In Situ Autonomous Biomechanical Characterization

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Summary. This paper presents a fully automated microrobotic system based on force/vision referenced control designed for cell mechanical characterization. The design of the prototype combines Scanning Probe Microscopy (SPM) techniques with advanced robotics approaches. As a result, accurate and non-destructive mechanical characterization based on soft contact mechanisms are achieved. The *in vitro* working conditions are supported by the experimental setup so that mechanical characterizations can be performed in biological environmental requirements as well as in cyclical operating mode during several hours. The design of the different modules which compose the experimental setup are detailed. Mechanical cell characterization experiments under *in vitro* conditions on human adherent cervix Epithelial Hela cells are presented to demonstrate the viability and effectiveness of the proposed setup.

Keywords: *In vitro* mechanical cell characterization; Scanning Probe Microscopy (SPM) techniques; human adherent cervix Epithelial Hela cells mechanical characterization.

1 Introduction

Nowadays robotics and microrobotics techniques play an important role for exploring the mechanical cell behaviour. The ability to study accurately the mechanical behaviour of individual cells is a key component in understanding the elementary biological functions that various biological cells exhibit. Furthermore, the individual mechanical cell characterization is a major step towards the knowledge and understanding the behavior of the complex mechanical tissues. Up to date, several experimental setups have been developed to identify the control mechanisms of the cell mechanical response [1]-[5]. Among these robotics and microrobotics systems, the most promising ones

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involves Scanning Probe Microscopy (SPM) techniques for nanoscale. These techniques have the potential to give an accurate quantitative information about local forces and contact mechanisms. The Atomic Force Microscope (AFM) has become a commonly used tool to measure mechanical properties of the biological samples [6]-[10]. In these studies, a flexible cantilever with low spring constant and an atomic sharp tip is brought in the vicinity of the biological sample. Deflection of the cantilever as a result of the mechanical interaction between the microindenter and the sample is monitored by a split photodiode and the use of a laser beam reflected on the back of the cantilever. The significant research involving AFM make possible the measuring of relevant cells mechanical properties (Young's modulus, bulk modulus, surface roughness, ...). However, most of these studies have not been performed in biological clean room environment. Since elementary biological functions and mechanical properties of biological cells are widely affected by the experimental conditions, identified properties may not be relevant. Furthermore, as the reaction of the biological samples to stress vary greatly in a given lapse time, it is important to monitor the characterization process continuously in an *in vitro* environment.
Moreover, some problems are commonly associated with the use of standard commercial cantilevers with a sharp tip for the soft sample mechanical characterization. The nanometers size dimensions of tips can cause important local strains which are higher than elastic domain. Furthermore, depending on the magnitude of the applied force on the soft samples, cantilevers tips as well as the samples can be easily damaged so that the local strain applied in the indented area becomes changed. To overcome these problems, Mahaffy et al. [11] suggest to attach a micro-sphere at the cantilever tip. However, besides the necessity of dexterous manipulations for accurate placement of the microsphere, this method needs to use well-know materials (geometry, mechanical properties, ...).

Another difficulty is associated with the mechanical characterization of biological samples using sharp cantilever. Usually, the spectroscopy curves collected with the AFM are used in conjunction with an appropriate analytical model to estimate Young's modulus, friction, wear and other material properties. According to the literature, the Hertzian model which describes the relationship between force and identation is the commonly approach used for fitting the experimental data. Also, two major assumptions are made : linear elasticity and infinite sample thickness. Some authors have showed that in the case of soft contact mechanisms, models derived from linear elasticity can lead to significant errors [12]. Moreover, due to the imperfections of the tip radius of curvature an ill-defined contact region results between the probe and the sample. Consequently, uncertainties are introduced for choosing the appropriate fitting analytical model. It has been also shown, depending on the applied force and on the sample thickness, that large errors may result when using infinite thickness models [13][14]. The authors compute force-displacement

curves for finite sample thickness to show that, for soft and thin sample the error in the estimated elasticity modulus can be an order of magnitude.

In our opinion, the cell mechanical characterization in *in vitro* environment using tipless cantilever seems to be a promising solution. As study involving such cantilever are less prone to problems associated to sharp tip cantilever, enhanced non-destructive cell mechanical characterization should be achieved. For this purpose, a Force Bio-Microscope *(FBM)* system has been developed which combines SPM techniques and advanced robotics approaches. A tipless chemically inert cantilever is used for this study. Contact mechanisms resulting from the biomechanical process are modeled with the DMT (Derjaguin, Muller and Toporov) contact theory which taking into account both adhesion forces and finite sample thickness. In order to demonstrate the accuracy of the DMT model in the case of soft contact mechanisms, the estimated force-deformation curve is compared to the one predicated by the Hertz theory.

2 Experimental Setup Overview

The automated Force Bio-Microscope *(FBM)* device is an hybrid AFM microscope associating both scanning microscopy approach and biological environment constraint. The *FBM* consists mainly of three units: the mechanical sensing unit which performs detection, positioning and sensing features, the imaging/grabbing unit for imaging and cell tracking features and the clean room *in vitro* unit which allows experiments to be conducted in biological environment.
As the *FBM* experimental setup provides suitable conditions for study in controlled environment, the mechanical measurements process can be done on the biological sample on an extended lapse of time. A master computer is used to drive the *FBM* in a automatic operating mode based on force/vision referenced control. A user-definable graphical interface has been developed in order to make configuration of the automatic cell mechanical characterization process easier. To avoid undesired mechanical vibrations during the cell characterization tasks, the *FBM* is installed on an anti-vibration table. The overall configuration of the *FBM* and the different working components are shown in figure 1(A).

2.1 Mechanical Sensing Unit

The mechanical sensing unit is based on the detection of the deflection of a cantilever by an optical technique. A four quadrant photodiode with internal amplifiers associated to a low power collimated laser diode (wavelength 650 nm) are used in order to perform both axial and lateral nanoNewtons forces measurements. The optical path of the Gaussian laser beam is optimized using a pair of mirrors and a glass aspheric condenser lens. The total

sensing area of the photodiode is 7 mm^2 with a spectral response from 400 to 1100 nm. The sensitivity of the optical detection device is 5 $mV/\mu m$.
A low spring constant (0.2 N/m) uncoated tipless silicon cantilever is used as probe for the cell mechanical characterization. The lever is 450 μm long, 90 μm large and 2 μm thick. The biological samples are accurately positioned below the cantilever by a 3 DOF's (x,y and z) micropositioning encoded stage with a submicrometer resolution (0.1 μm). The kinematics features of the micropositioning stage allows to achieve accurate mechanical measurements in a workspace of 25 x 25 x 25 mm^3 with a good repeatability. The configuration of the mechanical sensing unit including the optical detection device is presented in figure 1(B). A magnified picture of the cantilever with the focused laser beam on its reflective surface is shown in the same figure.

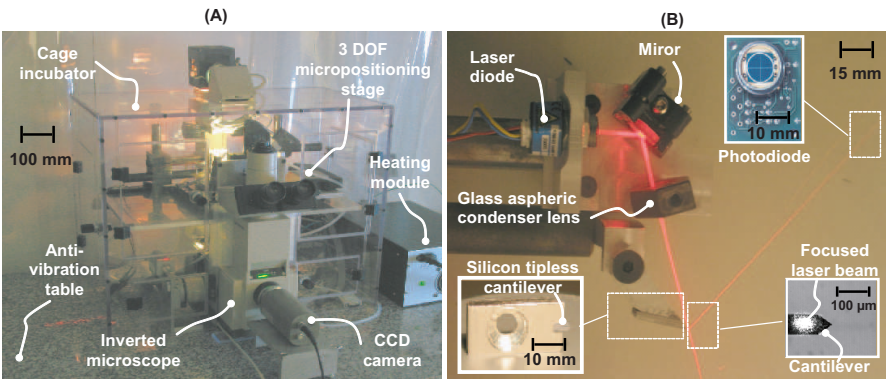

Fig. 1. (A) The *FBM* experimental setup overview. (B) The mechanical sensing unit.

For the preliminary study, we focused on force feedback control of cantilever flexural deflection. Thus, only the vertical micropositioning stage is servoed. By knowing the micromotors vertical position as well as deflection of the cantilever using the optical detection device, a optimized Proportional and Derivative (PD) controller was designed to ensure optimal control performance. The (PD) terms are optimized using the Ziegler-Nichols method. Figure 2(A) shows experimental results on the force referenced control approach for different desired forces.

2.2 Imaging/Grabbing Unit

The imaging/grabbing unit consists of an inverted microscope (Olympus IMT-2) with Nikon 10x and 20x objectives. A phase contrast device is mounted on

the microscope for precise contrast operation. The inverted microscope is fitted out with a CCD camera (754x488 pixels resolution). Using a frame grabber and a specialized imaging library package (Matrox Imaging) associated to the CCD camera, automatic mechanical characterization based on image features tracking is achieved (cf. figure 2(B)). The pixel to real world calibration of the CCD camera is achieved by means of a calibrated glass micro-array as well as calibrated micro-spheres (cf. figure 3).

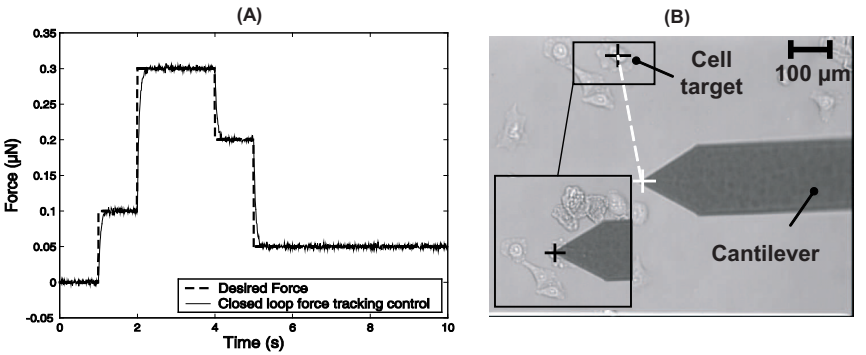

Fig. 2. (A) The force feedback control approach. (B) The vision feedback control approach.

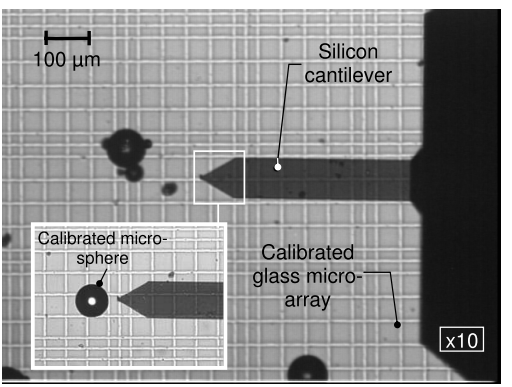

Fig. 3. Pixel to real world calibration of the CCD camera using the calibrated glass micro-array.

2.3 Clean Room *in vitro* Unit

The incubating system is formed with a controlled heating module which maintains temperature on the cage incubator at 37 ^oC using a single thermocouple probe. The cage incubator ensures a temperature stability within the 0.1 ^oC. A mixed stream composed by a 5% CO_2 and humidified air is fed into a small incubating chamber containing the biological samples, avoiding in this way condensation on the cage walls that could damage the mechanical parts of the microscope and the micropositioning stage. The whole system including the *FBM* is placed in a positive pressure clean room to protect the biological environment.

3 *In vitro* Mechanical Characterization Experiments

Experiments presented in this paper are focused on the mechanical stress response of human cervix Epithelial Hela *(EpH)* cells under controlled environment. In order to be valid, some assumptions are made in this regard: (i) the biological membrane is assimilated to a perfectly elastic spherical material; (ii) the enclosing liquid by the biological membrane of the *EpH* cells is considered as incompressible; (iii) the surfaces are assumed frictionless so that only normal forces are transmitted; (iv) the osmotic influence on volume modification is neglected.
The *EpH* cells can be assimilated morphologically to an elliptical cells with a thin surrounding biomembrane. In the present study, the average dimensions of the biological sample is 10 μm long, 9 μm large and 6 μm height (cf. figure 4).
The Epithelial Hela cells *(EpH)* are prepared on Petri dishes with specific culture medium formed by Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and L-glutamine components and 10 % of foetal bovine serum.

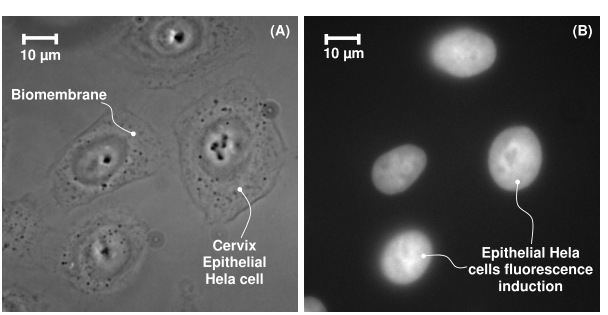

Fig. 4. (A) Magnified image of the cervix Epithelial Hela cells obtained with an 63x objective. (B) The cervix Epithelial Hela cells morphology observed by fluorescence techniques.

3.1 Cell's Mechanical Response Characterization

Figure 5(A) shows the experimental curves of the photodiode output as function of the sample displacement (Δz) performed on both single *EpH* cell and hard surface. The single step of the sample displacement is 200 nm and the total displacement is 8 μm. Deformations δ of the *EpH* cell are monitored by calculating the difference between the sample displacement Δz and the cantilever deflection Δd. The non-linear elastic behaviour of the *EpH* can be seen in the figure 5(B) which presents the sample deformation δ as function of the load force applied by the cantilever.

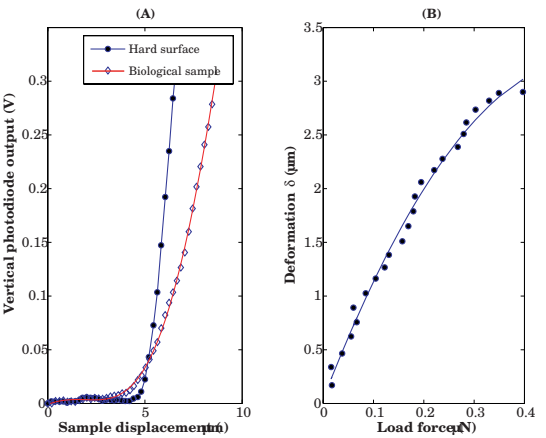

Fig. 5. (A) Experimental data of the photodiode output as function of the sample displacement performed on both single *EpH* cell and hard surface. (B) Experimental curve of the sample deformation δ as function of the applied load by the cantilever.

3.2 Viscoelastic Behaviour Characterization

The viscoelastic behaviour of the *EpH* cells are also investigated by the *FBM* device. Cyclical and automatic approach and retract experimentations were conducted on the same biological sample during 2 hours with 3 minutes intervals. For this given study, the motion amplitude and the single step of the vertical microstage are fixed to 8 μm and 200 nm, respectively. In order to reduce the cantilever damping oscillations during the mechanical characterization process, velocity of the sample positioning stage is chosen small (0.5 $\mu m/s$). Figure 6(A) shows 3 approaches and retracts curves monitored at different time intervals (t=0 mn, 40 mn and 80 mn) of the cyclical experiments. A single referenced approach and retract curves performed on hard surface are given in figure 6(B). According to the collected data, the *EpH* sample exhibit the same viscoelastic behaviour during all the experimentation.

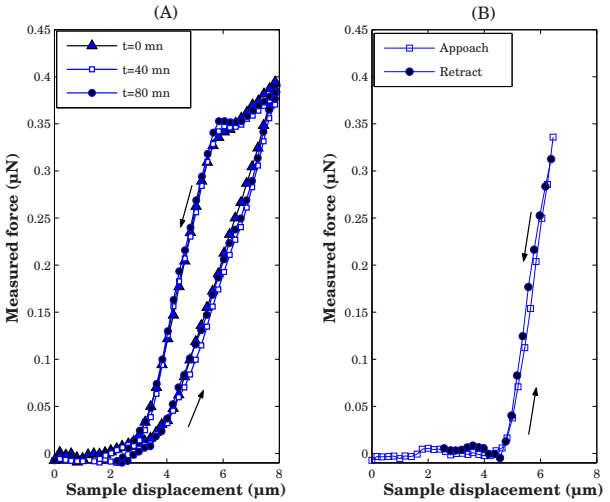

Fig. 6. (A) Experimental spectroscopy curves (approach and retract) performed on a single *EpH* cell at different time intervals (t=0 *mn*, 40 *mn* and 80 *mn*). (B) Single referenced approach and retract curves performed on hard surface.

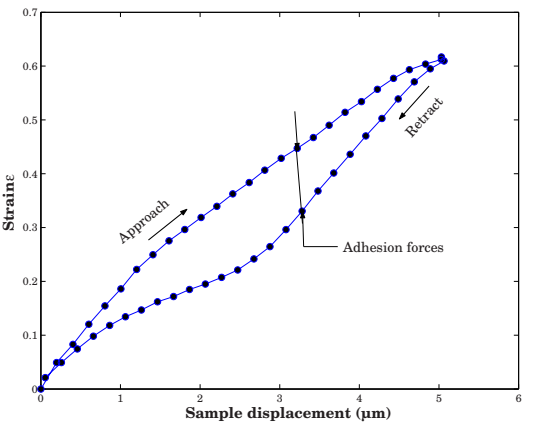

Fig. 7. Dimensionless strain per module length ε as function of the sample displacement Δz.

Compared to the approach and retract curves performed on hard surface (cf. figure 6(B)), the retract collected data performed on the *Eph* sample are different from the approach one. This suggests adhesion forces between the cantilever probe and the biological sample which modifies the total strain energy. Figure 7 shows the strain ε according to the sample displacement Δz. This figure emphasizes adhesion interaction between the cantilever and the biological sample.

3.3 *In vitro* Efficiency Approach for Cell Mechanical Characterization

In order to study correlation between the mechanical cell properties and the environmental culture conditions, we have experimented automatic and cyclical spectroscopy tasks on a single *EpH* cell during several minutes without the use of the incubating system. As the precedent study, the sample displacement and the single step of the vertical micropositioning stage are fixed to 8 μm and 200 nm respectively. Figure 8(A) shows evolution of the *EpH* cell mechanical behaviour of cyclical spectroscopy operation with and without the use of the incubating system. More specifically, curve (A) shows the approach and retract curves using the cage incubator. Curves (B), (C) and (D) show the mechanical behaviour of the studied *EpH* cell for different elapsed times t_0 once the cage incubator is turned off.
These mechanical characterization experiments obviously reveal that mechanical properties of the studied sample are affected by the temperature conditions. This difference suggests that the intra or extra-cellular matrix react to the variation of temperature.

3.4 Analytical Model for Sample Deformation Estimation

The deformation δ resulting from the mechanical cell characterization process is estimated using an appropriate analytical model. The DMT (Derjaguin, Muller and Toporov) theory is chosen for this purpose.
Noting R the radius of the biological sample (R=10 μm), w the adhesion work, P the load force applied by the cantilever and a_0 the radius of the contact area when $P = 0$, both contact area radius a and deformation δ can be expressed according to DMT [15] by

$$a^3 = a_0^3\left(\frac{P}{2\pi R w} + 1\right) \tag{1}$$

$$\delta = \frac{a^2}{R} \tag{2}$$

The DMT model suggests that adhesion work w can be expressed according to the pull-off force P_{off} needed to overcome adhesion forces as [15]

$$P_{off} = 2\pi Rw \tag{3}$$

As the pull-off force P_{off} and the contact area a_0 are accurately measured using the *FBM* ($P_{off} \simeq 20\ nN$, $a_0 \simeq 2\ \mu m$), the values of w and a_0 are introduced in equation 1 for the determination of a. Equation 2 gives the estimation of δ.

Figure 8(B) shows the estimation of the biological sample deformation δ as a function of the simulated load force P using the Hertz and the DMT theories. These analytical results are compared to the experimental measurements performed with the *FBM* and presented in section 3.1. As adhesion forces are not considered, large errors are observed between the experimental data and the predicated force-deformation curves (in order of 0.2 μm of magnitude) in the case of the Hertz model.

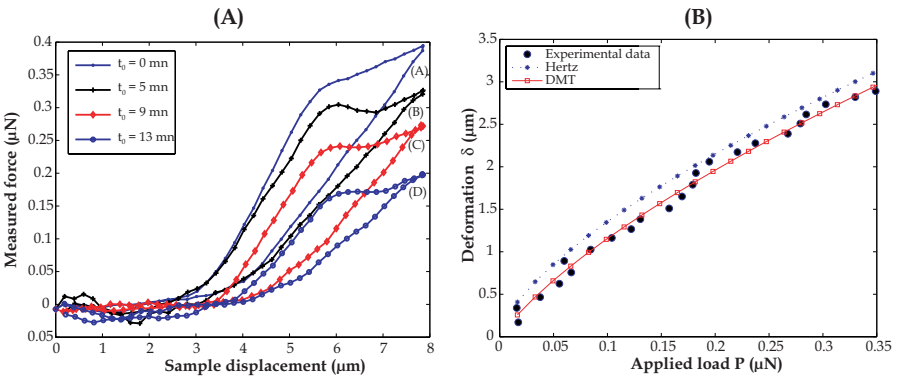

Fig. 8. Evolution of the measured force as a function of the sample displacement for different elapsed times $t_0 = 0, 5, 9$ and $13\ mn$. (B) Estimation of the biological sample deformation δ as a function of the simulated load force P using the Hertz and the DMT theories compared to the experimental data.

4 Conclusion

This paper has presented the development of a micro-force sensing system for *in vitro* mechanical cell characterization. The experimental setup combines Scanning Probe Microscopy (SPM) techniques with advanced robotics approaches. As the developed system operates in a fully automatic mode based on visual and force tracking control, effective mechanical characterization and reliable data acquisition are achieved. The Force Bio-Microscope

device *(FBM)* consists of three units with autonomous force sensing and measurements capabilities. Each unit is designed, calibrated or configured towards an effective *in vitro* mechanical cell characterization.
Mechanical characterization is conducted using the *FBM* on human adherent cervix Epithelial Hela cells. These experiments demonstrate the efficiency of the experimental setup developed to explore the mechanical properties in *in vitro* conditions of adherent biological samples. The contact mechanisms resulting form the cell mechanical characterization process are predicted using appropriate model (DMT) taking into account both adhesion forces and finite sample thickness.

5 Future work

Current work involves modelling the soft contact mechanisms using a tipless cantilever taking into account friction, osmotic influence and anisotropic non-linear elastic properties. Future work will be focused on exploring the mechanical transduction of living cells in *in vitro* environment conditions. One of the most promising results of this study will be the description of both mechanosensitivity and mechanical transduction mechanisms at the microscale for biological cells or tissue configuration.

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