Good Ultrastructural Preservation of Human Tissues and Cultured Cells by Glutaraldehyde Fixation, Sandwich Freezing, and Freeze-Substitution

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Summary Sandwich freezing (freezing the specimen rapidly with liquid propane by placing it between two copper disks) and freeze-substitution of living yeast cells has been used for observing exquisite close-to-native ultrastructure of cells. Glutaraldehyde fixation, sandwich freezing, and freeze substitution of bacteria and other microorganisms also yield close-to-native ultrastructure. Here, we have used glutaraldehyde fixation, sandwich freezing, and freeze substitution to observe human cells and tissues. We obtained clear and natural cell images of tissues sliced to 0.2 mm thickness. This is a remarkable result because, in the past, tissues as thick as 0.2 mm could only be frozen by high-pressure freezing. The present study has shown that it is possible to observe clear and natural cell structures in animal and human tissues anytime because glutaraldehyde-fixed tissues can be stored at 4°C for several months before freezing, and a sandwich-freezing device will soon become commercially available. Also, natural ultrastructure of cultured cells in suspension was found to be observed more clearly by glutaraldehyde-fixation, sandwich freezing, and freeze-substitution than sandwich freezing and freeze substitution of living cells. The present method should be used as a standard method to observe the close-to-native ultrastructure of animal and human tissues.

Keywords Buffy coat, Cultured cell, Freeze-substitution fixation, High-pressure freezing, Human skin, Sandwich freezing.

Chemical fixation with conventional dehydration procedure (CF-CD) has been used for observing ultrastructure of cells and tissues. This method, however, does not adequately preserve the ultrastructure of cells; hence, rapid-freezing of living cells and tissues followed by freeze-substitution (RF-FS), is becoming a popular method to observe close-to-native ultrastructure of cells.

There are three major methods for freezing cells rapidly: 1) cold metal block freezing is done by rapidly slamming cells or tissues onto a metal block cooled with liquid nitrogen or liquid helium (Dempsey and Bullivant 1976, Aoki et al. 1994); 2) high-pressure freezing is performed by freezing cells and tissues with liquid nitrogen under high pressure (Moor 1987, McDonald et al. 2007, Vanhecke et al. 2008); and 3) plunge freezing is performed by plunging specimens into a cooled cryogen such as propane or ethane. Plunge freezing is further divided into three methods: a) for freezing suspensions of viruses or molecules, microgrids with a thin layer of specimens are plunged into liquid ethane cooled with liquid nitrogen (Adrian et al. 1984, Yamaguchi et al. 2008); b) for observing fungal cells grown on a culture plate, small pieces of cellulose membrane support on which cells are grown are plunged into liquid cryogen cooled with liquid nitrogen (Howard and Aist 1980, Tanaka and Kanbe 1986); and c) for observing yeast or bacteria, cells collected by centrifugation are sandwiched between two copper disks and plunged into liquid propane cooled with liquid nitrogen (sandwich freezing) (Baba and Osumi 1987, Yamaguchi et al. 2009b).

We have been using sandwich freezing and freeze-substitution to study the dynamics of hepatitis B virus core antigen within yeast cells (Yamaguchi et al. 1988, 1991, 1994, 1996), the dynamics of yeast spindle pole bodies (Yamaguchi et al. 2002, 2003, 2009a, 2010), and

Chemical fixation by glutaraldehyde, sandwich-freezing, and freeze-substitution of microorganisms (chemical fixation-freeze substitution, CF-FS) was found to better preserve the ultrastructure of cells compared to CF-CD (Yamaguchi et al. 2005a, 2011b). The CF-FS has been applied to observe ultrastructure of not only microorganisms but also cultured cells with good results (Yamaguchi et al. 2012, 2016a, b, 2017, 2018, Yamada et al. 2014, Yamaguchi and Worman 2014, Kopecká

Fig. 1. The procedure of the CF-FS. (a) Buffy coat. (b) Skin. (c) Cultured cell.

In the present study, we applied the CF-FS to observe human cells and tissues and compared the ultrastructure with those of CF-CD. We also observed the ultrastructure of cultured cells using CF-FS and compared it with the ultrastructure obtained using RF-FS of living cells.

Materials and methods

Electron microscopy of human tissues

Blood samples were taken from a healthy adult man (34 years old) into heparin-treated centrifuge tubes and centrifuged at 2000 rpm for 10 min. Plasma was removed with Pasteur-pipet, and 2.5% glutaraldehyde-0.1 M phosphate buffer (pH 7.2) was carefully layered onto theuffy coat. Theuffy coat was cut into small pieces (about 0.5 mm × 0.5 mm × 2 mm) and kept at 4°C overnight (Fig. 1a). Normal skin was taken from the clavicle of a 42-year-old man with malignant melanoma of right thumb and immediately fixed in 2.5% glutaraldehyde-0.1 M phosphate buffer (pH 7.2) in an operation room. The skin was cut into small pieces (about 0.5 mm × 0.5 mm × 2 mm) and kept at 4°C overnight (Fig. 1b).

For CF-FS, theuffy coat and skin tissues were further sliced to a thickness of 0.1 to 0.2 mm with razor blades while soaked in the fixative under a stereomicroscope. The pieces of tissues in glutaraldehyde solution were placed on a copper disk (Veco, 0.1-mm single-hole grids), whose surface was made hydrophilic by glow discharge (Yamaguchi et al. 2016a,b), lightly stuck on a copper disc (Fig. 1c). Likewise, for RF-FS of living cells, the tissue suspension was centrifuged at 1400 rpm (400 G, swing rotor) for 7 min, and sandwiched between two copper discs (Fig. 1c). Likewise, for RF-FS of living cultured cells, cells were collected by centrifugation at 1400 rpm for 7 min and sandwiched between two copper discs.

The sandwiched cells were snap-frozen by plunging into melting propane cooled in liquid nitrogen. They were kept at 37°C for 10 min and stored at 4°C overnight. They were collected by centrifugation at 1400 rpm (400 G, swing rotor) for 7 min, and sandwiched between two copper discs (Fig. 1c). Likewise, for RF-FS of living cultured cells, cells were collected by centrifugation at 1400 rpm for 7 min and sandwiched between two copper discs.

The sandwiched cells were snap-frozen by plunging into melting propane cooled in liquid nitrogen. They were freeze substituted in ace tone containing 2% osmium tetroxide and freeze-substituted at -80°C for 2–4 days. They were gradually brought to room temperature, rinsed with acetone, embedded in epoxy resin (Quetol 812 10 mL, DDSA 2.8 mL, MNA 7.4 mL, DMP-30 0.3 mL; Nissin EM Co. Ltd., Tokyo), and polymerized at 60°C for 24 h. The specimens remained attached to the copper disks throughout the entire procedure (Fig. 1).

For CF-CD, the tissues in glutaraldehyde were washed with 0.1 M phosphate buffer, post-fixed with 1% osmium tetroxide at 4°C for 1 h, dehydrated with graded series of ethanol at room temperature, embedded in epoxy resin, and polymerized at 60°C for 24 h.

For CF-FS, the blocks were removed with razor blades, and the specimens embedded on the block surface were trimmed to a size of 0.25 mm × 0.25 mm by using an ultrasonic trimming blade (Nissin EM Co. Ltd., Tokyo) and razor blades under a stereomicroscope (Yamaguchi and Chibana 2018). The surface of the block was cut with a diamond trimming knife (Diatome Co. Ltd.) on an Ultracut S ultramicrotome (Leica Microsystems, Vienna). Ultrathin sections were cut directly to a thickness of 50 nm with a diamond knife (Diatome Co. Ltd.) without making semithin sections. They were picked up on single-slot grids (hole size: 2 mm × 1 mm) and placed on a Formvar support film mounted on an aluminum rack (Yamaguchi and Chibana 2018). The grids with sections were dried, detached from the rack, stained with uranyl acetate and lead citrate in a staining tube (Yamaguchi et al. 2005b), and observed in a JEM-1400 electron microscope (JEOL, Tokyo) at magnifications of 50 to 40000 at 100 kV (Fig. 1) (Yamaguchi and Chibana 2018). In order to examine the depth of good freezing, several blocks of tissues were cut perpendicular to the freezing surface.

The study’s protocol for human samples was approved by the Biomedical Research Ethics Committee of the Graduate School of Medicine, Chiba University (3085).

Electron microscopy of cultured cells

K562 cells, which are cell lines originated from human chronic myelogenous leukemia, were cultured in suspension at 37°C in RPMI-1640 medium (Nacalai Tesque, Kyoto) supplemented with 10% FBS (Life Technologies, Grand Island, NY) plus antibodies (Nacalai Tesque) (Hirao et al. 2018).

For CF-FS, glutaraldehyde was added directly to the cell suspension to a final concentration of 2.5%. They were kept at 37°C for 10 min and stored at 4°C overnight. They were collected by centrifugation at 1400 rpm (400 G, swing rotor) for 7 min, and sandwiched between two copper discs (Fig. 1c). Likewise, for RF-FS of living cultured cells, cells were collected by centrifugation at 1400 rpm for 7 min and sandwiched between two copper discs.

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The sandwiched cells were snap-frozen by plunging into melting propane cooled in liquid nitrogen. They were freeze substituted in acetone containing 2% osmium tetroxide for 2–4 days at -80°C and embedded in epoxy resin. Ultrathin sections were cut to a thickness of 50 nm with a diamond knife, picked up on single-slot grids, and placed on a Formvar film mounted on an aluminum rack (Yamaguchi and Chibana 2018). They were stained with uranyl acetate and lead citrate (Yamaguchi et al. 2005b), and observed in a JEM-1400 electron microscope (JEOL, Tokyo) at 100 kV as with human tissues (Fig. 1) (Yamaguchi and Chibana 2018).

Results

Ultrathin sections of human tissues

Figure 2 shows ultrathin sections of human tissues prepared by CF-FS. A platelet cell (Fig. 2a), a neutrophil in a buffy coat (Fig. 2b) and a keratinocyte of skin tis-
Fig. 2. Ultrathin sections of human tissues prepared by CF-FS. (a) A platelet cell. (b) A neutrophil cell. (c, d) High magnification images of platelet cells, (e) a lymphoid cell, and (f) keratinocyte of the skin. (g–h) High magnification image of (f). Note the clear and close-to native images of cells. The cells show higher contrast in spite of the sections being very thin (50 nm), and also show electron-lucent background in the cytoplasm. α, α granule; F, filamentous structure; K, Keratin fiber; M, mitochondria; Mt, microtubules; N, nucleus; NP, nuclear pore; PM, plasma membrane.
sue (Fig. 2f) were clearly observed. At high magnification, microtubules (Fig. 2c), alpha-granules (Fig. 2c, d), plasma membrane (Fig. 2d), mitochondria (Fig. 2d, g, h), nuclear pore (Fig. 2g), fibrous structure (Fig. 2e), and keratin fibers (Fig. 2g, h), were also clearly observed.

Figure 3 shows ultrathin sections of human tissues prepared by CF-CD. A platelet cell (Fig. 3a), a neutrophil (Fig. 3b), a keratinocyte of skin tissue (Fig. 3d), and mitochondria (Fig. 3c, e) could be observed. The cell images, however, were not very clear compared to those of CF-FS.

To examine the depth of good freezing of the human tissues, we cut the blocks perpendicular to the freezing surface (Fig. 4). Figure 4a shows a cross-section of the buffy coat, which is 0.06 mm thick. All the cells show good freezing, and no ice crystal damage was detected. Figure 4b shows a cross-section of the skin. The nuclei of the keratinocytes were photographed at high magnification to examine the presence of ice crystal damages because the nucleoplasm are very susceptible to ice crystal damage. No ice damage was observed in 12 cells throughout the whole section, which is 0.2 mm thick. Thus, sandwich freezing enables up to 0.2-mm-thick tissues to be frozen well using glutaraldehyde-fixed tissues.

**Ultrathin sections of cultured cells**

Figure 5 shows ultrathin sections of human cultured cells prepared by CF-FS. They show a homogeneous cell population at low magnification (Fig. 5a). The cell structure appeared to be well preserved and show

![Image](https://example.com/image.png)

**Fig. 3.** Ultrathin sections of human tissues prepared by CF-CD. (a) a platelet cell. (b) A neutrophil cell. (c) High magnification image of (b). (d) Keratinocyte of the skin. (e) High magnification image of keratinocyte. α, a granule; K, keratin fibers; M, mitochondria; N, nucleus; Nu, nucleolus. Note the images of cells are not very clear compared with the CF-FS (Fig. 2).
Fig. 4. Ultrathin sections of human tissues, cut perpendicular to the freezing surface, prepared by CF-FS to show good deep freezing. (a) Buffy coat. Leucocytes (L) were embedded in red blood cells. They showed clear cell structures with good contrast. (b) Skin. (c–n) High magnification images of nucleoplasm of keratinocytes indicated by c–n in b, showing no freezing damage. Arrowheads point to tissue surfaces that were facing the copper grid surfaces. This image indicates that up to 0.2-mm-thick tissues can be frozen by sandwich freezing without ice crystal formation.
high contrast in spite that the sections being very thin (50 nm). Several dividing cells were observed (D in Figs. 5a, 6). At high magnification, cell structures such as the nucleus, nucleolus, mitochondria, rough endoplasmic reticulum, ribosomes, and microtubules are clearly observed (Figs. 5b, c, 6).

Figure 7 shows ultrathin sections of human cultured cells prepared by RF-FS of living cells. They appear to show lower contrast (Fig. 7a) compared with the images of CF-FS (Fig. 5a), and some cell fragments were observed at a low magnification view (F in Fig. 7a). At high magnification, cell structures such as the nucleus,
nucleolus, mitochondria, and ribosomes were observed (Figs. 7b, c). The cell surfaces, however, appeared to be more or less rough-edged.

We enumerated the dividing cells in the cultured cell population (Figs. 5a, 7a). We found 12 dividing cells among 230 cells (5.21%) in the CF-FS sample and 4

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**Fig. 6.** Cells undergoing division observed in a sample prepared by CF-FS. (a, d) Whole cells. (b–c, e–f) high magnification images of cells. C, chromosome; CS, cell surface; Mt, microtubules. Note the clear and natural images of cells. Cell surfaces are smooth.
dividing cells among 220 cells (1.82%) in the RS-FS sample. Thus, there were fewer dividing cells in the RS-FS sample than in the CF-FS sample, a difference that was nearly significant ($\chi^2$ test, $p=0.0516$).

Discussion

Quality of images of human tissues prepared using CF-FS compared with conventional CF-CD

The images using the CF-FS (Figs. 2, 4) appear to be clearer than the images using the CF-CD (Fig. 3) because the former shows higher contrast and the background of the cytoplasm is more electron-lucent (Yamaguchi et al. 2017). The matrix of the mitochondria is electron-dense (Fig. 2d, g, h), and every single ribosome can be recognized in the CF-FS sample (Fig. 2e, g, h).

Aoki et al. (1994) observed human skin ultrastructure using cold metal block freezing and freeze-substitution and obtained exquisite micrographs of keratinocyte and mitochondria. By comparing their micrographs with our micrographs (Fig. 2f–g), it may be realized that the quality of the images obtained using CF-FS is high enough and comparable to the images obtained using RF-FS. Pokrovskaya et al. (2016) observed human blood platelets prepared by CF-FS using high-pressure freezing and obtained good micrographs of platelet cells. By comparing their micrographs with our micrographs (Fig. 2a–e), it may be realized again that the quality of the images obtained by CF-FS using sandwich freezing is good enough and comparable to the images obtained by CF-FS using high-pressure freezing.

The CF-CD is widely used for examining human cells and tissues because of its handiness. This method, however, does not yield exquisite cell images like the freeze-substitution method. In this method, the osmium tetroxide fixation and dehydration in ethanol series at room temperature appear to cause a collapse of ribosomes and other cell components, with the remnants of the cell components filling the cytoplasm, thereby making cell structures obscure. The present study showed that good freezing can be obtained for tissues as thick as 0.2 mm if the tissue has been fixed with glutaraldehyde. Since glutaraldehyde-fixed tissues can be stored at 4°C for up to several months (Yamaguchi et al. 2011b), the CF-FS can be performed anytime as necessary. Also, since the CF-FS preserves cell ultrastructure close-to-native, the method can provide new insights into the overall ultrastructure of every cell and tissue in humans and can prove useful in the pathological examination for precise diagnosis.

Depth of good freezing by sandwich method

Since no ice crystal damages were seen in ultrathin sections of living cultured cells that contain as big as 30 µm cells in diameter using the RS-FS (Fig. 7a), the sandwich method can, therefore, be used to freeze living cells with a diameter of at most 30 µm. Fixing tissues
with 2.5% glutaraldehyde allows extending the depth of good freezing to as deep as 0.2 mm (Fig. 4). This indicates that glutaraldehyde may have an anti-freezing effect (Walther et al. 2013). Also, since the morphology of the cells appears natural (Figs. 2, 4–6), holding the specimen with tweezers and plunge-freezing it by the sandwich method does not seem to cause any artificial force on the specimen.

High-pressure freezing after glutaraldehyde fixation is performed to observe fragile tissues like mouse brain (Sosinsky et al. 2008). This method gives better images than rapid freezing of raw brain tissues because the brain tissue is often damaged during the procedure to remove the tissues from the living body, and the ultrastructure of cells is altered. Although high-pressure freezing is an excellent method for freezing thick specimens, only a limited number of laboratories can use the method due to the prohibitive cost of the machine. Our results show that sandwich freezing, which is much less expensive, can now be performed for rapid freezing of fragile animal tissues as thick as 0.2 mm instead of high-pressure freezing.

Quality of images of cultured cells prepared using CF-FS compared with RS-FS

The aim of ultrastructure research is to observe cell and tissue structures as close to the native state as possible. Ultrastructure of cultured cells prepared by CF-FS show exquisite preservation of cell structure (Figs. 5, 6), and indicate better preservation than the cell images obtained by RF-FS (Fig. 7).

Cell fragments that may have been produced during the collection of cells by centrifugation were found in the RF-FS sample. Also, fewer dividing cells were found in the RF-FS sample than in the CF-FS sample. Since it took about 10 min to freeze cells by collecting, centrifuging, and placing the cells between two copper disks in the RF-FS, the cells could have undergone structural changes during the procedure (It may be worth a try to further shorten the time for collecting the cells; although it may not be possible to do it within a few minutes). In contrast, cultured cells were fixed immediately in a culture medium at the same temperature and the same culture condition in the CF-FS. Glutaraldehyde is reported to penetrate plant cells at 140 μm per min (2.3 μm s⁻¹) (Mersey and McCully 1978). Since the diameter of the cultured cells was 12.8 ± 0.2 μm (100 cells measured, Fig. 5a), glutaraldehyde could reach the center of cells within 2.8 s (6.4 μm/2.3 μm). Thus, the movement of cell components could stop within 2.8 s, preserving the cell structure as it is if they remained stationary within that period. Fixed cells could be more resistant to deformation caused by the force of centrifugation and other artificial forces. Considering these facts, therefore, we recommend fixing cultured cells of suspension culture in glutaraldehyde before rapid freezing, rather than rapid-freezing living cultured cells in order to observe the natural ultrastructure of cells.

The contrast of cell images by CF-FS is higher than that by RF-FS (Fig. 5a vs. Fig. 7a). Although this is an interesting result, we do not know the mechanism for the high contrast achieved through CF-FS, which is a topic, therefore, for future investigation.

Sandwich-freezing device

Thus far, we have been using a hand-made rapid freezing device, including in the present study (Yamaguchi 2007). It consists of an aluminum cap (28 mm in diameter, 40 mm deep) to be filled with liquid propane and a container (18 cm x 18 cm x 15 cm, made of Styrofoam) to be filled with liquid nitrogen to cool the liquid propane. The hand-made device works well and has been very useful, thus, it would be more convenient if the device is made commercially available. We are therefore planning to commercialize and sell the ‘Sandwich-freezing device’ in 2019 (Marine Works Japan, Ltd., Yokosuka, Japan, http://www.mwj.co.jp/en/).

Cell images obtained using conventional chemical fixation with conventional dehydration procedure are known to be distorted compared with those obtained using rapid-freeze freeze-substitution fixation. The previous and present studies showed that the distortion is mainly caused by the osmium tetroxide fixation and dehydration in ethanol series at room temperature (Yamaguchi et al. 2011b, 2017). The present study also demonstrated that tissues as thick as 0.2 mm can be frozen without ice crystal damages if tissues are fixed in glutaraldehyde beforehand. This is a significant discovery with a profound impact on the field of electron microscopy in life science because it was thought that the only method for freezing thick tissues is high-pressure freezing (Moor 1987, McDonald et al. 2007, Vanhecke et al. 2008). Based on this study, thick tissues can now be frozen by sandwich freezing using glutaraldehyde-fixed tissues. We are hoping that the CF-FS will become a standard procedure for observing biological specimens to obtain close-to-native ultrastructure of cells and tissues.

References


