Mechanical Characterization of Fixed Undifferentiated and Differentiating mESC

Anand Pillarisetti[†], Carol Keefer* and Jaydev P. Desai[†]
[†]Robotics, Automation, Manipulation, and Sensing (RAMS) Laboratory
*Department of Animal and Avian Sciences
University of Maryland, College Park, USA

Abstract— *Conventionally, biologists detect cell status visually based on protein markers. These assays are often qualitative and do not quantitatively define the outcome of a cell progression during differentiation. Consequently, we propose to develop an atomic force microscopy (AFM) based system that can be used to mechanically manipulate and characterize an individual cell. We have also connected a haptic feedback interface comprising of a PHANToM haptic feedback device to obtain a qualitative force feedback response when the AFM probe contacts the cell membrane. The system has the capability of measuring forces in nN range and provides a haptic display of the cell indentation forces in real time. We conducted experiments on mouse embryonic stem cells (mESC) roughly 10 µm in diameter and 7-10 µm in height at the interphase stage of the cell division process. Specifically, we performed single indentation studies of 2-2.5 µm on multiple fixed mESC, – early differentiating (6 days under differentiation conditions) and undifferentiated to determine the local elastic modulus of the cell membrane. Our experimental results indicate that the mechanical property of undifferentiated mESC differs from differentiating mESC in fixed cells.*

Index Terms **— Embryonic stem cells (ESC), Cell indentation, haptic feedback, Atomic Force Microscopy.**

I. INTRODUCTION

Mechanical manipulation and characterization of an individual biological cell is currently one of the most exciting research areas in the field of medical robotics applied to cellular level interactions. Single cell manipulation is a prevalent process in intracytoplasmic sperm injection (ICSI), pro-nuclei DNA injection, gene therapy and other biomedical areas. Furthermore, mechanical characterization of cells can be used as a potential biological marker to detect its state [1]. For example, quantifying the mechanical behavior of embryonic stem cells may lead to effective regenerative therapies [2]. Human embryonic stem cells (hESC) have strong potential for therapeutic use in treatment of disease (e.g., heart disease, Parkinson's and spinal cord injuries). However, successful therapy requires generation of pure populations of defined cell types. For clinical applications, efficient cell manipulation and quantification of the mechanical behavior of cells is needed as populations of ESC (both human and mouse) consist of mixed populations of cells (differentiated and undifferentiated). Generation of pure populations of defined cell types remains a challenge for stem cell biologists. The most common methods to monitor cell status are either to analyze mRNA levels or to visually monitor protein markers. Usually such measurements involve either destroying the cells or altering the conditions of the cells (e.g., cell staining protocols involving antibody binding). Furthermore, most assays involve assessment of cell populations and do not have the ability to monitor individual cell responses. Thus, we hypothesize that the mechanical property of undifferentiated mouse embryonic stem cells (mESC) differs from differentiated mESC. To address this hypothesis, we have developed a haptics enabled atomic force microscope (AFM) system that can be used to manipulate cells and quantify their mechanical behavior.

Several approaches are proposed in the literature to automate and improve the efficiency of cell manipulation systems. A detailed review on the existing techniques for cell manipulation is presented in [3]. One of the ways to improve the efficiency of biomanipulation tasks is the inclusion of force feedback. Few researchers have developed force feedback based cell manipulation systems [4, 5]. However, the experiments were performed on cells with dimensions in the range of 600µm – 1 mm. On the other hand, in the field of cell biology most experiments involve cells with dimensions less than 100 microns.

AFM, primarily developed as an imaging tool [6] has also emerged as a unique force sensor with nanometer resolution [7]. Lal and John have provided a detailed review of atomic force microscopy [8]. It has been used extensively to study the mechanical properties of biological materials [9]. On the other hand, robotics researchers are proposing new human machine interfaces and models to manipulate biological cells. Vogl et al [10] proposed a spline based surface model for nanomanipulation with 3D visual and force feedback using AFM. Another AFM based nanorobotics system was developed by Sitti et al [11]. However, both the spline based model and nanorobotics system have not been evaluated on biological systems. A nano-manipulation system consisting of an AFM and a haptic device has also been developed to provide force feedback from biological samples and carbon nanotubes [12]. In this set up the user does not feel the actual forces from the sample, but feels a surface representation that is simultaneously reconstructed during the AFM scan.

In this paper, we present an AFM combined with haptic feedback to characterize the mechanical property of cells. The system has the capability to reflect cell indentation force in real time to the user. Since, ESC have potential application in regenerative therapies, we chose to perform experiments on fixed, undifferentiated and differentiating mESC. We hypothesize that the mechanical property of undifferentiated mESC differs from early differentiated mESC, even though these differentiating cells had yet to undergo any major morphological alterations. In section II, we present the materials and methods used in our work. In section III, we present the results from our experimental work with mESC. Finally, in section IV, we present a discussion of our results.

II. MATERIALS AND METHODS

The haptic feedback interface consists of an Atomic Force Microscope (MFP-3D-BIO^{TM}, Asylum Research) and the PHANToM haptic feedback device (Sensable Technologies, Inc). The main part of AFM is the scan head which is integrated with phase contrast module and an inverted microscope (Model: TE2000U, Nikon, Inc). The entire set up is mounted on an active vibration isolation table manufactured by Herzan (see Figure 1). The phase contrast module enables imaging low contrast, transparent cells in fluid. XY stage (manual) allows the user to position the cell beneath the cantilever tip of AFM. The entire AFM set up is enclosed in an acoustic isolation chamber to prevent acoustic noise from interfering with the AFM measurements. The x and y-axes range of the scan head is 90 µm. The z-axis scan range is 40 µm. The AFM also has the capability to measure forces in the range of pN-nN. A silicon cantilever (AC240TS, Olympus, Inc) was used in our experiments. The cantilever geometry is shown in Figure 2. The spring constant of the cantilever was determined experimentally for each tip used in our studies using the IGOR software interface supplied by Asylum Research. The typical radius of curvature of the cantilever tip is below 10 nm.

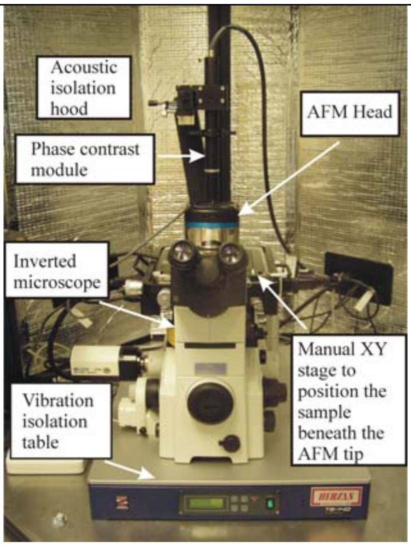

Figure 1. Experimental setup for AFM imaging.

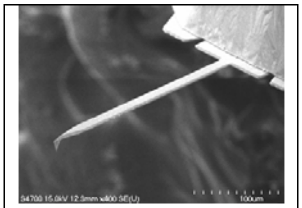

Figure 2. AC240TS silicon cantilever for mESC studies.

The AFM system is used to obtain force and cell deformation data from biological samples. The cantilever is moved by the piezoelectric scanner in the z direction towards the cell. The deflection of the cantilever is detected by a photodiode when the tip comes in contact with the cell. When the tip of the cantilever is in contact with the cell, the initial cantilever deflection, d_0, and movement in z direction, z_0, are obtained (see Figure 3). As the cantilever moves in the z direction and deforms the cell, the final cantilever deflection, d_1, and the movement, z_1, are obtained (see Figure 3). The actual cantilever deflection, d, and the movement, z, are given by:

$$d = d_1 - d_0 \quad (1)$$

$$z = z_1 - z_0 \quad (2)$$

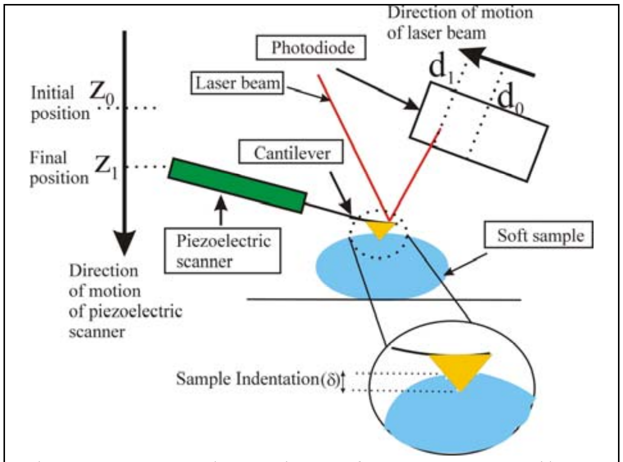

Figure 3. Schematic of AFM cantilever interacting with a cell. Point of contact: d_0 and z_0 represent the apparent cantilever deflection and movement respectively. Cell indentation: the cantilever further moves from the point of contact and indents the cell.

The difference between the actual cantilever movement, z, and actual cantilever deflection, d, represents the cell indentation, δ, and is given by (see Figure 3):

$$\delta = z - d \quad (3)$$

Substituting (1) and (2) in (3) we get:

$$\delta = (z_1 - z_0) - (d_1 - d_0) \quad (4)$$

Thus the cell indentation, δ, can be used to compute the elastic modulus of the cell based on the Hertz contact model. The force exerted on a biological cell and the corresponding deformation predicts its mechanical behavior.

A. Cell Preparation

Mouse embryonic stem cells (mESC) R1 (SCRC-1011, American Type Culture Collection [ATCC], Manassas, VA) were grown on 0.1% gelatin-coated plates in the absence of feeder cells. The ESC medium consisted of 1000 U/ml leukemia inhibitory factor (LIF, ESGRO, Chemicon, Temecula, CA), 15% fetal bovine serum (FBS) (Invitrogen), and basic medium that included Knockout Dulbecco's modified Eagle's medium (Invitrogen), 2 mM L-glutamine, 1x non-essential amino acids, and 0.1 mM mercaptoethanol. Differentiation was induced by removal of LIF from the medium for 6 days. Prior to experiments, cells were dispersed using trypsin to obtain single cells and were plated on 60 mm gelatin coated tissue culture petri dish. Fixed mESC were obtained by treating the live mESC with 4% formaldehyde for 10 minutes and were stored in phosphate buffered saline (PBS).

B. Mechanical characterization of a biological cell

The force exerted on a biological cell and the corresponding deformation predicts its mechanical behavior. Since the past decade, researchers have proposed different models to determine the mechanical properties of biological entities using AFM. Hoh et al [13] calculated the effective spring constant of Mandin-Darby canine kidney cells (MDCK) fixed by glutaraldehyde. A novel sample preparation method was proposed to measure the elastic properties of β-chitin fibers [14]. A micro-mechanics cell model was developed to determine the mechanical properties of human dentine [15]. Danti et al [16] used force modulation microscopy (FMM) to detect variations in mechanical properties of human mesenchymal stem cells (MSCs). However FMM imaging technique is not quantitative. It can detect only relative and qualitative elastic modulus differences between different cell surfaces. We are interested in quantifying the mechanical property of stem cells which would provide a better insight in developing an effective regenerative therapy. As an initial attempt towards this goal, we have used Hertz contact model to estimate the elastic modulus of undifferentiated and differentiating mESC in fixed state. Hertz contact model has been used extensively by physicists to quantify the mechanical property of biological samples using AFM [17-19]. The model assumes that: (a) the sample is elastic, isotropic, and homogenous, (b) the tip used for sample probing is infinitely stiff compared to the sample, and (c) no adhesion between the tip and the sample. For a conical tip indenting a sample, the relation between the indentation, δ, and the loading force (F) is given by:

$$F = \frac{2E\tan(\alpha)}{\pi(1-\nu^2)}(\delta^2) \quad (5)$$

where E and ν are the elastic modulus and Poisson's ratio of the sample respectively. α is the opening angle of the conical tip (35^0 for AC240TS). The spring constant and the elastic modulus of the cantilever are 2.00 N/m and 168.17 GPa respectively. Typically, the elastic modulus of cells is in the range of kPa [20]. Hence, the assumption (b) is valid in our analysis.

C. Haptic Interface

The force exerted on the cell and hence transmitted to the haptic feedback device is given by:

$$F = kd \qquad (6)$$

where k is the spring constant of the cantilever obtained initially through the IGOR software after exciting the AFM scanning tip in various modes. Substituting (1) in (6), we get:

$$F = k(d_1 - d_0) \qquad (7)$$

Thus, our haptics enabled AFM system obtains the relationship between the force exerted on the cell and the corresponding deformation. The force detected by the AFM during cell contact is acquired by a data acquisition board in real-time (model: dSPACE DS1103). The AFM is integrated with the PHANToM haptic feedback device. The interface allowed the user to feel the cell indentation force in real time. The force was amplified by a factor of 10^7 for the human operator to perceive the change in force during cell indentation by the AFM cantilever. Thus, haptics enabled AFM monitoring provides real time force information from an individual cell. This information can be used as a biological marker to detect the state of the cell.

III. RESULTS

We hypothesize that the mechanical property of undifferentiated mESC differs from early differentiated mESC. To address this hypothesis, we conducted single indentation studies on fixed mESC: undifferentiated and early differentiated. The phase contrast module enabled imaging/aligning the cantilever tip and an individual cell (Figure 4). The diameter of a cell is approximately 10 µm. Previous experiments have shown large standard deviation of the elastic modulus values for mESC [21]. One of the reasons for the large variation in modulus may be attributed to the variation in the stage of the cell cycle (M-phase or interphase) at the time of cell indentation. As a result, present experiments were performed on fixed mESC in one stage of the cell cycle, i.e., interphase. The cells were stained with Hoescht 33342 and were visualized by fluorescence microscopy (FM). In this microscopy, an individual cell in interphase is characterized by a blue circle (Figure 5). Thus, the FM allowed us to perform single indentation studies on fixed mESC in interphase. The cell indentation range was 2 – 2.5 µm. We obtained force-indentation data from 20 samples: 10 samples of undifferentiated mESC and 10 samples of differentiating mESC.

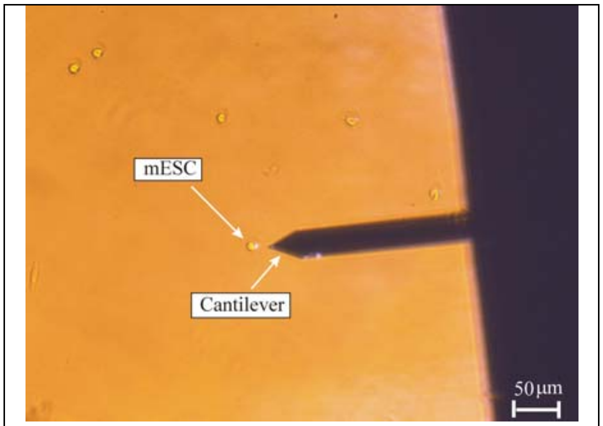

Figure 4. Phase contrast image of fixed differentiating mouse embryonic stem cells (mESC) and AC240TS cantilever (Olympus, Inc). The diameter of mESC is approximately 10 µm.

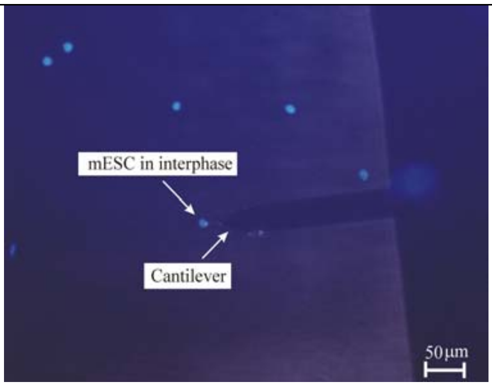

Figure 5. Fluorescence image (FM) of fixed differentiating mESC in interphase stained with Hoescht 33342.

A. Single indentation studies on fixed cells

Figure 6 and Figure 7 shows the force versus cell indentation, δ, for fixed undifferentiated mESC and fixed differentiating mESC respectively. From the figures, we observe that the undifferentiated mESC has a supple membrane compared to differentiating mESC. Based on the experimental data, we performed a least square fit on the dataset and the corresponding least square fit is shown in the figure. The R^2 value for fixed undifferentiated mESC was found to be 0.9141 and R^2 value for fixed differentiating mESC was found to be 0.8917.

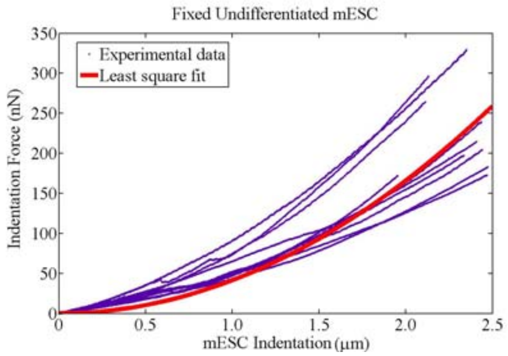

Figure 6. Force versus cell indentation, δ, for fixed undifferentiated mESC.

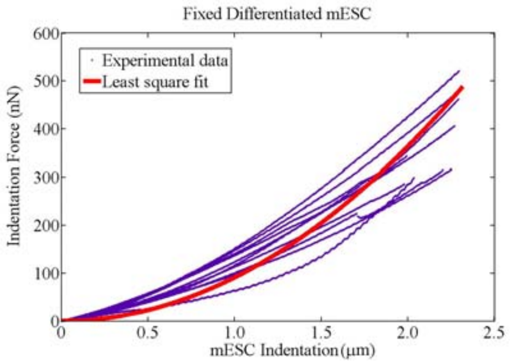

Figure 7. Force versus cell indentation, δ, for fixed differentiating mESC.

Figure 8 shows the average elastic modulus of the cell membrane for fixed undifferentiated and differentiating mESC. Equation (5) was used to compute the elastic modulus of the cell membrane for each mESC indentation task by assuming Poission's ratio of 0.5. The average elastic modulus is 78.26 kPa and 148.82 kPa for fixed undifferentiated and differentiating mESC respectively. The standard deviation is 23.349 kPa and 25.355 kPa for fixed undifferentiated and differentiating mESC respectively. The elastic modulus provides us a quantitative measurement of the cell membrane stiffness during haptic interactions. Thus the data is useful for mechanical property characterization of mESC. We also performed Kruskal-Wallis statistical test on the elastic modulus values for undifferentiated and differentiating fixed mESC. The p-value obtained was 0.0002, leading to a probability of greater than 99.99% that there was a significant difference between the two data sets.

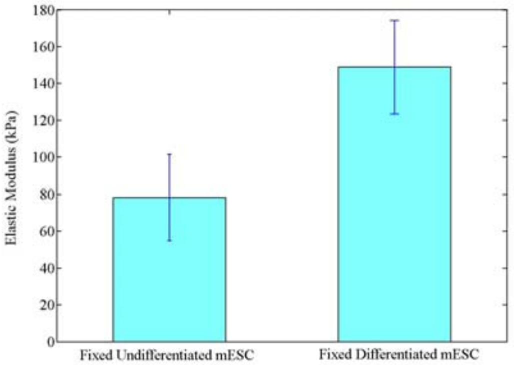

Figure 8. Elastic modulus of fixed mESC calculated from the Hertz contact model along with the error bar.

IV. DISCUSSION

In this paper, we have presented our work towards the development of eventually a haptics enabled atomic force microscopy system for mechanical property characterization of fixed mESC in differentiated and undifferentiated states with both qualitative as well as quantitative feedback. The results presented in the paper are primarily the quantitative data derived from the Hertz contact model. The haptic feedback obtained during cell indentation by the AFM tip was in real-time and the subject was able to feel the change in the stiffness during cell deformation. However, we still need to perform several human factors studies to correlate the cell stiffness change during indentation with the qualitative response experienced by the user.

We did studies on 20 fixed cells: 10 cells of undifferentiated mESC and 10 cells of differentiating mESC for single indentation tasks. The force curves obtained from each experiment were used to deduce the elastic modulus of the cell membrane using the Hertz contact model. Our results confirm that the mechanical property of undifferentiated mESC differs significantly from differentiating mESC in fixed cells (p value = 0.0002 from Kruskal-Wallis test).

The approach presented in this paper could be used to develop improved methods of targeted cellular differentiation of human embryonic and/or adult stem cells for therapeutic purposes, for development of new diagnostic procedures, and to monitor cellular responses to environmental stimuli. In future, we plan to conduct further studies on cells and extend our research hypothesis to live mESC.

REFERENCES

[1] G. Y. H. Lee and C. T. Lim, "Biomechanics approaches to studying human diseases.," *Trends in Biotechnology* vol. 25(3), pp. 111-118, 2006.

[2] A. G. Smith, "Embryo-derived stem cells: of mice and men," *Annual review of cell and developmental biology*, vol. 17435-462, 2001.

[3] J. P. Desai, A. Pillarisetti, and A. D. Brooks, "Engineering Approaches to Biomanipulation," *Annual Review of Biomedical Engineering*35-53, 2007.

[4] Z. Lu, P. C. Y. Chen, J. Nam, R. Ge, and W. Lin, "A micromanipulation system with dynamic force-feedback for automatic batch microinjection," *Journal of micromechanics and microengineering*, vol. 17314-321, 2007.

[5] A. Pillarisetti, M. Pekarev, A. D. Brooks, and J. P. Desai, "Evaluating the Effect of Force Feedback in Cell Injection," *IEEE Transactions on Automation Science and Engineering*, vol. 4(3), pp. 322-331, 2007.

[6] H. X. You and L. Yu, "Atomic force microscopy imaging of living cells: progress, problems and prospects," *Methods in cell science*, vol. 21(1-17), pp. 1-17, 1999.

[7] A. Alessandrini and P. Facci, "AFM: a versatile tool in biophysics," *Measurement science and technology*, vol. 16R65-R92, 2005.

[8] R. Lal and S. A. John, "Biological applications of atomic force microscopy," *The American Physiological Society*, vol. 266C1 - C21, 1994.

[9] W. R. Bowen, R. W. Lovitt, and C. J. Wright, "Application of atomic force microscopy to the study of micromechanical properties of biological materials," *Biotechnology Letters*, vol. 22893-903, 2000.

[10] W. Vogl, M. Sitti, and M. F. Zah, "Nanomanipulation with 3D visual and force feedback using atomic force microscope," in *4th IEEE Conference on Nanotechnology*, 2004, pp. 349-351.

[11] M. Sitti and H. Hashimoto, "Tele-Nanorobotics Using Atomic Force Microscope," presented at International Conference on Inteligent Robots and Systems, Victoria, B.C.,Canada, 1739-1746, 1998.

[12] M. Guthold, M. R. Falvo, W. R. Matthews, S. Paulson, S. Washburn, D. A. Erie, S. R., F. P. Brooks, Jr., and R. I. Taylor, "Controlled Manipulation of Molecular samples with the nanoManipulator," *IEEE/ASME Transactions on Mechatronics*, vol. 5(2), pp. 189-197, 2000.

[13] J. H. Hoh and C. Schoenenberger, "Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy," *Journal of Cell Science*, vol. 1071105-1114, 1994.

[14] W. Xu, P. J. Mulhern, B. L. Blackford, M. H. Jericho, and I. Templeton, "A new atomic force microscopy technique for the measurement of the elastic properties of biological materials," *Scanning Microscopy*, vol. 8499-506, 1994.

[15] Q. H. Qin and M. V. Swain, "A micro-mechanics model of dentrin mechanical properties " *Biomaterials*, vol. 255081-5090, 2004.

[16] S. Danti, M. D'Acunto, L. Trombi, S. Berrettini, and A. Pietrabissa, "A micro/nanoscale surface mechanical study on morpho-functional changes in multilineage-differentiated human mesenchymal stem cells.," *Macromolecular Bioscience*, vol. 7589-598, 2007.

[17] A. M. Collinsworth, S. Zhang, W. E. Kraus, and G. A. Truskey, "Apparent elastic modulus and hysteresis of skeletal muscle cells through differentiation," *American Journal of Physiology: Cell Physiology*, vol. 283 pp 1219 -1227, 2002.

[18] A. C. Fischer-Cripps, *Introduction to contact mechanics*, Second ed. New York: Springer, 2007.

[19] A. Touhami, B. Nysten, and Y. F. Dufrene, "Nanoscale mapping of the elasticity of microbial cells by atomic force microscopy," *Langmuir*, vol. 19 pp 4539 - 4543, 2003.

[20] M. Radmacher, "Measuring the elastic properties of biological samples with the AFM," *IEEE Engineering in Medicine and Biology*47-57, 1997.

[21] A. Pillarisetti, C. Keefer, and J. P. Desai, "Mechanical Response of embryonic stem cells using haptics-enabled atomic force microscopy," in *International Symposium on Experimental Robotics* Athens, Greece, 2008.