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Investigating live and fixed epithelial and fibroblast cells by atomic force microscopy

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Abstract

Purpose. To investigate the use of atomic force microscopy (AFM) to image live and fixed cell in culture. Rabbit corneal fibroblasts, Chang conjunctival cells, and transformed human corneal epithelial cells were chosen so that AFM parameters could be set for future use in toxicologic and pharmacologic studies of ocular cells.

Methods. Contact mode AFM was performed under air and in balanced salt solution (BSS) using live and fixed cells. All cell lines were imaged in the height mode for optimal resolution of cellular features.

Results. Images of fixed cells showed no discernible differences in surface features when visualized in air or under physiologic solution. Structural differences were observed, however, between fixed and live cells in BSS. Although the AFM technique provides high quality images of live cells under BSS, sub-membrane features of live cells are more well-defined compared to fixed cells. It was also possible to image live cells in air if imaging was completed within 10 minutes of removal of the cells from culture medium. Images of cytoskeletal features under air were similar to those obtained under BSS.

Conclusions. The atomic force microscopy technique can be used to study cells and provide sub-cellular details at resolution equal to or in some situations better than the scanning electron microscopy technique. However, parameters for imaging have to be tailored for individual experimental goals.

Keywords: atomic force microscopy; human corneal epithelial cells; Chang conjunctival cells; microtubules; actin; fibroblasts

Introduction

Within the past decade, atomic force microscopy (AFM) has established itself as a routine surface science technique amenable to investigating crystalline surfaces from near atomic resolution to mesoscales. Several recent reviews highlight the potential of this technique for studying biologically related problems.^{1–4} This is in part due to AFM's minimal sample preparation and non-invasive nature, as well as the potential for imaging living cells *in vitro*. Nonetheless, imaging biological surfaces with AFM does have limitations. Although resolution of several nanometers is routinely possible in most biological systems, sub-nanometer resolution is still a challenge.⁵ In general this lack of sub-nanometer resolution is attributed to the interaction between the solid AFM tip and the compliant biological surface. Sub-nanometer resolution, however, has been observed with biomolecules that are closely packed.⁶ Hence, biological systems that are less compact and more dynamic when imaged under typical loading forces of the AFM cause the generation of images with sub-optimal resolution.

A number of reports have been published on the imaging of living cells by atomic force microscopy.^{5,7–14} A majority of the reports show contact mode imaging, which displays image data in either height-mode or error-mode (deflection mode). Although error-mode imaging provides image quality similar to a scanning electron micrograph with a higher image contrast than images obtained in the height mode, it lacks information on the height of features in images. Furthermore, images obtained in the deflection or error mode tend to accentuate artifacts, especially, sub-membrane features. Surface features of cells observed in previous studies depend on the variations in the loading force applied by the AFM probe. Imaging live cells with low forces (<100 pN) provides fuzzy images with no discernible sub-membrane

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structural features.¹⁵ At intermediate and higher loading forces (>500 pN), sub-membrane cytoskeletal features are clearly visible suggesting that the probe indents the cell at higher loading forces. The most common features that have been reportedly observed are actin filaments, intermediate filaments, and microtubules. These are distinguishable by their relative sizes, which the AFM is capable of resolving. The dynamic changes of actin filamentous regions in living cells have also been observed with AFM.⁹ In addition to live cells, fixed cells and hydrated cells have also been visualized with the AFM.^{5,12,16} Better resolution is generally observed in fixed cells compared to live cells.^{5,12}

In this paper we make a structural comparison of three cultured cell types by AFM: primary rabbit corneal fibroblasts, Chang conjunctival cells, and a transformed human corneal epithelial (HCE-T) cell line. The goal of our investigation was to optimize imaging conditions for each cell type in the height mode and avoid the generation of artifacts typically visualized in the deflection mode technique.

Chang cells, corneal epithelial cells and corneal fibroblasts were chosen for this study because they are morphologically typical examples of epithelial and fibroblast type cells, respectively. Chang conjunctival cells adopt a polygonal shape typical of epithelial cells while fibroblasts are elongated. Because the morphologies of these cells are known from studies performed with light and electron microscopy (EM), a comparison between EM and AFM images allows for an assessment of the accuracy of the representation generated by AFM.^{17–20} The immortalized human corneal cell line used in this experiment was also examined because of its current use in *in vitro* ocular toxicology testing,²¹ and its potential use in our ongoing studies of artificial tear solutions for treatment of dry-eye syndromes. A long-term goal of the present study was to set parameters, especially in the height mode for the imaging of ocular surface cells so that AFM can be used in toxicologic and pharmacologic studies of these cells.

Materials and methods

Cell cultures

Chang conjunctival cells (ATC CCL-20.2) were obtained from the American Type Culture collection (Bethesda, MD) and were grown to confluence from frozen stock in T-75 flasks in Medium 199 (GIBCO BRL, Grand Island, New York) with 10% Fetal Bovine Serum (FBS). Primary rabbit corneal fibroblasts prepared as previously described²² were grown to confluence from frozen stock in T-75 flasks in Delbecco's Modified Eagle's Medium (GIBCO BRL) with 10% FBS. The cells were passed to 6-well plates (Corning Inc., Corning, New York) containing three Thermanox cover slips (NUNC Inc., Naperville, Illinois) per well at a density of 2×10^5 cells per well. The media were changed after 24 hours and every 2 to 3 days until the cells were about 75% confluent on the cover slips.

For culture of transformed human cornea epithelial cells (HCE-T), T-75 flasks were coated with 5 mL of Fibronectin/Collagen Mix (Biological Research Faculty & Facility, Ijamsville, Maryland). The HCE-T cells were a gift from Dr. Sherry Ward of Gillette Medical Laboratories (Needham, MA) and have been previously described.²¹ Cells were plated from frozen stock at 1×10^4 cells/cm² and grown to approximately 75% confluence in these T-75 flasks in Keratinocyte Basal Medium (KBM) (Clonetics, Walkersville, Maryland) containing Bovine Pituitary Extract (30 µg/ml), insulin (5 µg/ml), human epithelial growth factor (0.1 ng/ml), and hydrocortisone (0.5 µg/ml). The cells were passed at a ratio of 1:3 into 6-well plates containing three Thermanox cover slips coated with fibronectin/collagen mix. The medium was changed at 24 hours after plating and every 2 to 3 days until the cells were about 75% confluent on the cover slips.

Imaging with AFM

An atomic force microscope (Digital Instruments Nanoscope IIIa) instrument equipped with a liquid cell was used in all experiments. Contact mode and tapping mode (intermittent contact mode) methods were used to record images. For contact mode imaging, silicon nitride cantilevers (100-micron and 200-micron thin legged V-shape) were used as probes for raster scanning with a 125-micron scanner. In tapping mode imaging, a silicon cantilever is typically oscillated at its resonant frequency near the surface to provide intermittent contact. The spring constants of cantilevers were measured as previously described and typical spring constants were between 0.04–0.05 N/m.²³ Prior to imaging, probes were cleaned under a UV-lamp to remove contaminants (Tip Cleaner, Bio Force Inc.). Samples were typically equilibrated in the fluid cell for 15–30 minutes to avoid problems with drift. Data collection was at 1 Hz scanning rate on all large scans. While loading forces were kept between 0.5–1.0 nN prior to imaging, force curves were not monitored during image collection, instead loading forces were controlled by lowering or increasing the deflection setpoint voltage. All images were collected in the height mode and subsequently processed to remove tilt and slope. Images shown in this paper from fixed cells and those obtained under solution are representative images obtained from a minimum of five images collected per site on a given cell line. For imaging live cells in air, only a single image collection was possible within 10 minutes of removal of cells from solution, thus requiring several cell plates for live cell imaging in air.

Cells on a cover slip mounted on a metal disk were imaged under several different conditions. Live cells were imaged at 30°C both in air and using the liquid cell filled with Hanks balanced salt solution (BSS) at pH 7.4 (Gibco, Grand Island, NY). Care and time is of utmost importance when imaging live cells in air. Hence, live cells on a coverslip were removed from medium and placed in the scanning probe microscope

for immediate imaging by contact mode. All images of live cells in air were obtained within 10 minutes of removal from solution. Cells were also fixed by immersion in a solution of 1 mL of Medium 199 and 1 mL of 2.5% glutaraldehyde fixative in 0.1 M Na cacodylate buffer for 15 minutes. The discs were then transferred to a solution containing 1.5 mL of fixative and 0.5 mL of medium for an additional 15 minutes. This was followed by immersing the cells in 2 mL of 100% fixative for 2 hours. The cells were stored in 0.1 M Na cacodylate buffer until imaging. Several cover slips were separately analyzed to compensate for disc roughness in determining height measurements.

Results

Fixed Chang cells imaged in air are polygonal in shape ($\sim 15 \mu\text{m}$ wide) and show well-defined boundaries. All cells display a small bright region protruding from the surface, which we attribute to the cell nucleus. At high magnification, individual cells show remarkable clarity and details of a dense microtubule type network (Fig. 1). Although these microtubules and the nuclei are not surface features of the cells, the loading force applied (0.5–1.0 nN) by the tip dents the surface of the cell enabling cytoskeletal features to be observed. The measured height of a typical filament type feature in Figure 1 is approximately 25 nm, corresponding well to the diameter of a microtubule. The full-width at half height, however, is 120 nm, suggesting that tip convolution has a substantial effect in distorting the width of the microtubules in the lateral direction.

Prior to imaging live cells under air, it was necessary to determine how long these cells survive when removed from culture medium. Cells on coverslips were removed from wells, exposed to air for varying time periods and then stained with trypan blue to determine the number of live cells. We found that for both Chang cells and fibroblasts, 5% of the cells die within 5 minutes while 50% of the cells die during 10 minutes of exposure to air. Thus, we determined that image collection within 5–10 minutes of cell removal from wells provided a reasonable window for imaging live cells. Live Chang cells imaged under air (Fig. 2) are similar in size ($\sim 15 \mu\text{m}$ wide) to fixed cells. Peripheral cell features that were not observed on the fixed cells are more visible in the images of live cells. It is evident from these images that the borders of the cells form junctions with one another. When sub-membrane features were imaged, both live and fixed cells show very similar cytoskeletal features. Heights measured from the dense network of submembrane structures (Fig. 2B) are consistent with those obtained from Figure 1B and are thought to be microtubules.

Live Chang cells imaged under BSS (Fig. 3A) show remarkable clarity and detail, and appear larger in size ($\sim 25 \mu\text{m}$ wide) to the live Chang cells imaged in air (Fig. 2A). The apparent height of a live Chang cell in BSS ($\sim 0.8\text{--}1 \mu\text{m}$) appears to be smaller than that measured in air ($\sim 1.2\text{--}1.4 \mu\text{m}$)

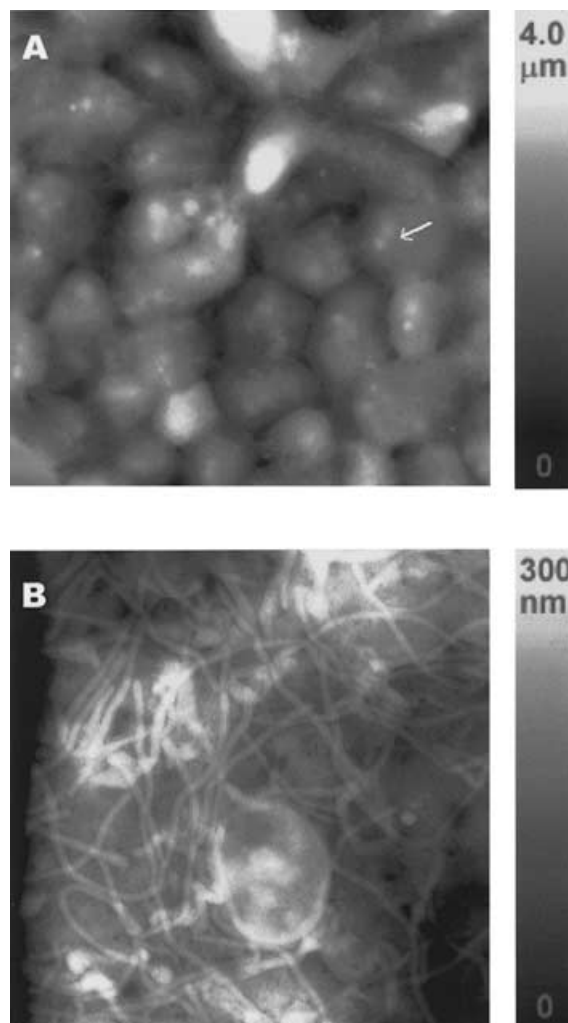


Figure 1. Fixed Chang cells imaged in air. (A) Chang cells in a $100 \times 100 \mu\text{m}$ window. (B) A high-magnification view of the area shown by the arrow in Figure 1A showing the nuclei and filamental features thought to be microtubules in an individual cell ($7.63 \times 7.63 \mu\text{m}$).

suggesting that the cells imaged in air may be curling up. Although polygonal cell shapes are observed when live cells are imaged in air and under BSS, the live Chang cells under a balanced salt solution display sharper boundaries between cell borders. This may well be a result of the lower loading forces applied under solution compared to air. Chemical fixation of Chang cells does appear to cause the cells to round up and shrink preventing the imaging of cell-cell junctions (Fig. 3B). However, fixed cells appear to be similar in shape both in air and under salt solution (Figs. 1A & 3B).

The elongated shape of cultured corneal fibroblast cells imaged by the AFM is evident in Figure 4. The short features observed on the fibroblasts (Fig. 4a) may be collagen fibers in the extracellular matrix. The fixed cells imaged under air show actin-like stress fibers (shown by the arrows in Figure 4A) beneath the cell surface that are similar to those seen in

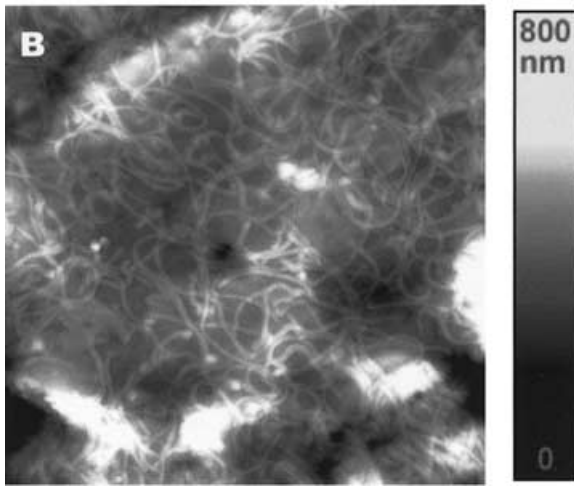
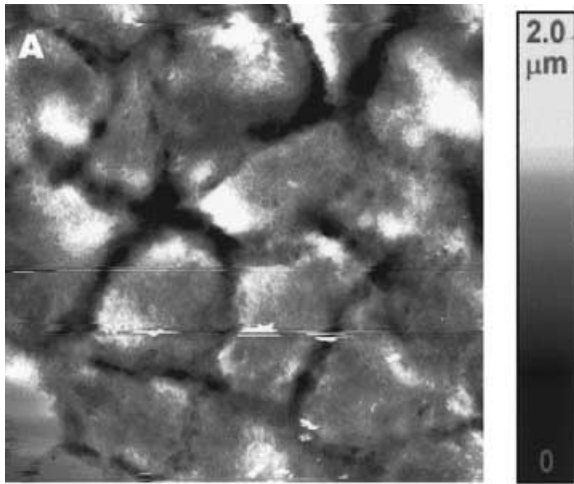


Figure 2. Live Chang cells imaged in air within minutes of removal from medium. (A) Several Chang cells in a $53 \times 53 \mu\text{m}$ window. (B) A dense network of microtubule like features from a single live Chang cell ($12.3 \times 12.3 \mu\text{m}$).

previous studies.^{10,24–25} Although no attempt was made to vary the loading force during imaging, the lower loading force applied when imaging the cells may result in the observation of features on the cell membrane while at the cell edges this same force may be higher, causing the appearance of cell features beneath the membrane. In AFM experiments, piezo drift will most likely cause changes in loading force during imaging causing the observation of features both on the membrane and beneath the membrane.

Live and fixed fibroblasts imaged under BSS show features similar to those seen in images obtained in air. In Figure 5, cell-cell connections are clearly visible, while the stress fibers that appear in Figure 5b are blurred in the height mode. Live fibroblasts imaged in either air or under BSS appear to be similar in size and smaller than fibroblasts that have been fixed.

The peripheral edges of human corneal epithelial (HCE-T) cells are shown in Figure 6, while whole cells are shown

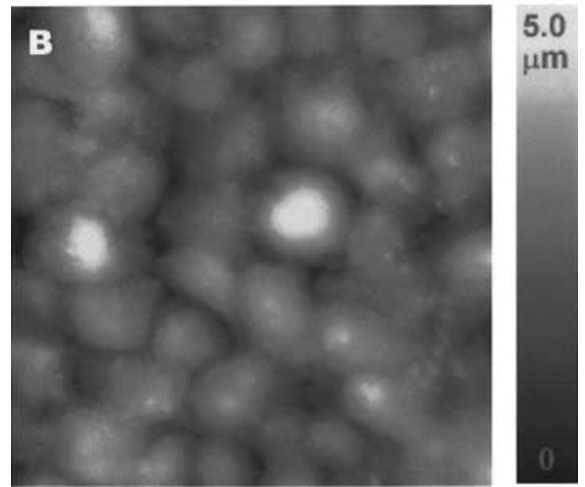
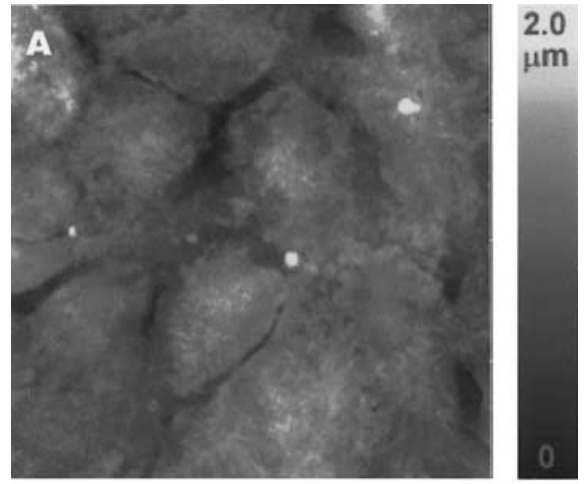


Figure 3. Comparison of live (A) and fixed (B) Chang cells imaged under balanced salt solution. Each image is a $100 \times 100 \mu\text{m}$ scan.

in Figure 7. These cell types are similar in shape to Chang cells, but are much larger ($\sim 50\text{--}60 \mu\text{m}$ wide). As shown in Figure 6, we were able to image the edges of migrating corneal epithelial cell membranes demonstrating filopodia and the lamellopodia. The filopodia are better resolved in the images of fixed cells and appear less resolved in the images of live cells under BSS.

Discussion

Imaging in air versus liquid

The present experiments demonstrate that live cells can be imaged in air within 10 minutes of removal from culture medium. However, only one or two images can be collected within that time (~ 5 min per image for a scan in 512×512 pixels at 1 Hz), after which a large percentage ($>50\%$) of the

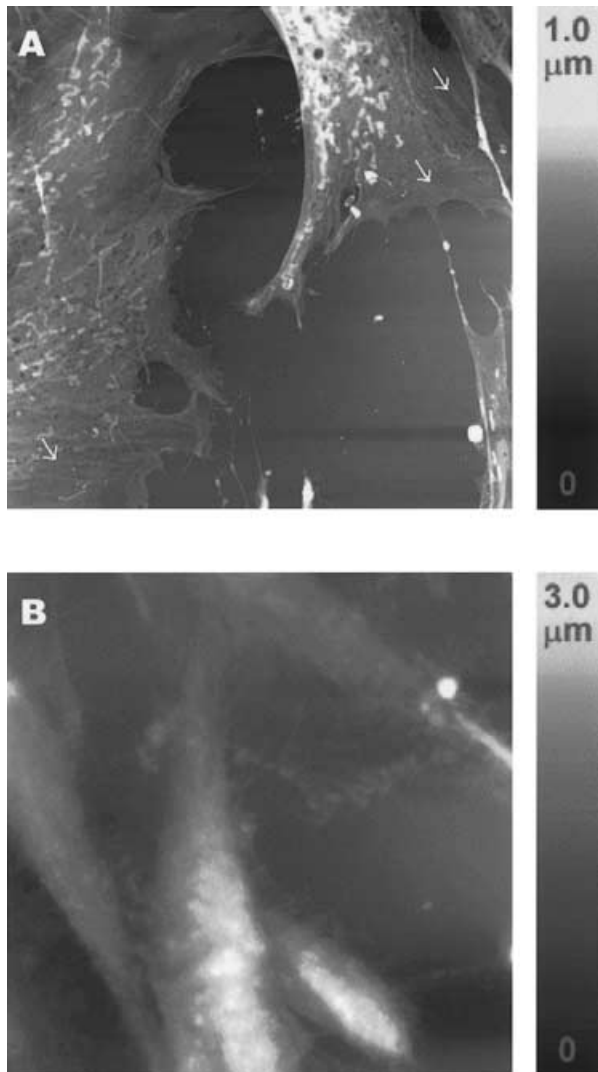


Figure 4. Corneal fibroblast cells imaged in air. (A) $100 \times 100 \mu\text{m}$ scan of fixed cells. (B) $100 \times 100 \mu\text{m}$ scan of live cells.

cells die, which is a drawback to this approach. Since it takes less time to setup an AFM experiment to be carried out in air, the advantage to imaging live cells in air within a short time of exposure to air, is to ascertain how well the cells are adhering to the coverslips. If cells are not strongly attached to the coverslips, it is virtually impossible to obtain a good image since cells may detach from the coverslip and attach to the probe. Images of cells obtained under air also provide the overall shape of the live cells, though cell dimensions will be smaller than cells imaged in BSS. When we imaged live Chang cells in air for a time period greater than 20 minutes, the cells tended to come off the surface and become round in shape compared to be polygonal shapes observed in Figures 2A and 3A. The round shape of a cell indicates cell death, and is largely due to osmotic changes and loss of cell junctional complexes. Such a change in cell shape can also be induced by variations in pH. Fixed Chang cells imaged in air and in BSS show no discernible differences: however,

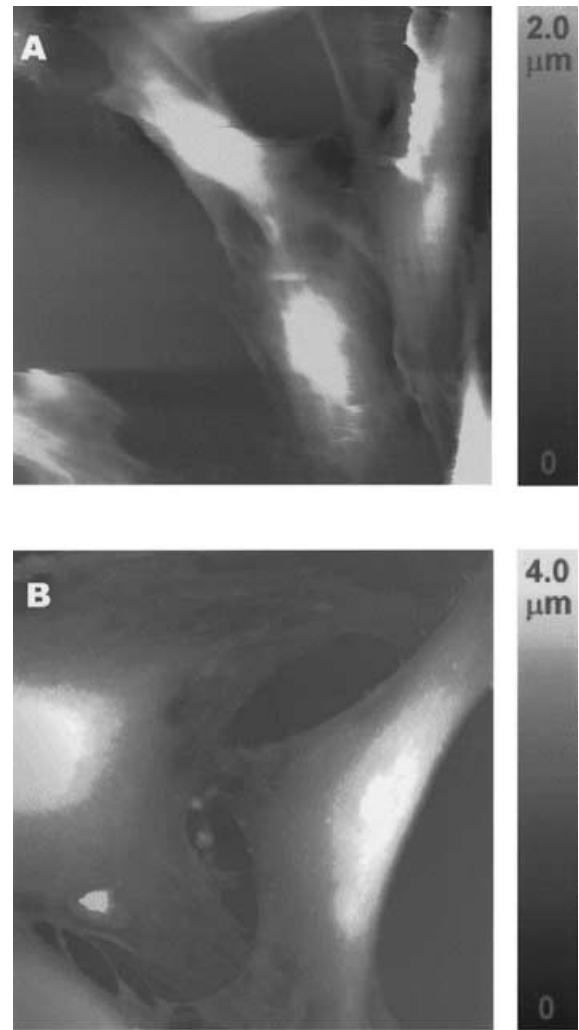


Figure 5. Live (A) and fixed (B) fibroblast cells imaged under balanced salt solution ($100 \times 100 \mu\text{m}$).

fixation does cause cells to shrink in size, which was observed in both air and under BSS. Another factor that is important in image interpretation in air and under liquids is the scanning force. The scanning force can be controlled in liquids and can be reduced by nearly two orders of magnitude compared to scanning forces in air. The effect of such scanning forces on image quality has been described elsewhere.¹⁵ When imaged in air, sub-cellular features of Chang cells are clearly discernible (Figs. 1A and 2A), while in BSS, with scanning forces $<500 \text{ pN}$, such features appear fuzzy.

A goal of the present study was to compare the quality AFM images of cells to images obtained with the electron microscope. The images of Chang cells in Figures 2A and 3A are qualitatively very similar to previously published scanning electron micrographs of ocular surface epithelial cells in culture.²⁰

The $100 \times 100 \mu\text{m}$ images of live fibroblast cells imaged in air or BSS (Fig. 5) show similar features with no dis-

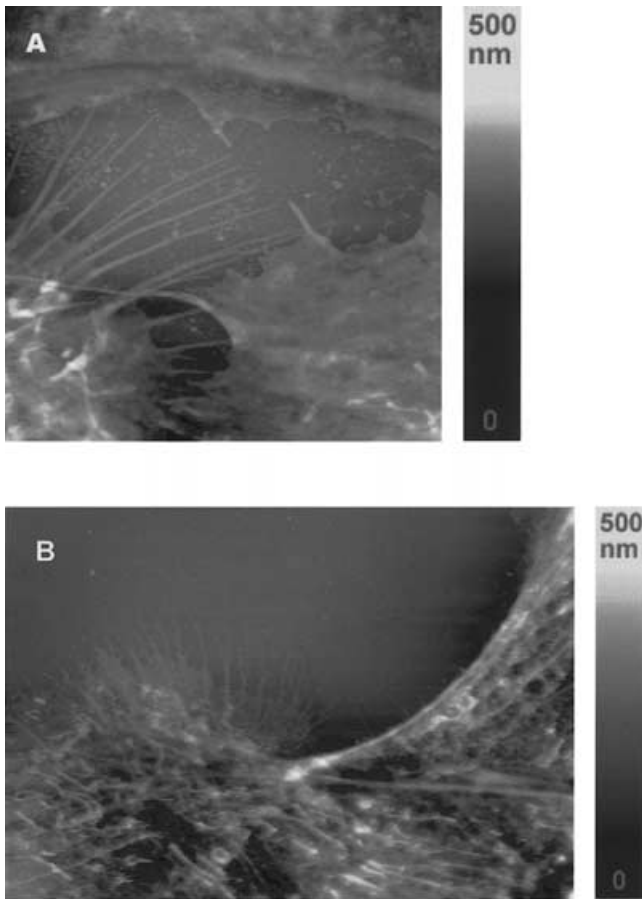


Figure 6. HCE-T cells fixed and imaged in air (A) and live and imaged under balanced salt solution (B). Scan sizes: (A) $16.5 \times 16.5 \mu\text{m}$; (B) $29.6 \times 42.5 \mu\text{m}$.

cernible differences. In fact, live fibroblasts could be imaged for several hours in BSS without much change in overall morphology. However, the sub-membrane features of live fibroblasts were difficult to image in the height mode, while under BSS stress fibers at the peripheral edges of the live cells were clearly visible. Scanning electron micrographs of fibroblast cells in culture are rare; however, the AFM image of a fixed corneal fibroblast in Figure 4A is very similar to published SEM images of fixed corneal fibroblasts in situ.¹⁹

The present data suggest that while AFM imaging of live cells under BSS is the most relevant for biological studies, imaging live cells in air within a short time of exposure to air is also possible and does have benefit in optimizing experimental parameters.

Imaging fixed versus live cells

As previous reports have shown, imaging fixed cells in air and under liquid is much easier than imaging live cells and provides greater resolution.^{5,12} It should be noted, however, that there are clearly surface structure differences between fixed and live cells. For example, images of fixed Chang cells

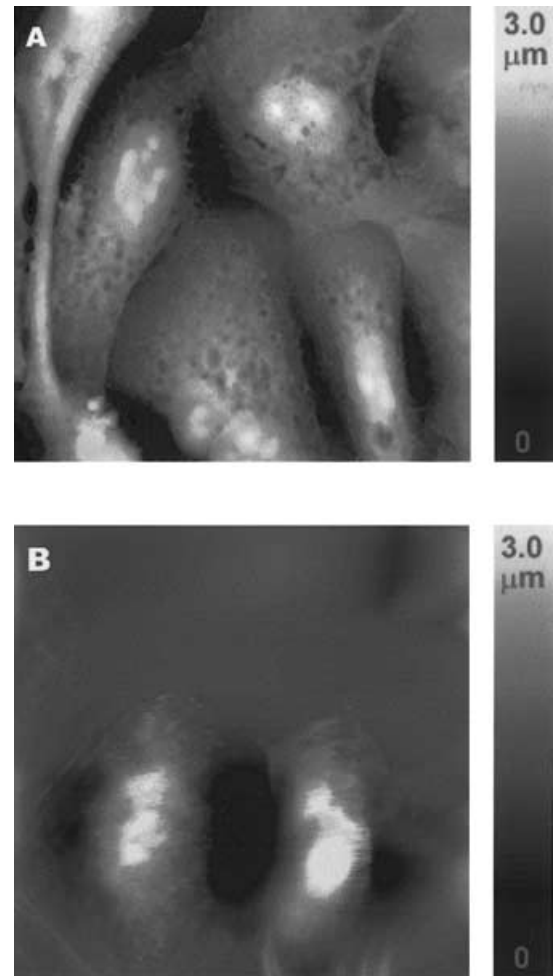


Figure 7. HCE-T cells. (A) HCE-T cells fixed and imaged in air ($100 \times 100 \mu\text{m}$). (B) Live HCE-T cells imaged under balanced salt solution ($100 \times 100 \mu\text{m}$).

show smaller polygonal shaped cells than images of live cells. In all three-cell types, fixed cell images show more clarity in the sub-cellular detail than live cells. The most striking example shown here is the fixed migrating HCE-T cell imaged in air (Fig. 6). The long filopodia extending from migrating cells contain actin filament bundles imaged at a resolution similar to that which has been previously observed in images of migrating corneal epithelium obtained by scanning electron microscopy.^{17,18} Non-migrating fixed HCE-T cells also present detailed images in air (Fig. 7A) that are similar to the SEM images published by Geerling *et al.*²⁰ These same features, however, look fuzzy when live cells are imaged under BSS, suggesting that they may easily be moved by the scanning tip under the loading force. Although the setpoint voltage was decreased and monitored during the imaging of the live cells, thus reducing the loading force, the fuzzy appearance of the filopodia is more likely due to the fact that they are not strongly attached to the surface of the coverslip. It is also conceivable that during imaging of live cells that probe may pick up debris from the cells

causing images to appear fuzzy. We have also noticed that over a period of 2 hours and under varying loading forces, fixed cells appear to be unchanged in BSS.

Because there are distinct advantages and disadvantages to each of the parameters we examined, imaging conditions should be selected based on the needs of a particular study. We found that all cell lines were easier to image under fixed conditions than under live conditions. If the goal of an experiment is to study the morphology of a cell line, chemical fixation is an appropriate avenue for data collection. If a physiological model is desired, cells should be imaged under live conditions.

In summary, we have compared three cell types for AFM imaging in height mode: the Chang conjunctival cells, human corneal epithelial cells, and rabbit corneal fibroblasts. We have shown that cells can be easily imaged in air and under BSS. Fixed cells were shown to be better for morphological studies, providing greater stability during AFM imaging and higher resolution. These images are often equal to the quality of scanning electron microscopic images^{17–20} with the advantage of simpler sample preparation. In comparison to the images of cells obtained in the deflection mode (error-mode),^{25–26} the height mode provides for better interpretation of cell surface features.

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