Staining and Embedding of Human Chromosomes for 3-D Serial Block-Face Scanning Electron Microscopy

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The high-order structure of human chromosomes is an important biological question that is still under investigation. Studies have been done on imaging human mitotic chromosomes using mostly 2-D microscopy methods. To image micron-sized human chromosomes in 3-D, we developed a procedure for preparing samples for serial block-face scanning electron microscopy (SBFSEM). Polyamine chromosomes are first separated using a simple filtration method and then stained with heavy metal. We show that the DNA-specific platinum blue provides higher contrast than osmium tetroxide. A two-step procedure for embedding chromosomes in resin is then used to concentrate the chromosome samples. After stacking the SBFSEM images, a familiar X-shaped chromosome was observed in 3-D.

Three-dimensional structural investigation of biological samples is an expanding area of research. Recently a number of new methods have been demonstrated to reveal the 3-D structure of chromosomes (1,2). The question of high-order human chromosome organization has been under scrutiny for nearly 50 years (3) and represents an inherently 3-D problem. Understanding these pivotal structures, the repository of all genetic material in 3-D, would fill a fundamental gap in our knowledge and allow scientists to examine the organization of the genome from a new perspective. However, imaging human chromosomes in 3-D has been problematic and still remains a challenge, partly because it is so hard to ensure preservation of the chromatin in its natural state.

Each one of the 23 pairs of chromosomes is unique and is composed of DNA (genes) and a number of complex proteins that make up chromatin. Our knowledge to date is limited above the level of the crystallographic structure of the nucleosome (>11 nm) or below the optical resolution images of the fully organized mitotic chromosome (4). This intermediate-level structure has not been resolved between 11 and 250 nm. For example, a big controversy surrounds the 30-nm structure, which is still debated (5,6).

Various microscopy methods have been used to study the different levels of chromosome compaction (7). Even though light microscopy can be used to image chromosomes at the mitotic stage of the cell cycle, the resolution is limited to about 250 nm and, therefore, does not provide information relating to the finer internal structure (4). More powerful fluorescence microscopy methods such as 2-D super-resolution imaging by photo-activated localization microscopy (PALM) on *Drosophila* chromosomes has been demonstrated (8), with no studies to date on 3-D human chromosomes. Imaging has also been achieved using scanning electron microscopy (SEM) on human chromosomes, but this method only visualizes the surface of the sample. Using this method, chromosomes are recognizable at low magnification. However, at increased magnification, SEM images have shown residual nucleoplasm that partially or completely conceals the chromosomes and prevents the high-resolution analysis of chromosome ultrastructure (9). Transmission electron microscopy (TEM) has provided high resolution, but it is limited to thin samples; even using high voltage systems, the sample thickness is limited to below 1 micron (10). This method cannot be used reliably to image the 2-micron-sized chromosomes because the electrons cannot penetrate through the sample (11). However, whole human chromosomes

METHOD SUMMARY

Procedures for cleaning, staining, and embedding human chromosomes for serial block face scanning electron microscopy (SBFSEM) have been optimized. This method shows X-shaped human chromosomes in 3-D and clear signs of internal structure. The work highlights the potential of SBFSEM as a useful tool in advancing chromosome structure research into three dimensions.

have been imaged and reconstructed in 3-D by TEM tomography after the sample was chemically prepared (12,13). An alternative method for tomography for 3-D imaging is to use TEM serial sectioning of the sample, as thicker volumes can be achieved after they are embedded in resin. Slices of chromosomes have been imaged (14); however, there is no report of 3-D reconstruction using this method, mainly because it is labor-intensive and time-consuming since the image sections have to be handled manually (12). Human chromosomes have been imaged in a pioneering X-ray study using 3-D X-ray diffraction imaging. This study showed 120-nm resolution in 3-D, but no significant internal structural detail was obtained after reconstruction (1).

Serial block-face scanning electron microscopy (SBFSEM) is an established 3-D method that has become an important technique for obtaining 3-D information on biological samples such as tendon (15) and brain (16); interchromosomal interphase chromatin organization from rat nuclei has also been obtained (17). The method is automated and uses a diamond knife to remove thin slices of samples using an ultramicrotome that is situated within an SEM. After each slice is removed, the fresh surface is imaged by collecting backscattered electrons and the sample is then sliced again to expose the next layer (18). Images collected by SBFSEM do not need to be aligned because the system has automated image acquisition (18,19). The method provides 3-D information from slices rather than projections, so it has an inherent advantage over TEM for visualizing 3-D structures.

SBFSEM has been found to work well for samples embedded in a suitably conductive composed epoxy resin. One of the challenges is obtaining sufficiently concentrated samples that fit within the limited 3-D field-of-view of the instrument. Appropriate staining is vital to success because the inherent electron contrast between the resin and chromatin is very low. Biological sample preparation methods for SBFSEM in the literature are mainly for tissue preparation (15–17), and, to date, there is no report of mitotic human chromosomes imaged in 3-D using this method. Here we present the optimization of sample preparation conditions for staining and embedding human chromosomes into resin, with the aim of preserving the internal structure.

Material and methods

Cell culture and mitotic chromosome preparation

A B-lymphocyte male Yoruba cell line (passage 4) was grown at 37°C in a 5% CO_2 atmosphere in RPMI medium supplemented with penicillin/streptomycin and 20% FBS (reagents purchased from Sigma-Aldrich, Dorset, UK). Mitotic cells were enriched through a thymidine block with the addition of 2-mM thymidine for 16 h and then arresting the mitotic cells after treating the cells with colcemid 0.2 μg/mL (Gibco Life Technologies, Paisley, UK) for 6 h. Polyamine mitotic chromosomes were prepared following the previously described procedures (20, 21). Briefly, the cells were treated with a pre-warmed, hypotonic 0.075 M KCl (VWR BDH Prolabo, Dublin, Ireland) solution for 15 min and then resuspended in the polyamine buffer. Other preparations were placed into methanol acetic acid (three washes in total) following a procedure described earlier (9). The polyamine prepared chromosomes were recovered after placing the suspension onto ice for 10 min and then vortexing for 2 min to allow the chromosomes to burst out of the cells. Chromosome preparations can be stored for 1 month at 4°C.

Chromosome fixation, staining, and embedding

Each step in the protocol was adjusted because no existing protocols were available. In-solution prepared chromosomes were centrifuged before and after each step, including fixation, staining, dehydration, and all washing steps prior to embedding the sample. The chromosome samples were kept wet during the whole preparation procedure to avoid any drying effects that might disrupt the structure. All steps were performed in 1.5-mL microcentrifuge tubes that were centrifuged at 1750 × *g* for 10 min before and after exchange of solution. Chromosomes were fixed in 2.5% (v/v) glutaraldehyde (Sigma-Aldrich) in 0.1 M cacodylate buffer (pH 7.2) for 1 h. The pellet was washed twice in 0.1 M cacodylate buffer (pH 7.2). The sample was stained with the required concentration of platinum blue stain for 30 min at RT. Platinum blue was prepared according to the published protocol (22). The sample was washed with Milli-Q water twice for 5 min each time. For embedding of platinum blue–stained samples, the chromosomes were dehydrated first in a series of ethanol baths (30%, 50%, 75%, then 100%) (Fisher

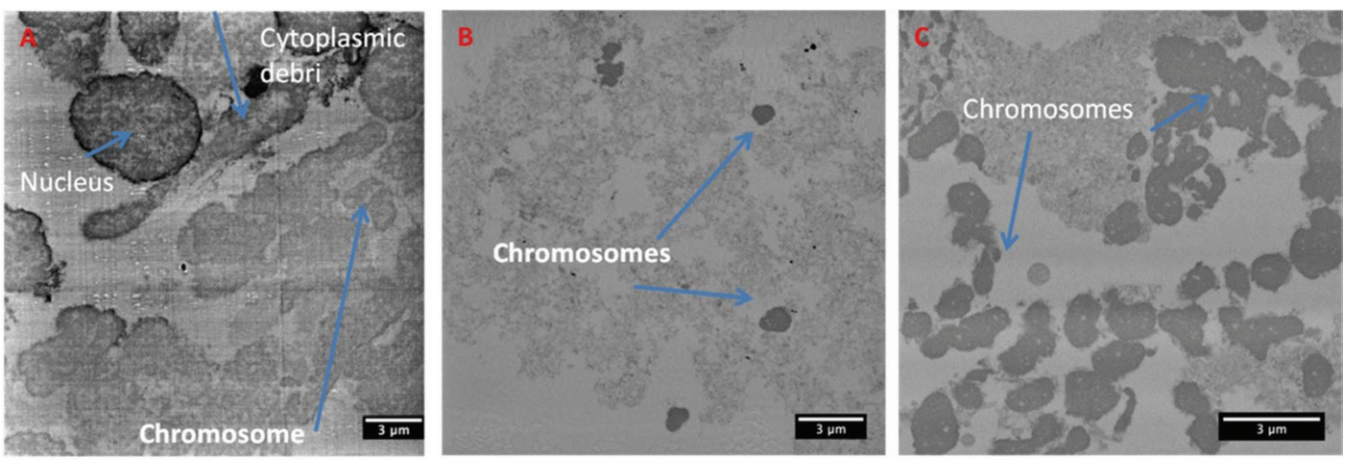

Figure 1. Cleanup of chromosome samples shown in sections after SBFSEM. (A) Shows sample with nuclei, cytoplasmic debris, and chromosome before filtration. (B) Sample after filtration using a 5 micron filter; a rich pool of chromosomes can be seen. (C) Chromosome samples could be further concentrated using a two-step resin embedding protocol. Samples were stained with 5 mM platinum blue.

Scientific, Loughborough, UK) for 15 min each. The sample was then infiltrated with Agar 100 resin (Hard) (Elektron Technology, Cambridge, UK) and was improved after applying a two-step protocol for embedding. The first step involved placing the sample in a small volume of 150 µL resin after dehydration and leaving it for 10 h at 60°C to cure. The second step involved layering the cured resin with 500 µL of additional fresh resin and leaving it for another 16 h to solidify.

SBFSEM sample block preparation using microtome

The sample block was prepared from the resin blocks by mechanical polishing and trimming with a conventional ultramicrotome (Leica Ultracut UCT; Leica, Buffalo Grove, IL). The mechanical polishing used 2000 grit paper to generate a pyramid shape with a block-face size of approximately 400 × 400 µm. The subsequent trimming was performed with a 45-degree glass knife at a clearance angle of 4 degrees to provide a selected area of dimensions of 200 × 200 µm.

Because of the blue color of the stained chromosomes, it was possible to position them very close to the pyramid tip visually. Once installed in the SBFSEM, the block-face was sliced by a 45-degree diamond knife for the required number of slices. A field emission gun (FEG) environmental SEM (QUANTA 250 FEGESEM; FEI, Hillsboro, OR) was used for imaging the block-face.

Alignment of image using software

The 3-D map was reconstructed by GATAN Digital Micrograph software (www.gatan.com/products/tem-analysis/gatan-microscopy-suite-software). The alignment of slices was performed by comparing the single gray value of two sequential images and calculating the difference of the gray values of both images. This automatically finds the X/Y alignment with the maximum correlation and minimum gray-level difference between the two images. 3-D map segmentation was performed using AVIZO software (www.fei.com/software/avizo3d/), and the length of the chromosome was taken as the maximum of the Feret Diameters of the object. The lengths of the arms were measured by the 3-D scale ruler in the software.

Results and discussion

In this study, the preparation conditions for human chromosomes were optimized for SBFSEM imaging. First, the concentration and purification of the starting material were optimized. As centrifuging led to sample loss, the concentration of the starting chromosomal material was determined after preparing a large-scale cell culture and pooling the samples together. A common problem during chromosome sample preparation is that the final preparation consists not only of chromosomes but also of intact (unburst) nuclei and cytoplasmic debris (Figure 1A). This problem has been well reported in the literature, and its solution has been problematic (21). A simple reverse filtration procedure was recently used to clean chromosome preparations (23). In this study, chromosome samples were cleaned after passing the suspension through a 41-micron-sized filter to remove large cytoplasmic debris, followed by a 10-micron filter to remove large nuclei, and, finally, a 5-micron filter for any smaller nuclei. A rich pool of chromosomes was obtained subsequently by filtering away any unwanted material using a 5-micron filter (Figure 1B).

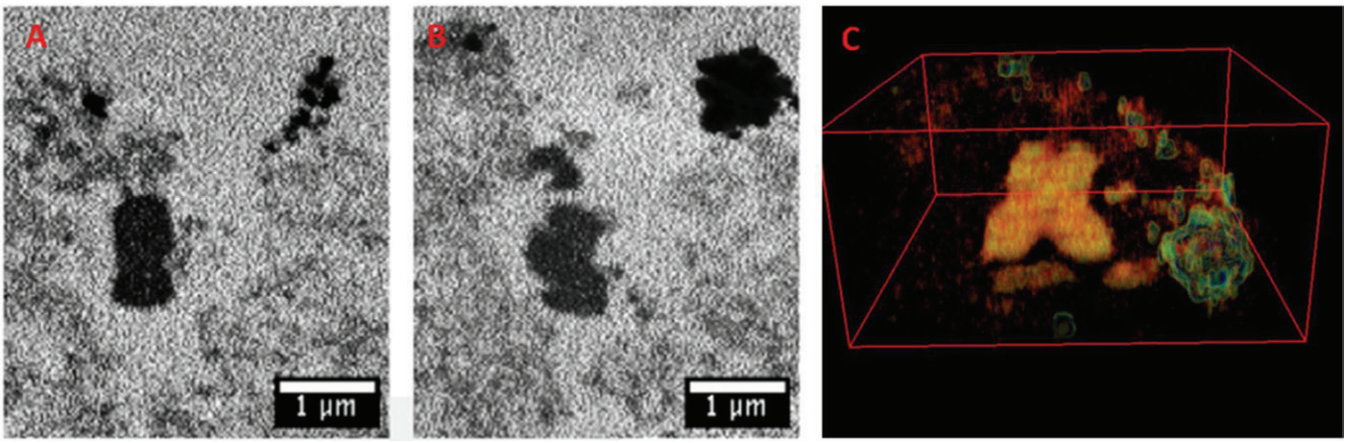

Figure 2. SBFSEM of human mitotic chromosome stained with 5 mM platinum blue. (A) Section No. 3 after SBFSEM of a whole chromosome. (B) Section No. 7, which is further down the series of the same chromosome shown in (A). (C) 3-D image showing X-shaped human mitotic chromosome after reconstruction of 13-nm × 100-nm sections. Box size: 4.253 × 3.741 × 1.6 microns.

Embedding of the sample was then optimized. Embedding into epoxy resin protects the samples against any structural collapse in the vacuum of the microscope chamber, thus preserving the 3-D structure. Problems were encountered whenever chromosomes were spaced too far apart within the resin, making imaging more time-consuming, as more time was spent on locating the sample during SBFSEM imaging. This was improved after applying a two-step protocol for embedding the sample that allows it to stay in the first part of the resin, where the knife cuts first in SBFSEM (Figure 1C).

Polyamine chromosome preparations can have intact nuclei, chromosomes, and/or debris in the final solution (23). While optimizing the chromosome preparation procedure and before embedding the samples into resin, parallel chromosome samples (as shown in Figure 1) were checked on glass slides by imaging using a fluorescence microscope. The DAPI fluorescent signals bound to DNA gave confidence that the samples were chromosomes (see Supplementary Figure S1).

Contrast from the sample can be increased using DNA-specific and electrically conductive stains before the embedding procedure. The heavy metal platinum contributes preferentially to the backscattered electron (BSE) signal. The contrast of platinum blue, a DNA-specific stain that has been used recently on human chromosomes (22), was explored after samples were embedded in resin. Using 2.5-mM platinum blue did not give sufficient contrast for the chromosomes embedded in the resin (Supplementary Figure S2A). For 2-D SEM imaging, 2 mM of platinum blue provided sufficient contrast after the chromosomes were prepared on glass slides. Different concentrations of platinum stain were then explored systematically to generate a sufficient and stable contrast. The 5-mM platinum blue–stained chromosomes provided sufficient contrast to the BSE images (Supplementary Figure S2B) and further increase of the platinum blue concentration to 8 mM showed little improvement of contrast in the generated images (Supplementary Figure S2C).

The contrast provided by platinum blue was calculated by taking the ratio between the average intensity of 1000 pixels from the chromosome and 1000 pixels from the background. The calculated contrast for platinum blue is 1.29 at 2.5 mM, 1.70 at 5 mM, and 1.57 at 8 mM. This shows that 5 mM is the optimum concentration of platinum blue for staining our chromosome samples. Increasing the concentration of platinum blue to 8 mM showed an increase in the intensity of the background. Advantages of using platinum blue include its solubility in water, shorter staining time, and easier handling as compared with other electron microscopy stains such as osmium tetroxide (OsO_4), which is highly toxic.

Images obtained after the slicing of the resin block also revealed uniformity of platinum staining among the different chromosomes. The contrast is constant throughout the entire slicing procedure, indicating uniform staining throughout the entire single chromosome. Sections of 100-nm thickness were taken from the block-face and imaged through the chromosome sample, as shown in slice 3 (Figure 2A) and slice 7 (Figure 2B).

The 3-D structure of a whole human chromosome was obtained by stacking a series of 13 slices together, with individual slice thicknesses of 100 nm (Figure 2C, Supplementary Video S1). The reconstruction shows the X-shaped mitotic chromosome where the chromatids are visible, including the q and p arms of the chromosome. The length of the chromosome is calculated to be 2.09 microns (Figure 2C and Supplementary Video S1). The diameter of each chromatid on the q arm was measured to be 0.53 microns and 0.41 microns for the p arm (Figure 2C and Supplementary Video S1). This also demonstrates that the filtration method preserves the X-shaped chromosome morphology.

Further trial experiments using SBFSEM were also performed on chromosomes prepared after methanol acetic acid fixation. Sections from the block-face showed internal structural details or cavities after being stained with 5-mM platinum blue (Figure 3, A–D). Methanol acetic acid prepared chromosomes tend to be less condensed. Studies have shown that treatment with methanol acetic acid removes part of the histones (24), with some studies showing that acid treatment removes histone H1 (21). In comparison, human polyamine chromosomes retain both their proteins and nucleic acid after preparation (20), as shown in Figure 1, A and B, and they are highly condensed, which is why they show up darker in the images. These chromosomes are shorter and more compact, showing no inner detailed structure in our current results, as has also been reported by Sone et

al. (21). This indicates that sample preparations need to be carefully considered when internal structure is investigated. As the sample is stained with platinum blue, which is a DNA-specific stain, it is probable that the cavities could be protein clustering regions that were not stained. No internal structural information from cross-sections of polyamine chromosomes was seen in Figure 1C, 2A, and 2B; however, to accommodate this, a more optimal condition for platinum blue staining of the chromosomes may be possible.

The SEM can potentially achieve resolutions down to 1–2 nm in the lateral x/y direction, but this might be limited by radiation damage or charging of the sample. However, the SBFSEM cutting mode limits the z-resolution to about 20 nm. We tested the resolution limit of chromosomes prepared in resin by performing finer pixel scans of 7 nm (data not shown) and found that the sample became changed after 5 continuous scans, suggesting 11-nm pixels is a safe limit for our samples.

It is clear that the SBFSEM is advantageous due to its automated registration of images of the slices. This work demonstrates that it is possible to access the full 3-D structure of single human chromosomes using SBFSEM. It demonstrates that platinum blue staining provides high-contrast images from samples embedded in resin. It may be possible to image chromosomes using intact mitotic cells if the platinum blue penetrates through the cell compartments and gives sufficient contrast. Here, we have demonstrated the advantage of concentrating chromosome samples with a two-step resin embedding procedure for chromosome imaging by SBFSEM.

Future work will involve obtaining 20– to 50–nm-thick sections to increase resolution in the slicing direction. The lateral resolution can be improved by addressing the charging and radiation damage issues. Also, the structure of chromosomes after different preparation procedures can be compared to study the steps of decondensation. This method would also be useful for investigating the 3-D structures of abnormal chromosomes, which would provide us with more understanding of 3-D chromatin organization and, furthermore, insights into disease-related genomes (e.g., cancer).

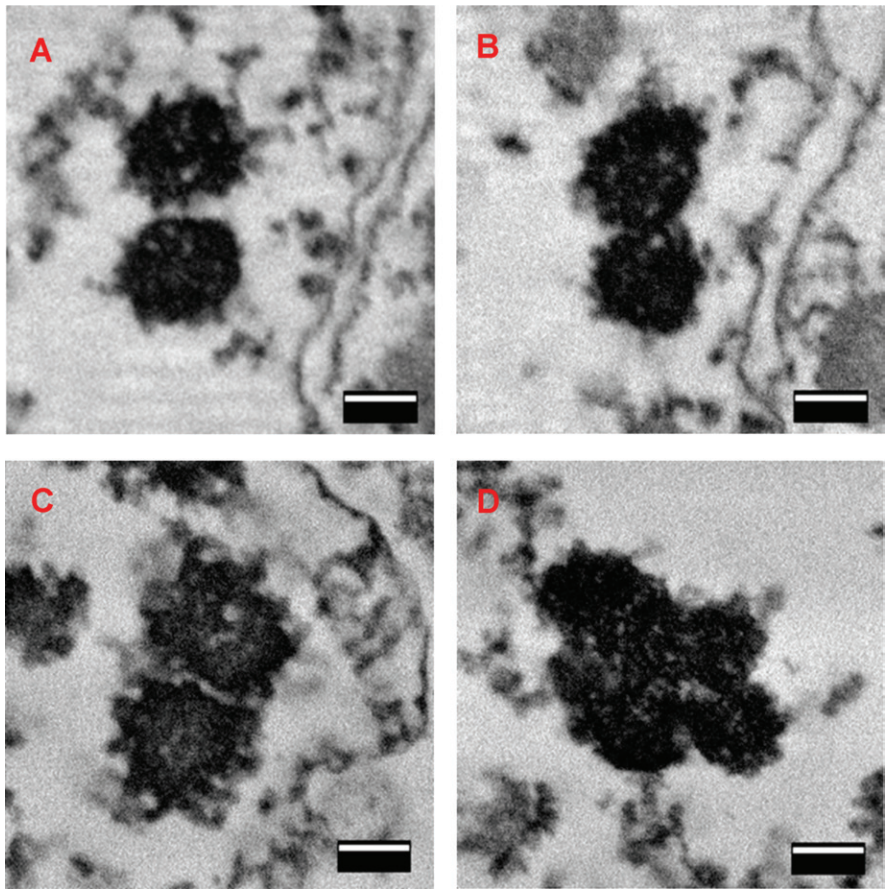

Figure 3. SBFSEM images of methanol acetic acid prepared human mitotic chromosomes. (A) A section showing two chromatids with cavities in a human chromosome. The distance between the two sections is 420 nm. (B) Another section showing the same human chromosome as in panel A. (C) A section from a second chromosome. (D) A section from a third chromosome. The scale bars are 500 nm. The chromosomes were stained with 5-mM platinum blue dye.

Author contributions

M.Y. wrote the manuscript and optimized all aspects of the sample preparation procedure. M.Y. and B.C. did the sample embedding in the epoxy resin. B.C .did the analysis of the chromosomes sizes and scale bars. B.C., T.H., and M.Y. prepared the block-face on the resin and performed SBFSEM measurements. A.K.E. synthesized the platinum stain and performed contrast calculations. All authors helped with manuscript preparation, amendments, and images for the manuscript. I.K.R. and G.B. supervised the study.

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Competing interests

The authors declare no competing interests.

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