available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/jmbbm

Tutorial

Characterization of cellular mechanical behavior at the microscale level by a hybrid force sensing device

Mehdi Boukallel[a], *Maxime Girot*[b], *Stéphane Régnier*[b,*]

[a] *CEA LIST, Service de Robotique Interactive, BP 6, 18 Route du Panorama, 92265 Fontenay aux Roses, France*
[b] *Institut des Systèmes Intelligents et de Robotique, Université Pierre et Marie Curie, CNRS FRE 2507, BP 61, 18 Route du Panorama, 92265 Fontenay aux Roses, France*

ARTICLE INFO

Article history:
Received 24 January 2008
Received in revised form
12 September 2008
Accepted 18 September 2008
Published online 9 October 2008

ABSTRACT

This paper deals with the development of an open design platform for characterization of mechanical cellular behavior. The resulting setup combines Scanning Probe Microscopy (SPM) techniques and advanced robotic approaches in order to carry out both prolonged observations and spatial measurements on biological samples. Visual and force feedback is controlled to achieve automatic data acquisition and to monitor process when high skills are required. The issue of the spring constant calibration is addressed using an accurate dynamic vibration approach. Experimentation on the mechanical cell characterization under *in vitro* conditions on human adherent Epithelial Hela cells demonstrates the viability and effectiveness of the proposed setup. Finally, the JKR (Johnson, Kendall and Roberts), the DMT (Derjaguin, Muller and Toporov) and Hertz contact theories are used to estimate the contact area between the cantilever and the biological sample.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

It is now well known that biological cells sense the mechanical disturbance from their environment. The resulting response to mechanical input is determinant in governing the cell's behavior, not only in cell culture, but also within the whole organism. The mechanical cell response is a complex phenomenon which involves different cell components ranging from the molecular to micrometer size level (nucleus, cytosqueletton, lipidic membrane, ...) inducing signals from the cell surface receptors to the nucleus. Understanding the mechanical behavior of the cell, first requires an accurate knowledge of both force and stress distribution within the cell membrane. This feature is crucial since determining the force/stress of various types of load can dictate the mechanical cell response. Moreover, it requires real-time observation of cell development (growth, motility, apoptose, ...) in order to correlate the molecular bio-chemical interactions with the mechanical response of the cell. Today, there is no prediction time model which can predict the morphological change of the cell due to external mechanical stimuli. Therefore, ongoing observations combined with an effective morphological tracking approach are required. Finally, mechanical cell characterization experiments need to be conducted in an *in vitro* environment so that the cell can be kept several hours in a living state for prolonged experiments. Therefore, reliable and automated measurements are required to reduce human involvement during the measurement process.

* *Corresponding author.*
E-mail addresses: mehdi.boukallel@cea.fr (M. Boukallel), maxime.girot@robot.jussieu.fr (M. Girot), stephane.regnier@robot.jussieu.fr (S. Régnier).

1751-6161/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jmbbm.2008.09.001

According to literature, much effort has been devoted to identifying the mechanism by which the cell senses the external mechanical stimuli. In fact, a variety of approaches have been developed – whether to mechanically stimulate cells, sense force distributions or to determine the mechanical properties of the cells – such as micropipette aspiration, atomic force microscopy, magnetocytometry or optical tweezers principles (Lukkari and Kallio, 2005). Among these approaches, the most promising ones involve Scanning Probe Microscopy (SPM) techniques for the nanoscale level. These techniques have the potential to give an accurate quantitative information relative to local forces and contact mechanics (Girot et al., 2006). The Atomic Force Microscope (AFM) has become a commonly used tool in the field of bioscience (Radmacher, 1997; Mahaffy et al., 2000). A flexible cantilever with a low spring constant (0.1–0.2 N/m) and an atomic sharp tip is usually brought in the vicinity of the biological sample. Deflection of the cantilever as a result of the interaction between the microindenter and the sample is monitored by a split photodiode and by using a laser beam reflected on the back of the cantilever. Some commercial solutions are available for performing experiments on Life Science (Veeco, Olympus, Andor, . . .) but only a few of them are effective studies. The most critical component of the commercial solution is the xyz scanning device based piezotube. Of course, the piezotube can achieve high spacial positioning features with Angstrom resolution (closed loop feedback) but cells are relatively large structures, several dozen of micrometers in diameter and several micrometers in height. Thus, the scanning range must be appropriate to this size scale. Typical AFM systems for cell imaging exhibit a lateral scan range about 100 μm and a vertical scan range of about 10 μm. At these extensions, the piezotube exhibits a high nonlinear behavior including, creep and hysteresis. Reproducible positioning is only possible with effective design based on expensive electronic circuits. We associate several problems with the use of a standard commercial cantilever with a sharp tip for life science requirements. In fact, the nanometer dimensions of the tip can cause significant local strain higher than those in the elastic domain. Furthermore, depending on the magnitude of the force applied to the soft samples, both the cantilever tip and the samples can be easily damaged, consequently the local strain applied in the indented area is modified. Of course, some authors have presented a hybrid AFM tip where spheres with a few micrometers radius are fitted to the tip, but this approach requires accurate placement of the micro-sphere as well as dexterous manipulation.

A sharp tip is commonly used since their precise shape can be determined using an SEM (Scanning Electron Microscope) and the contact mechanics between the tip and the cell can be analytically predicted. The Hertzian model which describes the relationship between force and indentation is the commonly used approach for a contact mechanics based prediction model. Some authors have shown that in the case of soft contact mechanics, models derived from linear elasticity can lead to significant errors. Moreover, due to imperfections of the curvature of the tip radius, an unknown contact region results between the probe and the sample. Consequently, uncertainties are introduced for choosing the appropriate fitting analytical model. The specific objective of this paper is the presentation of the development of an open platform where *in vitro* cell mechanical characterization can be conducted. This system, called the Force Bio-Microscope, combines SPM techniques and advanced robotic approaches. A tipless chemically inert cantilever is used in this study. The spring constant calibration using an accurate determination of the cantilever thickness is addressed in this paper. We use a dynamical frequency response method for the spring constant cantilever calibration. The contact mechanics is modeled with appropriate models taking into account the adhesion forces. More precisely, the JKR (Johnson, Kendall and Roberts), DMT (Derjaguin, Muller and Toporov) and Hertz contact theories are used to estimate the contact area between the tipless cantilever and the cell. In order to demonstrate the effectiveness of the developed system in the Life Science field, *in vitro* experiments have been conducted on Epithelial Hela cells *(EpH)*.

2. Experimental setup overview

The Force Bio-Microscope *FBM* device is an hybrid AFM microscope associating both scanning microscopy approaches and biological environment constraints. The *FBM* consists mainly of three units: the mechanical sensing unit which carries out 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 a biological environment (Fig. 1).

The *FBM* experimental setup provides suitable conditions for the study in a controlled environment so that the biological cells can be kept several hours in a living state by using a cage incubator. Therefore, the mechanical measurement process can be done on the biological sample for an extended time period.

A master computer is used to drive the *FBM* in an automatic operating mode based on referenced force/vision control. Data acquisition processing between the master computer and the *FBM* is achieved by the use of two specialized PCI cards (Matrox and National Instrument). A user-definable graphical interface has been developed in order to make configuration of the experiments easier. To avoid undesirable mechanical vibrations during the cell characterization process, the *FBM* experimental setup is installed on an anti-vibration table. The overall configuration of the *FBM* and the different working components are shown in Fig. 2.

2.1. *Mechanical sensing unit*

The mechanical sensing unit is based on detecting the deflection of a cantilever by an optical technique. A four quadrant photodiode (CentroVision) with internal amplifiers associated with a 650 nm low power collimated laser diode (Vector Technology) is used in order to perform both axial and lateral measurement of nanoNewtons forces. 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

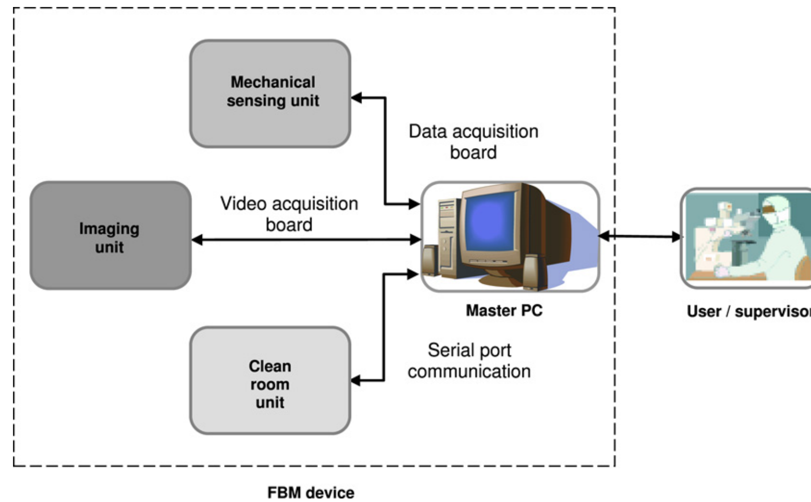

Fig. 1 – Block diagram of the Force Bio-Microscope *FBM* system.

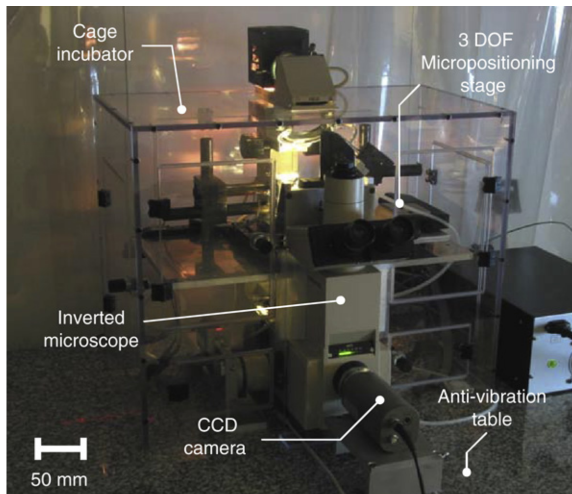

Fig. 2 – The *FBM* experimental setup overview.

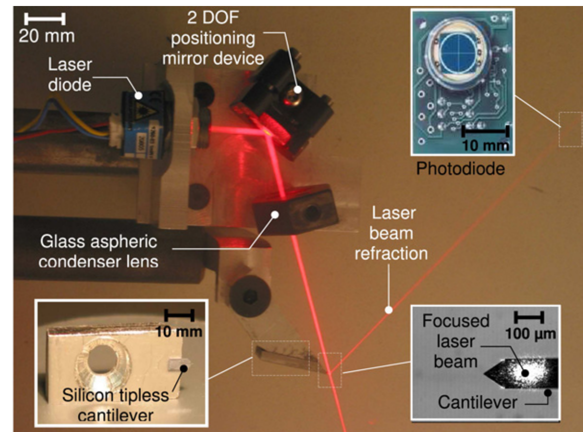

Fig. 3 – The mechanical sensing unit.

and an 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/µm.

A low spring constant (0.2 N/m), uncoated, tipless, silicon cantilever (Nanosensors) is used as a probe for the mechanical cell 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 (Physik Instrumente) with a submicrometer resolution (0.1 µm). The kinematic features of the micropositioning stages enables the achievement of accurate mechanical measurements in a workspace of $25 \times 25 \times 25$ mm^3 with a good repeatability. The configuration of the mechanical sensing unit including the optical detection device is presented in Fig. 3. 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 the 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, an optimized Proportional and Derivative (PD) controller was designed to ensure optimal performance control. The (PD) terms are optimized using the Ziegler–Nichols method.

2.2. *Imaging/grabbing unit*

The imaging/grabbing unit consists of an inverted microscope (Olympus IMT-2) with Nikon 10× and 20× objectives. A phase contrast device is mounted on the microscope in order to operate precise contrast. The inverted microscope is fitted out with a CCD camera (754 × 488 pixels resolution). Using a frame grabber and a specialized imaging library package (Matrox Imaging) associated with the CCD camera, automatic mechanical characterization based on image feature tracking is achieved. The pixel to real world calibration of the CCD camera is obtained by means of a calibrated glass micro-array as well as calibrated micro-spheres (cf. Fig. 4).

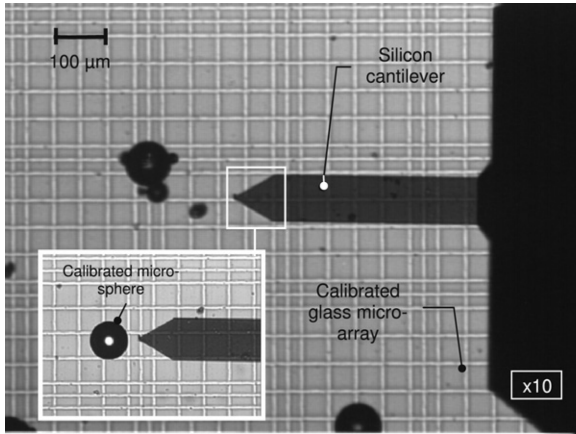

Fig. 4 – Calibration of the CCD camera based on geometrical calibration.

2.3. Clean room unit

The biological samples need specific requirements to be kept alive outside the *vivo* conditions, and to carry out prolonged observations. Besides the biological nutrition medium, biological cells need a temperature condition of 37 °C and 5% of CO_2 gas. The incubating system is formed with a controlled heating module which maintains the temperature at 37 °C using a single thermocouple probe. The desired temperature of 37 °C is reached in 2 h. The cage incubator ensures a temperature stability within the 0.1 °C. A mixed stream composed of 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 positive pressure clean room to protect the biological environment.

3. Cantilever spring constant calibration

The length and width of the cantilever are measured by an optical method using the same process as for the camera calibration. The values obtained for length and width ($L = 450$ µm and $l = 90$ µm) correctly correspond to those of the manufacturer. Knowing all the dimensions of the cantilever, the spring constant is then calculated using a static method.

3.1. 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 an undamped system:

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

where υ 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 the x axis, the other on time: $\upsilon(x, t) = f(x)g(t)$.

In order to solve Eq. (1) i.e. to calculate the solution's constants, the boundary conditions for the cantilever are needed. The fixed end of the cantilever must have zero displacement ($\upsilon(0) = 0$) 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 only accepts a solution only if the determinant is zero, which is equivalent to:

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

With $\mu = (\omega^2 \frac{\rho S}{E'I})^{\frac{1}{4}} L$, Eq. (2) gives one condition on μ to be respected, which defines the eigen frequency of the system. The first four 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 following equation:

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

with N the number of the measured eigen frequency. In our case, using the 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 prior to each experiment. Actually, the useful life of the cantilevers is very short (they can only be used once because of biological environment constraints), and the calibration process is repeated at every cantilever exchange.

3.2. Static approach for the determination of the spring constant cantilever

Knowing the dimensions of the cantilever and its material properties, the spring constant of a rectangular cantilever is given by $k = 3E'I/L^3$, 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 (µm)	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

Fig. 5 – Cantilever/sphere/cantilever contact.

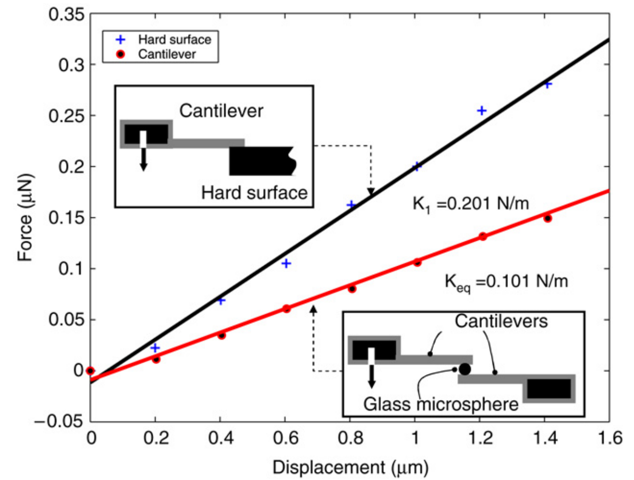

Fig. 6 – Experimental determination of the cantilever spring constant.

of the thickness all along the cantilever affects the eigen frequency of each mode differently.

The variations 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%. Compared with the spring constant announced by the manufacturer, the mean value is close for this batch, but the uncertainty is greatly reduced (3.7% instead of 90%).

3.3. *Validation of the experimental spring constant cantilever*

These experiments aim to validate the accuracy of the force measurements of the mechanical sensing unit, including the cantilever and the optical laser system. Two measurements are carried out. For the first, a cantilever that has been previously calibrated is pressed onto a rigid substratum. For the second, 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. Fig. 5).

The cantilever/substratum mechanical interaction is used to calibrate the whole system. The photodiode gives an output voltage corresponding to the translation (tilt) of the laser beam. As the cantilever has been previously calibrated, for a displacement of 1 μm, the sensed force is 0.2 μm. This technique allows one to avoid to calculate the laser optical path as well as the accurate calibration of the photodiode. In the case of the cantilever/cantilever interaction, the mechanical system is considered as two springs in sequence, with a respective spring constant of k_1 and k_2. The equivalent stiffness K_{eq} can be expressed as a function of k_1 and k_2 as: $1/K_{eq} = 1/k_1 + 1/k_2$. Fig. 6 shows the experimental force sensed by the measuring cantilever for both the rigid substratum and the cantilever/cantilever mechanical interaction. Since the spring constant corresponds to the gradient (slope) of the 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 keeping with the expected results.

4. *In vitro* mechanical characterization experiments

The Epithelial Hela cells *(EpH)* are prepared on Petri dishes with a 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 *(EpH)* cells can be assimilated morphologically to an elliptical cells with a thin surrounding biomembrane which has two functions: to ensure both the protection of the cytoplasm and the adhesion features on the subtractum. In the present study, the average dimensions of the biological sample are 10 μm long, 9 μm large and 6 μm height.

4.1. *Cell's mechanical response characterization*

Fig. 7(A) shows the experimental curves of the photodiode output as a function of the sample displacement (z) performed on both a single *EpH* cell and a 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 z and the cantilever deflection d. The non-linear elastic behavior of the *EpH* can be seen in the Fig. 7(B) which presents the sample deformation δ as a function of the load force applied by the cantilever.

The elastic behavior of the *EpH* cells is also investigated by the *FBM* device. Cyclical and automatic approaches and retract experiments were conducted on the same biological sample during 2 h with 3 min intervals (scan rate 0.05 $\mu m\ s^{-1}$). 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 μm/s). Fig. 8(A) shows 3 approach and retract curves monitored at different time intervals ($t = 0$ min, 40 min and 80 min) during the experiments. A single referenced approach/retract curves performed on a hard surface is given in Fig. 8(B). According to the data collected, the *EpH*