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W. C. de Bruijn
Centre for Analytical Electron Microscopy, Leiden, The Netherlands

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INTEGRATION OF X-RAY MICROANALYSIS AND MORPHOMETRY OF BIOLOGICAL MATERIAL

W.C. de Bruijn

Centre for Analytical Electron Microscopy, Laboratory for Electron Microscopy, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands

(Paper received March 15 1984, Completed manuscript received April 1 1985)

Abstract

It was investigated how to extract both morphometrical and X-ray elemental information from scanning electron microscopical (SEM) or scanning transmission electron microscopical (STEM)-images and how to integrate these two information streams either on line or off-line after storage.

Cytological reaction products in cell organelles in ultrathin sections are the biological structures of interest. In such organelles four different situations can be met: morphologically the structures are homomorph or heteromorph; chemically the elements are distributed either homogeneously or heterogeneously. A new program has been proposed and described, which permits determination of both the area and the mean net-intensity value of chemical elements, inhomogeneously distributed over heteromorph organelles. The value of this integration method is demonstrated by three examples of increasing complexity, starting with two elements which are more or less homogeneously distributed over one lysosome, the establishing of a platinum discontinuity in an acidophilic granule and finally the localization of two chemical elements inhomogeneously distributed over a rather heteromorph phagolysosome.

In two examples Chelex ion exchange beads, maximally loaded with the element also present in the structure of interest, are co-embedded with the tissue as internal standards. In such cases the absolute elemental concentration in the structures analysed can be established. The presence of such cross-sectioned beads in the ultrathin sections is also used: 1) to demonstrate their function as models to select the proper conditions for the digital-controlled raster analysis of the unknown cell- or tissue structures, 2) to prove the value of this method.

KEY WORDS: scanning transmission electron microscopy, X-ray microanalysis, morphometry, signal integration, biological tissue, Chelex ion-exchange standards, ultrathin sections, cytochemistry, image analysis

*Address for correspondence: Centre for Analytical Electron Microscopy, Laboratory for Electron Microscopy, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands Phone no. 31.71.148333/4666

Introduction

X-ray microanalysis (XRMA) of biological specimens gives information about the chemical elements present in structures of SEM or STEM or conventional transmission electron microscopical (CTEM) images, mostly recognized on the basis of morphological criteria. The elements present can be: naturally occurring, related to a disease, or deliberately introduced by a cytochemical reaction. Qualitatively, XRMA is widely used to collect information about: 1) absence or presence of chemical elements in various morphological structures, mostly recognizable cell organelles, provided the element is detectable by XRMA (Z>11); 2) chemical differences among morphologically identical structures; or 3) the ratio of several chemical elements within morphologically identical structures. Numerous examples are available in the literature. When there is a need for quantification, the general approach is to analyse a known standard with the unknown, and compare the relative net-intensities (P/B) under the same instrumental conditions. Fiori and Blackburn (13) and recently Roomans reviewed the literature on this subject (28). We have advocated the use of maximally loaded Chelex ion exchange beads as an internal standard which can be co-embedded with the biological tissue to be analysed (8,9,12). In this way quantitative chemical information is obtained about the elements in the morphological structures in cells or tissues. When the chemical element is homogeneously distributed over the structure, point analyses suffice. However, the chemical elements are frequently not uniformly distributed over the morphological structures and therefore problems arise in the quantification procedure. The same holds true when the morphological structures (like most cell organelles) are involved in dynamic processes, leading to changes in number or volume. In such cases morphometrical and chemical information has to be collected and both information streams have to be integrated. This signal integration process involves either the integration of two XRMA-signals or the integration of XRMA-signal(s) with electron-image signals. When multi-elemental X-ray maps are to be collected and quantitatively integrated, the acquisition step is rather complicated. Several regions of interest from a representative spectrum.
are to be processed to obtain net-intensity figures, to be displayed and/or stored for comparison. The use of multi-coloured images facilitates discrimination between the various grey-scale values or the different element localizations. Mathematical processing by filtering or by kernels ( = pixel masks) may be the next step in image-processing, followed by a reconstruction of the processed image on screen or printer. Examples of the application of mathematical morphology, at the light microscopical level, can be found in the literature (24,27,36). Recent examples in which chemical XRMA and morphometrical information are integrated can be found in metallurgy, in which various phases in an alloy are generally differentiated chemically and the total area occupied by each phase is established in relation to the total area (3,4,19,21,23,26,29,40). Other examples can be found in situations when in a population of (dust or asbestos) particles, one type has to be discriminated from the others. Individual particles are discriminated (based upon their chemical and morphological information) and placed in sub-populations (14,16,23,26,32).

This paper will report about efforts to determine from cytochemical reaction products in cell organelles, both the area occupied and the elemental concentration.

In EM-cytochemistry enzyme activity is converted in metal precipitates. Such metal precipitates can be present in cell organelles, such as endoplasmic reticulum, Golgi zones, lysosomes, or granules. Sometimes the precipitate is rather uniformly distributed over the entire organelle, mostly not. In some organelles more than one element can be present, either naturally (ferritin, containing iron and phosphorus) (25), or in a combination of a colloidal marker plus a cytochemical reaction product (7), or as two reaction products (1,33,34,35), or two markers. In the latter cases multiple point analyses are sometimes (qualitatively meaningful, but mostly not. When more than one element is present inside the structure, elemental mapping might disclose their presence and their relative topographic orientation (18,32).

By the method of digital-controlled raster analysis, both their areas occupied and each relative net-elemental concentration can be obtained and mutually related quantitatively by an integration procedure. As maximally loaded ion exchange beads (bearing the same element(s) in a known concentration as the precipitate to be analysed) are co-embedded with the cells or tissues, such standard analyses, with calibration beads can also be used as a model system to set analytical parameters optimally. Such an analytical approach has both hardware, software, microscopical and statistical aspects, which will be briefly outlined in the Materials and Methods, prior to the description of three examples, in increasing complexity, to illustrate the case.

Materials and Methods

The animal tissue used for this investigation was glutaraldehyde-perfusion-fixed rat kidney and liver or mouse peritoneal cells. After fixation and washing, the tissue was incubated in the media to detect acid phosphatase activity with either cerium or lead as the capture ions (according to Hulstaert et al., 20; and 39). The tissue was post-fixed with OsO4 plus K4Fe(CN)6 according to de Bruijn and den Breejen (6). The glutaraldehyde-fixed mouse peritoneal cells were incubated for peroxidase activity with H2O2 and Di Amino Benzidine as acceptor, which was made electron dense with 10% H2PtCl6 in 10% HCl for 1 h at room temperature, followed by a rinse in 10% HCl (de Bruijn et al., to be published).

These cells were not postfixed. Mixed with these cells, maximally loaded Chelex100 platinum beads were co-embedded. The rat liver tissue contained maximally loaded iron beads close to the tissue cubes. By neutron activation the iron concentration was determined to be 11.5% w/w, and the platinum concentration was determined by X-ray microanalysis in the SEM-mode at 20 kV, with 100% platinum as a reference, to be 18% w/w. The ultrathin 50 nm sections were observed without any post-staining.

The Philips EM 400 analytical electron microscope is equipped with a backscattered-electron ring detector, and a STEM-detector in combination with a Tracor Northern TN 2000 system. Spray apertures are present to reduce spurious X-rays, where possible. The ultrathin sections are mounted on Formvar-film covered 70 mesh copper grids in a beryllium low-background holder. In the specimen plate of the microscope, the side-entry goniometer and the X-ray detector are mounted in the same horizontal plane. The axis of the holder is situated perpendicular to the axis of the X-ray detector. The holder in the goniometer is tilted 18-24° towards the energy dispersive Sili-detector, mounted with 24° tilt with respect to the horizontal plane.

For the performance of the digital-controlled raster analyses, a TN 1310-unit is added to the computer system. With this unit the electron beam can be directed over the specimen in a line or raster pattern, by computer control. The acquired images are visualized on a colour screen. During the integration process of the electron-image- and X-ray net-intensity information the printer/plotter is used to relate the two images to each other (see below).

For the conventional X-ray analysis, the computer of the TN 2000 is loaded with the normal TN programs. Initially the TN-IPP (Image Processing Program) was used to solve the above-mentioned biological problem. The experiences gained stimulated us to make a new program called reduced raster (10). This program is mainly based upon the original IPP-ideas, but has additional advantages for biological material, in which the morphological information dominates over the chemical analysis.

The main arguments to do so were: 1) in ultrathin sections the grey-values of the various organelles are not sufficiently different to guarantee appropriate discrimination by the IPP program. Therefore this is now done manually by the analyst, by locating the reduced raster over the structure of interest to be analysed. 2) Once separated from the surroundings by that raster location, the analyst has to decide at which threshold value the structure is sufficiently isolated from the signals present in the cytoplasmic
obtained in Fig. 2. Mean Os net-intensity "inside" is 21.15 ± 6.37 c/p/s, Area array overlay some L in Fig. 3. Osmium net-intensity X-ray distribution is 21.15 + 6.06 c/p/s. Mean Os net-intensity "outside" is set by the electron image threshold 115, also colour print. Fig. 5.

Fig. 1. STEM-image of proximal tubule cell containing cerium precipitates from acid phosphatase activity in lysosomes and Golgi vesicles. M = 20,000 X unstained. L1 = lysosome to be analysed. See also colour print. Fig. 5.

Fig. 2. Electron array of 16 x 16 points in a reduced raster over lysosome L1 in Fig. 1. At threshold value: 115 c/p/s, the lysosome is well delineated from its surroundings. The mean electron value "inside" is 158.44 ± 19.86 c/p/s.

Fig. 3. Osmium net-intensity X-ray distribution array over lysosome L1 in Fig. 1. Particle delineation is set by the electron image threshold 115, obtained in Fig. 2. Mean Os net-intensity "inside" is 21.15 ± 6.06 c/p/s. Mean Os net-intensity "outside" is 18.85 ± 6.37 c/p/s, Area = 0.2980 /um².

Fig. 4. Cerium net-intensity X-ray distribution array over lysosome L1 in Fig. 1, at the same particle delineation threshold 115 c/p/s. Mean "inside" net-intensity is 6.02 ± 3.16 c/p/s.

Fig. 5-13: See colour plate on page 713.
matrix to permit morphometrical and chemical analysis of the structure. 3) Several instrumental parameters have to be judged while performing the analysis (spot-size, dwell time, magnification, interpixel distance) in relation to the signal to noise ratio of the structure of interest, and the final quantitative result.

In short, the reduced raster analysis program is performed as follows: 1) The SEM or STEM electron image is created on the screen (e.g. by photographic superimposition of the different delineations, the topographic relation of the various elements within the structure can be reconstructed. 2) The reduced raster is directed to the structure of interest, the size of it adopted to the structure and the spot size is chosen.

Now two approaches are operative:

a. If the structure is considered or expected to be chemically homogeneous then 3) the number of pixel points is set at 256 x 256 points per reduced raster area. On the colour screen (in 16 grey-level/pseudo colours) the electron image is obtained. Subsequently, the multi-coloured image is converted into a bi-coloured image, one for the "inside", one for the surroundings. The area of the "inside" part is determined as the percentage of pixels "inside", with respect to the total amount of pixels present in the whole area. As the area of the reduced raster is known, the inside area can be calculated. The original IPP "histogram" program assists in threshold setting and area calculation. By (multiple) point analyses inside the structure, the chemical content is acquired as net-intensity values, to be compared with an internal standard, present in the same ultrathin section.

If the structure is considered to be chemically inhomogeneous then after the initial area determination by the 256 x 256 electron image as described in 3), two extra acquisitions are made at 16 x 16 pixel points for the integration of the morphometric and chemical analysis as follows:

4) The 16 x 16 electron image of the reduced raster area is acquired. As a routine, prior to the digital-controlled raster performance, a line scan is made in the middle of the reduced raster area. The values of these 16 points are displayed graphically to judge the contrast of the structure with respect to the dwell time, which is set to assure the maximum separation between structure and surroundings. If necessary, the lower values can be cut off by raising the baseline in the graphic display. The 16 x 16 electron image is acquired and stored on disk. 5) Subsequently the 16 x 16 X-ray net-intensity images are acquired for the (maximally four) regions of interest in the spectrum set before. Also in this case a 16 points line scan in the middle of the area is performed and the net-intensity values of each of the elements present in the selected regions of interest are graphically displayed on the screen. Again by choosing the appropriate dwell time and the individually selected thresholding for each element, the conditions can be set, prior to the performance of the digital-controlled raster analysis of the entire selected area. Elements are acquired in one run and the net-intensity arrays are stored on disk. 6) The integration of the information is performed on the printer with either one of the X-ray net-intensity arrays and the 16 x 16 electron array or between two X-ray arrays (see top captions in Figs. 2-4). Prior to printing, each array can be displayed on the colour screen, threshold values can be introduced continuously backwards and forwards. Once an appropriate threshold has been reached and set, the array is printed. In the array the figures above the selected threshold value are separated by a line from those below that value in a way creating an "inside" area separated from its surroundings. By defaulting the array, the threshold boundary alone can also be printed. Moreover, this threshold boundary can be superimposed over other arrays. This enables boundaries, determined in the electron-image array, delineating the structure of interest, to be superimposed over net-intensity element arrays. Mean net-intensity values can be calculated from the figures present "inside" the structure.

The number of pixel points inside the delineated area is given, so the area occupied by the structure (or by the elements within the structure) can be calculated, as the total number of figures inside the whole reduced raster area, and its magnification is known. By photographic superimposition of the different delineations the topographic relation of the various elements within the structure can be reconstructed. 7) The vertical scale in the obtained arrays ranges from zero to 255. The grey-scale values in the electron array are divided between black and white into 255 steps (each grouped together in 16 pseudo-coloured classes including black and white at each end of the scale). In our specimens the rather heavy precipitates have a contrast range well within the 255 scale. The much longer dwell times during X-ray acquisition can create in the X-ray net-intensity arrays values by raising the baseline threshold in the line-scan graphics on the top 255 figures (e.g. from 500 to 755) can be printed in the array (such that 501 is represented by 1 and so on). Alternatively just the lower values can be printed, such that all values over 255 are printed as 255.

The conditions at which the integrated morphometrical and X-ray analyses are performed are rather important. In addition to the choice of the instrumental conditions (acceleration voltage, spatial resolution of the operation-mode selected, beam intensity and spot size), beam damage sensitivity and stability of the specimen in the microscope determine whether two separately acquired arrays can be compared with each other. Moreover, the signal to noise ratio of the electron and the X-ray signal determine whether the structure of interest will be recognized and measured. The heavy metal precipitates in the cytochemical examples described in this paper possess the required high signal to noise ratio, but other structures may fail to do so.

Several statistical tests have been applied ad hoc to the results obtained, to prove the "reliability" of the method. The co-embedded Chelex
Fig. 14. X-ray spectrum of a 100 sec. point analysis of an ultrathin cross-sectioned eosinophilic granule, visualized by a peroxidase reaction with DAB and H$_2$PtCl$_6$ (Fig. 15). The presence of platinum and chlorine is demonstrated.

Fig. 15. Enlarged portion of a normal STEM image of an ultrathin cross-sectioned eosinophilic granule, visualized by peroxidase/DAB/H$_2$PtCl$_6$ collected on a Formvar-film covered grid. The digitalized colour image is given in Fig. 7.

Fig. 16. Electron array of a 16 x 16 raster over the granule shown in Fig. 15. The threshold 99 c/p/s delineates the particle from its surroundings and the central core-area from the remainder of the granule. Particle area = 0.46 $\mu$m$^2$.

Fig. 17. The net-intensity platinum X-ray distribution array. Over the same area as shown in Fig. 16. The electron-image threshold 99 c/p/s shown in Fig. 16 also delineates the granule in this array.
Results

Analysis was carried out on lysosomes and golgi zones in proximal tubule cells of mouse kidney, eosinophilic granules, phagolysosomes in rat kupfer cells, and Chelex standard beads. For convenience, the results are summarised in Figs. 1-4 (mouse proximal tubule cells), in color prints Figs. 5 and 6 (mouse proximal tubule cells), Figs. 7, 8 (eosinophilic granules), Figs. 9, 10 (Chelex beads), Figs. 11-13 (phagolysosomes), and in Figs. 14-17 (eosinophilic granules), Figs. 18-21 (Phagolysosomes) and Figs. 22-26 (Chelex beads), respectively.

In the proximal tubule cells of mouse kidney, acid phosphatase is present in lysosomes and vesicles of the Golgi zones. The results are illustrated in Figs. 1-4 and colour prints Figs. 5 and 6. In Fig. 1 black precipitates are shown in these places in a CTEM-image of an unstained ultrathin section. By point analysis the presence of cerium and osmium is established both in lysosomes and the Golgi vesicles. In Figs. 2-6 the lysosome, marked L1 in Fig. 1, is shown after digital-controlled raster analysis. The non-uniform distribution of the precipitate visible in Fig. 5, the 256 x 256 binary electron image of the L1-lysosome, is averaged-out in the 16 x 16 binary electron image (Fig. 6). The out-print of this 16 x 16 electron image is shown in Fig. 2. The threshold, set at 115 c/p/s, separates the lysosomal particle from the cytoplasmic surroundings. The mean grey-level value inside the delineation is 158.44 ± 19.86 c/p/s (counts/point/sec), outside the delineation 67.03 ± 19.54 c/p/s (=131 pixelpoints). The area occupied is 125 pixelpoints "inside" from the total 256 = 0.298 µm². According to the Student t-test these two values are significantly different (p < 0.0005). In Fig. 3 the acquired osmium array is printed with the (threshold 115 c/p/s) electron-image boundary superimposed. The mean net-intensity osmium value is 21.15 ± 6.06 c/p/s "inside" the area (of 125 pixelpoints). Taking into account the spot size used (5 mm) and the dwell time per point (1 sec) the relative mean osmium net-intensity value is 1.1 ± 0.31 c/p/s/µm². The mean osmium net-intensity value of the cytoplasmic surroundings is 18.85 ± 6.37 c/p/s which is also significantly different from the "inside" value (p < 0.0005). In Fig. 4 the cerium distribution array is printed, with the same (threshold 115 c/p/s) electron-image delineation. The mean cerium net-intensity "inside" value is 6.02 ± 3.16 c/p/s; "outside" 2.23 ± 2.68 c/p/s; these values are significantly different (p < 0.0005). The relative mean cerium net-intensity value is 0.3 ± 0.16 c/p/s/µm².

As the program does not recognize "zero" points, the number of pixelpoints is incorrectly reported to be 116 for the "inside" area.

The platinum reaction product related to the peroxidatic enzyme activity of an eosinophilic granule is shown in the colourprints Figs. 7-8 and Figs. 14-17. In such granules a central area is present, known not to contain the enzyme peroxidase, and hence not containing the platinum reaction product. In Fig. 14 the spectrum of a point analysis of the granule is shown. In addition to copper peaks from the grid material in which the organ was assumed to derive from water in the knife-trough (as plain epon shows the peak too), platinum and chlorine from the reaction product are present. Fig. 15 shows the enlarged portion of the STEM-image micrograph, and Fig. 7 the 256 x 256 electron-image from the digital-controlled rastered area. In Fig. 8 the 16 x 16 Pt net-intensity distribution is shown, demonstrating that the nucleoid area still can be recognized. In Fig. 16 the electron-image grey-value array is printed with the particle delineation from threshold 99 c/p/s superimposed. This threshold readily separates both the particle from its surroundings and the nucleoid from the remainder of the particle, although a small part next to the particle is also included. The area occupied by the particle at threshold 99 c/p/s is 0.46 µm². The mean "inside" grey-value was 145.69 ± 35.03 c/p/s (129 pixel-points), the mean "outside" value 76.13 ± 13.57 c/p/s. The two sets of values were significantly different (p < 0.0005). In Fig. 17 the platinum net-intensity distribution array is printed, with the (threshold 99 c/p/s) electron-image delineation superimposed. The mean platinum net-intensity value for the "inside" part is 17.00 ± 8.51 c/p/s and for the "outside" part 11.48 ± 7.28 c/p/s (p < 0.0005). However, the mean Pt net-intensity value of the central core is 18.6 ± 7.15 c/p/s which is higher than the mean Pt net-intensity value inside the particle. Converted into a relative mean platinum net-intensity value: 0.9 c/p/s/µm² (excluding the points not demarcated by the black line). Also in this case the number of pixelpoints "outside" the particle is corrected for the zero points missed by the program.

A phagolysosome in a rat Kupfer cell is shown in the colour prints Figs. 11-13 and Figs. 18-21. By point analysis the presence of a gold signal from the clearly visible, ingested, colloidal gold particles was established plus the lead from the performed cytochemical reaction to demonstrate the acid phosphatase enzyme activity. Fig. 18 shows the enlarged portion of the original CTEM-image of the phagolysosome containing irregularly distributed colloidal gold particles are visible, surrounded by the osmiophilic lysosomal membrane, leaving a central area, without gold particles. The lead precipitate is hard to detect.

In Fig. 11 the 16 x 16 electron-image of the digital-controlled raster analysis of the area is shown. Fig. 12 shows the gold net-intensity distribution, and Fig. 13 the lead net-intensity distribution of the same area. At the thresholds indicated, most of the gold and lead points are located within the area marked by the electron image in Fig. 11. In Figs. 19-21 the gold and lead net-intensity elemental distribution arrays are printed. In Fig. 19 the delineation of the particle is set by the threshold 27 c/p/s in the gold net-intensity array. Visually the distribution coincides very well with the CTEM-electron image in Fig. 18. The area occupied by the gold is 1.03 µm² (1 µm² for the large particle marked L1 and 0.03 µm² for the
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Fig. 18. Shows CTEM-image of a phagolysosome with ingested colloidal gold particles non-uniformly distributed. The presence of lead in this lysosome is established by X-ray microanalysis. Magnification: 224,000X, unstained ultrathin section, 80 kV. and gold-particle-free areas.

Fig. 19. Gold net-intensity X-ray distribution array of phagolysosome shown in colour Figs. 11-13 and 18. The gold threshold 27 c/p/s delineates the gold particle area. (80 kV. 16 x 16 pixel-points). The mean net-intensity "inside" gold value is 49.09 ± 13.24 c/p/s.

Fig. 20. Lead net-intensity X-ray distribution array of the phagolysosome shown in Fig. 19. The gold threshold 27 c/p/s superimposed. The mean lead value "inside" is 13.71 ± 6.01 c/p/s. This value is significantly different from the "outside" value.
small particle in the top right corner marked L2 in Fig. 20). The mean gold net-intensity value for the L1 particle (at the threshold 27 c/p/s) is 49.09 ± 13.24 c/p/s. The mean "outside" net-intensity gold value is 7.21 ± 7.94 c/p/s, which values are significantly different (p < 0.0005). The area occupied 1.00 ± 4.7 ± 14.7 µm². The central area (Φ) not containing visually any colloidal gold particles has a mean gold net-intensity value of 17.53 ± 1.74 c/p/s/µm². The other (Φ*) area only one value 22 c/p/s. These values are significantly different from the gold containing area in the remainder of the lysosome (p < 0.005). The mean lead-intensity values inside the area (delineated by the threshold 27 c/p/s, gold-L1-particle) are calculated from the lead array shown in Fig. 20 to be 13.71 ± 6.01 c/p/s (for "inside") and 8.13 ± 4.44 c/p/s (for "outside" L1 particle, not including L2). These two values are significantly different (p < 0.0005). The mean relative net-intensity lead value for inside L1 becomes 0.17 ± 0.08 c/p/s/µm². The mean Pb net-intensity in the Central "white" area (Fig. 20) is 10.51 ± 4.43 c/p/s.

In Fig. 21, the threshold setting in the lead intensity array has been changed, into 15 c/p/s. The area occupied by the lead is 0.54 µm². The mean lead net-intensity value "inside" the threshold 16-Pb-delineation is 19.39 ± 2.56 c/p/s, the "outside" value is 7.96 ± 3.9 c/p/s (which two sets are again significantly different p < 0.0005). The mean relative net-intensity for the lead value at its threshold 16 c/p/s becomes 0.25 ± 0.03 c/p/s/µm². The mean lead values for threshold 18 as shown in Fig. 13) are for the "inside" L1 value 20.33 ± 8.16 c/p/s, the area occupied 0.33 µm²; the "outside" value 8.16 ± 4.15 c/p/s (excluding L2) and the mean relative lead intensity 0.26 ± 0.02 c/p/s/µm².

Model experiments with Chelex 100 beads

Chelex100 ion exchange beads loaded with either 11.5% iron or 18% platinum, are co-embedded with the tissues or cells to be analysed, and are present in the same ultrathin section. By a single point analysis (or a digital-controlled raster analysis of a small area in the cross-sectioned bead), the relative mean net-intensity values of the standard are compared with the values obtained under the same conditions in the unknown, and thus absolute values are obtained. In the colour print Figs. 9-10, at low magnification, an ultrathin sectioned platinum containing bead is analysed by digital-controlled raster analysis. In Fig. 10 the electron-image is shown, in Fig. 9 the platinum (top left Ma, binary; top right Ma plain), the chlorine (bottom left) and calcium (bottom right) element distribution is given. By comparison, the chlorine and calcium coincide well within the area occupied by the cross-sectioned bead. In Figs. 22-23 the platinum and chlorine arrays are printed with the electron-image boundary of the bead superimposed. The mean net-intensity values were: "inside" 29.1 ± 6.1 c/p/s and "outside" 4.9 ± 4.4 c/p/s. The mean chlorine intensities were: 21.2 ± 5.4 c/p/s for "inside" and "outside" 6.35 ± 5.12 c/p/s. For both the platinum and chlorine values the "inside" values were significantly different from the "outside" values (p < 0.005 for both). The calcium values (not shown) were respectively: "inside" 6.0 ± 3.4 c/p/s and "outside" 1.51 ± 1.79 c/p/s, which values were also significantly different. This element which was considered a contaminant, was not homogeneously distributed over the cross-sectioned bead.

In the arrays shown, the "outside" values were certainly not zero either in the electron grey-level distribution, or in the X-ray net-intensity distribution. Initially this fact was ignored, as the delineation of the particles was done subjectively by the analyst either on-line or ad hoc. To improve the "inside:" "outside" ratio (at a given signal to noise ratio situation) four "variables" are available: a) increase the dwell-time per pixelpoint (Figs. 22-23 are taken at a minimal spot size of 2 nm and 2 sec/point); b) increase the amount of electrons impinging onto the specimen, either by increasing the spot size, the beam intensity or both; c) for the X-ray net-intensities, improve or exaggerate the peak minus background calculation, and d) exclude, by thresholding, unwanted values "outside" the item of interest prior to performing the digital-controlled raster analysis. In Figs. 24-25 examples are given in which cross-sectioned beads are used for this purpose as models. In Fig. 24 the iron net-intensity array of an ultrathin sectioned platinum containing Chelex 100 bead is shown with the X-ray-Fe delineation of 65 c/p/s superimposed. This semi-lunar shaped part was analysed in the STEM-mode at 80 kV, with a 50 nm spot size at a beam intensity of 0.175 µ Amps (as set by the bias voltage) such that the iron net-intensity was below 255 c/p/s. The beam intensity was measured at the isolated 150 µm wide condenser-2 aperture. By varying the bias-voltage setting, the beam intensity (at 50 nm spot size) could be ranged from 0.175 to 1.048 µ Amps. The concomitantly acquired iron net-intensities "inside" the bead ranged from 119 c/p/s to the maximum of 508 c/p/s. The "outside" values were virtually zero, ranging from 0-6 c/p/s.

In Fig. 25 the iron net-intensity distribution is given of the same bead as in Fig. 24 with the X-ray Fe delineation of 50 c/p/s superimposed, but now at 20 nm spot size and 0.410 µ Amps at the C2 aperture. The "outside" values are very low indeed. In Fig. 26 the relation between the beam intensity (as measured at C-2) and the iron net-intensities are given (per sec in single point analysis) in the STEM-mode of a 22-23 nm cross-sectioned bead. Various spot sizes and bias voltage settings. The direct relation between the beam intensity and iron net-intensities is clearly shown. In these model-experiments in which actually no iron is present "outside" the bead area the iron values are virtually zero. In these two examples the threshold values of the single-scans were kept zero. By raising that threshold only a few counts/point the "outside" value could have been made zero throughout. In these types of experiments this is without danger, but in ultrathin sectioned cellular material, this "absence of element" condition cannot be guaranteed. However, the beads present in the same ultrathin sections can be used to create conditions favourable for the analysis of the unknown structures in the cells.

In the cross-sectioned bead in Fig. 24, the magnification chosen is rather low to analyse also the surrounding embedding medium. In doing so, the actual percentage of the area sampled by the spot diameter (in relation to the area covered by

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Fig. 22. Platinum net-intensity X-ray distribution array of a cross-sectioned Chelex100 ion exchange bead. The particle delineation superimposed is from the electron image array (shown, in Fig. 10).

<table>
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<th>Pt distribution</th>
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<td>Pt (c/2s) = 58.2 ± 12.2 (+ 21%)</td>
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**Fig. 23.** Gives the chlorine net-intensity X-ray distribution of the same Chelex100 bead. The delineation is again the electron-image threshold superimposed before, in Fig. 22.

**Fig. 24.** Ultrathin cross-sectioned Chelex100 bead with iron. (Spot size: 50 nm, 80 kV, 1 sec/point).

**Fig. 25.** Same semi-lunar shaped cross-sectioned Chelex100 ion exchange bead loaded with 11.5% iron (Spot size: 20 nm, 80 kV, 1 sec/point). Beam intensity = 0.410 µAmp. (at C2-aperture of 150 µm): "inside" maximum below 255 c/p/s.
the reduced raster area) is rather small (about 0.56%). By increasing the magnification and the spot size (or increasing the number of pixels points inside the analysed reduced raster area) this percentage can be augmented till the interpixel distance equals the beam diameter value. At that moment the whole area is completely covered by an interconnecting set of spots.

Discussion

In biological material, the X-ray signal to noise ratio can be rather low. However, in cytochemistry rather heavy metal precipitates are allowed to be generated at the enzyme-containing sites. Moreover, also OsO₄ post-fixation procedures (with or without complex-formation to increase membrane contrast) are generally applied (11,37,38). In such metal-containing cell organelles the enzyme-related metal (precipitate) can be detected by the electron image related metal (precipitate) from other elements present there. These elements can be present either naturally (e.g. ferritin in lysosomes) or artificially introduced as markers (ingested colloidal markers 7,25). Especially in the latter case the elements are certainly not homogeneously distributed over the organelle. Moreover, taking lysosomes as an example, each organelle in the population in the cell might have a different history and hence a different enzyme content. As such organelles are participating actively in cell processes both the number of the lysosomes in the population, their volume and/or their enzyme concentration might change. Whenever quantitative information can contribute to unravel such dynamic processes, from each individual organelle involved, the morphometrical information acquired has to be correlated with the chemical enzyme-related information.

In the present paper the results of the application of a new software program, designed to extract the desired morphometrical and chemical information and to integrate these two information streams, are reported. With respect to the results presented in the literature with such programs, a new aspect was the introduction of a manually located, reduced raster area around the structure of interest, to discriminate the area to be analysed from the elemental maps proposed by Jeulin (21) and Bauer et al. (3). In our program the electron image dominates over the X-ray images, which are considered secondary; however, the opposite approach can still be executed. As mentioned in the introduction, by integrating morphometrical and chemical information four different combinations are theoretically possible: homomorph or heteromorph. In practice two remain important: inhomogeneous or homogeneous, irrespective whether the structures morphologically are homomorph or heteromorph. Several aspects of the acquired results will be discussed: resolution, in both the electron and X-ray image, the integration process, the X-ray quantitative aspects, the minimal detectable limit and the statistics.

The electron-image resolution

Under normal S(T)EM-conditions the electron-image resolution is determined by the magnification, spot size, number of lines per frame and the line-time (in our case 1000 lines per frame; 64-1000 m sec/line). On our 125 x 100 mm screen at the proposed (basic) magnification of 12500x, a spot size of 10 nm suffices for 1000 lines per frame (when beam broadening is ignored temporarily) for 10 nm resolution. A digital-controlled raster analysis of the whole screen, using 1024 x 1024 pixelpoints, will maintain that resolution. By reducing the area to be analysed to 1/4, 1/8, 1/16 till 1/64 of the full screen, the number of pixelpoints can be reduced from 1024 x 1024, via 512 x 512, to 16 x 16, without losing the original resolution. In all cases the interpel distance (IPD) = spot size used. Whenever desired, the lateral resolution can be improved, and at 62500x (for 1000 lines/frame or 1024 x 1024 pixelpoints per full screen) our minimal spot size is reached. This resolution can again be maintained for smaller areas on the screen by reduced numbers of pixels per rastersquare, for the condition IPD = minimal spot size. A further increase of the magnification is possible but does not improve the resolution (empty magnification). Sometimes, under the condition IPD > spot size, the required resolution can be reached by reducing the area to be analysed somewhat further and/or by selecting a higher total number of pixelpoints, such that the condition IPD = minimal spot size is fulfilled again. For morphometrical analysis, as performed by our program, the resolution of the electron image is of less importance. The variation in magnification, or the number of pixelpoints (16 x 16 to 256 x 256) has a relatively small influence upon the calculated area.

However, under the condition IPD > spot size, the numerical information obtained from the electron image is fractional, and might, especially in inhomogeneously distributed electron scattering, lead to wrong conclusions. Moreover, when boundaries are analysed and converted into figures, these values are also less reliable. Although in such cases the use of a higher number of pixelpoints results in a better localization sharpness, the area calculated is influenced less. So far we have not used the numerical information of the electron image for quantitative purposes.

The X-ray image resolution

Under the condition IPD = spot size, the chemical X-ray information has the same lateral resolution as the electron image. Provided the minimal detectable limit for that element is exceeded. This is generally achieved by increasing the dwell-time per pixelpoint considerably. Under this condition the whole area to be analysed is covered by a network of joined circular spots, amounting to 70% of the total area within the reduced raster. When beam-broadening in the ultrathin, 50 nm thick section is taken into account the array of joined cylinders is transformed into an array of truncated cones. At which moment (ignoring the specimen tilt) the entire volume of material within the reduced raster area is analysed, at the cost of overlap.

Under the condition IPD > spot size, the analysed volume in the truncated cone represents only a fraction of the total volume to be investigated. When the elemental distribution is homogeneous, this does not create serious problems, but it certainly does when elements are
inhomogeneously distributed. An increase of the spot size can re-establish the condition IPD = spot size, with concomitant loss of resolution, but also with a gain in signal. However, the values obtained for such an area are averaged out and might give rise to false conclusions. The same holds true for the values obtained at boundaries where the "inside"/"outside" distribution over the analysed area can give rise to figures from just over the "outside" value to values just under the "inside" figure. Again this is of relatively little influence for the morphometrical conclusions but it is of influence for the chemical implications and for the integration process. In the examples shown in this paper the final magnifications chosen were higher than the maximum set by the resolution (62500 x). Moreover, for practical reasons related to the integration process, 16 x 16 points were used per reduced raster throughout. The IPD in Figs. 1-4 is 49 nm, in Figs. 16-17 = 59 nm and in Figs. 19-21 = 117 nm. The spot size was in all three cases 5 nm, and as a consequence the percentage of the total reduced raster area analysed was rather low. Increasing the spot size could have created the condition IPD = spot size, with the consequences discussed for the resolution and averaged mean elemental concentration. The electron image in Fig. 7, analysed at 256 x 256 pixel points, of 5 nm spot size deserved 512 x 512 points of 2 nm spot size to fulfill the condition IPD = spot size, for optimal resolution equal to the original STEM image Fig. 15.

Application of Goldstein's beam broadening equation to the 50 nm ultrathin sectioned Chelex100-Pt and Fe beads resulted in b-values of 7 and 3 nm respectively (15). As a consequence the d+b values for our spot sizes ranged from 5-103 (for Fe) and from 9-107 (for Pt) instead of 2-100 nm, and concomitantly the condition IPD = spot size (d) can be changed into IPD = (d+b). The increase in analysed volume can be calculated assuming that cylinders become truncated cones with top circles of d and bottom circles of (d+b):

\[ V_{\text{cylinder}} = \frac{\pi}{3} d^3 \]
\[ V_{\text{truncated cone}} = \frac{\pi}{3} h \left( \frac{d^3}{H} + \frac{b^3}{h} \right) \]

As a consequence the elemental concentration determined by digital-controlled raster analysis is based upon the presence in the same ultrathin section of a cross-sectioned standard to be analysed under the same conditions as the unknown structure, by a digital controlled raster.

\[ C_{\text{unknown}} = \frac{C_{\text{standard}} \cdot \frac{\pi}{3} d^3}{\frac{\pi}{3} h} = \frac{C_{\text{standard}} \cdot \frac{\pi}{3} b^3}{\frac{\pi}{3} H} \]

As the aim was to obtain net-intensity values, the original IPP-program idea was followed, to perform, on-line, a peak minus background subtraction per pixel point. However, the quantitative results are highly influenced by the choice, which region of the continuum is considered to represent best, the continuum under the three specific peak regions selected. Initially (Figs. 1-22) a background region in the continuum part of the spectrum was subtracted, that did not contain any peaks, according to the criteria generally accepted (30,31). Moreover, the residual figures outside the structures (as revealed by the line scan display prior to the raster analysis) were only sparingly cut off, by raising the threshold. Later we adopted a method in which the content of the chosen continuum contained so many counts, that after subtraction the resultant peak value was about equal to the value generated by a Tracor Northern program used for the quantitative analysis by hand calculation. In this way the discrimination between "inside" and "outside" the structure analysed in the reduced raster area is statistically improved (Figs. 24-25).

The first method underestimated the continuum under the three specific regions, the second has a tendency to overestimate it, as negative values are set zero.

Several other alternatives for this background subtraction problem are available which have not been tested so far. Until then the latter method is adopted, as the standard is present in the same ultrathin section and measured under the same conditions. For that reason, it had to be assumed that the section thickness and the specific mass of the standard area is the same as the area of the unknown structure, to express both mean relative net-intensity values as counts/sec/mm². The method applied demonstrates that heterogeneity in the elemental distribution in heteromorphic lysosomes can be measured and converted into figures. Only one example is shown (Figs. 13,20 and 21) (threshold 16-Pb-delineation: \( \frac{\pi}{3} \frac{\pi}{3} 0.25 + 0.03 \text{ c/s/mm}^2 \); threshold-18-Pb-delineation: \( \frac{\pi}{3} \frac{\pi}{3} 0.26 + 0.03 \text{ c/s/mm}^2 \) as compared to the gold threshold 27:

\[ \frac{\pi}{3} \frac{\pi}{3} 0.17 + 0.08 \text{ c/s/mm}^2 \] in which the selected thresholds produced Pb values which were mutually not highly significantly different.

However, the central area in the gold-containing lysosome (Figs. 18 and 19), not containing any gold particles visually, is significantly different from the mean gold value in the remainder of the lysosome (p < 0.005). (\( \Phi \) has a mean gold net-intensity = 17.53 + 1.74 c/p/s). Similarly has the outer (\( \Phi \) "white" area in the lysosome (gold value = 22 c/p/s) a significant difference with respect to the remainder of the lysosome. The mean Pb-net intensity inside the central area (10.5 + 7.4 c/p/s) is not significantly different from the mean "inside" Pb-value (13.71 + 6.01 c/p/s). The minimal detectable mass (minimal mass fraction)

The success of the application of the method of digital-controlled raster analysis is determined by the condition already mentioned, provided the minimal detectable limit is exceeded. For the minimal detectable mass (MMF) Joy and Maher (22) gave a formula: Minimal Detectable Mass = \( \frac{1}{\sqrt{T \cdot J}} \) where T is the counting time, J is current.
density impinging onto the specimen in the selected spot, and \( P \) is \( Q \cdot \omega \cdot r \cdot a \), detection efficiency (in which \( a \) = the fraction of the spectrum used). Again, \( T \) and \( J \) are the variables, making the MDM decrease when \( T \) and \( J \) are large. We had to select \( T \approx 1-10 \) sec., whereas initially (Figs. 1-4) the beam intensity at the chosen (too low) spot sizes was certainly not set at the maximum available value. When these conditions were set accordingly, the inside values were not only much higher, the outside values were virtually zero (Figs. 24-25).

According to Hall (17), the counting rate \( R \) (in c/sec) is determined by: \( R_x = (\text{number incident electrons/sec}) \times (\text{X-ray quanta generated/electron}) \times (\text{detection efficiency of the detector}) \) which becomes: \( R_x = (0.6 \times 10^{-13}) \times (\omega_x Q_x a S_x) \times (\text{detection efficiency}) \), in which \( I \) = probe current, \( Q_x \) = fluorescence yield, \( Q_x \) = cross section for ionisation, \( a \) = the fraction of the spectrum used, and \( S_x \) is the element fraction present in the area under the beam. Hall argued that \( a, \omega_x \), and \( Q_x \) are more or less constant, as was the detection efficiency in a certain instrument, so \( R_x = C.I.S_x \) (in which \( C = a \) constant value). A similar formula can be given for the embedding material not containing the element \( x \), \( B_x \), which becomes: \( R_x = C.I.S_x \) (see below). In our cases for the element \( x \), present in the structure of interest, the mean net-intensity value "inside" represents \( R_x \) and that in the surrounding cytoplasmic matrix ("outside") represents \( B_x \). \( B_x \) not only represents embedding material but might also contain the element \( x \). Adopting the equation for the Minimal Detectable Limit, as given by Chandler (5), we can formulate a criterion for the limit at which the mean elemental concentration "inside" the structure is too low, to be differentiated from the element in the surroundings. This criterion could be:

\[
\left[ \frac{X_{\text{in}}}{X_{\text{out}}} \right]_{\text{min}} \geq n \cdot \left[ \frac{X_{\text{in}}}{X_{\text{out}}} \right]_{\text{min}}^{\frac{1}{2}}
\]

(1)

in which \( X_{\text{in}} \) and \( X_{\text{out}} \) are the mean elemental values as obtained by the reduced raster application. Now the minimum concentration \( C_{\text{in}} \), at which the criterion works can be calculated. Considering the situation that an ultrathin sectioned standard is available and analysed by the digital-controlled raster method

\[
C_{\text{in}} = \frac{X_{\text{in}}}{X_{\text{out}}} \quad \text{and hence} \quad X_{\text{in}} = C_{\text{in}} \cdot X_{\text{out}}
\]

(2)

In this formula \( X_{\text{in}} \) = mean net intensity value in the standard \( C_{\text{in}} \) = concentration in the standard and \( X_{\text{out}} \) = minimal values. For the criterion (1), \( \frac{X_{\text{in}}}{X_{\text{out}}} \geq n \cdot \left[ \frac{X_{\text{in}}}{X_{\text{out}}} \right]_{\text{min}}^{\frac{1}{2}} \) two conditions are to be adopted:

1) \( \frac{X_{\text{in}}}{X_{\text{out}}} \min \approx \frac{X_{\text{in}}}{X_{\text{out}}} \geq n \cdot \left[ \frac{X_{\text{in}}}{X_{\text{out}}} \right]_{\text{min}}^{\frac{1}{2}} \) (in which \( \frac{X_{\text{in}}}{X_{\text{out}}} \) and \( \frac{X_{\text{in}}}{X_{\text{out}}} \) are the peak and background inside the structure under the minimum condition).

Equation (2) introduced into criterion (1) makes:

\[
C_{\text{in}} \geq n \cdot \left[ \frac{X_{\text{in}}}{X_{\text{out}}} \right]_{\text{min}}^{\frac{1}{2}} \cdot \frac{C_{\text{in}}}{C_{\text{st}}} \%
\]

When it is permitted to accept that \( \frac{X_{\text{out}}}{X_{\text{out}}} \) and \( \frac{X_{\text{out}}}{X_{\text{out}}} \) are more or less constant, the standard deviation are directly calculated by the program. For simplicity we excluded from the program the calculation of the mean "outside" values, which is regretted, as now it has to be done by hand, off line. The Student t-test was used to demonstrate that the mean "inside" values were significantly different from the outside values. Only in two examples (Figs. 19 and 20), the elemental heterogeneity was judged in the same way.

In our program only values between 0 and 255 are printed in the arrays. The linescan analysis, routinely performed prior to the digital-controlled raster analysis, is also used to trim down the vertical scale till below 255, generally by reducing the beam intensity. However, the opposite can be done too: at high X-ray yields, the baseline in the linescan display can be raised until it is 254 units below the highest value "inside" the structure: that value is set zero. In the latter case the conditions formulated by Barbi (2) to acquire at least 1000 counts/point/sec can be met and hence the accuracy level related to it. It is realized that the success of the acquisition process demonstrated in this paper is due to the high concentrations of an element present in the structure of interest. The cross-sectioned beads outside the cells or tissues can be used to set the instrumental and analytical conditions for correct analysis of the unknown structures. However, the cytoplasmic values for element X "outside" the structure are not necessarily zero, as the plain embedding medium outside the beads mostly is. By cutting these values off, information is lost.

The integration of the electron and X-ray images is performed after the two acquisition steps. In general, the stability of the specimen must be high enough to enable these two acquisitions, otherwise the integration, as performed now by calculating directly the area outside the structure, and the mean net-intensity value, creates nonsense values. When such instabilities occur, the obtained values can be...
"corrected" by hand calculation. Similarly, particles considered not to belong to the structure of interest can be excluded (Fig. 16).

The elemental maps are acquired in the same acquisition run and can be considered to be topographically alike. We will change the program in a way that the total dwell time per pixel point includes both the time needed to perform the electron analysis and the time for the X-ray acquisition.

The delineation process is multi-directional. The delineation lines, set by thresholds and e.g. created in one X-ray elemental array, can be superimposed over any of the other X-ray arrays or over the electron image array, or vice versa. By defaulting the arrays, only the threshold delineations can be printed. This enables the topographic relation between elements within one structure to be reconstructed. As the arrays are stored on disk, the integration process can be reconsidered completely, later on. So far, the decision where to create the boundary of the particle, or of the elemental location within it, is taken subjectively, by the analyst. Now that the "inside"/"outside" ratio has been improved, decisions about the particle delineation can be made more objective. However, this objectivity for the elemental concentration might be difficult and additional criteria have to be found. The presence of the ultrathin sectioned Chelex100 beads (containing the standard in a known concentration) next to the unknown particles in the same section enables direct quantitative analysis. Moreover, these beads can be used to judge homogeneity, now expressed as a mean figure plus or minus standard deviation, in relation to spot size and magnification. So far we have analysed cross-sectioned beads at rather low magnifications, small spots, and a relatively low amount of points within the bead have been measured, but there are no real barriers to do so at high magnification, and a high number of pixel points. It is to be realized that homogeneity of the cross-sectioned beads can be disturbed by inhomogeneous parts in the underlying film, or by material between film and ultrathin section. Moreover, the sectioning artefacts, although easily recognized, play an important role.

The main emphasis in the present program is to solve the problem starting from the non-uniform distribution of the elements inside the cell organelles. Three examples are given: 1) in which the relative small non-uniform cerium distribution in lysosomes is averaged out by the chosen 16 x 16 pixel points for X-ray analysis (Figs. 1 to 6), and also the osmium present appears to be rather uniformly distributed over the lysosome as discovered after the analysis; 2) in which a known naturally occurring discontinuity in the Pt-distribution within a granule is used to demonstrate at which level of resolution the program can discriminate between two areas (Figs. 7, 8, and 14-17); 3) in which the non-uniform distribution of colloidal gold particles in a phagolysosome are related to the lead precipitates of a cytochemical reaction to detect acid phosphatase activity. In: X-ray microanalysis in the electron microscope II. Histochem. J. 7, 205-229.

References


Acknowledgments

The assistance of Mrs. G.C.A.M. Spigt-v.d. Bercken and Mrs. J.M. Tjong Aklet in preparing the manuscript and the skillful support of Mr. J.J. Beentjes and Mr. L.D.C. Verschragen in preparing the micrographs is gratefully acknowledged. Prof. Dr. W.Th. Daems, Mr. H.K. Koerten and Mr. M.J. van Noord are thanked for their critical reading of the manuscript.
Integrated image analysis in biology

G.M. Roomans: The X-ray image resolution is not in the first place determined by the spot size, or 'cylinder' size, but by the volume of the 'truncated cone'. Although the author appears to be well aware of this, he creates in my opinion unnecessary confusion by first adhering to the 'cylinder' concept and then correcting to the 'truncated cone' concept. In the meantime, however, totally irrelevant calculations of X-ray intensities in c/sec/mm² have been carried out (results).

Author: Initially, the beam-broadening aspect as calculated by Goldstein (15) for thin metal foils was considered to play a rather unimportant role in biological ultrathin sectioned tissue. In a later phase, it was realized that specific gravity values had been published previously (7) for the Chelex standards also used in this investigation. This opened up the possibility to calculate the beam-broadening in such cross-sectioned beads. When performed, it was noticed that the initial point of view was not correct and consequently the cylinder concept was replaced by the truncated cone concept. For didactical reasons, the original cylinder concept was not withdrawn. As long as the truncated cones do not overlap, the X-ray resolution is determined by the IPD-values. The conversion of the X-ray net-intensity values into unit values (counts/points/sec/mm²) is independent of the resolution. It enables one to compare the net-intensity values acquired in the Chelex-standards with the values obtained in the 'unknown' in the cell. It is assumed, that in the two places, the volumes of the truncated cones and the specific mass therein, are identical and that the cones do not overlap during the reduced raster analysis.

A.T. Sumner: No mention is made of the stoichiometric aspects of either enzyme reaction. This is not in itself important for the demonstration of the procedures described in this paper, but the fact is that the results are given two decimal places with standard deviations attached. These figures are certainly measures of the amount of reaction product, but are not necessarily proportional to the amount of enzyme. If it is known that the amount of reaction product is an accurate measure of the quantity of enzyme, this should be stated; and if that is known, this limitation must also be mentioned.

Discussion with Reviewers

G.M. Roomans: In the case of values in the X-ray intensity arrays exceeding 255 (Materials and methods step 7), wouldn't it be better to scale all points relative to a maximum, i.e. for if the intensities range from 0-765, all intensities would be divided by 3?

Author: With the experience obtained with the program over the last period we now realized, that accepting the 255 counts as a maximum for the vertical scale can create a problem which is easily overlooked, when high intensity values per point are acquired. In the first revision of the program an increase to twice that value has been foreseen (see also the last question).

A.T. Sumner: It is not clear why the author used OsO₄ plus K₄Fe(CN)₆ postfixation after the acid phosphatase. No doubt the OsO₄ would be essential to give adequate image contrast, but the use of K₄Fe(CN)₆, which might be expected to stain glycogen perhaps needs further explanation.
not been established. Truncation, ad hoc was not applied, though possible. However, as the relation between enzyme activity and metal precipitate is now under investigation the accuracy can soon be adopted.

D.C. Joy: I would like to have seen some more details of the TN 2000 image store system, particularly with regards to its acquisition parameters (time per pixel, dynamic range, etc.), and some discussion of the problems of radiation damage - just how much dose is the sample exposed to in this procedure? Finally, I found the numerical presentation of data rather curious, in many places data is specified in the form 13.85 +/-6.37 etc. With error bars that large it is simply not sensible to give data to three significant figures. 14 +/-6 would have done just as well and actually have been more correct.

Author: Electron or X-ray net-intensity images are acquired per pixel point, ranging in number from 256 x 256 to 16 x 16 in a reduced raster area. The digitalized information is stored on disk, and can be re-introduced into the program at any time. In principle the acquisition in the reduced raster is not limited by the dwell-time per pixel point, but in practice the stability of the specimen (both in position and composition) restricts its acceptable exposure time. The radiation damage (per unit time) is not different from that present by a point analysis. Actually the total dose is spread over a larger area. I admit, that the numerical presentation is out of proportion, but when designing a program, decision have to be made without precise knowledge of the final result. Truncation, ad hoc, is always possible but not applied here.

R.P. Becker and G.M. Roomans: Can you comment on the significance of color intensity in Figures 5-13?

Author: By the computer, the acquired digitised grey-values, or net-intensity values ranging per point between 0 and 256, are divided into 16 categories (0-16; 17-32, etc.). To each of these categories a colour is assigned, dark colours for the low values, lighter colours for the higher values, white for the highest. As described, the line-scan, performed prior to the reduced raster analysis displays the acquired values in the sixteen points against the colour scale. By changing the beam conditions the position of the range of grey- (or net-intensity) values in the object analysed (per line-scan) can be shifted along the colour scale on the screen, more to the white range or more to the black side. Once fixed, the reduced raster area is analysed accordingly, and the final result displayed (and/or stored on disk). When the image is converted into a binary image with the help of the (original IPP) subroutine "Histogram" any colour can be selected for the two areas created. (In Figs. 5-6 the combination blue-white was selected). In the developed subroutine "Print" a second option was introduced, to have one colour for the surroundings and the full scale for the values inside the delineated area. One is free to choose the colour for the background (in Figs. 12-13 a dark red colour was selected). In short, the colours as used in this paper, only have artistic, no scientific values. The concomitantly introduded printed arrays represent the scientific part of the image, and show the calculations.

Captions for Figures 5-13

Dark colors indicate low values, light colors indicate high values (see Discussion with Reviewers).

Fig. 5. Binary electron image taken with 5 nm spot size at 256 x 256 pixel points from L 1 marked in Fig. 1. Cerium distribution is not uniform.

Fig. 6. Binary electron image taken from the same lysosome L 1 at 16 x 16 pixel points. In Fig. 2 this array is printed out. Cerium distribution uniform.

Fig. 7. Electron image of one eosinophilic granule visualized by a peroxidase/D.A.B./H 2 PtCl 6 method. Fig. 8. Net-intensity platinum X-ray distribution of the same particle shown in Fig. 7 at 16 x 16. Print out of this image in Fig. 17.

Fig. 9. Four net-intensity X-ray images from a cross-sectioned Chelex100 ion-exchange bead loaded with platinum. 2x Pt/C1/Ca. The Pt and Cl arrays are printed out in Figs. 22-23. (see also text.).

Fig. 10. Electron image of the same cross-sectioned Chelex 100 Pt-bead. Figs. 9 & 10 at same magnification.

Fig. 11. Electron image obtained by a 16 x 16 analysis of the phagolysosome, shown in Fig. 10. Fig. 12. Gold net-intensity X-ray distribution. Magnification is same as Figure 11.

Fig. 13. Lead net-intensity X-ray distribution of the same area, at the same magnification as Fig. 11.