

Optimization of Wet Fixation Methods for AFM Imaging of Human Fibroblast Cells

Gi Ja Lee^{1,2}, Yoon Kyung Uhm³, Yun-Hye Eo^{1,2}, Ji Hye Park^{1,2},
Ji Eun Lim^{1,2}, Tae Ho Jo⁴, Bum Shik Kim², Seok Keun Choi²,
Berm Seok Oh^{1,2,5}, Mu Hyoung Lee⁴ and Hun Kuk Park^{1,2,5*}

¹Department of Biomedical Engineering, School of Medicine, ²Healthcare Industry Research Institute, ³Department of Pharmacology, School of Medicine,

⁴Department of Dermatology, School of Medicine, ⁵Program of Medical Engineering, Kyung Hee University, Seoul 130-702, Korea

ABSTRACT

We investigated the effect by the chemical fixative on human fibroblast cells (HFCs) in order to make nano-scale images using by the atomic force microscopy (AFM). The cell fixation needed to be optimized as prerequisite step for the preparation before analysis. AFM imaging after optimal wet fixation can provide practical, simple and fast technique for scanning living cells. In this study, AFM images - topography and amplitude - and the optic images of HFCs which were fixed with phosphate buffered saline (PBS), 2 : 1 ethanol : acetic acid, 4% glutaraldehyde and 37% formaldehyde were compared respectively. The final effect by washing with PBS or distilled water (D.W.) was examined after 4% glutaraldehyde fixation. To determine the optimal fixation method for HFCs, we performed quantitative and qualitative analysis by the height profile, the presence of artifacts and the morphology of well-conserved fibroblastic topography image by AFM. From AFM image which showed fibroblastic cellular morphology and differential height value of cytoplasm (670 ± 47 nm, $n=10$) and nucleus (847 ± 32 nm, $n=10$) in HFCs, we proposed that wet fixation by 4% glutaraldehyde, followed by final washing with PBS, could be the most suitable preparation for AFM imaging of HFCs, which enable us to approach easily on living cells with the least shrinkage.

Key words: AFM, wet fixation method, fixative, human fibroblast cell

INTRODUCTION

Several microscopic techniques have been developed for depicting ultramicroscopic features of a

cell - fluorescence microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryo-electron microscopy (CEM), confocal laser scanning microscopy (CLSM) and etc.. To overcome the limitation of conventional microscopes to low resolution (≈ 200 nm), SEM has been widely used for higher resolution images of biological samples. However, SEM requires only fixed, dried and coated specimens for examination in the vacuum. Similarly, fluorescence microscope and TEM

*To whom correspondence should be addressed.

TEL: 82-2-961-0290, FAX: 82-2-961-5515

e-mail: sigmoidus@khu.ac.kr

Received June 2, 2008

Accepted for publication June 19, 2008

needed complicated preparation techniques. In particular, the atomic force microscopy (AFM) has become an important tool for non-invasive imaging of various cells and biological materials since its invention in 1986 by Binnig et al (1986). The major advantages of AFM over conventional optical and electron microscopes for imaging cells included the fact that no special coating and vacuum were required and imaging could be done in all environments - air, vacuum or aqueous condition. The AFM imaging of living cells under physiological condition is more complicated and challenging even for experts because of certain problems. The one is the detachment of cell from the substrate and the other is the contaminated scanning probe by cell during imaging procedure (Zimmermann et al., 1999). Although many researchers have tried in aqueous environment, the AFM imaging of cells is now performed mostly in the air. The relative facility of cell imaging in the air was attributed to increasing hardness of cell by the dehydration (Weyn, 1998). To prevent the cell from being dried, many researchers utilized cell fixation methods. However, as a result of the fixation, the artifact and depression were reported during the sample preparation or the measurement process (Thimm et al., 2000; Moloney, 2004). Moloney et al (2004) reported that fixation artifact in the air drying mainly consisted of the presence of coating, debris, depression and crystal as follows. Coating artifact occurred essentially at fixation process by forming a continuous layer of fixative over the sample surface during drying. The debris was considered as the results from cellular disintegration, post-fixation or the residual liquid from the final wash with phosphate buffered saline (PBS). The depression artifacts looked like volcano or valley, similar to cell dehydration. The crystal artifact was considered as salt crystal derived from the washing buffer (PBS).

Human fibroblasts were found in the dermis which consisted of connective tissue. The role of the fibroblast is to give strength to the skin by producing collagen and elastic fiber. Fibroblast is long and spindle-like cell with central nucleus (Braet et al., 2001). It was known that fibroblast could be imaged quite easily when cell was cultivated on the substrate treated with adhesion promoting factors (Murphy et al., 2006).

To obtain good image of cells in the air using the AFM, the optimization of cell preparation is the most important pre-requisite step and is determined by three main factors - the matrix for cell culture, the optimal concentration of cells to seed on the matrix and the fixation method of the cells (Moloney et al., 2004). In particular, the cell fixation is crucial factor for clear image without artifact because the fixatives can directly influence the topography of cell. Optimization technique for wet fixation makes easy access to living cell, even in the air condition. The application of AFM for tracking cell dynamics in vitro required stable and reproducible conditions for imaging. For this purpose, the AFM using periodically fixed cells can be performed less complexly in air than in liquid.

In this study, we measured the conditions of cell fixation for the AFM image with high clarity of human fibroblast cells (HFCs), close to living state. In previous research about cell fixation techniques by duplicate fixatives, the efficacy of the fixatives was simply evaluated by the presence of artifact and saturated signal (Moloney et al., 2004). However, we compared the AFM images - topography and amplitude - and the optic images of HFCs that were fixed with PBS, 2 : 1 ethanol : acetic acid, 4% glutaraldehyde and 37% formaldehyde respectively. The effect by final washing with PBS or distilled water (D.W.) was examined individually with each technique. From the height profile analysis about nucleus and cytoplasm in AFM image, we quantitatively evaluated the efficacy of wet fixatives. And we analyzed AFM images qualitatively by the absence of artifacts and the well-conserved fibroblastic cellular morphology. It was expected that this study could support users as a fundamental guide for successful imaging of cells and biomolecules with the AFM.

MATERIALS AND METHODS

Cell culture

Culture of normal human fibroblast was established from newborn baby foreskins obtained from Kyung Hee Medical Center after routine circumcision. Primary human fibroblast was maintained as monolayer culture in low-glucose DMEM (Dulbecco's modified Eagles's medium). All the cultures were grown in a humidified environment with 5% CO₂ atmos-

phere at 37°C.

Sample preparation

HFCs were seeded on sterilized mica substrates (15×15 mm) which were placed inside of the sterile culture dish (35×10 mm). After the cells were attached on the surface of mica, they were washed twice with filtered phosphate buffer saline (PBS, pH 6.8) and fixed as follows, except for the case of HFCs without any treatment:

1. HFCs without any treatment (No-fixed and washed HFCs): No fixatives were used, not even rinsed with PBS.

2. HFCs rinsed with PBS: Cells were washed three times in PBS to remove the culture medium without special fixative.

3. HFCs fixed with 2 : 1 ethanol : acetic acid, finally washed with PBS: Cells on mica substrate were pre-washed twice with PBS. Then cells were fixed for 20 min in 2 : 1 ethanol : acetic acid at room temperature, and finally washed three times in PBS.

4. HFC fixed with formaldehyde, finally washed with PBS: After pre-washing in PBS, cells were fixed for 20 min in 37% formaldehyde in PBS at room temperature. And then, they were washed three times in PBS.

5. HFC fixed with 4% glutaraldehyde, finally washed with PBS: After pre-washing in PBS, cells were fixed for 20 min in 4% glutaraldehyde in PBS at room temperature. And then, they were washed three times in PBS.

6. HFC fixed with 4% glutaraldehyde in PBS, finally washed with D.W.: After pre-washing in PBS, cells were fixed for 20 min in 4% glutaraldehyde in PBS at room temperature. And then, they were washed three times in D.W.

AFM measurements

The AFM images were obtained using the Nano-station IITM (Surface Imaging Systems, Herzogenrath, Germany) in non-contact mode. The Nano-station II was equipped with 92.5 μm XY / 6 μm Z scanner and Zeiss optical microscope (Epiplan 50x). The AFM was placed on top of the active vibration isolation table (TS-150, S.I.S., Herzogenrath, Germany), which was located inside of the passive vibration isolation table (Pucotech., Seoul, Korea) to eliminate external noise such as vibration. Data

acquisition and processing were performed by the SPIPTM (Scanning Probe Image Processor, version 4.1, Image Metrology, Denmark). The reflex coated silicon cantilevers for non-contact mode (PR-NC, S.I.S., Germany) had the following characteristics: (manufacturer's specifications: F=146~236 kHz, C=21~98 N/m, L=225 μm and R=0.01~0.02 Ohm·cm).

The images of cells, as seen by optical microscope, were captured with a camera, then images were digitalized and stored in the computer. Through the optical microscope, we selected the suitable area for AFM imaging.

To preserve the real morphology of the living cells, AFM measurement started within 10 minutes after the fixed cells were exposed to the air. Cells were scanned at the resolution of 512×512 pixel with scan speed of 0.5 line/s.

RESULTS

The most optimum fixative must preserve the cell similar to the in vivo condition as possible. Therefore, we preferred the wet fixation method which maintained cells in real morphological state, not dried state. Braet et al (2001) showed that AFM imaging of living cells revealed specific structure such as cytoskeleton, that was not observed by SEM. The prominent structure in the fibroblast was long and straight stress fiber. The fiber traversed the cytoplasm and were oriented in general parallel to the long axis of the cell.

To select the optimal fixation method for HFCs, we utilized the height profiles in AFM topography image, the absence of artifacts and the well-conserved fibroblastic cellular morphology. In the each fixation method, we displayed the optic image, non-contact mode AFM topography, amplitude images and height profile in topography images. The height difference between substrate and cytoplasm or nucleus was summarized in Table 1, respectively. Statistical analysis was performed using independent t-test (two-tailed).

HFCs without any treatment (Non-fixed and no-washed PBS)

Fig. 1A and B showed the non-contact mode AFM topography, amplitude images and optic image of HFCs which were exposed to the air without any

Table 1. The height difference value between the substrate and cytoplasm, nucleus or nucleoli (n=10, *: p<0.001)

	Height difference value (nm) (mean±S.D.)		
	Between the cytoplasm and substrate	Between the nucleus and substrate	Between the nucleoli and substrate
HFC without any treatment	284±29	303±22	Not determined
HFC rinsed with PBS	330±53	506±108*	605±15
HFC fixed with ethanol : acetic acid (final wash - PBS)	515±51*	623±50*	872±25
HFC fixed with formaldehyde (final wash - PBS)	551±47*	684±29*	896±91
HFC fixed with glutaraldehyde (final wash - PBS)	670±47*	847±32*	1,140±108
HFC fixed with glutaraldehyde (final wash - D.W.)	460±54*	499±44*	713±71

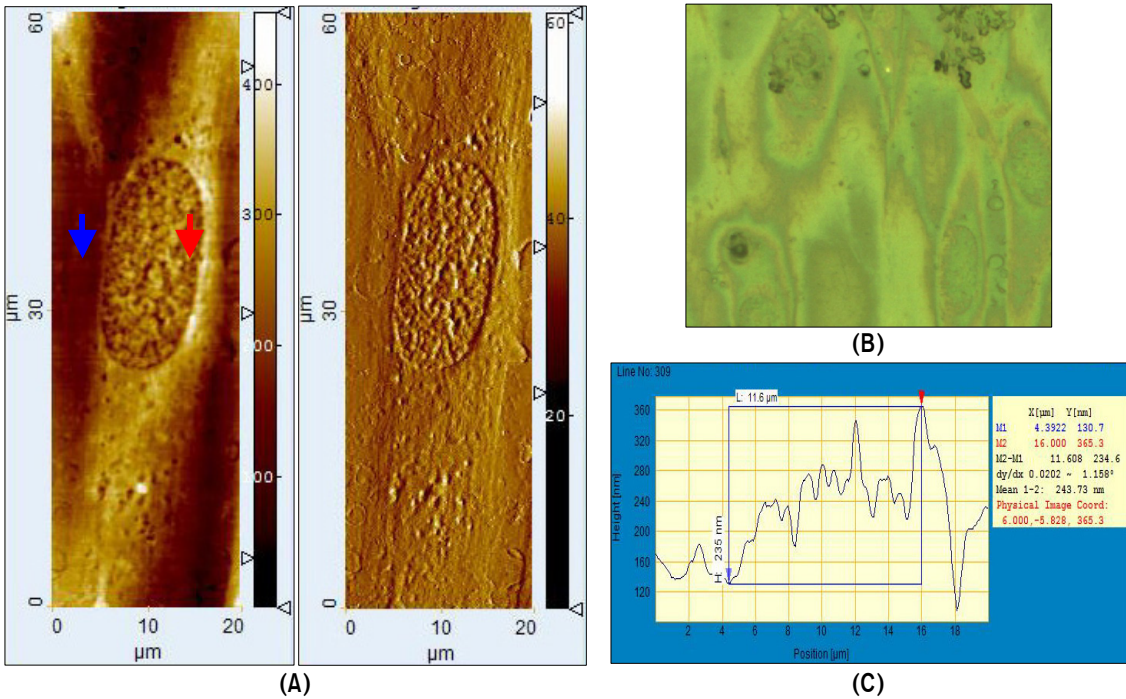


Fig. 1. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleus in topography images of HFCs which were exposed to the air without any treatments.

treatment. As can be seen in Fig. 1A, HFC was collapsed and hardly showed the fibroblastic morphology. There was no distinct height difference between cytoplasm and nucleus, as shown in Fig. 1C and Table 1. Even though, HFCs were slightly wet, most of the nuclear volume and cytoplasm of HFCs without any treatments started collapse as soon as they were exposed to the air.

HFCs rinsed with PBS

As shown in Fig. 2A, nucleoli, as well as nucleus, were found in the images of HFC rinsed with PBS, which were different from that of non-fixed HFC. And Fig. 2C showed that the height between

cytoplasm and nucleus of HFC was quite different. However, from the AFM topography and amplitude images, it was found that the cell surfaces were covered with debris and crystal artifact, which were known as common fixation artifacts. Therefore, it was thought that HFC was fixed by the only final wash with PBS buffer to some degree in short period, although it did not show good image.

HFCs fixed with ethanol : acetic acid, subsequently washed with PBS

Fig. 3A and B showed the non-contact mode AFM topography, amplitude image and optic image of HFCs which were fixed with 2 : 1 ethanol : acetic

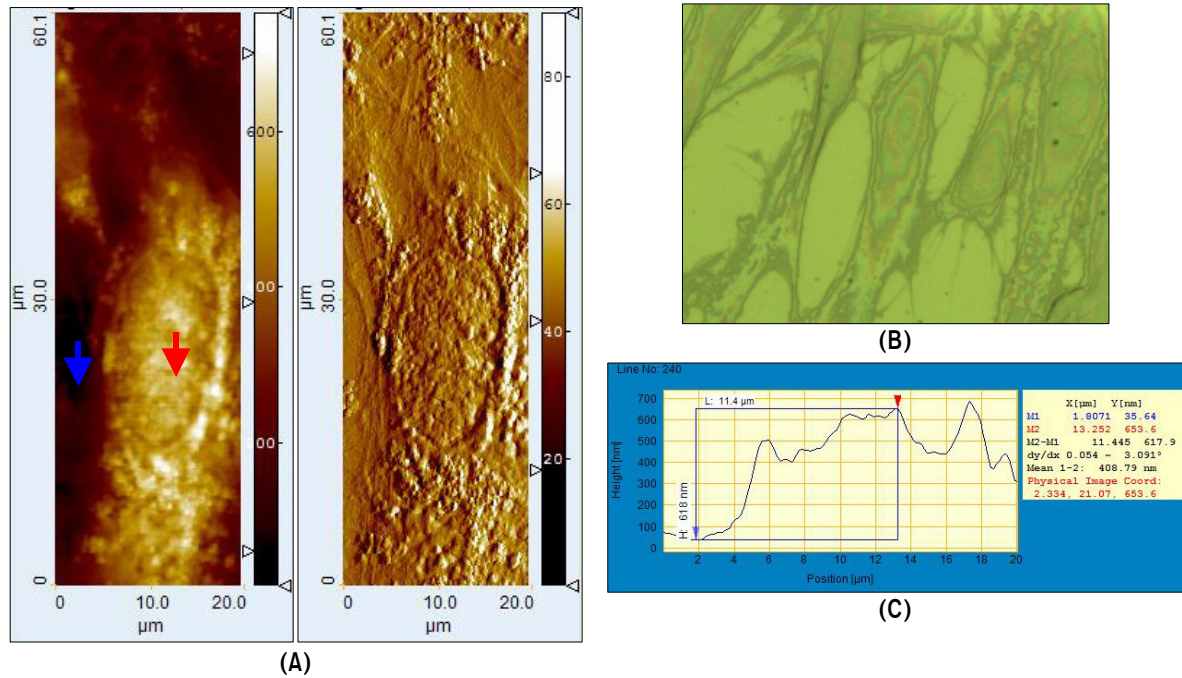


Fig. 2. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleoli in topography images of HFCs which were rinsed with PBS.

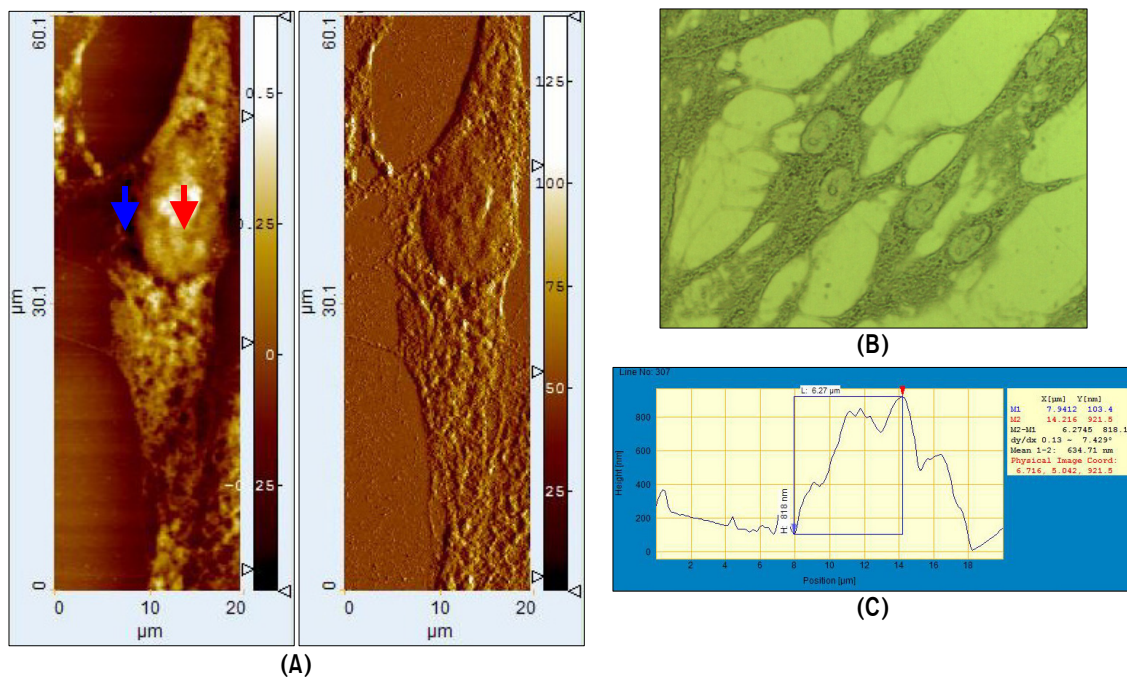


Fig. 3. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleoli in topography images of HFCs which were fixed with 2 : 1 ethanol : acetic acid, subsequently washed with PBS.

acid, subsequently washed with PBS. As shown in Fig. 3B, the optic image of HFC by this method was similar to the shaded normal contrast image of fixed fibroblast in the air (Bushell et al., 1999).

According to the previous study, in case of cells that had been fixed without dried, the nucleus and the densest part of the cytoskeleton around the nucleus remained as an intact structure about 500 nm

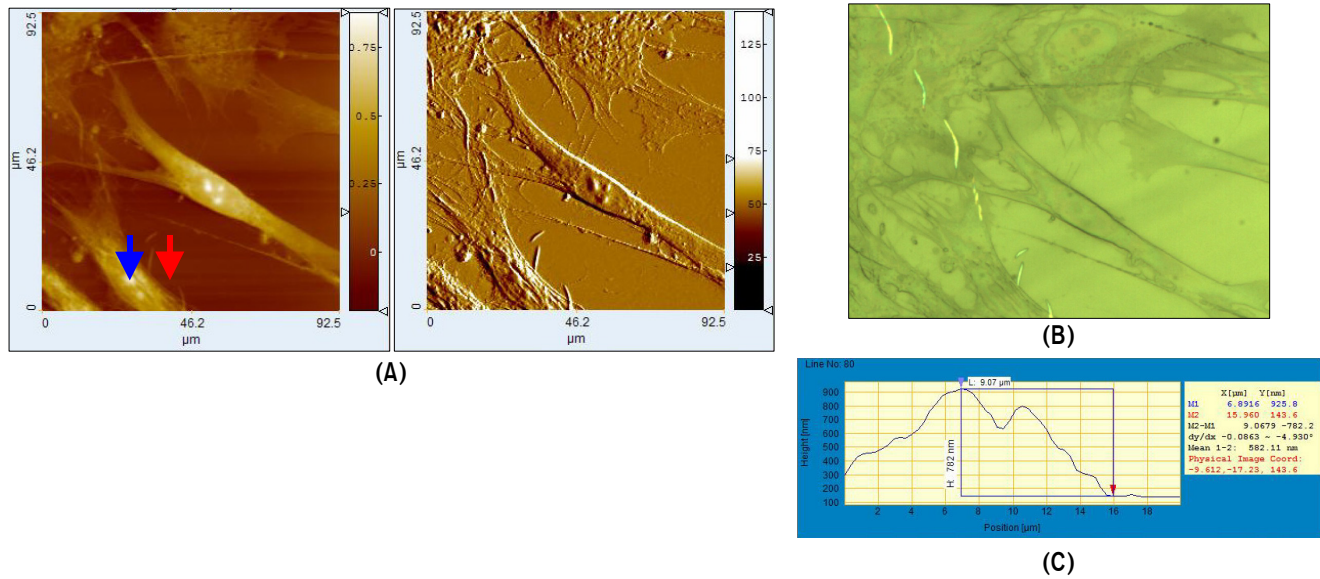


Fig. 4. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleoli in topography images of HFCs which were fixed with formaldehyde, finally washed with PBS.

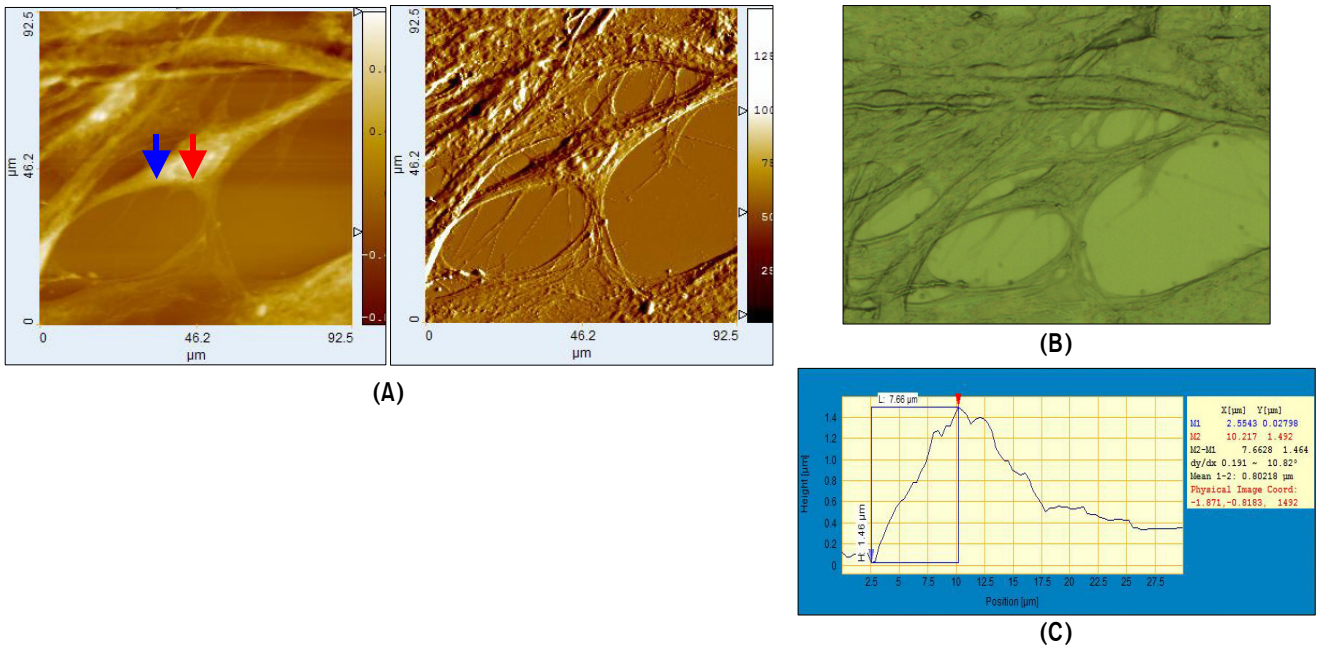


Fig. 5. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleoli in topography images of HFCs which were fixed with glutaraldehyde, finally washed with PBS.

in height along the z-axis, which was not significantly different from that of living cells. As can be seen from Fig. 3C and Table 1, both of cytoplasm and nucleus had more than 500 nm in height. No debris or crystal artifact was found. However, the cytoplasm looked rough with perforations, although the texture of the nuclear area seemed smooth.

These alterations in the cytoplasm were similar to the depressions by the dehydration which was also observed by Kalle et al (1996).

HFCs fixed with formaldehyde, finally washed with PBS

As shown in Fig. 4A and B, HFCs fixed with 37%

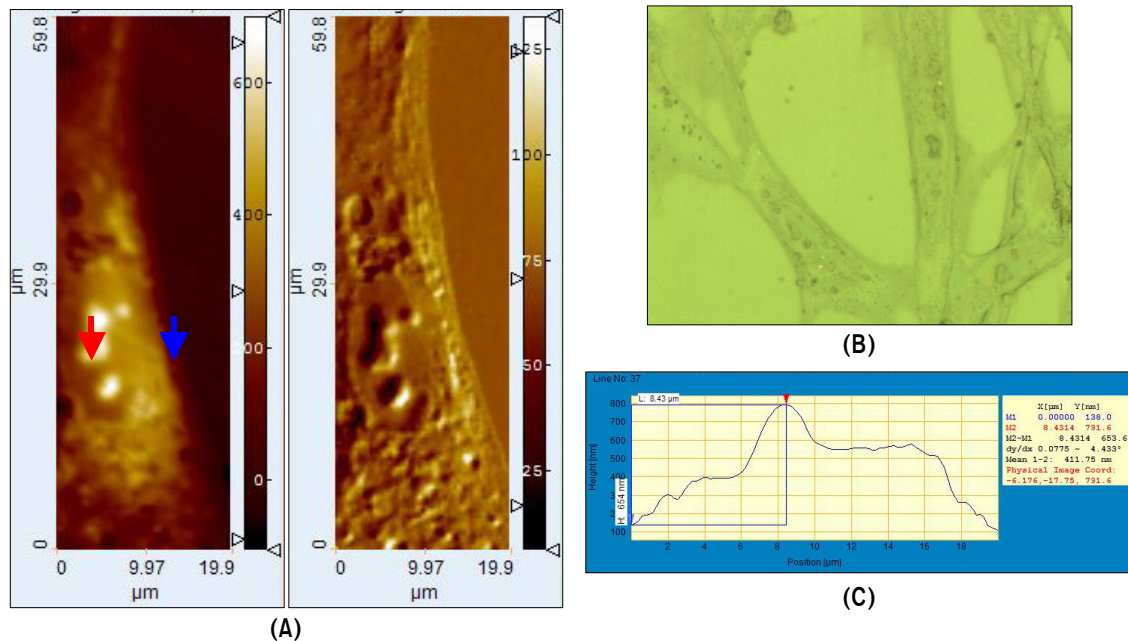


Fig. 6. (A) Non-contact mode AFM topography and amplitude images, (B) optic image, (C) height profile from substrate to nucleoli in topography images of HFCs which were fixed with glutaraldehyde, finally washed with D.W.

formaldehyde showed good image without debris and depressions and demonstrated clearly fibroblastic cellular morphology. Both of the cytoplasm and the nucleus had more than 500 nm in height which was similar to that of live cells (Bushell et al., 1999).

HFCs fixed with glutaraldehyde, finally washed with PBS

Fig. 5A and B showed the non-contact mode AFM topography, amplitude images and optic images of HFCs which were fixed with 4% glutaraldehyde in PBS, finally washed with PBS. As shown in Fig. 5, the cell showed fibroblastic cellular morphology and excellent image without debris, depression or crystal artifacts. Both of the cytoplasm and the nucleus had more than 500nm height which was similar to that of live cells (Bushell et al., 1999). From the results of Table 1, HFC by this fixation technique showed the least shrinkage and dehydration among all fixation methods.

HFCs fixed with glutaraldehyde, finally washed with D.W.

HFCs were fixed with 4% glutaraldehyde in PBS, subsequently washed with D.W. instead of PBS. As

can be seen in Fig. 6A, the nucleoli protruded approximately 200 nm in height from the nucleus. There were multiple perforations on the surface of cells. As shown in Table 1, there was relatively low height difference between cytoplasm or nucleus and substrate. From this result, it seemed that HFC fixed with 0.5% glutaraldehyde went through the partial dehydration by D.W.

DISCUSSION

AFM imaging of living cells under physiological buffer solution can be very complicated due to the detachment of cell and contaminated probe. AFM imaging of dried cell in the air showed many problems such as dehydration and shrinkage. Therefore AFM imaging by wet fixation technique were more practical, simpler and faster scanning technique for living cells. For this purpose, optimization of cell fixation methods, not dried, was the prerequisite step for AFM analysis.

It was reported that fixation could increase the rigidity of cells in comparison with living one, resulting in an improved image quality (Beckmann et al., 1994; Hoh and Schoenenberger, 1994; Schoenenberger and Hoh, 1994). Weyn et al (1998) sug-

gested that strong fixation induced flattening of the cytoplasm and lost nuclear structure while the morphology of hydrated cells showed overall 'rounding' of the surface with ill-defined structure. Bushell et al (1999) showed that the nucleoli remained the only structural feature protruding greater than 150 nm in height in fixed and dried cells. In the case of cells that had been fixed, but not dried, the nucleus and the densest part of the cytoskeleton around the nucleus remained structurally intact protruding over 500nm in height which was not significantly different to that of live cells.

From the results, both the nucleus and the cytoplasm had more than 500 nm height in all three fixatives - ethanol mixed with acetic acid, formaldehyde and glutaraldehyde. And the height difference values between the substrate and cytoplasm or nucleus were statistically significant in all three fixatives. However, HFC fixed with ethanol mixed with acetic acid showed the multiple perforations in cytoplasm which were attributed to the dehydration. Therefore, it is recommended both 4% glutaraldehyde and 37% formaldehyde as promising fixative combined with final PBS washing. Particularly, it is proposed that wet fixation by 4% glutaraldehyde and subsequent wash with PBS is the most suitable for AFM imaging of HFC from the point of the least shrinkage and minimum p value.

ACKNOWLEDGMENTS

This study was supported by the research fund from Seoul R&BD program (grant # CR070054).

REFERENCES

- Beckmann M, Kolb HA and Lang F (1994) Atomic force microscopy of peritoneal macrophages after particle phagocytosis. *J Membr Biol* 140:197-204.
- Binnig G, Quate CF and Gerber C (1986) Atomic force microscope. *Phys Rev Lett* 56:930-933.
- Braet F, Zanger RD, Seynaeve C, Baekeland M and Wisse E (2001) A comparative atomic force microscopy study on living skin fibroblasts and liver endothelial cells. *J Electron Microsc* (Tokyo) 50:283-290.
- Bushell GR, Cahill C, Clarke FM, Gibson CT, Myhra S and Watson GS (1999) Imaging and force-distance analysis of human fibroblasts in vitro by atomic force microscopy. *Cytometry* 36:254-264.
- Hoh JH and Schoenenberger CA (1994) Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *J Cell Sci* 107:1105-1114.
- Kalle WH, MacVill MV, Van De Corput MP, De Grooth BG, Tanke HJ and Raap AK (1996) Imaging of RNA in situ hybridization by atomic force microscopy. *J Microscopy* 182:192-199.
- Moloney M, McDonnell L and O'Shea H (2004) Atomic force microscopy of BHK-21 cells: an investigation of cell fixation techniques. *Ultramicroscopy* 100:153-161.
- Murphy MF, Lalor MJ, Manning FCR, Lilley F, Crosby SR, Randall C and Burton DR (2006) Comparative study of the conditions required to image live human epithelial and fibroblast cells using atomic force microscopy. *Microsc Res Techniq* 69:757-765.
- Schoenenberger CA and Hoh JH (1994) Slow cellular dynamics in MDCK and R5 cells monitored by time-lapse atomic force microscopy. *Biophys J* 67:929-936.
- Thimm JC, Burritt DJ, Ducker WA and Melton LD (2000) Celery (*Apium graveolens* L.) parenchyma cell walls examined by atomic force microscopy: effect of dehydration on cellulose microfibrils. *Planta* 212:25-32.
- Weyn B, Kalle W, Kumar-Singh S, Van Marck E, Tanke H and Jacob W (1998) Atomic force microscopy: influence of air drying and fixation on the morphology and viscoelasticity of cultured cells. *J Microsc* 189:172-180.
- Zimmermann H, Hagedorn R, Richter E and Fuhr G (1999) Topography of cell traces studied by atomic force microscopy. *Eur Biophys J* 28:516-525.