33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

A study on the cellular structure during stress solicitation induced by BioMEMS

Raffaella Fior, Stefano Maggiolino, Barbara Codan, Marco Lazzarino and Orfeo Sbaizero.

Abstract— **The investigation of single cells is a topic in continuous evolution. The complexity of the cellular matrix, the huge variety of cells, the interaction of one cell with the other are all factors that must be taken into consideration in the study of the cellular structure and mechanics.**

In this project, we developed different types of bioMEMS for cell's stretching, both transparent devices based on silicon nitride and non-transparent silicon based. While the use of silicon devices is limited to reflection microscopes, transparent bioMEMS can be used with transmission and reflection microscopes but can also be easily coupled with other tools such as patch clamp analyzers or atomic force microscope. This improvement will open brand new possibilities in the biological investigation field.

We used these two BioMEMS to stretch a single cell in a controlled way and, as a first investigation, we focused on its morphology. We noticed that during a controlled stretch, cells react to the applied deformation. A hysteretic behavior on the ratio between area and perimeter has been highlighted.

I. Introduction

THE understanding of cells and tissues physiology is a huge field in continuous expansion. The knowledge of single living cells could help finding a cure to different diseases and cancer.

The complexity of the cellular matrix, the differences in cell's type or the interaction between cells are all factors that must be considered in the study of the cellular structure and its mechanics. Single cell tests can give hints on some of the mechanisms involved in cell's mechanics: (i) morphological changes due to cytoskeleton rearrangements; (ii) Young modulus modifications after cyclic stretch or its variation compared to that of transfected cells; (iii) the activity of mechano-sensitive ion channels.

Many different techniques for the mechanical analyses of living cells are known. Most of them involve the study of a small portion of a cellular membrane. Atomic Force Microscope (AFM) indentation and optical tweezers, just to mention some common techniques, have been used to locally investigate the cell [1], [2]. These methods cannot give information about the behavior of the mechanics of a whole cell in a realistic time period and the given information involves a small section of the cellular membrane. On the contrary, bioMEMS represent fast and easy systems that can be useful for the study of cells in adhesion. A stretching protocol can last a couple of minutes and gives lots of data about cell mechanics and morphology.

In this project, we developed and employed two different bioMEMS for cell's stretching: non-transparent silicon based devices (first generation); and completely transparent silicon nitride/silicon dioxide (second generation), [3-4]. While the use of the first generation of devices is limited to reflection microscopes, transparent bioMEMS can be coupled with transmission and reflection microscopes but can also be easily coupled with patch clamp analyzers or AFM. This improvement allows three-dimensional studies on stretched cells or the evaluation of the activity of mechano-sensitive ion channels, just to mention some possible applications.

We used these two models of bioMEMS to stretch a single cell in a controlled way and, in this study we focused on the changes in morphology during the test trying to find a model for this behavior.

II. DEVICES REALIZATION

A. BioMEMS project

To carry out a single cell stretching, a preliminary condition is to realize biocompatible devices. Moreover, they must operate in liquid and avoid modifications in the physiology of living systems.

Micro fabricated devices can provide these requirements.

With these constrains, we designed and produced bioMEMS capable of single cell stretching.

The design is shown in Figure 1. Three are the main constitutive parts: (i) the cell measurement area, in which cells are stretched; (ii) the sensor, which allows the evaluation of the forces involved and (iii) the actuator, which ensures the prescribed stretch.

The sensing zone represents the most important element. The forces involved in cell stretch and deformation are very low so the sensitivity of the system should be appropriate. According to K. Addae-Mensah and J. P. Wikswo [5], to obtain an effective measurement of the mechanical

Manuscript received April 15, 2011.
R. Fior is with the Department of Industrial Engineering and Information Technology at University of Trieste, 34127 Trieste Italy (corresponding author; phone: +39 040 558 7974; fax: +39 040 572044; email: rfior@units.it)
S. Maggiolino is with the Department of Industrial Engineering and Information Technology at University of Trieste, 34127 Trieste Italy (email: smaggiolino@units.it)
B. Codan is with the Department of Industrial Engineering and Information Technology at University of Trieste, 34127 Trieste Italy (email: bcodan@units.it)
M. Lazzarino is with the IOM-CNR Laboratorio TASC 34012 Trieste Italy (email: lazzarino@tasc.infm.it)
O. Sbaizero is with the Department of Industrial Engineering and Information Technology at University of Trieste, 34127 Trieste Italy (email: sbaizero@units.it)

properties of a cell, the forces must be in the range of 500-1200nN. We used finite elements modeling (FEM) to accurately design this system.

The cell measurement area is composed by two platforms with 2µm gap between them. One is anchored to the substrate, while the other is suspended and can provide the cell deformation.

The actuator is the element of the device which links the motor (a piezo-electric actuator, external to the bioMEMS) to the platform.

The cell is plated in the middle of the testing area, the top section of the platform is anchored and the bottom can be actuated to provide the stretching.

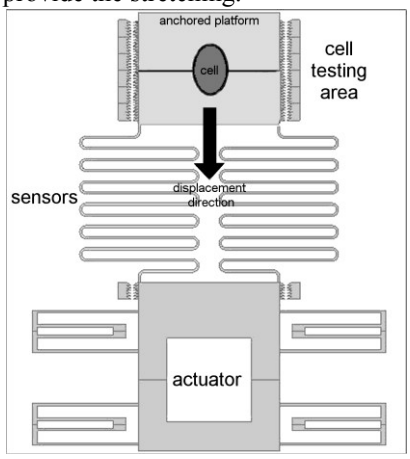

Figure 1: Design of the BioMEMS. From the top it is possible to see the cell testing area. A sketch of a cell represents the position in which it will be plated. The sensor is composed by two sensitive springs. From their deformation is possible to evaluate the forces involved in the stretching. The actuator is the area in which the piezo-motor is connected and from which the stretching is provided. The back arrow shows the direction of the displacement.

B. Silicon based bioMEMS

The first generation of devices has been made using the Multi-User Processes (MUMPs), a patented process that provides a three-layer polysilicon surface micromachining process [6].

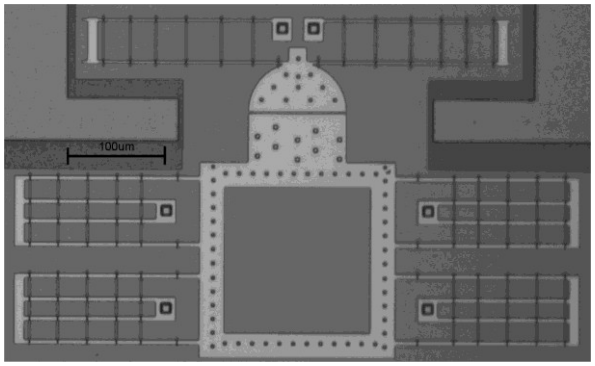

Figure 2: Optical image of a silicon based BioMEMS. From the top there are the sensors, the cell platform (100µm wide) and the actuation area.

Polysilicon is the structural material, the deposited silicon oxide is the sacrificial layer, and silicon nitride is used as electrical insulation between polysilicon and the substrate.

In Figure 2 a device of first generation is shown. All the suspended structures are in polysilicon. The resolution of the technique is 0.25µm and the smallest granted feature is 2 µm by 2 µm. This factor is very important in terms of reproducibility of single cell measures.

However, being a commercial technique, there are some limitations in the process such as the prescribed thickness, the materials that can be used and the order of the layers deposition. Above all these MEMS are not transparent.

C. Silicon dioxide/silicon nitride bioMEMS

In order to overcome the aforementioned problems, related to the silicon based bioMEMS, we developed a custom process to realize transparent bioMEMS.

For this second generation of stretchers, we focused on transparency. We are able to obtain structures with more the 80% of transmittance for the visible wavelengths.

These devices, obtained using micro-fabrication techniques, are silicon nitride based and the substrate is silicon dioxide. The suspended structures are realized on a sacrificial layer made of amorphous silicon that is removed before using the devices.

We have been able to realize geometries with a very high aspect-ratio. In particular, a single harm of the z-spring of the sensing elements, in 2 µm by 200 µm long and 2 µm thick. This result shows that the technique we developed is competitive with the commercial MUMPs technique used for the realization of the silicon devices.

In Figure 3 a transparent device capable of uniaxial and shear stretch is shown.

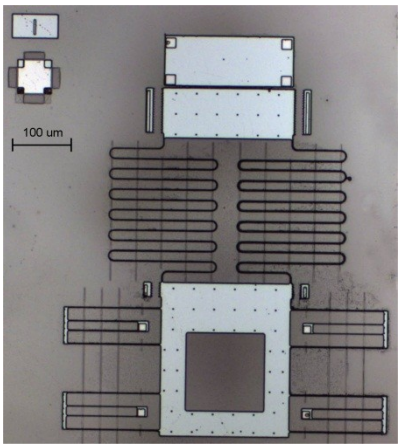

Figure 3: Optical image of the transparent device before the releasing process. This device has a cell testing area 250µm by 200µm and sensitive z-springs to evaluate the forces involved in the stretching.

III. Experimental Set Up

A. BioMEMS preparation

Before using bioMEMS for biological app lications we developed a protocol to release the suspended structures. All the details are shown in Table 1.

Once BioMEMS are stripped by the protective polymer and the suspended structures are released, they are sterilized

using UV irradiation and isopropanol and placed in a liquid cell. The system is rinsed three times with Phosphate Buffered Saline (PBS) solution to remove any alcohol residual.

		Silicon based	Silicon nitride based
i.	Stripping	Acetone 3 minutes D.I. Water 90 s Isopropanol 3 min Oven T=140ºC for 10 min	None
ii.	Release	HF [50%] 10 min D.I. Water 5 min Isopropanol 2 min Oven T=140ºC for 10 min	KOH [5M] T=70C 100 min D.I. Water 5 min Isopropanol 2 min Oven T=140ºC for 10 min

Table 1: Stripping and release protocols of silicon based and silicon nitride based devices.

B. BioMEMS stretching system

BioMEMS are actuated using a glass micro-wire linked to a piezo-electric motor. The bending angle of the wire is 70º, which is the optimal value to have access to the actuation area of the device.

Stretch mode	Linear
Theoretical Amplitude of each step	5µm
Velocity of Deformation	2µm/s
Time Delay between each step	20s
Number of stretching experiments for each cell	3
Time Delay between each experiment	60s

Table 2: Stretching protocol followed for fibroblasts solicitation.

Once the glass wire is in contact with the bioMEMS, the application of the prescribed protocol is granted by the piezoelectric motor controlled by custom made software, developed using National Instruments LabVIEW. Different stretching protocols can be applied: force controlled or displacement controlled movement. The cell can be stimulated with linear deformation or cyclic protocols as triangular, square wave, or sinusoidal stretching modes.

C. Cell preparation

The cells used for this set of experiments are NIH 3T3, mouse embryonic fibroblasts, growth using Dulbecco's Modified Eagle Medium (DMEM) added with 10% of Bovine Calf Serum (BCS) and 1% Penicillin-Streptomycin.

Cells are sub cultured in 35mm^2 flasks. The cell culture medium is removed and, after rinsing the cell layer with 5ml of PBS, 1ml of Trypsin-EDTA is added. The flask is incubated at 37ºC for 2/3minutes to facilitate the detach of the cells from the flask. After that cell culture medium with BCS is added to inhibit the enzyme effect and the solution is centrifuged to separate cells from the detachment solution. Cells are re-suspended in 3ml of cell culture media and gently pipetted to have cells well dispersed in the solution.

An aliquot of 500µl of fibroblasts in solution are then deposited on the bioMEMS. The liquid cell with the bioMEMS can be incubated to allow cells to attach on the measurement platform.

IV. RESULTS

A. Cell stretching, morphological data

After 12 hours, all cells are perfectly spread on the surface of the device and can be tested using the protocol shown in Table 2. For each stretching step we acquired an image that is used to evaluate the morphological changes of the mechanically deformed cell as shown in Figure 4 (steps 1-16).

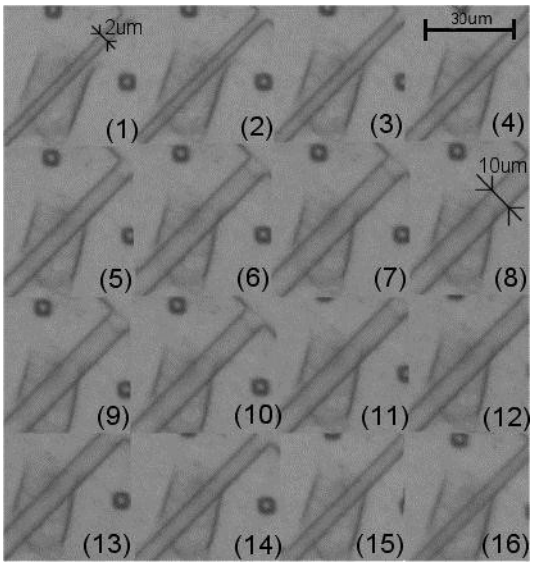

Figure 4: Sequence of the stretching protocol for a single fibroblast in adhesion, optical images. In the first image the cell is not stretched while the transition to the final deformation is shown in images 1- 8. The transition from maximum deformation to resting position is shown from imege 9 to 16. In resting position the gap between the platforms is 2µm and it increase up to 10µm at the maximum stretch.

Using a commercial software, we measured perimeter and area for each step. The values of both parameters increase, due to the deformation of the cell but the rate is different.

We analyzed the ratio between area and perimeter (A/P), we noticed that for any stretched cell there is a hysteretic behavior: the values of A/P are higher during the stretch than during the relaxation (Figure 5).

This pattern remains constant even if the same cell is stretched many times.

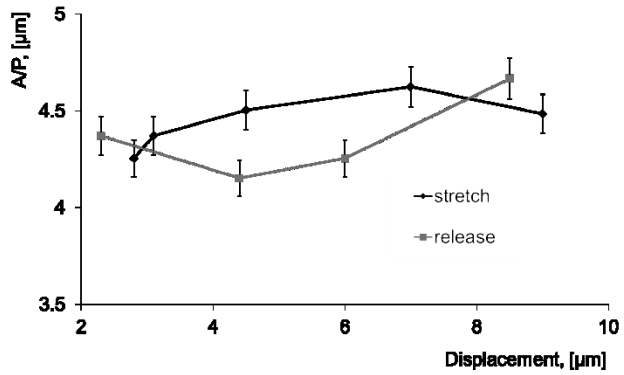

Figure 5: Example of a curve obtained stretching a living cell. During the relaxation the points of the curve follow a higher path than during stretch. This can be seen as a hysteresis like behavior for the ratio A/P.

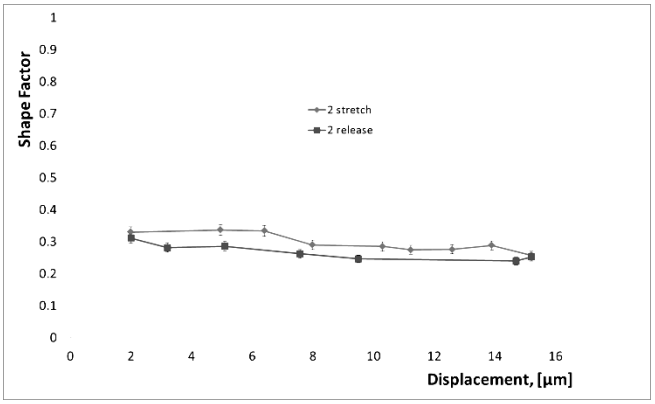

Figure 6: In the graph is plotted the typical trend of the shape factor during the stretching. SF values are basically constant which means that the cells can effort the stretching without suffer for the stimulation.

To check fibroblasts vitality during the experiments we evaluated parameter, proposed for the first time by Kearney et al. in 2008 [7], called Shape Factor (SF).

$$4\pi A/P^2 \qquad (1)$$

Shape Factor could span between 0 and 1 where 0 stands for a completely rounded cell (a sign that the cell is suffering) and 1 means that the cell edge is highly ragged. We noticed that, during the experiments, there are no significant changes in the value of SF: its values can be considered constant, which means that the applied stimuli do not affect the cell (Figure 6).

B. Cell stretching, improvements due to the second generation of bioMEMS

For this experiments we tested both devices, transparent and non-transparent. The high percentage of transmittance of the transparent devices (which is higher than 85% for the visible wavelengths), is very useful to decrease the error in the measurements. In fact with transparent bioMEMS the error passes from 5.1% to 2.24% for the ratio A/P.

With transparent devices will be possible to use two different optics for the microscope, one from the top, in reflection, to control the actuator, and one from the bottom, in transmission, to record at high magnification the modifications of the cell's internal structure, in particular for tubulin and actin.

V. CONCLUSION

We have realized two different bioMEMS for single cell stretching. These devices have been used for stretching single fibroblasts following a prescribed protocol.

During the analyses of the data referred to the morphological changes, a hysteretic behavior for the ratio area/perimeter has been noticed.

This behavior can be explained with a different growth rate of the area and of the perimeter due to changes in the raggedness of the cellular membrane. This is a response to the applied stress. Cells' internal structure changes as a response to the stimuli. Recently, has been shown that a locally applied deformation to the cellular membrane induces local actin polymerization [8].

We expect modifications not only in two-dimensional properties but also in three-dimensional characteristics. For this reason further investigation will be assessed and the bioMEMS will be coupled with AFM imaging for a more complete morphological characterization.

REFERENCES

[1] E. Ahassan, W. Heinz, M. Antonik, N. Dcosta, S. Nageswaran, C. Schoenenberger, and J. Hoh, "Relative microelastic mapping of living cells by atomic force microscopy," *Biophysical Journal*, vol. 74, Mar. 1998, pp. 1564-1578.

[2] J. Dai and M. Sheetz, "Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers," *Biophysical Journal*, vol.68, Mar. 1995, pp. 988-996.

[3] N. Scuor, P. Gallina, H. V. PAnchawagh, L. R. Mahajan, O. Sbaizero, and V. Sergo, "Design of a novel MEMS platform for the biaxial simulation of living cells," *Biomedical Microdevices*, vol. 8, Sep. 2006, pp. 239-246.

[4] R. Fior, S. Maggiolino, M. Lazzarino, and O. Sbaizero, "A completely transparent MEMS for mechanical properties evaluation of a single living cell," *Microfluidics, BioMEMS, and Medical Microsystems IX, Proc. of SPIE*, Vol. 7929, 792906-1, Mar. 2011.

[5] K. Addae-Mensah and J. P. Wikswo, "Measurement techniques for cellular biomechanics in vitro," *Experimental biology and medicine*, Vol. 233, Jul. 2008, pp. 792-809.

[6] D. Koester, A. Cowen, R. Mahadevan, M. Stonefield, and B. Hardy, "PolyMUMPs design handbook," MEMSCAP, 2003 [Online]. Available: www.memscap.com/mumps/documents/PolyMUMPs.DR.v11.pdf

[7] E. M. Kearney, P. J. Prendergast, and V. A. Campbell, "Mechanism of strain-mediated mesenchymal stem cell apoptosis," *Journal of Biomechanical Engineering*, Vol. 130, Dec. 2008, 130(6):061004.

[8] A. Trache, and S. Lim, "Live cell response to mechanical stimulation studied by integrated optical and atomic force microscopy," Journal of Visualized Experiments, 44, 2010. http://jove.com/index/Deails.stp?ID2072