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Microfluidic Tissue Model for Live Cell Screening

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Abstract

We have developed a microfluidic platform modeled after the physiologic microcirculation for multiplexed tissue-like culture and high throughput analysis. Each microfabricated culture unit consisted of three functional components: a 50 μ m wide cell culture pocket, an artificial endothelial barrier with 2 μ m pores, and a nutrient transport channel. This configuration enabled a high density of cancer cells to be maintained for over a week in a solid tumor-like morphology when fed with continuous flow. The microfluidic chip contained 16 parallel units for “flow cell” based experiments where live cells were exposed to a soluble factor and analyzed via fluorescence microscopy or flow-through biochemistry. Each fluidically independent tissue unit contained ~500 cells fed with a continuous flow of 10 nl/min. As a demonstration, the toxicity profile of the anti-cancer drug paclitaxel was collected on HeLa cells cultured in the microfluidic format and compared with a 384 well dish for up to 5 days continuous drug exposure.

Introduction

The development of microfluidic technologies offers many promising applications for cell based research¹. The major advantages of microfabricated platforms are the ability to design the cellular microenvironment, precisely control fluid flows, and to reduce the time and cost of cell culture experimentation^{2–4}. Broadly, microfluidics technology involves the use of small scale plumbing (<100 μ m) to transport liquids, particles, and cells through specialized compartments. The application of this methodology for cell culture has been demonstrated in various forms over the past few years^{5–15}. While each of these approaches presents novel concepts for improved microfluidic cell culture, the overall challenge of realizing a practical, cost effective, and commercially available platform has yet to be solved. Importantly, key factors such as the cellular environment, micro/macro interface, sample preparation, fabrication reliability, experiment throughput, and user interface need to be addressed to further advance the field¹⁶.

There is growing demand in the life science research community to study biological events in the context of living cells^{17–20}. The success of technologies in the fields of genomics and proteomics has prompted researchers to demand similar high throughput techniques for cell biology. While advances in automation tools and robotic systems can provide large scale live cell screening systems,^{21, 22} the fundamental limitation has been the difficulty in maintaining cells *in vitro* in a state that is relevant to the physiologic condition. It is generally accepted that cell phenotype is highly dependent on the local culture conditions; therefore it is important to develop a screening platform better suited to microenvironment control. One approach would be to replicate the physical structure of cells in living tissues. Since most tissues units are organized on the micron scale, microfabrication methods are well suited for this task^{23–27}. From a mass transport perspective, there are three hallmarks of living tissues: 1) parenchymal cells reside at a very high volumetric density, 2) long distance transport is facilitated by convection through blood vessels and, 3) short distance transport is mediated by diffusion controlled by endothelial layers.

Currently, the most significant advances in cellular microenvironment research are in the field of cancer biology. There is convincing evidence that factors such as cell-matrix contacts, cell-cell interactions, and the local signaling microenvironment can be strong regulators of cell behavior^{28–32}. Since the cancerous phenotype is manifested on the cellular level, it is important to develop *in vitro* screening models relevant to *in vivo* tumors. A well studied approach is the use of multi-cellular cancer spheroids³³. This method consists of culturing cancer cell aggregates of ~100µm diameter, and has been shown to recapitulate key properties of solid tumors, such as morphology, signaling, and drug transport³⁴. However, the difficulty in establishing, maintaining, and analyzing these units in suspension culture have prevented more wide scale application.

In this current work, we demonstrated a 16 unit microfluidic chip for live cell cancer toxicity screening. This core culture unit was based on a previously developed artificial endothelial barrier structure that was able to maintain high density cell aggregates with improved nutrient transport properties³⁵. The key contribution of this current manuscript was to develop a multiplexed cell culture screening system that was easily operated. This device allowed the user to input a cell suspension and drugs of interest and observe the effect on cultured cells using fluorescent microscopy or by collection of the effluent. Each culture unit consisted of a tumor like cell mass fed by continuous flow of medium across a microfluidic perfusion barrier. By carefully designing the microfluidic structures, it was possible to recreate the key mass transport properties of an archetypical tissue unit. Furthermore, the ability to localize cells in specified culture chambers separated from the nutrient flow paths allowed a high degree of uniformity across the array. In order to make this microfluidic technology more accessible to the research community, the entire system was designed to enable repeatable operation without specialized equipment or training.

Results

Microfluidic design

The basic culture unit was designed with three main components: a cell culture area, a microfluidic perfusion barrier, and a fluid flow channel (Figure 1). The flow channel and the cell culture region (50W×50H µm) were connected through the perfusion barrier, which consisted of a network of 5W×2H µm channels. Each culture unit contained 4 fluidic ports: a cell inlet, a flow inlet, a cell outlet, and a flow outlet. During operation, cells from suspension were introduced via the cell loading port, and subsequently fed via a continuous flow through the transport channel. The bottom surface of the culture channel was a glass coverslide, facilitating high magnification microscopy. Each inlet/outlet port was microfluidically routed to a reservoir well on the top surface of the chip, allowing up to 20 µl of solution to be directly filled by pipet.

Mass transport

The perfusion barrier was designed to mimic key transport properties of a natural endothelial barrier. The premise was to create a barrier between the cultured cells and the nutrient transport vessel that could 1) prevent cells from crossing over, 2) insulate the cells from flow stresses, and 3) allow diffusion of soluble factors. Due to the nature of microscale flows, the fluidic resistance of microfluidic networks can be precisely calculated based on channel geometry. The flow rate through each branch then takes the form of Ohm's Law, such that $Q = P/R$, where Q is the volumetric flow rate, P is the pressure drop, and R is the fluidic resistance. Since the microfluidic barrier consisted of channels with a much smaller cross section than the flow channels, this formed the dominant resistor in this network (Figure 2). When the pressure drop was applied from the flow inlet to outlet, there was virtually no convective flow across the barrier. Based on calculations of fluidic resistance from the laminar flow equations, the

convective velocity of fluid across the barrier was 90 times smaller than flow through the main channel. However, due to the time scale of diffusion across short distances, the cells still received adequate nutrient exchange across the barrier. This principle was demonstrated by visualizing the exchange of a soluble fluorescent dye in the microfluidic unit. Here, both channels were initially filled with a fluorescent dye (7-hydroxy-coumarin). A pressure drop was then introduced from the flow inlet filled with a non-fluorescent buffer. This resulted in a rapid exchange ($t_{1/2}$ = 3 sec) in the flow channel as convective transport carried the buffer through the channel. Since there was virtually no convective transport across the barrier, it took much longer ($t_{1/2}$ = 20 sec) for the dye in the cell culture area to diffuse out. The finite element simulation depicted in figure 2 was consistent with this theoretical value. This simulation was prepared with Femlab software as a 2D model, with mathematical adjustments made to approximate the change of channel height. While the full 3D flow profile was not recapitulated, the ratio of convective flow rates across the barrier to flow through the main channel was preserved.

Cell culture experimentation

Three main steps were required to perform cell culture experiments in the microfluidic format: 1) cell loading into the culture area, 2) continuous nutrient exposure, and 3) fluorescence based assay. For the first step, a branched loading channel was implemented to facilitate cell localization (Figure 3). During loading, cells either entered the culture region (where they became trapped) or flowed out towards the outlet. While this approach sacrificed a large portion of the initial population, it allowed the removal of non-localized cells from the fluidic circuit by flushing with a buffer solution. This design also relieved pressure buildup on the loaded cells to minimize membrane damage during loading. Since the cell size was greater than the size of the perfusion barrier channels, the cells were localized and concentrated in the cell culture region. Once the desired cell density was attained, continuous perfusion of culture medium through the flow channel sustained cell proliferation for over 2 weeks. Figure 3 depicts the growth of MCF-7 cells into an aggregate structure, highlighting the ability of the microfluidic device to be applied to different cancer cell lines. Fluorescent labeling of the cultured cells was achieved by introducing the appropriate dye through the transport channel and imaging on an inverted microscope.

Screening chip

This microfluidic design was multiplexed by placing 16 parallel units on a single 24×60mm chip (Figure 4). In this configuration, a single cell inlet well allowed uniform loading of all 16 culture regions. Each culture unit had a separate flow inlet and outlet reservoir, allowing 16 different solutions to be used on a single chip. The fluidic resistance through each culture unit was designed to be identical, ensuring uniform flow across the entire array (see supplementary figure 1). Operation of the chip was handled with a custom built interface (see supplementary figure 2). After the desired solutions were filled into the chip reservoirs, it was placed into the manifold where controlled pneumatic pressure provided the driving force for flows through each culture unit. This was primarily used for cell loading, rapid solution exchange, and introduction of fluorescent probes. For long term culture, the chip was placed in a CO₂ incubator on an incline such that gravity driven flow through the culture unit was ~10 nl/min. For a 15 μ l well, this was sufficient to sustain 24 hours of growth before the wells had to be replenished.

Paclitaxel toxicity

To demonstrate a live cell screening application in the microfluidic format, the chemotherapeutic drug paclitaxel was exposed to HeLa cells at 0, 0.1, 1.0, 5.0, and 100 nM (Figure 5). The cells were loaded into the devices and cultured in the microfluidic tissue space

for 4 days with continuous flow of culture medium, resulting in the high density morphology. A control experiment was prepared in a glass bottom 384 well culture dish. On day 4, the cells were exposed to paclitaxel (diluted in culture medium) at the specified concentrations and incubated at 37°C and 5% CO₂. Cell viability was determined using a LDH membrane integrity assay as well as fluorescent live/dead staining followed by microscopy. After 24 hours of exposure, HeLa cells cultured as a monolayer on the dish showed a clear loss of viability in the dosage range tested. In the microfluidic tumor model, there was no statistical difference between the various drug concentrations and the control ($P > 0.3$). At the highest dosage (100 nM), there was a clear difference between cell response in the monolayer culture and in the microfluidic model ($P < 0.001$). HeLa cells in the microfluidic model were then continuously exposed to paclitaxel and analyzed each day for up to 5 days. In the first two days, there was no statistical difference in the different drug concentrations, however, after 72 hours of drug exposure, a characteristic dose-response curve was observed.

Discussion

Extensive work has been conducted in recent years to develop microfluidic solutions for cell culture experimentation, yet there is currently no widely available method for general laboratory use. A major goal of this current project was to demonstrate a reliable and easy to use cell culture and imaging chip by taking advantage of the key aspects of microfluidic technology. On the micro scale, careful design of the fluidic network and perfusion barrier enabled localization of cells into high density, high viability aggregates at specified culture regions. The use of a separate flow channel for nutrient perfusion ensured uniform exchange regardless of the cell density or culture time. On the macro scale, the system was designed to fit with conventional research tools. Wells were directly machined on the disposable chips such that cells and solutions could be dispensed by pipet. Culture was performed in a standard CO₂ incubator, and the cells were analyzed using an inverted microscope or plate reader. The entire operation process took roughly the same amount of time as using a 12-well dish while offering the advantages of continuous flow exposure, high cell density, and improved optical imaging. We believe that this current prototype represents a first step towards advancing microfluidic cell culture tools into the biological laboratory.

One of the major challenges facing the field of cell biology is to provide an *in vitro* cellular microenvironment to model *in vivo* behaviors. While there is high demand to conduct large scale cellular level screens for pharmaceutical development and systems biology, if the cells are kept in an irrelevant state, the data are likely to also be irrelevant. As biologists continue to reveal the role of the microenvironment in cell function, improved culture methodologies should be developed to supplant the incumbent technologies. A critical aspect of this approach is the use of microfluidics to address the need for mass transport control on the micron scale.

In this current work, the implementation of a biomimetic tissue culture architecture showed altered cell behavior. This was likely caused by the high degree of cell-cell contact that was made possible with the microfluidic design. Unlike in standard monolayer cultures, cells in the microfluidic device exhibited an altered morphology characteristic of a solid tumor. Cell boundaries became obscured, their size was compacted, and multiple cell layers were evident in the aggregate. Preliminary results show that when medium was limited over 4 days of culture, there appeared to be onset of cell death in the center of the cell culture channel, indicating the possibility that the microfluidic model recapitulated the mass transport properties of solid tumors (see supplementary figure 3).

To demonstrate one possible application of this system, we performed a drug toxicity assay on cultured cells with the model compound paclitaxel. The major goal of this experiment was to show the ability to utilize the microfluidic platform to perform multiplexed live cell

experimentation. When cultured in the microfluidic format, HeLa cancer cells rapidly adopted a high density configuration. This appeared similar to the *in vitro* spheroid cultures that have been previously reported with cancer cells³⁴. Additionally, when exposed to the anti-cancer drug paclitaxel, cells in this tumor-like state were more resistant to cell death, presumably due to the protective properties of the altered morphology. The effect of drug on the microfluidic tumor culture appeared to have no effect for the first 48 hours, with onset of cytotoxicity at day 3. The dose-response profile on day 3 of the microfluidic model was similar to a 24 hour exposure in the standard monolayer format, and the day 5 microfluidic data resembled the 48 hour monolayer response. This is consistent with previous findings that 3D cultured cancer cells exhibit a phenomenon called “multicellular resistance,” with properties similar to clinical tumor resistance³⁶. It is believed that improved methods for solid tumor-like cultures *in vitro* will lead to a better understanding of clinical resistance of anti-cancer drugs. While the biological consequences of this specific microfluidic culture format will require additional investigation, it is promising to note that the engineering innovations presented in this work have resulted in a usable cell culture and screening platform.

In summary, we have designed a novel microfluidic tissue model for cell culture and multiplexed experimentation, with initial results on the practical performance of the system. This work represents a proof-of-concept that microfluidic engineering can offer unique advantages for *in vitro* tissue culture experimentation. When applied in combination with methods such as ECM patterning, soluble factor engineering, co-culture, and 3D culture, it becomes possible to envision a complex microenvironment that more fully resembles an *in vivo* tissue. The continued development of microfluidic culture technologies will enable new experimental methods for the cell biology community.

Methods

Microfabrication

The microfluidic chip was constructed by replicate molding of lithographically defined features. First, SU-8 photoresist was patterned on a silicon wafer to define the microfluidic channels. Multiple height features (e.g. 2 μ m and 50 μ m) were exposed in separate steps to form the perfusion barrier and flow channels. The mold was replicated with poly-dimethyl-siloxane (PDMS) polymer (Dow Corning Sylgard 184) as indicated in the product sheet. The polymer structures were then cut to size and covalently bonded to a #1 thickness borosilicate glass coverslide and sealed to an acrylic piece with machined reservoirs.

Fluidic interface

A custom chip manifold was machined to facilitate fluidic control. The manifold formed a seal over the reservoir channels, with each channel routed to a separate air line. These control lines were connected with pneumatic tubing to a set of 3-way valves downstream of a pressure regulator. Pressurizing the fluidic reservoirs at 0.5-3.0 psi was sufficient to induce rapid cell loading and liquid exchange through the chip. Cell loading was stopped by switching off the pressure to the cell channel, leading to an abrupt stop of cell flow.

Cell culture

Prior to loading into the microfluidic chip, cell suspensions were prepared by incubation with trypsin. The cells were collected, centrifuged, and resuspended in CO₂ Independent Medium (Invitrogen) supplemented with 10% FBS. Each microfluidic chip was pre-filled with culture medium and equilibrated in a CO₂ incubator. Roughly 20 μ l of this solution was pipetted onto the chip and loaded into the culture regions using the custom manifold (~1 psi, 60 seconds). The inlet wells were then filled with 15 μ l of culture medium (DMEM, 10% FBS) and placed in a CO₂ incubator for culture. Since the PDMS walls are gas permeable, gas exchange within

the chip was not limited. Fluid reservoirs were replenished when necessary to maintain cell viability.

Toxicity experiments

For drug toxicity experiments, HeLa cells were first cultured for 24 hours in fresh medium and checked for proper attachment. After the desired multicellular morphology was observed, the inlet reservoir was replaced with DMEM containing paclitaxel diluted from a 5 mM stock solution in DMSO. Concentrations from 0.1 nM to 100 nM were prepared by serial dilution, with fixed 0.02% v/v DMSO content. As a comparison, 384-well glass bottom plates were similarly prepared and screened for drug toxicity. Analysis was performed on each well using a LDH release assay (Promega) and measured on an absorbance plate reader. Additionally, cell cultures were analyzed by microscopy with a live/dead assay kit (Invitrogen) containing calcein AM, ethidium homodimer-1, and Hoechst 33342.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Microfluidic Design. (a) A simplified model of physiologic cell culture includes a high cell density tissue space, a porous endothelial barrier, and a nutrient transport vessel. This arrangement allows a high density of cells to be maintained without being subjected to flow stresses. Convective flow allows rapid long distance transport of nutrients, while local exchange into the interstitial space is mediated by diffusion over a short length scale. (b) A microfluidic analog to this tissue microstructure replicates the key functional parameters for cell culture. Each unit consisted of a cell culture region, a microfabricated barrier, and a nutrient flow channel. The microfluidic barrier consisted of a network of channels with a $2 \times 5 \mu\text{m}$ cross section, insulating the cells from shear stresses while enabling nutrient diffusion. This filter also served as a cell concentrator, allowing a high initial cell density to be loaded from suspension. Here, two units are depicted in parallel, demonstrating the ability to fabricate a fluidically independent culture array. (c) HeLa cells cultured in this microfluidic design at low density and high density. After the cells were introduced to the culture region, a continuous flow of medium through the flow channel enabled sustained proliferation until the $50 \times 50 \times 500 \mu\text{m}$ culture region was completely filled. Scale bar represents $50 \mu\text{m}$.

Figure 2.

(a) The mechanics of micro-flows allows the fabrication of high resistance regions that mimic endothelial barriers. Flow through microchannels is laminar in nature, resulting in well defined fluidic resistances that can be analyzed in the same manner as electric circuits. The small cross section of the barrier channels creates a high resistance, preventing convective flow into the cell culture area, as verified by a finite element simulation. This maintains the cells in a low stress environment with continuous nutrient exchange via diffusion. (b,c) The kinetics of fluid exchange in the flow channel and cell area was visualized with a fluorescent dye. Note the fast convective transport in the flow channel (■) and slower diffusive transport (●) into the cell area.

Figure 3.

(a) Schematic diagram of the branched cell loading design. As cells entered the culture region, they became localized and concentrated. The second branch of the loading channel exited to waste, allowing removal of cells from upstream regions by flushing with a buffer solution. (b) HeLa cells loaded into the microfluidic cell culture area to a high initial density. The microfluidic design allowed rapid cell loading without significant cell damage. (c) MCF-7 cells cultured in the microfluidic format showed a densely packed morphology that was sustained with continuous perfusion.

Figure 4.

(a) The microfluidic chip contains 20 inlet wells (left) and 17 outlet wells (right). A single cell inlet well is used to fill all 16 culture regions, and 16 individual drug inlets each lead to separate microfluidic channels and out to a secretion well. Three additional wells can be used to treat all 16 chambers for functions such as washing and fluorescent staining. (b) Channel layout of the entire chip. Each individual culture unit is accessed by three fluidic paths: a cell inlet, a nutrient inlet, and a nutrient outlet. (c) Expanded view of the 16 parallel culture regions depicting cell channels (blue), drug flow paths (red), and the perfusion barrier (yellow). The optical transparency of the multiplexed chip enables cell analysis via microscopy.

Figure 5.

Cell toxicity of the anticancer drug paclitaxel. (a) HeLa cell viability as a function of paclitaxel dose compared in the microfluidic tissue model (solid bars) and for a monolayer culture (hatched bars) after 24 hours exposure. The data plots the average viability and SEM of 4 culture units exposed to paclitaxel at concentrations of 0.1, 1.0, 5.0 and 100 nM. (b) Five day drug toxicity profile in the microfluidic tumor model at 0.1 (■), 1.0 (□), 5.0 (▲), and 100 (△) nM. Average and SEM plotted for 4 culture units at each time point. Phase contrast images show the difference in cell morphology in a dish (c) and the microfluidic tissue model (d) at the same magnification.