Atomic Force Microscopy of height fluctuations of fibroblast cells

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We investigated the nanometer scale height fluctuations of 3T3 fibroblast cells with the atomic force microscope (AFM) under physiological conditions. Correlation between these fluctuations and lateral cellular motility can be observed. Fluctuations measured on leading edges appear to be predominantly related to actin polymerization-depolymerization processes. We found fast (5 Hz) pulsatory behavior with 1–2 nm amplitude on a cell with low motility showing emphasized structure of stress fibres. Myosin driven contractions of stress fibres are thought to induce this pulsation.

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I. INTRODUCTION

The motility of animal cells is dominated by actin-myosin-based contraction and actin polymerization-based protrusion. The two basic types of protrusions, lamellipodia and filopodia are driven by actin polymerization-depolymerization processes [1, 2, 3]. The physical theory of such biological motilities employing the tools of statistical physics [4, 5] gives an idea how it works but it still needs to be developed.

A number of cellular activities can cause height fluctuations on the time and distance scales that we investigated. Actin polymerization and actin-myosin based contractions represent only one class. The rearrangement of the structure built up from intermediate filaments (IF) under the plasma membrane can be another source of vertical fluctuations. IF-s provide mechanical stability to animal cells. Any significant weakening of the IF array alters at least locally the elastic properties of the cell leading to increased susceptibility to intrinsic or extrinsic forces. Assembly or disassembly of large protein complexes in the plasma membrane or their lateral motion under the AFM tip will also result in vertical motility, not to mention endo- and exocytotic activity. Intracellular transport processes can have an effect on the vertical fluctuations, as well.

Cellular motility on the micrometer scale has been extensively investigated with video-microscopy ([6, 7, 8] and references therein). Spatial resolution provided by optical microscopy of living cells, however does not enable researchers to observe nanometer scale motion and rearrangement of cell components. AFM is an adequate tool for such measurements [9, 10, 11, 12]. Stress fibres (contractile bundles of actin filaments and myosin-II) play an important role in the control of cell shape and the adhesion of cells to the extracellular matrix through focal contacts. These characteristic cytoskeletal elements can be imaged with the AFM due to their high elastic modulus [13, 14]. AFM is capable not only for recording high-resolution topographic images of living cells but also for measuring the elastic properties of them simultaneously [15] and investigating cellular dynamics [16, 17].

By repetitively scanning on the surface of a cell time-lapse images can be recorded [18, 19]. The analysis of subsequent images yielding a movie is highly informative in terms of the kinetics of the cytoskeleton. Although fast cellular motility can not be examined by the repetitive scanning procedure due to the minute-range of scanning time, nanometer scale fast motion can be probed by positioning the tip on the area of interest. In this way vertical fluctuations can be investigated almost without a limitation of the time scale.

Close to the molecular scale the motion of cells is dominated by stochastic fluctuations. With the AFM we tried to shed light on the linkage between microscopic fluctuations and organized motility.

II. MATERIALS AND METHODS

A. Cell Culture

3T3 mouse fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (GIBCO), 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.75 µg/ml Amphotericin B (SIGMA) at 37 °C, in a 5% CO₂ atmosphere. A few days before AFM measurements cells were subcultured on 13-mm glass cover slips.

B. Atomic Force Microscopy

We investigated the nanometer scale motion of 3T3 mouse fibroblast cells in culture. Different cells (C6 rat
A commercial AFM (TopoMetrix Explorer, Santa Clara, CA) with custom-made sample heating control system and fluid chamber was used. Measurements were carried out at 37 °C in CO2 independent medium containing 10% fetal calf serum (GIBCO). We used soft silicon nitride cantilevers (Thermomicroscopes, Coated Sharp Microlevers, Model # MSCT-AUHW, with typical force constant 0.01–0.03 N/m, 20 nm radius of curvature). Topographic and deflection images were acquired in contact mode. High-resolution images were acquired at a scanning frequency of ~4 Hz. Non-destructive low force scanning provided stable sustained imaging of living cells for 8–10 hours. After AFM experiments cells were maintained in the same medium for 1–2 days and found to be normal. We could not achieve high-resolution imaging on a portion of cells due to their increased height or softness.

Considering that small details of cellular components can be observed at the best quality on shaded deflection mode images we present our experimental data in this format. Topographic images provide height information but with poor contrast.

On the basis of consecutive images local lateral velocity of cells was calculated. At the edges of cells we measured the average displacement of the contour. In the middle of cells we chose some structures with characteristic shape and the lateral displacements of these structures were measured.

The measurement of local height fluctuations of cells was started at least 1/2 hour after mounting the sample into the fluid cell. In this way thermal transient effects could be eliminated. After each scanning the tip was positioned onto the point of interest with the same force and feedback parameters and we captured the DC voltage of the z-piezo by a digital oscilloscope (Tektronix TDS 210) for 22.5 seconds with 100 Hz sampling rate.

### III. RESULTS

Distinct types of cellular motility could be examined by the measurement of vertical fluctuations.

FIG. 2 displays 2 images of a movie showing the slight motility of the rear edge of a cell with a 4.5 minutes time shift. This cell was almost quiescent during the experiment with a highly stable structure of cytoskeletal fibres and moderate lateral motility. The rear edge is being pulled by the stress fibres: see the parallel set of curved fibres anchored to the edge of the cell. In the same time cell-matrix junctions or nonspecific contacts adhering the rear of the cell to the support weaken and break. We also observed a typical retracting triangular shaped 20 µm wide contact (image not shown) of the same cell at the rear edge. The contact was broken a few minutes after recording the vertical fluctuations.

Typical vertical fluctuations registered on these two locations are presented in FIG. 2. We suppose that the apparent difference between vertical fluctuations originates in the different biological activities of the two regions. While the entire region of the cell shown in FIG. 2 was extremely stable with a lateral velocity of about 2 nm/s, the edge beside the retracting triangular shaped contact moved with a speed of about 11 nm/s.

To analyze height fluctuations we calculated the power spectrum and the height-height correlation function with a maximal τ=5 s time shift of each $x(t)$ height-time curve:

$$y^2(\tau) = \frac{1}{N} \sum_{i} \left( x(t) - x(t+\tau) \right)^2, \quad t = i\Delta t, \quad i = 1..N,$$  \hspace{1cm} (1)

$$N\Delta t = 22.5 - 5 s$$  \hspace{1cm} (2)

where $\Delta t$ (10 ms) is the sampling time.

This function can characterize stochastic height fluctuations by giving the average change of height as a function of time. Curves are presented in FIG. 2. the number of measured height-time curves $n$ is indicated. The lateral velocity of each location seems to correlate with the saturation value of the height-height correlation function measured at that location confirming our assumption that height fluctuations are related to local biological activity (motility). The starting slopes of the curves give the speed of fast fluctuations. Curves saturate with different characteristic (saturation) times. There is an apparent difference between curves (a) and (b) in the saturation value. The characteristic time (∼2 s) of curve (a’) is approximately double those of the other two curves. Curve (a’) was registered on the middle region (cell body) of the quiescent cell. (See Table 2). Characteristic time and saturation value are related to the average duration and amplitude respectively of an upward or downward motion.

The analysis of power spectra (FIG. 2) of the height-time curves acquired on each location of this quiescent cell revealed sustained periodic fluctuations during the experiment (1.5 hours). We found a characteristic peak at 4.9 Hz with a width of 3.5 Hz. The area of this peak gives an average amplitude of 1.5 ± 0.4 nm. Cells without apparent stress fibres nearby lack this peak. The origin of the sharp peaks in the spectrum is electric noise.

FIG. 3 shows the contours of a leading edge of a motile cell from consecutive images. Note the bright spot (S) appearing on the cell surface close to the edge in the middle of the second image. It appears in less than 7 minutes and disappears soon after. A similar one can be observed on the upper part of the last image. These features seem to be linked to the ends of curved filaments. In many cases micrometer sized unidentified nodes were found on stress fibres. They might be large protein complexes attached to F-actin.

FIG. 4 displays the height-time curves captured on the leading edge (c) and close to that on the cell body
Table I: Comparison of the lateral velocity and parameters characterizing vertical fluctuations of different locations on the quiescent and the motile cell. Parameters of the height-height correlation function are calculated on the basis of curves presented in FIG. 3. Starting slope and saturation value were determined by linear fitting in the (0.3 s, 1 s) and (3 s, 5 s) intervals respectively, r: correlation coefficient of fitting, SD: Standard Deviation, n: number of measured height-time curves.

| Curve       | Starting Slope [nm/s] | r     | Saturation value ± SD [nm] | n  | Lateral Velocity ± SD [nm/s] |
|-------------|-----------------------|-------|-----------------------------|----|-----------------------------|
| Quiescent cell | a 6.3 0.94           | 10.5 ± 0.2 | 10          | 2.3 ± 0.4 |
|             | a’ 8.4 0.97          | 17.0 ± 0.2 | 10         | 3.2 ± 0.5 |
|             | b 16.7 0.97         | 28.1 ± 0.3 | 8          | 11.4 ± 2.5 |
| Cell in motion | a 20.3 0.998     | –      | 10         | 5.3 ± 2.9 |
|             | b 28.7 0.999       | –      | 9          | 5.3 ± 2.9 |

FIG. 1: Shaded deflection mode images with a 4.5 minutes difference showing stable actin-myosin cables at the rear of a quiescent cell. Arrow in the lower left corner indicates the direction of motion. SF: stress fibres.

IV. DISCUSSION

The analysis of height fluctuations acquired at different locations allows a sensitive monitoring of the motility of cellular components. Both actin-myosin based contractions and actin polymerization-based filopodial and lamellipodial protrusions can be examined by this method. We found a correlation between the characteristics of vertical fluctuation and organized lateral locomotion.

We explain the observed 5 Hz pulsation of a cell with the periodic contractions of stress fibres. This type of oscillation cannot be easily identified by other techniques due to its low amplitude. Although the frequency of mechanical pulsation of cardiomyocytes is in the same frequency range (~1.25 Hz), its amplitude is 2 orders of magnitude higher [17]. Spontaneous oscillatory contractions of muscle fibers with a period of a few seconds are widely known for several years (e.g. [21]). Theoretical models can explain spontaneous oscillation under certain conditions [21].

Slow pulsation of non-muscle cells has been observed in several cases. Microtubule depolymerization can induce rhythmic actomyosin-based contractility with a period of ~50 s in fibroblasts [22] and oscillatory activity in the cortical microfilament system of lymphoblasts [23]. Shape oscillations of leukocytes driven by cyclic actin polymerization has been studied by several groups [24]. The period of this process is about ~8 s.

The cortical tension of non-muscle cells generated by myosin-II can drive a change of shape [25]. Myosin molecules cycle about 5 times in a second in muscle [26]. Based on the above mentioned facts, we think that a synchronized behavior of myosin molecules in stress fibres may cause the observed pulsation. Myosin synchronization has been theoretically predicted close to the isometric condition in highly organized actin structures [27, 28]. Further experiments are needed to elucidate the background of this phenomenon. Using drugs affecting a specific system of the cytoskeleton will help to distinguish their roles in the nanometer scale fluctuations of cells.

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FIG. 3: Averaged height-height correlation functions of fluctuations measured on the surface of the quiescent and the motile cell. Curves (a, n=10); (b, n=8); (c, n=10) and (d, n=9) are the corresponding correlation functions of vertical fluctuations shown in FIG. [5]. Curve (a', n=10) was registered on the middle region of the quiescent cell. There is an apparent difference between the behavior of curves belonging to the quiescent and the motile cell. Saturation disappears on the scale of several seconds in case of the motile cell. This fact indicates the presence of vertical motility on this time scale. Significant difference between graph (a) and (b) is attributed to the dynamics of the retracting contact at the rear edge. See the value of lateral velocity of locations at each curve. 50 Hz noise on curves can be observed.

FIG. 4: Power spectrum (n=10) of height fluctuations measured at the location shown in FIG. 1. The peak at 4.9 Hz can be found in each power spectrum of height fluctuations captured on the surface of the quiescent cell. The power spectra of height fluctuations of cells without apparent stress fibres nearby lack this peak.

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FIG. 5: Consecutive shaded deflection mode images of a leading edge. Approximately 7 minutes elapsed between images. Arrow indicates the direction of motion. Note the bright spot (S) appearing on the cell surface close to the edge in the middle of the second image. L: lamellipodium, F: filopodium. Contour lines (extreme right) display the forward motion of the edge. The standard deviation of lateral velocity was found to be higher than in the case of a less mobile edge due to extensions growing with high speed, such as the extension on the lower region of the last contour line corresponding to the right hand image.
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