Backscattered electron imaging of cultured cells: application to electron probe X-ray microanalysis using a scanning electron microscope

E. FERNÁNDEZ-SEGURA,* F. J. CAÑIZARES,*† M. A. CUBERO,* F. REVELLES* & A. CAMPOS*
*Department of Cell Biology, School of Medicine, University of Granada, E-18071 Granada, Spain
†Institute of Neurosciences, University of Granada, E-18071 Granada, Spain

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Summary
We report a simple method to study the elemental content in cultured human adherent cells by electron probe X-ray microanalysis with scanning electron microscopy. Cells were adapted to grow on polycarbonate tissue culture cell inserts, washed with distilled water, plunge-frozen with liquid nitrogen and freeze-dried. Unstained, freeze-dried cultured cells were visualized in the secondary and back-scattered electron imaging modes of scanning electron microscopy. With backscattered electron imaging it was possible to identify unequivocally major subcellular compartments, i.e. the nucleus, nucleoli and cytoplasm. X-ray microanalysis was used simultaneously to determine the elemental content in cultured cells at the cellular level. In addition, we propose some improvements to optimize backscattered electron and X-ray signal collection. Our findings demonstrate that backscattered electron imaging offers a powerful method to examine whole, freeze-dried cultured cells for scanning electron probe X-ray microanalysis.

Introduction
Cultured cells provide a model to study the composition, distribution and transport of ions in defined physiological states by electron probe X-ray microanalysis with the electron microscope. Different investigators have used this technique to determine the elemental composition of primary cultures of vascular smooth muscle cells (James-Kracke et al., 1980), chondrocytes (Wroblewski et al., 1983), fibroblasts (Abraham et al., 1985), cardiac myocytes (Buja et al., 1985), renal epithelial cells (Larsson et al., 1986), hepatocytes (Zierold & Schäfer, 1988), endothelial cells (Hall et al., 1992), airway smooth muscle cells (Warley et al., 1994) and glandular epithelial cells (Hongpaisan et al., 1996). In addition, several studies have investigated the element content of established cell lines such as glioma C6 cells (Zierold, 1981), HeLa S3 cells (Warley et al., 1983), colon carcinoma cell lines (von Euler & Roomans, 1992; von Euler et al., 1993) and A6 epithelial cells (Borgmann et al., 1994). These cells were adapted to grow on solid substrates (plastic and glass coverslips, graphite discs) or thin films (Pioloform-coated gold and titanium grids) to determine the elemental content at the cellular level. In contrast, high-resolution X-ray microanalysis at the subcellular level requires ultrathin cryosections of cultured cells (James-Kracke et al., 1980; Warley, 1994).

Although a variety of studies have documented the usefulness of X-ray microanalysis of whole cultured cells using SEM (Zierold, 1981; Abraham et al., 1985; Larsson et al., 1986; Hall et al., 1992; Borgmann et al., 1994; Hall et al., 1994), this method has some methodological problems. One is the need to remove the culture medium which may overlie the surface of the cells after freezing and freeze-drying (Wroblewski & Roomans, 1984). In addition, difficulties can arise with absolute quantitative analyses of elemental content because the substrate may contribute to the background, decreasing the peak-to-background (P/B) ratio (Roomans, 1981). Moreover, because of its inferior spatial resolution, SEM X-ray microanalysis does not permit the precise identification of subcellular structures or the measurement of elements in different compartments of cultured cells (Zierold, 1981).

To overcome these problems and enhance lateral resolution, Zierold (1981) adapted freeze-etching methods for X-ray microanalysis of cultured cells with SEM. This method made it possible to recognize the major intracellular compartments, i.e. the nucleus, cytoplasm and mitochondria. Another potential way to localize subcellular structures with SEM is to use backscattered electrons (BSE).
which produces an image with z contrast which provides useful structural information from a significant depth within the sample (Richards & ap Gwynn, 1995). Different studies have reported the utility of this method to examine the subcellular structures in cultured myocardial cells (LeFurgey et al., 1983) and in bulk, hydrated human tissue (Oates et al., 1984). Echlin & Taylor (1986) used simultaneous BSE imaging and X-ray microanalysis to examine and measure element concentrations in bulk frozen hydrated vacuolar plant tissue with a combined BSE/X-ray detector. In addition, backscattered electron signals in combination with quantitative electron probe X-ray microanalysis has been used to measure the dry mass fractions of biological specimens at the subcellular level (Stols et al., 1986).

Recently, different investigators have used BSE and X-ray microanalysis to obtain simultaneous structural and compositional information on mineralized tissues (Kaufman et al., 1990; Kodaka et al., 1995; Roschger et al., 1995; Tjaderhane et al., 1995). However, this combined method has not been used to study the elemental content of whole, freeze-dried cultured cells with SEM.

We present here a simple method with X-ray microanalysis and scanning electron microscopy with BSE imaging to study the elemental composition at the cellular level in unstained, freeze-dried cultured cells. In addition, we propose some improvements in specimen preparation and instrumentation for BSE and SEM X-ray microanalysis of whole cultured cells.

Material and methods

Materials

Dulbecco’s modified Eagle’s essential medium (DMEM) and fetal bovine serum (FBS) were obtained from Sigma (St Louis, MO, U.S.A.). Tissue culture flasks (75 cm²) and 24-well tissue culture plates were from Nunc (Roskilde, Denmark). Sterile 0.4-μm pore size polycarbonate (Millipore-PCF) tissue culture plate cell inserts were obtained from Millipore (Bedford, MA, U.S.A).

Cell culture

The MCF-7 (human breast cancer) and the LLC-PK1 (pig kidney) cell lines were used throughout this study. The cultures were maintained in DMEM supplemented with 10% FBS at 310 K in a humid atmosphere containing 5% CO₂.

Cells were subcultured every 7 days in 75-cm² tissue culture flasks, and the culture medium was changed every 48 h.

Sample preparation for X-ray microanalysis

For X-ray microanalysis, MCF-7 and LLC-PK1 cells were prepared by a modified method of Wroblewski & Wroblewski (1993). Briefly, MCF-7 and LLC-PK1 cells were subcultured using trypsin-EDTA at a density of 2·5 × 10⁶ cells mL⁻¹ in polycarbonate tissue culture plate well inserts placed into 24-well tissue culture plates. Cells were grown in DMEM supplemented with 10% FBS at 310 K in a humid atmosphere containing 5% CO₂. After 48 h, cultured cells were in the logarithmic growth phase and cell viability was >95% as determined by trypan blue exclusion. At this time the cells were washed with ice-cold distilled water for 5 s to remove the extracellular medium. This washing solution was more effective than 0.15 M ammonium acetate or 0.3 M sucrose in removing the culture medium and preserving the morphology of cultured cells (not shown). After washing, excess fluid was drained from the surface and the polycarbonate membrane filter was immediately plunge-frozen in liquid nitrogen (Abraham et al., 1985; Warley et al., 1994). After cryofixation the polycarbonate membrane filter was placed in a precooled aluminium specimen holder at liquid nitrogen temperature and freeze-dried at 213 K for 20 h in an E5300 Polaron freeze-drier apparatus equipped with a vacuum rotary pump system (Polaron, Watford, U.K.). The samples were left in the chamber until they had reached room temperature. Freeze-dried membrane filters were fixed to adhesive graphite lamina in SEM holders, carbon-coated in a high-vacuum coating system (Hitachi, Tokyo, Japan) and stored in a desiccator cabinet.

Instrumentation and analytical conditions

Electron probe X-ray microanalysis of the specimens was performed using a Philips XL30 scanning electron microscope (Philips, Eindhoven, The Netherlands) equipped with a conical objective lens, an ultrathin window (UTW) energy-dispersive detector (EDAX International) and a solid-state backscattered electron detector (KE Development, Cambridge, U.K.) positioned directly under the conical lens. The geometric arrangement of this configuration makes it possible simultaneously to detect high-deflected BSE, which provide z contrast, and to perform X-ray microanalysis with a high take-off angle. The samples were examined with SEM using a combination of secondary electron (SE) and BSE imaging modes. For BSE imaging, the microscope was operated at different accelerating voltages to optimize the collection of images, with a tilt angle of 35° and a free working distance of 10 mm. The BSE images were observed and photographed with reverse signal polarity. Micrographs were made using Ilford FP4 125 film.

For X-ray microanalysis, the analytical conditions were: tilt angle 35°, take-off angle 60° and working distance 10 mm. All spectra were collected in the spot mode at 10 000× for 200 s live time, and the number of counts per second by the detector was about 500. To determine total ion content, we used the peak-to-local-background (P/L) ratio method (Statham & Pawley, 1978; Roomans, 1981;
Boekestein et al., 1984) with reference to standards composed of 20% dextran containing known amounts of inorganic salts (Warley, 1990). Student’s t-test was used to evaluate the statistical significance of the differences between means; a value of $P<0.05$ was considered statistically significant.

Results and discussion

BSE imaging of whole, freeze-dried cells

Figure 1a shows a scanning electron micrograph of whole freeze-dried MCF-7 cells as seen with SE. MCF-7 cells were flattened and polygonal in shape; some spindle-shaped and rounded cells were also seen. The LLC-PK₁ cells displayed similar cell shapes (not shown). Because the SE imaging mode does not allow the accurate identification of any subcellular structures in whole, freeze-dried cells, we used BSE which provides structural information within samples. When unstained freeze-dried cultured cells were observed with BSE, the major subcellular compartments, i.e. nucleus, nucleoli and cytoplasm, were readily identifiable (Fig. 2b,c).

To optimize BSE signal collection, the emission current was increased to induce a higher production of BSE, which improved contrast (Richards & ap Gwynn, 1995). In addition, we compared the images obtained at different accelerating voltages; this allowed us to assess the depth in the specimen from which the BSE emerged and thus obtain information from different depths in the whole, freeze-dried cells (Fig. 2). Figure 2a shows a backscattered electron micrograph of whole, freeze-dried LLC-PK₁ cells at 7 keV. At this accelerating voltage, no subcellular structures were noted. In contrast, when unstained, cultured cells where examined with BSE at 9–13 keV, it was possible to identify the nucleus, nucleoli, and cytoplasm (Fig. 2b–d). However, as accelerating voltage increased (15–17 keV), the edges of the cytoplasm of freeze-dried cells faded, revealing the polycarbonate membrane filter (Fig. 2e,f). These findings suggest that moderate accelerating voltages (9–11 keV) can provide structural information within cells when the BSE imaging mode is used, because of the different densities of the subcellular compartments in unstained, freeze-dried cultured cells. However, we caution that these conditions will need to be tested and confirmed for each particular type of cell under investigation.

To cultivate MCF-7 and LLC-PK₁ cells we used polycarbonate tissue culture plate cell inserts. This thick growth substrate does not interfere with the elements of the cellular spectra, provides stability of the element content and cell morphology during culture, and makes it possible to detect changes in these features during pharmacological assays (Wroblewski & Roomans, 1984; Hall et al., 1994). In addition, it does not absorb significant numbers of BSE, thanks to its low $z$ value. Therefore, polycarbonate
membrane filters are an excellent, growth-compatible, low-z matrix that make it possible to obtain information on the structural elements of unstained cells, with good image contrast when the cells are viewed with BSE.

**Fig. 2.** Backscattered electron micrographs of LLC-PK₁ cells cultured on polycarbonate tissue culture plate well inserts at increasing accelerating voltages. (a) 7-keV accelerating voltage image. Scale bar: 10 μm. Note that the major intracellular compartments cannot be recognized. (b) 9-keV and (c) 11-keV accelerating voltage images. Note that the subcellular compartments, i.e. nucleus, nucleoli and cytoplasm, are recognizable. (d) 13-keV accelerating voltage image. At this voltage subcellular structures can be recognized, but fine details of the cytoplasm are lost. (e) 15-keV and (f) 17-keV accelerating voltage images. Note that the image of cytoplasm has disappeared, revealing the microporous polycarbonate membrane filter below. Some details of the nucleus are also lost. Micrographs were taken with reverse signal polarity. Samples were processed as indicated in Fig. 1.

X-ray microanalysis of whole freeze-dried cells

The application of the BSE imaging to freeze-dried cultured cells plated on polycarbonate tissue culture plate well inserts allowed us to investigate the elemental composition at the
cellular level, i.e. the nucleus and cytoplasm together, by SEM X-ray microanalysis. However, penetration of the electron probe is a problem in SEM X-ray microanalysis of cells cultured on a thick substrate (von Euler & Roomans 1992). The dependence between beam penetration and accelerating voltage has been documented in several SEM X-ray microanalysis studies of bulk, hydrated specimens (Echlin & Taylor, 1986) and freeze-dried specimens (Abraham et al., 1985; Hall et al., 1992; Borgmann et al., 1994). These studies demonstrate that the use of higher accelerating voltages to analyse cells on a thick support induces overpenetration into the solid support, leading to a decrease in the detection of cellular elements.

To test for the possible overpenetration in whole, freeze-dried cultured cells, we calculated the theoretical maximum penetration depth of the electron probe into the specimen at 10 and 15 keV accelerating voltages based on the Bethe range equation:

$$R_B = 70 \left( \frac{E^{1.66}}{\delta} \right),$$

where $E =$ incident energy in keV and $\delta =$ specimen density (kg cm$^{-3}$). These accelerating voltages provide structural information from the depth of cells with BSE imaging, and are sufficient to achieve critical excitation energy for Cl and K. To compare maximum penetration depths, we also calculated the thickness of the LLC-PK$_1$ cell line. Transmission electron micrographs of LLC-PK$_1$ cells showed that the mean thickness was 8.95 $\mu$m (not shown). Wroblewski & Wroblewski (1993) reported that the thickness of cultured monolayers is about 5 $\mu$m, rarely exceeding 10 $\mu$m. If we assume that the density of freeze-dried cytoplasm is $\approx 0.5$ kg cm$^{-3}$ (Echlin & Taylor, 1986), the theoretical maximum overpenetration in whole, freeze-dried cells is 12.5 $\mu$m at 15 keV and 6.3 $\mu$m at 10 keV. Taken together, these data indicate that an accelerating voltage of 10 keV does not induce overpenetration of the electron probe in X-ray microanalysis of whole, freeze-dried adherent cells on polycarbonate tissue culture plate well inserts.

We also tested the elemental content in whole, freeze-dried MCF-7 and LLC-PK$_1$ cells at 10 and 15 keV. To analyse cultured cells, the static spot probe was positioned above the nucleus imaged with BSE. Figure 3 shows X-ray spectra from whole, freeze-dried cultured cells. A spectrum from a polycarbonate membrane filter washed with distilled water is also shown. Our results demonstrate that in MCF-7 and LLC-PK$_1$ cells washed with distilled water, Na, Mg, P, S, Cl and K can be detected. However, Ca was detected only in damaged cultured cells, which were also characterized by their high Na and Cl contents. Our results show that MCF-7 and LLC-PK$_1$ cells were characterized by high K/Na ratios when analysed at 10 and 15 keV. MCF-7 cells had K/Na ratios of 11 ± 1 and 10 ± 1, and LLC-PK$_1$ yielded K/Na ratios of 13 ± 1 and 12 ± 1, respectively. These analytical results, together with the good morphological appearance of MCF-7 and LLC-PK$_1$ cells, suggest that our method of preparing cultured cells for electron probe X-ray microanalysis yields satisfactory results.

Table 1 compares the elemental composition in MCF-7 and LLC-PK$_1$ cells analysed at 10 and 15 keV. Use of an accelerating voltage of 10 keV in freeze-dried cells causes a significant increase in the intracellular concentrations of Na, P, Cl and K. The fact that these elemental contents did not increase at 15 keV suggests that the electron probe penetrated the thick support, which thus contributed to the X-ray spectra. In contrast, at 10 keV the X-ray signals detected were generated exclusively from the cells.

In addition, elemental concentrations were related to the P content as an indicator of dry mass of the cell analysed (Abraham et al., 1985; Larsson et al., 1986). Figure 4 shows
Table 1. Elemental composition of whole, freeze-dried cultured cells at accelerating voltages of 10 and 15 keV.†

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n‡</th>
<th>Na</th>
<th>Mg</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 (clone BUS)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>10 keV</td>
<td>25</td>
<td>38 ± 2</td>
<td>33 ± 1*</td>
<td>285 ± 5**</td>
<td>78 ± 2</td>
<td>101 ± 3**</td>
<td>395 ± 8*</td>
</tr>
<tr>
<td>15 keV</td>
<td>25</td>
<td>39 ± 2</td>
<td>37 ± 1</td>
<td>260 ± 5</td>
<td>78 ± 2</td>
<td>87 ± 3</td>
<td>367 ± 7</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td></td>
<td></td>
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<tr>
<td>10 keV</td>
<td>20</td>
<td>32 ± 2*</td>
<td>26 ± 1*</td>
<td>239 ± 4**</td>
<td>76 ± 3</td>
<td>84 ± 5**</td>
<td>383 ± 9**</td>
</tr>
<tr>
<td>15 keV</td>
<td>20</td>
<td>28 ± 2</td>
<td>30 ± 5</td>
<td>202 ± 5</td>
<td>77 ± 2</td>
<td>63 ± 3</td>
<td>296 ± 9</td>
</tr>
</tbody>
</table>

† Data given in mmol kg⁻¹ dry weight ± SE; ‡ n, no. of cells analysed; *P < 0.05, **P < 0.01.

the content ratios for Na/P, Cl/P and K/P in LLC-PK1 cells analysed at 10 and 15 keV. Our results demonstrated that the concentration ratios of K/P obtained at 10 keV were significantly higher (P < 0.01) than the corresponding ratio obtained at 15 keV. Taken together, these results are compatible with previous reports that accelerating voltages of about 10–11 keV should be used to analyse the elemental content in whole, freeze-dried cells plated on thick supports (Abraham et al., 1985; Hall et al., 1992; Borgmann et al., 1994). In addition, we demonstrate that this range of accelerating voltages was high enough to provide structural information from a depth of several micrometres within unstained whole, freeze-dried cells viewed with BSE.

Conclusions

We describe a method to measure the elemental content in whole cultured adherent cells by X-ray microanalysis with SEM, using backscattered electron imaging. This imaging mode provides useful structural information from subcellular compartments, i.e. the nucleoli, nucleus and cytoplasm, and makes it possible to analyse the elemental composition of cultured cells at the cellular level. We believe that backscattered electron imaging will prove useful as a complementary technique to analyse whole, freeze-dried cultured cells with scanning electron probe X-ray microanalysis.

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References


