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Microbioreactor arrays for controlling cellular environments: design principles for human embryonic stem cell applications

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Abstract

We discuss the utilization of microbioreactor arrays for controlling cellular environments in studies of factors that regulate the differentiation of human embryonic stem cells. To this end, we have designed a simple and practical system that couples a microfluidic platform with an array of microbioreactors, and has the size of a microscope slide [1]. The system allows quantitative studies of cells cultured in monolayers or encapsulated in three-dimensional hydrogels. We review the operating requirements for studies of human embryonic stem cells (hESCs) under steady-state and dynamic conditions, and the related control of the mass transport and hydrodynamic shear. We describe the design and fabrication of the individual bioreactor components, and the criteria for selecting the bioreactor configuration and operating parameters, based on the analysis of the characteristic times and scales of reaction, convection and diffusion. To illustrate the utility of the bioreactor, we present a “case study” of hESC cultivation with detailed experimental methods and representative biological readouts.

1. INTRODUCTION

Novel cell culture technologies developed in recent years mimic the *in vivo* cellular microenvironments with an increasing fidelity [2], through improved control and the provision of cascades of multiple regulatory factors. Miniaturization of the culture systems is an important step towards accurate control of the cultured cells and tissues. Some of the most interesting outcomes come from the optimization and accurate use of microfluidic platforms [3,4]. Small transport distances are key for enabling fast responses to environmental stimuli in studies involving spatial and temporal gradients of factors. Since its first appearance decades ago, microfluidics have been adapted to many different applications; several excellent reviews give insights into the operating principles for system configurations of interest [5-8]. Microscale technologies were designed for applications ranging from studies at a single cell level [9] to the recreation of more complex 3D tissues [10] and the development of diagnostics platforms [11,12]. High-tech platforms involving integrated microdevices such as micro-

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valves, injectors, pumps or mixers [13] are also being considered for use in live cell experimentation.

Our laboratory has been interested in developing a micro-bioreactor array that would allow high throughput studies of the most challenging cell source: human embryonic stem cells (hESCs). To this end, we have designed an efficient, multiplexed device that couples a microfluidic platform with an array of micro-bioreactors and allows quantitative studies of hESCs in two-dimensional (2D) and three-dimensional (3D) settings [1]. This system enables quantitative measurement of performance and accurate control over the culture microenvironment with a relative simplicity, not requiring additional integrated devices such as micro- valves, pumps or mixers. In this paper, we first summarize the design requirements for systems of this kind. We review the operating requirements for studies of cultured cells under steady-state and dynamic conditions, and the related control of the mass transport and hydrodynamic shear. The design and fabrication of the individual bioreactor components and the system assembly are described in detail. Then we describe the design specifications for important operating parameters and the principles for their optimization at the microscale level that are based on the analysis of transport rates and characteristic times of the involved phenomena. Finally, we present a “case study” of hESC cultivation, with detailed experimental methods and representative biological readouts.

2. OVERALL REQUIREMENTS

2.1 Biomimetics

Our body is composed of highly organized tissues formed by multiple cell types and sustained through complex structure-function relationships at many hierarchical levels. Short and long range communication between single cells and/or tissues occurs through an enormous cascade of signaling pathways, most of which involve mechanisms, intermediate steps and connections that are not fully understood. Ideally, biological studies should be performed under conditions that are *controllable* and at the same time *capture the complexity of the native environment*.

2.2 Scale

When it comes to scaling down to the biologically relevant lengths and time scales, the conventional methodologies of the so-called “flat biology” fail. Standard culture methods involving cell culture in Petri dishes cannot be representative of the real state of physiologic systems and therefore often result in unrealistic and uncontrollable biological readouts. The relatively large volume of medium in contact with cells and the batch-wise operation associated with the periodic medium exchange does not allow for the generation and control of precise patterns of stimulation such as spatio-temporal gradients. Petri dishes are therefore not suitable for use in studies of either steady-state or dynamic system. The batch processes have unpredictable timescales, are diffusion-limited and, most important, are intrinsically uncontrollable. Macroscale observations of averaged cell/tissue properties cannot be reliable in defining complex biological systems and must thus be replaced by more precise and realistic microscale observations. In this respect, the microscale culture technologies help overcome most of the above limitations, and to operate at the characteristic time and length scales of biological phenomena.

2.3 Time constants

At the microscale - inside the microchannels and culture chambers - the dominating forces change and differ from the most known, macroscale ones. Flow is always laminar (Reynolds numbers are well below the turbulence threshold, and in most cases $Re < 100$) the inertial forces are therefore dominated by viscous forces, and the transport is dominated by molecular diffusion or by convective regime of well-defined hydrodynamic profile. Reducing the

characteristic dimensions to a *microscale level* allows a more rigorous control of the operating parameters involved, due to the very short transport distances, which are in turn associated with very short time constants. As a result, biological responses are not limited anymore by the slow kinetics of physical phenomena.

2.4 Multi-parametric analysis

The miniaturization of the system also results in the reduction of the amounts of cells, culture media and supplements and thereby helps reduce the cost and time involved in cell culture, and enables the high throughput quality of the data. The possibility to control multiple factors, molecular and physical, allows multi-parametric analyses, thus again reducing both time and cost of experimentation.

2.5 Imaging compatibility

Most micro-bioreactors are optically transparent and fully compatible with conventional imaging techniques. They allow online analyses and real time experimentation such as time-lapse monitoring the state of the system and the time-course of specific phenomena.

3. SPECIFIC REQUIREMENTS

3.1 Steady state conditions

Perfused systems offers the advantage of working under well-defined and stable steady-state conditions. Standard culture systems, such as Petri dishes, can be compared to batch reactors in which conditions are precisely defined only at time zero, and then continuously vary until next medium exchange. Working at steady-state conditions maintains, by definition, the parameters of interest at their constant levels. Precise perturbations of the system may then be introduced to investigate the dynamic biological responses of the cells.

3.2 Fast transients in space and time

It is often necessary to accurately modulate transients in space and time to recreate precise stimulation patterns in the form of concentration gradients, and deliver particular signals. Working with small volumes (order of a few μL) generally leads to fast-response systems. This means that the switch between defined sets of conditions can be fast and still controllable. For example, operating parameters can be changed by a simple variation of the medium flow rate. At the microscale, one can design the system to provide tightly controlled, orderly conditions of flow and mass transport.

3.3 Control of mass transport and flow shear

Due to the well-defined geometry, short transport distances, and fast transients, transport phenomena occurring in microdevices can be more easily subjected to theoretical analysis and precise control than those in larger scale systems. Accurate predictions of the velocity gradients and shear stresses are indeed very important because of the profound effects of flow environment on biological systems. Again, the versatility of microscaled systems, allows decoupling the effects of mass transport phenomena such as the generation of specific concentration patterns from physical phenomena such as the application of mechanical forces.

4. TECHNICAL DESCRIPTION OF THE MICROBIOREACTOR PLATFORM

This section contains the description of the main components of the micro-bioreactor array culture system: the cell culture chamber, the microfluidic platform, the gas-exchange unit, the tubing and connections, and the flow circulation system. The described elements can be visualized in Figure 1, keeping in mind that their application can be extended to different

configurations. Briefly, panel **A** shows a top view of the micro-bioreactor array assembly with its functional units, which will be treated more in detail in the following paragraphs. Panel **B** presents an image of the culture system set up in the incubator, while the lateral views in panel **C** clarify the layered structure of the micro-bioreactor array exemplifying the two configurations used.

4.1 Culture chamber – 2D and 3D settings

The cell culture chambers were designed to provide (i) culture of a statistically significant number of cells for biological assays, (ii) easy and rapid access to the chamber for application of standard protocols (i.e. coating, fixing, staining), (iii) the maintenance of versatility in choosing proper characteristic dimensions (chamber height and diameter), and (iv) high throughput characteristics of the system (e.g., a 96-well plate or 384 well plate format), and (v) easy adaptation to 2D and 3D culture configurations.

The chambers must also ensure rapid establishment of the desired medium composition in the surroundings of the cultured cells. The chamber is shaped as a cylindrical well with a 3 mm diameter, with PDMS walls and a glass bottom, by coring the PDMS layer using a stainless steel biopsy punch. The height of the chamber depends on the chosen configuration (2D or 3D), and in either case is determined by the height of the PDMS slab. Fitting the device to a standard microscope slide, an array of 4×3 culture chambers is formed. The open wells are reversibly sealed on the top by using a thin, gas permeable PDMS membrane that is fixed with four thumbscrews within a polycarbonate-aluminum frame. The membrane isolates the cells and medium from the external environment while allowing for gas exchange. The 96-well configuration further enables easy interface of the bioreactor slide with robotic system for fluid manipulation and fast analysis and readout.

4.2 Microfluidic platform

The microfluidic platform was designed to flow the culture medium and any supplements in and out the culture chambers. Consideration was given to the time and length scales of diffusion and convective transport inside the microfluidic channels and cell culture chambers. Responding to the need for uniform hydrodynamic conditions inside the culture chambers and over the cultured cells, the fluid flow rates and the geometry of the microfluidic channels need to be accurately determined. Computational modeling of the steady-state momentum balance and time-dependent mass transfer balance within the micro-bioreactors and the channels, provided the quantitative basis for system design. The modeling had crucial role in analyzing the mass transport and in defining the relative contributions of convection and diffusion as a function of the medium flow rate.

The microfluidic platform was fabricated by standard soft lithography. In most cases the channel cross section is 100 μm × 100 μm . These dimensions and aspect ratio (height versus width) enabled the formation of structurally stable channels with great precision. As can be seen in Figure 1, the platform is composed of three independent inlets that deliver the culture medium to a 4×3 array of chambers; consequently, each entering stream must be connected to a set of four chambers. A flow splitter was thus designed to ensure that each well received the same amount of medium with the same flow rate. Each fluid stream traveled the same path-length from the inlet to the outlet of the microfluidic platform, thereby providing equal flow resistance in each of the three channels. The inlets and outlet ports were cored from the PDMS slab using stainless steel biopsy punches.

4.3 Gas exchange

The gas exchange unit ensures the maintenance of the correct, physiological levels of gases inside the culture chambers: carbon dioxide to regulate the pH of the culture medium, and

oxygen to ensure the correct metabolic activity of cells. The gas exchange depends on the permeability coefficient of each gas through the top membrane, geometry of the system, temperature, composition of the gas atmosphere and the flow rate which determines the residence time of the fluids in the channels.

The only gas-permeable material used is PDMS; the system is maintained at a temperature of 37 °C, 95% humidity and 5% carbon dioxide partial pressure; the cross section of the channels is 100 μm $\times$ 100 μm and the height of the PDMS platform is set at 3 mm. The fresh culture media, injected at the inlet of the system, must be perfectly equilibrated at the physiological values before entering the culture chambers, thus the shape and dimensions of the gas exchanger are designed based on the assumption of gas equilibrium. From the performed calculation, using coefficient values from the literature, the length of 20 mm was determined to be sufficient. We chose a serpentine-like shape of the gas exchanger channels to maintain the arrayed configuration of the platform.

4.4 Other components

The PDMS layer with the microfluidic channels, the gas exchanger, the punched ports and culture chambers is irreversibly bonded to the glass slide by air-plasma surface treatment. The assembled unit is connected to the pump by Tygon tubing. A syringe pump is used to control the medium flow rate at a steady level and with extreme accuracy; in particular, the pump served the purpose to control a motor fluid (water) that would transmit the motion to the culture media through a flow transducer. The complete set up, shown in Figure 2, has two advantages: (1) the culture medium could be maintained at a low temperature to prevent denaturation of delicate components such as growth factors, and (2) the syringes containing the culture media are protected from contamination or contact with external components. The assembled micro-bioreactor and the connecting tubing are placed inside an incubator (37°C, 95% humidity, 5% carbon dioxide), the discarded culture medium is collected in reservoirs that are placed 20 cm above the platform plane and connected to the chamber outlets.

It is worth describing in more detail the 2D and 3D configurations that our platform allows. The 2D setting allows for two different configurations; in the first configuration, the cell layer is at the same level as the microfluidic channels, directly adhering to the glass slide; in the second configuration, an additional PDMS layer with the punched chamber-wells is used to set the distance between the cell culture-level (still adhering to the glass slide) and the microfluidic channels, adhering to the PDMS layer. For the 3D culture setting, the second configuration is used and the cells are encapsulated in a volume of hydrogel (e.g., photopolymerized hyaluronate). The assembled micro-bioreactor platform is entirely optically transparent and can be easily interfaced with microscopes for real-time observation and experimentation.

5. Operational parameters

The operational parameters affecting the pool of variables in the cell micro-environment can be divided into two categories: the initial parameters that are normally set before the experiment (such as the characteristic dimensions of the culture chamber, the cell seeding density, the medium composition), and operative parameters that can be controlled during the experiment (such as the medium flow rate). In this section, we discuss the principles guiding the selection of the optimal variables and their values through the analysis of characteristic times of fundamental phenomena such as cellular uptake and release, and diffusional and convective mass transport rates.

The configuration of the culture chamber is mainly determined by the chamber diameter and height. The selection of the chamber diameter is mainly dependent upon the minimum number

of cells required for obtaining biological data on a relevant population size. Typical number of 10^3 cells per square millimeter is required to apply most biological assays. The height of the cell culture chamber has great influence on the cell micro-environment; large chamber heights reduce the average velocity for a given flow rate and decrease the rates of mass transport. Also, a deep chamber acts as a reservoir for exogenous factors and causes dilution of endogenous factors, at the rates that depend on the kinetics of cellular processes and the number of cells per unit volume.

The ratio of the time scales of uptake at the bottom of the culture chamber (by cultured cells) τ_{UP} , to species diffusion from the medium, τ_D , can be estimated. For example, assuming a maximum uptake reaction rate expressed by a mass action law for a ligand-receptor association kinetics [14], the above mentioned time scale ratio can be expressed by:

$$\frac{\tau_{UP}}{\tau_D} = \frac{D}{h\rho_C k_{on}[R]} \quad 1$$

where D is the diffusion coefficient of the chemical species, h is the height of the culture chamber, k_{on} is the ligand-receptor association rate constant, $[R]$ is the number of receptors on cell surface and ρ_C is the surface cell density. The values for all parameters used are listed in Table 1.

Figure 3 shows the calculated ratio of the time constants at the bottom of the culture chamber between the reaction, τ_{UP} , and diffusion, τ_D , as a function of cell seeding density. The ratio is shown for two representative molecules: oxygen (the most important upon small molecules, panel A) and albumin (a protein representative of larger growth factors, panel B). At low cell densities, the characteristic time scale for reaction is longer than that for diffusion. Therefore, mass transport at low cell densities is dominated by uptake reaction rates. As cell density increases up to its maximum value (i.e. at confluence), the time constant for diffusion becomes comparable with that for reaction. It is interesting to note that the height of the culture chamber only slightly influences the ratio of the two time constants. This semi-quantitative analysis suggests that for higher cell seeding densities, medium perfusion is required to enhance mass transport rates and establish a kinetic-limited operating regime.

In this context, the most important variable that can be precisely controlled is the medium flow rate; selecting the syringes volume, the tubings cross section and using accurate syringe pumps, it is possible to provide precise volumetric flow rates down to 10 nL/min. The flow rate affects the local cellular micro-environment both by inducing the shear stresses and by altering the mass transport rates.

Shear stress results from the tangential forces applied to cell surfaces, and can be mathematically represented by the Newton's law of viscosity. The shear stress depends on the velocity gradient at the surface and for constant flow rate it linearly depends on the height of the culture chamber. The values of shear stress can quickly be estimated assuming that the flow profile in the middle of the culture chamber would be well-approximated by a parallel Poiseuille flow. The maximum amount of shear that can be tolerated by the cells will depend on the cell type since certain cells (*e.g.*, mesenchymal and endothelial) require shear stress to differentiate and develop properly while other cells are affected by shear stress without the change in their phenotype [15]. Most cells tolerate shear stress, $\bar{\tau}_{shear}$, up to 1 dyne/cm, which are generated in the culture chamber and are calculated assuming parabolic flow between parallel plate:

$$\bar{\tau}_{shear} = \frac{6\mu Q}{h^2 d} \quad 2$$

where μ is the medium viscosity, Q is the flow rate, h and d are the height and the diameter of the chamber, respectively. Again, the values for all parameters used are listed in Table 1.

The analysis of the influence of the medium flow rate on mass transport is carried as follows. The ratio of the time scales of diffusion in the culture chamber, τ_D , to convection, τ_C (residence time of the medium in the culture chamber), can be defined as:

$$\frac{\tau_D}{\tau_C} = \frac{hQ}{Dd^2} \quad 3$$

where D is the diffusion coefficient of the chemical species, h is the height of the culture chamber, Q is the medium flow rate and d is the culture chamber diameter.

Figure 4 shows the ratio of the time scales of diffusion to convection as a function of medium flow rate, for different heights of the culture chamber. In the presented logarithmic graphs, values greater than 1 represent culture conditions that are reaction controlled, whereas <1 values correspond to the convection-limited regimes, and values of ~ 1 indicate the competition between the two rates. From these graphs we can conclude that for small metabolites with high diffusion rates in the 4 mm diameter culture chamber, flow rates >1 $\mu\text{L}/\text{min}$ are necessary to cross from a diffusion- to a convection-controlled regime. In addition, it is possible to adjust the mass transport phenomena at a given flow rate by changing the height of the culture chamber. The chamber geometry thus provides an additional degree of freedom for setting the proper culture conditions.

Figure 5 shows the distance z from the bottom of the culture chamber at which the diffusion and convection time scale are comparable as a function of the flow rate. Introducing the above mentioned variable z , and traducing the flow rate Q in terms of the linear velocity v_z , the following equation for the ratio of τ_D and τ_C was obtained.

$$\frac{\tau_D}{\tau_C} = \frac{(h-z)^2}{D} \frac{v_z}{d} \quad 4$$

Assuming that the flow profile would be well-approximated by parallel-plate Poiseuille flow, v_z can be expressed as:

$$v_z = 1.5 \langle v \rangle \left(1 - \left(\frac{z}{h} \right)^2 \right) \quad 5$$

where $\langle v \rangle$ is the mean channel velocity.

The data in Figure 5 were obtained by simultaneously solving equations 4 and 5 for $\tau_D/\tau_C = 1$. At low flow rates, the values of z correspond to the height of the culture chamber, indicating that diffusion dominates at all levels. By increasing the flow rate, z decreases and takes values

between the top and bottom of the chamber. In turn, increasing the height of the culture chamber decreases the transport rates at the cell culture surface and thereby decreases the wash-out of endogenous factors. This latter aspect can be relevant to cultures of stem cells, where the preservation and recreation of the natural diffusion-limited cell niche is of fundamental importance.

The above discussion is generally applicable to steady state conditions. Microfluidic bioreactors can also be used in principles to modulate and control transients in environmental conditions to mimic some of the processes to which the cells are exposed *in vivo*. However, it is rather difficult to replicate short and fast episodes of biochemical stimulation. In principle, the residence times of culture media in the microfluidic channels and inside the culture chamber should be shorter than the characteristic times for the phenomena involved in step-wise biochemical stimulation.

6. CASE STUDY: CULTURE OF HUMAN EMBRYONIC STEM CELLS IN A MICRO-BIOREACTOR AT STEADY STATE CONDITIONS

This section describes the design and detailed protocols for hESC culture in a micro-bioreactor array. Two different bioreactor configurations have been chosen for this case study, to assess the influence of cell density and mass transport regime on stem cell differentiation [1].

6.1 Design criteria for hESC culture

Human embryonic stem cells (hESC) can give rise to any of the cell types in the human body. However, the pluripotency and plasticity of hESC are the reason why these cells are difficult to control in culture. It has been shown that poorly defined combinations of factors in serum and even the differences between different serum lots could be a source of variability and difficulties in reproducing hESC culture outcomes. These problems can be partially overcome by the use of serum-free media and well-defined biochemical stimuli to direct proliferation or induce differentiation [16]. However, defined medium compositions are not sufficient, because they only provide the consistency of average conditions in the culture system, whereas the conditions in the cellular environment are further mediated by the spatio-temporal gradients. The spatial organization of hESC, the local cell density and hESC clustering, the uptake of exogenous factors and release of endogenous factors are all involved in creating the hESC micro-environment that strongly influences cell fate. Recently, a niche-dependent effect on hESC colonies has been demonstrated, proving that cell density and the agonistic/antagonistic local effects of endogenous/exogenous factors play important roles in determining cell differentiation [17].

Bioreactors can be used for modulating and controlling the hESC niche by controlling the thickness of the diffusional layer (Figure 5) and consequently, the transport and the accumulation of endogenous and exogenous factors. Bioreactors can be continuously perfused with fresh medium, to culture hESC at steady-state conditions without the periodic and dramatic environmental changes associated with the conventional batch-wise culture in Petri dishes. The experimental setup discussed below allows the application of precise hESC cultivation protocols with tight control of mass transport at the cellular level. By medium perfusion at small operating volumes, the system enables rapid establishment of steady-state conditions, at either low shear (diffusion-limited transport) or high shear (convection-limited transport).

6.2 Micro-bioreactor assembly

The micro-bioreactor, reported in Figure 1, consists of (1) a microfluidic PDMS platform with culture chambers, (2) an additional PDMS slab with punched holes for an optional increase in

the height of the culture chambers, (3) a smooth PDMS membrane, (4) a supporting glass slide, (5) a polycarbonate-aluminum frame with four thumbscrews, (6) a set of Tygon tubing and stainless steel tubing for the connections, (7) a set of plastic syringes, (8) a syringe-based flow transducer, and (9) a syringe pump.

The microfluidic platform is used in conjunction with two micro-bioreactor configurations, with different geometries and transport regimes. In the first configuration, the microfluidic platform is positioned at the height ($z = 0$) of the cell monolayer at the bottom of punched culture chamber:

- $z = 0$: supporting glass slide with PDMS layer ($h = 4$ mm) with the microfluidic platform and the punched culture chambers;
- $z = 4$ mm; PDMS membrane sealing the tops of culture chambers.

In the second configuration, the microfluidic platform is positioned 4 mm above the cultured cells:

- $z = 0$: supporting glass slide with culture chambers in the $h = 4$ mm high PDMS slab;
- $z = 4$ mm: PDMS layer with the microfluidic platform and punched holes aligned with the culture chambers;
- $z = 6$ mm: PDMS membrane sealing the tops of culture chambers.

For each micro-bioreactor configuration, all layers except for the PDMS top sealing membrane are first irreversibly bonded by plasma treatment, and the functionality of the bioreactor is then verified. The microfluidic platform is tested to exclude the presence of clogging in the channels and/or failures in the hydraulic sealing of the channels and the culture chambers. Ethanol may be used to render the channels more hydrophilic prior to sterilization. The entire assembly, the membrane and all connections and tubings are then sterilized via autoclave treatment. It is helpful to arrange the components in a way that facilitates subsequent operations in a laminar hood.

6.2.2 Cell culture set up and analysis—Once the micro-bioreactor is assembled, tested and sterilized, the cell culture steps include: (i) surface coating, (ii) cell seeding, (iii) setting the medium flow rate, and (iv) biological data readout and analysis.

Surface coating and treatment: The glass surface of each culture chamber was treated with a solution of 4% 3-aminopropyltrimethoxysilane in acetone for 15 min. The solution was removed, the chamber rinsed with PBS and coated with 0.05 mg/ml collagen IV for 1 h at room temperature. Particular attention must be taken in keeping the collagen solution at $+4^{\circ}\text{C}$ in ice before dispensing it over the glass surface.

Cell seeding: The excess collagen solution was removed and the cell suspension was applied to the coated substrate to achieve a desired initial density. Bubble formation must be avoided in this phase, as bubbles would affect the subsequent steps.

Medium flow: The microfluidic channels were filled culture medium, and the PDMS membrane was positioned on top of the other PDMS layers and held in place with the polycarbonate-aluminum frame and secured with four thumbscrews. Again, bubble formation inside the culture chambers and the microfluidic channels should be avoided.

Cells were allowed to attach to the coated surface for 24 hours before starting media perfusion. To initiate medium perfusion, the syringes and the tubing were filled with the amount of fresh culture medium necessary for the entire experiment. Any air bubbles inside the syringes or the

tubing were removed. The syringes were kept at +4°C and coupled to the flow transducer; the tubings were connected to the inlet ports of the micro-bioreactors microfluidic channels, and the syringe pump was set to the chosen flow rate.

Biological analysis: The cultured cells were evaluated by on-line bright field imaging and by immunofluorescence for specific markers. Primary staining included Smooth Muscle Actin (SMA; Dako, Troy, Michigan, USA) or Oct4 (R&D Systems, Minneapolis, MN, USA), and DAPI for nuclei (Sigma–Aldrich, St. Louis, MO, USA). Secondary staining included Cy3 or the FITC-conjugated antibodies (Sigma–Aldrich, St. Louis, MO, USA). Automated image analysis via custom-adapted scripts in MATLAB (The MathWorks, Natick, MA, USA) was used to quantify the percentages of differentiated and undifferentiated cells, in form of FACS-like data, as previously reported [1].

6. 3 Results

The entire set of results obtained using the micro-bioreactor array for hESC cultures was reported in Figallo et al. [1]. In this section, to prove the validity of our methods and procedures, we discuss some of the results to illustrate the application of the above principles for designing micro-bioreactors for hESC culture. We report an example for evaluating the effects of transport regimes for two micro-bioreactor configurations on a specific cell type (hESC).

At all cell densities studied, homogeneous surface coating of a given extracellular matrix protein was achieved that resulted in homogeneous distribution of hESC over the entire surface of each culture chamber (Figure 6 A-C).

The two different bioreactor configurations described above, result in two completely different transport regimes at the same flow rate of 0.3 $\mu\text{L}/\text{min}$. Specifically, for the configuration corresponding to $h = 0$ mm, a convection-dominated mass transport regime was established with relatively high shear stress at the cell surface. In contrast, for the configuration corresponding to $h = 4$ mm, a diffusion dominated mass transport regime was established with negligible shear stress effects. The same micro-bioreactor arrays were also used without perfusion, to serve as static controls.

The effect of shear stress on hESC cell morphology is shown in Figure 6 D-F, where the shear stress increases from left to right, from statically operated micro-bioreactors, to the low-shear and high-shear perfused configurations. The low-shear condition appeared to have the sole effect of removing poorly adhering cells, and the cell morphology changed only after exposure to high shear stress.

Notably, cell differentiation depends on cell density (Figure 6 G-H), and this is due in part to the changes in the transport vs. reaction limitation of the system. Cell density and the bioreactor configuration each determine the rates of reaction and transport. As a result, the limiting phenomena (transport vs. reaction, Figure 3) can change and influence the biological responses of the cultured cells.

Figures 4 and 5 suggest that the static and low-shear perfused conditions can lead to similar transport regimes. This is consistent with the results of cell differentiation studies (Figure 6 L), where the statically operated and low-shear perfused conditions (both groups in the $h = 4$ mm configuration) gave statistically indistinguishable results. In contrast, an increase in shear stress that shifts the controlling transport regime from diffusion to convection (in the second configuration $h = 0$ mm), led to significantly different cell outcomes. The increased vascular differentiation of the cultured hESC cells at higher shear stress could be due to the dilution and depletion of endogenous factors and the constant supply of exogenous factors.

7. Conclusion and further developments

Micro-bioreactors are versatile as they allow one to vary the geometrical, configuration-related and operating parameters, and to explore with a high accuracy a broad range of cell cultivation conditions. Even though the exploration of signaling pathways is leading to constantly increasing levels of understanding of stem cell behavior, the properties of compounds produced or required by living cells are still far from being fully known. Thus, the use of perfused micro-bioreactors designed and operated using rational principles outlined in this paper would be beneficial for gaining more control over culture parameters for answering specific biological questions.

Tight control over transport phenomena allows the modulation of regulatory signals in space and time, and helps mimic *in vitro* the physiological patterns found in living organisms. The integration with high-tech components such as micro pumps or micro valves could further improve the quality and the highthroughput of the data.

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Figure 1. Micro-bioreactor array

Panel A shows the top view of the micro-bioreactor array assembly with its functional units: the separate inlet ports for each chamber, the serpentine-shaped channels of the gas exchanger, the splitter dividing the flow into 4 equal parts, the array of culture chambers, and the outlet ports. The 12 independent culture chambers (diameter $d = 3.5$ mm) are arranged in a 4x3 array (8 mm vertical and 7 mm horizontal center-to-center spacing); the microfluidic channels (100 μm wide by 100 μm high) are printed in a PDMS mold attached via plasma treatment to a glass slide or to a punched PDMS slab of thickness h . The inlets, outlets and culture chambers are all punched through the PDMS. Panel B shows the culture system set up, with connections and tubings, inside the incubator. The lateral views in panel C clarify the layered structure of the

micro-bioreactor array in the two configurations used. The glass slide supports the cells culture and allows the irreversible sealing of PDMS via an air-plasma surface treatment while the open culture chambers are reversibly sealed by the PDMS membrane on top of them. In the first presented configuration (**left**), the microfluidic channels are positioned at the height of $h = 4$ mm above the glass substrate. In another configuration (**right**), the microfluidic channels are positioned at the level of the glass substrate where the cells are cultured ($h = 0$). Both configurations support cell culture in 2D setting, and the first also supports the culture of hydrogel-encapsulated cells in a 3D setting. A contrast dye is used in images **D-F** to show the fluid paths in both axonometric and lateral views.

Figure 2. The microbioreactor platform

A syringe pump transmits the motion of the motor-fluid (water) to the culture medium, without direct contact between the two fluids. The culture medium is kept at controlled temperature (4°C) to prevent the degradation of medium components such as growth factors, and pumped into the inlet ports of the bioreactor residing inside incubator (37°C, CO₂ 5%); the waste medium from the outlet ports is collected in a container.

Figure 3. Diffusion and reaction time constants as a function of cell density and culture chamber geometry

The ratio of characteristic times of the uptake reactions and molecular diffusion, τ_{UP}/τ_D , is shown as a function of the cell density, for three values of the culture chamber height (see Fig. 1): $h = 1, 2$ and 4 mm; $d = 4$ mm. Panel **A** shows data for a small metabolite, such as oxygen while panel **B** shows data for albumin representative of large growth factors. The values for all the parameters used are listed in Table 1.

Figure 4. Diffusion and reaction time constants as a function of medium flow rate and culture chamber geometry

The ratio of characteristic times of the uptake reactions and molecular diffusion, τ_{UP}/τ_D , is shown as a function of medium flow rate, for three values of the culture chamber height. All parameter values are as specified in Fig. 3. Panel **A** shows data for a small metabolite, such as oxygen while panel **B** shows data for albumin representative of large growth factors. The values for all the parameters used are listed in Table 1.

Figure 5. Characteristic length scale as a function of medium flow rate and culture chamber geometry

The distance from the bottom of the culture chamber at which the ratio of the diffusion and convection time constants is equal to one is shown as a function of the flow rate and culture chamber geometry $h = 1, 2$ and 4 mm; $d = 4$ mm. Panel **A** shows data for a small metabolite, such as oxygen while panel **B** shows data for albumin representative of large growth factors.

Figure 6. Effects of cell density and transport regime on cell morphology and differentiation
A-C Bright field images of the culture wells with three different cell densities (increasing from left to right: 60, 160 and 314 cells/well). Cell nuclei are stained with DAPI. The coordinates of each nucleus is individuated by automated system analysis. Phase contrast images (**D-F**) and fluorescence images (**G-I**) of a static (non-perfused) bioreactor, a low shear bioreactor ($h = 4$ mm) and a high shear bioreactor ($h = 0$). **J** The fractions of cells expressing smooth muscle actin (SMA), a marker of vascular cell differentiation, as a function of cell density and bioreactor configuration.

Table 1**Parameters used**

The table reports a list of the parameters and variables used for the performed calculations with their values and ranges. When applicable, references are reported in the last column.

Variable	Values	Units	Reference
D_{O_2}	3.30E-09	m^2/s	[1]
D_{Alb}	7.00E-11	m^2/s	[1]
h	$0 \leq h \leq 0.004$	m	[1]
ρ_c	$1.0E+7 \leq \rho_c \leq 4.0E+10$	cells / m^2	this study
k_{on}	1.00E+07	1/M s	[14]
$[R]$	1000	Molecules/cell	[14]
μ	0.0007	Pa s	[1]
d	0.004	m	[1]
Q	$0 \leq Q \leq 2.3E-10$	m^3/s	this study

Table 2**Parameters involved and their main targets**

The main geometrical and operative parameters are summarized in the table, listing also the corresponding symbols and the main targets of their variations.

Parameter	Symbol	Description	Phenomena
Design	L_g	Channel length	Gas-liquid mass transfer
	h	Chamber height	Cell-liquid mass transfer
	d	Chamber diameter	Total number of cells
	$h \times d$	Chamber cross section	Velocity field; Mass transport
Operation	ρ_c	Seeding density	Total uptake and release rates
	Q	Total flow rate	Velocity field; Shear stress; Mass transfer