Towards a Non-Destructive *In Vitro* Biomechanical Characterization

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Abstract—**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 interactions mechanics 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 and calibration of the different modules which compose the experimental setup are detailed. Experimentation on the mechanical cell haracterization under *in vitro* conditions on human adherent cervix Epithelial Hela 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; Human adherent cervix Epithelial Hela cells mechanical characterization.**

I. Introduction

Robotics and microrobotics techniques can play an important role for addressing the study of mechanical cell response. Up to date, several experimental setups have been developed to identify the control mechanisms of the mechanical cells response [1]-[5]. These significant research efforts make possible the measurement of relevant mechanical properties (Young's modulus, bulk modulus, surface roughness, ...). However, most of these studies have been performed in non-biological clean room environment. Since elementary biological functions and mechanical properties of biological cells are widely affected by the experimental conditions, some identified properties may not be relevant. In fact, due to the structural complexity of various adherent cells (such as the deformable cytoskelton formed by a three dimensional intercellular network of interconnected filamentous biopolymers), significant differences on the cell mechanical responses can be observed.
Furthermore, there is recently a great interest in the field of biopolymers, for the quantification of the viscoelastic behavior of biological samples. To suite this need it is necessary to perform an accurate automatic mechanical characterization process as well as supervised micromanipulation tasks in a cyclical operating mode. Moreover, since 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.
In the next section, the context and motivations of these works are presented. The experimental device produced, called Force Bio-Microscope *(FBM)*, is presented and detailed in the section III. The cantilever spring constant calibration is explained in section IV. Section V is devoted to the description of our preliminary experimentation and analysis of cell mechanical characterization using the automated *FBM* under *in vitro* conditions on human adherent cervix Epithelial Hela cells. Finally, planned future work on the cell mechanical characterization using the autonomous force sensing and measurements capabilities of the *(FBM)* are introduced in section VI.

II. SPM Techniques for the Cell Mechanical Characterization

Among the robotics and microrobotics systems developed during the last decade to perform mechanical characterization of biological samples, the most promising ones involves Scanning Probe Microscopy (SPM) techniques for nanoscale. These techniques have the potential to give an accurate quantitative information on local forces and mechanical interactions at the nanoscale. The Atomic Force Microscope (AFM) has become a commonly used microindenter tool to measure mechanical properties of the biological samples [6]-[11]. Despite the AFM's multiple operating modes, the mechanical characterization of biological samples is mainly performed in contact mode. In this case, a flexible cantilever with low spring constant and an atomic sharp tip is brought in contact with the biological sample. Deflection of the cantilever which results of the interaction between the microindenter and the sample is monitored by a split

photodiode and a laser beam reflected on the back of the cantilever.

Some problems are commonly associated with the use of standard commercial cantilevers carrying usually sharp tip for soft materials characterization. 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. Consequently, uncertainties are introduced for choosing the appropriate fitting analytical model and the initial contact point is difficult to maintain on the sample during the mechanical characterization process. Even, the standard commercial cantilevers carrying usually sharp tips may cause irreversible damages on the external lipids cell biomembranes. Hence, non-destructive and effective approaches for the cell mechanical characterization are more suitable.

In our opinion, the mechanical characterization of adherents biological cells using tipless cantilevers are rare and need to be investigated. Moreover, experiments conducted with sharp indenter have been performed in non-biological environment. Since elementary biological functions and mechanical properties of biological cells are affected by the experimental conditions, the mechanical characterization process should be done in real biological environments. The issue of the *in vitro* mechanical characterization of biological cells using a tipless chemically inert cantilever is addressed in this paper.

III. 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. The overall configuration of the *FBM* and the different working components are shown in figure 1.

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) are 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
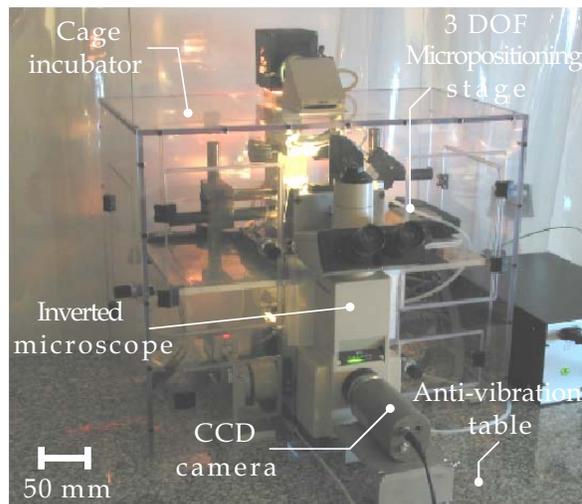

Fig. 1. The *FBM* experimental setup overview.

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. Hence, a sensitive and accurate detection device is produced for the aim of our study. 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 sample to be studied is accurately positioned below the cantilever by a 3 DOF's (x,y and z) micropositioning encoded stages with a submicrometer resolution (0.1 μm). The kinematics features of the micropositioning stages 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. A magnified picture of the cantilever with the focused laser beam on its reflective surface is shown in the same figure.

For the preliminary study, we focused on force feedback control of cantilever flexural deflection. Thus, only the vertical motion of micropositioning stage is servoed. By knowing the vertical position of the micromotors as well as the 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.

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
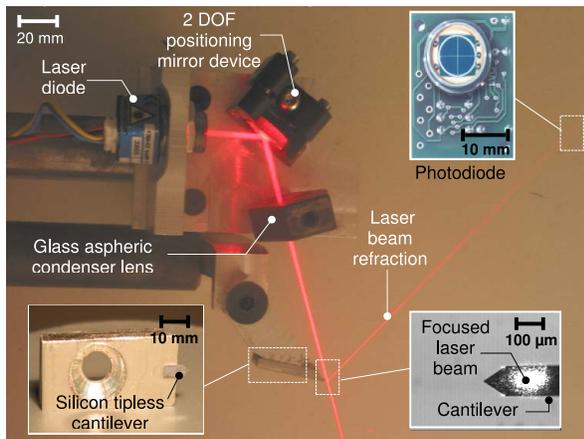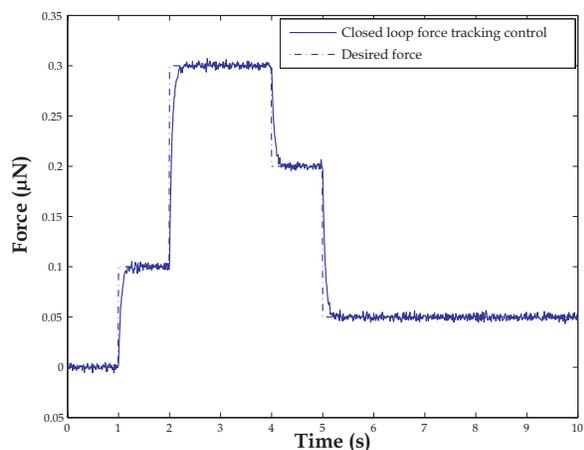
Fig. 2. The mechanical sensing unit.

Fig. 3. Experimental results on the force feedback control approach.

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. 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 (Figure 4).

C. Clean room 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. The incubating system is formed with a controlled heating module which maintains temperature at 37 ^oC using a single thermocouple probe. The desired temperature of 37 ^oC is reached in 2 hours. 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 stages. Temperature control is achieved by means of a configurable PID controller communicating with a water bath via serial port to the master computer. The whole system including the *FBM* is placed in a negative pressure clean room to protect the biological environment.

IV. CANTILEVER SPRING CONSTANT CALIBRATION

Since the beginnings of Scanning Force Microscopy (SFM), many methods for the spring constant calibration have been developed and studied. These methods agree to discard the use of the cantilever's dimensions since the determination of the thickness is problematic. To overcome this problem, we use a dynamical frequency response method for the thickness determination. As this method is quite accurate, the spring constant calibration is done according to the dimensions of the cantilever.
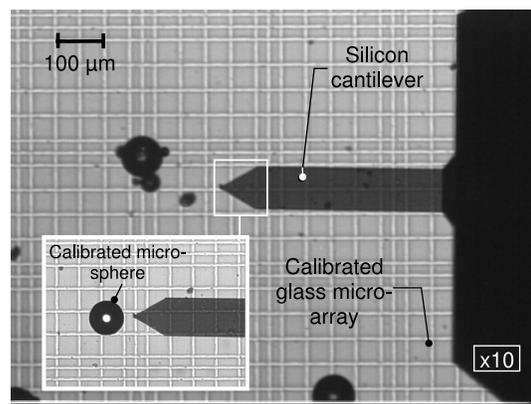

Fig. 4. Cantilever length and width measurements, $L = 450 \mu m$ and $l = 90 \mu m$.

The length and width of the cantilever are measured by an optical method using a glass calibrated micro-array. Figure 4 shows the calibration process under the microscope. The obtained values for length and width are in good agreement with those of the manufacturer. Knowing all the dimensions of the cantilever, the spring constant is then calculated by a static method.

A. Frequency response method for the determination of cantilever's thickness

Let us consider a cantilever of uniform section S, density ρ, Young's modulus E', and inertial moment I. Each point of the cantilever should validate the classic wave equation for a beam in vibration, under the hypothesis of undamped system:

$$\rho S \frac{\partial^2 v}{\partial t^2} + E' I \frac{\partial^4 v}{\partial x^4} = 0 \quad (1)$$

where v is the instantaneous deformation of the beam depending on time and position. The displacement can be

written in two parts; one depending on the position along x axis, and another one on time: $v(x,t) = f(x)g(t)$.

In order to solve equation (1) i.e. to determine the solution's constants, boundary conditions for the cantilever are needed. The fixed end of the cantilever must have zero displacement ($v(0) = 0$) and zero rotation ($\theta(0) = 0$). The free end of the cantilever cannot have a bending moment ($M(L) = 0$) or a shearing force ($T(L) = 0$).

The system of boundary equations accepts a solution only if the determinant is zero, which is equivalent to:

$$1 + \cos\mu \cosh\mu = 0 \qquad (2)$$

With $\mu = (\omega^2 \frac{\rho S}{E'I})^{\frac{1}{4}} \alpha L$, equation (2) gives one condition on μ to be respected, which defines the eigen frequency of the system. The four first solutions of this transcendent equation, determined numerically, are listed below:

μ_1	μ_2	μ_3	μ_4
1.875	4.693	7.854	10.995

Given these solutions, if the length, and the experimental eigen frequency of the cantilever are known, the mean value of the thickness can easily be calculated by the flowing equation:

$$<h> = \frac{1}{N} \sum_{i=1}^{N} \omega_i \frac{L^2}{\mu_i^2} \sqrt{\frac{12\rho}{E'}} \qquad (3)$$

With N the number of the measured eigen frequency. In our case, the use of eigen frequency to determine the last dimension of the cantilever improves the accuracy, in comparison to the optical method, by a factor of 100. Moreover, this method can be achieved before each experimentation. Actually, the useful life of the cantilevers is very short (they can only be used once because of biological environment contraints), and the calibration process is repeated at every cantilever exchange.

B. Static approach for the spring constant cantilever determination

Knowing the dimensions of the cantilever and its material properties, the spring constant of a rectangular cantilever is given by $k = 3EI/L^3$, with the inertia momentum $I = lh^3/12$.

All the results for different modes (experimental results of mode 3 are unexploitable, because some mechanical parts of the microscope start resonating) are summarized in the following table:

	Modes number			
	1	2	4	
μ	1.875	4.693	10.995	theory
$f(kHz)$	12.63	82.4	446	measured
$h(\mu m)$	1.516	1.579	1.557	estimated
$k(N/m)$	0.187	0.211	0.202	estimated

The difference on the value of k can be explained by the error on the measured eigen frequency, but also because the estimated thickness is a mean value. Actually, the variation of the thickness all along the cantilever affects differently the eigen frequency of each mode.

The variation from the mean value of k are weak and acceptable, the logarithmic error is about 3.7%, with a contribution of the thickness for this error of 1.9%. In comparison with the spring constant announced by the manufacturer, the mean value is near for this batch, but the uncertainty is deep lower (3.7% instead of 90%).

C. Spring constant cantilever validation

These experiments aim to validate the force measurements accuracy of the mechanical sensing unit, including cantilever and optical laser system. Two measurements are performed. In the first one, a previously calibrated cantilever is pressed on a rigid substrate. For the second one, another calibrated cantilever (from the same batch) is pressed against the other one. A silicon sphere is placed between the two cantilevers to avoid adhesion effects and to guarantee punctual contacts on both sides (cf. figure 5).
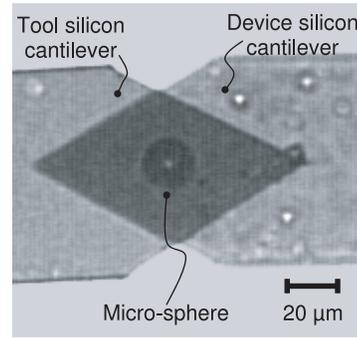

Fig. 5. Cantilever/sphere/cantilever contact.

The cantilever/substrate mechanical interaction is used to calibrate the whole system. The photodiode gives an output voltage corresponding to the translation of the laser beam. As the cantilever has been calibrated before, for a displacement of 1 μm the sensed force is 0.2 μm. This technique allows to avoid to calculate the laser optical path, and the accurate calibration of the photodiode. In the case of the cantilever/cantilever interaction, the mechanical system is considered as two springs in serial, with a respectively spring constant k_1 and k_2. The equivalent stiffness K_{eq} can be expressed as function of k_1 and k_2 as: $1/k_{eq} = 1/k_1 + 1/k_2$. Figure 6 shows the experimental force sensed by the measuring cantilever for both rigid subtract and cantilever/cantilever mechanical interaction. Since the spring constant corresponds to the gradient of curves, the cantilever/cantilever curve leads to a value of K_{eq}=0.101 N/m on average. As the measuring cantilever is calibrated (k_1=0.201 N/m), we found k_2=0.203 N/m which is in adequacy with the expected results.
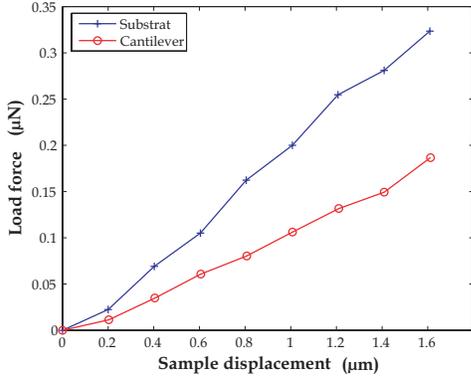
Fig. 6. Cantilever/substrate mechanical interaction.

V. *In vitro* MECHANICAL CHARACTERIZATION EXPERIMENTS

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. The cervix *(EpH)* cells can be assimilated morphologically to an elliptical cells with a thin surrounding biomembrane which has two functions : ensuring both protection of the cytoplasm and adhesion feature on the subtract. In the present study, the average dimensions of the biological sample is 10 μm long, 9 μm large and 6 μm height.

A. Cell's mechanical response characterization

Figure 7(A) shows the experimental curves of the photodiode output as a 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 is 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 7(B) which presents the sample deformation δ as function of the load force applied by the cantilever.

B. In vitro *efficiency approach for cell mechanical characterization*

In order to adress either the efficiency of the *in vitro* clean room unit or how mechanical cell properties can be affected by the environmental culture conditions, we have experimented automatic and cyclical spectroscopy operation 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. Since the purpose of this study is to observe the difference which can occur on mechanical behaviour of the studied biological sample, experimentation is initially conducted using the incubating system. Figure 8
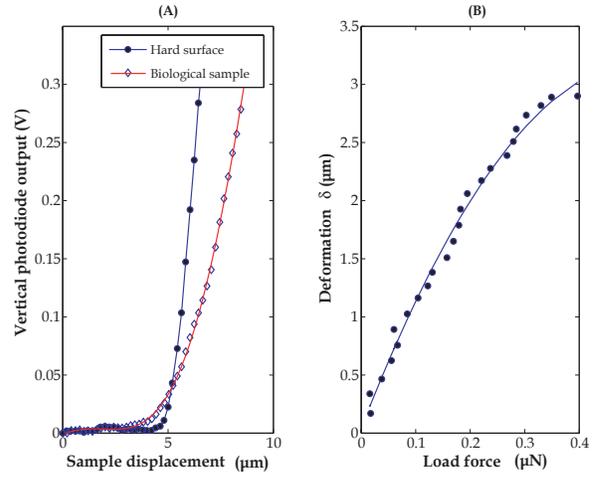

Fig. 7. (A) Experimental data of the photodiode output as function of the sample displacement performed on both single *EpH* cell and hard surface. (B) Experimental curve of the sample deformation δ as function of the applied load by the cantilever.
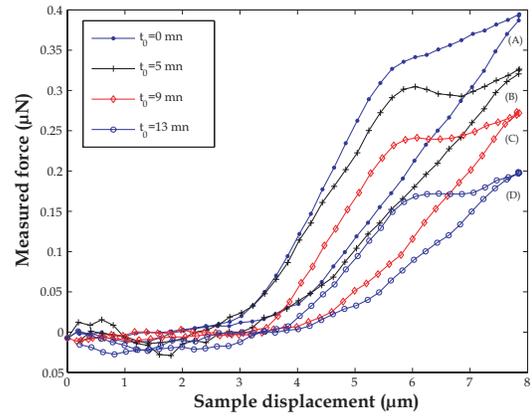

Fig. 8. Evolution of the measured force as a function of the sample displacement for different elapsed times $t_0 = 0, 5, 9$ and $13\ mn$.

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 environmental culture conditions. This difference suggests that the intra or extra-cellular matrix react to the variation of temperature.

VI. CONCLUSION

This paper has presented the development of a micro-force sensing system for *in vitro* single cell mechanical characterization. The experimental setup combines Scan-

ning 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 modules with autonomous force sensing and measurements capabilities. Each module is designed, calibrated or configured towards an effective *in vitro* cell mechanical characterization.

Experimentation on the cell 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.

VII. FUTURE WORK

Current work involves modelling the contact mechanics resulting from the cell mechanical characterization process using a tipless cantilever. Some important issues will be addressed such as friction, osmotic influence, non-linear elastic behaviour and adhesion forces. Relevant mechanical properties of biological samples will be extracted using an appropriate fitting model derived from soft contact mechanics theories. 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 mechanosenitivity and mechanical transduction mechanisms at the microscale for biological cells or tissue configuration.

REFERENCES

[1] Y. Sun and B.-J. Nelson, "Microrobotic cell injection", Proc. *Int. Conference on Robotics and Automation*, Seoul, Korea, pp. 620-625, May 2001.
[2] K.-J. Van Vliet, G. Bao and S. Suresh, "The biomechanics toolbox: experimental approaches for living cells and biomolecules", *Acta Materialia Journal*, vol. 51, pp. 5881-5905, August 2003.
[3] D.-H. Kim, S. Yun and B. Kim, "Mechanical force response of single living cells using a microrobotic system", Proc. *Int. Conference on Robotics and Automation*, New Orleans, USA, pp. 5013-5018, April 2004.
[4] J. Guck, R. Ananthakrishnan, C.-C. Cunningham and J. Kas, "Stretching biological cells with light", *J.Physics:Condens. Matter*, vol. 14, pp. 4843-4856, May 2002.
[5] M. Lukkari and P. Kallio, "Multi-purpose impedance-based measurement system to automate microinjection of adherent cells", Proc. *Int. Sympos. on Computational Intelligence in Robotics and Automation*, pp. 20-26, 2005.
[6] M., Radmacher, "Measuring the elastic properties of biological samples with AFM", *IEEE Engineering in Medicine and Biology*, pp. 47-57, 1997.
[7] O. Sahin, G. Yaralioglu, R. Grow, S. -F. Zappe, A. Atalar, C. Quate and O. Solgaard, "High-resolution imaging of elastic properties using harmonic cantilevers", *Sensors and Actuators A*, vol. 114, pp. 183-190, February 2004.
[8] S. Park, K. Costa and G. Ateshian, "Microscale frictional response of bovine articular cartilage from atomic force microscopy", *Journal of Biomechanics*, vol. 37, 1687-1697, Februrary 2004.
[9] Y. Sun and B.-J. Nelson, "MEMS for cellular force measurements and molecular detection", *Journal of Information Acquisition*, vol. 1, no. 1, pp. 23-32, January 2004.
[10] E.-K. Dimitriadis, F. Horkay, J. Maresca, B. Kachar and R.-S. Chadwick, "Determination of elastic moduli of thin layers of soft material using the atomic force microscope", *Biophysical Journal*, vol. 82, pp. 2798-2810, May 2002.
[11] X. Yao, J. Walter, S. Burke, S. Stewart, M.-h. Jericho, D. Pink, R. Hunter and .-J. Beveridge, "Atomic force microscopy and theoretical considerations of surface properties and turgor pressures of bacteria", *Colloids and Surfaces B: iointerfaces*, vol. 23, pp. 213-230, May 2002.