



Biological: Full-length

Use of the unroofing technique for atomic force microscopic imaging of the intra-cellular cytoskeleton under aqueous conditions

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Abstract Atomic force microscopy (AFM) combined with unroofing techniques enabled clear imaging of the intracellular cytoskeleton and the cytoplasmic surface of the cell membrane under aqueous condition. Many actin filaments were found to form a complex meshwork on the cytoplasmic surface of the membrane, as observed in freeze-etching electron microscopy. Characteristic periodic striations of about 5 nm formed by the assembly of G-actin were detected along actin filaments at higher magnification. Actin filaments aggregated and dispersed at several points, thereby dividing the cytoplasmic surface of the membrane into several large domains. Microtubules were also easily detected and were often tethered to the membrane surface by fine filaments. Furthermore, clathrin coats on the membrane were clearly visualized for the first time in water by AFM. Although the resolution of these images is lower than electron micrographs of freeze-etched samples processed similarly, the measurement capabilities of the AFM in a more biologically relevant conditions demonstrate that it is an important tool for imaging intracellular structures and cell surfaces in the native, aqueous state.

Keywords atomic force, cytoskeleton, actin, freeze-etching, unroofing, electron microscopy

Received 25 May 2012, accepted 28 June 2012; online 7 August 2012

Introduction

Atomic force microscopy (AFM) is a unique method for observing the surface structure of cells, as well as protein molecules, in liquid and in air [1]. The resolution of the measurement of biological samples in liquid is not as high as measurements performed in air, but AFM is the only microscopy technique that is capable of detecting topological features on the surface of live cells in aqueous media. In contrast, the scanning electron microscope is generally used to image the surface structure of cells at a high

resolution, but biological samples require a complex preparation process that consists of pre- and post-fixation followed by dehydration, drying and metal shadowing, which are pre-requisites to observation. Such procedures may distort the native structures. Although freeze-etching, another method for analyzing the topological features on the cell surface as well as intracellular structures, employs rapid freezing instead of chemical fixation in order to maintain fine structures close to their native state, metal shadowing is necessary to enhance the contrast. Of

these two methods, only AFM requires minimal, non-intrusive sample preparation, which enables it to be used in liquid conditions and makes it a very important tool for investigating the structure and function of living cells. Indeed, extensive topological data of the cell surface have been accumulated with the AFM technique [2–13]. However, to date, AFM has not been utilized for the *in situ* measurement of intracellular structures such as organelle surfaces, actin filaments, etc., except for a few cases using detergent-extracted or isolated samples [14–22]. Although there are several reports on the imaging of cytoplasmic stress fibers (bundles of actin filaments), the images were obtained indirectly, that is, most filamentous images were recorded as linear undulations of the cell surface caused by actin filaments located just beneath the cell membrane [2–12], and resolution was therefore inevitably restricted. The limited number of studies is partly because there are only a few methods available exclusively for the preparation of biological samples for AFM. Permeabilization of membranes with detergent is a useful method for revealing the cytoplasmic cytoskeleton, but significant interactions between the membrane and cytoskeleton are not preserved because the membrane is completely dissolved by the detergent. In contrast, for isolated samples, the shapes of myosin molecules moving on actin filaments in liquid has been captured in milliseconds [23,24] by AFM using an innovative image-retrievable speed development method. However, the application of AFM to *in situ* or *in vivo* analysis of intracellular structure is limited. In this study, we employed the unroofing technique, which is a preparation method used for observing the membrane cytoskeleton in freeze-etching electron microscopy, in order to observe the intracellular cytoskeleton *in vivo* by AFM. Consequently, we were able to describe the membrane cytoskeleton and the cytoplasmic surface of the cell membrane under aqueous conditions.

Materials and Methods

Normal rat kidney (NRK) cells cultured on uncoated coverslips (2.5 × 2.5 mm) were used for subsequent experiments.

Unroofing methods

The unroofing procedure used is summarized in Fig. 1. To observe the intracellular cytoskeleton, we employed an unroofing (deroofing) method involving ultra-sonication [25,26]. Cells on cover slips were washed once with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-based Ringer's solution consisting of 155 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM glucose in 5 mM HEPES buffer (pH 7.4), and then with Ca⁺⁺-free Ringer's solution. Subsequently, the cover slips were soaked for about 10 s in poly-lysine solution (0.5 mg ml⁻¹ poly-lysine dissolved in Ca⁺⁺-free Ringer's solution), and then washed three times for a few seconds each in hypotonic Ringer's solution prepared by mixing one part of Ringer's solution with two parts of distilled water (DW). This induced cell swelling, which enabled the cells to burst easily following ultrasonic stimulation. Immediately after immersing in hypotonic solution, the cells were exposed to a small bubble jet by weak ultrasonic vibration in isotonic KHMgE buffer (30 mM HEPES, pH 7.4, 70 mM KCl, 3 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 0.1 mM AEBSEF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride)) [25]. Cells unroofed by the bubble jet were washed briefly in fresh KHMgE buffer and immediately fixed for 10 min with 0.5% glutaraldehyde and 1% paraformaldehyde in KHMgE buffer. Fixed samples were washed twice with KHMgE buffer and used for AFM measurements.

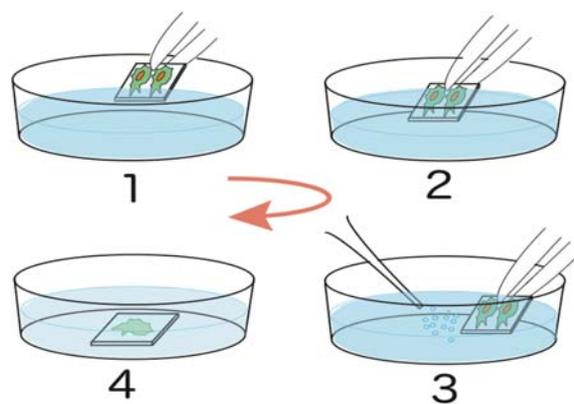


Fig. 1. Schematic diagram of the unroofing process. Incubate for 10 s with 0.5 mg ml⁻¹ poly-L-Lysine in Mammalian Ringer's solution (Ca). Wash three times for a few seconds each in hypotonic HEPES-based Mammalian Ringer's solution prepared by 3-fold dilution with distilled water. Unroof by ultrasonic stimulation (to remove the apical cell membrane together with the cytoplasm) in KHMgE buffer. Fix with 0.5% glutaraldehyde and 1% paraformaldehyde in KHMgE buffer for 10 min.

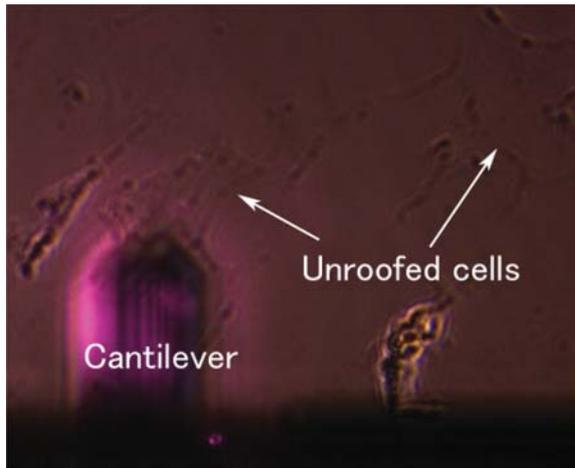


Fig. 2. Unroofed cells mounted on the AFM combined with a phase contrast inverted microscope. Images were recorded while observing the target cells.

AFM measurement

Samples were placed in a shallow Petri dish, 6 cm in diameter, containing DW, and then positioned in the XE-Bio AFM apparatus (Park Systems Inc. Suwon, Republic of Korea). As shown in Fig. 2, the cantilever (Biolever or Biolever mini, Olympus Inc., Tokyo, Japan) was depressed slowly, while targeting unroofed cells precisely under the phase-contrast microscope. Measurements were performed using the complete non-contact mode.

Freeze-etching electron microscopy

NRK cells cultured and unroofed as described above were rapidly frozen by plunging them onto a copper block cooled with liquid helium. Frozen samples were placed in the freeze-etching device (FR 9000, Hitachi, Mito, Ibaraki, Japan), and then excess ice covering the samples was removed with pre-chilled glass knives prior to etching (slight freeze-drying). Etched surfaces were shadowed with platinum and carbon at -93°C under vacuum at 5×10^{-6} Pa.

For immuno-labeling, unroofed cells were fixed with 0.1% glutaraldehyde in KHMgE buffer for 10 min and then washed once with phosphate buffered saline (PBS). Subsequently, the samples were immersed in anti-actin antibody diluted 200 times with 1% bovine serum albumin in PBS and incubated for 2 h. After being washed three times with PBS in Petri dishes, samples were further incubated

with a gold-conjugated secondary antibody for 1 h, and were fixed again with 1% glutaraldehyde for 10 min and washed twice with DW (5 min each) prior to rapid freezing.

Results

We successfully imaged intracellular structures for the first time in aqueous buffer using AFM combined with an excellent inverted phase contrast microscope and innovative sample preparation method. A cantilever was placed precisely and easily onto unroofed cells to scan their surfaces, while observing the cells by light microscopy (Fig. 2). Unroofing enabled observation of the cytoplasmic surface as well as intracellular structures. In general, however, unroofed cells are too thin to be detected by normal light microscopy, because only the ventral cell membrane and membrane cytoskeleton remain as a result of removing the nucleus and cytoplasm by ultrasonic washing. The combination of AFM with phase contrast microscopy made it possible to visualize such thin membrane samples and to record the cytoskeleton attached to the cytoplasmic surface of the cell membrane as described below.

Internal view of the cell membrane

The membrane cytoskeleton was observed as many fine filaments attached to the cytoplasmic surface of the ventral cell membrane. In the cortical area, particularly in filopodia, the filaments often extended in parallel, as shown in Fig. 3. In other regions such as the proximal cortical area and the central area, however, fine filaments extended in various directions on the membrane (Fig. 4). Observation of the filaments at higher magnification revealed a characteristic periodicity (about 5 nm intervals) probably due to the assembly of G-actin monomers (Fig. 3, see the inset). These filaments were identified as actin filaments by antibody labeling (Fig. 5, data not shown). As shown in Fig. 4, loose bundles of filaments were observed to aggregate and disperse at several points, which eventually divided the membrane surface into several domains. Because many filaments did not terminate or originate at these points but passed over them, the aggregation and dispersion points indicate

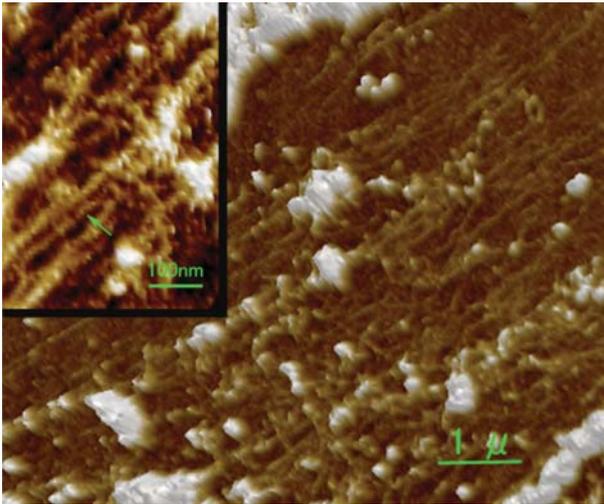


Fig. 3. Membrane cytoskeleton (mainly actin filaments) in the cortical area viewed from the cytoplasmic side. The filaments typically extend in parallel. The inset shows a high-power view of fine filaments containing characteristic periodic striations (5 nm interval) that are probably due to G-actin molecules assembling into actin filaments.

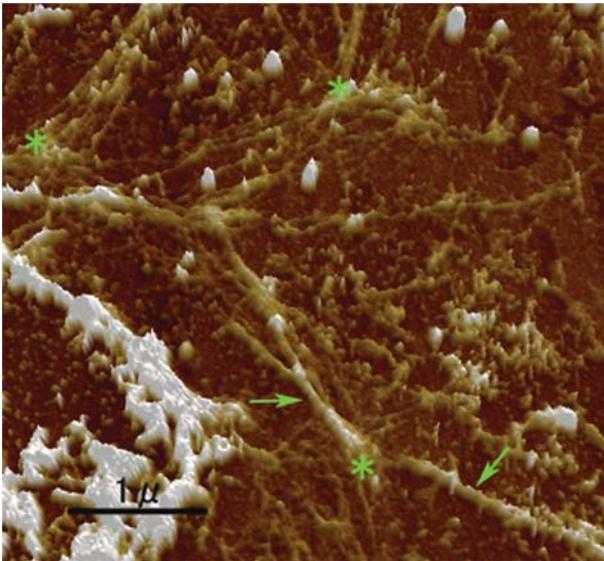


Fig. 4. Atomic force image of the cytoplasmic surface of the ventral cell membrane scanned from the cytoplasmic side. Actin filaments aggregate and disperse at several points (asterisks). Microtubules are also clearly observed (arrows) to be tethered onto the membrane surface as fine filaments with a twig-like morphology.

intersections formed by the filaments. A similar spatial structure formed by the arrangement of the filaments on the membrane was also confirmed by freeze-etching electron microscopy (Fig. 5), and therefore seemed to be a ubiquitous spatial structure of the membrane cytoskeleton. This sample

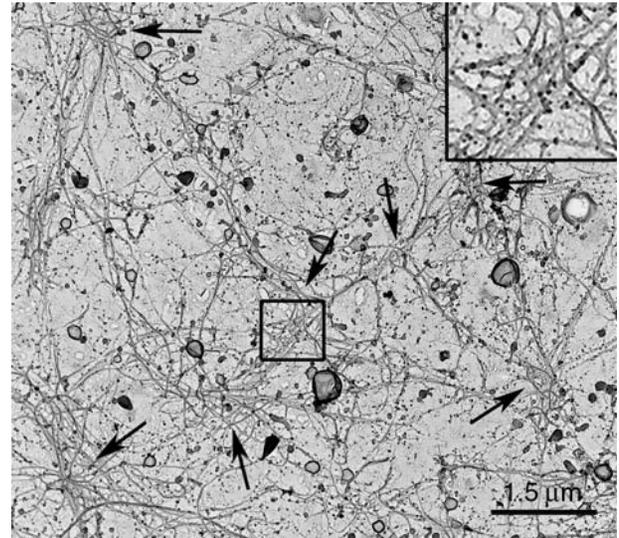


Fig. 5. Freeze-etching electron micrograph showing the cytoplasmic surface of the ventral cell membrane similar to that shown in Fig. 4. Arrows indicate the aggregation and dispersion points of actin filaments (or intersection points). This sample is labeled with a gold-conjugated anti-actin antibody. The inset shows an enlarged image of the boxed area, in which immuno-gold labeling is clearly visible as black dots.

was also labeled with a gold-conjugated anti-actin antibody. Gold labeling was clearly detected at higher magnification (Fig. 5, see the inset), enabling these fine filaments to be identified as actin filaments. Only a few actin filaments appeared to originate from the intersection points, and the appearance was not typical of the focal contacts from which stress fibers (compact bundles of many actin filaments) originate. It is unknown why and how many actin filaments met at certain points and passed over to form the intersections. Frequently, microtubules also passed over the intersection points (Fig. 4). In many cases, microtubules crawling along the membrane surface were tethered by short fine filaments (which were identified as actin filaments by immuno-freeze-etching in other experiments; data not shown). A careful probe scanning detected several single actin filaments that were firmly in contact with the membrane surface (Fig. 6). Those actin filaments extended randomly and did not form bundles. High-resolution scanning also revealed several clathrin coats, which are involved in endocytosis (Fig. 6). The fine structure of these clathrin coats was identical to the image revealed by the freeze-etching method, but not at the same resolution.

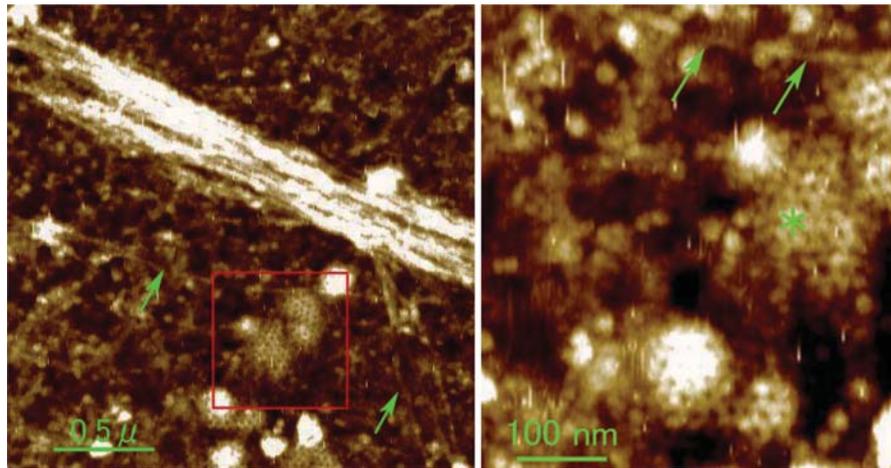


Fig. 6. The figure on the left shows a high-power atomic force image of the cytoplasmic surface of the plasma membrane. In addition to the stress fibers, solitary actin filaments (probably single filaments) extending along the cytoplasmic surface of the membrane (arrows) are detected by careful observation. Clathrin-coated pits are also clearly observed (boxed area). The figure on the right shows an enlarged view of the boxed area in the figure on the left. The surface structure of the clathrin coats is clearly visible. Arrows indicate solitary actin filaments.

Discussion

AFM has not yet been applied for the intracellular measurement of membrane cytoskeletons *in vivo*. The currently accepted appearance of the cytoskeleton was determined indirectly from measurements on the outside of the cell. Inevitably, only stress fibers (bundle of 20 or more actin filaments) could be detected at an unsatisfactory resolution by AFM [2–12]. In the present study, we successfully imaged various types of intracellular actin filaments directly at higher resolution by the combination of AFM with unroofing techniques, and thereby enabled a new and important application of AFM in biology. It should be noted that the cantilever was able to directly scan the cytoplasmic surface of the cells in aqueous buffer. Therefore, we were able to detect clathrin coats intimately attached to the membrane that cannot be observed when detergent was used for sample preparation. Unfortunately, however, our experiments also present important issues for the application of AFM to biology. At present, imaging at a sufficient resolution takes approximately 30–40 min. Such a recording speed is obviously too slow to depict the surface of living or moving cells. Because cells are easily maintained for a few hours in HEPES-based Ringer's solution containing serum, imaging of the living cytoskeleton is possible with further improvement of the scanning speed.

Removal of the cytoplasm by unroofing with sonication is conventionally used in freeze-etching

electron microscopy. The images obtained from samples prepared in this way must therefore be comparable between different observation methods (compare Figs. 4 and 5). Confirmation of results by two different observation methods is very important for determining the real structures. In general, AFM imaging has not yet reached the technical level in order to guarantee its result without artifacts, so this requires verification from other imaging methods. In this case, AFM imaging can directly be compared with freeze-etching electron microscopy using the unroofing preparation method thus allowing different imaging techniques to provide complementary data on biological samples.

Our experiments revealed that actin filaments forming a complicated meshwork and clathrin coats on the cytoplasmic surface of the membrane. However, these results were of lower resolution than those obtained by electron microscopy. The cytoplasmic surface of the cell membrane has only been observed by freeze-etching electron microscopy to date, so the technical advance presented here that enables the observation of intracellular structures under aqueous condition is a major step moving forward in AFM imaging applications.

The thickness of actin filaments other than stress fibers varied from 7 nm (single filaments) to 20 nm (bundles of a few filaments). Such measurements were also true for samples imaged with freeze-etching electron microscopy. Bundle formation of a

small number of actin filaments recognized in the freeze-etched replica was considered to be an artifact caused by ice crystal formation during the freezing and etching (slight freeze-drying) process or during platinum shadowing. However, as the current AFM imaging studies performed under aqueous conditions revealed the same bundling of a few actin filaments, it is very likely that small bundle formation may in fact occur in the living state. Indeed, actin filaments were attached each other too tightly to enable the number of actin filaments to be counted until they were allowed to naturally disperse on the membrane.

Funding

This study was supported by a Grant-in-Aid for Scientific Research (B) 22370056 from the Japan Society for the Promotion of Science (to J.U.). Instrument development was also sponsored by the Industrial Source Technology development Program (ISTDP 10033633 to S.J.C) in Ministry of Knowledge Economy in Korea.

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