Enhanced Near-Field Force Probing for *In Vitro* Biomechanical Characterization

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***Abstract*— This paper presents a fully automated micro-robotic system based on force/vision referenced control designed for mechanical cell 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 characterization 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 is detailed. Mechanical cell characterization experiments under *in vitro* conditions on human cervix Epithelial Hela adherent cells are presented to demonstrate the viability and effectiveness of the proposed setup.**

***Index Terms*— *In vitro* mechanical cell characterization; Scanning Probe Microscopy (SPM) techniques; Mechanical characterization of human cervix Epithelial Hela adherent cells.**

I. 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 complex mechanical behaviour of tissues. Up to date, several experimental setups have been developed to identify the control mechanisms of the cell mechanical response [1]-[6]. Among these robotics and microrobotics systems, the most promising ones 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 [7]-[11]. 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 mechanical characterization of soft samples. The nanometers size dimensions of tips can cause important local strains which are higher than elastic domain. Furthermore, depending of 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. [12] suggest to attach a micro-sphere at the cantilever tip. However, besides the necessity of dexterous manipulations for accurate placement of the micro-sphere, 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 [13]. 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 of the applied force and on the sample thickness, that large errors may result when using infinite thickness models [14][15]. 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 studies 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 cantilevers are used in this study. Contact mechanisms are estimated with appropriate models taking in account both adhesion forces and finite sample thickness. More precisely, the JKR (Johnson, Kendall and Roberts) and the DMT (Derjaguin, Muller and Toporov) contact theories are used to estimate both sample deformation and the contact area radius resulting from the mechanical characterization process. In order to demonstrate the accuracy of the JKR and the DMT models in the case of soft contact mechanisms, the estimated force-deformation curves are compared to the one predicated by the Hertz theory.
The rest of the paper is organized as follows. Section II is devoted to the description of the experimental device produced. First experiments on mechanical cell characterization using the automated Force Bio-Microscope *(FBM)* under *in vitro* conditions on human cervix Epithelial Hela adherent cells are presented in section III. Finally, conclusions and future work on the mechanical cell characterization using the autonomous force sensing and measurements capabilities of the *(FBM)* are respectively introduced in section IV and section V.

II. 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.

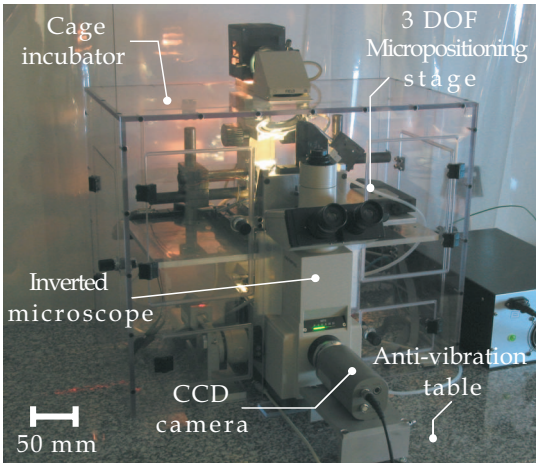

Fig. 1. The *FBM* experimental setup overview.

A. 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) is used in order to perform both axial and lateral nanonewtons forces measurements. The total sensing area of the photodiode is 7 mm^2 with a spectral response from 400 to 1100 nm. The optical path of the Gaussian laser beam is optimized using a pair of mirrors and a aspheric condenser glass lens. The sensitivity of the optical detection device is 5 $mV/\mu m$.

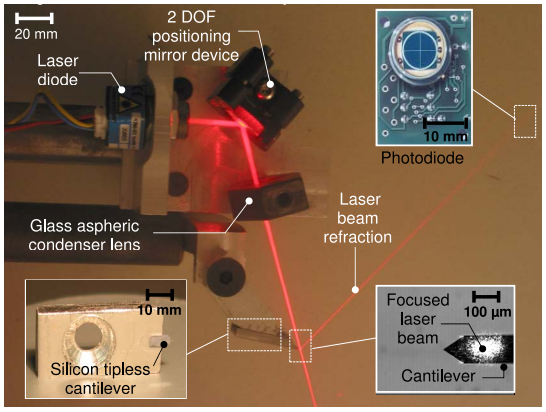

Fig. 2. The mechanical sensing unit.

A low spring constant (0.2 N/m) uncoated tipless silicon cantilever is used as probe for the mechanical cell 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 2. 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 3 shows experimental results on the force referenced control approach for different desired forces.

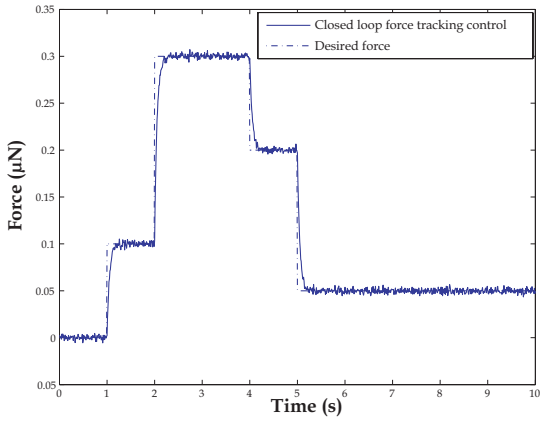

Fig. 3. The force feedback control approach.

B. 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 4). 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.

C. *Clean room* in vitro *unit*

The biological samples need specific requirements to be kept in live outside the *vivo* conditions to carry out prolonged observations. Besides the biological nutrition medium, biological cells need 37 ^oC temperature condition and 5% of CO_2 gas. Our 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 heating controller 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

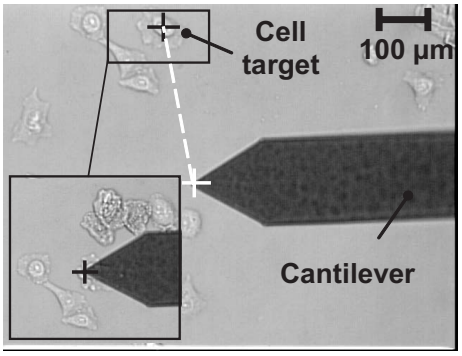

Fig. 4. The vision feedback control approach.

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.

III. *In vitro* MECHANICAL CHARACTERIZATION EXPERIMENTS

Experiments presented in this paper are focused on the mechanical stress response of human cervix Epithelial Hela *(EpH)* adherent 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 pressure 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 samples is 10 μm long, 9 μm large and 6 μm height (cf. figure 5(A) and (B)). 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.

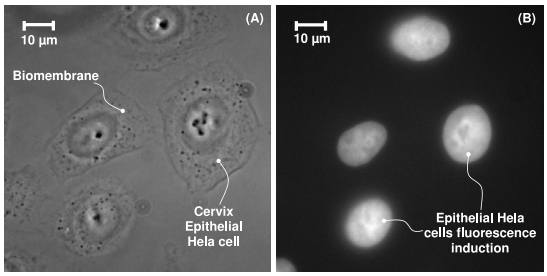

Fig. 5. (A) Magnified image of the cervix Epithelial Hela cells obtained with an 63x objective. (B) The cervix Epithelial Hela cells morphology observed by fluorescence techniques.

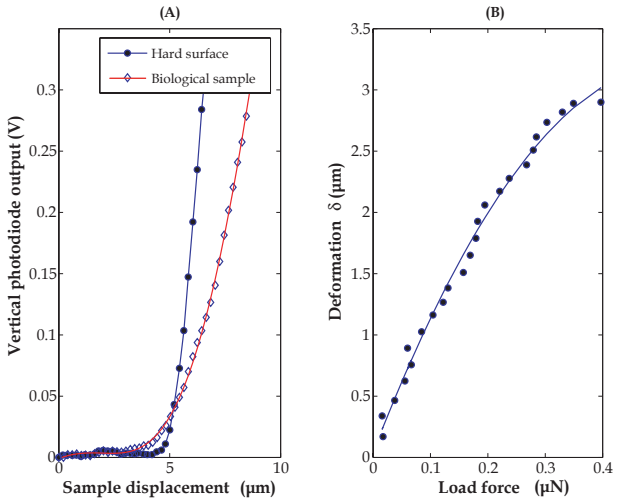

Fig. 6. (A) Experimental data of the photodiode output as function of the sample displacement performed on both single *EpH* cell and hard surface. (B) Experimental curve of the sample deformation δ as function of the applied load by the cantilever.

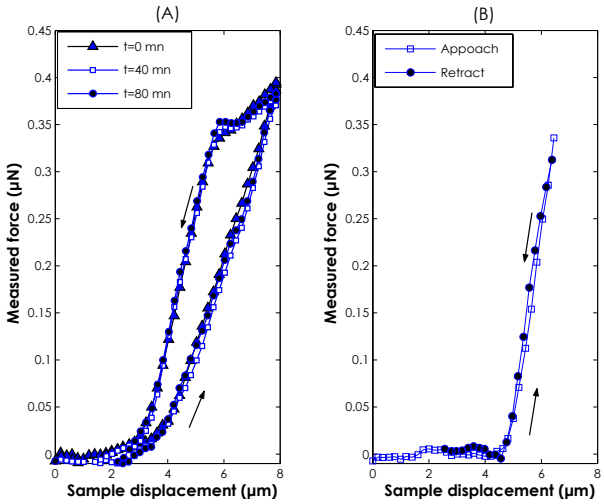

Fig. 7. (A) Experimental spectroscopy curves (approach and retract) performed on a single *EpH* cell at different time intervals (t=0 mn, 40 mn and 80 mn). (B) Single referenced approach and retract curves performed on hard surface.

A. *Cell's mechanical response characterization*

Figure 6(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. Deformation 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 6(B) which presents the sample deformation δ as function of the load force applied by the cantilever.

B. *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, the sample positioning stage velocity is chosen small (0.5 $\mu m/s$). Figure 7(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 7(B). According to the collected data, the *EpH* sample exhibit the same viscoelastic behaviour during all the experimentation. Compared to the approach and retract curves performed on hard surface (cf. figure 7(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.

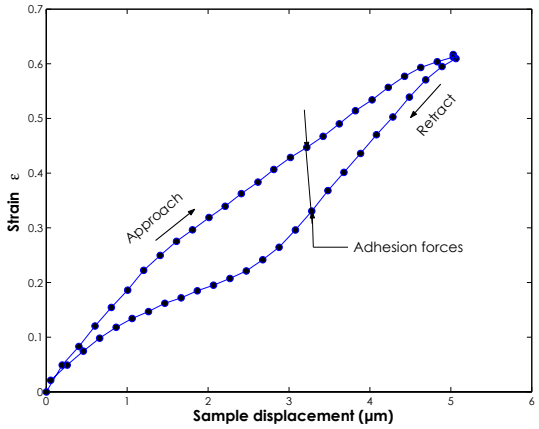

Fig. 8. Dimensionless strain per module length ε as function of the sample displacement Δz.

Figure 8 shows the strain ε according to the sample displacement Δz. This figure emphasizes adhesion interaction between the cantilever and the biological sample.

C. 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 9 shows evolution of the *EpH* cell mechanical behaviour of cyclical spectroscopy operation with and without the use of the incubating system. More

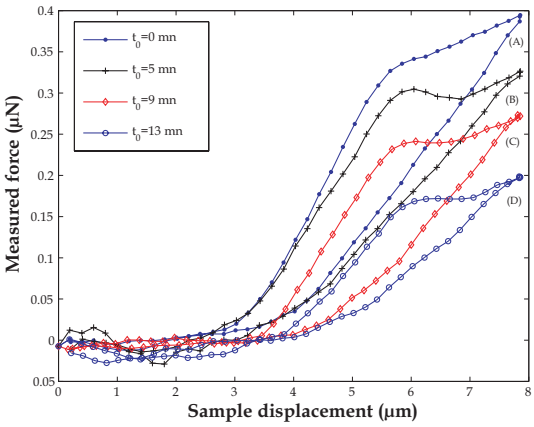

Fig. 9. Evolution of the measured force as function of the sample displacement for different elapsed times $t_0 = 0, 5, 9$ and $13\ mn$.

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 temperature variation.

D. Analytical model for sample deformation estimation

The sample deformation δ as well as the contact area radius a resulting from the *EpH* cells mechanical characterization process is estimated using an appropriate analytical fitted models. Three analytical models are chosen based on the Hertz, the JKR (Johnson, Kendall and Roberts) and the DMT (Derjaguin, Muller and Toporov) theories. The chosen models are fitted to sample deformations where elastic linear properties are satisfied. According to figure 6(B) the quasi linear elastic behaviour is satisfied for load P less then 0.15 μN.

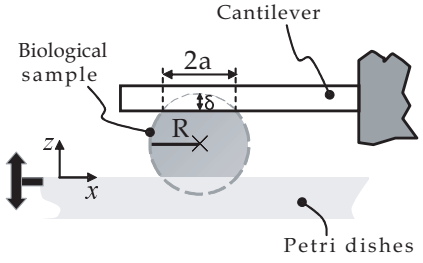

Fig. 10. Mechanical interaction scheme between the silicon tipless cantilever and the biological sample.

Figure 10 presents the mechanical interaction between the silicon tipless cantilever and the biological sample. Noting R the radius of the biological sample (R=5 μm), w the adhesion work and P the load force applied by the cantilever, the contact area radius a can be expressed respectively according to the Hertz, the JKR and the DMT theories by [16]

$$a^3 = \frac{RP}{K} \quad (1)$$

$$a^3 = \frac{R}{K}\left(P + 3\pi Rw + \sqrt{6\pi RwP + (3\pi Rw)^2}\right) \quad (2)$$

$$a^3 = \frac{R}{K}(P + 2\pi Rw) \quad (3)$$

Where K is the effective Young's modulus of the two materials which are in contact. K is expressed according to either the Hertz, the JKR or the DMT models as

$$\frac{1}{K} = \frac{3}{4}\left(\frac{1-\nu^2}{E} + \frac{1-\nu'^2}{E'}\right) \quad (4)$$

ν and ν' are respectively the Poisson's coefficients of the *EpH* cells (ν=0.5) and the silicon cantilever. The manufacturer data gives the Young's modulus of the silicon tipless cantilever and the Poisson's ratio as E'=140 GPa and ν'=0.17.

The JKR and the DMT theories suggest that adhesion work w can be expressed by two ways according to the pull-off force P_{off} needed to overcome adhesion forces as [16]

$$P_{off} = \frac{3}{2}\pi Rw \quad (JKR) \quad (5)$$

$$P_{off} = 2\pi Rw \quad (DMT) \quad (6)$$

As the pull-off force P_{off} is accurately measured using the *FBM* ($P_{off} \simeq 20\ nN$), the adhesion work w is introduced in equations (2) and (3) to estimate a.

The deformation δ of the elastic body are expressed respectively using the Hertz, the JKR and the DMT analytical models as [16]

$$\delta_{Hertz} = \delta_{DMT} = \frac{a^2}{R} \quad (7)$$

$$\delta_{JKR} = \frac{a^2}{R} - \sqrt{\frac{8\pi wa}{3K}} \quad (8)$$

Figure 11 (A) shows the estimation of the biological sample deformation δ as function of the simulated load force P using Hertz, JKR and DMT theories. These analytical results are compared to the experimental measurements performed with the *FBM* and presented in section III-A. Figure 11 (B) shows the estimated contact area radius as function of the load force P.

These results emphasize, in our case, that the Hertz model is not appropriate for estimation of contact mechanisms in the case of soft materials at the microscale. Since adhesion forces are not considered, large errors are observed between the experimental data and the predicted force-deformation curve (in order of 0.2 μm of magnitude). Even, the contact

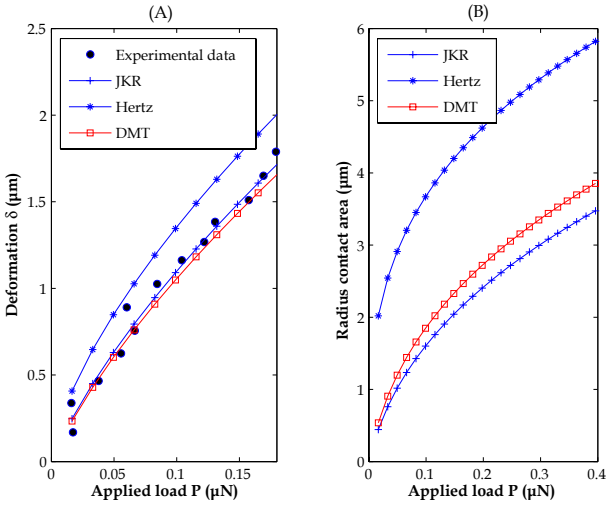

Fig. 11. (A) Estimation of the biological sample deformation δ as function of the simulated load force P using Hertz, JKR and DMT theories compared to the experimental data. (B) Estimation of the contact area radius a using the Hertz, JKR and DMT theories.

area radius a in the latter case is approximately twice greater than predicted by the JKR and the DMT models. We have observed small deviation between the JKR and the DMT models for force-deformation prediction. In the case of the contact area radius estimation with large deformations, deviations have been observed in order of 0.3 μm of magnitude. According to the literature, the JKR theory in opposition to DMT theory is applied for soft materials, high energy adhesion and large radius [16], which is the case in this study.

IV. 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 cervix Epithelial Hela adherent 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 from the mechanical cell characterization process are predicted using appropriate models taking in account both adhesion forces and finite sample thickness.

V. Future work

Current work involves modelling the soft contact mechanisms using a tipless cantilever taking in 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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