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Atomic force microscopy imaging and 3-D reconstructions of serial thin sections of a single cell and its interior structures

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Abstract

The thin sectioning has been widely applied in electron microscopy (EM), and successfully used for an in situ observation of inner ultrastructure of cells. This powerful technique has recently been extended to the research field of atomic force microscopy (AFM). However, there have been no reports describing AFM imaging of serial thin sections and three-dimensional (3-D) reconstruction of cells and their inner structures. In the present study, we used AFM to scan serial thin sections approximately 60nm thick of a mouse embryonic stem (ES) cell, and to observe the in situ inner ultrastructure including cell membrane, cytoplasm, mitochondria, nucleus membrane, and linear chromatin. The high-magnification AFM imaging of single mitochondria clearly demonstrated the outer membrane, inner boundary membrane and cristae membrane of mitochondria in the cellular compartment. Importantly, AFM imaging on six serial thin sections of a single mouse ES cell showed that mitochondria underwent sequential changes in the number, morphology and distribution. These nanoscale images allowed us to perform 3-D surface reconstruction of interested interior structures in cells. Based on the serial in situ images, 3-D models of morphological characteristics, numbers and distributions of interior structures of the single ES cells were validated and reconstructed. Our results suggest that the combined AFM and serial-thin-section technique is useful for the nanoscale imaging and 3-D reconstruction of single cells and their inner structures. This technique may facilitate studies of proliferating and differentiating stages of stem cells or somatic cells at a nanoscale.

Keywords

Atomic force microscopy; Mouse embryonic stem cells; Transmission electron microscopy; Serial thin sections; 3-D reconstruction

1. Introduction

Ultrastructural analyses of cells appear to evolve into a fascinating new era, in which the three-dimensional (3-D) structures of biomolecules can be visualized and reconstructed [1]. Since its invention in the 1930s, high-resolution electron microscopy (EM) has played a dominant role in morphological studies of cells and their ultrastructure. Recent progress in EM tomography, high-pressure freezing and other techniques has made it possible to image or reconstruct 3-D structures of single cells and even their inner structures [2]. Recently, high-resolution scanning probe microscopy (SPM) including scanning tunneling microscopy (STM), atomic force microscopy (AFM), near-field scanning optical microscopy (NSOM) and other derivative microscopies have been emerging as powerful tools for studying cell structures and single molecules at a nanoscale [3–7]. In fact, AFM has been used to observe structures on thin sections of various cells or tissues, including pancreatic cells [8], kidney and liver cells [9], cartilaginous tissue [10] and others [11–15]. AFM is also used to compare different material-embedded sections [9] and sections created using various knives [16]. Furthermore, AFM has been employed to image thin-sectioned skeletal muscle [17], semi-thin-sectioned human trigeminal ganglion [18], human oculomotor nerve [19] and epithelial cells of rat kidney [20]. The capacity of AFM to display clear inner structures of cells and/or tissues raises the possibility to visualize nanostructures on serial thin sections of a single cell as well as to perform the 3-D reconstruction of single cells or their inner structures.

To date, AFM imaging of serial thin sections and 3-D reconstruction of a single cell have not been studied. We therefore employed AFM technology to image nanostructures on serial thin sections of single cells and to reconstruct their 3-D structures. Given the potential importance of stem cell research, we focused on serial thin sections of a mouse embryonic stem (ES) cell line. Our studies showed that AFM was able to image not only the clear inner structures of the single ES cell, but also the texture of a series of adjacent sections of the same cell. The AFM images on serial thin sections allowed us to validate and reconstruct 3-D models of morphological characteristics, numbers and distributions of interior structures of the single ES cells.

2. Materials and methods

2.1. Cell cultures

Inner cell mass (ICM) from BALB/c mice blastocysts was isolated and cultured, and an embryonic stem (ES) cell line was established. It was stably maintained in an undifferentiated state with the typical morphology of an ES cell line, expressing the embryonic stem cell marker alkaline phosphatase, and displaying a normal diploid karyotype. The mouse ES cell line and its culture method were described previously [21]. Briefly, BALB/c mouse ES cells were grown in DMEM (high glucose, more than 4.5 g/l) supplemented with 15% fetal bovine serum (FBS; Hyclone, USA), 0.1mM non-essential amino acids, antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin), 2mM L-glutamine, 0.1mM 2-mercaptoethanol and 1000 U/ml leukemia inhibitory factor (LIF). The cultures were examined daily and passaged by trypsinization every 2–3 days. Actively proliferating colonies of cells closely resembling ES cells were apparent from an early stage. Before experiment, the cells were trypsinized and centrifuged at $1000 \times g$ for 5 min. The extracted ES cells were then diluted in phosphate buffered saline (PBS) at a density of about 1×10^6 cells/ml. The vast majority of cells obtained were single cells.

2.2. Preparation of thin-section specimens and TEM

The medium was removed from the flasks and the mouse ES cells were washed twice with PBS. To each flask 5ml 1% trypsin solution was added and 2 min later, the cells were washed with PBS and then re-suspended in medium. Cell suspensions were centrifuged at $50 \times g$ for

10 min. Blocks of cells were separated. The cells were immediately fixed overnight in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) at room temperature. The cells were washed and equilibrated in PBS 3 times. They were then postfixed for 2 h at room temperature in 1% osmium tetroxide. PBS washing dehydration was then done with increasing concentrations of ethanol and finally with acetone. The specimens were infused overnight at 4 °C with an acetone–araldite resin solution and, the following morning, with an araldite resin mixture at 4 °C for 2 h while being periodically stirred. After infusion, the samples were embedded in a fresh mixture of araldite resin and kept at 60 °C for 72 h. Thin sections (60 nm) were poststained with lead citrate and examined in the transmission electron microscope. For AFM observation, thin sections were plated on cleaned cover slips. For TEM imaging, thin sections were placed on copper meshwork.

Tens serial thin sections approximately 60nm thick were cut, from which six sections were imaged using AFM. The positions of the six sections in a cell and the surfaces of the sections imaged by AFM were indicated by the numbers and letters in Fig. 1.

2.3. AFM

The cover slip was mounted on an AFM stage, and the integral video camera was used to locate the regions of interest. The thin sections were observed with an AutoProbe CP AFM (Thermo-microscopes, USA) in tapping mode, in which mode the vibrating cantilever tip was brought close to the sample so that at the bottom of its travel it just barely taps the sample. Therefore, tapping mode AFM was less likely to damage the sample than contact mode AFM because it eliminated lateral forces (friction or drag) between the tip and the sample. That is, during the detection, the surface structures of thin sections imaged by tapping mode AFM do not deform due to scanning of AFM tips. In our experiment, we used microfabricated silicon nitride cantilevers (Park Scientific Instruments) with sharp tips with a tip radius of curvature 10 nm and a force constant of approximately 2.8 N/m. Observation was carried out in air at room temperature. The scan speed of the tip was 0.5–1 Hz. The AFM images were planar leveled using the software (Thermo-microscopes Proscan Image Processing Software Version 2.1) provided with the instrument. Using the line analysis function of the software, the average width and the average height of the regions of interest were determined.

2.4. Three-dimensional reconstruction of serial thin sections

This study used the “3-D Slicer” [22,23] software program to reconstruct the inner structures of part of a single mouse embryonic stem cell. The software permitted the segmentation of structures in the 2-D slices by means of various techniques. The segmented 2-D slices were then put together to form a segmentation volume. This segmentation volume was used to create 3-D models using the Visualization ToolKit (VTK), a software program offering a variety of visualization algorithms to display geometric data. Data acquisition was performed by tracing the contours of the desired structure on the AFM images on a digitizing tablet. Before tracing the contours, reference points were marked at the geometric center of the profiles of at least three granules appearing in at least two successive sections. The program uses these points of reference to align the sequence of the material that has been sectioned in serial manner, and to address the shift effect of the AFM images caused by scanning in directions of minor differences. The profile volume in the section was thus the product of the thickness of the section multiplied by the area. The total volume was obtained by adding together the sectional volumes.

3. Results

To facilitate studies of structures on serial thin-section samples, we first investigated AFM images and sizes of a mouse ES cell deposited on a glass cover slip. The mouse ES cell on substrates was oval in shape, with the thickness of 2–5 μm and diameter of approximately 10

μm (Fig. 2). It was noted that ES cells in various developmental stages differed in diameter and thickness. Actually, because an ES cell was sunk partly due to its deposition on a substrate, the diameter of the cell in suspension may be less than 10 μm.

We then investigated the images of thin-section samples using TEM (Fig. 3a) and AFM (Fig. 3b–d). The AFM topographic images were compared side by side with the TEM images. In the low-magnification AFM image (Fig. 3b), the cross section of the cell was elliptical with a width of approximate 7 μm and a length of approximately 9 μm. This 7 × 9 μm size was close to the diameter of an un-sectioned mouse ES cell (around 10 μm). Detailed analyses indicated that this cross section lied nearly at the center of the cell, as shown in Fig. 1. In both the TEM and AFM images, certain inner structures of the single mouse ES cell were clearly evident, which included cell membrane, cytoplasm, mitochondria, nucleus membrane and linear chromatin (Fig. 3b and c). In this cross section of the cell, there were about eight mitochondria with diameters of 0.5–1.5 μm, and most of them exhibited round-to-elongated micrographs (Fig. 3b). Ultrastructure of a round mitochondrion could be visualized by a higher-magnification AFM topographic image of the cross section (Fig. 3d). The outer membrane, inner boundary membrane, cristal membrane (the inner boundary and cristal membranes were components of the inner mitochondrial membrane, and both constituted one continuous surface joined at tubular connections or crista junctions) and typical contact sites between the outer membrane and inner boundary membrane of the mitochondrion were clearly distinguishable.

Next, we undertook imaging studies of serial thin sections of the single ES cell. Shown in Fig. 4 were the AFM topographic images of the surfaces of six serial thin sections of the same ES cell as described above. The surfaces shown at Fig. 4a–f were consistent with those surfaces a–f of the serial thin sections shown in Fig. 1. At a first glance, these images at Fig. 4a–f appeared to be derived from a same thin section. However, a detailed analysis of the images identified the section-associated changes in the number and morphology of the mitochondria. One mitochondrion indicated by green arrows became smaller and smaller on the serial images revealed from the first through the last thin section. Three mitochondria (green arrowheads) became smaller and disappeared on the image from the fourth section. Other two mitochondria (blue arrows and arrowheads) emerged on the image from the fourth section and became increasingly larger over the subsequent images, but the mitochondrion indicated by the blue arrow underwent changes more quickly than the other indicated by the blue arrowhead.

To demonstrate the schematic changes in the number and morphology of the mitochondria on serial thin sections of the ES cell, we performed the 3-D surface reconstruction of mitochondria (Fig. 5). Two 3-D models viewed in two different directions (Fig. 5b) provided information on the orientation of mitochondria within the cell. In the 600-nm-thickness volume, there were 12 mitochondria (marked by numbers 1–12 in Fig. 5a), in which mitochondria 5, 7 and 9–11 were extracted and reconstructed (Fig. 6). The morphological changes of mitochondria in the 600-nm-thickness volume could be clearly demonstrated based on these 3-D models in Fig. 6. Mitochondrion 5 underwent morphological changes with a consistent diameter in the sections from top to bottom (Fig. 6). Mitochondria 10 and 11 were absent on the first section but viewable at the last section, whereas mitochondria 7 and 9 exhibited smaller changes (Fig. 6). The schematic representation (Fig. 6d) of 3-D structures in Fig. 6c roughly showed which parts of these mitochondria were sectioned.

4. Discussion

Since mouse and human ES cell lines were successfully established [24–27], numerous papers describing the proliferation and differentiation of ES cells have been published. However, little has been done for studying the inner structures of ES cells using EM imaging, let alone the AFM technology. In the present study, we have conducted in situ observation of the inner

structures of a single mouse ES cell using both TEM (Fig. 3a) and AFM (Fig. 3b–d). Our work provides recognizable images of cell membrane, cytoplasm, nucleus membrane, mitochondria and linear chromatin (Fig. 3b, c). In addition, the outer membrane, inner boundary membrane, cristal membrane and typical contact sites between the outer membrane and inner boundary membrane can be distinguished in a single mitochondrion (Fig. 3d). A good resolution of these structures is achieved through overcoming the potential artifacts caused by AFM tips [28]. Our study employed an AFM tip with a radius of curvature 10 nm, and this made it possible to resolute structures 5–10 nm in size. Our studies on thin sections suggest that AFM is able to identify ultrastructure in section samples that are usually analyzed using TEM. In fact, AFM allows us to identify not only large structures such as nuclei but also small ones such as mitochondria and linear chromatin. One similar paper has recently described the clear AFM observation of small structures of rat kidney cells, such as nuclear pores and nucleolus [20]. The results from these two independent studies imply the potential application of AFM to the in situ observation of inner structures in cells.

Our studies represent the first attempt to conduct AFM imaging of ultrastructure on serial thin sections of a single ES cell. Recent studies have demonstrated that EM tomography can allow one to visualize and process 3-D reconstruction of single cells or their interior structures. Our experiments indicate that ultrastructural observation of inner cell structures on serial thin sections can be similarly done by AFM technology. AFM images on six serial 60 nm sections of an ES cell clearly demonstrate that mitochondria undergo predictable changes in the number, morphology and distributions in the cellular compartment (Fig. 4). The AFM images on six thin sections in Fig. 4a–f reveal 10, 10, 9, 9, 9 and 8 mitochondria, respectively. However, a total of 12 mitochondria are noted, because some mitochondria are not viewable in certain thin sections. In addition to the changes in the number and morphology of mitochondria in serial thin sections, the relative locations of each of 12 mitochondria can be determined in the cellular compartment (Fig. 5a). These changes and distributions can be displayed more visually when the 3-D surface reconstruction is employed (Fig. 5b and Fig 6). Our data on serial thin sections suggest that AFM imaging on a series of adjacent sections is useful for developing 3-D reconstructions of ultrastructure in a single cell. These 3-D reconstructions will facilitate studies of the identification, proliferation and differentiation of ES cells or other stem cells.

The AFM technique offers several advantages for studying ultrastructure of a single cell in comparison to EM tomography. (i) AFM does not exert any damage resulting from high voltage, low temperature, vacuum, electron beam and others. Take electron beam-induced distortions for example, plastic sections often collapse under the action of an electron beam. In the section's plane shrinkage is modest (5–10%), but along the direction of the beam it is 40–50% [2]. Although ice sections do not show the shrinkage, the preparation of such specimens are more difficult than plastic sections. The embedding ice tends to crack during sectioning, and sample compression is usually significant. However, AFM of thin sections does not have those problems, since AFM obtains images on sample surface via weak interaction (the force is generally in the order of nN and even pN) between the tip of AFM probe and surface of thin sections on substrate. (ii) AFM is applicable to cells of various sizes, whereas EM cryotomography is currently limited to small cells [2]. AFM allows one to observe structures of various sizes, ranging from large trigeminal ganglion [18] or mouse ES cells (this study) to small nuclei/inner structures [20]. (iii) Serial sections of optional thickness can be visualized using AFM technique. Without a need for the beam's electrons, AFM is able to scan serial sections of optional thickness depending on the objective of research. In contrast, EM requires electron-based multiple scattering events that can seriously degrade image quality. Virtually, AFM allows us to readily observe the morphological changes of a mitochondrion by scanning serial sections as thin as 60 nm. Nevertheless, TEM imaging of thin resin sections usually does not yield high quality images. It is worth mentioning that the AFM imaging of thinner sections may be possible once a potentially innovated technology is able to cut such

super thinner sections (<20nm) of a single cell. AFM probes the surface of a sample through a sharp tip, which is located at the free end of a cantilever. Atomic forces between the tip and the sample surface cause the cantilever to bend or deflect. As the tip is scanned over the sample, a detector measures the cantilever deflections and generates a map of surface topography. Therefore, thickness of thin sections on a substrate generally does not affect the AFM resolution. This unique feature of AFM may allow AFM to more readily reconstruct 3-D inner structures of a cell at a nanoscale than EM does.

We certainly realize that there are some limitations for the AFM-based serial-thin-section imaging approach. Unlike EM, AFM is unable to use multiple tilted views. Thus, if one hopes to reconstruct an entire intermediate cell with an approximate thickness of 10 μm , more than 100 serial sections 100 nm thick are required for scanning and imaging using AFM. In addition, it is difficult to find the different cross sections of the same cell hiding among lots of cells at different slices. Furthermore, it is still quite challenging to obtain better resolution of serial-thin-section images using the AFM/resin-sectioning technique. Currently, AFM does not yield good resolution of thin sections as EM does, although the AFM resolution of molecules has exceeded EM. While multiple factors may contribute to the relatively low AFM resolution of thin sections, the convolution effect of AFM tips is one of the important reasons. The convolution effect often makes it difficult to determine the exact dimensions of imaged structures, and has been regarded as an outstanding problem since the invention of AFM. Sharpening of AFM tips has been shown to partly circumvent the convolution problem. Further studies are certainly needed to improve AFM resolution of thin resin sections of cells.

The combined AFM and serial thin section technique may facilitate studies of differentiating or differentiated cells. Stem cells or somatic cells at various stages are different from each other in the number or morphologies of certain interior structures such as nuclei/chromatins and mitochondria. Currently, conventional optical microscopy (OM) and scanning electron microscopy (SEM) are widely applied to identify non-differentiated ES cells from those ES-cell-derived stem cells or somatic cells. However, when the size and shape of the ES cells and the ES-cell-derived cells are similar, it will be difficult for OM or SEM to distinguish those cells in different stages. In situ observation of interior ultrastructure and 3-D reconstructions may be useful for characterizing differentiated and non-differentiated cells at a morphologic level. Therefore, AFM imaging of serial thin sections of a single cell or molecule [29] can certainly have a potential role in identification of proliferating and differentiating stages of stem cells or somatic cells at a nanoscale.

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Fig 1.

Schematic representation of the relative positions of the serial thin sections of a single cell imaged by AFM. Ten serial thin sections each about 60 nm thick with (marked by numbers 1–10) were generated from approximately 600 nm central portions of a single ES cell (marked by a gray circle). The ES cell had a diameter of approximately 10 μ m. The surfaces (red) of sections 1,2,4, and 8–10 (yellow) were imaged using AFM. Therefore, the distance between the two surfaces of two adjacent thin sections was approximately 60 nm, and the distances between surface a and surfaces b–f were 60, 180, 420, 480 and 540 nm, respectively.

Fig 2.
An AFM topographic image and a cross-sectional height profile of a single mouse ES cell on a glass cover slip. The full-width at half-maximum amplitude (FWHM) of the cross section is approximately 10 μm. Scanning area: 18 μm × 18 μm.

Fig 3.

TEM (a) and AFM (b–d) images of single thin sections of mouse ES cells. In these images, the cell membrane (M), cytoplasm (Cy), nucleus (N), chromatin (C) and several mitochondria (Mt) were clearly evident. The image shown at (c) was a higher magnification of that at (b). Shown at (d) was the AFM topographic image of a single mitochondrion, showing the typical contact sites (CS) between the outer membrane (OM) and inner boundary membrane (IBM). Note that the inner boundary membrane and cristal membrane (CM) were clearly distinguishable. In (b), the bright extracellular regions (indicated by arrowheads) were the embedded araldite resin. The lines drawn in parallel fashion (arrows) were not artifacts of scanning. These lines originated from the ultramicrotome and represented the direction of cutting. Bars: 2 μm (a) Scanning areas: (b) 12 μm × 12 μm, (c) 5 μm × 5 μm, (d) 1.3 μm × 1.3 μm.

Fig 4.

AFM images of serial thin sections of a mouse ES cell showed the number and morphological changes of mitochondria. The green arrows (or arrowheads) showed the mitochondria that became smaller at different sections, whereas the blue arrows (or arrowheads) showed those that were increasingly larger. In (a–f), the number of mitochondria was 10, 10, 9, 9, 9 and 8, respectively. However, the total number of mitochondria was 12, because some mitochondria could be seen but others were not viewable in the six serial thin sections.

Fig 5.

Quantitation and distribution analyses of mitochondria within cells. (a) A schematic representation of the quantity and distribution of mitochondria within the 600-nm-thick volume of the mouse ES cell. Twelve mitochondria emerged and distributed over the volume (indicated by numbers 1–12, respectively), but mitochondria 7, 9 and 10 were absent from the slice depicted. (b) The superior (upper) and anterior (lower) views of the 3-D model of reconstruction showed a distribution of 12 mitochondria within the cell. The arrow indicated the embedded araldite resin and the arrowhead indicated the cell membrane.

Fig 6.

Shape changes of individual mitochondria and the spatial distribution of mitochondria in the vertical direction of cutting. Shown were 3-D models of five mitochondria in the P-view (a), A-view (b), and AR-view (c) orientation, respectively. (d) A schematic representation of spatial distribution and the sectioned parts of the five mitochondria. The numbers in (c) corresponded to the numbers in Fig. 5a and showed the reconstructed mitochondria.