Effects of dehydration and drying steps on human chromosome interior revealed by focused ion beam/scanning electron microscopy (FIB/SEM)

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Keywords: Chromosome, Critical point drying (CPD), Chromosome inner structure, Focused ion beam/scanning electron microscopy (FIB/SEM), Ionic liquid (IL)

Introduction

The compaction process of chromatin fibers into a mitotic chromosome and the resultant higher order structure of chromatin fiber are still controversial even though the chromosome was discovered more than 100 years ago. Although, researchers have been trying to elucidate the higher order structure both by biochemical and visualization means, a concrete model of chromosome higher order structure has not yet been established (Wanner and Formanek 2000, Fukui and Uchiyama 2007, Nishino et al. 2012).

Electron microscopy has been used for chromosome study because of its superior resolution and magnification. Most reports by electron microscopy focused on the surface structure and little has been known about the interior to date. Recently, focused ion beam/scanning electron microscopy (FIB/SEM) has been introduced for biological studies as a tool for observation of the detailed interior. In this hybrid SEM system, samples could be dissected using Ga+ beam irradiated from a perpendicularly equipped FIB gun against the tilted sample stage, and the surfaces of sections could be simultaneously observed by SEM. The image data obtained by serial slice-and-view technique allows the reconstruction of 3D models of the samples such as a whole cell or cultured tissues (Bushby et al. 2011). However, biological samples must be exposed to high vacuum environment in an electron microscope, which causes rapid water evaporation followed by destruction of structures. For this reason, biological samples are subjected to many preparatory steps such as dehydration and drying before observation. In order to minimize destruction of structures, several mild drying methods such as freeze drying and hexamethyldisilazane (HMDS) method have been developed (Lee and Chow 2012).

Critical point drying (CPD) is the most popular methods to dry up biological samples using supercritical fluid avoiding structural destruction. As supercritical fluid has almost no surface tension, detailed structures in samples can be maintained in their original structure. However, it was also reported that CPD, including pre-dehydration with organic solvent, may cause certain shrinkage or other artifacts in biological samples (Boyle and Macconnachie 1979, Bahr and Engler 1980, Nordestgaard and Rostgaard 1985, Ris 1985).

To examine the effects of CPD on the chromosome structure, we applied ionic liquid (IL) method, which are...
being recently introduced for biological sample observation by electron microscopy. IL is salt in liquid state at room temperature which has a high ionic conductivity and no volatility, meaning IL is in liquid state even exposed to high vacuum environment in an electron microscope. By covering biological samples with IL, they could be observed in close-to-native state omitting conventional dehydration, drying by CPD and metal/carbon coating (Kuwabata et al. 2006, Arimoto et al. 2008, Dwiranti et al. 2012).

So far, chromosome structure has been studied by using FIB/SEM with a combination of CPD or IL. Barley and human chromosomes dried by CPD showed sections with and without cavities, while IL coated chromosomes always had no cavities inside the body (Schroeder-Reiter et al. 2009, 2012, Hamano et al. 2014). This manuscript reports the presence or absence of cavities of human chromosomes under the different conditions of dehydration, drying by CPD and IL treatment.

**Materials and methods**

**Preparation of human chromosomes**

Human mitotic chromosomes were isolated from HeLa S3 cells by the polyamine (PA) method (Hayashihara et al. 2008). The PA chromosome stock solution (-20°C in glycerol) was applied on aluminum substrates on ice,

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*2% OsO$_4$

**3-metylbutylacetate treatments were commonly applied to all the treatments after dehydration by ethanol series.

**Figure 1.** Whole images (a and d) and section images (b, c, e and f) of isolated human chromosomes dried by CPD. Chromosomes were dissected by FIB at the positions indicated by white lines (a and d). Cavities were observed both in SE (b and e) and BSE (c and f) images regardless of cutting directions. Bars = 1 µm (a and d) and 500 nm (b, c, e and f).
which had been hydrophilized and coated with 0.01% poly-L-lysine in advance. After 10 min, samples were washed with XBE2 buffer (10 mM HEPES, 2 mM MgCl2, 100 mM KCl, 5 mM EGTA, pH 7.7) to remove excess glycerol stock solution. Chromosomes were pre-fixed with 2.5% glutaraldehyde and 0.2% tannic acid for 30 min at room temperature and washed with XBE2 buffer for 3 times, 5 min each. Post-fixation was performed with 2% osmium tetroxide (OsO4) for 10 min, followed by wash with MilliQ water for 3 times, 5 min each.

**Dehydration and drying by CPD**

For gradual dehydration, ethanol series was carried out with 50 to 100% of ethanol (Table 1), followed by further dehydration with ethanol : 3-methylbutylacetate = 1 : 1 and 3-methylbutylacetate 100% treatment, 15 min each. Drying by CPD was performed on dehydrated chromosomes using carbon dioxide. In order to increase the image contrast, dried chromosomes were coated with OsO4, 3 nm in thickness.

**IL treatment**

1-butyl-3-metylimidazolium tetrafluoroborate (C8H15BF4N2, BMI-BF4, MERCK) was used (Dwiranti et al. 2012). IL was diluted with MilliQ water to 0.5% (v/v) and mixed up using voltex for 1 min and incubated at 99°C for more than 1 h in advance. Details of the sample preparation were shown in Table 1. For IL treatment, IL solution was applied onto samples for 1 min at room temperature, and subsequently excess IL solution was removed using filter paper and a blower. In order to replace remaining water in the chromosomes with IL, samples were placed in near-vacuum condition by pumping for more than 1 h.

**FIB/SEM observation**

Aluminum substrates with chromosome samples on were cut into suitable sizes and attached to silicon wafer with electron conductive carbon tape. After insertion of samples into FIB/SEM system (Helios 660, FEI Inc.), chromosomes were tilted at 52°, and dissected by focused Ga+ beam. Images of sections were obtained both in secondary electron (SE) and back scattered electron (BSE) modes using the accelerating voltage at 2.00 kV.

In SE mode, structural information can be obtained by detecting electrons which are emitted from the surface of samples. On the other hand in BSE mode, the atomic

Figure 2. Whole images (a and d) and section images (b, c, e and f) of isolated human chromosomes dehydrated for longer duration and dried by CPD. White lines in whole images (a and d) indicate the dissected positions. Many cavities were observed both in SE (b and e) and BSE (c and f) images regardless of cutting directions. Bars = 1 µm (a and d) and 500 nm (b, c, e and f).
number information can be obtained by detecting electrons with high energy emitted from deeper position of samples (Hamano et al. 2014).

Results and discussion

The effects of dehydration

First, we checked the effects of the dehydration step on the chromosome structure by observing the inner structure of human chromosomes prepared by two different duration of ethanol series (50, 60, 70, 80, 90, 95 and 100% for 10 min or 30 min each, Table 1). The whole images of chromosomes observed by SE mode showed rough surfaces consisting of many particles and fibers with several nanometers in width (Fig. 1a and d). By SE mode, the cross section images of chromosomes showed many cavities with different sizes randomly distributed inside of their bodies (Fig. 1b), which was consistent with the previous result (Hamano et al. 2014). BSE image also showed many cavities on the same positions to those shown in the SE image (Fig. 1c). Both the SE and BSE images were quite similar to those obtained and reported by Schroeder-Reiter et al. (2009). Existence of cavities was investigated using five sections from two chromosomes on the same aluminum substrate. As a result, many cavities were observed in all the cross sections. A chromosome longitudinally dissected also showed many cavities inside of the body, indicating that such cavities more or less evenly distributed within the chromosome (Fig. 1e and f). Moreover the cavities showed the similar shapes and size ranges between cross and longitudinal sections.

The interiors of chromosomes dehydrated for longer duration were also observed (Fig. 2). The whole images showed rough surfaces (Fig. 2a and d), and both cross and longitudinal section images showed many cavities (Fig. 2b, c, e and f). Comparison of the two chromosome samples prepared by different duration of dehydration by ethanol series showed almost no significant differences in the shapes and size ranges of the cavities. Because the similar cavities were observed regardless of the difference in dehydration durations, this step seems not to have much effect on appearance of cavities.

The effects of CPD

The IL method was combined with post-fixation, dehydration and drying by CPD. In the previous report,
all the chromosomes covered with IL just after pre-fixation and metal staining had no cavities in the chromosomal body (Hamano et al. 2014). 0.5% IL was applied onto chromosome samples after post-fixation, dehydration or drying by CPD (Table 1), and the interiors were observed by FIB/SEM (Fig. 3 and 4). Chromosomes treated with IL after post-fixation or dehydration showed smooth surfaces (Fig. 3a and d). Moreover both in SE and BSE cross section images, the chromosomes had no cavity in their body as shown in Fig. 3b, c, e and f. However, these chromosome images were comparatively less detailed than the chromosome images prepared by CPD. Thus it may still be too early to conclude that fixation and dehydration steps are not related to the appearance of cavities based on the data obtained only in this study.

Then, chromosomes treated with IL after drying by CPD were also observed by FIB/SEM (Table 1, Fig. 4). OsO4 coating was omitted in the case of chromosomes treated with IL, and the cross and longitudinal sections were directly observed after drying by CPD and subsequent IL treatment. Although the chromosomes showed the similar smooth surfaces between those with and without drying by CPD (Fig. 3a and d, 4a and d), both the SE and BSE images of the cross sections showed dark regions in the same positions (Fig. 4b and c, white circles) which were not observed in the cross section images of the former two samples without drying by CPD. These regions were more clearly observed in the BSE images than the SE images, indicating that the dark regions were filled with the lighter elements. Thus the most probably, the dark regions in the cross sections were filled with permeated IL with lighter elements than OsO4 which distributed more or less evenly in the chromosome body after OsO4 fixation step. IL was thought to be trapped at cavities in chromosomes and appeared as dark regions. In other words, in this sample, cavities could be recognized as dark regions. A longitudinally dissected chromosome also showed the dark regions in the sections, suggesting that the cavities were randomly distributed in the chromosome (Fig. 4e and f, white circles). The shapes and size ranges of dark regions were almost the same regardless of cutting directions.

In conclusion, cavities were observed inside of chromosomes only when drying by CPD was carried out regardless of IL application but were not observed after

![Figure 4. Whole images (a and d) and section images (b, c, e and f) of isolated human chromosomes treated with IL after drying by CPD. Chromosomes were dissected in two directions indicated by white lines in whole images (a and d). Chromosomes showed smooth surfaces similar to Figure 3, however section images showed dark regions at the same positions both in SE and BSE images regardless of cutting directions (b, c, e and f, white circles). Bars = 1 µm (a and d) and 500 nm (b, c, e and f).]
fixation and dehydration steps. Although the number of cavities observed in the samples dried by CPD and treated with IL (Fig. 4) was smaller than those in dried by CPD samples without IL treatment (Fig. 1 and 2), it may be appropriate to think that small cavities in samples were filled up with IL and not be visualized by electron microscopy. Although IL treated chromosomes are expected to maintain the structure in a close-to-native state, it is still difficult to conclude the origin and/or the appearance of cavities in chromosomes based only on the comparison between CPD and IL treated chromosomes because the effect of IL on chromosome is not fully understood. However, the fact that the larger size cavities appeared in CPD treated chromosomes could be seen after IL treatment (Fig. 4b, c, e and f, white circles), indicates that IL does not cover the larger cavity structure. The chromosomes treated with IL after post-fixation or dehydration did not show such dark regions, indicating that they did not have serious structural changes like large cavities shown in chromosomes dried by CPD. Taking this fact into account, both the post-fixation and dehydration seem not to strongly affect chromosome structure compared to drying by CPD. It is also noteworthy that most cavities in the chromosomes prepared by CPD showed bubble-like spherical shapes with similar ranges of diameters regardless of cutting directions. This might suggest that those cavities are not authentic structures of chromosome, but rather were induced or enlarged during drying step by CPD.

CPD method has been thought to well maintain the ultrastructure of the samples to date. Induction of shrinkage and other artifacts, especially to biological samples (Boyd and Maconnachie 1979, Bahr and Engler 1980, Nordestgaard and Rostgaard 1985, Ris 1985) has also been reported. Moreover, supercritical fluid of carbon dioxide is widely used for the extraction of organic substances and the generation of tiny bubbles within materials (Williams 1981, Arora et al. 1998). Taking all these points into account, cavities in chromosomes might be caused by, 1) shrinkage of chromatin fibers, 2) elution of biomolecules from chromosome and/or 3) generation of carbon dioxide gas bubbles during the CPD treatment. If the cavities are produced by shrinkage, the observed structure might reflect the original chromosome internal structures such as chromatin fibers. As chromosomes were fixed with glutaraldehyde and tannic acid, it seems less appropriate to conclude that cavities are formed by extraction of biomolecules such as proteins and/or DNA in chromosomes. On the other hand, it may be understandable that cavities are formed by the rapid volume increase of carbon dioxide when its phase changes from supercritical fluid to gas. The third one is further supported by the fact that most cavities have bubble-like spherical shapes. As we discussed above, different causes could still be considered, it is impossible to identify the specific cause for cavity formation now, however, the CPD method may be the most probable candidate among them based on the discussion above. We should be careful to use this method for quite small fragile biological samples, such as chromosomes. For further investigation of the appearance of cavities, it is necessary to examine the inner structure of chromosomes dried by the other methods, such as freeze drying, HMDS, etc.

Acknowledgments

This research was technically supported by Research Center for Ultra-high Voltage Electron Microscopy, Osaka University, and the authors are grateful for Dr. Tomoki Nishida for his kind technical support. This study was financially supported by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (A) Grant Number 25252064 to K. F.

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