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Microactuator device for integrated measurement of epithelium mechanics

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Abstract

Mechanical forces are among important factors that drive cellular function and organization. We present a microfabricated device with on-chip actuation for mechanical testing of single cells. An integrated immersible electrostatic actuator system is demonstrated that applies calibrated forces to cells. We conduct stretching experiments by directly applying forces to epithelial cells adhered to device surfaces functionalized with collagen. We measure mechanical properties including stiffness, hysteresis and visco-elasticity of adherent cells.

Introduction

The cell cytoskeleton is a dynamic network of polymers, motors and other regulatory proteins that not only provide structural stability but also integrate several biological functions such as adhesion, motility, contraction and sensing [1]. How cells generate, sense and transduce mechanical forces is important for understanding various physiological functions of the cellular machinery. The mechanics of cells is also important since many pathological states are manifest in the mechanical properties of the cells [2,3].

The various techniques to analyze mechanics in cells include micropipette aspiration [4], rheology [5,6], magnetic twisting cytometry [7], atomic force/scanning force microscopy [8–10], microplate manipulation [11–13], and optical tweezers [3]. These methods address measurements over a wide range of distances and forces, suitable for systems from single molecules to single cells and whole tissues [14–17]. While many of these techniques measure the material properties of cells, few examine the dynamics of cells under directly applied forces, in part due to the lack of suitable measurement tools. Here, we address the need to develop systems that can apply calibrated forces to adherent cellular systems and measure their response.

While deformations by glass rods, substrates, cantilevers and even protein filaments can be utilized as force sensors, there are few actuators that can apply calibrated forces or displacements directly to cellular components. Typically, force probes are used in conjunction with actuator stages to apply forces to cells [9,12,18,19]. However, the piezoelectric stage or manipulator used for actuation acts from outside the liquid media, thus

limiting the approach to the samples. Moreover, cantilevers measure forces in a direction perpendicular to the substrate, while many forces of physiological interest are known to act in the plane of the cell [20,21]. Cantilever-based methods require extensive experimental modifications to measure in-plane forces. Furthermore, piezoelectric stages are large compared to the cell samples and hence cannot be integrated into measurement systems, thus limiting the approach and spatial positioning relative to cells.

To achieve greater spatial resolution and better integration of sensing and actuation, the actuator needs to be scaled down with the measurement device. Microelectromechanical systems (MEMS) actuators have been increasingly applied to biological measurements due to suitable forces, displacement and scalability [22–24]. However, completely immersed actuation integrated with force sensing has not been demonstrated, especially in cell culture media. Recent advances in MEMS devices have shown electrostatic actuators to operate in aqueous ionic solutions such as biological buffers [25,26]. By combining such an immersible electrostatic actuation system with calibrated force sensors we demonstrate in-plane force measurements on single adherent cells. Integrating the sensing and actuation mechanism on-chip dramatically shrinks the force sensing and delivery system, providing spatial access to in-plane forces at the single cell scale. This approach further increases the potential for integrating mechanical testing into microfluidic systems for diagnostic purposes [27]. Here, we measure the static and dynamic mechanical properties of adherent epithelial cells cultured on the device. The resolution, dynamic range and response of such devices are well within the range for studying single cell mechanics. We present the design, implementation, calibration and testing of the integrated device.

Integrated device approach

One of the critical limitations of immersible actuation design is the presence of aqueous ionic solutions such as biological buffers and media. Operation of actuation mechanism in biological media is critical to applying and measuring forces on living cells under physiologically relevant conditions. The integration is achieved by the implementation of electrostatic comb-drive actuator designs that can function in aqueous ionic solutions [26]. Modulation of the actuation voltage at high frequency overcomes the ionic shielding effects and electrochemical corrosion. Electrostatic comb-drive actuators offer the force and displacement ranges and the response times that are suited for cellular mechanics studies. The electrostatic force generated in a comb-drive actuator is

$$kx = \frac{N\epsilon\epsilon_0 b}{d} V^2 f(\omega), \quad (1)$$

where k is the stiffness of the suspension, N is the number of finger pairs, ϵ is the relative permittivity of the medium, ϵ_0 is the permittivity of free space, b is the thickness of the device, d is the gap spacing and V is the applied voltage between the electrodes. $f(\omega)$ denotes the frequency dependence of electrostatic actuation in aqueous solutions [28]. The actuation signal is modulated at a frequency of 25 MHz to achieve actuation while immersed in the biological media. Further details of actuator design and characterization are described elsewhere [26].

Consider the design schematic shown in Figure 1. A cell is stretched across the gap between two suspended substrates. One of the substrates (actuator) is actuated by electrostatic forces to apply a mechanical load to the cell, while the other substrate is suspended by a sensing springs. The cell response can be calculated from the deflection and stiffness of the beam (sensor) at the base of this suspension. The force-displacement relationship for the cell is evaluated from the equivalent representation of the spring network. Note that the stiffness relationship for the cell is typically visco-elastic. The cell is represented as a linear spring

here to evaluate the force-displacement relationship, which is valid for any sample. If the force experienced by the cell is represented as F_{cell} , the sensor and actuator displacements as δ_s and δ_a , respectively, the displacement of the cell δ_c is

$$\delta_c = \delta_a - \delta_s. \quad (2)$$

The force experienced by the cells is directly balanced by the sensing beam of stiffness k_s .

$$F_{cell} = k_s \delta_s \quad (3)$$

The total force exerted by the actuator F_a is given by,

$$F_a = k_a \delta_a + F_{cell}, \quad (4)$$

or

$$F_a = [k_a \delta_a + k_s \delta_s], \quad (5)$$

or in terms of the effective cell stiffness k_c ,

$$F_a = \left[k_a + \frac{k_s k_c}{k_s + k_c} \right] \delta_a. \quad (6)$$

From Eq. 6, we see that the effective stiffness of the device changes according to the stiffness response of the cell. Thus, by characterizing the force-displacement relationship of the actuator we can establish the same for the cell sample under test. A few special cases are considered for practical application. In many cases, the flexure is designed such that one of the suspensions is much stiffer. In Eq. 6, if we assume that $k_s \gg k_c$, then we get

$$F_a = [k_a + k_c] \delta_a. \quad (7)$$

Here, the system simply reduces to two springs in parallel and the cell stiffness can be easily discerned from the change in stiffness of the system. The relationships in Eqs. 6 and 7 highlight some design constraints for the beam suspensions. If the actuator stiffness is much higher compared to the cell stiffness, the range of forces exerted on the cell is small. In the extreme case, the forces are below the resolution for the apparatus. In the other extreme, if the cell is much stiffer than the suspension the displacements are greatly reduced and could be below the detection limit. To avoid these extremes, the stiffness should be chosen to be in the same order of magnitude as the expected sample stiffness. Since reported measurements of forces in cell mechanics range from 1nm-1μN in a displacement range of 0.1–10 μm [9,12,19], the stiffness of our designs ranges from 20–300 nN/μm with an actuation range of at least 10 μm.

Calibration

Force measurement is dependent on the calibrated values of the suspension stiffnesses. For this method we employ electrostatic force to calibrate the devices. The devices are observed under a 50× objective at a frame averaging time of 100 ms yielding a resolution of about 20 nm. The devices are actuated with a DC voltage signal in air and the displacements are measured. The displacement-voltage relationship for electrostatic actuators is used to measure the suspension stiffness (Eq. 1).

From a measured plot (Figure 2) of displacement (x) vs voltage (V^2), we obtain the suspension stiffness k . Both the sensor (k_s) and actuator (k_a) stiffness are calibrated for each device before the experiments. We note that the sources of error for such a calibration technique arise from the measured slope of the calibration curve, the device geometric factors (b, d) and the applied voltage (V). Of these, the largest error comes from the device gap d , due to the variations in the side wall flatness and planarity from the silicon deep reactive ion etch step during fabrication [26]. The process introduces scalloped edges that deviate from the assumption of uniform electric field between flat plates. If s denotes the slope of the measured calibration curve, the stiffness k is calculated as,

$$k = \frac{N\epsilon_0 b V^2}{ds}. \quad (8)$$

The maximum error appears in the lateral dimension d , which is defined by the etch. For a conservative error estimate we take the geometric error to be 500 nm. From this, the maximum error estimated is about 25 % of the stiffness value. Figure 2 depicts a typical calibration curve for an actuator. The measured stiffness value for all devices ($n=14$) compares well with the theoretical values calculated from fixed folded beam designs [29]. The tolerance on calibration is comparable to other techniques for cantilevers found in literature [30].

Materials and Methods

Microscopy

All measurements are performed with a Leica DMRXA2 upright microscope under either a 50 $\times$ air objective or 63 $\times$ water immersion objective. The images are recorded with a Leica DFC350FX camera at a frame averaging rate of 100 ms. The microscope is encased in a custom built acrylic incubator to maintain the cells at 37°C. The incubator box is connected to an AirTherm heater-controller to maintain a constant temperature in the unit. The petri dishes containing cells are kept in a humid environment created by wet tissue.

Cell culture

Madin-Darby canine kidney (MDCK) cells are grown in standard tissue culture dishes in low glucose Dulbecco's Modified Eagle's Medium (DMEM) with 110 mg/L Sodium Pyruvate, glutamine and 5g/L Sodium Bicarbonate supplemented with 5% v/v fetal bovine serum (FBS) and 1% v/v Penicillin-Streptomycin-Kanamycin (PSK) solution. The cells are dissociated by incubating in 0.05% Trypsin in Ethylenediaminetetraacetic acid (EDTA) solution. The trypsin is neutralized in equal volume of the above mentioned media and centrifuged. The cells are resuspended and seeded onto tissue culture plates or actuator devices.

Cell Placement

The devices are cleaned in de-ionized (DI) water and detergent solution. The surfaces are coated with Collagen I solution (10 μ g/ml in 0.1% acetic acid) to facilitate cell adhesion. The devices are rinsed in 0.1% acetic acid solution and further with DMEM. It is important to selectively pattern the cells in the binding region to prevent fouling of the electrodes and mechanical suspension with either cells or debris, as they would inhibit the functioning of device components. To overcome this, a micropipette is used to capture and place cells with a micromanipulator (Eppendorf Micromanipulator 5171). An Eppendorf CellTram vario piston pump is used for suction. The cells are captured onto a micropipette and placed over

the binding regions. The microscope stage is maintained at 37°C during the incubation period of 2–3 hours when the cells attach and spread on to the substrate.

Mechanics

Cell Stiffness

We measure the cell stiffness by stretching the cell between the suspended substrates by electrostatic force (Figure 3). The plot in Figure 3(b) indicates the comparison between the displacement of the device with and without the cell. As described in the previous sections, this difference is used to measure the forces experienced by the cell at each displacement according to Eq. 6. The measurements are taken at an average rate of about 100 nm/s, with a time of 5 s between each step. Figure 4 indicates loading (blue) and unloading (red) measurements on the sample. The cell is stretched from a nominal gap size of 2 μm to a final gap of 8 μm. The measurement indicates non-linear stiffness, with significant stiffening, but no rupture at large strains. Similar mechanical behavior was also reported for fibroblasts [31]. The cells exhibit an average stiffness of 70.0 ± 12.8 nN/μm ($n=8$) when stretched at a rate of 100 nm/s. We did not observe a significant change in the stiffness at a slower strain rate of 20 nm/s, suggesting that the strain-dependence is not prominent at these rates. Though the cells did not rupture at high strains, there were a few cases where the cells lost traction on the substrate during stretching. The images shown in Figure 5 demonstrate cell slipping as seen by the outline of the cell during stretch. The slip is due to weakening adhesion forces between the cell and substrate. The strength of adhesion can be measured from the peak force measured just before slipping occurs. Such slip phenomenon was observed only in 3 cases in 12 experiments at forces between 160–230 nN. In the other 9 cases, we did not notice a higher incidence of slip even at forces close to 1 μN, indicating that the recorded cases of slip were likely due to anomalous weak cell-substrate adhesion. In the future these experiments could be carried out with live cell imaging of focal adhesions or cytoskeletal components to identify patterns of slip and reorganization.

Viscoelasticity

To further characterize the dynamic properties of cellular response, we subjected the cells to a step displacement. A fixed actuation signal is applied in the cell response is recorded over time. Displacements are recorded at a rate of 10 frames/s over a time of 10 s and continued at a slower rate of 0.2 frame/s over 60s. The short term (few seconds) and long term (few minutes) responses are analyzed in Figure 6 for two different actuation signals of 10 and 15 V_{pp} . The inset in Figure 6 shows a standard linear model for viscoelasticity (Kelvin model) which can be fitted to the creep function to obtain the viscoelastic parameters k_0 , k_1 and τ_1 ($\tau_1 = \mu_1/k_1$). k_0 is the static stiffness which dominates at low strain rates, k_1 is the dynamic stiffness that dominates at high strain rates and τ_1 is the viscous time constant. Though biological samples have been shown to have different relaxation times in different strain regimes, the creep function has been shown to equilibrate over a few minutes [9]. Thus a slow measurement would indicate a stiffness closer to k_0 while higher strain rates would indicate a higher stiffness depending on k_1 and τ_1 .

The creep function for this model is given by [32],

$$k(t) = [k_0 + k_1 e^{-t/\tau_1}]. \quad (9)$$

Fitting traces from three experiments yields the following values for the parameters: $k_0 = 79.6 \pm 22.8$ nN/μm, $k_1 = 307.5 \pm 193.8$ nN/μm and $\tau_1 = 6.88 \pm 3.77$ s. The linear stiffness k_0 compares well with static measurements made on the samples suggesting that the static measurements were 'slow' and close to equilibrium.

Discussion

We have presented the design and development of an integrated microscale testing system for studying cell mechanics. The device utilizes microfabricated force sensors and a novel actuation system to make the measurement on-chip. The system provides a high resolution (300 pN) and a large dynamic range (1 μ N), well suited for single cell measurements. The resolution and dynamic range may be modified by design to suit different applications. We have demonstrated the measurement of dynamic properties by directly interfacing with epithelial cells through functionalized surfaces. Slip measurements may be useful in other applications such as measuring extracellular and intercellular adhesion through in-plane stretching. This device is the first step towards a fully controllable system that can dynamically interact with adherent epithelium under physiological conditions, opening the door to measurements coupled with morphology and parameters affecting cell adhesion. The system could also be adapted for studying the influence of external forces on the cytoskeleton when combined with live-cell imaging using fluorescent tagged proteins; thus such platforms will enable the observation of the behavior of subcellular structures and the adhesive plaques under quantitative and controlled dynamic load profiles. The device also allows for integration into microfluidic networks that could potentially utilize mechanics for diagnostic purposes.

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Fig. 1.

(a) Schematic of on-chip cell mechanics testing system comprising two suspended platforms separated by a small gap. The cell adheres to both platforms, straddling the gap. One of the suspensions is actuated by application of electrostatic forces. The cell is stretched between the substrates and the forces are measured from the deflection of the calibrated sensor beams. (b) A scanning electron micrograph (SEM) of the integrated device indicating the cell-binding platforms suspended by flexures in silicon and actuation electrodes.

Fig. 2.

A sample calibration curve of an actuator beam obtained by measuring displacements vs applied voltage in air. The slope of the linear fit to the curve is used to obtain the stiffness of the beam suspension ($R^2 = 0.99$).

(a)

(b)

Fig. 3.

(a) MDCK cells attach and spread on the binding pads. Application of the actuation force stretches the cells across the gap. (b) A comparison of the displacements of the actuator with and without the cell samples shows an increased stiffness due to the cell. The stiffness of the cell is obtained from this curve using Eq. 6.

Fig. 4. Hysteresis measurements on cell sample indicating loading (blue) and unloading (red) paths. The cell exhibits non-linear stiffness and hysteresis effects. There was no sign of rupture even at large displacements, as observed from the lack of discontinuities in the force curves.

(a)

(b)

Fig. 5.

(a) Stretch causes slipping of the cell adhesion points as indicated by the arrows at the deformed cell boundary (highlighted for clarity). (b) Slippage is evidenced by a discontinuity in the Force-displacement curve.

Fig. 6.

The sample is subjected to step in displacement by applying actuation voltages of 10 V_{pp} and 15 V_{pp} and the cell deformations are recorded over time. The displacements indicate an exponential rise during the step, followed by a long term creep. (Inset) Schematic of Kelvin model components.