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# Periodic "flow-stop" perfusion microchannel bioreactors for mammalian and human embryonic stem cell long-term culture

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**Abstract** The present study examines the use of automated periodic "flow-stop" perfusion systems for long-term culture of mammalian cells in a microchannel bioreactor. The method is used to culture Human Foreskin Fibroblasts (HFF) and Human Umbilical Vein Endothelial Cells (HUVEC) for long periods of time (>7 d) in a microchannel (height 100 μm). Design parameters, mass transport and shear stress issues are theoretically examined via numerical simulations. Cell growth and morphology are experimentally monitored and an enhanced growth rate was measured compared to constant perfusion micro-reactors and to traditional culture in Petri dishes. Moreover, we demonstrate the use of the method to co-culture undifferentiated colonies of human Embryonic Stem Cells (hESC) on HFF feeder cells in microchannels. The successful hESC-HFF co-culture in the microbioreactor is achieved due to two vital characteristics of the developed method—short temporal exposure to flow followed by long static incubation periods. The short pulsed exposure to shear enables shear sensitive cells (e.g., hESC) to withstand the medium renewal flow. The long static incubation period may enable secreted factors (e.g., feeder cells secreted factors) to accumulate locally. Thus the developed method may be suitable for long-term culture of sensitive multi-cellular complexes in microsystems.

**Keywords** Micro-bioreactors · Perfusion system · Human embryonic stem cells · Cell culture in microsystems

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## 1 Introduction

Micro-bioreactor systems may be valuable for cellular bioassays and tissue engineering (Beebe et al. 2002; El-Ali et al. 2006). Designing reliable systems for long-term culture of cells in micro-devices with defined culture conditions, is required in order to utilize these techniques for a broad range of cell biology applications (Khademhosseini et al. 2006). Generally, micro-systems are characterized by an increased surface area to volume ratio compared to macro-systems; hence the average medium available per cell is significantly reduced in micro-systems. While traditional macro-culture systems are based on infrequent (>=once a day) medium exchange, in micro-systems medium should be refreshed constantly. Most common method to provide adequate supply of medium to cells in microdevices is to use a constant perfusion system(Andersson and van den Berg 2004).

The perfusion of medium affects local solute concentration and shear stress acting on the cells. The perfusion system should be designed according to cell viability requirements (Mehta and Linderman 2006). This is a challenging task, particularly for long term culture of mammalian cells, since mammalian cells have high nutrient requirements and are sensitive to culture conditions such as: pH, oxygen supply, etc. In order to culture mammalian cells for long periods of time, several complicated approaches have been applied, such as: micro-grooved substrates (Park et al. 2005), internal membrane oxygenators (Roy et al. 2001) and orthogonal networks (Tourovskaia et al. 2005). In general, constant perfusion systems maintain better-defined culture conditions by continuously removing metabolic byproducts and supplying fresh medium. Culturing cells in a microfluidic environment under continuous perfusion offers the ability to control cell-media interactions by producing a steady state chemical gradient in contrast to regular culture methods in

which the chemical composition varies with time (Kim et al. 2006). However, the cells are constantly exposed to shear stress, which may be undesired, and the convective flow washes out secreted factors, that may be relevant to cell function and signaling (Walker et al. 2004).

As opposed to constant flow protocols, when large microchannels (e.g, 250 μm in height) and/or insensitive cells (e.g., insect cells) are used, the average medium available per cell is sufficient to culture the cells for a day or more and medium can be infrequently and manually replaced (Walker et al. 2004, Yu et al. 2005). Under static conditions in microscale cell culture systems, diffusion becomes the dominant mass transport mechanism. Hence, secreted factors may transport locally in the cells' microenvironment in a similar manner to the *in vivo* case. Several studies have shown that cell proliferation rates in static (no flow) microsystems vary from rates observed in macroscale culture devices. For example, murine embryos cultured in microchannels proliferate more rapidly than in conventional embryo culture methods (Raty et al. 2004). Recently, Yu et al. (Yu et al. 2007) showed that as medium exchange frequency decreases normal mouse mammary gland epithelial cells proliferation increases. Their results indicated the importance in considering the physical differences between microchannels and open well culture systems when designing microchannel cell culture experiments. Thus, further signifying the need to analyze and study design parameters, mass transport and shear stress issues in microchannel bioreactors. By properly addressing these issues, it may be possible to develop, optimize and understand the unique abilities of pulsed perfusion systems—particularly for human—and stem cells which have high nutrient requirements, being sensitive to culture conditions, while their culture in microsystems is problematic.

The present study examines the use of automated periodic "flow-stop" perfusion for long-term culture of human cells in a microchannel bioreactor, presents design tools for analysis of the system and demonstrates the unique abilities of the method to culture sensitive cells in a micro-reactor. In the method we developed, cells are cultured under static incubation conditions in a microchannel and are regularly infused every several hours with medium for a short period of time (1 min). The method is used to culture Human Foreskin Fibroblasts (HFF) and Human Umbilical Vein Endothelial Cells (HUVEC) for long periods of time in a microchannel. Design parameters, mass transport and shear stress issues are theoretically examined via numerical simulations and their effect on cells' growth, morphology and biology are experimentally examined, discussed and compared to constant perfusion micro-reactors and traditional culture in Petri dishes. The ability of the method to culture sensitive cellular complex is demonstrated by successfully co-culturing Human Embryonic Stem Cells (hESC) in undifferentiated colonies on HFF feeder cells. Recently, hESC were cultured in a multi-well perfusion bioreactor inside millimeter sized (3.5 mm diameter×2 mm deep) wells (Figallo et al. 2007). In order to utilize the physical and engineering advantages associated with microfluidics and microscale approaches for controlling culture conditions and performing high-throughput experimentation it is necessary to culture the cells in microsystems (Beebe et al. 2002; Andersson and van den Berg 2004). However, as mentioned by Figallo et al., the insufficient medium hold-up within a microchannel reactor without perfusion, on one hand, and the relatively high shear stress required for medium perfusion, on the other hand, hinder hESC culture inside a microchannel bioreactor. Our method enables, for the first time, hESC culture in microchannel bioreactors using a controlled automated simple flow system. Maintaining human embryonic stem cells in the undifferentiated state is a fundamental step towards utilizing microfluidics methods to study and control hESC biology.

## 2 Methods

### 2.1 System design and fabrication

Microchannels were fabricated using conventional soft-lithography techniques, as previously described (Korin et al. 2007b). Briefly, Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) was poured over a 4″ silicon wafer containing an SU-8 pattern of the microchannel in relief and teflon inserts ($\phi$3×3 mm) which were glued to the wafer at the channels inlets and outlets. The microchannel dimensions used were 100 μm (height)× 1 mm (width)×2 cm (long), unless stated otherwise. In order to enable reuse of the microfluidics device the PDMS replicas were reversibly bonded to glass slides 76×50× 1 mm (Marienfeld, Germany). Thereafter they were thoroughly cleaned using a solution of 1% Liquid-Nox (Alconox, USA) in DI water and rinsed with DI water. The glass slides were cleaned using acetone, ethanol and isopropanol followed by immersion in an ultrasonic bath (Elma S10H, Germany) filled with DI water for 15 min at 35°C. The PDMS replicas were then immersed in 0.1N HCl solution at 80°C for 2 h. Both slides and replicas were rinsed in DI water, thoroughly dried and pressed together. The devices were then left on a hot plate at 80°C for 5 h.

The perfusion system comprised a syringe pump (KDS 210, KDS Scientific), a custom-made oxygenator, a PDMS microchannel and a waste container. The microchannel, oxygenator (1-m long, 1 mm ID, 1.3 mm OD, Silastic ® tube, Cole-Parmer), and waste container were placed in an

incubator. Medium was delivered to the cells in a controlled automated manner using a computer program which managed the syringe pump. Thus the perfusion "flow-stop" protocol parameters (static incubation time; flow period, flow rate, etc.) were computer controlled. The microchannels were sterilized with 70% ethanol. The channels were coated with 50 μg/ml human fibronectin solution (Sigma Aldrich), prior to cell seeding.

## 2.2 Cell culture

All cells were maintained in a 37°C humidified environment with 5% CO2. Human foreskin fibroblast (HFF) were cultured in medium containing: Dulbecco's Modified Eagle's Medium (DMEM+4,500 mg/l Glucose+L-glutamine—Pyruvate, Gibco) supplemented with 10% bovine calf serum (Hyclone), 0.1 M nonessential amino acids, 0.1 M β-mercaptoethanol. The cells were passaged when 95% confluent, using a solution of trypsin with 3.8 g/L EDTA (Invitrogen). HUVECs (Clonetics) were grown in EGM-2 medium (Cambrex, Walkersville, MD).

Undifferentiating hESC (H7, passages 20–40) were grown on an inactivated HFF feeder layer and maintained on hESC medium consisting of 80% knockout-DMEM supplemented with 20% knockout-serum, 0.5% L-glutamine (200 mM in 0.85% NaCl), 1% non-essential amino acids, 0.2% mercaptoethanol (55 mM in PBS) and 5 ng/ml of basic Fibroblast Growth Factor (bFGF) (Invitrogen). The hESC medium was daily exchanged and the cells were passaged every 4–6 days using collagenase type IV (2 mg/ml, Invitrogen) for 30 min. The cells were passaged on a ratio of 1:3. Additionally, hESC transfected with enhanced green fluorescence protein (EGFP), under the control of murine Rex1 promoter were used to mark undifferentiated cells (Eiges et al. 2001) (from Nissim Benvenisty, Hebrew University, Jerusalem).

Cell counting was assessed inside the microchannel within defined areas. Cells were observed every day under an inverted microscope (Nikon TS100, ×10 objective) and pictures were acquired at defined areas. The number of cells in each picture was counted manually. The results of all pictures taken at the same time was averaged and divided by the picture area (in $cm^2$) to obtain the measured cell density. For comparative purposes, static cell cultures were also maintained in conventional Petri dishes and monitored in a similar manner.

## 2.3 Shear and transport modeling

### *2.3.1 Shear stress estimation*

Assuming no slip conditions, a fully developed laminar flow regime, a Newtonian incompressible fluid, and a rectangular cross section with w>>h, where w is the channel's width and h is the channel's height the following equation can be used to evaluate the shear stress on the bottom of the channel (Deen 1998).

$$\tau_w = 6\mu \frac{Q}{h^2 w} \tag{1}$$

where: Q is the flow rate, $\tau_w$ is the shear stress at the walls, $\mu$ the fluid viscosity, w is the channel's width and h is the channel's height.

### *2.3.2 Mass transport*

For adherent cells a diffusion-convection equation may be used to evaluate the mass transfer of various soluble factors (such as: oxygen, nutrients, and growth factors).

The obtained reaction diffusion equation is outlined in Eq. (2)

$$\frac{\partial C}{\partial t} + v \cdot \nabla C = \nabla (D \cdot \nabla C) + R \tag{2}$$

where $C$ is the concentration, $D$ is the effective diffusivity, $R$ reaction rate, $v$ is the velocity vector.

During static incubation the convective term diminishes and mass transport is governed by diffusion.

In the present analysis we focus on oxygen transport. Oxygen is the most essential nutrient, and oxygen transport studies are widespread (Allen and Bhatia 2003).

### *2.3.3 Analytical oxygen model*

For a thick (w>>h), infinitely long microchannel (L>>H) a one dimensional transient diffusion model may be formulated.

$$\frac{\partial c}{\partial t} = \left(\frac{D}{H^2}\right) \frac{\partial^2 c}{\partial y^2} \tag{3}$$

where $c$ is the dimensionless concentration with respect to the oxygen concentration at the initial conditions. $y$ was normalized by the microchannel's height ($H$) and $D$ is oxygen diffusivity.

Initially, a constant homogenous level of oxygen is assumed:

$$c(y, t = 0) = C_0 \tag{4}$$

where: $C_0$ is the initial oxygen concentration.

Since the cells are cultured on the glass substrate covering the microchannel and the cell free surface in the microchannel is made of a very thick (>5 mm) layer of PDMS, oxygen flux from the surround air is neglectable. Assuming oxygen uptake at the cell surface

according to Michaelis–Menten kinetics, zero oxygen flux at the cell free surface, the boundary conditions can be defined as:

$$\frac{\partial c}{\partial y}(0,t)=0,\frac{\partial c}{\partial y}(1,t)=Da\frac{c}{k_{\mathrm{m}}+c} \tag{5}$$

$$Da=\frac{\rho\cdot V_{\max}\cdot H}{D\cdot C_0} \tag{6}$$

where Damkohler number ($Da$), is the dimensionless oxygen flux defined as the ratio of the oxygen uptake rate to the diffusion rate, $\rho$ is the cell density; $V_{\max}$ is the maximum oxygen uptake rate and $k_{\mathrm{m}}$ is the non-dimensional Michaelis–Menten constant defined as $K_{\mathrm{m}}/C_0$ ($K$m—Michaelis–Menten constant).

The postulated analytical model may serve as the worst case scenario for cells' oxygen supply, since it neglects the oxygen influx from the inlets/outlets of the microchannel.

#### 2.3.4 Finite elements model

A 3D finite element mass transport model (Comsol 3.3, Comsol, Burlington, MA, USA) was applied in order to evaluate design parameters (e.g., effects of the inlets and outlets) and to examine the validity of the analytical model. A 3D model consisting of the microchannel geometry and the inlet and outlet geometry was assembled. The boundary and initial conditions were similar to the ones assumed in the analytical model. Zero Neumann boundary conditions were imposed at all surfaces, besides the cell surface—where oxygen uptake is according to Michaelis–Menten kinetics. The initial conditions assumed a constant homogenous level of oxygen in the microchannel and the inlets/outlets. The computed results confirmed that the solutions were grid independent.

#### 2.3.5 Cell growth rate

The logistic growth model, which accounts for inhibition of cell proliferation as the cell density increases, was used to evaluate the proliferation rates of the cells under different culture conditions.

The model results in the following cell growth equation:

$$\phi=\frac{\phi_0 e^{kt}}{1-\frac{\phi_0}{\phi_\infty}(1-e^{kt})} \tag{7}$$

where: $\phi$ is the cell density, $\phi_0$ is the initial cell density, $\phi_\infty$ is the final (steady state) cell density and $k$ is a growth parameter. A least square fit was used to evaluate the growth parameter $k$, attained under different culture conditions.

## 3 Results

### 3.1 Theoretical

#### 3.1.1 Cell type

The analytical model enabled the estimation of oxygen levels in microchannels of different heights as a function of the period of time following fresh medium replacement. In order to avoid hypoxia (oxygen level <10 mmHg) in the micro-reactor, medium should be replaced before hypoxia levels are reached at the cell surface. The period of time to reach hypoxia in the microchannel at static conditions following medium exchange, was evaluated for different cell types and various microchannel heights. The parameters used for the model are shown in Table 1 and the model results are presented in Fig. 1.

#### 3.1.2 Finite elements model

The finite element model was applied to evaluate design parameters for HFF and HUVEC culture (model parameters see Table 1). The finite element analysis enabled the estimation of oxygen level in the microchannel as a function of time and location in the microchannel for different bioreactor configurations. The analysis was done on 100 μm (in height) microchannels, as used in the experimental part. Figure 2 shows a typical result for a 2 cm long microchannel having an inlet $\phi 3\times 3$ mm. The lowest oxygen level is at the most distant point from the inlets/outlets, at the center of the microchannel. The time to reach hypoxia at this point, in a 2 cm long microchannel, was hardly affected by the inlet size (no inlet up to $10\times 10\times 10$ mm—less than 5% variations). Additionally, for microchannels longer than 1.5 cm, the time to reach hypoxia was unaffected by the microchannel length and was similar to the one derived by the analytical model.

#### 3.1.3 Experimental

Typically 100 μm×1 mm×20 mm long microchannels with 3 mm×$\phi$3 mm cylinders inlets/outlets were used in the experiments. Different perfusion "flow-stop" protocol

**Table 1** Oxygen model parameters (Korin et al. 2007b, Park et al. 2005)

| Cell | $V_{max}$ nmol/s/Mcells | $K_m$ mmHg | ρ cells/cm$^2$ |
|---|---|---|---|
| HFF | 0.03 | 1 | $2\times10^4$ |
| HUVEC | 0.04 | 0.5 | $1\times10^5$ |
| Hepatocyte | 0.38 | 5.6 | $1.7\times10^5$ |

*Vmax* Maximum $O_2$ uptake, *Km* Michaelis–Menten constant, $\rho$ cell density

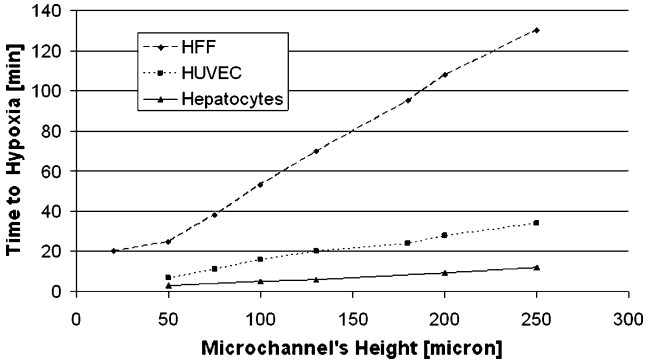


**Fig. 1** Period of time to reach hypoxia in the microchannel at static conditions following medium exchange, as a function of the microchannel height. The different lines represent different cell types

parameters (static incubation time; flow period, flow rate, etc.) were examined.

### *3.1.4 HFF and HUVEC culture*

Static incubation periods of 2–4 h (<1 h or >8 h were unsuccessful) were found to be suitable for long-term culture. Medium exchange was performed by flowing medium at a flow rate of 1–2 ml/h (shear stress 100 mPa, see eq. 3), for 1–2 min. Hence, the medium perfusion period was shorter than 1% of the static incubation period. HFF and HUVEC cells were cultured using the "flow-stop" method in the micro-reactor for up to 14 days. Figure 3 shows HFF cultured using the "flow-stop" method (a) compared HFF cultured under constant flow (0.05 ml/h) in the microchannel (b) and to static plate culture (c).

Figure 4 presents the cell growth kinetic in a microchannel using the perfusion flow-stop method compared to regular culture dishes and to constant perfusion protocols in the microchannel. Table 2 summarizes the logistic growth kinetics parameters obtained using least square fit to the data shown in Fig. 4. It can be observed that the maximum cell density is similar to the maximum cell density obtained for cells cultured in a regular culture plate. However, the proliferation kinetics are different: the cells in the microbioreactor have a higher growth rate compared to regular culture conditions.

### *3.1.5 hESC culture*

Experiments conducted under a constant flow regime at very low shear stress (shear stress <1 mPa which is substantially lower than 20 mPa suitable for HFF and HUVEC) were unsuccessful and hESC were washed out. However, by using the "flow-stop" method, hESC colonies were maintained and cultured in the microchannel bioreactor. Figure 5 shows the culture of hESC on HFF feeder cells. Additionally, Rex1-EGFP hESC were used to mark undifferentiated colonies (when medium was not exchanged differentiation of colonies was observed within 24 h). It can be seen that the colonies grow well and attain the normal shape of undifferentiated hESC colonies.

## 4 Discussion

### 4.1 Cell type and ability to culture

The use of the "flow-stop" method may be suitable for culturing various cell types in a microchannel bioreactor. Figure 1 shows the time to reach hypoxia as a function of the microchannel's height. As shown in the figure for confluent culture of cell having a high oxygen consumption

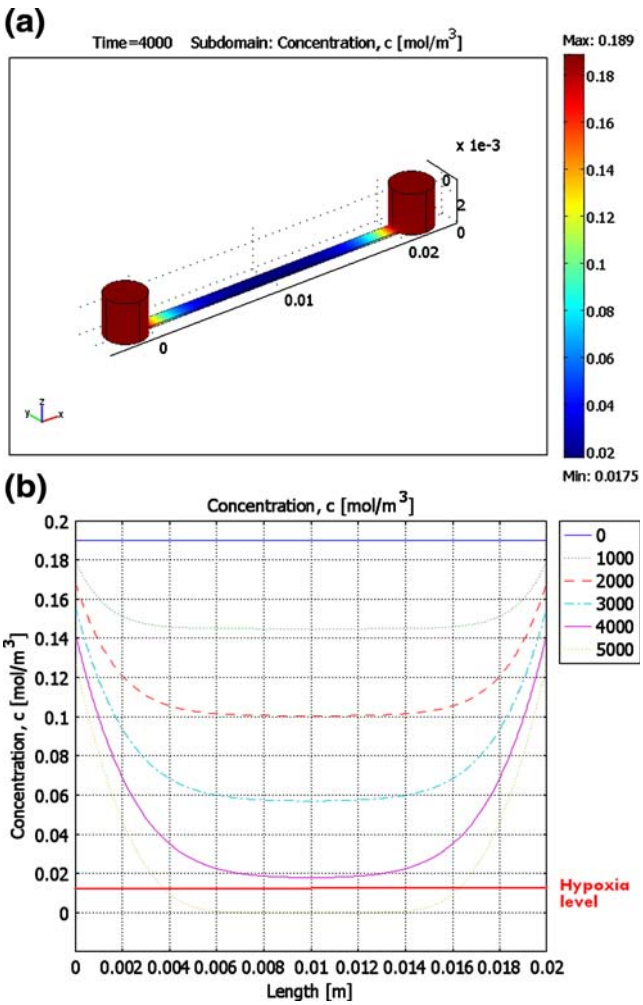


**Fig. 2** A typical simulation result for oxygen level in a 2 cm long microchannel (1 mm width×100 μm in height) having an inlet $\phi 3 \times 3$ mm. (**a**) 3D color plot of the oxygen level in the microchannel after 4,000 s at static condition for HFF cells (see Table 1), (**b**) plot of the oxygen level along the centerline of the microchannel bottom—the different lines represent different incubation periods

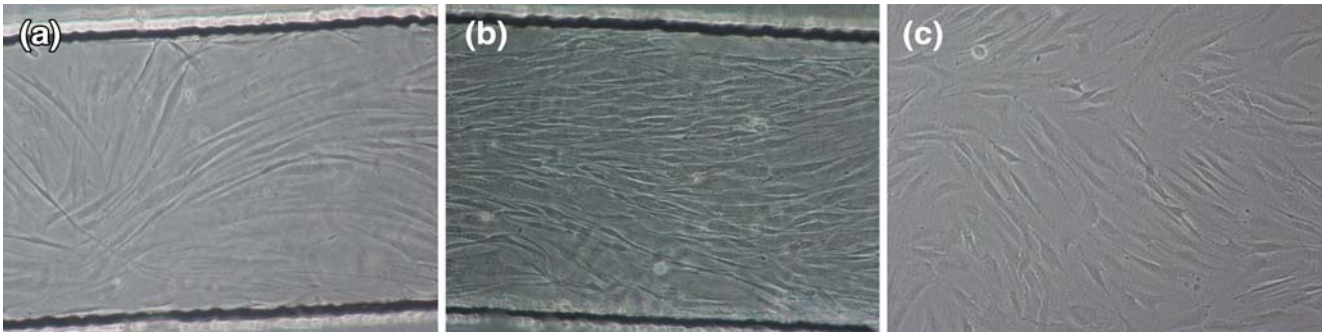


**Fig. 3** HFF cultured using the "flow-stop" method (**a**) compared to HFF cultured under constant flow (0.05 ml/h) in the microchannel (**b**) and static plate culture (**c**). Pictures (**a**) and (**c**) were taken at day 4 of culture and picture (**b**) at day 6 of culture

rate (e.g., hepatocytes) medium should be refreshed every several minutes even at 250 μm microchannels. Hence, a constant perfusion system is more appropriate for these cell types. However, for cells having low oxygen consumption (e.g., HFF, HUVEC) and/or cells at moderate/low cell densities, medium can be replaced every 30–90 min—depending on cell type and the microchannels' height. Additionally, cells may be cultured under partial hypoxic conditions, which will not be harmful to cell growth (e.g., undifferentiated hESC (Ezashi et al. 2005)). Thus, the "flow-stop" protocol should be designed and implemented according to cell culture requirements.

### 4.2 Cell growth

HUVEC and HFF were cultured in the microchannel for a period >7 days using the "flow-stop" method. The protocol applied medium renewal every 1–2 h. Though the mass transport model results imply that the cells in the middle of the channel may have been under hypoxia conditions for a short interval of time (<30 min), the cells proliferated well within the micro-bioreactor. This may be related to the ability of the cells to endure partial hypoxic conditions.

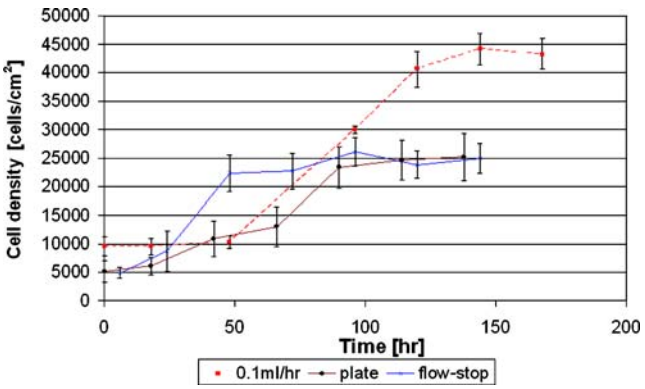


**Fig. 4** HFF growth kinetic in the microchannel bioreactor cultured using the "flow-stop" method (2 h stop, 2 min flow at 1 ml/h), using a constant flow perfusion protocol (flow rate 0.1 ml/h), and at static conditions in a regular culture plate

The measured HFF growth kinetics (Fig. 4) shows that the cell growth rate is enhanced in the microchannel ("flow stop" regime) compared to regular culture plates. This may be attributed to the enhanced supply of medium in the micro-bioreactor (medium exchanged every 1–2 h) compared to regular culture protocols (>daily medium exchange) and/or to the enhanced removal of growth inhibiting factors (Yu et al. 2007). Frequent medium exchange and enhanced supply of nutrients has been shown to improve growth kinetics in macro-systems as well. Additionally, the growth kinetics data (Table 2) shows that the maximum cell density achieved using the flow-stop method is similar to the cell density in regular culture dishes. However, the maximum cell density obtained under constant flow conditions in the microchannel is higher than under static conditions. Furthermore, cell morphology under flow differed from the one obtained in regular culture dishes and in the "flow-stop" method (Korin et al. 2007b). Under flow conditions the cells align with the flow direction (Fig. 3(b)). Fibroblasts in static culture conditions (culture dishes/microchannel) at high density orient as well, however the direction of orientation is random and alters within the culture area (Fig. 3(a) and (c)). Cell alignment may result in a more compact cell spreading and lead to increase in the maximum cell density. The fact that under the "flow-stop" protocols the cells resembled regular culture dishes characteristics as opposed to the characteristics of constant flow may be related to the short time

**Table 2** Cell growth constants obtained under different culture conditions (100 μm microchannel)

| Culture conditions | $\phi_0$ [cells/cm$^2$] | $\phi_\infty$ [cells/cm$^2$] | k [1/h] |
|---|---|---|---|
| Plate | 7,000 | 24,000 | 0.036+0.003 |
| "Flow-stop" method | 3,000 | 25,000 | 0.084+0.012 |
| Flow 0.1 [ml/h] | 10,000 | 45,000 | 0.043+0.003 |

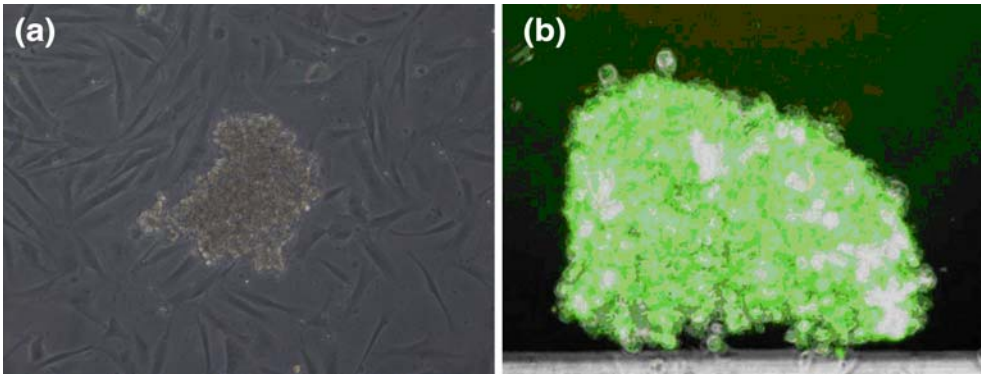


**Fig. 5** hESC co-cultured with HFF in the microchannel (**a**) hESC colony cultured for 2 days (**b**) Rex1-EGFP hESC colony (24 h culture). The GFP image is imposed (in *green*) on the colony phase contrast image. When medium was not exchanged differentiation of colonies was observed within 24 h. Colonies size: $1–2\times10^4$ μm$^2$

interval of flow (<5%) compared to the period of static culture in the "flow-stop" protocols. Hence, the "flow-stop" method may be used for long-term culturing of cells in a micro-bioreactor in a way minimizing undesired flow effects (e.g, shear stress).

### 4.3 Shear stress

The medium infused over adhered cells exerts shear stress on the cells. Above a defined shear stress threshold, cells will detach from the substrate (Lu et al. 2004). This threshold is a function of several parameters such as: cell type, substrate and time. In constant flow systems, shear stress is constantly applied on the cells. In the "flow-stop" method it is applied during a short interval of time accompanied by a long period of static incubation. We have previously shown that HFF cell culture in the same microchannels under a constant flow regime for long periods of time require shear stress lower than 20 mPa (Korin et al. 2007b). However, using the current method long term HFF culture was achieved even at a shear stress of 100 mPa (for 2 min every 2 h). This is due to the dependence of the shear stress threshold on the duration of shear. Cell may endure shear stresses for short periods of time (e.g., minutes) several orders of magnitude higher than the ones endured under steady flow. For example, fibroblasts can endure a shear stress of 100 Pa for a few minutes (Lu et al. 2004) while for long term culture under constant flow they cannot withstand a shear stress higher than 0.1 Pa.

Though the shear stress applied on the cells during perfusion by the "flow-stop" protocol is higher than the ones exerted by the constant flow protocol, the fact that it is applied only for a short period of time compared to the static incubation period may minimize long term shear stress effects on cell biology. As mentioned, cell morphology in the "flow-stop" method resembled the static incubation morphology in contrast to constant perfusion protocols. For example both HFF and HUVEC cells cultured using the "flow-stop" method did not align with the flow direction and/or became elongated in the flow direction, as opposed to cell cultured under constant flow regimes (Korin et al. 2007b). The fact that shear stress is applied only for short time intervals may be valuable for culturing cells (most cell types) in which shear stress effects are undesired.

### 4.4 Growth factors

As mentioned, microscale cell culture systems are normally based on constant perfusion systems, which are governed by convective mass transport. The infusion of medium induces the clearance of cell secreted factors. On the other hand, due to the restricted mixing characteristic of microsystems, static (no flow) culture in microchannels is governed by diffusion, consequently enabling the accumulation of secreted factors (e.g., growth factors) in the cells microenvironment (Walker et al. 2004). *In vivo* the cell micro-environment, is highly influenced by diffusion mechanisms, which play a key role in cell behavior and cell signaling.

In the "flow stop" method a short pulse of flow is followed by a long period of static incubation. Though the short flow pulse withdraws secreted factors from the cells micro-environment, the long static incubation enables the recovery of secreted factors and cell signaling events. Hence, the flow-stop method may be fruitful for culture/co-culture Microsystems where diffusion and secreted factors are significant. The recovery of a growth factor after its removal by the short flow pulse is a function of the factor's diffusion properties and secretion parameters. Hence, different "flow-stop" protocols may be helpful in understanding cell signaling and growth factors bio-physical characteristics (such as: time constants). The understanding of microenvironmental cues regulating cell fate is of key challenge in stem cell biology and stem cell tissue engineering (Chin et al. 2004).

### 4.5 hESC culture

Micro-Bioreactors have great potential for stem cell and hESC biology studies due to the length scales involved. It

has been shown that the culture of mammalian embryos in microchannels under static conditions results in more natural developmental kinetics (Raty et al. 2004). Despite its prospective studies involving hESC culture in microsystems have been rarely performed.

In order to maintain their pluripotency human ES cells are routinely grown on a layer of feeder cells (Draper et al. 2004). Traditionally mitotically inactivated mouse embryonic fibroblasts (MEFs) or inactivated HFF (Amit et al. 2003) are used to support hESC growth and maintenance. The feeder layer secrets factors which are essential for hESC to remain in their pluripotent state. Culture under a constant flow regime may result in the washout of the factors required for hESC pluripotency state. Additionally, hESC are highly sensitive to shear stress, even at low flow rates colonies will detach (Korin et al. 2007a). Therefore, the use of perfusion micro-reactor is problematic for hESC culture. Abhyankar et al. (Abhyankar and Bittner 2003) showed the feasibility of culturing hESC in bulky 250 μm in height microchannels by daily exchanging medium manually. Here, an automated "flow-stop" system is used to culture hESC colonies on HFF feeder cells in 100 μm microchannels. Two important features of the developed "flow-stop" method are demonstrated and exploited in the culture of hESC on HFF feeder cells: a) short temporal exposure to shear stress and b) long static incubation period. The short pulsed exposure to shear allows for shear sensitive cells such as hESC to withstand the medium influx. The long static incubation period allows for secreted factors (e.g., feeder cells secreted factor) to accumulate. Thus enabling the long-term culture of a sensitive cellular complex, such as the culture of hESC on HFF feeder cells, in a microchannel.

## 5 Conclusions

The use of an automated periodic flow system was shown to be successful in culturing human cells for long periods of time. The "flow-stop" protocol should be engineered according to cell culture requirements. The mass transport and fluid mechanics characteristics of a short flow pulse followed by a long static incubation period may be suitable for developing unique culture systems. The ability to culture hESC on HFF feeder cells demonstrates the unique abilities of the "flow-stop" method. This method may be fruitful for culture/co-culture microsystems where diffusion, secreted factors and cell signaling are significant. Such complex multi-cellular reactors may be valuable for studying and controlling cellular processes, such as: stem cell proliferation and differentiation.

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