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Study of long-term viability of endothelial cells for lab-on-a-chip devices

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ABSTRACT

Biosensors that employ live mammalian cells as sensing elements require precise information about the cell longevity. These biosensors could be stored in an incubator and used during the lifetime of the cells. This paper is a study of the longevity of bovine aortic endothelial cells (BAECs) that are used as sensorial component for cell-based biosensors. Different types of polydimethylsiloxane (PDMS) cell culturing chambers along with the culturing conditions required for BAECs to survive long term in lab on a chip systems are presented. The electric cell-substrate impedance (ECIS) technique was used to monitor cell viability over extended time periods. Media was automatically recirculated over the cells by a portable pump, in order to create the conditions required for testing the sensor in the field. It was demonstrated the BAECs could survive up to 37 days.

1. Introduction

Cell-based biosensors employ living cells as sensors and have the capability to monitor the reaction between cells and analyte without the need of a priori knowledge of the analyte's chemistry. Biosensors incorporating mammalian offer important information about the physiological effect of an analyte on the mammalian cells [1,2]. The ability to operate and screen for unknown analytes provides benefits in numerous applications including: toxic industrial water and chemicals, pharmacological drugs or pathogens monitoring [3–15]. The signal readout maybe configured for electrical (electrical potential or impedance spectroscopy techniques) or optical (fluorescent, colorimetric or luminescent) sensing [3,10].

The observation of the cell viability is important for the cell-based biosensors, where cell death is an indicator of the analyte potential threat to human health [16,17]. Bovine aortic endothelial cells (BAECs) are one cell type that can be used in cell-based toxicity sensing as they respond to a variety of toxicants and demonstrate relatively long-term survivability [16–18]. In addition, they are commercially available and their large size and stable morphology provide useful features for cell-based sensing applications.

To develop a field portable cell-based sensor, there is a need to develop a miniaturized cell culture chip that has the capability to maintain long-term cell viability automatically with low cell media consumption. Having exact information about the BAECs longevity is important, because the biosensor shelf life is limited by the life time of the cells. All the experiments using a sensor based on live cells have to be performed during the sensorial cells life time. Long-term cell maintenance requires the device to provide suitable physiological conditions for cells, including cell culture media composition, pressure, shear stress, temperature, pH, and chemical and geometrical microenvironment [19–22].

This paper presents a study of the longevity of BAECs cultured in a polymer-based chip, specially fabricated for these experiments. This study provides information about the length of time interval when the BAECs are viable and should be employed as sensing elements. The portable sensor could be used for sensing, only during the time interval when the cells remain viable. In this research we demonstrated that the BAECs cultured inside the polymer-based chip remained morphologically viable for 37 days. Observing the integrity of the cells with the microscope over 37 days or performing live/dead stain to determine cell viability could be a lengthy process. Measurements and cell preparation should be made at each endpoint, making

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the process labor-intensive and expensive. In this research, to simplify the BAECs longevity monitoring for long time, electric cell-substrate impedance (ECIS) technique was used. ECIS is a real-time, label-free, impedance-based method generally used to study the activities of cells grown in culture [23–37].

Cell-based sensors could be used in the field for water toxicity testing or other similar tests [16–19]. In the field the person using the sensor will not have the laboratory conditions to change the media manually and also will not be able to transport large quantity of media required for maintaining high cellular viability. In the experiments presented in this paper the BAECs were kept alive for 37 days and the media was automatically recirculated over the cells during this time by a portable pump, in order to create the conditions required for testing the sensor in the field. The automatic media perfusion provides physiological cell culture conditions in terms of media composition, pressure and shear stress.

The cell culture chip presented in this paper, conceived to keep the cells alive for long time, was designed as an enclosed perfusion cell culture chamber containing multiple parallel microchannels. To appropriately design the dimensions of microchannels and chambers in the chip, finite element analysis (FEA) was performed to simulate its fluidic properties. After the enclosed cell culture chamber was fabricated with soft lithography, cell viability in the chamber was investigated. To evaluate the performance of enclosed culture chamber for long-term cell maintenance, a parallel experiment with an open-chamber was performed to mimic the standard cell culture method. At the end of this paper, our enclosed perfusion chamber was integrated onto ECIS sensors and cell longevity studies were performed with the ECIS technique. We successfully demonstrated the possibility of long-term cell maintenance on the ECIS toxicity sensors for field applications. This is the first time when the BAECs longevity is assessed with ECIS measurements and automatically recirculated media.

2. Materials and methods

2.1. Enclosed perfusion chamber and open chamber

Two types of cell culture chambers were used in this study. The first type of culturing chamber, illustrated in Fig. 1(a), was fabricated as a simple open rectangular well with thick PDMS walls to mimic the standard cell culture method, and with similar culture area as the enclosed cell culture chamber. The second type of cell culture chamber, illustrated in Fig. 1(b), is enclosed in a PDMS layer, and incorporated a tree-like network of microfluidic channels (for the media perfusion) leading to the cell culturing chamber and leading away from the chamber (media evacuation). The enclosed cell culture chamber is important for this research, because it allows the media to be automatically flowed with the help of a pump.

The enclosed cell culture chamber integrated with automatic media perfusion was designed to meet two requirements for improving long-term cell viability: (1) minimize flow related shear stress effects below the level which can impair cell function and, (2) control cell media flow for equality at all points in the cell culture chamber. A series of perfusion microchannels on both sides of cell chamber were designed to provide equal flow to all perfusion channels [20,37]. As shown in Fig. 1(b), the microfluidic chamber and microchannels were fabricated in two different planes. By locating the media inlets and outlets above the culturing surface the fluid flow path reduces excessive shear stress at the cell surface. The perfusion barriers were used to evenly distribute media over the cell culture area. In addition to the perfusion inlet and outlet, an extra inlet is provided for cell seeding. The small dimensions of the perfusion channel provides high flow resistance, thus low flow rate control is achieved by appropriate fluidic dimensions for a given head pressure with a concomitant low shear stress applied to the cells during any flow related procedure. With this microfluidic device design, cell media can be automatically perfused into the culturing chamber in an even fashion while maintaining shear stress induced by media perfusion lower than the level that can impair cell longevity.

2.2. Finite element analysis of fluidic properties of the enclosed perfusion culture chamber

Finite element analysis (FEA) was performed to design and evaluate the fluidic properties of the enclosed perfusion culture chamber. For this simulation, the stationary flow module of COMSOL Multiphysics based on Incompressible Navier–Stokes equation was used [38,39]:

$$\rho\left(\frac{\partial V}{\partial t} + V \cdot VV\right) = -\nabla P + \nabla \cdot T + f \quad (1)$$

where V is the medium flow velocity, ρ is the medium density, P is the pressure, T is the stress tensor, and f is the body forces per unit volume. A 2-D finite element model, which contains inlet and outlet, microchannels and culture chamber, was developed to evaluate the uniformity of media perfusion through the cell culture chamber, Fig. 1(b). The diameter of inlet and outlet ports are 4 mm, the width of the inlet and outlet main channel is 3 mm, the width of six branch channel is 1 mm, perfusion microchannel and spacing is 1 mm in length and 0.3 mm in width. There are 10 barriers on each side of the chamber. The dimensions of culturing chamber are 10 mm × 6.3 mm. To ensure the negligible shear stress on cells during media perfusion, another 2-D model to simulate the flow velocity profile within the cross-section of the culture chamber was developed. The heights of inlet and outlet are 0.1 mm, respectively. Two values of the culture chamber depth (1 mm and 3 mm) were used in the simulation. For these two simulations, the property of medium flow is incompressible, and the temperature was set as 310 K. No-slip boundary conditions were applied to the walls of the channel. A parabolic velocity profile with an average speed of 500 μL/h perfusion rate was used at the inlet and the pressure at the outlet was set as zero.

2.3. Fabrication of cell culturing chambers

Two types of cell culturing chambers were fabricated from polydimethylsiloxane (PDMS) on microscope glass slides (25 mm × 75 mm) (Fisher Scientific). The fabrication procedure for the open PDMS culturing chamber is straightforward. The PDMS culturing chambers shown in Fig. 1(a) were fabricated with cubic dimensions of: 10 mm × 6 mm × 10 mm. The PDMS used in this research was Sylgard 184 Elastomer Kit (Dow Corning Inc.), with a mixing ratio of monomer to curing agent of 10:1. As a viscous liquid, the PDMS was poured onto a regular silicon wafer to a thickness of about 10 mm. The PDMS coated silicon wafer was then introduced into a vacuum oven to remove any gas bubbles and cured at 80 °C for 4 h. The cubic culturing chambers with thick walls were cut with a surgical knife from the cured PDMS material. Three cubic culturing chambers were then bonded on a microscope glass slide using a high frequency generator (BD-10AS, Electro-Technic Products). These open PDMS culturing chambers are illustrated in Fig. 1(a).

The enclosed perfusion PDMS microfluidic chamber was fabricated by soft lithography. Two PDMS layers were used to fabricate the culturing chamber at the bottom and the microfluidic channels in a different plan above the culturing chamber. The bottom PDMS layer contained only the culturing chambers whereas the top PDMS layer contained the microfluidic channels, the perfusion barrier, culturing chamber, and the inlet and outlet connections along with

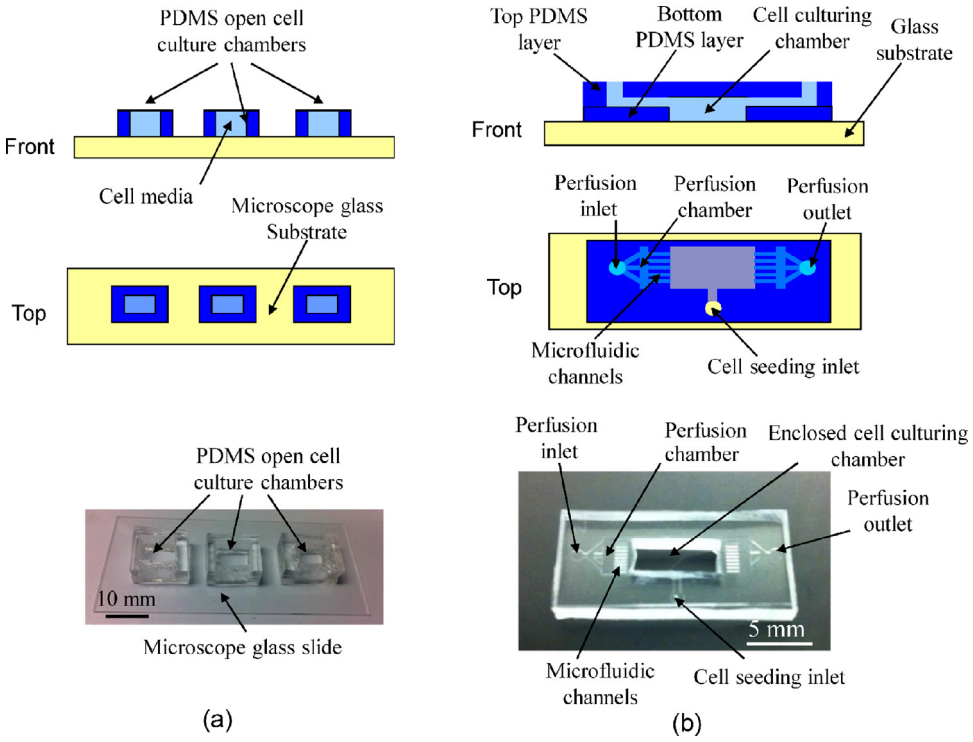

Fig. 1. Schematic view and photo of open cell culture chambers (a) and a enclosed perfusion microfluidic cell culture device (b).

the cell seeding inlet. The PDMS layer containing the culturing chamber was fabricated using a similar approach. The geometry of the culturing chamber was fixed at 10 mm × 6 mm × 3 mm ($l \times w \times t$). For the top PDMS layer a SU-8 mold was fabricated using standard photolithography. Into this mold, PDMS was poured, and after curing this, the PDMS specimen was removed. The width and spacing of the microchannels in barriers were designed at 300 μm. The depth of microchannels was fixed at 80 μm. After the top and bottom PDMS layers were fabricated, the one with microchannels that constitutes the top of the culturing chamber was bonded to the bottom cell culture chamber layer with oxygen plasma. The experimental parameters used for plasma bonding were as follows: power: 50 W; pressure: 200 mT; O_2: 98 sccm; and plasma time duration: 15 s. Subsequently both the top and bottom of this structure were bonded either to a microscope glass slide using a similar plasma procedure or glued to commercial ECIS electrodes (Applied Biophysics, Troy) using a silicone glue (DAP Products, Inc.; Baltimore, MD) and allowed to dry for 1 h. The overall dimension of the PDMS microfluidic chip was around 25 × 12 × 8 mm. Fig. 1(b) shows a photograph of a completed PDMS microfluidic chamber with perfusion capabilities. Fig. 2 shows the perfusion microfluidic device fixed to commercial ECIS electrodes.

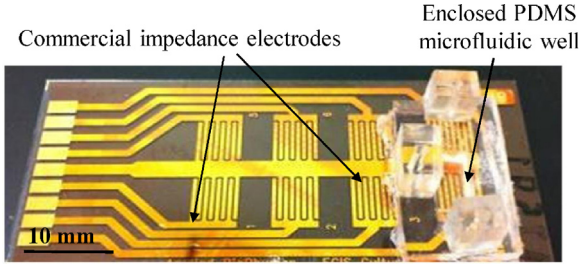

Fig. 2. Photograph of enclosed PDMS microfluidic device glued onto a commercial chip with ECIS electrodes.

2.4. Device preparation and cell culture

BAEC longevity tests were performed in open PDMS culturing chambers (Fig. 1(a)) and enclosed perfusion PDMS chambers (Fig. 1(b)) bonded on microscopic glass slides. Experiments performed with the open cell culture chambers were carried out in a Petri dish filled with deionized (DI) water to prevent media evaporation. Experiments performed with the enclosed perfusion chambers were achieved by connecting to a commercially available peristaltic pump with variable flow (Fisher Scientific), used to drive media over the cells in the culturing chamber. Tubing and a media bag were connected to the microfluidic chambers and micropump.

Before any tests were performed, all tubing, connectors, and culturing chambers were sterilized in an autoclave. In order to completely sterilize the microchannels and the microchamber, during the autoclave process, the enclosed perfusion chamber device was filled with water and maintained at 121 °C in the autoclave for 30 min. Subsequently, the chips were exposed to UV light for 10 min. Tubing was then connected to the perfusion culturing chamber and the micro pump. For all the experiments, fibronectin was used as an extracellular matrix coating. Before seeding the cells, the fibronectin solution with the concentration of 30 μg/mL was filled into the cell culture chamber and incubated for 1 h. Next, the fibronectin solution was removed and replaced with BAECs (VEC Technologies, Inc.) with desired densities. The media used for cell cultures was MCDB-131 with 10% FBS, 1% L-Glutamine and 1% Pen-Strept (VEC Technology, Inc.).

2.5. Viability and longevity testing of BAECs

BAEC viability studies were performed in triplicate in open PDMS culturing chambers, with three identical open chambers attached to a single glass slide, Fig. 1(a). It is likely that some of the dead cells are flushed away when the media is changed. Based on our cell viability experiments, we found that cell death is not dependent on the initial cell density (Data not shown in this paper). The initial cell density used in these experiments was

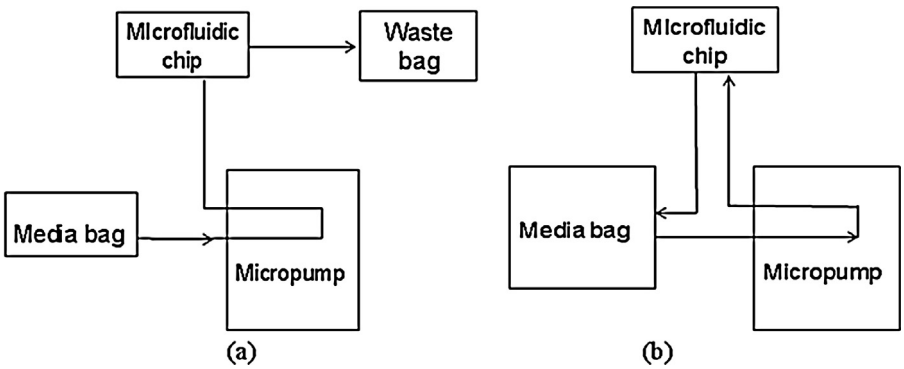

Fig. 3. Schematic of long-term cell culture microfluidic device with (a) the non-circulating and (b) recirculating perfusion modes.

chosen as 10,000 cells/cm^2. Six glass slides each containing three open chambers were used in the experiment. In accordance with normal culture protocol, cell media was manually changed every three days during the long-term viability studies. Cell morphology was observed every day under microscopy to characterize cell viability.

For testing the viability and longevity of BAECs cultured in enclosed perfusion culture chambers, two modes of media perfusion were used: nonrecirculating and recirculating, see Fig. 3(a) and (b). In the noncirculating perfusion mode (Fig. 3a), the media was delivered from the source bag through the perfusion culturing chamber to waste so that any secreted factors from the BAECs or waste products were not re-exposed to the cells. In this nonrecirculating mode, the pump was operated every two days during the first six days, with the flow rate of 1 mL/min for 5 min. Once the cells reached the confluence, the pump was operated each day with a flow rate of 1 mL/min for 5 min. The size of the enclosed perfusion microfluidic chamber used in this experiment was 10 mm × 6 mm × 3 mm and total volume of the microchamber was 180 μL.

In the recirculating perfusion mode (Fig. 3b), the pump was operated continuously moving the media through the system, into the perfusion chamber and back into the source media bag. The recirculating micropump was connected to the inlet of the perfusion microfluidic chamber to circulate the media from the bag through the culturing chamber. The outlet of the culturing chamber was connected back into the media bag for recirculation. The recirculation flow rate was maintained at 500 μL/h, which allowed the microchamber volume to be swept around three times/hour. Secreted factors from the BAECs and waste products were diluted into the total culture media volume before recirculating back to the cell chamber. With the geometry of the perfusion microchannels positioned 1 mm above the cell culture, the shear stress observed by the cells was minimized to a level where they were unaffected by fluidic flow procedures. Fig. 4 shows the image of the biological hood containing the experimental test-bed (perfusion microfluidic culture chamber glued glass slides, tubing, media bags and pump for media circulation) with non-circulating mode. In non-circulating mode there is one media bag containing fresh media and another media bag for storing the wasted media. In recirculating mode, there is only one media bag that initially contains fresh media and the waste is later transported to this bag. Media and waste are circulated over the cell culture.

For both the non-recirculating and recirculating perfusion modes, the perfusion was initiated by using the pump one day after the cells were plated in the chamber. After one day all the cells were firmly attached to the fibronectin-coated substrate surface. Cell morphology was observed each day by optical microscopy to characterize cell viability.

2.6. Electric cell-substrate impedance (ECIS) measurements of BAECs longevity

The BAECs viability over one month was monitored with commercial ECIS electrodes. Custom made PDMS microfluidic chambers with perfusion capability were fabricated for this experiment and glued on the ECIS electrodes, see Fig. 2.

The microfluidic perfusion chamber containing BAECs was connected to the media bag in a closed circuit with a pump. The pump flow rate was 500 μL/h and the media was constantly flowing from the media bag over the tested BAECs and back into the media bag. The media and waste was automatically circulated over the cells during the entire experiment.

During impedance measurements the cell suspension was introduced in the perfusion microfluidic cell culturing chamber using the cell seeding inlet and impedance values were recorded as a function of time. The working and counter electrodes are coupled by the electrolytic cell culture medium. The amplitude of ac voltage signal was 10 mV peak-to-peak. The frequency used in this experiment was 64 kHz. In these experiments, the microfluidic chambers were seeded with BAECs at an initial cell density of 80,000 cells/cm^2. As before, the cells were seeded on fibronectin as the extracellular matrix. During the experiments, the impedance values of BAECs monolayer were constantly monitored during 37 days to confirm that the cells were alive. Impedance measurements

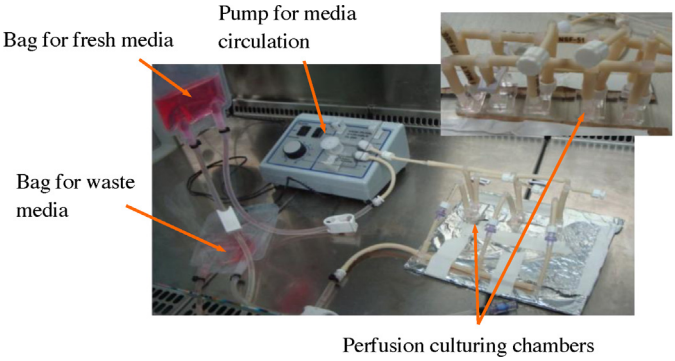

Fig. 4. The experimental test-bed (perfusion microfluidic culture chamber glued glass slides, tubing, media bags and pump for media circulation) using non-circulating mode. In this non-circulating mode there is a media bag for fresh media and a media bag for waste. The waste is not recirculated over the cell culture. The media is automatically circulating by the pump.

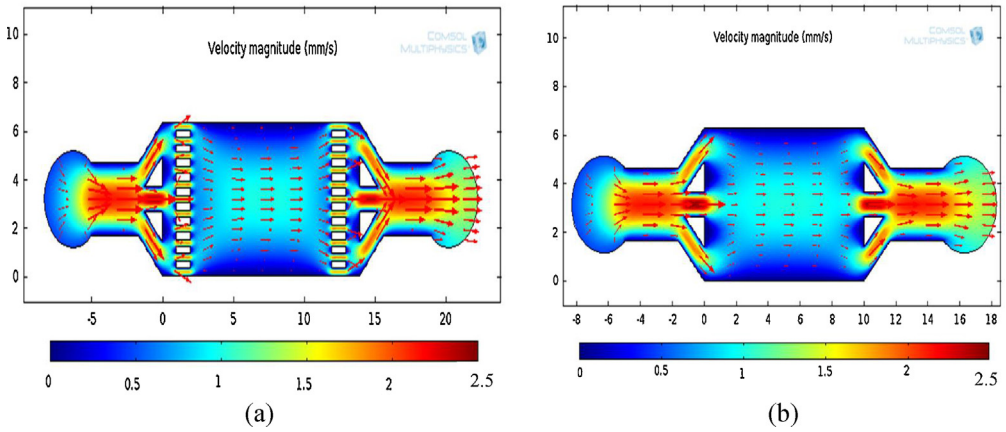

Fig. 5. The medium flow velocity field in horizontal plane (length × width plane) of (a) a chamber with microchannels; (b) a chamber without perfusion microchannels.

with a control culturing chamber filled with cell media only, were also performed during 37 days.

3. Results and discussions

3.1. Finite element analysis of fluidic properties of the enclosed chamber

Fig. 5(a) shows the medium flow velocity field in a horizontal plane (length × width plane) of a culturing chamber with perfusion microchannles. In Fig. 5(a), the medium was introduced from the left port and flowed into three branch channels before entering into the main culturing chamber. The barriers designed at the left and right side of the culturing chamber allow a more uniform medium flow into and out of the culturing chamber. The medium flows out of the perfusion device through three branch channels and the output port designed at the right side. The arrows indicate the flow direction and velocity field intensity. Fig. 5(b) shows the flow velocity field in a horizontal plane of a chamber without perfusion microchannels. In this case the flow profile is less uniform than in the chamber containing microfludic barrier channels. Therefore these barrier microchannels in the enclosed culture chip significantly enhance the homogeneity of the media flow. It is expected that the more and narrower the microchannels are, the more uniform the media flows through the chamber is. However, the small dimensions of microchannels induce the complexity and more cost for the fabrication. In our simulation, with the configuration of the width and spacing of microchannles in barriers of 300 μm, the flow velocity field is uniform through the majority of the chamber, which provides the cells with a homogenous culture environment.

Fig. 6(a) and (b) show the flow velocity profile in the cross-section (length × height plane) of chambers with two heights of 1 mm and 3 mm. Fig. 6(c) and (d) show the flow velocity profile on the middle lines (shown in Fig. 6(a) and (b)) of these two chambers. With the constant flow rate through chambers, the maximum velocity at the middle line in the chamber is inversely proportional to the height of the chamber. The deeper the chamber, the smaller the flow resulting velocity. Usually, if the inlet and outlet have the same cross-section with that of the chamber, the flow velocity in the chamber has a parabolic profile due to the laminar flow. Since in our study, the inlet and outlet are located close to the upper surface of the chamber, the maximum velocity is not at the central point of the line, but shifts to the upper region. For example, for the 3 mm deep chamber with 0.1 mm deep inlet and outlet, the maximum velocity is located at 1.6 mm above the bottom surface.

The resulting shear stress produced by the fluid flow on the cells was calculated using a mathematical model that assumes a Newtonian fluid with the shear tensor proportional to the velocity gradient, namely shear rate [40].

$$\tau = \mu \frac{du}{dy} \tag{2}$$

where μ is viscosity, u is the fluid velocity and y is the distance form the surface, and du/dy is the velocity gradient. The parabolic flow profile in the cell culturing chamber, in the case of laminar flow (also known as Poiseuille flow), has the shear stress at the wall estimated as; [38],

$$\tau = \frac{6\mu Q}{h^2 w} \tag{3}$$

where Q is the flow rate, h is the chamber height, and w is the chamber width. In our enclosed chambers, since the flow profile is not symmetric, the velocity gradient at the bottom surface of the chamber is smaller than that at the top surface. Therefore, the shear stress on cells cultured on the bottom surface of chambers is less than the value given by Eq. (3). With the flow rate of 1 mL/min used in non-recirculating mode, chamber width of 6 mm and chamber height of 1 mm, the shear stress by Eq. (3) is 0.2 dyne/cm^2, which is orders of magnitude below the stress level (4–25 dyn/cm^2) at which adverse effects are observed on the cells [38].

3.2. Cell longevity in open and enclosed culturing and chambers

Fig, 7 shows images of cells cultured in the perfusion microfluidic device with recirculating mode (7a) and nonrecirculating mode (7b) and in open chambers (Fig. 7c) at different time intervals. By visual inspection, the cell images indicate that regardless of the type of culturing chamber or the media circulating mode, the BAECs can be maintained viable for approximately one month. Fig. 7(a), (b) and (c) show the cells after the 3rd and 22nd day in both types of devices. By the 3rd day, a monolayer of cells attached to the substrate is forming and all the cells appear viable. There are no apparent differences between the cells seeded in the perfusion microfluidic device with circulating mode or noncirculating mode or in the open chambers. By the day 22nd, the cell images also show no apparent differences between the cells cultured in the two different devices. However, by the 32nd day, the visual appearance of the cells in the perfusion microfluidic and open chamber devices is markedly different with significant evidence of cell death in the open chamber when compared with the enclosed perfusion microfluidic device.

For the same cell culture, the enclosed microfluidic chambers and open chambers exhibited cell maintenance times, of 37 and

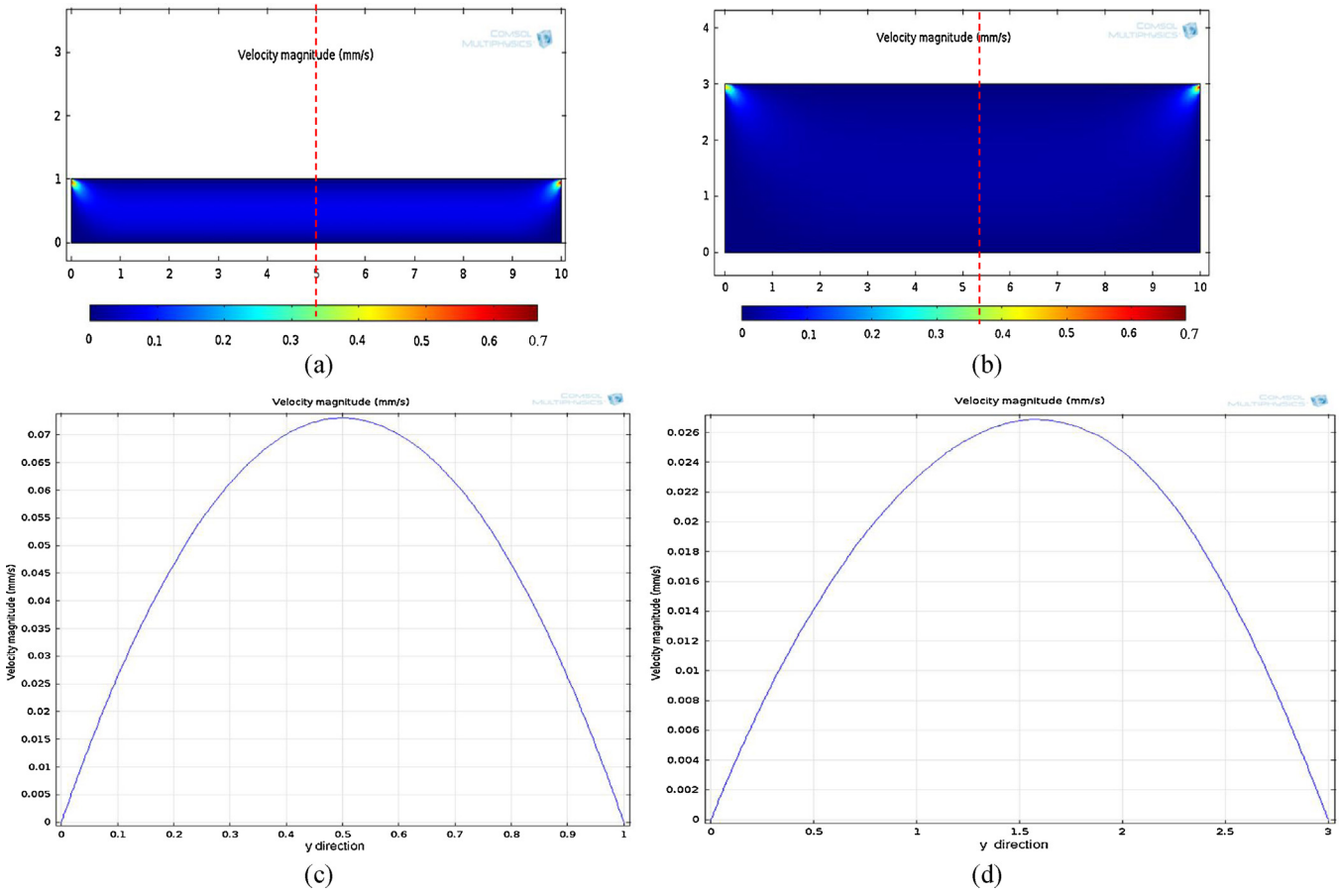

Fig. 6. The flow velocity profile (a) in the cross-section (length × height plane) of a 1 mm-deep chamber; (b) in the cross-section (length × height plane) of a 3 mm-deep chambers; (c) on the line shown in Fig. 6(a) and (d) on the line shown in Fig. 6(b).

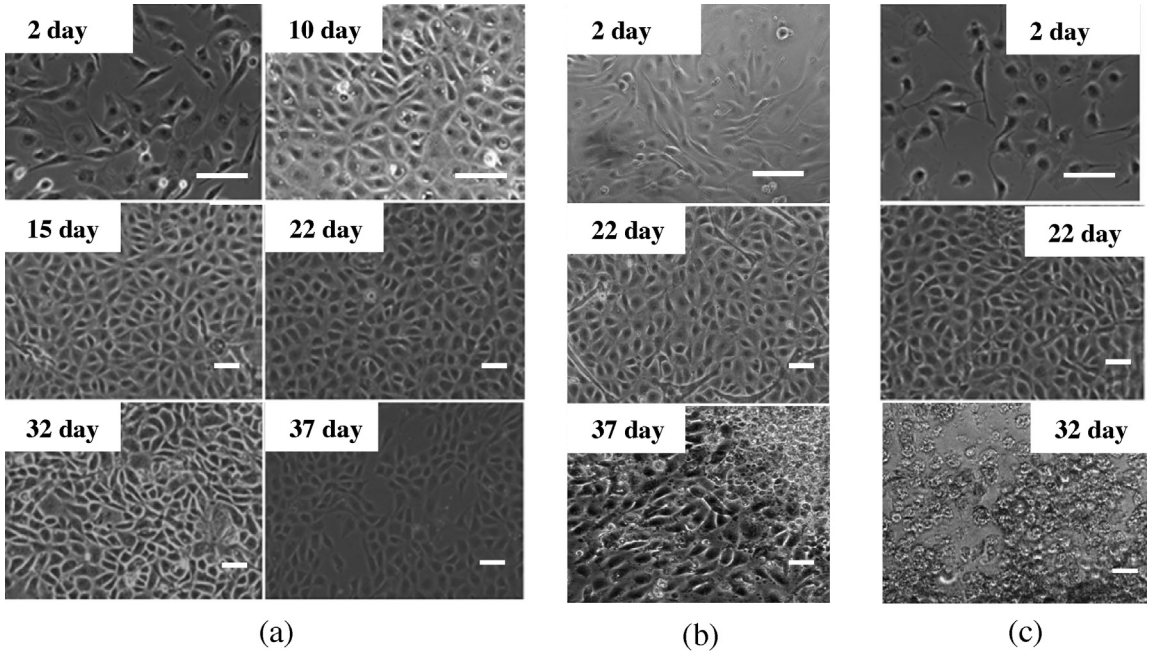

Fig. 7. Images of cells cultured at different times in: (a) enclosed perfusion microfluidic device and recirculation mode, (b) enclosed perfusion microfluidic device and noncirculation mode and (c) in the open culturing chambers. These images demonstrated that the cells can be maintained in the enclosed microfluidic devices and in the open culturing chambers for more than one month. On the enclosed perfusions microfluidic device the cells start dying after 37 days and on open culturing chambers the cells start dying after 32 days. All the scale bar have the dimensions 20 μm.

32 days respectively. The slightly longer time to maintain cells healthy in enclosed chambers than in open chambers shows that our enclosed microfluidic perfusion culture chip has compatible or even higher capability to maintain cells than standard cell culture methods. The automatic media perfusion provides a supply of nutrients and metabolic waste removal for cell maintenance. Unlike the standard cell culture method, in which media is replaced every three or four days manually, the enclosed culture chips integrated with perfusion function offer the possibility for automatic cell culture without the need for operator intervention. Moreover, the enclosed configuration reduces the risks of contamination in cells as if prevents the cells from coming into direct contact with the incubator air.

For the enclosed microfluidic chips, the devices were operated under two media perfusion modes: non-recirculating and recirculating, as shown in Fig. 3. In the non-recirculating mode, which is more similar to standard cell culture method, the media is replaced regularly with perfusion method to supply nutrients and remove waste. In recirculating mode, media perfusion is continuous and the secreted factors and waste products are diluted into total culture media volume and recirculate back to cells. Comparing these two modes, continuous perfusion culture with recirculating mode increases nutrient delivery. However, it may influence the local concentration of secreted factors. The method to overcome this limitation in recirculating mode is to replenish media in a controlled manner. The media consumption in non-recirculating mode is determined by culture media residence time, which is defined as the time needed for one complete change of culture media in the cell culture chamber. If assuming the fluidic velocity is uniform, the residence time can be calculated by dividing the chamber volume by the fluid flow rate. However, based on our simulation, although the flow rate is considered as uniform in the horizontal plane (length × width plane), it is not uniform along the height direction. Therefore, the true culture media residence time increases with a given flow rate, which increases media consumption. Increasing the uniformity of the flow rate along height direction is needed to reduce media consumption in the non-recirculating mode. In the recirculating mode, the media consumption is independent of the flow rate and uniformity of the flow velocity, and only determined by cell density and culturing time.

In our experiments, a benchtop incubator provides a temperature of 37 °C for cell culture in both open and enclosed chambers. Due to the high permeability of PDMS, the incubator also provides humidity and a 5% CO_2 atmosphere to prevent the evaporation of cell media and maintain suitable media pH values (7.2–7.4) for cell culture. Although an incubator was used in this study, for field applications, the enclosed cell culture chip could be easy modified to eliminate the need of an incubator. In this case the cell culture chamber could be fabricated on a hotplate with controlled temperature. Coating the PDMS layer with a low-permeable material, such as Parylene C, will prevent the evaporation therefore eliminates the humidification of surrounding atmosphere [38]. In addition, with such an enclosed perfusion chip having low gas permeability, the pH value of media can be maintained at an appropriate level by pre-equilibrating the cell culture media in a 5% CO_2 environment before perfusion [38].

In summary, longevity experiments were conducted to study the time interval the BAECs are viable and could be employed as a sensing monolayer. The experiments were conducted in open and enclosed culturing chambers. The enclosed culturing chamber offered a more sterile environment for the BAECs, which were viable for 37 days. The cell viability was assessed by optical inspection with a microscope. Two modes of operating the media during the longevity experiments were considered; recirculating and noncirculating mode. The enclosed culturing perfusion chip with the tubing, pump and media bag is illustrated in Fig. 4. In this figure the noncirculating mode is observed where two media bags were used; one bag contained fresh media and the second bag contained only waste media. The recirculating mode, which is not showed in the Fig. 4, included only one media bag, which initially contained fresh media and during the time of the experiment contained media and waste. The longevity experiments concluded that the BAECs longevity with recirculating and noncirculating mode is the same, 37 days. The optic inspection of BAECS vitality is laborious and in these studies ECIS technique was used to automatically monitor the BAECs over long period of time. When we started to study the BAECs longevity open culturing chamber were used due to their simple fabrication. The open culturing chambers required the media to be changed manually, could not be used for portable cell-based sensor and as illustrated in Fig. 7(c) the BAECs life is shorter on this type of chambers. Since the cell-based sensor is intended to be field portable, only the recirculating mode and the enclosed culturing chamber were used for the ECIS experiments.

3.3. ECIS measurements

The cell longevity tests described above revealed that BAECs can remain viable for at least one month. To minimize the need for visual observation we analyzed the use of the impedance spectroscopy technique to replace the intermittent visual observations with a continuous electrical signal measurement.

For this experiment, the chip with impedance electrodes illustrated in Fig. 2 was used. The PDMS enclosed culturing chamber was glues on the impedance electrodes to create the conditions required for automatic media recirculation over the BAECs culture. The impedance measurements over 37 days are graphed in Fig. 8, initial BAECs density was 80,000 cells/cm^2 (magenta color). As shown in Fig. 8(a), during the first ten hours of cell culture, the impedance value continuously increased to 2500 Ω and remained constant at that value. The increase in the impedance values suggests the cell attachment and spreading on the sensor. The ECIS measurements are possible because the viable cells membrane has dielectric properties. When the cells attach and spread on the ECIS electrodes, due to their dielectric properties, they block the current flow from the electrode into the electrolyte media. The increase in the impedance values is caused by the changes in cell morphology and cell adhesion strength to the sensor surface during cell attachment and spreading. Constant impedance values indicate that the BAECs formed a monolayer. According to Fig. 8(a) the cell monolayer was formed ten hours after seeding, when cell morphology and cell adhesion strength to the substrate remained stable.

Fig. 8(b) shows impedance measurements that were recorded for 40 h on day 28. According to Fig. 8(b) the impedance values essentially remained around 2500 Ω, and were similar to the value recorded in the second day of this experiment (See Fig. 8(a)). The constant impedance value shows that after 28 days of culture in the enclosed perfusion chamber, the BAECs were viable and remained in a compact monolayer. The impedance values started to decrease on day 28. The impedance measurements at this stage decreased at a rate of 200 Ω over the 40 h time interval. During days 28 and 29 of the experiment, the impedance values were sufficiently high for performing toxicity testing. The BAECs were further monitored by ECIS until day 37 at which point cell death started and the corresponding impedance values decreased. The cell membrane potential is only a characteristic of viable cells. When cells die they lose the dielectric properties and there will be a decrease in the measured membrane impedance. When the value of the impedance approaches zero, this is an indication of total cell death. The impedance values for the last 60 h of the experiment are shown in Fig. 8(c). When these measurements were started in day 34, the

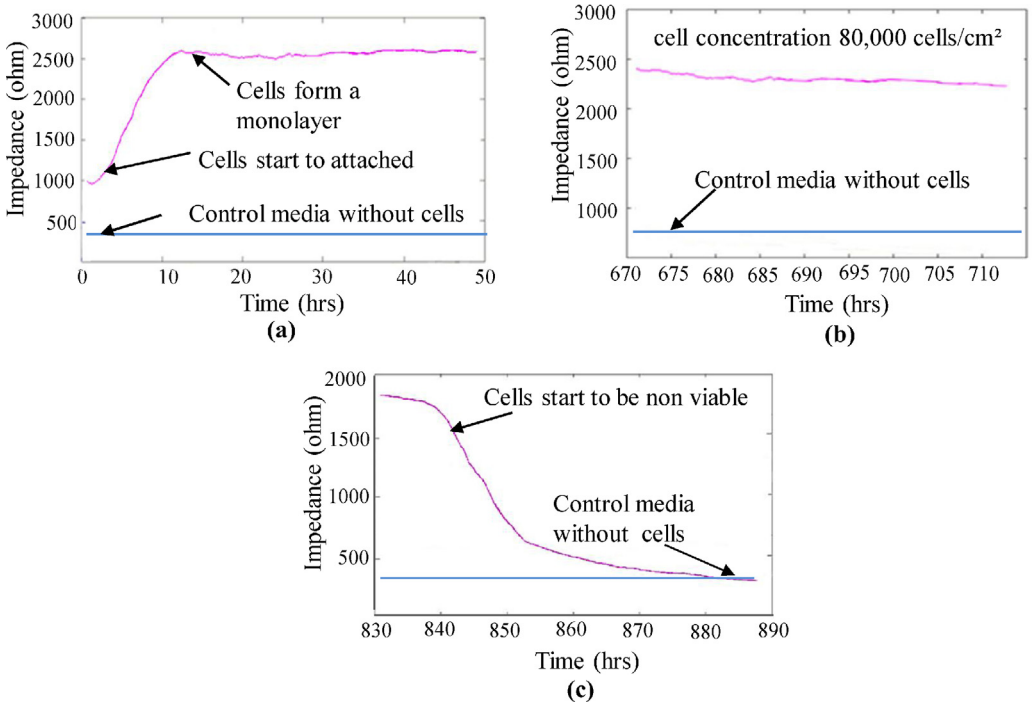

Fig. 8. Impedance measurements (magenta color) taken over 37 days, during the BAECs viability experiment. Initial BAECs density was 80,000 cells/cm^2. Impedance measurements of control media without cell is a straight line (blue color). In this experiment the recirculating mode was used. Media was automatically renewed using a micropump and media and waste were steadily flowed over the cells. (a) After about 10 h in culture, the cells formed a uniform monolayer with constant impedance values of 2500 Ω. (b) Impedance measurements starting on the twenty eight day of the experiment. The impedance values were at 2400 Ω, as a proof that the cells were still viable and formed a uniform monolayer. The impedance values were decreasing with small values over the time interval of 40 h. (c) Impedance measurements in the last 60 h of the long term BAECs viability experiment. The impedance decreased with larger values as a sign that the BAECs were not alive. The end of the experiment was during the day 37, when all the cells were not viable and the impedance value of 300 Ω was generated only by the media. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

impedance values had already dropped from the initial value of 2500 Ω to 1900 Ω. This illustrates the onset of apoptosis in a fraction of the BAECs, but at this time a large number of BAECs remained viable. After another 10 h the impedance values abruptly decreased and in the last 40 h of the experiment all the BAECs were not viable. The impedance values of 300 Ω measured at the end of the experiment, see Fig. 8(c), corresponds to the impedance of the cell media. The straight line (blue color) represents impedance measurements of control media without cells. Media was automatically renewed using a micropump and recirculating mode, and media and waste were steadily flown over the cells.

During the longevity experiment no BAECs passage was performed. As BAECs are physiologically contact inhibited, they do not grow over each other once they reach confluence. Furthermore, they slow down their division and growth once they reach confluence. The BAECS do divide. Initially the mitosis rate is high and decreases as the layer becomes confluent, eventually becoming a constant number. We have measured mitosis rate on these cells

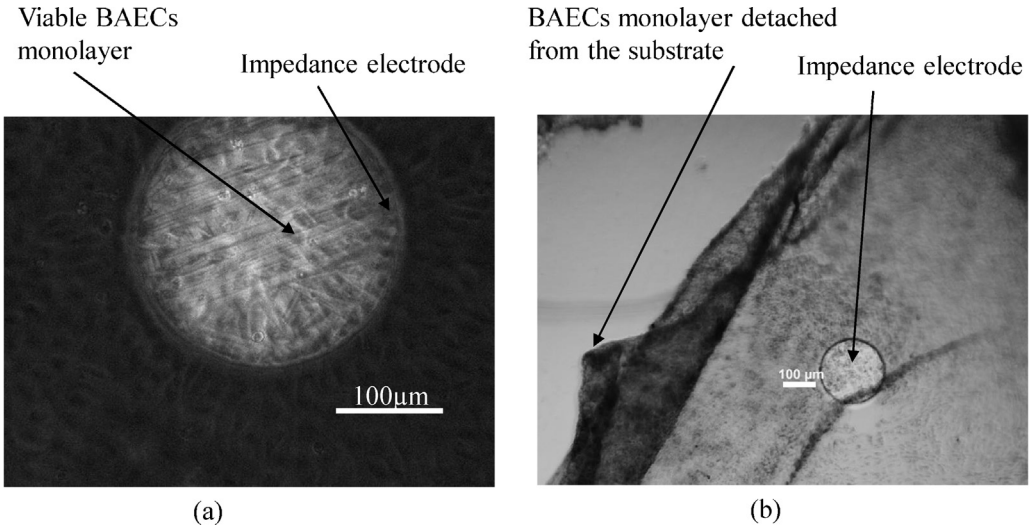

Fig. 9. Images of BAECs cultured on the chip with impedance electrodes. (a) Image of viable cell monolayer taken on day 20. (b) On day 37 the cells are not viable and the cell monolayer is detached from the substrate.

from day 1 to day 6, when the cells are fully confluent in large petri dishes. The rate is from about 1.1% on day 1 to about 0.1% on day 4 and then it is constant and equal to 0.008–0.093 on day 6 [41].

In this experiment, in order to create the conditions required for testing the sensor in the field media and waste was automatically recirculated over the cells by a portable pump during 37 days. It is possible that after one month of being kept in an incubator at 37 °C, media lost its nutritive qualities. When the cells died at the end of the experiment their death was the result of a combinations of factors such as: the cells were forming the monolayer for long time and this monolayer started to detach and also media after long time storage in the incubator lost its nutritional and septic qualities.

The substrate of the chip with the commercial ECIS electrodes was fabricated from polycarbonate, the electrodes from gold and the culturing chamber from PDMS (Fig. 2) and allowed microscopic observation of the cells. The impedance measurements were performed during 37 days. From time to time microscopic images of the cells were also taken. In this case the impedance measurements were stopped for a short time, to allow the microscopic observation of the cells. Fig. 9 shows the cells cultured on the chip with impedance electrodes on day 20 and day 37. On day 20 the cells were viable and were attached on the chip substrate in a compact monolayer, see Fig. 9(a). On day 37 the cells were apoptotic and the cell monolayer was detached from the substrate, Fig. 9(b).

This work demonstrates that it is possible to monitor cell viability over a long period of time using the ECIS technique. The impedance measurements provide useful information about the viability of BAECs.

Since the BAECs could resist at least 37 days on the fabricated chip and demonstrate high values of the impedance, these cells could be further used for cell-based sensors. After 30 days of cell culturing inside the microfluidic device the impedance values started to decrease with large values. The conclusion of these tests was that the biochips seeded with BAECs could be used for chemical or toxicity testing up to 30 days after cell seeding. After this time the impedance values start to decrease with large values and that is a sign that the cells monolayer started to detach, and this will affect the accuracy of the testing.

4. Conclusions

In conlusion, this research demonstrated that it is possible to monitor the cell longevity over long period of time using a low-cost optically transparent polymeric chip with integrated perfusion microfluidics and ECIS method. Microfluidic chambers were fabricated from PDMS to be transparent for microscopic observations and were glued on commecial impedance electrodes. BAECs were seeded in these microfluidic chambers and were monitored with ECIS technique during 37 days. To maintain the cells alive for 37 days the chip was connected in a fluidic system with the media bag and a commercial pump. During the experiment, the media and the waste media was perfused over the cells monolayer by the pump. This experiment of long term BAECs culture demonstrates that the cell viability on the chip could be up to 37 days. The ECIS approach is a label free, non-invasively and automated technique that allows the cell culture to be monitored in real-time, compared to techniques that require sacrificing a cell culture for each time point.

Although currently we have not demonstrated that BAECs can be maintained within the microfluidic chambers much longer time than in open chambers, we believe that in the future the cell maintenance time in the microfluidic chips could be improved by optimizing perfusion rate and the dimensions of the microchannels and chamber.

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Ali Khademhosseini is an Associate Professor at Harvard-MIT's Division of Health Sciences and Technology (HST), Brigham and Women's Hospital and Harvard Medical School. He also directs a satellite laboratory at the World Premier International-Advanced Institute for Materials Research at Tohoku University, Japan. In 2005 he received his PhD in Bioengineering at MIT. He received his undergraduate and master degrees, both in chemical engineering at University of Toronto in 1999 and respectively 2001. He has carried out research in the area of biomedical microdevices and biomaterials. He has developed a number of methods for controlling the stem cell microenvironment using microscale devices and to engineer biomaterials for tissue engineering. He has published extensively in the area of biomedicine and stem cell bioengineering with over 200 peer-reviewed publications, more than 150 invited presentations and 14 pending patents.