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A microfluidic chip for real-time studies of the volume of single cells[†]

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Abstract

We report a microfluidic chip that is capable of measuring volume changes in single cells in real-time. Single eukaryotic cells were immobilized in the sensing area and changes in volume in response to hypotonic challenges and drugs were measured using the electrical impedance method. Experiments on MDCK cells showed that the maximum swelling and the time course of swelling vary between individual cells following hypotonic stimulation. The microfluidic chip allows, rapid and convenient change of solutions, enabling detailed studies of various drugs and chemicals that may play important role in cell physiology at the single cell level.

Introduction

The transport of ions and organic solutes across a cell membrane reflects cellular metabolism, ^{1,2} neurotransmitter activity, ³ as well as interaction with extracellular environment. ^{4,5} Studies include the effector mechanisms responsible for transport activities and the role of surrounding environment on transport kinetics. ⁶ Since transport can change intracellular and extracellular osmolarity, it leads to changes in cell volume. Thus, real time measurements of cell volume provide direct access to transport activities and cell physiology.

The effectors responsible for cell volume regulation and ion transport have generally been studied in cell populations. ^{7–9} Those experiments enable observation of collective response of cells to a given physiological condition. In contrast, single-cell experiments expose heterogeneities in cell properties that are otherwise masked by averaging across a population. In addition, time-resolved response of a single cell under controlled environment provides the explicit correlation between a specific effector to a given stimulation, offering insights into possible sensory and signaling pathways for membrane transport.

Most commonly used techniques for studying cell volume include cytometry, ^{10,11} electrophysiology, ^{12,13} and fluorescence microscopy. ¹⁴ Coulter counter technology can address steady state population variance by providing histograms of cell size. ^{15,16} However, it is unable to acquire time-resolved properties of populations or that of an individual cell.

[†]Electronic supplementary information (ESI) available: **SI-I.** Movie showing the sequential captures of three single cells; **SI-II.** Movie showing the swelling of single cell in response to an osmotic challenge from 326 to 187 mOsm; **SI-III.** Movie showing the shrinking of a single cell in the chamber in response to a change in extracellular solution from 187 to 326 mOsm; **SI-IV.** Volume change of a single NIH 3T3 cell in response to a hypotonic stimulation. The solution in the channel was changed sequentially from isotonic (326 mOsm) to hypotonic solution (187 mOsm) and back to isotonic. The volume was measured using single-cell sensor chip with an input current of 2 μ A RMS.

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Electrophysiology, such as patch clamp, enables the measurement of ion transport across cell membrane at single ion-channel level. However, it entails cumbersome experimental procedure, is time consuming, and is not suitable for screening multiple changes in extracellular solutions. Fluorescence methods to explore individual cell volume involve intrusive dye loading, photo-oxidation, and expensive microscopes and cameras.

The microfluidic chip approach enables convenient analysis of single cells on a chip in real-time, and provides precise control over test solutions; it is an ideal platform for studying cellular physiology at the single cell level.¹⁷ Various microfluidic devices have been developed to facilitate cell physiology studies,¹⁸ including lab chip-based patch clamp in electrophysiology,^{19–21} microfluidic platform for mass spectroscopy,²² multilayered microfluidic chambers to measure neural activities,²³ and microfluidic shear devices to observe intracellular Ca^{2+} activities in response to varying shear stresses.²⁴ In addition, the microfluidic approach has been utilized to provide accurate control of extracellular environment (such as temperature or chemistry) for electrophysiology studies.²⁵

Several strategies have been employed to immobilize individual cells on a chip.^{26–28} A bifluidic channel design was used to study mitochondrial movement in single cells and correlated with the disruption of actin filaments due to the local treatment with latrunculin A.²⁹ A similar design was used to study the effect of environmental temperature on differentiation in embryonic cells.³⁰ Single cell assays have been used to record extracellular potentials,³¹ protein counting,³² chemical release³³ and for measuring cell volume using optical observation.³⁴

This paper addresses the design and performance of a new lab-chip strategy that can measure the change in volume of a single cell in real-time. The chip utilizes audio frequency electrical impedance to directly measure changes in cell volume independent of cell shape. The chip allows a rapid change of solutions enabling high throughput screening while simultaneously permitting optical microscope observation of the cells under study. Although the present study focuses on volume measurements of animal cells, the same sensing strategy can be used to measure any organelles defined by non-conductive membranes.

Materials and methods

Design and simulation

In our impedance-based sensor, cells are placed in a small fixed-volume chamber.⁹ As cells swell or shrink, the cross-sectional area of the extracellular fluid in the chamber changes and the measured resistance across the chamber changes. The impedance of cells in a saline medium has a relaxation frequency above 200 kHz so the cells appear as insulating objects at lower frequencies.³⁵ The sensor sensitivity depends on the volume fraction of the chamber occupied by the cells between the electrodes that define the axial dimension of the chamber. A detailed analysis of the relationship between volume change and chamber resistance is discussed in a previous publication.^{9,36}

In this work, a narrow sensing region was created along the fluidic pathway within which a chevron like feature was designed to capture suspended cells in the solution, as shown in Fig. 1. To increase the sensitivity, the sensing region was made 125 μm wide, which is eight-fold narrower than other part of fluid channel (see Fig. 1). A reduction in fluid cross-section area in the sensing region increases the ratio of cell volume to the total volume of the sensing region (thereby increasing the sensitivity). Once a cell is captured, various solutions can be readily perfused through the channel and the resulting changes in cell volume can be followed *via* impedance measurements in real-time.

To evaluate the perturbing effect of the chevron-like feature on the flow characteristics, we evaluated the sensor region using finite element analysis. A 3D model was built to imitate the actual dimensions of the prototype chip. The flow velocity distribution in the sensing zone was calculated numerically using computational fluid dynamics (CFD) software (CoventorWave). The model used Newtonian flow with nonslip boundary conditions and a constant flow velocity of 1 mm/s was assigned at the inlet channel. The flow field and the shear stress distribution in the sensing region were calculated and are discussed in the following section.

Fabrication and experimental details

The single-cell sensor chip in Fig. 1 was constructed using the photosensitive polymer (SU-8) on a Pyrex glass substrate. As shown in Fig. 1, the main flow channel is 1000 μm wide, and the channel width tapered to 125 μm at the sensing zone. The two outer platinum electrodes (100 μm wide) in the broader channel provided constant current. The two narrower inner electrodes (20 μm) within the sensing region (shown zoomed in Fig. 1) were used for voltage measurements. The length of the sensing region between the voltage electrodes was 170 μm , and the depth of the channel was 17 μm . The zoomed view in Fig. 1 shows the details of the chevron feature used for cell capture. It had an opening angle of 60 degrees and a 3 μm gap at the tip of the 'V'.

A standard optical lithography technique was used to fabricate the sensor chip. Using the lift-off technique, a 200 nm thick platinum film was deposited on the glass wafer by e-beam deposition to configure the electrodes. The fluidic channel was then constructed using SU-8 photoresist following the standard processing protocol. A programmed temperature ramp-up hard-baking process was applied after lithography helped to heal micro cracks in the SU-8. Two through-holes were drilled through the glass at each ends of the channel to enable fluid perfusion (not shown). The sensor chip was mounted on an acrylic platform using PDMS. The acrylic platform contained three fluid inlets and one outlet aligned with the chip inlet and outlet holes, respectively, for changing test solutions. For experiments the chamber was covered by a glass slide coated with a thin PDMS layer that served as a tight fluid seal; the chamber and the PDMS-covered glass slide were pressed together using a clamp fixture.

For impedance measurements, a constant current source was used to supply 50 Hz, 2 μA RMS sinusoidal current to the two current electrodes. The voltage between the two inner electrodes was measured using a homemade differential amplifier connected to a lock-in amplifier (Model 5210, EG & G Princeton Applied Research). A Zeiss Axio upright fluorescence microscope with a high-resolution Zeiss digital camera (AxioCamMR) was used for *in-situ* imaging. The time-lapse experiments were performed using imaging software (AxioVision by Zeiss).

Solution preparation

Isotonic and hypotonic solutions consisted of 75 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 , and 10 mM HEPES, pH 7.4. The osmolarity of the solutions was adjusted using mannitol to allow osmotic perturbation at constant ionic strength. Prior to the experiments, the conductivity of the two solutions were titrated to be equal using NaCl. The final osmolarity was then measured using an osmometer (Model 303, Advanced Instrument).

Cell culture

Madin-Darby Canine Kidney (MDCK) epithelial cells were grown in 35 mm Petri dishes to 80–90% confluence in Dulbecco's modified Eagle medium (DMEM) containing fetal bovine serum and penicillin and streptomycin. For suspension, the cultures were trypsinized and suspended in 1 mL isotonic solution. The cell suspension solution was further diluted in a 1:20 ratio in isotonic solution.

Coulter Counter measurements

The mean cell volume was measured using a Coulter counter (Beckman Coulter, Multisizer™-3) with a 100 μm aperture. The instrument was calibrated using 20 μm latex beads (Beckman Coulter) prior to the experiments. The cells were cultured in 50 mL cell culture flasks to 80–90% confluence, trypsinized for suspension, centrifuged at 1000 rpm for 2 min and re-suspended in 8 mL isotonic solution. To measure resting cell volume, half of the cell suspension was diluted by 1:40 in an isotonic solution. The histogram of cell size was obtained by passage of ~7000 cells through the aperture and the measurements were performed every 1.5 min. To obtain the cell volume change upon hypotonic stimulation another half was diluted by 1:40 in a hypotonic solution and sampled at the same rate starting at 1.5 min after hypotonic challenge. The signal was recorded using Multisizer™-3 software (Beckman Coulter). Relative volume change over time was expressed by normalizing mean volume to the mean baseline volume obtained in isotonic solution.

Results and discussion

The flow characteristics in the sensing zone were modeled and are shown in Fig. 2. Fig. 2a shows the distribution of flow velocity in the sensing region. The amplitude of the flow velocity along the center of the fluidic channel (dashed line) and the side of the fluidic channel (solid line) are plotted in Fig. 2b. This data shows the expected minimum velocity gradient along the center of the flow channel and a sharp increase in flow velocity along the side. The geometry of the cell immobilizer displaces most of the fluid flow to either side of the channel and provides a slow and uniform flow field at the cell capture location inside the chevron. The 3 μm downstream aperture of the chevron joint provides an outlet to maintain flow through the chevron.

The single cell sensor was first tested by measuring the impedance change due to the capture of a single cell. The cell capture in the microfluidic channel was simultaneously monitored using an optical microscope. To minimize the adhesion of the cells to the chamber, the channel was first flushed with cell culture media containing serum that coated the surfaces. A dilute suspension of MDCK cells in isotonic solution was then perfused through the channel at a flow rate of 1.02 $\mu\text{L}/\text{min}$ giving a linear velocity of approximately 1 mm/s through the center region of the chevron. Whenever a cell was captured there was a stepwise increase in chamber resistance characterized by a voltage drop. The typical voltage drop due to a single cell capture was 8 to 14 mV, depending on the original size of individual cells. Fig. 3E shows the sensor output for the sequential capture of three cells which generated three steps in the output, indicated by steps 'B', 'C', and 'D'; legend 'A' in Fig. 3E indicates the baseline sensor output without any cell. Fig. 3A is the optical image without any cell while Figs. 3B–D are the corresponding optical images taken at steps 'B', 'C', and 'D', respectively. The sequential cell capture process was recorded as a video and is submitted in the ESI (SII).[†] In this experiment, approximately 1 out of 40 of the inflow cells was captured. The calibration of the sensor to absolute cell volume was estimated by comparing the sensor output before and after cell capture to the cell volume estimated from the optical image assuming a spherical cell, giving a converting factor of 180 μm^3 per mV (volume change per sensor output). Note that the cell membrane is not smooth and most cells have irregular shapes. Optical observations have limited spatial resolution and are not confined to the bilayer since that is below the resolution

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limit. Thus the impedance probe provides higher resolution and more accurate data than optical observations. For example, using an impedance-based sensor we are able to measure the cell volume change in response to 1 mOsm challenge.⁹ This small change is not accessible with the optical microscope. In addition, more accurate calculation of volume using optical microscope requires a three dimensional image stack for each data point, deconvolution to reduce blurring, and integration of the image over densities above background arbitrarily set by the user. The dimensions of the cell are small compared to the space constant of the extracellular fluid so stimulus is constant over the cell. In this case, the impedance of a biological cell suspension depends primarily on the volume concentration, and is nearly independent of cell shape.³⁷ During fluid flow, cells also invariably passed by at high speeds on either side of the chevron. These fast transient signals were suppressed by low pass filtering of the data at about 2 Hz.

The cell volume change in response to changes in extracellular solution was recorded as a function of time. When a cell was arrested in the sensing zone, the solution was switched to a plain isotonic solution containing no cells in order to establish a baseline representing the resting cell volume with minimal cell interference. The channel was then perfused with hypotonic solution to cause cell swelling. The volume change of the single cell in response to hypotonic challenge was measured in real-time, and the images of cell volume were simultaneously recorded using optical microscopy. Figs. 4A and B show respectively optical micrographs of a suspended MDCK cell in an isotonic solution (326 mOsm) and the cell swollen in a hypotonic solution (187 mOsm). A movie of the cell's response is shown in the ESI, SI-II and SI-III.[†] The sensor output in Fig. 4C provides a precise record of the volume change in response to changes in osmolarity of extracellular solution. The cell swelled by more than 90% of its resting volume in approximately 1 min followed by a slow volume decrease. When cells are exposed to a hypotonic extracellular fluid, an imbalance in the osmolarity between the intracellular and extracellular solutions generates water flux across the cell membrane causing the cells to swell. This volume change activates homeostatic regulatory mechanisms that transport osmolytes across the membrane reducing internal osmotic pressure. The gradual release of osmolytes causes the cell to reduce its volume towards its original size. This change is called the regulatory volume decrease (RVD).^{7,8} In MDCK cells, RVD is related to a loss of K^+ , Cl^- and amino acids through both K^+ channels and anion channels.³⁸ As seen in Fig. 4C, the subsequent perfusion of the channel with isotonic solution brings the cell to a state that is slightly lower than its original volume, illustrating the loss of osmolytes during RVD. Fig. 4D shows a control experiment where the empty chamber was perfused with isotonic, hypotonic and isotonic solutions following the same protocol. The change in chamber resistance subjected to solution exchange was 1.5 mV, which is less than 10% of the sensor output at the maximum cell swelling. The sharp spikes in the measured signal are a result of switching the solutions.

We have tested other cell types, such as NIH3T3 fibroblasts, and observed similar results. The volume change of a single NIH3T3 cell in response to hypotonic stimulation is shown in the ESI (SI-IV).[†] Since the resting volume of fibroblasts is larger than MDCK cells we observed a larger output signal or equivalently higher sensor sensitivity.

The volume change of single-cells measured using sensor chip was compared to the average cell volume change from large cell populations using a Coulter counter (Fig. 5). The solid lines in Fig. 5 show the relative volume change of three individual cells subjected to a step change of osmolarity from 326 to 187 mOsm. The relative volume percent change for a given cell was obtained by normalizing the measured voltage change during osmotic challenge to the voltage drop due to the capture of that cell. For comparison, the data obtained using the Coulter counter is shown in Fig. 5 (triangular data points), and the error bars indicate the variation over four runs. As seen in Fig. 5, the time resolution of the Coulter experiments is much lower than the

chip (for the Coulter counter the cell were sampled every 1.5 min). The Coulter counter provides a size assay of a population of cells, producing a histogram of the cell size distribution. The assay requires a large number of cells and large volumes of solution making the assay slow relative to many of the cell volume kinetics. In a sense, the Coulter counter provides a time averaged sample of cell volume in the time that it takes to measure a statistically meaningful number of cells. Thus, the coulter analyses can't represent the true time course of the swelling. The response of individual cells to osmotic challenge showed a large variation in both amplitude and kinetics. As shown in Fig. 5, the maximum swelling of individual cells are different from cell to cell (65%, 90% and 100%) even though the osmolarity in extracellular solution was the same. In contrast, the Coulter counter measured the mean cell volume change, showing an approximately 80% increase in mean volume after 4 min of hypotonic challenge. The MDCK cell line is a heterogeneous population of cells with diverse physiological properties.³⁹ Cells differ in resting size, physiological properties and potentially different expression of transporters. The maximum swelling reflects a kinetic balance of all transports governed by regulatory mechanisms including cytoskeletal stresses. The variation in maximum volume in different cells (Fig. 5) indicates that there is considerable cellular variability. The time course of volume change of three single cells was obtained by curve fitting the rising phase with a single exponential, and the time constants of volume increase were found to be 0.8 min, 0.4 min, and 0.5 min, respectively. In contrast, the time constant of mean volume increase measured using the Coulter counter was 1.6 min. The time course of initial swelling reflects the osmotic water permeability of the membrane and aquaporins (AQPs) can greatly increase the permeability of the bilipid membrane to water.^{40–42} Collecting duct cells of the kidney that MDCK cells emulate have abundant AQP-2 and AQP-3.⁴³ Differences in swelling rate may be the result of differences of AQP expression in the MDCK cells. While many factors can affect cell volume, these factors can be assayed by treating the cells with selective inhibitors of suspected transporters or from genetic knockouts or knockdowns of suspected transporters. Since the initial swelling of the cells is much faster than can be measured by the Coulter counter, these aspects of cell physiology are best done in a microfluidic sensor chip. Although this work is focused on the volume measurement of mammalian cells, the technique can be used to measure any organelles defined by non-conductive membranes. For example, the same sensor strategy can be utilized to measure the volume change of yeast and bacteria; in such cases, a fully sealed chamber with much smaller dimensions is needed to further increase the sensitivity of the device.

Conclusions

We have developed an impedance based sensor that can measure the volume change of a single cell in real time. We have demonstrated that the impedance based sensing provides the required sensitivity and resolution to detect single-cell volume change. Using the sensor, we have measured the response of MDCK cells to osmotic challenges showing that both the maximum volume and the time course of the swelling vary between cells. The single cell sensor allows consecutive perfusion of various solutions, and can measure the volume response in real time with changes in cell morphology being simultaneously monitored using optical microscopy.

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Fig. 1. SEM image of the single-cell sensor. The narrow rectangular region with two sensing electrodes defines the sensing region. The enlarged micrograph shows the details of chevron-shaped structure in the sensing region to capture cells. The two outer electrodes in the wider section supply current and the inner two electrodes measure voltage.

Fig. 2. (A) Numerical simulation of the flow field in the sensing region. (B) Velocity distribution of flow in the center of the channel [along the dashed line in (A)] and the sides of the channel [along the solid line in (A)]. The inlet velocity at the entrance of the main channel (not shown) was set as 1 mm/s.

Fig. 3. Real-time recording of sequential captures of three single cells. Upper panels: Optical micrograph of the sensing region before (3A) and after each cell was captured (3B–D). 3E (lower panel): Resistance change during capture of three single cells. The input current was 2 μ A RMS. The arrows indicate the time when the corresponding optical images (B–D) were taken.

Fig. 4.

Single cell response to hypotonic challenges. (A–B) Optical images of a captured single cell in an isotonic and hypotonic solution, respectively. (C) Volume change when the solution in the channel was changed sequentially from isotonic (326 mOsm) to hypotonic solution (187 mOsm) and back to isotonic. (D) The sensor output without cells in the channel when the solutions were perfused following the same protocol as in (C).

Fig. 5.

Comparison of the time resolved volume change of single cells (solid lines) and the average volume change measured using the Coulter counter (triangles). The error bars indicate the variation from four Coulter counter measurements. For the single cell measurements, the relative volume change was determined by comparing change in resistance during cell swelling to the change in resistance due to the capture of the same cell.