

Sweat gland coil cells. Normal human skin samples were obtained from patients (20-50 years old) undergoing mastectomy or abdominal surgery. The isolation of the sweat gland coil has been described in detail in Hongpaisan *et a*l. (1996). The coil was cultured in 25 cm^2 tissue culture flasks (Costar, Cambridge, MA) containing 800 µl culture medium. The culture medium consisted of William's E medium, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), insulin (10 µg/ml), transferrin (10 µg/ml), hydrocortisone (5 ng/ml), epidermal growth factor (10 ng/ml), trace element mix (0.5% v/v; Gibco BRL/Life Technologies, Paisley, UK) and 20 mM HEPES, to which 1% fetal calf serum was added. When a cellular outgrowth was seen, additional culture medium was added. After 7-14 days, the cultured cells were incubated with 1 ml dispase (Boehringer, Mannheim, Germany)

for 30-45 min. Sheets of cells detached from the floor of the culture flasks were allowed to recover in culture medium and then seeded out on 75 mesh titanium grids (Agar Scientific, Stansted, UK). The grids had been covered with a Formvar (Merck, Darmstadt, Germany) film and coated with a thin carbon layer. The grids were sterilized under ultraviolet light before use. The cells were allowed to attach and spread for 3-7 days at 37°C in a humidified atmosphere of 5% CO_2/95% air in a culture chamber.

NHBE cells. Normal bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA) were used. Cell cultures were established at Clonetics Corporation's cell culture facilities from normal human tissue obtained from a 16-year old female. The cells were cultured in plastic culture flasks (Costar) in bronchial epithelial basal medium (BEGM) (Clonetics) supplemented according to the manufacturer's instructions with recombinant human epidermal growth factor (0.5 µg/ml), insulin (5 mg/ml), hydrocortisone (0.5 mg/ml) transferrin (10 mg/ml) adrenalin (0.5 mg/ml), triiodothyronine (6.5 µg/ml), bovine pituitary extract (13 mg/ml), retinoic acid (0.1 µg/ml), gentamicin (50 mg/ml) and amphotericin B (50 mg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The culture medium was changed every 48 h. From these cultures, cells were seeded out on titanium grids as described above, and allowed to grow for 2-3 days.

Preparation and analysis. The cells on the grids were rinsed briefly in cold distilled water (4°C), frozen in liquid nitrogen-cooled liquid propane (-180°C), freeze-dried in vacuum overnight at -130°C and slowly brought to room temperature under vacuum. Finally, the freeze-dried specimens were coated with a conductive carbon layer.

X-ray microanalysis was performed at 100 kV in the STEM mode of a Hitachi H7100 electron microscope with an Oxford Instruments ISIS energy dispersive spectrometer system (Oxford Instrument, Oxford, UK). Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background (Roomans, 1988a) and by comparing the spectra from the cells with those of a standard (Roomans, 1988a). Spectra were acquired for 100 seconds and only one spectrum was obtained from each cell.

Methodological Aspects and Discussion

The experience with analysis of the two types of specimens showed four important differences:

(1) Cells grown on a solid substrate have to be analyzed at low accelerating voltage to avoid overpenetration of the beam, and even then, overpenetration cannot always be avoided. If the cells grow in a single layer, the freeze-dried specimen is in effect only about 3 µm thick and easily penetrated already by a 10 kV beam. This means

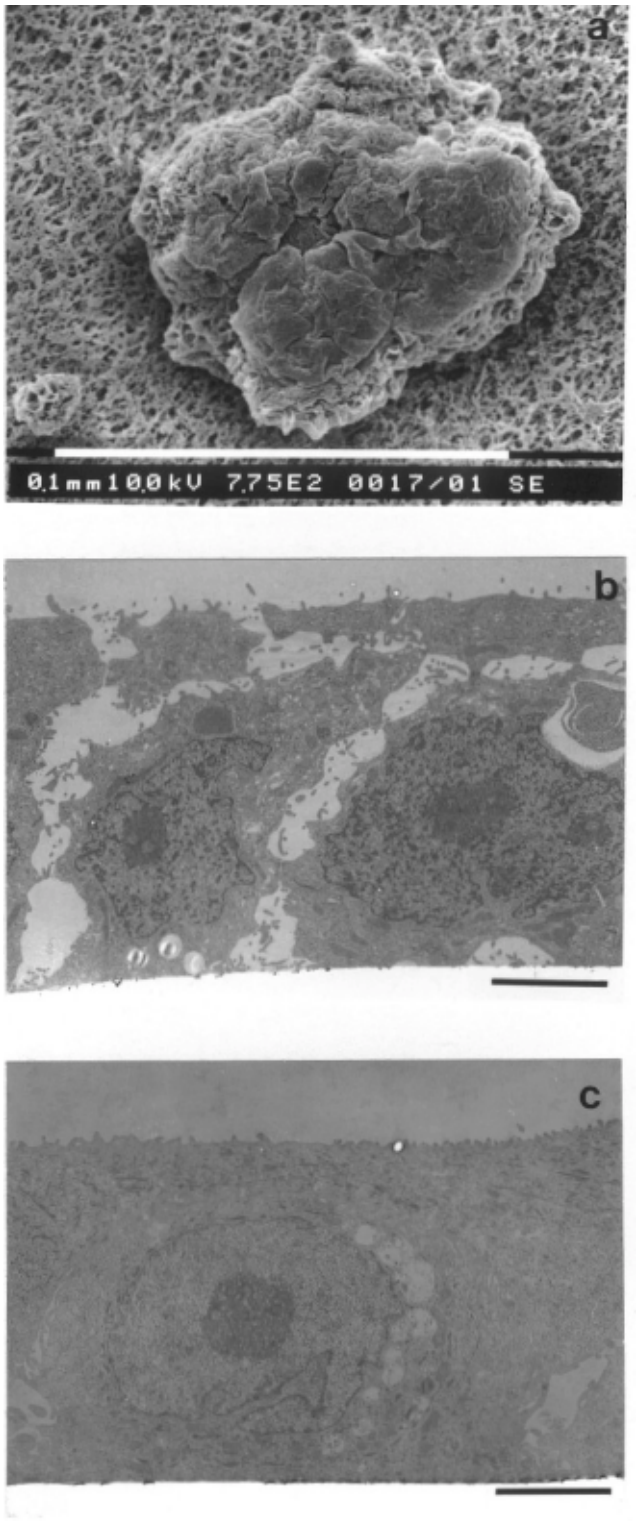

Figure 1. (a) Scanning electron micrograph and (b) transmission electron micrograph of HT29 cells growing in multilayers; (c) transmission electron micrograph of NHBE cells growing as a single layer. Bar = 100 µm in (a) and 5 µm in (b) and (c).

that apart from the cell, also the substrate is excited. The substrate only contains elements with an atomic number < 10 which are not seen by a conventional detector, but it "dilutes" the elements present in the specimen. This results in lower peak-to-background ratios. Cells grown on thin films on grids can be analyzed at high accelerating voltage, which gives an optimal signal and peak-to-background ratio, since the peak-to background ratio increases with increasing accelerating voltage. This increases the sensitivity of the analysis.

The HT29 cells grow several cell layers thick (Fig. 1a). The effect of overpenetration can be monitored by carrying out the analysis at different accelerating voltages. Therefore, the HT29 cells were analyzed at accelerating voltages ranging from 10 to 20 kV, but this did not affect the results. It could therefore be concluded that, since the HT29 grow as multilayers, there is no appreciable overpenetration, even at 20 kV. In contrast, the NHBE cells are very thin and grow in a single cell layer (Fig. 1b).

(2) To obtain the best possible signal, analysis of cells grown on a solid substrate must, in practice, always be carried out over the thickest part of the cell, i.e., the nucleus. In cells grown on thin films, analysis of the cells can be carried out both over the nuclear area and on cytoplasmic areas where no nucleus is present. This allows data on potential differences between nucleus and cytoplasm to be collected.

(3) The specimens where cells are grown on a solid substrate are sturdier and less likely to be damaged during preparation and analysis. The films on the grids may be broken during washing, freezing, freeze-drying and analysis. This is particularly annoying if the experiment is designed to check the time course of a reaction, or the concentration dependency of the effects of a physiological or pharmacological agonist or antagonist (see e.g., W. Zhang and Roomans, 1997). In such an experiment loss of a few grids may invalidate the entire experiment. This led Zhang (1997) to conclude that for this reason alone, solid substrates would be preferable to thin substrates.

(4) The analysis of cells on thin substrates is complicated by the presence of the extraneous background, i.e., the background caused by film, the grid, and possibly the specimen holder and parts of the electron microscope in the immediate surrounding of the specimen. The role of the extraneous background in quantitative analysis of thin sections was first pointed out by Gupta and Hall (1979) and formalisms were developed by Gupta and Hall (1979) and by Roomans and Kuypers (1980). The formalisms have been incorporated in the software for quantitative biological microanalysis provided by manufacturers of energy-dispersive X-ray analysis systems. The correction for extraneous background is in principle carried out in the following way: measurements are carried out (1) on a bare grid without film or specimen, (2) on the film beside the specimen, and (3) on the specimen. A background area without peaks is selected for determination of the background intensity. The corrected background for the specimen B_{sp} is given by:

$$B_{sp} = W_{sp} - W_f - [(G_{sp}-G_f)/G_g]W_g \quad (1)$$

where W_{sp} is the measured background on the specimen (measurement 3), W_f is the measured background on the film (measurement 2), W_g is the measured background on the bare grid (measurement 1), G_{sp} is the net peak for the grid metal measured on the specimen (measurement 3), G_f the net peak for the grid measured on the film (measurement 2) and G_g the net peak for the grid metal measured on the bare grid (measurement 1).

The problems with this correction are well known and have been dealt with in several publications (Roomans, 1988b; von Euler *et al*., 1992; Kopstad, 1993). The correction is based on a very simple model of electron-specimen interaction. Experience in practice shows that this simple model does not hold, and that serious errors may occur unless one carefully matches the site of measurement on specimen and film with regard to position relative to grid bar and to specimen holder. Often, specimen holders made of low atomic number material (beryllium, carbon) are used and a proper correction for the contribution of these holders to the background cannot be calculated. Finally, if the specimen is thin, the factor $W_f - [(G_{sp}-G_f)/G_g]W_g$ in equation (1) may be in the same order of magnitude as the factor W_{sp}. Since each factor in the equation has a statistical error, and these errors are propagated, the resulting statistical error in B_{sp} may become relative large. As a consequence of this problem, one may encounter considerable variation in the calculated intensities, due to variations in the correction for extraneous background. This variation is a major problem in analysis of thin sections in general and also in STEM analysis of cultured cells.

When cells grown on a solid substrate are analyzed, there is also an extraneous contribution, namely from the substrate, and possibly from the specimen holder and the microscope. These latter two factors are probably not very important. The specimen chamber in the SEM is much larger than in a (scanning) transmission electron microscope, so there is no metal directly surrounding the specimen. Whether there is a substantial contribution from the substrate or specimen holder surrounding the specimen is difficult to determine when they are made of low atomic number material, as is usually the case. The contribution of the substrate below the specimen may be considerable and may, in addition, vary with the thickness of the specimen.

Since the advantages and disadvantages on first sight appear to balance, experimental data were collected to de-

Table 1. X-ray microanalysis of cultured cells on different substrates.

HT29 cells (analyzed in the SEM on a thick substrate)

experiment	P	sd%	K	sd%	P/K	sd%
1	519	24	362	19	1.44	17
2	538	14	391	27	1.49	35
3	537	10	317	16	1.71	11
4	519	20	407	11	1.28	21
5	607	10	352	11	1.75	19
average	544	7	367	10	1.53	13

NHBE cells (analyzed in the STEM on a thin substrate)

experiment	P	sd%	K	sd%	P/K	sd%
1	692	13	894	18	0.79	13
2	802	17	777	34	1.15	47
3	693	16	800	30	0.92	31
4	803	27	729	40	1.16	26
5	751	21	658	28	1.19	25
6	804	21	688	32	1.23	24
average	758	7	758	11	1.07	16

human sweat gland coil cells (analyzed in the STEM on a thin substrate)

experiment	P	sd%	K	sd%	P/K	sd%
1	718	19	941	24	0.79	21
2	599	18	872	18	0.70	15
3	396	22	530	17	0.74	10
average	571	29	781	28	0.74	6

P: net intensity for phosphorus, K: net intensity for K, P/K: ratio of phosphorus to potassium
sd%: standard deviation in %

termine (1) the extent of the statistical variation under different conditions, (2) whether these variations are less in STEM or in SEM, and (3) whether the variations are larger in the measurement of the elemental peaks compared to measurements of elemental ratios. In principle, the elemental ratios should be insensitive to errors in the determination of the extraneous background in the analysis of cells on grids, and also insensitive to the contribution of the substrate to the background in analysis of cells on a solid substrate. The results of these analyses are given in Table 1.

The data in Table 1 allow some interesting and surprising conclusions. The errors are in the same order of magnitude for the SEM and for the STEM measurements and the data do not seem to favor one method over the other. The errors in the measurements of the elemental peaks are much larger than the statistical counting error. The statistical counting error should be equal to the square root of the number of counts, and in the range of 700-1000 counts, the error should only be about 3-4%. The error in the elemental ratios is in the case of the NHBE cells and the HT29 cells equal to or even larger than the error in the individual elemental intensities. This indicates that the variation cannot be always explained by the complications introduced by the extraneous sources (background or substrate). It would therefore appear that biological variation between the analyzed cells is a very important factor determining the spread in the results.

It should be pointed out, that the HT29 cells grow in multilayers, which is optimal for SEM analysis, since problems with overpenetration are minimized. On the other hand, the analytical volume will contain more than one cell, and also some intercellular space. Rinsing of the cells will probably remove most of the solutes from the intercellular space, but this is difficult to monitor. It should, therefore, be considered to analyze such multilayered cell cultures after sectioning, rather than as whole mounts.

Conclusions

X-ray microanalysis of cultured cells as "whole mounts" is a relatively fast method to determine cellular elemental contents and changes therein due to physiological and pathological processes. Cells growing in single cell layers should preferably be grown on thin plastic films on a suitable support and analyzed in STEM. Cells growing in multilayers may be grown on solid substrates and analyzed in SEM. If care is taken to standardize analytical conditions, the main source of variation seems to be the biological variation between cells.

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References

Abraham EH, Breslow JL, Epstein J, Chang-Sing P, Lechene C (1985) Preparation of individual human diploid fibroblasts and study of ion transport. Am J Physiol **248**: C154-C164.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem **260**: 3440-3450.

Gupta BL, Hall TA (1979) Quantitative electron probe microanalysis of electrolyte elements within epithelial compartments. Fed Proc **38**: 144-153.

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 Microscopy **6**: 753-763.

Hongpaisan J, Roomans GM (1996) Preparation of cultured smooth muscle cells from human myometrium for X-ray microanalysis. Scanning Microsc **10**: 1192-1200

Hongpaisan J, Roomans GM (1998) Extracellular UTP regulates Na, Cl and K transport in primary cultures from human sweat gland coils. Cell Struct Function **23**: 239-245.

Hongpaisan J, Mörk A-C, Roomans GM (1994) Use of in vitro systems for X-ray microanalysis. Scanning Microsc Suppl **8**: 109-116.

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.

Jin Z, Roomans GM (1998) X-ray microanalysis of uterine epithelial cells in culture. Micron **28**: 453-457.

Kopstad G (1993) Quantitative X-ray Electron Probe Microanalysis of Thin Biological Sections. Doctoral Thesis 1993:65. University of Trondheim.

Mörk AC, Hongpaisan J, Roomans GM (1995) Ion transport in primary cultures from human sweat gland coils studied with X-ray microanalysis. Cell Biol Int **19**: 151-159.

Roomans GM (1988a) Quantitative X-ray microanalysis of biological specimens. J Electron Microsc Techn **9**: 19-44

Roomans GM (1988b) The correction for extraneous background in quantitative X-ray microanalysis of biological thin sections: some practical aspects. Scanning Microsc **2**: 311-317.

Roomans GM, Kuypers GAJ (1980) Background determination in quantitative X-ray microanalysis of biological thin sections. Ultramicroscopy **5**: 81-83.

Roomans GM, Sevéus LA (1977) Preparation of thin cryosectioned standards for quantitative microprobe analysis. J Submicrosc Cytol **9**: 31-35.

Roomans GM, Hongpaisan J, Jin Z, Mörk AC, Zhang A (1996) *In vitro* systems and cultured cells as specimens for X-ray microanalysis. Scanning Microsc Suppl **10**: 359-374.

Sagström S, Roomans GM, Wroblewski R, Keulemans JLM, Hoogeveen AT, Bijman J (1992) X-ray microanalysis of cultured respiratory epithelial cells from patients with cystic fibrosis. Acta Physiol Scand **146**: 213-220.

Verkman AS (1990) Development and biological applications of chloride-sensitive fluorescent indicators. Am J Physiol **259**: C375-C388.

von Euler A, Roomans GM (1991) X-ray microanalysis of cAMP-induced ion transport in cystic fibrosis fibroblasts. Cell Biol Int Rep **10**, 891-898.

von Euler A, Roomans GM (1992) Ion transport in colon cancer cell cultures studied by X-ray microanalysis. Cell Biol Int Rep **16**: 293-306.

von Euler A, Wróblewski R, Roomans GM (1992) Correction for extraneous background in X-ray micro-

analysis of cell cultures. Scanning Microsc **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.

Warley A (1994) The preparation of cultured cells for X-ray microanalysis. Scanning Microsc Suppl **8**: 129-138.

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

Warley A., Cracknell KP, Cammish HB, Twort CH, Ward JP, Hirst SJ (1994) Preparation of airway smooth muscle cells for study of element concentrations by X-ray microanalysis. J Microsc **175**: 143-153.

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

Wroblewski R, Wroblewski J (1994) X-ray microanalysis of endocrine, exocrine and intestinal cells and organs in culture: technical and physiological aspects. Scanning Microsc Suppl **8**: 149-162.

Zhang AL, Roomans GM (1997) Ion transport in cultured pig tracheal submucosal gland acinar cells studied by X-ray microanalysis. Eur Resp J **10**: 2204-2209.

Zhang W (1997) Regulation of Chloride Secretion in HT29 Cells Studied by X-ray Microanalysis. Doctoral Thesis, University of Uppsala.

Zhang W, Roomans GM (1997) Evidence for muscarinic-3 receptor mediated ion transport in HT29 cells studied by X-ray microanalysis. Cell Struct Function **22**: 379-385

Zhang W, Roomans GM (1998) Volume induced chloride transport in HT29 cells studied by X-ray microanalysis. Microsc Res Techn **40**: 72-78.