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Observation on Backscattered Electron Image (BEI) of a Scanning Electron Microscope (SEM) in Semi-thin Sections Prepared for Light Microscopy

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In order to examine semi-thin section for light microscopy with the backscattered electron mode (BE mode), identical sites in tissue sections were comparatively observed with both light microscopy and BE mode. Tissue blocks (ca. 3×3×1 mm) were fixed in glutaraldehyde or combined formaldehyde-gluta raldehyde solution. After dehydration in alcohol, they were embedded in Kushida's GMA-Quetol 523. 1.0 μm sections on glass slides coated with indium oxide were stained with hematoxylin-eosin or toluidine blue or by the Giemsa method, and then treated with osmium tetroxide vapor or aqueous $KMnO_4$ solution or uranyl acetate-lead citrate solution.

The identical places of such sections could be examined with the accelerating potential of 6 kV and the probe current of 8×10^{-10} A using a JSM-35C SEM with BEIS BE detector. Photographs were taken with the 2500-line resolution cathode ray tube and the time of exposure was 100 sec. The sections were placed at a distance of 5mm from the BE detector.

BE images from osmium tetroxide vapor staining showed a distinctly improved contrast especially when the sections were previously stained with hematoxylin and eosin. The cellular structure was clearly demonstrated under the electron microscope in the BE mode. Identical sites in tissue samples could be compared exactly with both light and electron micrographs.

(Key Words: Light and Scanning Electron Microscopy, Backscattered Electron Mode, Semi-thin Section, GMA-Quetol 523 Embedding, Indium oxide-coated Slide Glass)

INTRODUCTION

Recently, backscattered electron images (BEI) have been applied to the study of histochemical sections (1,2,3,4,8,16,19). The BE signal has become sufficiently detectable under the same observation condition as for secondary electron images by use of a high-sensitivity BE detector (BEIS) (8,16). Hartman and Nakane (1981) revealed that the antigenic sites may be localized with high sensitivity on tissue sections mounted on carbon-coated glass slides by detecting osmium tetroxide-DAB complexes using the BE mode. Furthermore, the improvement of the BEI method was presented by Kushida *et al.* (1982), who applied it to the study in sections mounted on indium oxide-coated glass slides from tissue samples embedded in GMA-Quetol 523 and revealed detail of the cellular structure.

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A mixture of GMA and Quetol 523 has been introduced in the fields of semi-thin section by Kushida (1977). Identical parts of such sections have been observed with both light and transmission electron microscopy after staining with basic and acidic dyes, followed by osmium tetroxide vapor treatment (9,10). In these experiments, the cellular components, particularly mitochondria, Golgi apparatus, endoplasmic reticulum and cytoplasmic granules have been easily identified in sections mounted on grids. On the other hand, it follows that BEI allows the use of sections mounted on glass slides. It is therefore necessary to establish a direct correlation between tissue structure studied in the light microscopy and the intracellular organs identified in the BE mode.

The purpose of the present study is to further develop the BEI method and to find direct correlations with light microscopy. Significant improvements in contrast, resolution, stainability and tissue preservation have been made. The cellular structure was clearly demonstrated under the SEM in BE mode. Thus, photomicrographs and electron micrographs of identical sites in tissue sample could be compared exactly.

MATERIALS AND METHODS

Tissue blocks (ca. $3 \times 3 \times 1$mm) were fixed in 2.5% phosphate buffered glutaraldehyde or combined formaldehyde and glutaraldehyde (14). Fixation was achieved by immersion for 2—3 hr followed by washing with phosphate buffer. After dehydration in alcohol, the blocks were embedded in GMA-Quetol 523M* resin mixture with QCU-1* as catalyst, according to the method described by Kushida *et al.* (1982). All steps were carried out on a shaker at room temperature (6). Gelatin capsules (no. 0 or 00) with flat bottoms were filled to the brim with GMA-Quetol 523M with QCU-1. Polymerization was carried out in an oven for 12 hr at 60°C (when 0.05g of QCU-1 was added to 100ml of GMA-Quetol 523M) or for 36 hr at 39°C (when 0.4g of QCU-1 was added to 100ml of GMA-Quetol 523M) as usual.

Sections 1.0μm thick were mounted on glass slides coated with indium oxide by water flotation. Indium oxide coating provided adequate protection against surface charging (8). After staining with Giemsa method or toluidine blue or hematoxylin-eosin without removal of embedding matrix, they were observed under a light microscope using a 40× NCG or 100× NCG objective lenses and photomicrographs were taken using a Nikon microflex UFX. For the observation of BE images, such sections were exposed to osmium tetroxide vapor for 3hr or slightly stained with 0.5% aqueous $KMnO_4$ solution for 3 min or stained with 5% aqueous uranyl acetate solution for 15 min and Reynold's lead citrate solution for 5 min. Preparations were then examined at low magnification with a JSM-35C SEM using an appropriate detector, a high-sensitivity BE detector (BEIS). The contrast of BE images was electrically reversed. An accelerating potential of 6kV and the probe current of 8×10^{-10} A with probe size of 1000 Å were used. Photographs were taken with the 2500-line resolution cathode ray tube, and the time of exposure was 100 sec. A reduced working distance of 5mm from

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the BE detector was usually employed. Scanning electron micrographs were taken at low magnification and enlarged photographically.

RESULTS AND DISCUSSION

A direct correlation with both light microscopy and BE mode has been made. The identical sites in semi-thin sections of 1.0μm were examined and the photomicrographs were compared with the electron micrographs of BE images. The stained sections gave sharp images with brilliant colors satisfactory for conventional light microscopy. Indium oxide coating did not interfere with stainability or color images.

Tissue samples fixed in a formaldehyde-glutaraldehyde mixture, dehydrated in alcohol and embedded in GMA-Quetol 523M mixture, are illustrated in Figs, 1—4. Fig. 1A is a photomicrograph of mouse small intestine and Fig. 2A is a photomicrograph of mouse seminiferous tubule in the testis. These 1.0μm sections were treated with osmium tetroxide vapor after hematoxylin and eosin staining. Figs. 1B and 2B show electron micrographs of the reversed contrast BE images of identical sites in the same sections shown in Figs 1A and 2A, respectively. Photomicrographs and electron micrographs of the reversed contrast BE images of identical sites on the sections could thus be exactly compared. Figs. 1C and 2C are enlargement from Figs. 1B and 2B, respectively. Fig. 3 shows mouse small intestine stained with toluidine blue, while Fig. 4 shows cortex of mouse kidney stained with hematoxylin and eosin. They were examined after osmium tetroxide vapor treatment for 3 hr.

For BEI observation, osmium tetroxide vapor staining showed a distinctly improved contrast which was specifically based on the previous staining dyes (Figs. 1B, C, 2B, C, 3 and 4). BE images of sections treated with osmium tetroxide vapor exhibited enhanced basic dyes compared to either $KMnO_4$ or uranyl acetate-lead citrate staining. Aqueous solution of $KMnO_4$ and uranyl acetate-lead citrate uniformally increased the contrast of BE images and failed to preserve the cellular structure of tissue specimens. Among the histological staining studied in this paper, it has been revealed that osmium tetroxide vapor after hematoxylin and eosin staining selectively identified the tissue components and so facilitated cytological studies using SEM in BE mode. Reversed contrast of BE images has a similar contrast to that of images taken with a conventional transmission electron microscope (TEM). The specific contrast at the intracellular boundaries also distinguished each cell and cytoplasmic components were clearly recognizable. Combined glutaraldehyde and formaldehyde fixation (14) preserved the cytoplasmic structure in the BEI along with good stainability in light microscopy.

The use of the BEI method to the semi-thin sections embedded in resins has been developed in recent years by Abraham and DeNee (1973, 1974), Backer and De Bruyn (1976), Tannenbaum *et al.* (1978), Backer and Sogard (1979) and Ogura and Hasegawa (1980), who pioneered the possible application of BEI in cell and tissue research. Soligo and De Harven (1981) studied cytological methods to BEI mode by use of TEM and SEM control. The BE mode was also employed for immunohistochemical localization of antigens by Hartman and Nakane (1981) and Nakane *et al.* (1982). Problems pro-

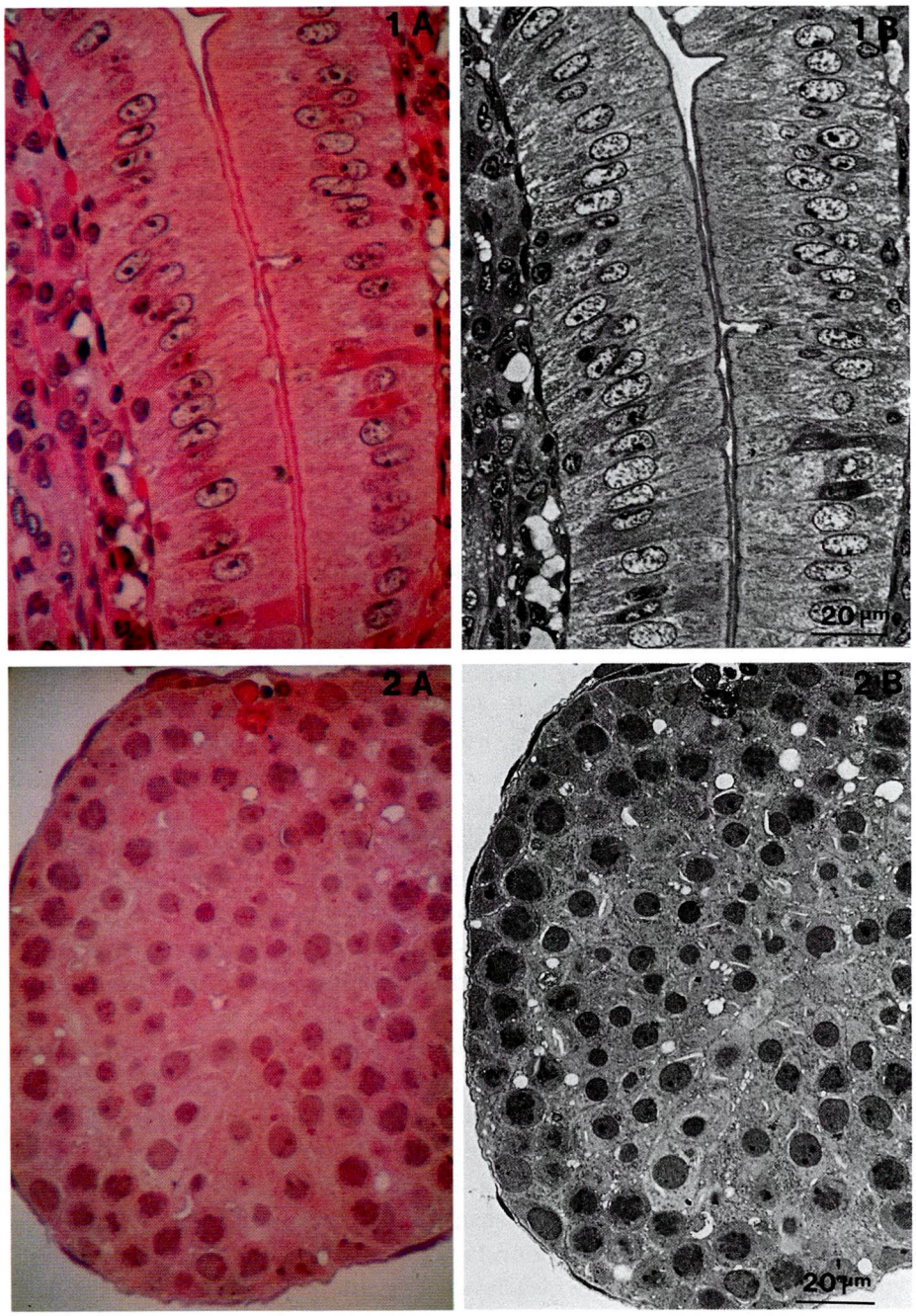
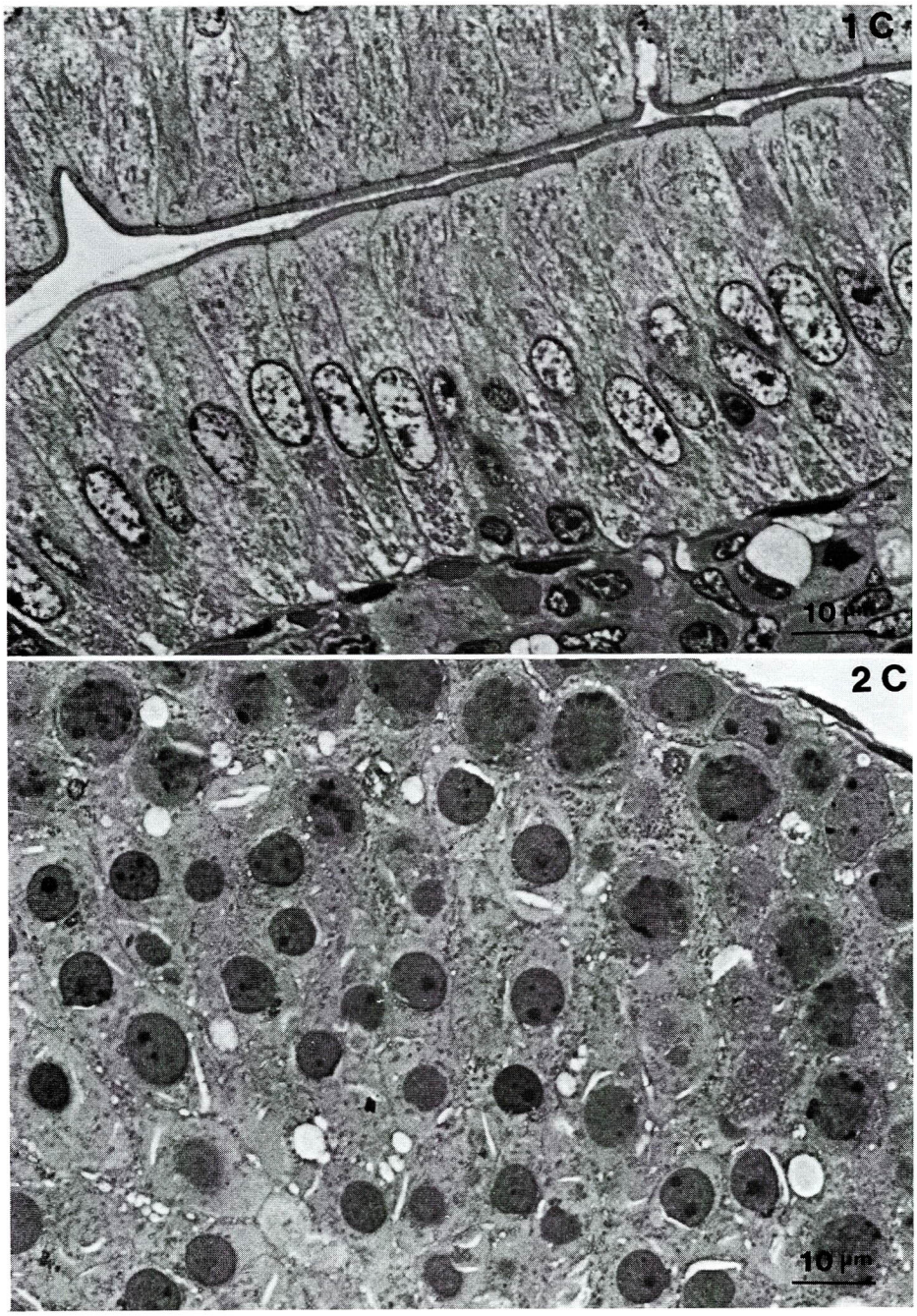


Fig. 1A Photomicrograph of mouse small intestine, stained with hematoxylin and eosin. ×770.
Fig. 2A Photomicrograph of mouse seminiferous tubule, stained with hematoxylin and eosin. ×770.
Figs. 1B and 2B Electron micrographs of reversed contrast BE images of the same sites in sections as Figs. 1A and 2A, respectively, treated with osmium tetroxide vapor after hematoxylin-eosin stain.



Figs. 1C and 2C Electron micrographs of reversed contrast BE images, enlarged from Figs. 1B and 2B, respectively. ×1600.

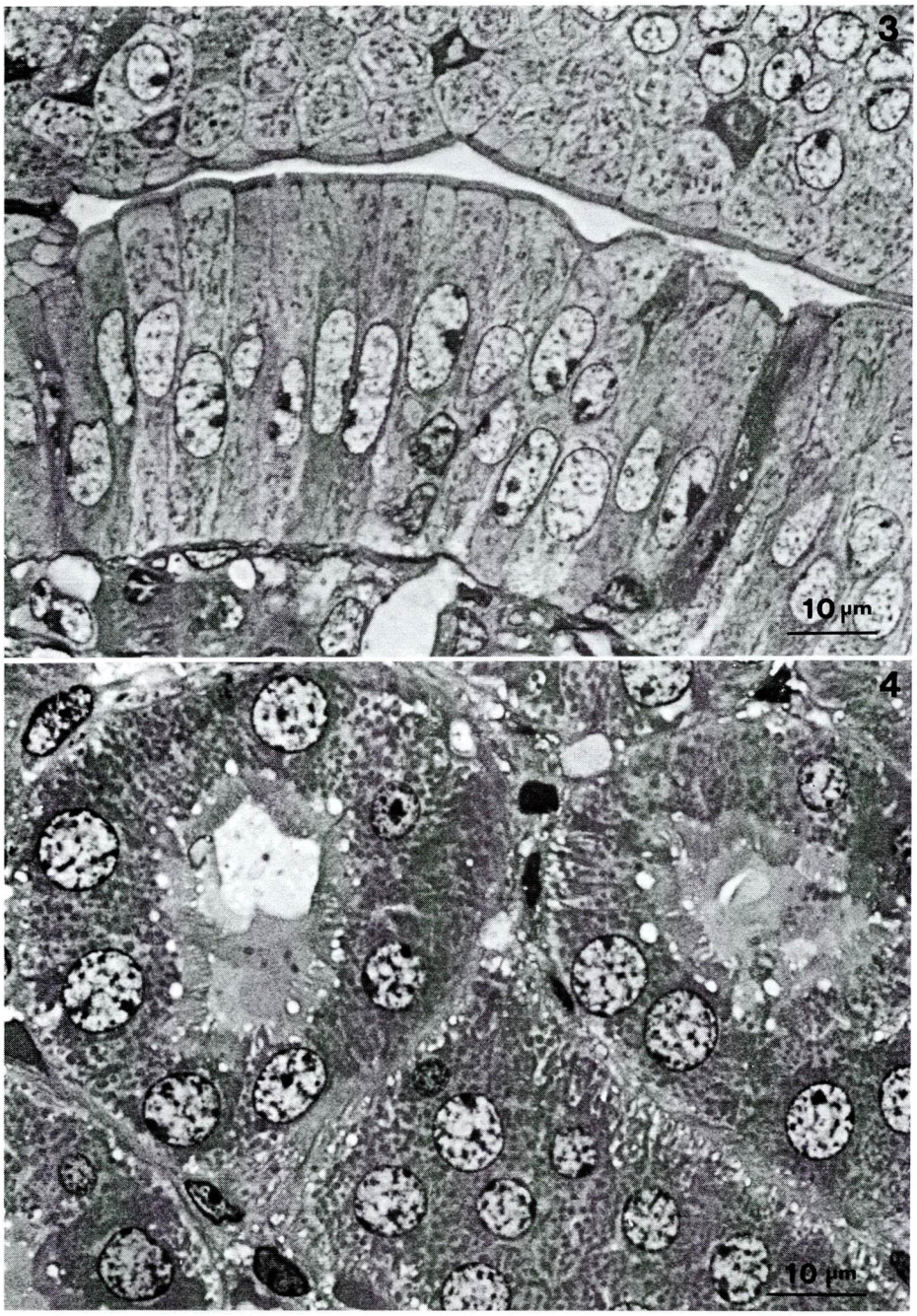


Fig. 3 Electorn micrograph of reversed contrast BE image of mouse small intestine, treated with osmium tetroxide vapor after toluidine blue stain. ×1700.

Fig. 4 Electron micrograph of reversed contrast BE image of mouse kidney, treated with osmium tetroxide vapor after hematoxylin-eosin stain. ×1900.

bably remain in the areas of contrast, resolution and direct light microscopy controls of BEI, and more importantly in the lack of suitable embedding medium. Kushida *et al.* (1982) improved the BEI method by means of the GMA and Quetol 523 resin mixture, and suitable BEI detector (BEIS). The first reports empolying the GMA and Quetol 523 as an embedding medium applied to the fields of semi-thin sections were presented by Kushida (1977) and Kushida *et al.* (1977), who stained histological sections with osmium tetroxide vapor and visualized identical sites of cellular components under both the light microscope and TEM. Quetol 523 was a light-colored, flexible, water-miscible methacrylate. It has a low viscosity and reacted with GMA to become an integral part of the polymerized system. QCU-1 was readily miscible with this methacrylate. This mixture has also proved suitable for staining in histochemistry, enzyme-histochemistry and immunohistochemistry (9,10,11,12,13,14,17).

This paper further developed the BEI method in contrast, resolution, stainability and tissue preservation of the samples embedded in GMA-Quetol 523. Osmium tetroxide vapor treatment after hematoxylin and eosin stain improved the contrast and resolution, and clearly showed the intracellular organellae. The direct correlation between intracellular structures seen in the BEI mode and staining affinity for various dyes of the specific structures in cell and tissues can, therefore, be established at the level of 1.0μm sections mounted on glass slides. The direct correlation encourages the use of this method for other histochemical stains.

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