Freeze-Fracture of Biological Specimens Prior to Conductive Staining*

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Summary. Liver, kidney, spleen and other organs of the rat were fixed with glutaraldehyde, substituted with absolute ethanol or dimethyl sulfoxide (DMSO), freeze-fractured in liquid nitrogen, stained by the rapid tannin-osmium thiocarbohydrazide-osmium (TaOTO) method (staining with each agent for 10 min), critical-point-dried with liquid carbon dioxide, and observed with the scanning electron microscope. The absolute ethanol or DMSO freeze-fracture method provided flat fracture surfaces (without regard to cell boundaries) of the samples and allowed a good visualization of their inner structures. The fracture surfaces were suitably stained by the rapid TaOTO method, and could be scanned with no charging. Neither maked damage nor undesired dislocation of tissue elements was noted on the freeze-fractured and TaOTO-stained surfaces. This procedure, freeze-fracture prior to conductive staining, has an advantage of eliminating the bulk charging effects that tend to occur in specimens fractured after staining. When substituted with 75% DMSO aqueous solution, the samples spontaneously fractured without any need for razor blades. Fracture planes in this spontaneous fracture sometimes ran along the cell boundaries and allowed a clear visualization in the SEM of the enfaced surfaces of closely associated cells such as hepatocytes.

Biological specimens conductive-stained by tannin-osmium, thiocarbohydrazide-osmium or other ligand-mediated osmium impregnation methods produce good scanning images with no charging (Murphy, 1978, 1980). The conductive-stained specimens are usually freeze-fractured to expose their inner surfaces or structures (Tokunaga et al., 1974; Muto, 1976; Umetani, 1977). This freeze-fracture process, however, sometimes causes undesired bulk charging in the core or central areas of the fracture surfaces because of incomplete osmium tetroxide or ligand penetration into the specimen (Tokunaga et al., 1974). Recent experiments in our laboratory have indicated that performing freeze-fracture prior to conductive staining eliminates such bulk charging effects of the fracture surfaces.

MATERIALS AND METHODS

Liver, kidney, spleen and other organs of the rat were washed by vascular or needle-

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puncture perfusion with physiological saline or Ringer solution, and fixed by vascular or needle-puncture perfusion with 2–4% glutaraldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.0–7.4) (Tokunaga et al., 1974; Itoshima et al., 1978a). The samples were then cut with a razor blade into relatively large blocks (3 × 3–5 × 5 × 5 mm), and refixed for 3–6 hrs in a buffered 2–4% glutaraldehyde solution.

The refixed blocks were dehydrated through a series of five ascending concentrations of ethanol or dimethyl sulfoxide (DMSO) (25, 50, 75, 90, and 100%; for 30 min at each step), frozen for 3–5 min in a small metal tureen containing liquid nitrogen, fractured with a razor blade in this tureen (Tokunaga et al., 1974) (Fig. 1), thawed in 90% ethanol or DMSO solution, immersed for 30 min in this 90% ethanol or DMSO solution, rehydrated through a triple series of ethanol or DMSO (75, 50, and 25%; again 30 min each), and washed for 1 hr in 0.1 M phosphate or cacodylate buffer (pH 7.0–7.4).

The blocks were then immersed for 30 min in a buffered (0.1 M phosphate or cacodylate, pH 7.0–7.4) mixture of 2–4% glutaraldehyde and 1–2% tannic acid (Ta), washed for 1 hr in 0.1 M phosphate or cacodylate buffer (pH 7.0–7.4), rinsed for 10 min in 1–2% osmium tetroxide (O), washed for 20 min in distilled water, rinsed for 10 min in thiocarbohydrazide-saturated solution (T), washed for 20 min in distilled water, rinsed for 10 min in 1% osmium tetroxide (O), and washed for 20 min in distilled water (Murakami and Jones, 1980).

The specimens thus fractured in liquid nitrogen and stained by the rapid TaOTO method were conventionally treated by dehydration through a graded series of ethanol, critical point-dried with liquid carbon dioxide (Lewis and Nemanic, 1973), and observed with a scanning electron microscope (Hitachi, HHS-2R) using 10–30 kV accelerating voltage.

**RESULTS**

Vascular or needle-puncture perfusion with physiological saline or Ringer solutions before fixation sufficiently eliminated any blood and tissue fluid which might form undesired deposits during fixation or TaOTO staining and mask the detailed structures of the tissue. Successive vascular or needle-puncture perfusion with glutaraldehyde resulted in a prompt fixation of the specimens and facilitated the removal with razor blades of appropriate blocks or areas of interest from the samples with minimized tissue damage. Immersion in the glutaraldehyde and tannic acid mixture after freeze-fracture sufficiently stabilized the fracture surfaces and allowed intense, ligand-mediated osmication with no marked damage in the fine structure of the surfaces.

Freeze-fracture in 100% or absolute ethanol or DMSO almost always provided flat
fracture surfaces of the tissues (Fig. 2-6). No marked ice crystal damage of tissues was noted in either the ethanol- or DMSO-substituted specimens.

Ethanol freeze-fracture usually caused fracture planes running through the cells without regard to intracellular elements (Fig. 2, 3, 5). On the other hand, fracture planes in the DMSO freeze-fracture tended to progress along the nuclear and mitochondrial envelopes or between the outer and inner layers of these envelopes. Thus, the nuclei and mitochondria were usually demonstrated as protrusions from or convavities in the fracture surfaces (Fig. 4, 6). Non-cellular elements caused some different fracture patterns. Basement membranes were sharply fractured in both the ethanol and DMSO methods, their fractured edges almost always in the same plane or level as those of the fractured cells (Fig. 2). Collagen fibers and their bundles were usually torn off, and their edges irregularly protruded over (Fig. 3) or sank deep below the fractured surfaces. Only rarely did fracture planes run along or between the cell boundaries in either the ethanol or DMSO methods.

Though repeatedly dipped or rinsed in many solutions in rehydration, refixation, TaOTO staining and dehydration, the fracture surfaces remained well preserved, showing neither marked damage nor undesired dislocation of tissue elements. Thus, the fine structures on the fracture surfaces, exposed intracellular and extracellular elements, free surfaces of vascular or tubular linings and other tissue components were clearly visible after both ethanol and DMSO fracture methods (Fig. 2–6). Even blood cells in passage through the sinusoidal walls in the spleen could be observed with

**Fig. 2.** Scanning electron micrograph (SEM) of a glutaraldehyde fixed, 100% ethanol freeze-fractured, and TaOTO stained rat kidney block. G glomerulus, T urinary tubule. ×540. **Inset.** E endothelium of glomerular capillary, arrow: fenestration of the endothelium. ×11,000
no dislocation (Fig. 5). The macrophages suspended in the hepatic sinusoids could also be observed with no detachment (Fig. 3).

The fracture surfaces were adequately stained by the rapid TaOTO method, and scanned with good contrast and no charging at 10–30 kV acceleration voltages (Fig. 2–6). The mural micropores of the kidney glomerular endothelium and those of the liver sinusoidal endothelium were thus clearly visible with no coated metals (Fig. 2, 3).

DISCUSSION

The present paper confirms that the tannin-osmium-thiocarbohydrazide-osmium (TaOTO) conductive staining method produces well contrasted scanning images of biological specimens without the use of coated metals (Murakami and Jones, 1980), and proves that rapid treatment with each agent for 10 min stains the fracture surfaces sufficiently for non-metal-coated scanning observations.

Freeze-fracture is a useful adjunct to specimen preparation for biological scanning electron microscopy. This process was initially used in blood vascular casts (Murakami, 1971) and then in animal and plant materials (Tanaka and Ino, 1972; Humphreys et al., 1974; Haggis and Phipps-Todd, 1977). The freeze-fracture of conductively stained samples was initiated by Fujita and his associates (Tokunaga et al., 1974). They stained spleen, kidney, bone marrow and other specimens by the tannin-osmium method and then fractured them in liquid nitrogen or Freon after substitution with ethanol.
or DMSO or before critical-point-drying (Fujita, 1974; Muto, 1976; Umetani, 1977).

The present paper also contends that the freeze-fracture method, even when it is followed by conductive staining or ligand-mediated osmication, allows a good, vivid visualization of the inner structures of organs and tissues. Freeze-fracture prior to conductive staining may be of great value for eliminating the bulk charging effects that are usually observed on surfaces prepared by fracture after conductive staining (Tokunaga et al., 1974) (Fig. 7).

Freeze-fracture prior to conductive staining was initially used in scanning electron microscopy on small samples to expose intracellular organelles, especially the Golgi's complex and endoplasmic reticulum (Tanaka, 1981; Tanaka et al., 1983). In these studies, specimens fixed with osmium tetroxide (1%) (Tanaka, 1981), glutaraldehyde (0.1–0.5%) (Tanaka et al., 1983) or paraformaldehyde (0.1–0.5%)-glutaraldehyde (0.1–0.5%) (Tanaka et al., 1983) were freeze-fractured on a metal plate chilled with liquid nitrogen after immersion in 25% and 50% DMSO solutions, digested in 0.1% osmium tetroxide solution at room temperature (20–22°C) for 2 days or longer, conductively stained or hardened by the tannin-osmium method, critical-point-dried with liquid carbon dioxide or dry ice, thinly coated with gold or other metals, and observed with a scanning electron microscope. The procedure described here is not suited for demonstration of such intracellular details because the intracellular matrices firmly fixed with glutaraldehyde at a concentration of 1% or more are insufficiently digested with osmium tetroxide (Fig. 6).

The use of osmium tetroxide is not always recommended as the primary fixative in our procedure, the main reason being that the penetration of osmium tetroxide is
too slow to sufficiently or thoroughly fix our relatively large blocks. Such a low concentration of glutaraldehyde and/or paraformaldehyde fixation as used in exposing cell organelles (Tanaka et al., 1983) is also undesirable in our procedure because it does not always stabilize the specimens. It also, at times, causes undesired damage or dislocation of tissue elements during rehydration before the conductive staining.

It has well been confirmed that the substitution or infiltration of biological specimens with ethanol, DMSO, and other anti-crystal agents or solvents before freeze-fracture prevents ice crystal damage of tissues, and that this fracture using pure or absolute solvents almost always produces flat fracture surfaces without regard to cell boundaries (Tanaka and Iino, 1972; Tokunaga et al., 1974; Humphreys et al., 1974; Haggis and Phipps-Todd, 1977). It has also been well confirmed that in the DMSO freeze-fracture, the fracture planes usually run along the nuclear and mitochondrial envelopes or between the outer and inner layers of these envelopes (Tokunaga et al., 1974).

Our preliminary experiments in this study have shown that the spleen, kidney, liver and other glutaraldehyde fixed specimens, except for bony tissues, spontaneously fracture without any help of tools when substituted with 75% DMSO or 50% ethanol and rapidly frozen in liquid nitrogen, and that the fracture planes in this spontaneous fracturing run both along and across the cell boundaries (Fig. 8). However, the use of 50% ethanol freeze-fracture is not recommendable because ice crystal damage of tissues was observed (Fig. 9). In our preliminary experiments, such spontaneous fracture preferentially useful for exposure of cell boundaries did not occur when the specimens

![Fig. 5. SEM of a glutaraldehyde fixed, 100% ethanol freeze-fractured, and TaOT stained rat spleen block. B blood cell in passage through the wall (W) of sinusoid (S), M macrophage in the Billroth's cord (C), R rod cell of the sinusoidal wall (W). × 4,300](image-url)
Fig. 6. SEM of a glutaraldehyde fixed, 100% DMSO freeze-fractured, osmium tetroxide digested, and TaOTO stained rat liver block. The specimens were fixed with 2% glutaraldehyde for 6 hrs, infiltrated with 100% DMSO, fractured in liquid nitrogen, and allowed to be digested for 3 days at room temperature (20–22°C) prior to the TaOTO staining. E endoplasmic reticulum, F cast-off mitochondrion, M mitochondrion, N nucleus. ×16,000

Fig. 7. SEM of a TaOTO stained and 100% ethanol freeze-fractured rat kidney block. Note that the central or core area (C) shows marked charging because of incomplete penetration of ligand or osmium tetroxide into this area. ×90
Fig. 8. SEM of a glutaraldehyde fixed, 75% DMSO freeze-fractured, and TaOTO stained rat liver block. Note that in the 75% DMSO freeze-fracture, fracture planes tend to run along the cell boundaries. In this 75% freeze-fracture, the specimens spontaneously fractured without any help of tools (see text). B bile canaliculi, D hepatocyte surfaces enfacing the space of Disse, R red blood cell suspended in the liver sinusoids (S). × 3,250

Fig. 9. SEM of a glutaraldehyde fixed, 50% ethanol freeze-fractured, and TaOTO stained rat liver block. Note that severe tissue damage (coagulation of cell matrices or breakage of cell membranes) (arrow) is observed in the 50% ethanol freeze-fractured specimens. E endothelium of sinusoid, M micropore or fenestration of the endothelium. × 110,000
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were substituted with 25% DMSO, 50% DMSO, 90% DMSO, 100% DMSO, 75% ethanol, 90% ethanol, or 100% ethanol.

It has recently been shown that direct breaking or snapping of the tannin-osmium stained and critical-point-dried specimens are useful for the exposure of cell boundaries and intracellular structures. Some authors reported that the cell surface and intracellular organization of muscle fibers were best visualized when the tissues were fixed with tannic acid-osmium tetroxide and torn after critical-point-drying (SAWADA et al., 1978), and others beautifully demonstrated the bile canaliculi or the enfaced surfaces of the hepatocytes by snapping the tannin-osmium stained and critical-point-dried specimens (ITOSHIMA et al., 1978b; FUJITA et al., 1981). As described above, the 75% DMSO freeze-fracture after glutaraldehyde fixation can be used for demonstration of such enfaced surfaces of the closely associated cells, including the hepatocytes. In this connection, it may be worthwhile to add our further preliminary finding that rat liver, kidney, spleen and other blocks fixed with osmium tetroxide or stained by the TaOTO method also spontaneously fractured to expose cell boundaries when they were substituted with 75% DMSO and placed into liquid nitrogen.

REFERENCES

Fujita, T.: A scanning electron microscope study of the human spleen. Arch. histol. jap. 37: 187–216 (1974).

Fujita, T., K. Tanaka and J. Tokunaga: SEM atlas of cells and tissues. Igaku-Shoin Ltd., Tokyo, 1981 (p. 144–157).

Haggis, C. H. and B. Phipps-Todd: Freeze-fracture for scanning electron microscopy. J. Microsc. 111: 193–201 (1977).

Humphreys, W. J., B. O. Spurlock and J. S. Johnston: Critical point drying of ethanol-infiltrated, cryofractured biological specimens for scanning electron microscopy. Scan. Electron Microsc. 1974/I: 275–282 (1974).

ITOSHIMA, T., K. YOSHINO, K. YAMAMOTO, F. MNETOMO and T. MURAKAMI: Cleaning of scanning electron microscope specimens by puncture perfusion. Microsc. Acta 80: 207–210 (1978a).

Lewis, E. R. and M. K. Nemanic: Critical point drying techniques. Scan. Electron Microsc. 1973: 767–774 (1973).

MURAKAMI, T.: Application of the scanning electron microscope to the study of the fine distribution of the blood vessels. Arch. histol. jap. 32: 445–454 (1971).

MURAKAMI, T. and A. L. JONES: Conductive staining of biological specimens for non-coated scanning electron microscopy: double staining by tannin-osmium and osmium-thiocarbohydrazide-osmium methods. Scanning Electron Microsc. 1980/I: 221–226 (1980).

Murphy, J. A.: Non-coating techniques to render biological specimens conductive. Scan. Electron Microsc. 1978/II: 175–194 (1978).

———: Non-coating techniques to render biological specimens conductive/1980 update. Scan. Electron Microsc. 1980/I: 209–220 (1980).

Muto, M.: A scanning and transmission electron microscope study on rat bone marrow sinuses and transmural migration of blood cells. Arch. histol. jap. 39: 51–66 (1976).

SAWADA, H., H. ISHIKAWA and E. YAMADA: High resolution scanning electron microscopy of frog sartorius muscle. Tiss. Cell 10: 179–190 (1978).
Tanaka, K.: Demonstration of intracellular structures by high resolution scanning electron microscopy. Scan. Electron Microsc. 1981/II: 1-8 (1981).

Tanaka, K. and A. Iino: Frozen resin cracking method for scanning electron microscopy and its application to cytology. In: (ed. by) C. J. Arceneaux: Proceedings of 30th Annual EMSA Meeting. Claitor’s Publishing Division, Baton Rouge, USA, 1972 (p. 408–409).

Tanaka, K., A. Mitsushima, Y. Kajima and H. Fukutome: Revised osmium digestion method using aldehyde prefixation for scanning electron microscopy. Acta anat. nippon. 58: 95–96 (1983).

Tokunaga, J., M. Edanaga, T. Fujita and K. Adachi: Freeze-cracking of scanning electron microscope specimens. A study of the kidney and spleen. Arch. histol. jap. 37: 165–182 (1974).

Umetani, Y.: Postcapillary venule in rabbit tonsil and entry of lymphocytes into its endothelium: a scanning and transmission electron microscope study. Arch. histol. jap. 40: 77–94 (1977).