Low vacuum scanning electron microscopy for paraffin sections utilizing the differential stainability of cells and tissues with platinum blue*

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Summary. The present study introduces a novel method for the direct observation of histological paraffin sections by low vacuum scanning electron microscopy (LVSEM) with platinum blue (Pt-blue) treatment. Pt-blue was applied not only as a backscattered electron (BSE) signal enhancer but also as a histologically specific stain. In this method, paraffin sections of the rat tongue prepared for conventional light microscopy (LM) were stained on glass slides with a Pt-blue staining solution (pH 9) and observed in a LVSEM using BSE detector. Under LVSEM, overviews of whole sections as well as three-dimensional detailed observations of individual cells and tissues could be easily made at magnifications from ×40 to ×10,000. Each kind of cell and tissue observed in the section could be clearly distinguished due to the different yields of BSE signals, which depended on the surface structures and different affinities to Pt-blue. Thus, we roughly classified cellular and tissue components into three groups according to the staining intensity of Pt-blue observed by LM and LVSEM: 1) a strongly stained (deep blue by LM and brightest by LVSEM) group which included epithelial tissue, endothelium and mast cells; 2) a moderately stained (light blue and bright) group which included muscular tissue and nervous tissue; 3) an unstained or weakly stained (colorless and dark) group which included elastic fibers and collagen fibers. We expect that this method will prove useful for the three-dimensional direct observation of histological paraffin sections of various tissues by LVSEM with higher resolutions than LM.

Introduction

Platinum blue (Pt-blue) is a deep blue aqueous complex prepared from the reaction of cis-Pt with thymidine. Using Pt-blue, a novel conductive staining method was developed for the observation of hydrous biological specimens by low vacuum scanning electron microscopy (LVSEM) (Tanaka and Inagaki, 1993), as Pt-blue effectively enhances backscattered electron (BSE) signals from specimens under the LVSEM. We demonstrated the usefulness of LVSEM for cytohistology by revealing the three-dimensional intracellular structure of hydrous HeLa cells, including the nucleus, mitochondria, secretory granules, and microfilaments (Tanaka et al. 1997). This method is also very useful for fine structural observations of chromosomes because of the strong affinity of Pt-blue to DNA (Tanaka et al. 1998, Inaga et al. 2002).

We also recently learned that Pt-blue can be used as a heavy-metal stain for transmission electron microscopy (TEM) and have proposed its use as an alternative...
to uranyl acetate (UA) not only for the conventional positive staining of ultrathin sections but also for the negative staining of bacteria (Inaga et al., 2007a). In that study, we mentioned differences in staining effects between Pt-blue and UA in ultrathin sections of hepatic parenchymal cells. For example, the electron densities of glycogen granules and the mitochondrial matrix in resin sections stained with Pt-blue were higher than those seen with UA, while in our additional TEM study (Inaga et al., 2007b), the electron densities of zymogen granules in pancreatic acinar cells and collagen fibers in the connective tissue were low even in staining en bloc with Pt-blue. These results indicated that the affinity of Pt-blue might be selective not only for cell organelles but also for tissues—although the staining mechanism of Pt-blue is still unelucidated. In order to investigate precisely the characteristic stainability of different cells and tissues with Pt-blue, we compared different Pt-blue staining conditions for electron microscopy (Inaga et al., 2008) and obtained the best results with a Pt-blue staining solution adjusted to pH 9.

We next conceived the idea of directly observing tissue paraffin sections stained with a properly adjusted Pt-blue solution by LVSEM at high magnification. For BSE imaging of paraffin sections under conventional high vacuum SEM, metal coating is usually essential to avoid charging, as previously reported in the literature on enzyme-histochemistry (Kato et al., 1990). However, the present method using LVSEM and Pt-blue staining enabled the BSE imaging of paraffin sections without the metal coating. In this study, we evaluate the differential stainability of cells and tissues with Pt-blue, and show the usefulness of this stain for obtaining detailed BSE images from paraffin sections of rat tongue, which is composed of various kinds of cells and tissues.

Materials and Methods

Tissue preparation

The tissue sample was obtained from an adult male Wistar rat tongue, which was fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) by perfusion through the left ventricle, cut into small pieces, and immersed in the same fixative for one day. Paraffin sections were prepared as follows: A piece of the fixed tissue was embedded in paraffin according to the standard preparation method for light microscopy (LM). The paraffin block was cut into thin sections (5–8 μm) and mounted on glass slides. The sections on the slides were deparaffinized with xylene and transferred to distilled water after an alcohol descending series. Sections were then stained with a Pt-blue solution, which was adjusted to pH 9 by adding a small volume of ammonia solution (TI-blue staining kit: Nisshin EM Co. Ltd., Tokyo), for 10–15 min at room temperature. After washing with distilled water for 1–2 min, sections were observed by LM with coverslips followed by LVSEM without coverslips.

Low vacuum scanning electron microscopy (LVSEM)

The LVSEM used in the present study was a Hitachi TM-1000 table-top microscope (Hitachi Co. Ltd., Tokyo) which has previously been used for the observation of non-conductive samples. This microscope has a high-sensitivity semiconductor BSE detector with a charge-reduction mode which allows the direct imaging of non-conducting biological specimens and a wide low vacuum chamber accommodating a whole glass slide of the usual

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**Fig. 1.** LM and LVSEM images of the same paraffin section from the rat tongue stained with Pt-blue. At low magnification, the general overview observation is easily performed on both micrographs. Tissue components of the rat tongue may be distinguished by the intensity of the blue color under LM (a), and the contrast of BSE signals under LVSEM (b). The epithelial tissue (E) and the muscular tissue (M) appear blue by LM and bright by LVSEM, but the submucosa (SM) between them appears colorless by LM and dark by LVSEM, respectively. Bar = 100 μm

**Fig. 2.** Higher-magnification LVSEM image of the central part of the rat tongue stained with Pt-blue. Nervous tissue (N), blood vessels (arterioles and veins: A and V), muscular tissue (M), connective tissue (C) and mast cells (red arrows) are clearly distinguished by the differing contrasts of BSE signals. Bar = 100 μm

**Fig. 3.** Mast cells in the connective tissue observed by LM and LVSEM at higher magnifications. The characteristic small granules of the mast cells are clearly observed under LVSEM (b) although these granules cannot be recognized individually under LM (a) in the deep blue-colored cytoplasm. Bars = 10 μm (a), 5 μm (b).
Fig. 1–3. Legends on the opposite page.
size (76 mm × 26 mm) without requiring its cutting into smaller pieces. The paraffin sections stained with Pt-blue were observed without metal coating and photographed under LVSEM at an acceleration voltage of 15 kV with 30 Pa.

Results and Discussion

Overview images of the Pt-blue stained sections from the rat tongue obtained at low magnification by LVSEM compared well with those obtained by LM; more detailed observations at higher magnification were easily performed under LVSEM by zooming up from × 40 to × 10,000. Each tissue component could be clearly distinguished under LVSEM because different yields of BSE signals provided different contrasts of images under LM (Fig. 1). At low magnification, the epithelial tissue (stratified squamous epithelium) and the muscular tissue of the rat tongue appeared bright but the submucosa was dark. In general, we observed that the bright tissues giving abundant BSE signals have a high affinity for Pt-blue, while the dark tissues have a low affinity for Pt-blue. Thus, Pt-blue was effective not only as a BSE signal enhancer but also as a histologically specific stain at improving the performance of LVSEM.

In the central part of the tongue of the same section, blood vessels (arterioles and small veins), nervous tissue, muscular tissue, fibrous connective tissue, and some kinds of cells could be distinguished by their differing BSE contrasts at higher magnifications (Fig. 2). Among these tissues and cells, mast cells were particularly bright and prominent. As shown in Figure 3, characteristic small granules in the cytoplasm of each mast cell, which were strongly stained with Pt-blue, could be observed distinctly under LVSEM (Fig. 3b) although these granules generally could not be recognized individually in the blue-colored cytoplasm under LM (Fig. 3a).

Figure 4 shows magnified transverse sections of an arteriole, a peripheral nerve fiber, and a longitudinal section of muscle fiber under LVSEM. The three-layered structure of the arteriole could be clearly recognized due to the differing yield of BSE signals in the layers (Fig. 4a). The endothelial cells of the tunica intima were brightest (strongly stained with Pt-blue) while the internal elastic lamina was dark (unstained). The smooth muscle of the tunica media was bright (moderately stained) while the tunica adventitia, composed of collagen fibers, was somewhat darker (weakly stained). Interestingly, the dark internal elastic lamina appeared transparent due to the absence of any BSE signal, as if there were no material at all. The structure of the peripheral nerve could be also observed in the transverse section at a higher magnification (Fig. 4b). Individual axons and Schwann cells were comparatively bright, while the myelin sheaths, which were often extracted and altered during the preparation procedure of paraffin sections, were slightly dark, especially in the myelinated nerve fibers which were preserved well. Some blood vessels and mast cells were recognized in the endoneurium. When striated muscle fibers were observed at the highest magnification (× 10,000) (Fig. 4c), not only the characteristic striated pattern of myofibrils (e.g. Z lines within I bands and M lines within A bands), but also spherical mitochondria could clearly be observed among myofibrils in the longitudinal section. Thus, LVSEM observations of paraffin sections stained with Pt-blue revealed the differential stainability of various cells and tissues.

We roughly classified the cells and tissues observed in the rat tongue into three groups according to the staining strength of Pt-blue, represented as the differing intensity of blue color under LM and the differing brightness under LVSEM, respectively. The strongly stained group (deep blue under LM and brightest under LVSEM) included mast cells and epithelial tissues such as the stratified squamous epithelium and the endothelium. The moderately stained group (light blue and bright) included muscular tissue, such as smooth muscle and striated muscle, as well as nervous tissue. The unstained or weakly stained group (colorless and dark) included fibrous connective tissues such as the elastic fibers and collagen fibers.

In conclusion, we here introduce a simple and useful method for the three-dimensional direct observation of paraffin sections with high resolution by LVSEM, utilizing the selective stainability of cells and tissues by Pt-blue. We anticipate that this method will allow the rapid and detailed analysis of pathological paraffin sections such as renal biopsy samples due to the increased resolution relative to LM.

Acknowledgements

The authors are grateful to Prof. Tatsuo Ushiki, Niigata University, for editing the manuscript; and to Mr. Osamu Tanaka and Mr. Yasuo Yamamoto, Hitachi High-Technologies, for their helpful assistance.
**Fig. 4.** Higher-magnification LVSEM images of each component of the tissue shown in Figure 2. **a:** Transverse section of an arteriole. The three-layered structure of the arteriole is clearly delineated by the different yields of BSE signals. The endothelial cells (open arrow) of the tunica intima are brightest while the internal elastic lamina (arrow) is dark or transparent due to absence of BSE signals. The smooth muscle of the tunica media (asterisk) is moderately bright, while the tunica adventitia (white arrow) is somewhat darker. **b:** Transverse section of a peripheral nerve. Individual myelinated nerve fibers (white arrows), blood vessels (big arrows), and a mast cell (M) in the endneurium are recognized. As shown in the inset, the axon (black arrow) and the Schwann cell (arrow head) are comparatively bright, and the myelin sheath (asterisk) is slightly dark in the well-preserved myelinated nerve fiber. **c:** Longitudinal section of a striated muscle fiber observed at the highest magnification (direct magnification is ×10,000). Not only the characteristic striated pattern of myofibrils, such as Z lines (Z) and M lines (M), but also spherical mitochondria (arrows) among the myofibrils can be identified. Bars = 10 μm (a, b), 1 μm (c).
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