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CORRECTION FOR EXTRANEOUS BACKGROUND IN X-RAY MICROANALYSIS OF CELL CULTURES

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Abstract

Some practical aspects of the X-ray microanalysis of cell cultures have been investigated. Cells were cultured on titanium grids covered with Formvar films and analyzed at 100 kV either in the scanning transmission (STEM) or transmission mode (TEM) of the electron microscope. Different holders, grids and configurations were compared with respect to the relative contribution of different factors to the extraneous background in the X-ray spectrum. When low atomic number holders are used, the contribution to the spectrum of electrons scattered through high angles, may be negligible. In practice this may result in negative values for the contribution of these scattered electrons to the background. Computer programs for correction of the extraneous background should ignore these negative values and replace them by zero. When a brass holder is used, the contribution to the spectrum from electrons scattered through high angles becomes more important than that of the uncollimated radiation. The position of the analyzed cell relative to the grid bars is more important than the choice of grid or holder type. The data show that for the specimens used in the present study the correction for extraneous background is of little importance and can be neglected.

Key Words: X-ray microanalysis, quantitative methods, Hall-method, extraneous background, cell culture.

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Introduction

Cell cultures are for a variety of reasons increasingly used to study physiological and pathological processes (Phillips and Gilchrest, 1992). X-ray microanalysis of cultured cells allows the investigator to gain information on individual cells. This can in some cases show that a particular cell culture contains different populations of cells, that can react in a different way to certain stimuli (von Euler and Roomans, 1992). Methods for X-ray microanalysis of cultured cells have been reviewed by Wroblewski and Roomans (1984). Cells can be cultured on a variety of substrates, that from the point of view of quantitative X-ray microanalysis can be defined as thin or thick. Thick substrates, e.g., a filter or the plastic bottom of a culture dish, cannot, by definition, be penetrated by the electron beam. Since these substrates generally also are relatively thick in comparison to the cells, they contribute the majority of the background radiation to the spectrum, and decrease the sensitivity of analysis (von Euler and Roomans, 1991). Thin substrates, e.g. a thin plastic film, are more suitable for quantitative analysis according to the Hall-method (Hall, 1989; Roomans, 1990), but are fragile and may break during preparation. In our experience (von Euler and Roomans, 1991), Formvar films on titanium mesh grids mostly withstand freezing and freeze-drying, even though part of the specimens may be lost during the preparative procedure.

A consequence of analyzing the cultured cells on grids is that the grid bars contribute to the spectrum. The problem of extraneous radiation in quantitative microanalysis of thin sections has been considered before (Gupta and Hall, 1979; Roomans and Kuijpers, 1980; Roomans, 1988; Hall, 1989; Roomans, 1990). A formal solution for the problem has been worked out, in which it is assumed that the contribution of the grid to the spectrum is due to two factors: [1] uncollimated radiation, consisting of electrons outside the main beam, and hard X-rays generated in the condenser aperture [2] electrons that are scattered over such an angle (close to 90°) in specimen and substrate film that they collide with the grid bars. In practice the correction for extrane-

ous background appears more complicated than the theoretical model suggests (Roomans, 1988), in particular since more complicated scattering patterns may occur inside the specimen area of the electron microscope. Instrumental conditions such as grid type and geometry of the electron microscope therefore may have effects that are difficult to describe in a formal way.

The correction for extraneous background, first proposed by Gupta and Hall (1979) is now generally used to correct for the extraneous background. The method of Roomans and Kuijpers (1980), is formally equivalent to that of Gupta and Hall (1979), but allows in addition the explicit calculation of the different factors contributing to the background. This can provide information that may help the operator choose between different practical alternatives to carry out the analysis. Since we were faced in our ongoing studies of cultured cells with a number of decisions on practical details of analysis, we decided to use the method of Roomans and Kuijpers (1980) to compare grid types, specimen holders, and electron microscopes with respect to the extraneous part of the background in the X-ray spectrum.

Materials and Methods

As test specimens, cultures of the human colon cancer cell line Colo 205 or of the human breast cancer cell line MDA231 were used. Cultures were seeded on titanium grids as described for fibroblasts by von Euler and Roomans (1991), and after about 6 hours of growth, the culture medium was washed off with 0.15 M ammonium acetate (Colo 205 cells) or distilled water (MDA231 cells). The specimens were then rapidly frozen in liquid nitrogen and freeze-dried. Two types of titanium grid were used, 300x75 mesh and 75x75 mesh. Prior to analysis, the grids were coated with a conductive carbon layer in a Balzer's CED020 carbon evaporator.

Analysis was carried out in a JEOL 1200 EX TEMSCAN electron microscope in the STEM mode at 100 kV, with a Tracor 5500 energy-dispersive analysis system. The detector of this system is mounted horizontally, i.e., normal to the electron beam. The 300x75 mesh titanium grids were mounted either with the long sides of the rectangles parallel to the detector surface ("horizontal position"), or with the short sides of the rectangles parallel to the detector surface ("vertical position"). Also the 75x75 mesh grids were mounted so that the grid bars were parallel to the detector surface. Two specimen holders were compared: the standard, brass holder, and a graphite holder (Liljesvan and Roomans, 1976). The specimens were tilted 35°.

Analysis was also carried out in a Philips 400 TEM with field emission gun and twin lens, using a LINK QX200 energy dispersive detector system. The geometrical conditions were kept the same as described above for the JEOL microscope.

Measurements were carried out as follows: [1] on the cell (nucleus or cytoplasm), [2] on the support film (outside the cell), [3] on an empty grid, in the middle of the grid square "hole count", [4] on an empty grid, so close to the grid bar that a reasonably high count rate in the Ti peak was obtained. The background in the region of 6.7-7.7 keV was used for all calculations. With this choice of region, small instrumental peaks of Fe and Cu were avoided. Unless otherwise stated, all measurements were carried out close to the middle of the grid (Roomans, 1988).

The observed continuum in the first measurement (that on the specimen) (W_1) consists of contributions from the specimen itself (W_{sp}), the film (W_f), scattered electrons hitting grid bars ($W_{1,sc}$) and uncollimated electrons (W_{uc}):

$$W_1 = W_{sp} + W_f + W_{1,sc} + W_{uc} \quad (1)$$

The various contributions to the continuum were calculated as follows (Roomans and Kuijpers, 1980):

The background due to uncollimated radiation (W_{uc}) is obtained in measurement [3]:

$$W_{uc} = W_3 \quad (2)$$

The background due to high-angle electron scattering in the film ($W_{2,sc}$) is obtained from

$$W_{2,sc} = r\,(P_2 - P_3) \quad (3)$$

where

$$r = W_4/P_4 \quad (4)$$

and P with subscript refers to the net intensity of titanium in the measurement as numbered above.

Likewise, the background due to high-angle electron scattering in the specimen (including the underlying film) ($W_{1,sc}$) is given by

$$W_{1,sc} = r\,(P_1 - P_3) \quad (5)$$

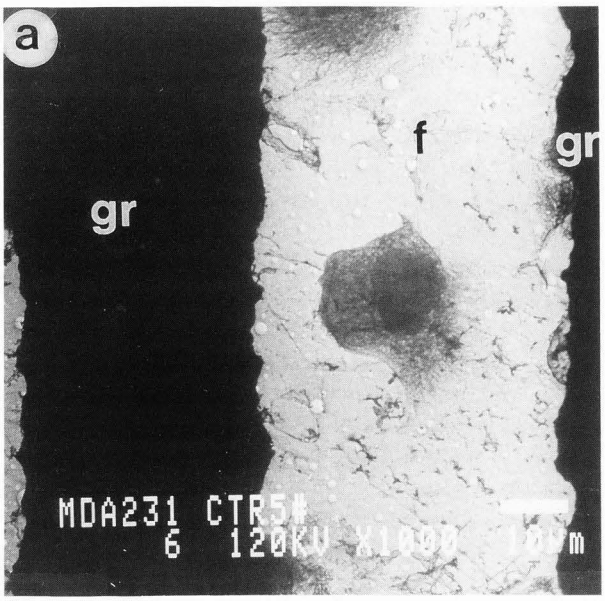
Hence, the background from the film proper (W_f) is given by:

$$W_f = W_2 - W_{2,sc} - W_{uc} \quad (6)$$

and finally, the background from the specimen proper (W_{sp}) is given by:

$$W_{sp} = W_1 - W_f - W_{1,sc} - W_{uc} \quad (7)$$

The values for the film include the contribution from the conductive carbon coating.



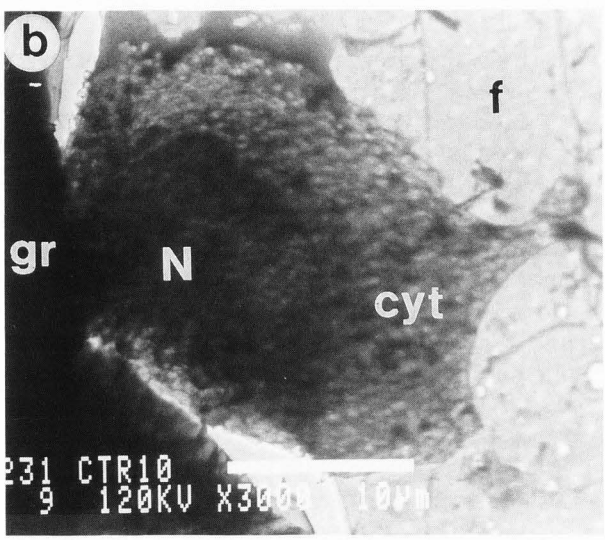


Fig. 1. STEM micrographs of MDA231 breast cancer cells: (a) low magnification showing the position of the cell relative to the bars of the 300x75 mesh titanium grid *(gr)* covered by a Formvar film *(f)*; (b) higher magnification showing that the nucleus *(N)* can easily be distinguished from the cytoplasm *(cyt)*. Bar = 10 μm.

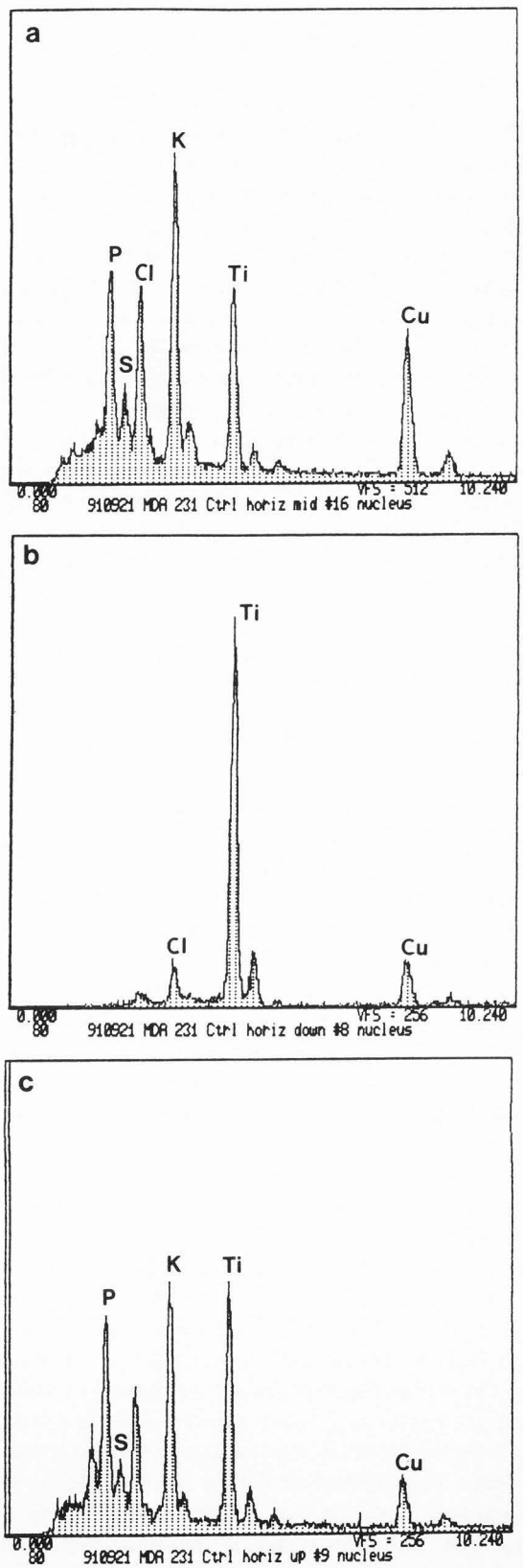


Fig. 2. Typical spectra of MDA231 breast cancer cells, (a) taken in the middle of the grid rectangle, (b) taken close to the grid bar closest to the detector, (c) taken close to the grid bar furthest from the detector. In (b) absorption of low-energy X-rays is evident

Results and Discussion

In the first series of measurements in the JEOL 1200 EX electron microscope, the 300x75 mesh titanium grids were used and mounted in a brass holder. A micrograph of a typical specimen is shown in Fig. 1a,b, a typical spectrum taken in the middle of the grid rectangle is shown in Fig. 2a. As already pointed out by Roomans (1988) analysis close to the edge of the specimen holder, or close to the grid bar closest to the detector, carries the risk of serious absorption artifacts. Fig. 2b shows that under improper conditions the low-energy end of the spectrum may be virtually completely absorbed. If the spectrum is taken close to the grid bar far from the detector, absorption artifacts may be avoided (Fig. 2c) even though the contribution of the grid metal to the spectrum increases. Hence, for optimal comparison of instrumental parameters, measurements were carried out in the middle of a grid square or rectangle, and close to the middle of the grid. Analysis of the data according to equations (1) to (7) showed that about 90% of the background observed in the spectra of the cells (nucleus or cytoplasm) was due to the specimen itself (Table 1). As judged from the corrected background intensity, the mass of the analyzed volume of cytoplasm is less than half of that of the analyzed volume in the nucleus, which is in accordance with the smaller relative contribution of W_{sp} for the cytoplasm to the total spectrum (Table 1). Film, high-angle scattering, and uncollimated radiation give only minor contributions to the background. The difference in background radiation between the "horizontal position" and the "vertical position" was not significant. When the film only is analyzed, the contributions of scattered and uncollimated electrons are relatively larger, since the film is much thinner than the freeze-dried cell (Table 2).

Similar results were obtained when the 75x75 mesh titanium grids were analyzed (Table 1). Here, different positions with regard to the grid bar were chosen, and the results confirm earlier data (Roomans, 1988) that the relative contribution of extraneous background varies significantly with the position of the beam relative to the grid bar (Fig. 3). This is important in practice, since both the background generated by the specimen itself and the extraneous background are subject to statistical variation (equal to the square root of the count rate). If the extraneous contribution to the background is relatively large, the resulting statistical error in the corrected background may be very substantial. The variation caused by choosing different positions within a grid rectangle or square appears of much more significance than the choice of grid or material.

When the graphite specimen holder was used, the

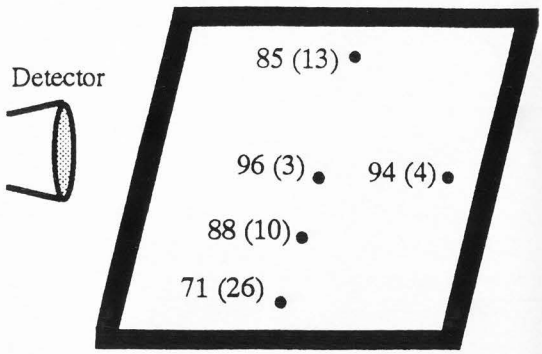


Fig 3. Relative contribution of the specimen itself (W_{sp} in % of W_1) and of the extraneous background due to high-angle scattering ($W_{1,sc}$ in % of W_1), in parentheses, for different positions within a grid square of a 75x75 mesh titanium grid.

value for the net intensity of the grid in the "hole count" measurement (P_3) was in the same order of magnitude as the net intensity of the grid when the film was analyzed (P_2), This occasionally resulted in a negative value for $W_{2,sc}$, which is nonsense from a physical point of view. It must be concluded that negative values for $W_{2,sc}$ mean that the contribution to the spectrum by electrons, scattered by the film over such an angle that they hit the grid bars, is negligible. The negative value results from the statistical uncertainty and possibly from small variations in instrumental conditions. In a computer program correcting for extraneous background, negative values for $W_{2,sc}$ should be replaced by zero or a very small positive number.

The mass of the graphite holder is somewhat less than that of the brass holder, and more importantly, the atomic number (Z) of carbon is much lower than that for copper and zinc. It is therefore likely that the brass holder produces a larger number of backscattered electrons that, possibly after first hitting the polepiece, generate X-rays from the grid. This would support the use of low-Z specimen holders for quantitative X-ray microanalysis. Problems with extraneous background correction when graphite specimen holders were used have, however, been noted earlier (Roomans, 1988) but were mainly ascribed to geometrical problems and absorption of low-energy X-rays by the rather thick holder.

Also analysis in the Philips 400 electron microscope with a beryllium holder resulted in P_3 being not much different from P_2. The results from the Philips microscope were basically the same as those from the JEOL microscope, despite the fact that a different way of imaging was used (TEM with twin lens in the Philips

Table 1. Relative Contribution of Different Factors to Total Background from the Specimen

Conditions	Relative Contribution (%) Spec	Film	Sc e^-	Unc e^-
JEOL/brass holder, vertical pos/300x75				
nucleus	92	3	4	1
cytoplasm	86	8	5	1
JEOL/brass holder, horizontal pos/300x75				
nucleus	91	3	5	1
cytoplasm	87	6	6	1
JEOL/brass holder/75x75				
nucleus	87	1	11	1
JEOL/graphite holder, vertical pos/300x75				
nucleus	93	3	4	1
cytoplasm	85	8	3	3
JEOL/graphite holder, horizontal pos/300x75				
nucleus	92	3	5	1
cytoplasm	87	7	3	3
Philips/beryllium holder, vertical pos/300x75				
nucleus	90	3	5	2
cytoplasm	81	8	5	6
Philips/beryllium holder, horizontal pos/300x75				
nucleus	92	2	4	2
cytoplasm	88	4	3	5

The table gives the relative contributions of the specimen itself (W_{sp}), the film (W_f), the scattered electrons ($W_{1,sc}$) and the uncollimated electrons (W_{uc}) to the total measured background from the specimen (W_1) according to equation (7). Data are averages from 5 measurements.

versus STEM in the JEOL).

The difference between the brass holder and the low-Z material holders is shown even more clearly in Table 2. In the measurements carried out in the brass holder, the relative contribution from the scattered electrons to the background is larger than that from the uncollimated electrons; for the low-Z material holders, the situation is the reverse.

Table 2. Relative Contribution of Different Factors to Total Background from the Film

Conditions	Relative Contribution (%) Film	Sc e^-	Unc e^-
JEOL/brass holder, vertical pos/300x75	76	14	10
JEOL/brass holder, horizontal pos/300x75	66	22	12
JEOL/brass holder/75x75	49	37	14
JEOL/graphite holder, vertical pos/300x75	71	0	29
JEOL/graphite holder, horizontal pos/300x75	62	9	30
Philips/beryllium holder, vertical pos/300x75	53	3	44
Philips/beryllium holder, horizontal pos/300x75	43	0	57

The table gives the relative contributions of the film itself (W_f), the scattered electrons ($W_{2,sc}$) and the uncollimated electrons (W_{uc}) to the total measured background from the specimen (W_2) according to equation (6). Data are averages from 5 measurements.

In view of the fact that under the experimental conditions used in this study the specimen contributes about 90% of the background, it may be questioned whether the correction for extraneous background is relevant for this type of specimen. This particular specimen type may also be considered as semi-thick specimens, where the ratio of the net intensity to the background under the peak is used for quantitation (Roomans, 1990). Comparison of the values for the element potassium show that this alternative deviates less than 5% from the value obtained by quantitative analysis according to the method for thin specimens including correction for extraneous background. This is less than the biological variation in cultured cells and, given the known uncertainty in the correction for extraneous background (Roomans, 1988), a fully acceptable alternative.

The present paper demonstrates that the method of Roomans and Kuijpers (1980) can be used not only for correction of the background for extraneous contributions, but also for an objective evaluation of the sources

of the extraneous background under different instrumental conditions.

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Discussion with Reviewer

T. von Zglinicki: If there are other extraneous elements apart from Ti, how would that affect the background subtraction routine?

Authors: In the factor giving the contribution of the scattered electrons to the background ($W_{1,sc}$ or $W_{2,sc}$), the different sources should be summed. E.g., for n extraneous sources, equation (5) becomes

$$W_{1,sc} = \sum_n r_n (P_{1,n} - P_{3,n}) \quad (8)$$

T. von Zglinicki: Scattered electrons hitting the grid bars are surely the major, but not the only source of "scatter background". With your choice of measuring point 4 to scale the scatter background correction you exclude all the scattering hitting the holder or any other parts. Even if this is not important in the rather favorable case here, it will play a role as soon as one moves closer to the edge of the grid. How would you suggest to include this effect in the background correction?

Authors: One consequence of electrons hitting the holder or other parts of the microscope is that there will be more extraneous elements than the grid metal in the spectrum. As pointed out above, this can, in principle, be taken care of by equation (8). However, the problem is the accurate determination of the factor r_n, since the electrons hitting e.g., the specimen chamber indirectly, may have lost energy and generate X-rays with a lower peak-to-background ratio (higher value of r_n) than when they would have hit the metal directly. In addition, there are absorption effects to be considered, that depend in part on the angle under which the electrons hit the grid, specimen holder, or specimen chamber. Actually, because in practice the grid is not only hit directly by scattered electrons, but also indirectly (by electrons that first hit the specimen chamber) the value of r used in the present procedure also may be doubtful. It will be extremely difficult to make a model that provides for all these complex interactions of scattered electrons with structures surrounding the specimen. This is, as pointed out in the paper and in Roomans (1988, text reference), one of the reasons why the correction for extraneous background may fail if the extraneous background constitutes a relatively large part of the background. It is therefore generally agreed that the extraneous background should be kept as low as possible.

T. von Zglinicki: How does W_{uc} change if one goes close to the grid bar?

Authors: In the model as described above, W_{uc} is measured in the middle of the grid square and the same value of W_{uc} is used for all calculations irrespective of the position of the measurement in relation to the grid bar. As a consequence, the increase in external background when one analyzes close to the grid bar is ascribed to an increase in the contribution by scattered electrons. In reality, this is not quite correct; measurements of the "hole count" close to the grid bar give higher continuum values than "hole count" measurements in the middle of the grid square.