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Freeze Cracking of Scanning Electron Microscope Specimens. A Study of the Kidney and Spleen

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Summary. Freeze cracking methods for the preparation of the scanning electron microscope specimens are described and the results obtained in the rat kidney and human spleen are demonstrated. Fixed tissue pieces immersed either in ethanol, isoamyl acetate, or in 40% dimethyl sulfoxide were quench-frozen in liquid nitrogen or in Freon-22 cooled by liquid nitrogen and cracked. Freeze cracking in ethanol and isoamyl acetate produced clean and flat fracture surfaces causing excellent visualization of the lining surfaces of the opened vessels, tubules and tissue spaces. Freeze cracking in dimethyl sulfoxide tended to cause fracture along cell surfaces and intracellular membranes.

In scanning electron microscope (SEM) studies of tissues and organs, it is often desired to cut them open, not only to see the inside of some cells, but, more importantly, also to visualize some interior surfaces enclosed by cells and other elements, e.g., hepatic sinusoids, splenic sinus, urinary canalicules and capillary endothelial surfaces.

Cutting the tissue simply with a razor blade (cf. FUJITA, TOKUNAGA and INOUE, 1971) is often useful but damage to the delicate wall of those enclosed structures are usually more or less conspicuous. Stiff structures such as collagen fibrils may not be cut cleanly and may tear or dislocate adjacent soft elements. Moreover, no information can be expected on the cut surface structures of cells after using this method.

Tearing or cracking of fixed tissues either before or after drying generally gives better information of the tissues as far as the cell surfaces are concerned, because in this method fracture tends to occur along the intercellular phase. This method may thus be effective in the studies of the construction of epithelial cells. Beautiful images of the lateral surfaces of retinal cells (STEINBERG, 1973), epithelial cells of the intestine, gallbladder and renal tubules (MILLER and REVEL, 1974) and hepatic cells (MOTTA and PORTER, 1974) could be demonstrated by cracking the tissues either before or after critical point drying. This method, however, generally is inferior to the razor blade cut in preserving the wall structures of cavities as the fibrous elements are difficult to cut and may damage soft parts.

Freeze-fracture has been applied by some authors in the studies of retina (HAGGIS, 1970; GERMINARIO and McALEAR, 1971) and liver (BROOKS and HAGGIS, 1973). The tissues were snap frozen in Freon-22 and fractured at -150°C , and then processed to freeze-drying. The surface structures of cells were preserved well after this method, but the cut surface of the cells revealed severe damage by ice crystallization.

TANAKA (1972) invented a "frozen resin cracking method." The tissues were

embedded in Cemedine 1500 (epoxy resin) without catalysts, hardened at -30°C and cracked. After resin is removed with propylene oxide, the fractured surfaces reveal well preserved structures including some intracellular organelles (TANAKA et al., 1973; KOBAYASHI, 1973; TAKATA, 1974).

We realized it more reasonable to avoid the embedding and resin removal in TANAKA's method for a simple and prompt procedure and for a better preservation of the tissue, and rather adopted modified freeze-fracture methods. We attempted to eliminate the destruction of cellular structure by ice crystal formation in freezing, by using ethanol, isoamyl acetate and dimethyl sulfoxide (DMSO) as the freezing media. The results obtained by these methods are reported in this paper.

Materials and Methods

Use was made of rat kidneys and a human spleen excised from a 39-year-old man with gastric cancer. The organs were perfused with warmed Ringer solution and then by 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4). They were kept in the same fixative for a few days and then cut into elongate pieces.

The tissue pieces were dehydrated in an ascending ethanol series and in isoamyl acetate. In the different stages of this process, i.e., infiltrated either in 70, 90, 95 or 100% ethanol or in isoamyl acetate, the tissue pieces were frozen in Freon-22 cooled by liquid nitrogen or directly in liquid nitrogen, and fractured, generally at the middle of the elongate piece, with a chisel and hammer or with a specially devised breaking instrument. A part of the fixed tissue pieces were immersed in 40% DMSO and freeze-cracked.

The fractured specimens were then processed to higher concentration of ethanol (to 70% ethanol in the case of DMSO) or to isoamyl acetate, both adequately cooled, to be successively dried by the CO_2 critical point method.

The dried pieces were adequately trimmed, mounted on the metal stub, fractured surface upward, with conductive paste, and evaporated with gold-palladium.

A part of the spleen specimens were immersed, before dehydration and fracturing, in a solution of 2% glycine, 2% sodium glutamate and 2% sucrose for 16 hrs, in 2% tannic acid for 24 hrs and in 2% OsO_4 for 6 hrs, according to the method of MURAKAMI (1974). The specimens thus amply impregnated with osmium could be observed in SEM either completely without or with only slight metal coating.

The microscopes used in this study were a conventional type SEM (JSM-2, Japan Electron Optic Co.) and a new, field emission type SEM (HFS-2, Hitachi Manufacturing Co.). All the micrographs shown in this paper were taken in the latter instrument.

Results

Freeze-fracture in isoamyl acetate and absolute ethanol gave essentially identical results. The fractured surfaces were mostly smooth and the intracellular structures such as nucleus and organelles generally were only slightly discernible. Only rarely one encountered a cell in which the fracture occurred along the membranes, thus revealing the round surfaces of the nucleus and some organelles. The cytoplasm and nucleoplasm after this cryotechnique were compactly preserved without formation of discernible artifacts.

Fig. 1. A and B. A pair of micrographs of complementary fracture planes of a rat glomerulus. Ethanol(100%)-freeze fracture. $\times 600$

Fig. 2. A and B. A pair of compensatory fracture planes through a rat glomerulus. The complementary fracture surfaces of an endothelial cell and of a podocyte process (*P*) are labeled. Ethanol(100%)-freeze fracture. $\times 4,000$

The fine structures of cell surfaces, the linings of vascular and tubular lumina and of other tissue spaces were very well preserved after this method, because the sharp and even fracturing has caused neither damage nor dislocation in the cellular and fibrillar tissue elements.

Quench-freezing in liquid nitrogen gave good preparations in the case of the spleen but not in the kidney which often became fractured or fragmented in freezing. This damage did not occur when the tissues were quenched in Freon-22 previously cooled by liquid nitrogen.

Opposit or complementary fracture surfaces could be obtained relatively easily. In Figures 1 and 2, the surface A is compensatory to the surface B, respectively. The information obtained in a fracture surface could be enriched and extended by observing the corresponding structures in the complemental surface.

In the kidney, the Bowman's capsules and glomeruli were fractured flat at different levels. The fine structure of the glomerulus could be plastically observed with partially or totally cut-open aspects. The podocyte end-feet interdigitating on the basement membrane and the fenestrated capillary endothelium on the other side were clearly demonstrated (Fig. 3-7). The fine structure of the renal tubules could be precisely observed after this fracturing method. The epithelial cells were fractured flat and only vacuolar structures in the cytoplasm were clearly visualized (Fig. 8, 9). The nuclei and basal infoldings of the cells were generally recognized only faintly. On the other hand the microvilli, cilia and other organelles forming the cell surfaces were beautifully shown. Cony pits in the brush border of the proximal convolution

Fig. 3. A part of a rat glomerulus showing, in the middle, a podocyte extending its processes to the capillary walls. Isoamyl acetate-freeze fracture. $\times 5,000$

Fig. 4. A part of a rat glomerular capillary. DMSO-freeze fracture. Note the different fracture surfaces of an erythrocyte (*E*) and a leucocyte (*L*). Podocyte end-feet and pored endothelium are seen. $\times 12,000$

Fig. 5. Fracture view of a rat glomerular capillary. DMSO-freeze fracture. In the fracture plane of a leucocyte (*L*) the nucleus and some cytoplasmic granular and vesicular structures are shown. *E* endothelial cell, *P* podocyte processes. $\times 10,000$

Fig. 6. High power view of the glomerular capillary, showing the luminal surface of the fenestrated endothelium, fractured basement membrane (*B*) and podocyte processes. Ethanol (100%)-freeze fracture. $\times 20,000$

Fig. 7. A stereoscopic pair of micrographs of a fracture view of a rat glomerulus. 70% ethanol-freeze fracture. The subcellular damage caused by the use of ethanol of this low concentration is not noticed at this magnification. *E* endothelial cell, *P* podocyte processes, *R* red blood cell. $\times 4,300$

Fig. 8. A part of a rat proximal convoluted. Note some cytoplasmic vesicles. Ethanol (100%)-freeze fracture. $\times 3,000$

Fig. 9. A closer view of the apical cytoplasm and microvilli of the proximal convoluted. Note the cony pits in the microvilli and three cilia (arrows). $\times 10,000$

Fig. 10. A portion of a rat proximal convolution after DMSO-freeze fracture. Note the swollen surface of a nucleus (*N*), the clear intercellular space and basal interdigitations. Above left is a macrophage also fractured. $\times 7,000$

Fig. 11. A portion of a rat distal convolution after 90% ethanol-freeze fracture. Note the caveolar structure of cytoplasm caused apparently by ice crystal growth in the cells. $\times 2,000$

A

B

Fig. 12. A. Fractured distal convolution showing a round nucleus. DMSO-freeze fracture. In a closer view (B) possible nuclear pores are seen as button-like discs (arrows). *M* probable mitochondria. A. $\times 10,000$ B. $\times 20,000$

(Fig. 9) and attachment upon it of urine globules of unknown nature were observed, which detailed observations will be published elsewhere.

In the spleen the sinuses and splenic cords were cleanly cut open, revealing their well preserved fine structures (Fig. 13-19). The white pulp and arteries were also cleanly fractured but the intercellular spaces appeared to contain more or less amounts of proteinic precipitations, thus disturbing the images of cell contours (Fig. 13).

Cryofracture in ethanol of lower densities (95-70%) also produced clean surfaces, but it was apparent that some cells were damaged in their cytoplasmic structure showing loosened and vacuolated texture (Fig. 11). This damage was most conspicuous in 70% ethanol.

Cryofracture in dimethyl sulfoxide gave quite different images of fractured surfaces. They were not flat as in the cases of ethanol and isoamyl acetate but the fracture tended to occur along the boundaries of cells and intracellular structures (Fig. 10, 12). The nucleus was often exposed as a round swelling on the fractured surface. The outer leaflet of its envelope likely tended to be removed in fracturing, as apparent nuclear pores often were represented by elevated, button-like discs (Fig. 12). Mitochondria and other intracytoplasmic bodies generally revealed their shapes either by a projected or by an indented relief.

Images deserving special mention were the basal interdigitations of the epithelial cells in the proximal convolution of the kidney. Because of uneven fracture levels between the adjacent cell processes and because of the apparently artificial widening of intercellular spaces, the interdigitation of the cells was visualized much more plastically and intractively than in the transmission electron microscope images of thin sections (Fig. 10).

Some cells, after DMSO-freeze fracture, appeared loosened in cytoplasmic texture, possibly indicating certain subcellular damages in this method.

Discussion

Cutting the fixed tissues with a razor blade was widely applied to the SEM studies of the kidney (BUSS and KRÖNERT, 1969; FUJITA, MIYOSHI and TOKUNAGA, 1970; ARAKAWA, 1970, 1971; MIYOSHI, FUJITA and TOKUNAGA, 1971; ANDREWS and PORTER, 1974; BULGER, SIEGEL and PENDERGRASS, 1974). The Bowman capsule was easily opened by this method and the surface of the glomerulus could be successfully observed. The glomerulus itself often was cut but the cut surface was not clean enough. Nor was the cut surface of the renal tubules clean and the observation of the interior structures of the epithelial cells was only poorly attainable.

Fracturing or tearing the fixed tissues before or after drying is often a valuable method and some of the above mentioned authors and MILLER and REVEL (1974) applied this method to the kidney also. As the fracture in this method tends to occur between the cell boundaries, such structures as the basal interdigitations of proximal tubule cells may be demonstrated (ANDREWS and PORTER, 1974; MILLER and REVEL, 1974). However, stiff structures such as collagen fibrils may be pulled out in fracturing, and may even destroy adjacent soft elements. The "long slender tendrils" spanning the lumen of the proximal convolution observed by ANDREWS and PORTER (1974) may possibly be fibrils pulled out of the interstitial connective tissue.

Fig. 13. An overview of the fracture surface of human spleen. The specimen was treated by the tannin-osmium method by MURAKAMI and then ethanol(100%)-freeze fractured. No charging effects may be seen in this non-coated material. A arteries, S sinuses, W white pulp. $\times 200$

Fig. 14. A part of the splenic red pulp showing fractured cords of Billroth and the luminal surface of sinuses. Tannin-osmium method, ethanol(100%)-freeze fracture, slightly coated with gold-palladium. $\times 800$

Fig. 15. A splenic sinus, cross-sectioned, is surrounded by the cord of Billroth containing numerous round cells (mostly neutrophils in this micrograph). Specimen preparation as in Figure 14. $\times 2,200$

Fig. 16. A stereoscopic pair of micrographs from a non-coated specimen. No charging effects are seen because of the previous tannin-osmium treatment. When stereoscopically viewed, the cytoplasm, especially its thinner microprojections look transparent in this preparation. S sinus lining cells (nuclear portion), E erythrocytes, M macrophages. $\times 1,300$

Fig. 17. A part of Billroth cord after DMSO-freeze fracture. In a leucocyte (probable neutrophil) the surface of the nucleus is exposed (*L*). *M* macrophage fractured, *P* blood platelet, *R* reticulum cells. $\times 6,600$

Fig. 18. A stereoscopic pair of the views of Billroth cord intercalated between the sinus endothelia (*S*). A macrophage (*M*) reveals a large phagosome in its fracture surface. *P* blood platelets, *R* reticulum cells. Ethanol (100%)-freeze fracture. $\times 3,700$

Fig. 19. Closer view of the macrophage, platelets (*P*) and reticulum cell (*R*) shown in Figure 18. $\times 7,500$

Razor blade cutting was applied also to the spleen (MIYOSHI, FUJITA and TOKUNAGA, 1970; MIYOSHI and FUJITA, 1971). Although the free surfaces of cells could cleanly be exposed, the cut surfaces were more or less severely damaged. Fracturing and tearing methods seem not suited to the spleen, as the reticular cells forming the delicate network shift their position in fracture and the pulled collagen fibrils also may injure the cells.

TANAKA (1972) succeeded in producing clean fracture surfaces by his "frozen resin cracking method." As the tissue is embedded in resin and sufficiently hardened by a low temperature (*vide supra*), fracture occurs homogeneously through hard and soft, stiff and fragile components of the tissue. The fracture surface after this method may be flat but more often may be along cell surfaces and intracellular membranes.

Freeze-fracture followed by freeze-drying has been applied to SEM studies (HAGGIS, 1970; GERMINARIO and McALEER, 1971). The free surfaces of cells were well preserved after this method but the cut surfaces indicated intracellular damage by ice crystal formation such as the cavernous appearance of cytoplasm and nucleoplasm.

For the elimination of the occurrence of ice crystals in the tissue, the use is known of some organic solvents as the freeze media. Recently HAMANO et al. (1973) and HUMPHREYS et al. (1974) reported the utility of ethanol as the freeze medium. Both research groups quench-frozen ethanol-infiltrated tissue pieces in liquid nitrogen and cracked them. Their studies were characterized by adopting critical point drying after freeze-fracture. HAMANO et al. (1973) demonstrated mitochondrial cristae of renal tubule cells and the lamellae of endoplasmic reticulum in pancreatic acinar cells. HUMPHREYS et al. (1974) demonstrated beautiful micrographs of the cut

surfaces of the hepatic cells and renal glomeruli, though the fracture planes were too flat to show intracellular structures plastically. MIYAI, WAGNER and RICHARDSON (1974), on the other hand immersed the tissues in Freon 113 through acetone, and froze them in liquid nitrogen; the tissues were dried by the critical point method. The cell fine structures were well preserved and reliefs of some organelles could be observed. They further attempted freeze-fracture of paraffine-embedded tissue followed by deparaffinization and critical point drying. By this technique they reported that the structural and positional distortion of tissue elements was better prevented than in the former method.

Independently from and simultaneously with the above studies, we were attempting a simple and consistent technique of fracture for SEM studies of tissues and cells. After a series of trials, our selections were isoamyl acetate, ethanol and 40% DMSO.

As indicated in this study isoamyl acetate and ethanol as freeze media seem identical in the nature of fracture planes and structure preservation. The planes are generally flat, independent of cell boundaries and membrane systems, but occasionally fracture occurs along these structures. It is an undecided problem what factors determine the nature of the fracture plane. The structure damage of cells by ice crystallization in freeze-fracture through water can be eliminated by using absolute ethanol or isoamyl acetate instead. The use of ethanol of lower concentrations seems to cause certain ice crystal formation as the cell damages and vacuolation of the cytoplasmic architecture are resulted.

DMSO has been known as one of organic solvents which prevents ice-crystal growth by the hydrogen-bonding of water. The use of this substance in the present study caused good preservation of cell structures and, further, showed the characteristic nature of the fracture surfaces. They were rarely flat and usually they corresponded to cell surfaces and boundaries of nucleus and smaller intracellular structures.

It is stressed in this study that the merits of the freeze-fracture of solvent-infiltrated tissues are: 1) it prevents the dislocation and damage of fragile elements caused by uneven forces acting on the tissue during fracturing, and 2) the intracellular fine structures are well preserved by prevention of ice crystallization. The ease in obtaining large complementary fracture surfaces may be an additional merit in this method. Combined with the subsequent critical point drying, this new technique is able to provide SEM specimens whose finest structures remain in their natural positions. Further, by adopting the osmium impregnation technique of MURAKAMI (1974), one can observe the surfaces of the tissue elements with very thin metal coating or even without any coating. The high resolution of the field emission SEM actually needs this kind of preparation.

走査電子顕微鏡試料の凍結割断法. 腎臓と脾臓による研究

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走査電子顕微鏡の試料作製法として凍結割断法を考案し, ラットの腎臓とヒトの脾臓において得られた結果を示した. 固定した組織片を エタノールまたはイソアミル アセ

テート,あるいは40%ジメチル スルフォクサイド に浸し,液体チッ素 または 液体チッ素で冷やしたフレオン22の中に入れて急速に凍結し,切断した. エタノールとイソアミル アセテートでの切断は清潔で平坦な切断面を生じ,開かれた脈管や小管や空隙の内面構造がよく観察できた. ジメチル スルフォクサイドを用いての切断では 細胞表面や細胞内の膜系に沿って割れる傾向が強かった.

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