

Imaging of biological samples in liquid environment

Tibor Füzik, Pavel Ulbrich, Tomas Ruml Institute of Chemical Technology Prague, Department of Biochemistry and Microbiology

This short review describes the possibilities of imaging of biological materials using atomic force microscopy (AFM) in liquid environment. During the years, AFM became a promising method for imaging of properly prepared soft materials, that were usually hardly visualized by other methods. Atomic force microscopy can give new insight on biological matter, because it can work in environment close to native for the living cells, bacteria and viruses.

INTRODUCTION

This application note focuses on scanning of biological samples in liquid environment. It covers the basics of proper tip selection, scanning modes and procedures needed to properly tune the cantilever in semi-contact mode as well as the necessities that

are needed to be done before successful imaging in liquid. These advices can be followed also for non biological samples. In the second part of the document, we focus on the possibilities of the AFM usage for imaging of biological samples in liquid.

CONTACT VS SEMI-CONTACT MODE

The most common AFM techniques used in imaging of biological samples in liquid are the contact and semi-contact (amplitude modulation) modes. Whereas scanning in contact mode works very similar like in air, the semi-contact mode needs more skills to achieve the desired result. The contact mode is used mainly for stiff and firmly attached samples, or samples where the features under soft layer can be visualized using this method. However, because of soft and fragile nature of the majority of biological samples the semi-contact mode is more suitable for these kinds of samples.

Nowadays the two major ways of scanning are scanning with tip or scanning with sample. In some cases, the scanning with tip is the only reasonable method, when the sample cannot be attached to the scanner or the configuration of AFM with an optical microscope is used. This scanning technique can adopt wide range of samples and sample holding surfaces

(i.e. Petri dishes) where the lateral size of the sample is often not a limiting factor. Scanning with tip usually provides a less stable environment, and is less suitable for high resolution imaging. However, where the resolution and the thermal stability is not the key of the experiment, scanning with tip can be successfully used.

Scanning with sample is preferred method for high resolution imaging. In this case the tip holder with the tip remains steady and the scanning movement is provided by the scanner with sample. The wet cell attached to the scanner is designed in a way to minimize the movement of the sample inside the cell and to decrease the thermal instabilities. The design of the liquid cell often limits the observation of some samples that cannot fit into it, nevertheless, for high resolution imaging the properly designed liquid cell greatly decreases the noise affecting the final measurements.

PROBES

The proper selection of AFM probe is the most important for any type of scanning. In case of scanning of biological sample in liquid it is crucial to select the probe that is the most suitable for the particular experiment.

For scanning in air, the most widely used probes are manufactured from silicon. These silicon tips with silicon cantilevers provide a very well defined conical shape with tip height of $\sim 10\text{-}15\ \mu\text{m}$. The radius of the tip apex is usually 10 nm or less. This provides very sharp and well defined images while scanning in air, however, these tips are usually not suitable for scanning in liquids. The main drawback is the stiffness of the cantilever. The high spring constant of the silicon cantilever does not allow light semi-contact imaging in the liquid.

For this reason the silicon nitride cantilevers with pyramidal silicone nitride tips are the preferred

probes in liquid environment imaging. The silicone nitride cantilevers have very low spring constant that allows not only semi-contact scanning but contact mode scanning as well. Silicone nitride has great durability and the tips suffer less of wear than silicon tips, however, the drawback of these tips is the height of the tip ($\sim 2,5\ \mu\text{m}$) and the sharpness of the tip apex, that is usually $\sim 20\text{-}60\ \text{nm}$ for non sharpened tips, which limits the resolution. The solution is the use of oxide sharpened silicon nitride tips where the tip apex radius goes down to $\sim 10\ \text{nm}$ and great results with high resolution can be achieved.

The last class of probes suitable for liquid imaging are the newly developed silicon/silicone nitride probes. These probes combine the advantages of both above mentioned probes. The cantilever of the probe is fabricated of soft silicon nitride and the tip is made of silicon. Declared sharpness of the tip goes well below 10 nm which ensures great resolution.

TUNING OF CANTILEVERS FOR SEMI-CONTACT MODE

For semi-contact mode in liquid, the first option to optimize is the proper choose of feedback signal. In liquid the preferred is the RMS signal from the photo detector, because it gives less noise at low frequencies than the magnitude signal which is amplified by lock-in amplifier (see Fig. 1). To ensure proper range of RMS values, preamplifier can be used ($\times 10$), which rises the values of the RMS to the level that the set point value can be adjusted more precisely.

Also for the excitation of the cantilever in liquid environment a higher generator voltage must be applied (from 1-10 V), to achieve proper cantilever resonance. Too low generator voltage can lead to improper tuning, that's why, maximum value is recommended. Afterwards, it can be lowered to the desired amplitude.

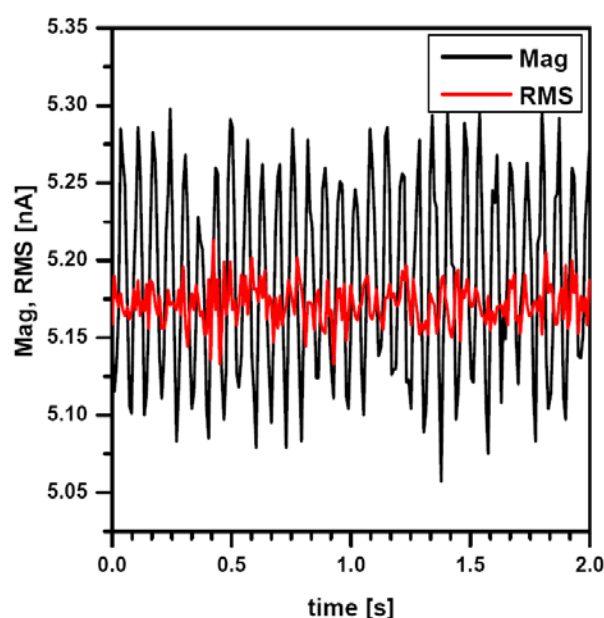


Figure 1. Comparison of RMS and Mag signal stability in liquid environment. This 2-second oscillogram shows that at low resonance frequencies in liquid (10 kHz) the RMS signal from the photo detector is less noisy than the lock-in amplifier amplified Mag signal.

The resonance frequency of the cantilever in liquid is usually an order of magnitude lower than in air and is located below 20 kHz. Auto-tuning is not recommended in liquid, because in contrast with resonance frequency in air there is not only a single peak in liquid environment (see Fig. 2). After initial frequency sweep (from 5-20 kHz), multiple peaks appear on the plot. It must be noted that the resonance frequency of the tip in liquid is not only a property of the tip, but rather of the entire environment. The selection of proper peak can be a challenge for beginners and would resemble more art than science. There are several recommendations for the proper selection:

- choose a peak that is higher than the majority of the peaks (but not always the highest peak is the best)
- after landing in contact mode and when the tip is still in contact with the sample, check that the height of the peak decreased
- once the proper peak was selected for the cantilever, it usually remains very similar for the other cantilevers of the same type (and the same instrument setup) - this can serve as a guide to select the proper peak for the next time.

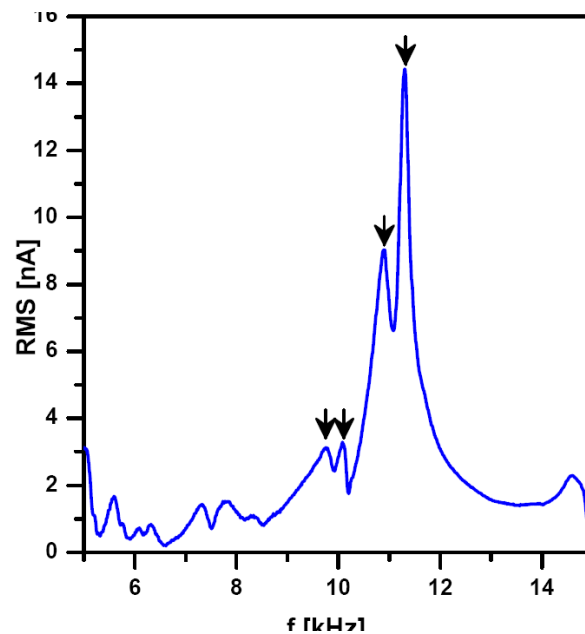


Figure 2. Cantilever frequency response plot in liquid environment. This multiple-peak response is typical in liquid environment. Peaks marked with arrow may be chosen for imaging (in this case the third one from the left was the most suitable - as was found by trial error). Frequency sweep (5-15 kHz) on triangular silicon nitride cantilever was done with 10 V generator voltage and 10x RMS preamplifier.

APPROACHING THE SAMPLE SURFACE IN LIQUID

Approaching the sample surface can be also challenging in the liquid environment. While the procedure of approaching the sample surface does not differ significantly from the approaching in air in contact mode, in semi-contact mode it can be quite challenging. For beginners it is recommended to approach the surface in contact mode, than to switch to semi-contact mode, however, sometimes (in case of soft and sticky samples) this procedure can lead to tip contamination. To avoid this problem, approaching the sample surface in semi-contact mode is recommended.

Approaching in semi-contact mode in liquid differs from the approaching in air. At the beginning the

amplitude of the cantilever starts to rise while getting closer the surface, than it starts to decrease. That is because the intensity of the acoustic waves in the liquid differs when the tip holder and the tip is closer to the sample surface. Thus, during the approaching process the amplitude gradually increases until a certain point where the tip reaches the liquid in close surrounding of the sample, while it is still not in contact with the sample. This causes a decrease of amplitude, however, this decrease is not suitable for imaging. After getting even closer the tip comes in contact with the sample with a sudden drop of its amplitude.

Fig. 3, a typical response curve of the cantilever (RMS signal) can be seen during the process of approaching. At the beginning, the rising amplitude of the cantilever slows down the approaching speed (controller switches to ne approach mode) and that is why the approaching procedure needs to be several times restarted (marked with arrows). When the cantilever comes very close to the surface the amplitude starts to decrease.

This mild decrease does not mean that there is a contact with the sample surface. At the end, there is a sudden drop in the amplitude, hitting the applied set point, which means that the tip reached contact with the sample. As well as the tuning, the landing procedure needs also some training and a good sense to set a proper set point and feedback gain.

After proper approach to the surface has been done, it is advisable to retune the cantilever which can slightly change its resonance frequency, when it is very close to the surface.

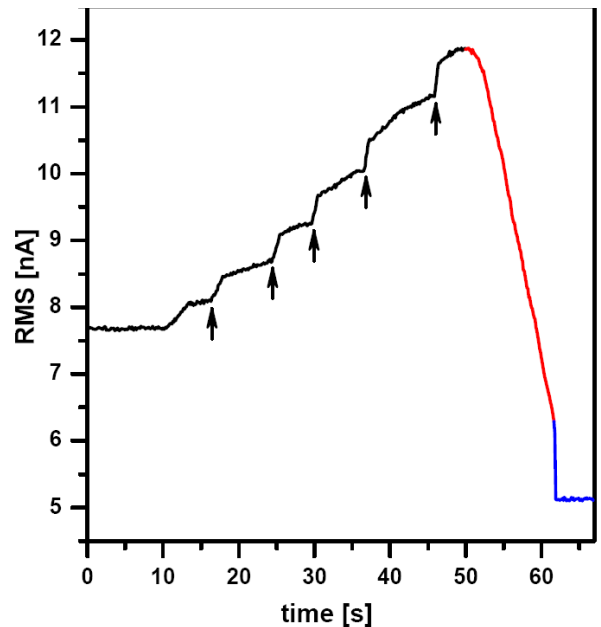


Figure 3. Cantilever resonance response during the surface approaching procedure in liquid environment. Typically, at the beginning the signal rises (black) by getting closer to the surface. When the probe is very close to the surface the amplitude starts to decrease (red), however, in this region the set point would not be proper for imaging. At the end, when the tip comes in contact with the sample, there is a sudden drop in amplitude to the set point. Black arrows show where the approaching procedure required restarting, because the instrument switched to ne approach mode.

AMPLITUDE OF THE CANTILEVER

To check the amplitude of the cantilever in liquid a similar procedure as in air can be performed. A basic amplitude distance curve can help to set the generator output level to set the proper amplitude of the cantilever. Therefore, also the force applied by the tip to the sample during the scanning. The

amplitude distance curve may also help to determine whether the tip is in proper contact with the surface. If the amplitude does not change with the distance, there is not a proper contact with the sample. There are only small differences between the amplitude distance curves in air and in liquid (see Fig. 4).

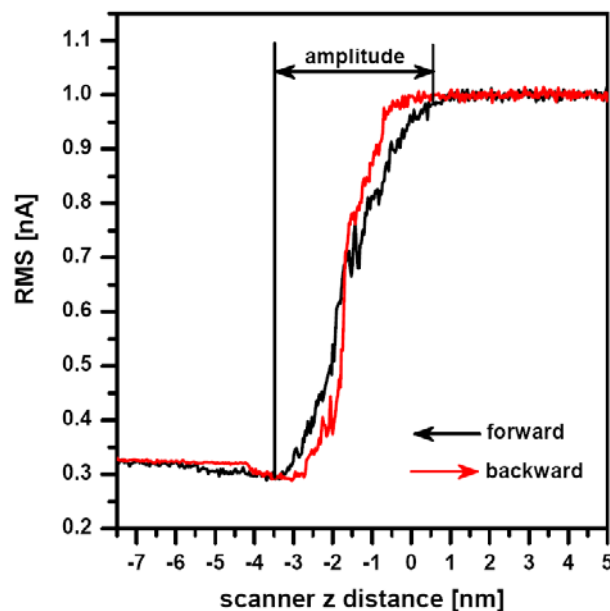


Figure 4. Amplitude distance curves in liquid environment. A typical amplitude distance curve measured in liquid environment. Please note that in liquid, the amplitude signal (RMS) never reaches zero as it is usual in air. Rather the lowest point is considered as the base for amplitude measurement. From the image above it is also clear that there is a very weak sticking of the tip to the surface (little differences in slope of the forward/backward curve), which does not affect the imaging.

PARAMETERS OF IMAGING IN LIQUID ENVIRONMENT

Typical tuning of scanning parameters in liquid does not differ from the scanning in air. Proper set of feedback gain, set point, amplitude and speed of scanning are the key factors for the best results.

As it was mentioned in the previous chapter, cantilever amplitude can be estimated using amplitude/distance curves. The proper amplitude for the scanning depends on the height of features on the surface. The higher the features of the scanned sample, the higher amplitude is recommended, however, the higher amplitude the higher force on the sample is applied, which can be critical for the soft biological samples. For relatively flat and stable samples 10-50 nm amplitude is a good starting point.

Set point and the feedback gain are maybe the most important parameters of scanning during the imaging process. For a good non sticky sample the feedback gain can be set up to 1, however the range of values 0.5-0.7 are more reasonable for the majority of samples. In case of some samples, it must be extremely decreased, but on small scanning range it is not necessarily a problem. As well as the amplitude of the scanning, the set point also defines the force applied to the sample by the tip. The lower the set

point the higher the force applied. Usually 80-90% of the free amplitude is a good starting point, or you can gradually decrease the set point until clear image appears.

Often, the set point must be repeatedly decreased during the scanning to keep the image stable. This is caused by the bending of the cantilever in the liquid. A cantilever that is not thermally equilibrated has a tendency to bend during the scanning process. This leads to movement of the laser spot from the center of the photodetector, which causes decrease of the amplitude signal (not the real amplitude!), therefore the set point must be decreased to maintain the same scanning force. To avoid this drift, a longer equilibration time is recommended before the high resolution scanning takes place.

The speed of scanning depends not only on the reaction time of the feedback loop (causing tailing), but also the stickiness of the sample must be considered. On some samples a slow scanning speed causes permanent sticking of the tip to the surface, which leads to problems of imaging. In this case a faster scanning can solve the problem, although sometimes at the cost of resolution.

BASIC PREPARATION OF BIOLOGICAL SAMPLES

Viruses

Size of the viruses predetermines them to be investigated mainly by electron microscopic methods. However, these methods rarely investigate the virus in its native form and often undesirable staining and dehydration of the sample is needed. The main advantage of atomic force microscopy in such

samples is its ability to perform the scanning in liquid environment. In liquid, the pressure applied by the tip to the virus particles can be precisely controlled and often the structure of the sample surface can be directly visualized (see Fig. 5).

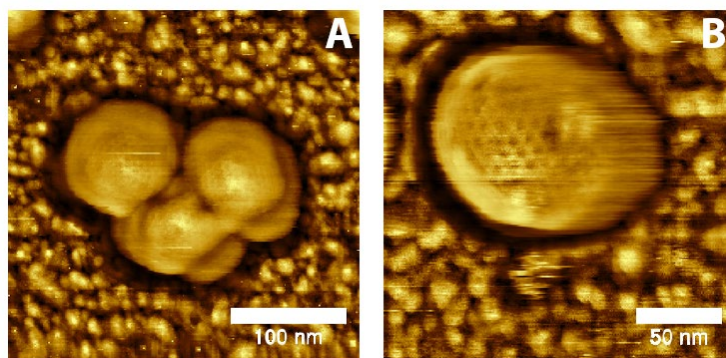


Figure 5. AFM images of virus-like particles scanned in liquid. These *in vitro* self-assembled retrovirus structural proteins form particles of about 80 nm. Fine structure of the particle surface can be clearly resolved. Observed hexagonal network correlates with electron microscopic analysis. Images were obtained by scanning in semi-contact mode in liquid environment using oxide sharpened silicon nitride probes. (Images obtained with AFM by NT-MDT SI).

To date, many articles describing the scanning of the virus particles from the smallest (i.e. rhinoviruses [1]), to the biggest ones (the giant mimivirus [2]) have been published. These papers investigate not only the surface structure of the viruses, the process of crystallization of viral particles [3, 4] which is often visible, but also mechanical properties [5].

The main problem in scanning of viral particles is usually their attachment to the scanning surface. This is usually based on electrostatic principle, achieved

by polylysine coated surface [2, 6] or multivalent metal cation [7]. Attachment to silane modified mica surfaces [8], or silane modified silicon wafers with immobilized antibodies [9] has been also reported.

The buffered environment with controlled ionic strength and pH can not only serve to achieve high resolution images [10], but also to investigate the stability of particles in different conditions, and to simulate the release of their genome depending on the properties of the environment [1].

Bacteria

The size of bacteria is usually almost an order higher than the size of virus particles. The easiest way to visualize bacteria by AFM is scanning in air. Sample immobilization is as simple as to dry the suspension of bacteria onto a glass cover slip or mica surface.

Using this method the bacteria are immobilized rmlly enough to scan them even in contact mode, however, semi-contact mode is preferred where even the amplitude or phase can be recorded (see Fig. 6).

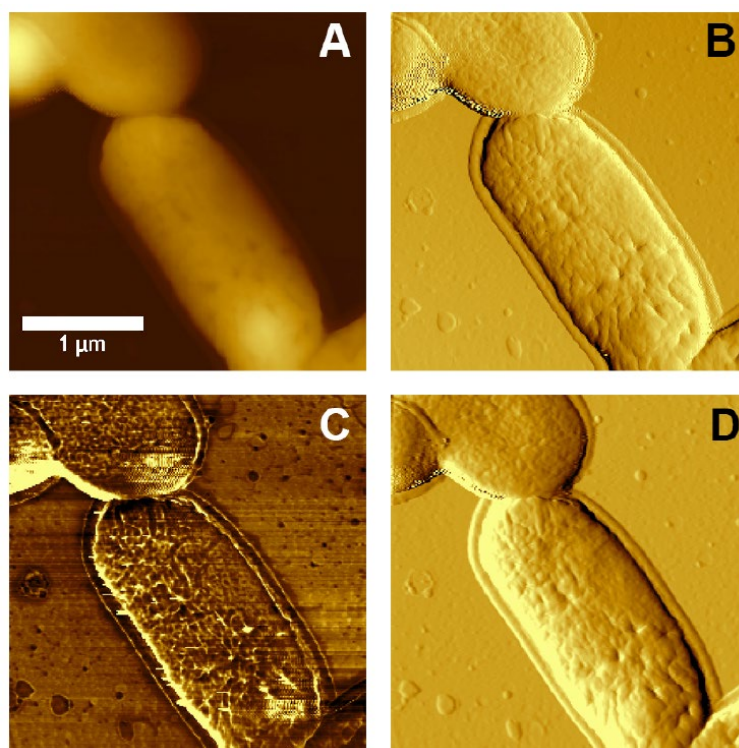


Figure 6. AFM image of *Escherichia coli* scanned in air. These bacteria were simply dried on the glass surface, then scanned in semi-contact mode in air with silicon probes. Features on the cell wall can be resolved on the amplitude (B), phase image (C) as well as on the derivative (D) of the topography (A). (Images obtained with AFM by NT-MDT SI).

To immobilize the bacteria for scanning in liquid environment special surfaces must be prepared. Immobilization techniques using gelatin coated mica surfaces has been reported [11], however, probably the most straightforward way is to immobilize them on polylysine coated glass or mica [12]. Although usually the resolution of biological samples is better in liquid than in air [13], in the case of bacteria this is not true. It seems that in liquid environment, the cell wall of bacteria is more smooth and less striae can be found on it than in images obtained in air (Fig. 7).

This can be explained by the fact that during the drying process some part of the cell wall dries out and

thus is more rigid than in natural liquid environment, where the dynamics of the surface compartment can be preserved.

Also the geometry of silicon nitride tips introduces scanning artefacts at the steep edges of the bacteria, where tip-broadening happens [14].

While AFM scans only features on the surface of the bacteria, which is coated by cell wall, there have been several reports of using it to check effect of antibiotics [15] on the cell wall or in situ treatment of cell wall with lysozyme [12].

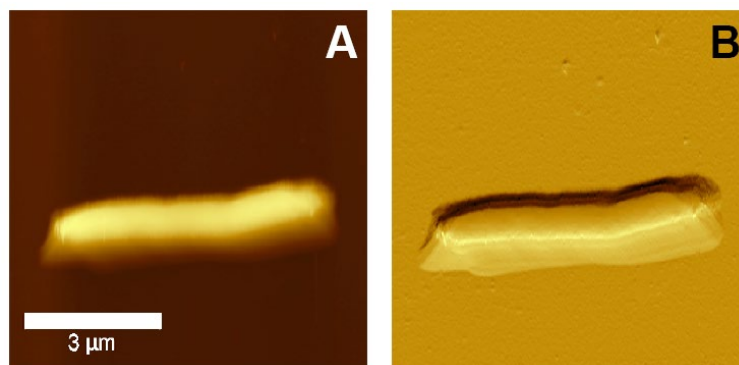


Figure 7. AFM image of *Escherichia coli* scanned in liquid buered environment. *E. coli* was immobilized on polylysine coated mica surface and visualized in liquid environment using semi-contact mode with silicon nitride probe. The are almost no features visible on topography(A) nor on derivative (B) of topography. Also distortion at the bottom of the bacteria is visible, which is caused by the pyramidal tip shape. (Images obtained with AFM by NT-MDT SI).

Tissue cell cultures

Cell cultures are usually the biggest samples used in AFM investigations of biological samples. Their lateral size exceeds tens of μm and vertical size depending on the cell line can be several μm . They are one of the softest materials for scanning because they lack cell wall and the naked plasmatic membrane comes in contact with the AFM probe. Because of the fragility and the nature of tissue cells, these samples should be measured in liquid environment, because during drying they loose their natural features.

The diculty of sample immobilization depends on the nature of the cell line. Adherent cell lines can be easily immobilized by growing them directly on a glass cover slip. For the attachment of suspension cell lines polylysine coated glass surfaces can be used.

Because the cell membrane is very soft, less sharp probes (i.e. non sharpened silicon nitride probes) are recommended for imaging of living unxed cells to prevent tearing of the membrane. Softness of the membrane allows even visualization of the

cytoskeleton underneath the cell membrane (Fig. 8). This can be achieved easily in contact mode, where the increasing force applied to the tip enables visualization of the cytoskeleton under the cell membrane appears [16], but also in semi-contact mode by great reduction (40%) of free amplitude [17].

To enhance the stiness of the cells, xation methods could be applied. The most common is to x proteins using formaldehyde or glutaraldehyde (see Fig. 8) and the lipids in the cell membrane by OsO_4 . The later makes the membranes stiff enough to visualize their surface structure [18], while in unxed state the membrane is too fluid and soft for visualization of small details. These dierences could be detected not only on the topography image but on amplitude, phase and on second harmonics image as well [19].

Using AFM, release of viral particles from tissue cells can be visualized [18], and also real-time monitoring of virus release through the cell membrane can be monitored [20].

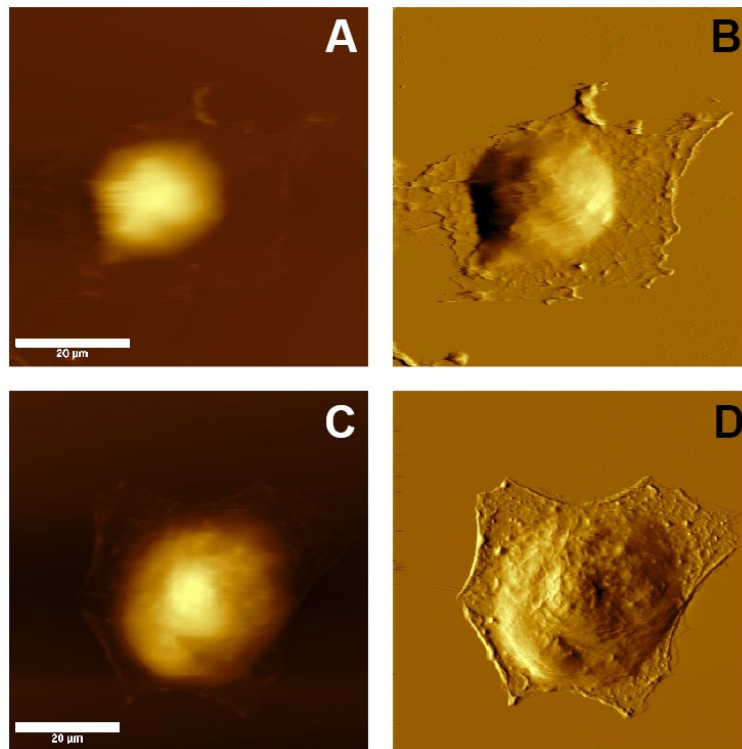


Figure 8. AFM images of living and fixed Cos1 tissue cells scanned in liquid buffered environment. This adherent cell line was scanned in liquid environment in contact mode with soft silicon nitride probes. The properly set pressure of the tip enabled to visualize the cytoskeleton under the membrane of the living cells (images A and B), yet prevented tearing of the cell membrane. The cells were more rigid after the fixation with 4% formaldehyde (images C and D). Features on the membrane as well around the center of the cell can be more clearly resolved than on unfixed cells. Topography (A, C) and deflection (B, D) images are shown. (Images obtained with AFM by NT-MDT SI).

CONCLUSION

Although atomic force microscopy was developed mainly to study surfaces of rigid and well defined materials, during the years of development it has become an invaluable tool for investigating soft

biological materials. In this application note we briefly described possibilities of imaging of biological material as virus particles, bacteria and cell cultures using atomic force microscopy in liquid environment.

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