Viscoelasticity of Living Cells Allows High Resolution Imaging by Tapping Mode Atomic Force Microscopy

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ABSTRACT Application of atomic force microscopy (AFM) to biological objects and processes under physiological conditions has been hampered so far by the deformation and destruction of the soft biological materials invoked. Here we describe a new mode of operation in which the standard V-shaped silicon nitride cantilever is oscillated under liquid and damped by the interaction between AFM tip and sample surface. Because of the viscoelastic behavior of the cellular surface, cells effectively "harden" under such a tapping motion at high frequencies and become less susceptible to deformation. Images obtained in this way primarily reveal the surface structure of the cell. It is now possible to study physiological processes, such as cell growth, with a minimal level of perturbation and high spatial resolution (~20 nm).

INTRODUCTION

The study of biological processes at very high spatial resolution is one of the potential key areas for application of the atomic force microscope (AFM; Binnig et al., 1986). Although this was recognized early on, the number of reported studies on living cells has remained rather limited up to this moment. The most spectacular result obtained so far is undoubtedly the observation of the exocytosis of a virus from an infected cell using an AFM (Häberle et al., 1992), where the tip is pressed on the cell surface (contact mode AFM). Other studies showed the dynamics of actin filaments in large (up to 150 μm in diameter) glial cells (Henderson et al., 1992; Parpura et al., 1993) (with the tip in continuous contact) and the activation of platelets (Fritz et al., 1993). Until now studies of living cells were hampered by the problem of imaging the soft surfaces, such as the cell membrane, without deformation. In all the cases mentioned before it was not clear whether disruption of the cellular surface was present or not. Several explanations have been put forward. First, the normal force applied to the membrane results in large deformation of the cell (Weisenhorn et al., 1993), making it hard to get any high resolution images on living cells. It may even result in breaking of the membrane followed by resealing after passage of the tip (Henderson et al., 1992). It is quite possible that the filaments of the cytoskeleton of the cells could only be observed because of this tip penetration through the membrane. Second, lateral or shear forces exerted by the tip cause surface features to be swept aside and smeared out. In this report we show the successful application of tapping mode AFM under liquid. Because of the viscoelastic properties of living cells, the cells "harden" under a tapping motion a high frequencies. Since the cells are no longer soft, they are less susceptible to deformation and high-resolution imaging of cellular processes, with a minimal level of perturbation, becomes feasible.

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MATERIALS AND METHODS

Cells

Monkey kidney cells can be grown on glass slides to form a monolayer. For the growth medium Iscove's medium is used with fetal calf serum and 200 mmol of L-glutamine added, resulting in a final ratio of 100:10:1. For our experiments cells were taken out of a batch according to a standard procedure used in our laboratory. 3×10^5 cells were put on a microscope cover glass in a petri dish and stored at 37°C under 5% CO. In a full monolayer, obtained after ~48 h of incubation, an area of 1000–3000 μm will be occupied per cell.

Atomic force microscope

An existing stand-alone AFM (Putman et al., 1993; Van der Werf et al., 1993) has been modified by positioning a multilayer piezoelectric actuator (AE0203D08, NEC, Tokyo, Japan) in the cantilever holder. A function generator supplies a sine wave to this piezo to excite the cantilever. The AFM is combined with an inverted optical microscope (Axiovert 135, Zeiss, Oberkochen, Germany), enabling quick localization and selection of cells. In this AFM the tip is scanned while the sample stays stationary, so as to prevent displacement of cells due to inertial movements.

RESULTS AND DISCUSSION

In measuring the surface of a living cell with AFM one has to take into account the viscoelastic properties of the cellular membrane and its underlying organelles. This is illustrated in Fig. 1 where we measure the response of the cell surface following a sudden downward movement of the AFM tip while it rests on the cell surface. We interpret this as the viscoelastic behavior of the cell (Schmid-Schönbein et al., 1981; Tran-Son-Tay et al., 1991; Radmacher et al., 1992; Hochmuth et al., 1993). Due to this viscoelasticity the cell surface relaxes slowly under the applied pressure to a new equilibrium. From these data it can be concluded that with an interaction time between tip and cell surface smaller than ~1 ms, the surface acts as a "hard" material. In standard

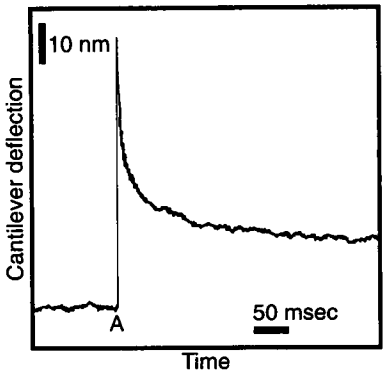

FIGURE 1 Time-dependent relaxation of a monkey kidney cell surface in medium upon deformation. The graph depicts the position of the cantilever as a function of time. During period before A the tip is in contact with the cell (*center*). At A a step voltage is applied to the piezo actuator giving a displacement of 125 nm (k cantilever, 0.58 N/m). Due to the viscoelastic properties, the tip feels an initial strong resistance, but under the load of the cantilever the cell relaxes during the period after A. Due to mechanical vibrations the response time to step functions of our setup is ~2 ms. In these first 2 ms the cell is already relaxed over a distance of 65 nm (125–60 nm).

contact mode AFM, however, the interaction has a continuous nature (a feedback loop actually keeps the applied force at a constant level) and the cells undergo large deformations. In equilibrium (end of the response curve) the deformation is ~100 nm, in good agreement with values reported previously (Weisenhorn et al., 1993). The results shown in Fig. 1 have led to the suggestion that a soft surface may act as if it is effectively hardened when imaged while oscillating the cantilever. In addition, the lateral forces are minimized if the oscillating tip is going in and out of contact each cycle, as known from tapping mode AFM in air (Zhong et al., 1993). In tapping mode AFM in air a stiff cantilever (force constants of 10 to 100 N/m) oscillates with a frequency between 250 and 400 kHz and an amplitude between 50 and 100 nm. Upon approaching the surface, the tip goes into contact at the bottom of each swing and the oscillation is damped. This creates the possibility to use the decreased amplitude in a feedback system for topographic imaging of the surface. During each cycle the tip is in contact with the surface for only a short time, therefore the highly destructive lateral forces, originating from the relative movement of the tip with respect to the sample (and not from slopes of surface features), are minimized.

While driving the piezo actuator supporting the weak cantilever (force constants of 0.38 and 0.58 N/m; Digital Instruments, Santa Barbara, CA) at frequencies raising from 10 kHz to 100 kHz in air (*dashed line*) and in liquid (*solid line*) we observed several resonances (Fig. 2 *a*). The oscillation frequency of the cantilever in air is 53 kHz. The other resonance peaks in air are from resonances excited in the complex mechanical construction close to the tip. This includes the piezo tube, a aluminum plate connecting the piezo tube and the additional piezo actuator, a steel wire holding down the cantilever onto the steel cantilever holder and an adjustable plate inside the piezo tube carrying the laser diode and two

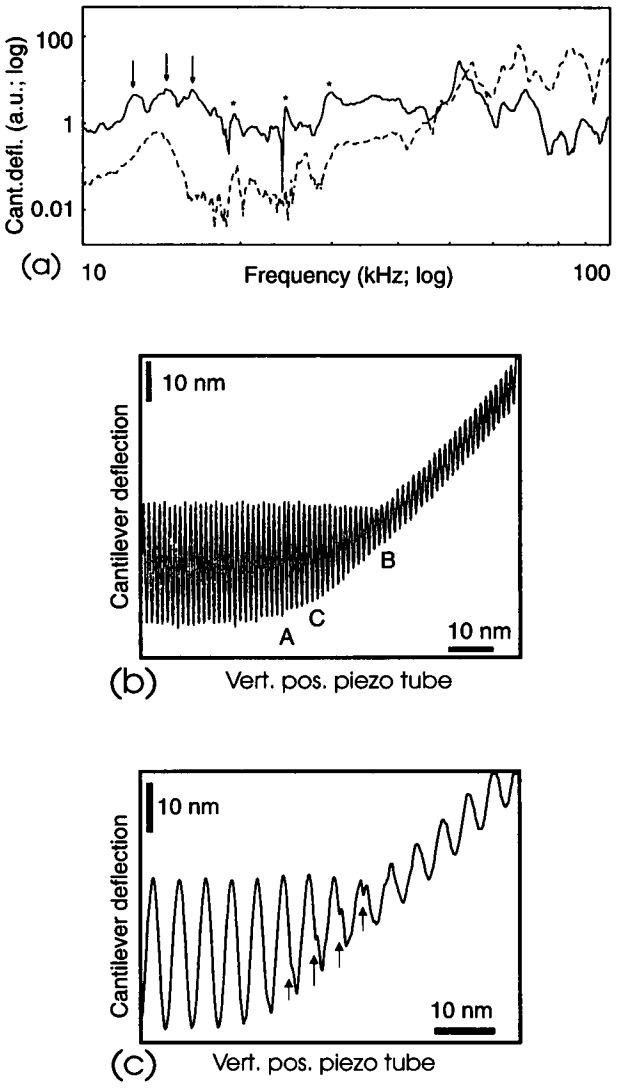

FIGURE 2 Frequency characteristics and approach curves. (*a*) Frequency characteristics of the cantilever movement in air (*dashed line*) and in liquid (*solid line*) in the 10–100 kHz range. The arrows denote peaks which can be used for tapping and the asterisks label peaks not useful for tapping. (*b*, *c*) Approach curves at an oscillation peak (14.1 kHz), meeting the requirements for tapping mode AFM in liquid, depicting the cantilever deflection as a function of the vertical position of the piezo tube (from left to right: tip movement toward the sample). *Arrows:* see text.

springs under tension (Van der Werf et al., 1993). In liquid we observe an increased cantilever movement up to ~50 kHz, because the coupling between the cantilever and the acoustic modes present in the mechanical construction is more efficient in liquid than in air. Especially in the 10–30 kHz region new resonance peaks appear, some of which may be used for feedback (marked by arrows) and thus for imaging (Fig. 2, *b* and *c*). Starting with an oscillation amplitude of 30 nm, the tip is lowered toward the surface by applying a voltage to the piezoelectric tube used for scanning. Coming closer to the surface, the tip starts touching the surface and the oscillation is damped (point A). Decreasing the tip-sample spacing further, results in more damping and at a certain point (B), the tip is continuously in contact with the

surface. Between points A and B, in this case separated by 25 nm, a feedback loop can be activated, which keeps the amplitude at a constant level. In the region between A and B this amplitude contains contributions of two modes of cantilever movement: 1) the angular deflection at the end of the cantilever due to a free movement of the cantilever in the liquid, and 2) the angular deflection of the cantilever while in contact due to a rotational movement of the cantilever with the tip as the pivoting point. Kinks in the sine wave (arrows in Fig. 2 *c*) indicate the positions where the tip gets in contact with the surface. Beyond point B the amplitude is solely due to the rotational movement of the cantilever (no vertical displacement of the tip). Tip displacements in our AFM are detected with optical beam deflection (Amer and Meyer, 1988). Optical beam deflection does not measure vertical tip displacements directly in the way an interferometer does, but it detects angular deflections which can be translated to vertical tip displacements. Taking the contribution of the rotational movement into account, the maximum and the average loading forces between tip and surface can be calculated (Putman et al., 1994). At C the maximum force is 3.8 nN and the average force 0.55 nN ($k = 0.38$ N/m). Oscillation peaks not applicable for tapping (marked by asterisks in Fig. 2 *a*) have a small amplitude of free oscillation in relation to the amplitude while in contact. In that case there is no or only a very limited range where a feedback loop can be activated.

Measurements indicate that the driving mechanism behind the cantilever movement is a combination of the mechanical excitation by the piezo actuator and—more importantly—the excitation by acoustic waves. At some frequencies, resonances are excited by the piezo actuator within parts of the mechanical construction. These acoustic resonances couple over to the cantilever via the liquid, resulting in the frequency spectrum with resonance peaks (Fig. 2 *a, solid line*). No resonances were observed when the cantilever was replaced by a rigid mirror. So the weak cantilever acts as a pick-up device for the acoustic waves. This idea is supported by the observation that the geometry of the tip environment, including the fluid cell and the liquid level in it, appears to influence the exact position of the resonances observed. For instance, very close to the steel walls of the fluid cell (the bottom is being formed by a 0.2-mm-thick microscope cover glass) the frequency spectrum is somewhat different when compared with the center of the fluid cell; some peaks shift up to 500 Hz. In ethanol the resonance peak at 11.8 kHz (in water) shifts to 12.5 kHz, while the magnitude stays more or less constant; the viscosity of the liquid influences the frequency characteristics. Moreover, in a petri dish with a 1-mm wall thickness, the exact locations of the peaks deviates from the situation in our standard fluid cell. Thus, for obtaining cantilever resonance peaks, acoustic modes within parts of the mechanical construction and the fluid cell coupling over to the cantilever via the liquid are of importance. This has been demonstrated even more convincingly in a separate experiment in which the piezo actuator supporting the cantilever was disconnected from the sine generator. A similar piezo was positioned at the edge of the fluid cell, partly immersed in liquid, and connected to the generator. Even though there is no direct mechanical contact between cantilever and piezo actuator, it is possible to excite the cantilever and operate the AFM in the tapping mode as described above. Although the exact frequency characteristics may change from experiment to experiment, there are always resonance peaks meeting the feedback requirements (Fig. 2, *b* and *c*).

Fig. 3 *A* shows an image a monkey kidney cell using the AFM in the regular contact mode. The cytoskeleton is clearly observed in this mode, suggesting that the cell membrane is deformed by the tip. If instead we image the same cell surface part using the tapping mode at a frequency of 14.1 kHz and an amplitude of 25 nm (starting out with an undamped oscillation amplitude of 30 nm) we obtain the result shown in Fig. 3 *B*. The data clearly demonstrate that when the tapping mode is applied the cell behaves as a "rigid" material during the short interaction time, yielding a picture of the cell surface and much less of the structures underneath. Accordingly, the average height of the cell observed is larger than the height of the cytoskeletal fibers. Figs. 3, *C* and *D*, show other images illustrating this major difference between contact and tapping mode AFM.

Since these results indicate that tapping mode AFM may be extremely suitable to study the cell surface of living cells without disrupting them, we next tried to image growing monkey kidney cells. A series of tapping mode AFM images

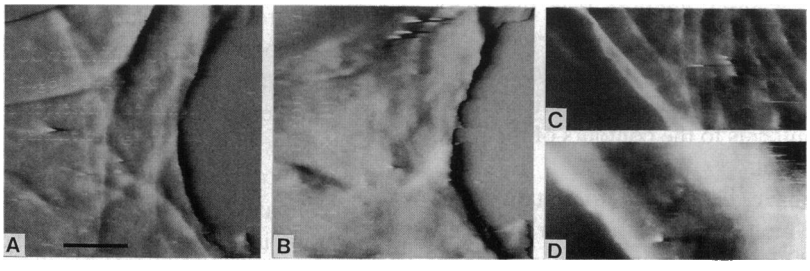

FIGURE 3 Contact vs. tapping mode AFM on cells in medium. Contact mode AFM images (*A, C*) and tapping mode AFM images (*B, D*) of the same monkey kidney cells. There is an offset between images in (*C*) and (*D*). In the case of tapping (at 14.1 kHz), the average loading force was ~0.5 nN and in the case of contact mode AFM it was ~10 nN (required to obtain stable imaging). Scale bar = 5 μm (for all images). Scan speed: 60 μm/s (500 × 500 data points). The data were collected in the height mode and high-pass filtered to enhance surface features. The height of the fibers in *A* varies from 50 to 125 nm, whereas the height of the cell as measured (*B*) varies from 150 to 250 nm. The cell in *D* is somewhat flatter (average height 150 nm) and the cytoskeletal fibers lower (*C:* 40–100 nm).

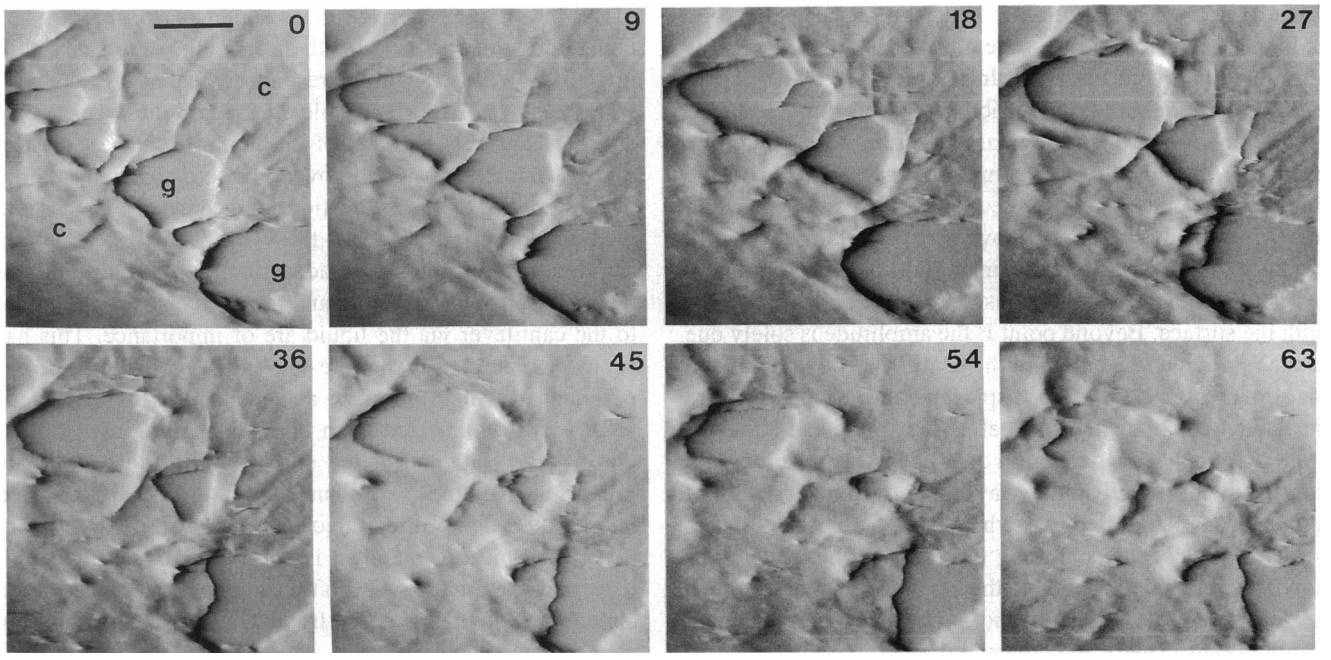

FIGURE 4 Temporal sequence of tapping mode AFM images of two adjacent monkey kidney cells (c) on a microscope cover glass (g). The maximum loading force and the average loading force were 3 and 0.4 nN, respectively. The oscillation frequency was 13.6 kHz and the scan speed 60 μm/s (500 × 500 data points). The data were collected in the height mode and high-pass filtered to enhance surface features. Scale bar = 5 μm (for all images).

of this growth process, ~24 h after incubation, is shown in Fig. 4. We see many details of the cells, which over a period of ~1 h grow toward each other, while slowly filling in the gaps on the surface of the glass slide. At the start of the sequence thin filopodia (with widths of ~100 nm) are running from the left cell to the cell at the right hand side. Some of these filopodia retract while others grow out to form bridges between the cells. At time 63 min almost no empty glass surface is left. Initially the cells look very smooth, but at 27 and 36 min the cell on the left shows an increasing number of bumps on the surface. This may be related to the rate of cell growth, because it coincides with the period in which the left cell grows fastest. The images clearly reveal that the cell on the right grows over the edge of the other cell. Primarily the cell surface and not the submembrane structures are imaged, although some cytoskeletal features are faintly visible on the cell on the right in Fig. 4, pointing in the direction of cell growth. Imaging the same cell with an increased loading force (set point closer to B in Fig. 2 *b*), results in a lower apparent average height of the cell. Additionally, as a result of the increased force the cytoskeletal features become now more pronounced. This indicates that the flexible membrane conforms to the contours of the more rigid underlying cytoskeleton.

Different approaches have been used to obtain high-resolution images of soft biological materials. At low temperatures cells stiffen and high-resolution imaging becomes feasible (Prater et al., 1991). Cells also becomes stiff after chemical fixation (Butt et al., 1990). These circumstances, however, can hardly be called physiological. Cantilevers with much lower force constants than currently commercially available will also result in a lower deformation. Still the problem of the lateral forces will remain.

In this report we have used the standard silicon nitride cantilevers and not the stiffer silicon cantilevers (available from Nanoprobe, Aidlingen, Germany) routinely used in tapping mode AFM in air. Although these stiff cantilevers (20–100 N/m) did meet the feedback requirements as illustrated in Fig. 2, imaging of cells was not possible. The increase in the force constants of the cantilevers can probably not be compensated by an increase in cell stiffness by oscillating at higher frequencies.

From the data presented here we conclude that this tapping mode AFM is much more gentle to cells than standard contact AFM. This is caused by two major differences. Firstly, the lateral forces are minimized. Secondly, we employ the intrinsic viscoelastic properties of the cells. The soft cells are stiff and are no longer soft on the time scale used in tapping mode AFM. This viscoelastic phenomenon is also at the basis of the success of tapping mode AFM in air on soft samples (Zhong et al., 1993). Thus tapping mode AFM under liquid primarily reveals the surface structure of the cell, whereas contact mode AFM shows underlying submembrane structures, such as the cytoskeletal fibers, due to local deformation of the cell envelope. The lateral resolution obtained on monkey kidney cells using tapping mode AFM is on the order of a few tens of nanometers (data not shown).

In conclusion, this new AFM technique is capable of high-resolution imaging of the cellular surface without disturbing the growth of the cell. Other processes, such as cell migration and phagocytosis, are now finally within reach.

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