Optimizing the reaction temperature to facilitate an efficient osmium maceration procedure

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ABSTRACT

The osmium maceration method is a powerful technique for observing the three-dimensional ultrastructure of cellular organelles by scanning electron microscopy. In the conventional osmium maceration method, tissues are immersed in a diluted osmium tetroxide solution for several days at 20°C to remove soluble cytosolic proteins from the freeze-cracked surface of cells, and the optimal duration of this process is dependent on the cell type. To improve the efficiency of the osmium maceration procedure, we have examined systematically the relationship between the reaction temperature and time of the osmium maceration procedure. Treatment at temperatures higher than 20°C drastically shortened the time required to remove cytosolic proteins from the freeze-cracked surface of specimens with optimal durations for the osmium maceration of hepatocytes at 30, 40, 50 and 60°C being 30, 15, 5 and 1 h, respectively. Considering the stability and reproducibility of the macerated specimens, we concluded that the most appropriate temperature was 30 to 40°C. This rapid osmium maceration procedure was used successfully to observe the 3D ultrastructure of Purkinje cells in the cerebellum and proximal convoluted tubule cells in the kidney. This simple and reproducible rapid osmium maceration protocol should find wide appeal for the 3D analysis of cellular organelles in various cell types.

INTRODUCTION

Scanning electron microscopy (SEM) has been used for more than a half century in the field of biology because it has an advantage over conventional transmission electron microscopy of ultrathin sections; SEM directly reveals the three-dimensional (3D) surface topology of tissues and cells. However, the conventional sample preparation method used for SEM analysis did not enable observation of subcellular structures buried in cytosolic matrices. This limitation was overcome by using osmium maceration techniques (i.e., the osmium-DMSO-osmium (O-D-O) method and the aldehyde-osmium-DMSO-osmium (A-O-D-O) method) developed by Tanaka and colleagues (Tanaka and Naguro 1981; Tanaka and Mitsushima 1984). By using these methods, the 3D ultrastructure of cellular organelles including the Golgi apparatus, endoplasmic reticulum (ER) and mitochondria could be observed directly by SEM without any reconstruction processes. Subsequent studies using the osmium maceration techniques have elucidated the 3D morphological characteristics of various cellular organelles (Hanaki et al. 1985; Tanaka et al. 1986; Lea and Hollenberg 1989; Riva et al. 2010).

We have utilized the osmium maceration method for exploring detailed structural information of the Golgi apparatus because the spatially complex structure of this organelle within a wide variety of highly differentiated cells in tissues is an ideal target for
the method (Koga and Ushiki 2006; Watanabe et al. 2012; Koga et al. 2017). As we recently demonstrated, a novel SEM technique combining osmium-impregnation and -maceration methods (the osmium impregnation/maceration method) enabled visualization of the cis-trans polarity of the Golgi apparatus three-dimensionally in osmium-macerated specimens (Koga et al. 2016). By combining the osmium maceration technique with the cryo-sectioning method, the molecular distribution in target cells could be correlated with the 3D ultrastructure of membranous cell organelles (the correlative light and scanning electron microscopy method; Koga et al. 2015).

To remove cytoplasmic proteins from the cracked-surface of cells selectively and concomitantly leave the membranous organelles preserved in situ, tissues firmly fixed with 1% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer (PB, pH 7.4), with (the A-O-D-O method) or without (the O-D-O method) prefixation by aldehydes, should be immersed in a diluted osmium solution (usually 0.1% OsO₄ in 0.1 M PB), termed the osmium maceration procedure. The osmium maceration procedure under conventional temperature conditions of 20°C takes a considerably long time, usually several days, and the optimal duration for preparing macerated specimens is dependent on the cell type (Hanaki et al. 1985; Koga and Ushiki 2006; Koga et al. 2012). However, in the aforementioned osmium impregnation/maceration method, 24 h treatment with a 0.1% OsO₄ solution in 0.1 M PB was sufficient for the maceration step after prolonged osmication treatment at higher temperature for osmium impregnation (with 2% OsO₄ at 40°C) (Koga et al. 2016), suggesting that the reaction at temperatures higher than 20°C accelerates the osmium maceration process. In this study, we examined whether a higher reaction temperature than the conventional 20°C can shorten the reaction time for the osmium maceration procedure. Based on the results, we propose a novel protocol for the rapid osmium maceration procedure, which will provide a simple and useful tool for investigating the 3D ultrastructure of cellular organelles in a wide variety of cells.

**MATERIALS AND METHODS**

**Animals.** Twenty male adult Wistar rats purchased at 8 weeks of age from Sankyo Laboratory Service Co., Ltd. (Tokyo, Japan) were used in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources; National Research Council, Washington, DC, 1996) under the permission of the experimental animal welfare committee of Asahikawa Medical University (permission #19099-2).

**Osmium maceration method.** Under deep anesthesia with ketamine/xylazine (100 : 10 mg/kg) rats were perfused with physiological saline followed by 200 mL of a mixture of 0.5% paraformaldehyde (PFA) and 0.5% glutaraldehyde (GA) in 0.1 M PB (pH 7.4). Livers were excised from the animals, cut into small pieces and post-fixed with 1% OsO₄ in 0.1 M PB for 2 h at 4°C. After rinsing with the buffer solution for 1 h, the specimens were immersed in 25% and 50% dimethyl sulfoxide (DMSO) in distilled water for 30 min each. The specimens were subsequently frozen on a metal plate pre-cooled with liquid nitrogen and were cracked into two pieces by a screwdriver and hammer. The split pieces were then replaced in a 50% DMSO solution for thawing at room temperature and rinsed in the buffer solution until the DMSO was completely removed from the specimens. For removal of soluble cytosolic proteins from the cracked surface of cells, the two split pieces of tissues were placed in glass vials containing 10 mL of 0.1% OsO₄ solution (0.1 M PB, pH 7.4) for 72, 96 or 120 h at 20°C, as the conventional temperature condition for the osmium maceration procedure. To determine the optimal conditions for osmium maceration, we altered both the temperature and duration of the osmium maceration process. Vials containing 10 mL of a 0.1% OsO₄ solution were pre-warmed at 30, 40, 50 and 60°C in a water bath and kept at the same temperature throughout the osmium maceration process ranging from 2 to 30 h. The macerated specimens were further immersed in a 1% OsO₄ solution (0.1 M PB, pH 7.4) for 1 h at 4°C, rinsed in the buffer solution (0.1 M PB, pH 7.4) for 1 h, and treated with 1% tannic acid (Nacalai Tesque, Kyoto, Japan) in 0.1 M PB for 1 h at 20°C. After rinsing in the buffer solution for 1 h, the specimens were further immersed in 1% OsO₄ in 0.1 M PB for 1 h at 4°C. The samples were then dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, 100%, 30 min each), transferred to isoamyl acetate and dried in a critical point dryer (HCP-2; Hitachi, Tokyo, Japan). The dried specimens were mounted onto aluminum bases, coated with platinum-palladium at thickness of 3 nm in an ion-sputter coater (E1010; Hitachi Koki Co., Ltd) and observed under a field emission scanning electron microscope (S-4100; Hitachi High-Technologies, Tokyo, Japan). The described procedures for osmium maceration are summarized...
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Treatment of specimens with a 0.1% OsO₄ solution for 96 h at 20°C greatly improved removal of soluble cytosolic proteins (Fig. 2b), but a small amount of residual cytosolic materials still interrupted the detailed observation of membranous organelle on the cracked-surface of cells. Treatment for 120 h was sufficient to fully remove the cytosolic proteins to reveal the 3D ultrastructure of cell organelles in hepatocytes (Fig. 2c) with ribosomes clearly visible on the surface of cisterns of the rough ER. These results indicated that treatment with a 0.1% OsO₄ solution for 120 h at the conventional temperature of 20°C is necessary to expose properly the 3D ultrastructure of cell organelles in hepatocytes for SEM analysis.

RESULTS

Optimal duration of the osmium treatment at the conventional temperature of 20°C
To establish optimal conditions for the osmium maceration procedure, we first varied the duration of the osmium maceration process under the conventional temperature condition of 20°C (Fig. 2). Maceration treatment for 72 h at 20°C (Fig. 2a) caused exposure of the mitochondrial cristae on the cracked-surface of hepatocytes but did not reveal the fine 3D ultrastructure of other organelles; the profiles of the Golgi apparatus and ER on the cracked surface were still masked with residual cytosolic proteins. The treatment of specimens with a 0.1% OsO₄ solution for 96 h at 20°C greatly improved removal of soluble cytosolic proteins (Fig. 2b), but a small amount of residual cytosolic materials still interrupted the detailed observation of membranous organelle on the cracked-surface of cells. Treatment for 120 h was sufficient to fully remove the cytosolic proteins to reveal the 3D ultrastructure of cell organelles in hepatocytes (Fig. 2c) with ribosomes clearly visible on the surface of cisterns of the rough ER. These results indicated that treatment with a 0.1% OsO₄ solution for 120 h at the conventional temperature of 20°C is necessary to expose properly the 3D ultrastructure of cell organelles in hepatocytes for SEM analysis.
treatment with a 0.1% OsO₄ solution to find optimal conditions for osmium maceration.

Specimens treated at 30°C for 10 h with a 0.1% OsO₄ solution was insufficient for removing the soluble cytosolic proteins from the cracked-surface of the cells, and organelles such as the Golgi apparatus and rough ER were still embedded in the residual cytosolic materials of hepatocytes (Fig. 3a2), even though the nuclei and mitochondria could be identified in the low magnification images (Fig. 3a1). Longer treatment with the 0.1% OsO₄ solution, however, improved the efficiency of maceration. Twenty-hour treatment at 30°C exposed the profiles of the Golgi apparatus and rough ER (Fig. 3b1) with only a small amount of residual cytoplasmic matrix still discernable within hepatocytes when observed at the higher magnification (Fig. 3b2). The 30 h treatment at 30°C removed the cytosolic proteins sufficiently to reveal details in the 3D ultrastructure of cell organelles such as the Golgi apparatus with the stack of cisterns, tubular structure of smooth ER, sheet-like cisterns of rough ER, ribosomes on the ER cisterns, mitochondria with cristae and glycogen granules within hepatocytes (Fig. 3c1, 3c2).

The efficiency of the maceration process was noticeably accelerated when the specimens were treated at 40°C (Fig. 4). Although the residual cytosolic matrix was still distinguishable around organelles in specimens treated with 0.1% OsO₄ for only 10 h at 40°C (Fig. 4a2), 15 h treatment with the OsO₄ solution satisfactorily removed the cytosolic proteins to unveil the 3D ultrastructure of organelles in hepatocytes (Fig. 4b2). Thus, the optimal duration of the osmium maceration process at 40°C was twice and twelve times shorter than that at 30 and 20°C, respectively, indicating that treatment at higher temperatures reduces the time required for osmium maceration.

Tissues were treated with the 0.1% OsO₄ solution at 50 and 60°C to evaluate the upper temperature limit suitable for osmium maceration (Fig. 5a, b). Soluble cytosolic proteins were removed from hepatocytes after 5 h treatment with the 0.1% OsO₄ solution at 50°C (Fig. 5a), while treatment for less than 5 h was insufficient for removing the cytosolic proteins from the cracked-surface of the cells (data not shown). Treatment of specimens for only 1 h at 60°C with the 0.1% OsO₄ solution was sufficient to expose the ultrastructure of membranous organelles (Fig. 5b). However, specimens treated with the 0.1% OsO₄ solution at 50 and 60°C were extremely fragile, and the cracked-surface of tissues often collapsed during subsequent sample preparation.
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OsO₄ solution, usually with 0.1% OsO₄ in 0.1 M PB (pH 7.4), after tissues are directly fixed with OsO₄ by immersion (the O-D-O method; Tanaka and Naguro, 1981) or fixed both by perfusion with an aldehyde fixative and subsequently by immersion with OsO₄ (the A-O-D-O method; Tanaka and Mitsushima 1984). Under the conventional temperature condition of 20°C the osmium maceration method usually takes at least 72 h to remove the

DISCUSSION

The osmium maceration method has enabled the direct observation of the 3D ultrastructure of membranous cell organelles by SEM without any reconstruction processes. As the core process of this method, tissue specimens are treated with a diluted

Fig. 3 Corresponding pairs of low (a1, b1 and c1) and high (a2, b2 and c2) magnification SEM images of hepatocytes macerated at 30°C for 10 h (a1 and a2), 20 h (b1 and b2) and 30 h (c1 and c2). M: mitochondria, Go: Golgi apparatus, RER: rough endoplasmic reticulum, SER: smooth endoplasmic reticulum, G: glycogen granules, N: nuclei.
soluble cytosolic proteins from the cracked-surface of the specimens, and the optimal duration for removing the soluble cytosolic proteins is dependent on the cell type (Hanaki et al. 1985; Koga and Ushiki 2006; Koga et al. 2012). To overcome the drawbacks of the relatively long and unpredictable reaction time under conventional conditions, we reconsidered in the present study whether changes in the reaction temperature could improve the efficiency of the osmium maceration process.

It has long been believed that the temperature of the solution for osmium maceration should be kept at 20°C throughout the reaction to avoid thermal damage to intracellular structures (Tanaka 1989). The optimal reaction time was found to be 120 h for preparing osmium-macerated specimens of rat hepatocytes at the conventional temperature of 20°C (Fig. 2c). Increasing the reaction temperature drastically shortened the reaction time necessary for complete removal of the soluble cytosolic proteins from the specimens (Figs. 3–5). The optimal reaction times for osmium maceration were reduced to 30, 15, 5, and 1 h at 30, 40, 50, and 60°C, respectively. When the specimens were treated with the 0.1% OsO₄ solution at 50 and 60°C, however, tissue specimens were too fragile and often collapsed before observation by SEM. Taking into consideration these results, treatment of specimens with a 0.1% OsO₄ solution at 30 or 40°C, which we term the rapid osmium maceration procedure, are recommended to reproducibly prepare specimens appropriate for observation by SEM.

To verify whether the rapid osmium maceration procedure established in the present study is also suitable for use with other cell types, Purkinje cells in cerebellum and renal tubular cells in kidney were treated with a 0.1% OsO₄ solution at 30 or 40°C, which we term the rapid osmium maceration procedure, are recommended to reproducibly prepare specimens appropriate for observation by SEM.

Fig. 4 Corresponding pairs of low (a1 and b1) and high (a2 and b2) magnification SEM images of hepatocytes macerated at 40°C for 10 h (a1 and a2) and 15 h (b1 and b2). M: mitochondria; Go: Golgi apparatus, RER: rough endoplasmic reticulum, SER: smooth endoplasmic reticulum, N: nuclei.
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Besides the reaction temperature, other parameters and/or procedures possibly affect the efficiency of osmium maceration. Hotta et al. (1990) reported that membranous organelles including mitochondria and rough ER were clearly observed both in hepatocytes and renal tubular cells osmium-macerated for 60 or 120 min under microwave irradiation. The osmium-ethanol-osmium (O-E-O) method introduced by Sasaki (1988), where tissues are directly fixed with osmium and freeze-cracked by using 70% ethanol instead of 50% DMSO, can remove the cytosolic matrix from the cracked surface of specimens by incubating the sample in a diluted OsO₄ solution for only 14 h at room temperature; although, the nuclear envelope and the nucleoplasm appeared to be poorly preserved by this method. Application of the microwave maceration procedure and the O-E-O method, which were both applied to specimens fixed solely with OsO₄, to specimens prepared by the A-O-D-O method has not been examined; the ultrastructure of the specimens pre-fixed with aldehydes is generally much better preserved than those without prefixation. In contrast, the ultrastructures of membranous cell organelles including the Golgi apparatus, ER, mitochondria and nucleus prepared by the rapid osmium maceration procedure developed in the present study were appropriately preserved for detailed observation by SEM because the tissues were properly pre-fixed with a mixture of 0.5% PFA and 0.5% GA in 0.1 M PB.

In summary, the present study clearly showed that osmium maceration at higher temperatures than the conventional 20°C, especially at 30 to 40°C, drastically shortened the reaction time with the diluted OsO₄ solution without deterioration of the ultrastructure of cells and tissues. The simple and reproducible protocol for the rapid osmium maceration method established herein should find wide appeal for 3D analysis of cell organelles in various cell types.

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Fig. 5 The effects of extremely high temperature for the osmium maceration procedure. High magnification SEM images of hepatocytes macerated at 50°C for 5 h and 60°C for 1 h are shown in (a) and (b), respectively, and a low magnification SEM image of a liver tissue block macerated at 60°C for 1 h is shown in (c). Although the freeze-cracked surface of the tissue had mostly collapsed and lost from the specimen during the maceration procedure, some areas of the properly freeze-cracked surface remain (indicated with stars). M: mitochondria, Go: Golgi apparatus, RER: rough endoplasmic reticulum, SER: smooth endoplasmic reticulum.
CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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Figs. S1 and S2  High magnification SEM images of a Purkinje cell in the cerebellum (S1) and a proximal convoluted tubule cell in the kidney (S2) prepared by the osmium maceration procedure at 30°C for 30 h. Note that the Golgi apparatus (green), mitochondria (blue) and rough ER (RER) are clearly recognized three-dimensionally in the Purkinje cell, and numerous mitochondria (blue) located between the basal infoldings of the plasma membrane (purple) are vividly observed in the renal tubule cell.