

Mechanical Characterisation of HeLa Cells using Atomic Force Microscopy

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Abstract: Recently, atomic force microscopy (AFM) has been shown to be a suitable tool for imaging biological structures and their modification, adding to accurate morphological and cytomechanical information. AFM is capable of simultaneous nanometer spatial resolution and piconewton force detection, allowing detailed study of a cell surface morphology and monitoring of cell-tip interactions. Modern AFM techniques allow solving a number of problems of cell biomechanics due to simultaneous evaluation of the local mechanical properties and the topography of the living cells at a high spatial resolution and force sensitivity. We present a method that images and studies mechanically characterized cells using atomic force microscopy. We used a HeLa cell line (virus epithelioid cervix carcinoma), growth media DMEM as a scanning surrounding, atomic force microscopy Bruker Bioscope Catalyst for imaging and cytomechanical measurement. Local mechanical characteristics of cells has been analysed with focusing on the cell nucleus. The Young's modulus of elasticity for intact cells can indicate mechanical changes as main properties of cells. We determined the mode and the median of Young's modulus on 35 kPa during nucleus area of the HeLa cells.

Keywords atomic force microscopy; living cells, Young's modulus

1. Introduction

AFM provides excellent topographical information of prokaryotic and eukaryotic cell surfaces, and the extracellular material produced by the cells. It is an excellent tool for use in cell biology applications, as it provides nanometer resolution, and can be performed in a variety of media, including liquids such as buffers, allowing detailed analysis of cells in their natural state [1]. In addition, AFM has been widely used as a nanomechanical tool to probe the elasticity of living intact or damaged cells, because standard methods for characterizing cell elasticity are still imperfect. AFM provides a great opportunity to study individual living cells at the nanometer level under biocompatible conditions.

Biomechanical properties of cells have been identified as an important factor in a broad range of biological processes [2]. The elasticity of a cell provides important information on the state, function and health of the cell, and these information can be obtained using the AFM via force curve measurements over an extended area (sometimes referred to as force volume) [3]. It is well known that abnormal tissue stiffness is an indication of a wide range of diseases such as osteoporosis, atherosclerosis or cancer. Detection of changes in cell elasticity, measured by AFM, has been used to distinguish metastatic cells from benign cells, thus identifying disease states of cancer. All above mentioned features have demonstrated that AFM can be potentially developed as a diagnostic tool for detecting human disease states with fairly high efficiency and accuracy [2].

New view of the AFM signal interprets a range of features that can be characterized from a single scan or $F-d$ curves of scan. At this time, the AFM apparatus can display the mechanical properties of cells at each point of scan with high resolution. From one usual $F-d$ curve can be counted several mechanical parameters describing cell surface or subsurface area. The most common parameters from Peak Force Quantitative NanoMechanics (PF QNM developed by Bruker) mode are Peak force error, DMT modulus, Adhesion, Deformation, Dissipation or mathematical recount this parameters for example Log DMT modulus. These measurements are based on Peak Force Tapping mode. Because this mode controls the force applied to the sample by the tip, sample deformation depths are small and the effect of the substrate on the measured modulus is decreased. The cantilever tuning is not required, because this mode does not resonate the cantilever. This is particularly advantageous in fluids.

This study is focused on quantitative cytomechanical measurements of adherent cervix cancer cells under physiologically relevant conditions (nontreated substrate, nonfixated cell, with using the heating stage and the incubation flow cell) by Bioscope Catalyst AFM containing Peak Force Quantitative NanoMechanics force mapping mode. The biomechanical characteristic can be an important step for a deeper understanding of cervix cancer. The mechanics of cancer cell deformability and its interactions with the extracellular physical, chemical, and biological environments offer an enormous potential for significant new developments in disease diagnostics, therapeutics and drug efficacy assays [4]. It is expected that the approaches described in this paper for studying cells by AFM will also be relevant to investigations of other cell types including health cells of various tissues, cancer cells or damaged cells after antitumor treatments.

2. Material and Methods

2.1 Material and Instruments

In our experiments, we used a cell line HeLa (Virus epithelioid cervix carcinoma) as a biological material. Chemicals used were Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Sigma) and 2% 200 mM L-glutamine and 0.4% penicillin/streptomycin (Sigma). Measurements were carried out on AFM Bioscope Catalyst with PSI module (Bruker) and with transmission microscope IX81 (Olympus), DNP-10 tip (Bruker). Further we used sterile Petric Dish with glass bottom (Will-co) as substrates for cells.

2.2 Cell Culture

2×10^5 HeLa cells (virus epithelioid cervix carcinoma cells) were cultivated in DMEM medium under a humidified 5 % CO₂-atmosphere on glass bottom Petric dish Will-co for 24 h. After incubation, cells were scanned in perfusion cell (PSI module) in fresh DMEM growth medium in 37 °C. Mechanical and topographical mapping was done on non-fixed living cells.

2.3 AFM imaging

Cells were imaged with a scan rate of 0.1 Hz. We used DNP-10 tip with a resonant frequency of 12 - 24 kHz and a force constant of 0.06 N.m⁻¹. AFM surface images were acquired in a Peak Force QNM imaging mode. All images were processed by Nanoscope Analysis (Bruker). Bioscope Catalyst had heated stages and PSI perfusion cell at the time of measurements. Thus the experiments were not limited in time. After recording scan, the tip was placed on the identified cell and force curves (ramp) were measured. Approximately two measurements were recorded on the twenty cells. The modulus E^* was obtained by fitting the retract curve using the Derjaguin, Muller, Toropov (DMT) model [5].

3. Results and Discussion

Our results show intact HeLa cell imaged by Quantitative NanoMechanics mapping in various mechanical characterisations determined from $F-d$ curves in high resolutions. PF QNM performs a very fast force curve at every pixel in the image. The peak interaction force of each of these force curves is then used as the imaging feedback signal. Peak Force Tapping mode modulates the Z-piezo at ~2 kHz with a default Peak Force Amplitude of 150 nm. Analysis of force curve data is done on the fly, providing a map of multiple mechanical properties that has the same resolution as the height image. Untreated and nonfixed Hela cells spreaded onto glass bottom Petri dish are imaged in Figure 1. after 24h incubation. The six signals were reconstructed from each $F-d$ curve. Peak Force error (Figure 1A) produces a map of the peak force measured during the scan. Because the PeakForce QNM mode uses peak force as the feedback signal, this channel is essentially the Peak Force Setpoint plus the error. Peak Force Error data comes from the differential signal off of the top and bottom photodiode segments relative to the Peak Force Setpoint. This is commonly referred to as the error-signal. The error-signal provides a sensitive edge detection technique and can be very helpful in visualizing fine details in topography that are difficult to see in regular height data. On the relevant scan 1A is imaged the fine structure of glass substrate. On the other hand, cell structure is rougher than glass substrate and cell adhesion to the substrate is visualized. Lamellipodias and filopodias connected to another cell are imaged. The height data are visualized in Figure 1B. Parameters of the pictures are 76 μm × 76 μm, resolution 512 pixels, scan rate 0.1 Hz. The cell size was 57.6 μm in lenght (incomplete cell body in the Figure 1), 26.6 μm in wide and 0 (light field) – 3.16 μm height (dark field) as can be seen in line profile in Graph 1.

The DMT modulus (elasticity) is imaged in Figure 1C. The reduced elastic modulus E^* was obtained by fitting the retract curve using the Derjaguin, Muller, Toropov (DMT) model [5]:

$$F = \frac{4}{3} E^* \sqrt{Rd^3} + F_{adh}, (1)$$

where F is the force, R is the tip radius, d is the separation, F_{adh} is the adhesion force, and E^* is the reduced elastic modulus. By assuming infinite elastic modulus for the tip (E_{tip}) and knowing the Poisson's ratio of the sample ν_s , the Young's modulus for the sample (E_s) can be calculated using following equations [6]:

$$E^* = \left(\frac{1-\nu_s^2}{E_s} + \frac{1-\nu_{tip}^2}{E_{tip}} \right), (2)$$

DMT modulus determined the HeLa cell elasticity. Stiffness range is specified in Arb relative units. Elasticity reached of 15.6 mArb on the cell nucleus (the most soft part of cell) to 1 900 mArb on the glass substrate. If we

calibrate the AFM by the standard sample, we get an absolute unit of Young's modulus in kPa. The darker part of image, the softer area of the cell. On the other hands, lamellipodias and filopodias exhibit a higher stiffness compared to the body cells. Energy dissipation (Figure 1E) is given by the force times the velocity integrated over one period of the vibration. The energy dissipation is therefore the hysteresis between the load and unload curves. Pure elastic deformation has no hysteresis which corresponds to very low dissipation. Energy dissipated is displaced in eV (after calibration by standard samples) as the mechanical energy lost per tapping cycle. Energy dissipation on the glass substrate reached values 26.6 Arb and on the cell body between 0 – 8.6 Arb.

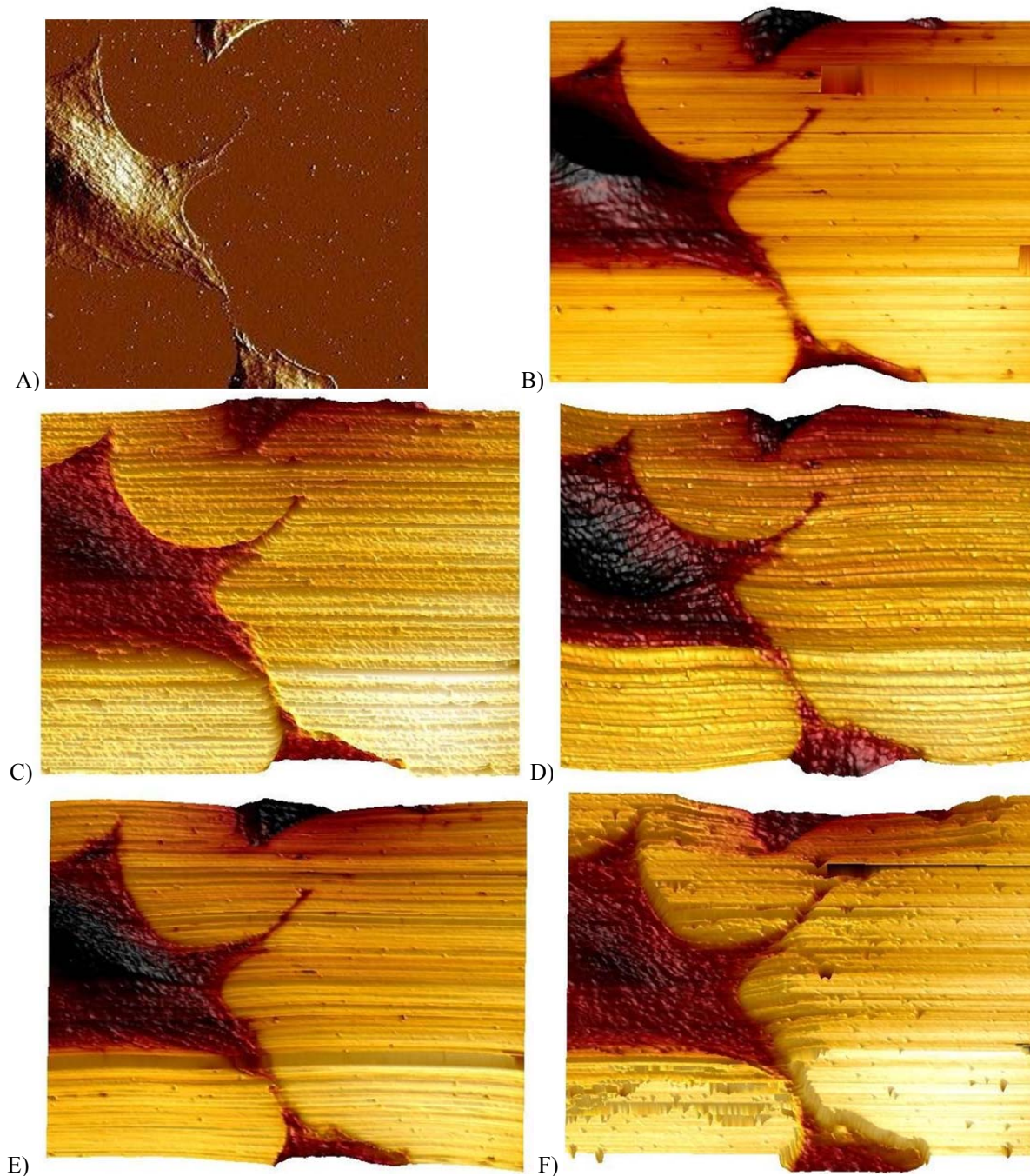
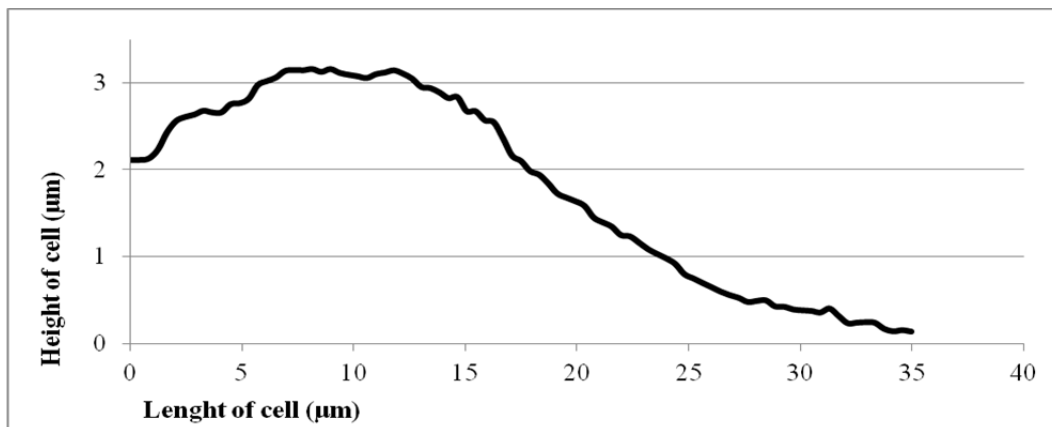


Fig. 1 The image of Hela cell line. Scans were obtained by Peak Force QNM imaging mode. A) Peak force error, B) topography signal, C) DMT modulus (elasticity), D) adhesion, E) energy dissipation and F) deformation. Parameters of the pictures are $76 \mu\text{m} \times 76 \mu\text{m}$, resolution 512 pixels, scan rate 0.1 Hz. The cell size was $57.6 \mu\text{m}$ in length, $26.6 \mu\text{m}$ in wide and 0 (light field) – $3.16 \mu\text{m}$ height (dark field).

Elasticity of small portions of a cells could not represent the entire cell property and may lead to invalid characterization. For this reasons, the scanning over whole cell including the substrate, is a promising model for cell

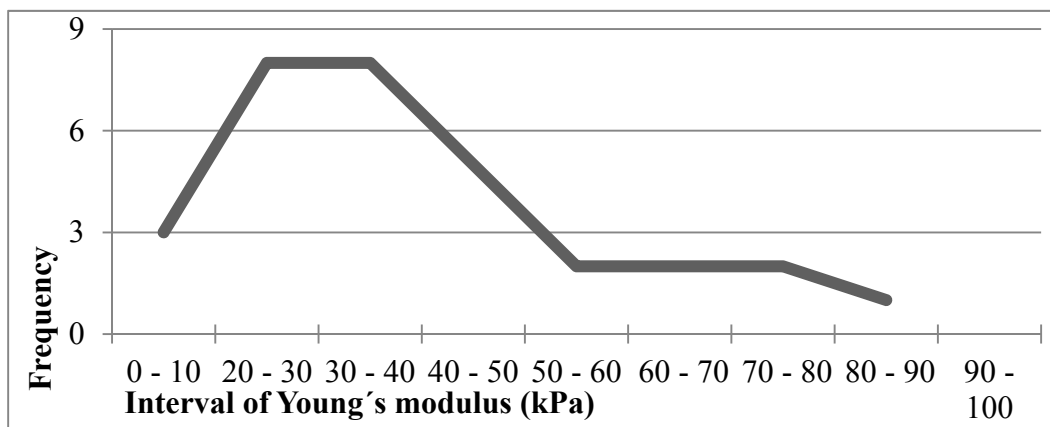
stiffness determination (see Graph 2 and Figure 1C). However, the substrate can affect the overall stiffness and height of cells. Guo et al. showed that the elasticity and height of human aortic endothelial cells on a softer substrate have higher value, then on a harder substrate [1]. The substrate contributions to the Young's modulus can be neglected if the AFM tip never indented more than 10 % of the cell thickness [7]. For this reason we made the stiffness measurement on the cell nucleus. The maximum deformation of the sample (see Figure 1F) is defined as the distance from the base of the deformation fit region position to the peak interaction force position caused by the probe. The indentation of cell is visualized on Figure 1F. A total value of the cell deformation was determined to 325 nm, which it is approximately 10 % of the height of the cell.



Graph 1 The line profile over the cell body. The highest part of cell is nuclear region with height 3.16 µm. The cell nucleus was determined as place for Young's modulus measurement.

The living cells are rather soft and delicate for their AFM probing under physiological conditions. Their drying, freezing and fixing with chemical agents improve the AFM images and AFM indentation results. However, these procedures change cell structure, viability and elasticity. For this reason, we imaged untreated and nonfixed HeLa cells. Cells are the highest in this area and contribution of substrate can be disregard [7].

The Young's module is expressed in Graph 2. The mode and the median of Young's modulus measurements were determined on 35 kPa during nucleus area of the HeLa cells.



Graph 2 The frequency incidence of the of Young's modulus value. The mode (modus) and median of Young's modulus data set was determined on 35 kPa on the nucleus area of HeLa cells. What is the Young's modulus increases, the cell is stiffer.

It is known that differences in the Young's modulus between normal and cancerous human cells were found to be due to a different organization of the cell cytoskeleton [4]. For example, the MCF7 breast adenocarcinoma cell line behaves as a complex linear viscoelastic material in applied power of 0.5 - 4 nN [8]. Metastatic cells are softer than healthy cells [4]. The softest part of the cell is nuclear region. The Young's modulus of mouse NIH3T3 fibroblasts in the core area is 4 kPa and the area outside the core reaches the stiffness to 100 kPa [9]. Young's modulus measurement of different types of living mammalian cells is summarized in Table 1. It shows that the elastic modulus value of living cells varies in wide range [3]. The data of Young's modulus are were various and dependent on the AFM parameters, tip parameters and applications of the numerical model. The analysis of almost a decade progress in this area allows

generalizing some methodological factors having a significant influence on Young's modulus value. Literature survey demonstrated that under a change of the external conditions, the elasticity of cell membranes is changed much stronger. We therefore maintain the natural environment of cells in exchange fresh medium by flow cell and heated stage. We do not use the substrate treatment and cell fixation.

Table 1 Young's modulus of mammalian cells [3].

Cell type	Young's modulus E (kPa)
HUVEC endothelial cells	10 – 11
BPAEC endothelial cells	0.2 – 2
HL60 - leukemia myeloid cells	0.2 – 1.4
Jurkat cells - leukemia lymphoid	0.02 – 0.08
Outer hair cells of Corti organ	300 – 400
Mouse outer hair cells of Corti organ	2 – 4
Osteoblasts	0.3 – 20
Migrating 3T3 fibroblast cells	3 – 12
L 929 fibroblast cells	4 – 5
Epidermal keratocytes	10 – 55
Platelets	1 - 50
Murine C2C12 myoblasts	11 - 45
Rat cardiocytes	32 – 42
Chicken cardiocytes	5 – 200
Erythrocytes	19 - 33

Concentration and molecular architecture of the various components of the cytoskeleton determines the total deformability and the overall mechanical response of cells with chemomechanical environment that ensures the interaction of cells with other cells and ECM. Any changes in a cell function caused by biochemical processes, such as the invasion of foreign organisms or the development of diseases can significantly affect the mechanical properties of cells [10]. These changes can be used as indicators of biological states of the organism and can offer valuable information about the pathogenic basis of disease.

Cytoskeleton polymers play the key role on cell biomechanics. Internal scaffolding comprise a complex network of biopolymeric molecules, primarily determine the cell's shape and mechanical deformation characteristics [10]. The cytoskeleton includes a comprehensive network of biopolymer molecules and mainly determines the shape of the cell and its mechanical resistance. The three basic cytoskeletal biopolymers have different responses to mechanical action: F-actin in microfilaments has a high resistance to deformation up to a certain critical value of the local pressure and the Young's modulus was determined up to 2.5 GPa. A high concentration of F-actin is in cells of the cortex, where it forms a kind of polymer network, but it is easily fluidized at high shear stress which facilitates cell movement. Actin stress fibers contribute to viscoelastic properties of cells, including chondrocytes, fibroblasts and hepatocytes. Disrupting actin can decreased the elastic modulus of cells. Nanoscale topography may, through an effect on actin organization, regulate cell compliance and potentially cellular response to environmental clues, especially mechanical signals [11]. Vimentin in intermediate filaments has the Young's modulus up to 5 GPa. These fibers are resistant to a medium deformation only, but they are highly resistant to the shear deformation and maintain the structural integrity of cells. Tubulin in microtubules has not alone sufficient strength and rigidity. However, tubulin operates together with other cytoskeleton biopolymers and contributes to the overall stability of the cytoskeleton. The Young's modulus of 1.9 GPa was determined [10].

Defects in the structure of the cytoskeleton affect the number of diseases, including various types of tumors. If we focus on the cytoskeleton and modify structural, mechanical and biochemical functions of damaged cells, we can open a new way for drug development of cancers or another diseases. It is now well accepted that cell functions are essentially determined by its structure. At different hierarchical levels, the structural organization of cells is characterized by certain mechanical properties [3] AFM probing of whole cells is an effective tool for studying membrane and sub-membrane cell structures and can be effectively used in the investigation of cytoskeleton characteristics and dynamics.

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