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Real-Time Microfluidic System for Studying Mammalian Cells in 3D Microenvironments

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We describe a microfluidic system that can control, in real time, the microenvironments of mammalian cells in naturally derived 3D extracellular matrix (ECM). This chip combines pneumatically actuated valves with an individually addressable array of 3D cell-laden ECM; actuation of valves determines the pathways for delivering reagents through the chip and for exchanging diffusible factors between cell chambers. To promote rapid perfusion of reagents through 3D gels (with complete exchange of reagents within the gel in seconds), we created conduits above the gels for fluid flow, and microposts to stabilize the gels under high perfusion rates. As a biological demonstration, we studied spatially segregated mouse embryonic stem cells and mouse embryonic fibroblasts embedded in 3D Matrigel over days of culture. Overall, this system may be useful for high-throughput screening, single-cell analysis and studies of cell−cell communication, where rapid control of 3D cellular microenvironments is desired.

This paper demonstrates a microfluidic chip for studying the real-time growth and communication of systems of cells in three-dimensional (3D) microenvironments. This chip contains a 4 by 4 array of individually addressable microchambers, inside which small groups of mammalian cells are grown. By turning on and off sets of microvalves, the 3D extracellular microenvironment inside each chamber, along with the interconnecting pathways that connect the cell chambers, can be modified independently and in real time.

The development of microfabricated systems for biological applications has resulted in methods that allow for exquisite control over cellular microenvironments.[1–3] For example, cells can be spatially positioned to micron resolution, and chemicals can be delivered to cells to subcellular spatial resolution.[4–6] Many different cell types (including bacteria and mammalian cells) can be routinely grown and patterned inside a poly(dimethylsiloxane) (PDMS) microchip, but typically on flat 2D surfaces.[7–9] Recent studies indicate that, for many biological processes (such as fibroblast motility, progression of melonoma tumors, and the polarity and differentiation of mammary epithelial cells), 3D microenvironments may mimic physiological context better than 2D surfaces in terms of cell morphology, adhesion, proliferation, and migration.[10–12] Whereas bulk 3D extracellular matrices (ECM) (such as collagen I, Matrigel, alginate, hyaluronic acid, agarose, gelatin, and acrylate-based hydrogels) are commonly used *in vitro* systems for providing mechanical and chemical stimulation to cells, studies of mammalian cells in microfabricated 3D matrices are only emerging. These studies include the encapsulation of cells in microchannels to mimic tissue architecture and to study mammalian cells in perfusion cultures.[13–19]

Our technical approach uses pneumatically actuated microvalves, which were previously developed by the groups of Quake and others[1,20,21] for a broad range of applications, including the analysis of live cells (such as cell sorting and long-term monitoring of bacteria). In this work, we aim to use microfluidics to impart real-time, dynamic control over the 3D environments of an array of mammalian cells while also incorporating the ability to study chamber-to-chamber communication of diffusible factors. Specifically, we aim to develop microvalves to study mammalian cells in 3D environments (to enhance biological relevance, we focus on Matrigel, a naturally derived ECM that is used to study cells

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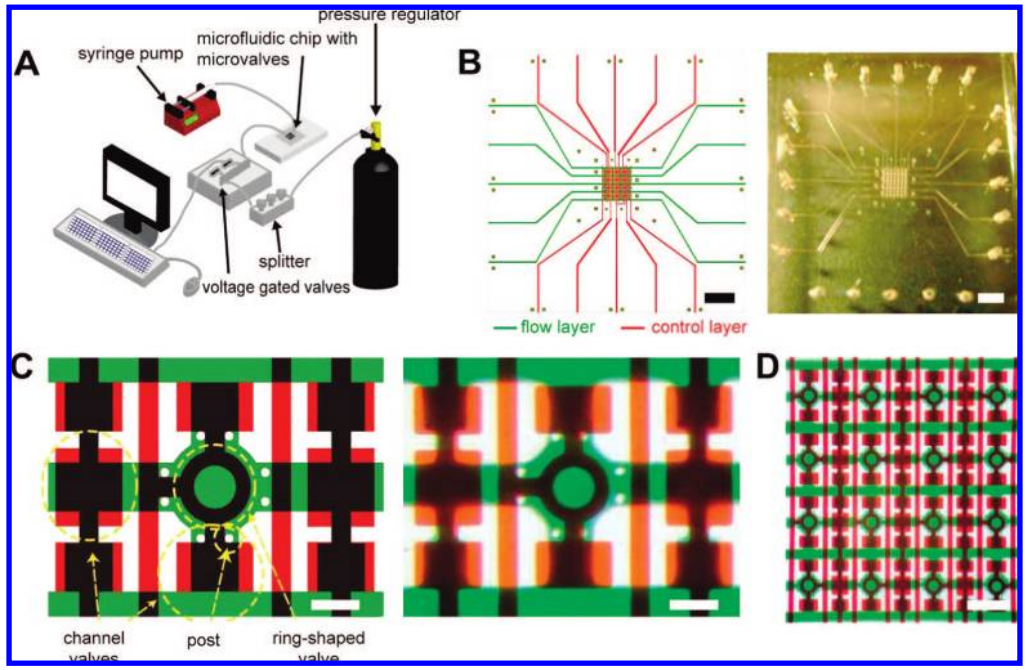

Figure 1. Design and structure of the microfluidic setup. (A) A schematic diagram of the experimental setup. A two-layered PDMS chip is connected to a regulated gas tank via voltage-gated valves. For media perfusion through all microchambers, a syringe pump is connected to the chip. For injection of reagents or cell-matrix mixtures into specific microchambers, the path of the fluid flow is controlled through the switching of the voltage-gated valves. (B) Design of the microfluidic chip as shown by an overlay of both the control and flow layers (left), and picture of the corresponding chip after assembly (right). Scale bars = 2 mm. (C) Design of a single chamber (left), and brightfield image of the device loaded with two different food coloring dyes (green in fluidic flow layer and red in control layer) (right). The microvalves are located at enlarged crossings of a control channel with a flow channel when viewed from the top. Scale bar = 100 μm. (D) Brightfield image of the 16 microchambers loaded with green dye in the flow layer and red dye in the control layer. Scale bar = 300 μm.

in 3D[22–24]), and to demonstrate their use for studying long-term culture of undifferentiated mouse embryonic stem cells.

MATERIALS AND METHODS

Device Design and Fabrication. We designed a microfluidic device composed of two layers of PDMS elastomer: a fluidic flow layer on the bottom, and a pneumatically actuated control layer (filled with air or water) on the top, with the two layers separated by a 5 to 10 μm thin PDMS membrane (Figure 1). In the fluidic flow layer, channels were 100 μm wide, whereas microposts and chambers were 20 μm and 200 μm in diameter, respectively; this geometry of the chamber along with the microposts prevented the 3D gel from being displaced from the chamber. In the pneumatic control layer, channels over the actuation areas were 140 μm wide. In addition, in the pneumatic control layer, we designed ring-shaped valves over the chambers to create shallow conduits above 3D gels for rapid media perfusion; these valves were 40 μm wide and 100 μm in diameter. Due to their thinner width than the regular actuation valves, the ring-shaped valves do not deflect completely to the bottom of the fluidic channel under standard operating pressures (from 20 to 50 psi).

We fabricated both layers of PDMS using soft lithography followed by replica molding.[20,25] We designed photomasks using CleWin (WieWeb Software, The Netherlands) and printed them at a high resolution using a commercial service (20,000 dpi; CAD Art Services Inc.). The master for the pneumatic control layer was fabricated using a 15 μm tall layer of epoxy-based negative photoresist SU8-2015 (MicroChem, Newton, MA) on a silicon wafer. The master for the fluidic flow layer was fabricated using an 18 μm tall positive photoresist SPR220-7 photoresist layer; after development, we reflowed the photoresist by heating the wafer on a hot plate at 140 °C for 15 min to form channels with a round geometry (thus allowing complete closure of the channel upon actuation of valves). Both masters were then exposed to a vapor of trimethylchlorosilane for 1 h (to aid PDMS removal), and replica molded with PDMS (Dow Corning Sylgard 184) made from a 10:1 ratio of base to curing agent; the PDMS mold was thick (~0.5 mm) for the pneumatic control layer, and thin (~30 μm; spin-coated at 2500 rpm for 1 min) for the fluidic flow layer. We treated the two PDMS layers with oxygen plasma, and very gently aligned the two layers using a microscope with the aid of 16 alignment marks built into the two layers. We finished the alignment within 2 min, such that the PDMS surfaces remained hydrophilic; once aligned, compression by hand of the two pieces irreversibly bonded the two layers. The two PDMS layers were placed on a hotplate at 90 °C for 15 min to facilitate bonding. Then, the two layers were cut out from the wafer and interconnecting holes were punched through. Finally, the resulting device was mounted onto a clean glass slide with the channel side facing downward under the assistance of oxygen plasma treatment for 1 min, and then heated on a hotplate at 95 °C for 15 min. The assembled microchip is shown in Figure 1B.

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To set up the equipment for operating the valves, we connected a compressed nitrogen gas tank to a splitter, then to two tubing manifolds, split into voltage-gated solenoid valves (Lee Co, LHDA2433115H) to form multiple independent pressure sources, and then connected to the microfluidic chip via polyethylene tubings. This configuration allowed the valves in the control layer to be individually controlled. We operated the microvalves either by manually connecting the voltage switches on the breadboard where a compressed nitrogen tank can be placed nearby, or by manually applying positive pressure via syringes (for example, in the cold room); in all cases, the control layer was filled with water. We have also used a LabVIEW program to control the voltage-gated solenoid valves using a digital acquisition card (AT-DIO-32HS, National Instruments) for high-speed operations of a microfluidic chip containing a 10×10 array (unpublished results).

Culture of Mammalian Cells and Preparation of Cell-Encapsulated 3D ECM. We isolated primary mouse embryonic fibroblasts (MEF) from CF-1 strain mice at 13 days gestation (Charles River Laboratories) using the established protocol from WiCell Research Institute. We cultured the MEFs in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum, supplemented with 1% nonessential amino acids (all reagents from Invitrogen) in 5% CO_2 and 37 °C. Before injecting MEFs into the chip, we used a Cs-137 irradiator at 8000 rads to irradiate the MEFs to prevent them from dividing as a feeder layer.

For stem cells, we obtained mouse (strain R1) embryonic stem (ES) cells that express GFP under an Oct4 promoter from the Vunjak-Novakovic laboratory (original source from the Zandstra laboratory). We first cultured them in 5% CO_2, 37 °C, in complete ES cell growth media: knockout Dulbecco's minimal essential medium (KO-DMEM; Gibco) supplemented with 0.01% of LIF (Chemicon), 15% ES qualified fetal bovine serum (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Next, we cultured the mouse ES cells in flasks coated with 0.1% gelatin (Sigma), and passaged them every 2–3 days (depending on the morphology and size of colonies), with a change of complete ES cell growth media every day and 3 h before passages. Differentiation media was prepared by supplementing the complete ES cell growth media, in the absence of LIF, with 1 mM retinoic acid (RA) (Sigma).

To prepare cell-encapsulated ECM, we thawed Matrigel (BD Biosciences) overnight at 4 °C in a refrigerator. We trypsinized the MEFs, centrifuged them, and resuspended them at 10^8 cells/mL in ice-cold Matrigel. Similarly, we trypsinized colonies of mouse ES cells, centrifuged them, and resuspended the cells as small clumps at 10^8 cells/mL in ice-cold Matrigel.

Operation of Microfluidic Chip: Loading of Cells and ECM and Perfusion of Reagents. To load cell-encapsulated ECM into the microchambers, we first sterilized the PDMS chip by dipping the chip in ethanol followed by air-drying, and moved the chip and cell-Matrigel mixtures to a cold room. For a PDMS chip with an $m \times n$ array of microchambers, there are $(m + 1)$ flow channels and $(2n)$ control channels (for a 4×4 array of microchambers, 5 inlets/outlets for flow channels and 8 inlets for control channels).[20] Initially, we closed all control valves. To seed the cell−Matrigel mixture into a microchamber at a specific position (ith row, jth column), we release the air pressure at the $(2j - [i]_{mod2})$th inlet of the control layer, and a moderate amount of 4 °C cell−Matrigel mixture is pipetted through the ith inlet of the flow layer [for example, to seed the mixture at the (2,2) position, we release the fourth inlet of the control layer, and inject the cell−Matigel mixture through the second inlet of the flow layer; see Supporting Information for the definition of flow and control layer inlets' numbering]. We flowed the liquid mixtures (and all other fluids into the microfluidic chip) by positive pressure via a syringe or syringe pump (Genie Plus infusion/withdrawl pump, Kent Scientific). After observing by microscopy that cells have been loaded inside the chamber, we reapplied pressure to the inlet of the control layer. We repeated this procedure to place all types of cell−Matrigel mixtures into designated microchambers. After the seeding process was done, we moved the chip to room temperature while keeping the valves closed for 30 min, to allow the cell−Matrigel mixtures to gel. We then opened all the valves, and mESC medium was perfused into the whole PDMS chip at a flow rate of 0.1 μL/min.

We placed the microfluidic chip on a microscope stage and inside a stage-top environmental chamber (at 37 °C, 5% CO_2, and constant humidity) with constant perfusion of fresh media. To perfuse reagents through the ECM in the microchambers (such as the experiments on determining time for complete media perfusion of a microchamber, and staining cells *in situ*), we delivered reagents (such as media, green and red dyes from Ateco Spectrum Food Colors, or PBS) by positive pressure using a syringe pump at 0.1 μL/min. We visualized dyes by taking images through the eyepiece of an Olympus SZ61 stereoscope at 45× under brightfield conditions with a Canon PowerShot S2 IS. To photopolymerize 3D microstructures in our chip, we used a Leica CTR 6000 inverted fluorescent microscope (Leica Microsystems, IL) equipped with an automated motorized stage and InVitro acquisition software (MediaCybernetics, MD). The prepolymer consisted of 40% w/v of PEG-diacrylate (Sigma), 2% w/v of Irgacure 2959 (Ciba Specialty Chemicals, Tarrytown, NY) as photoinitiator, and 20 mM CellTracker Blue(CMAC, Invitrogen). Exposures were carried out with a mercury arc lamp operating at 365 nm for 10 s.

Modeling of Perfusion. Details of the modeling of the perfusion through the 3D hydrogel, as simulated by fluid flow through a porous media, are provided in Supporting Information.

Fluorescence Microcopy. To assess cell viability, we perfused the ECM in the microchambers with the Live/Dead assay kit (Invitrogen), followed by incubation at 37 °C for 30 min. We acquired fluorescent images using a Leica DMI6000B inverted microscope equipped with a Qimaging Retiga 2000R camera and commercial image acquisition software (InVitro).

For the 3D reconstruction images, we prepared 4 mg/mL type I collagen and 8.3 mg/mL samples and let them gel in a 37 °C incubator for 1 h before imaging. We imaged the samples using an Olympus Fluoview 300 IX70 inverted confocal microscope with a HeNe laser at 488 nm excitation/532 nm emission and 40× magnification. In Fluoview, the chambers were imaged by taking z-stacks every 1 μm throughout the whole gel. The raw images were then reconstructed and processed in ImagePro using a 0.46 μm voxel size.

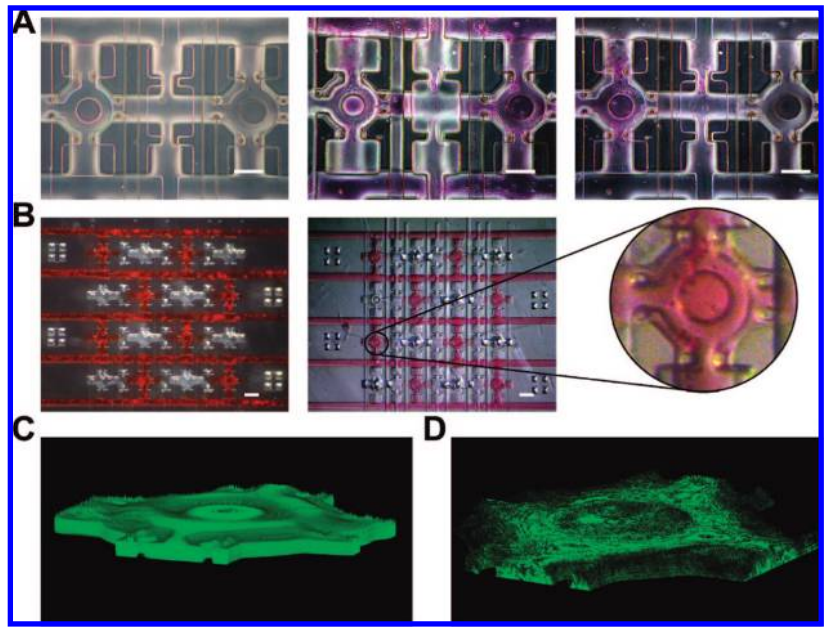

Figure 2. Demonstration of actuation of valves. (A) Phase-contrast images showing the loading of a single chamber with Matrigel encapsulating MEFs. The cell−Matrigel mixture is loaded into the two empty chambers (left), valves surrounding the left chamber are closed (center), and the right chamber is purged (right). The Matrigel is gelled after the right chamber is purged. Scale bar = 100 μm. (B) Demonstration of ability to individually address chambers, by loading red dye (left) and cell−Matrigel mixture (right) into a checkerboard pattern. The pattern is created by first closing four sets of valves to prevent the mixtures from entering the chamber (while leaving the rest of the valves open). Then the solution is injected into the top left inlet. Scale bar = 200 μm. (C) 3D reconstruction of a confocal image (taken at 40× magnification) of a chamber loaded with 3D Matrigel, with visible conduits above the 3D gel created by partial deflection of a ring-shaped valve positioned above the chamber. (D) 3D reconstruction of a confocal image of a chamber loaded with a 3D collagen I matrix, with visible conduits.

RESULTS AND DISCUSSION

Design and Fabrication of Microfluidic System. Integrating 3D ECM into microfluidic chips poses special design considerations, such as effective perfusion of medium and reagents without displacing the gel from the chamber. We used the pneumatically actuated valves developed by Quake and others, and designed small posts and valve features specifically for working with 3D matrices (Figure 1). The 3D ECM in each chamber in the array can be individually loaded and perfused in real time, and the behavior of cells inside the 3D chambers can be monitored by phase-contrast or fluorescence microscopy.

Our microfluidic chip contains two PDMS layers (attached to a glass substrate). The *fluid layer*, where cells and ECM are placed, contains 16 individually addressable microchambers (200 μm diameter and 18 μm tall) organized in a 4 × 4 geometry and spaced 330 μm apart from each other. Five inlets and five outlets are used to load and purge the chambers. A water-filled *control layer* on top is pressurized by a compressed nitrogen source (to deflect the PDMS membrane downward to seal off the fluid channel underneath); each channel in the control layer is independently controlled by an external solenoid valve that can be adjusted manually or by voltage (Figure 1A,B). Four valves surround each chamber in the fluid layer; controlling the opening and closing of channels in the fluid layer (to allow exchange of diffusive factors between adjacent chambers) can potentially produce 16 different 3D microenvironments since each chamber has access to a different set of neighboring chambers.

Compared to previous work,[20] our procedure for fabricating and operating the chip features two notable differences. First, to build the two-layer device, we treated the two PDMS layers with oxygen plasma before alignment and bonding to allow actuation of the valves at high pressure. Compared to devices using the bonding of PDMS layers made from 1:5 and 1:20 ratios (these devices typically withstand pressures up to 20 psi), plasma treatment provides tight bonding of the two layers while leaving the device intact for pressures up to 70 psi; this higher pressure was needed to close taller fluidic channels than previous studies (18 μm vs 9 μm[20]) in order to achieve a sufficiently thick ECM (at least 15 μm to be considered 3D[26]). Our results demonstrated that the two-layer device can be aligned and produced to high spatial fidelity using the plasma-bonding method (Figure 1C,D). Second, to facilitate working with 3D ECM that gels at room temperature, we loaded the chambers with liquid ECM in the cold room (by actuating the valves manually using syringes or via gas tanks) before moving the devices to room temperature in order to gel the Matrigel.

To facilitate the perfusion of media or biochemical reagents through the 3D ECM inside the microchambers, we incorporated two sets of new design elements. First, in the control layer, we designed ring-shape microvalves that are positioned on top of each individual chamber of the fluid layer (Figure 1C). Thus, after loading of the liquid ECM into individual chambers, actuation of the ring-shaped valve (followed by gelling of the ECM, and opening of the ring-shaped valve) will result in the formation of small conduits above the gelled ECM to allow for facile medium perfusion; we ensured that these valves do not deflect completely to the bottom of the fluid channel by decreasing their width to 40

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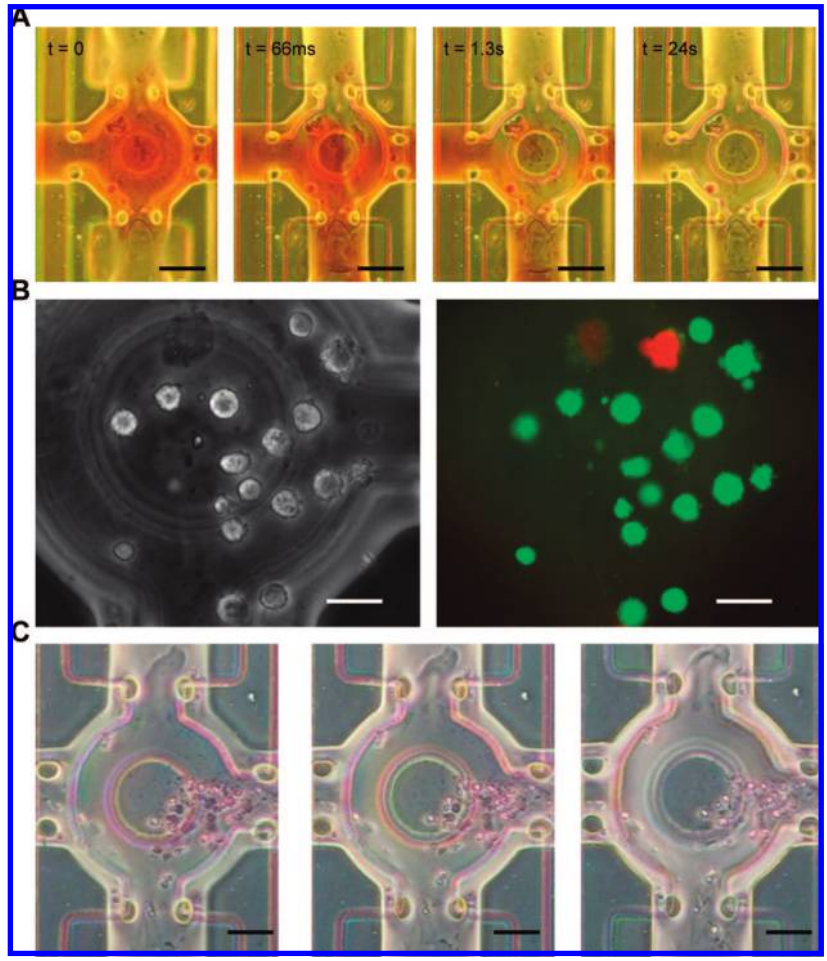

Figure 3. Characterization of ring-shape microvalves and microposts. (A) Time-lapse brightfield images showing perfusion of water through a single chamber loaded with a 3D Matrigel and red food coloring dye (some debris remained lodged in the gel). When water flows into the chamber, the red dye is washed away in the conduit (as shown at $t = 66$ ms). The remaining dye in other regions slowly washes away and is completely cleared after 24 s. The movie of this process is available in Supporting Information as Video S-1. Scale bar = 100 μm. (B) Phase contrast (left) and fluorescent (right) images of MEFs that are stained *in situ* using the Live/Dead assay. After the MEF−Matrigel mixture is gelled within the chamber, it is perfused with the Live/Dead reagents for 30 min. The cell viability rate is about 90%. Scale bar = 25 μm. (C) Phase-contrast images of a single chamber under different flow rates of 1 μL/min (left), 10 μL/min (center), and 100 μL/min (right). With the microposts anchoring the 3D gel, the Matrigel remains firmly in the chamber at high flow rates. Scale bar = 50 μm.

μm. Second, we designed small posts around each chamber to act as a fence to maintain the integrity of the gel during perfusion.

Loading of 3D ECM and Cells into Microchambers. For the 3D ECM in this study, we used Matrigel, a reconstituted basement membrane matrix (composed mainly of laminin and collagen IV[27]). Matrigel is a naturally derived ECM used in previous studies to mimic natural 3D environments for mammalian cells, and has been used to encapsulate mouse ES cells.[23,28] As an example of a real-time sequence of controlling microvalves, we briefly describe the loading and purging of a cell−Matrigel mixture into desired chambers. We loaded the desired chambers with cell−Matrigel mixtures by first flowing the solution into the chip without closing any valves. We could load all 16 chambers within 20 s of injection. Next, we closed the valves surrounding the loaded chambers, and purged the remaining chambers with PBS. For two neighboring chambers denoted (by row and column) (2,2) and (2,3), we released the $2 * 3 - [2]_{mod2} = 6$th inlet of the control layer to flush the (2,3) chamber with PBS through the second flow layer inlet (Figure 2A). Since each chamber contains a volume of 0.2 nL, we used a cell density of 10^8 cell/mL to achieve on average 20 cells/chamber (in practice, we observed clumping of cells). Thus, by opening and closing different microvalves in an appropriate sequence, we loaded 3D cell-laden ECM into individual selected chambers, and could selectively purge cells and ECM out of other chambers (Figure 2B).

Although Matrigel has a viscosity of 10–15 cP[29] (about 10 to 15 times the viscosity of water), we easily loaded it into the chambers as long as the temperature was maintained around 4 °C during the loading process. By loading of the Matrigel into a chamber, actuation of the ring-shaped valve, gelling of the Matrigel, and release of the valve, we aimed to produce a small conduit above the gelled structure. We verified the 3D nature of the ECM inside the microchambers and visualized the conduits

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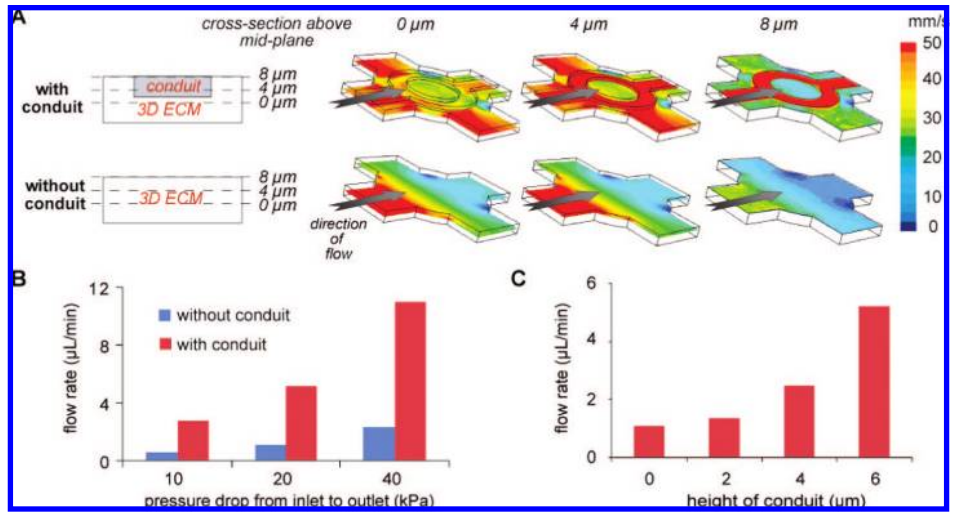

Figure 4. Fluid modeling of perfusion efficiency. (A) Flow velocities across the microchambers with and without a 6 μm tall conduit. Vertical cross sections of the channel indicate the position of the three horizontal cross-sectional planes relative to the 3D Matrigel and open conduit, in chambers with and without the conduit. The images on the right show the local velocity distribution for the cross-sectional planes at 8, 4 and 0 μm above the midplane with a pressure drop of 20 kPa between the inlet and outlets. Chambers with conduits exhibit flow rates of about 5-fold higher and more uniform velocity distribution than chambers without conduits. (B) Comparison of the flow rate within chambers with and without conduits, under pressure drops of 10, 20, and 40 kPa. (C) Comparison of flow rates within chambers with different conduit heights under constant pressure drop of 20 kPa. Under the same pressure, an increase in conduit height from 0 to 6 μm is modeled to increase the flow rate about 5-fold.

using confocal microscopy followed by 3D reconstruction (Figure 2C). We also loaded collagen I, another commonly used 3D naturally derived ECM, [24] into the microchamber with a conduit for media perfusion using this technique (Figure 2D).

Perfusion through 3D ECM. Using this setup, we aimed to demonstrate that gels could readily be perfused *in situ* with media and reagents reliably and quickly (Figure 3A, and a real-time movie in Video S-1, Supporting Information). In an initial experiment, we loaded a chamber with a mixture of liquid Matrigel and red food coloring dye, partially depressed the chamber valve to create a conduit, and gelled the mixture. Using time-lapse microscopy, we observed that with a flow rate of 1 μL/min of buffer, we could purge the dye out of a single chamber filled with 3D Matrigel in about 2 s (Figure 3A).

We explored our ability to analyze cells *in situ* inside 3D matrices by perfusing the gels with analytical reagents. In this experiment, we cultured and stained MEFs in Matrigel by performing a viability assay. We continuously perfused the MEFs inside the Matrigel with a Live/Dead solution for 30 min. We observed cell viability of around 90%, which is comparable to the viability observed for mammalian cells in other experiments using 3D ECM[14,15] (Figure 3B).

Rather than a fixed perfusion rate, we also investigated the integrity of the gel structures (stabilized in their positions by the microposts) at high flow rates (up to 100 μL/min). As we increased the flow rate by 10 μL/min increments, the pressure buildup due to resistance from the small dimensions of the channel appeared to expand the PDMS microchannels. Nonetheless, even at the highest flow rate attempted (100 μL/min), the gel and cells remained in the chamber (Figure 3C).

In order to verify our claim that the conduits facilitate the perfusion of media through the gel, we used a computational fluid dynamics (CFD) modeling approach to analyze fluid behavior in the chamber. We modeled flow through the 3D ECM in the chamber as flow through a porous media. (see Supporting Information for further details of the modeling).

Figure 4A shows the local velocity distributions for three different cross-sectional planes, with and without conduits above the 3D ECM inside the chamber. The cross-sectional plane at midplane cuts through the 3D ECM, and hence represents the rate of perfusion through one portion of the 3D matrix. For this plane (and all other planes), the velocity is higher for the chamber with the conduit than without the conduit. Overall, the flow rate is higher for the chamber with a conduit (5.2 μL/min) than without a conduit (1.1 μL/min). The approximate 5-fold increase in flow rate can be achieved for a range of applied pressures (Figure 4B). To achieve even higher flow rates in the chamber, one can increase either the inlet pressure or the height of the conduit (Figure 4C). The model also showed that the conduit helped produce a uniform velocity distribution in the 3D gel. Finally, since we observed that pressures in the control layer of greater than 70 psi tended to disrupt the bonding of the microfluidic chips, it may be advantageous to alter conduit heights to achieve the desired interstitial flow rate[30] rather than applying high pressure.[31]

Culture and Staining of Mouse Embryonic Stem Cells inside 3D ECM. As a demonstration of a biological application, we explored the ability of our microfluidic chip to culture embryonic stem (ES) cells. The ability of ES cells to maintain an undifferentiated state is highly sensitive to their 3D microenvironments (such as growth factors, presence of neighboring cells, and the size of the embryonic stem-cell cluster): for example, mouse ES cells differentiate in plain media without supplementa-

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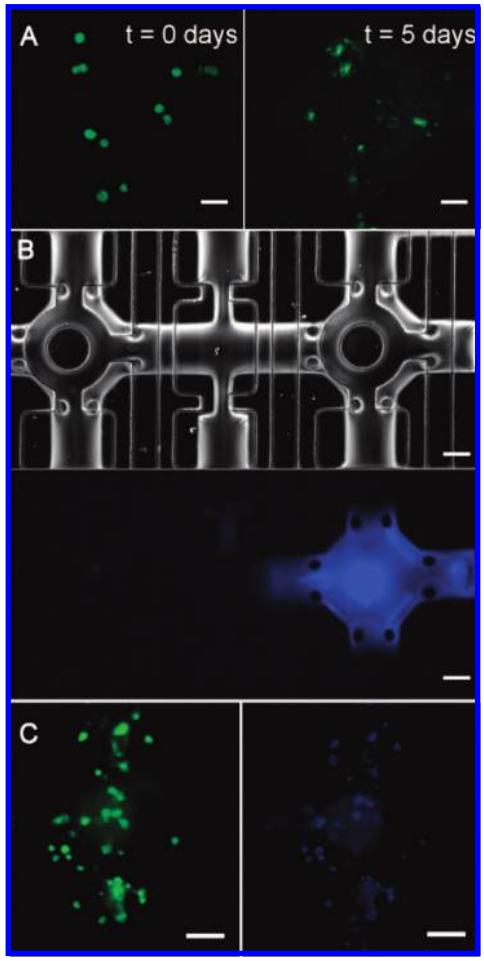

Figure 5. Study of mouse ES cells in chambers of 3D Matrigel inside microfluidic chip. (A) Fluorescence images of mouse ES cells encapsulated within 3D Matrigel immediately after gelling of the Matrigel, and after five days of culture. (Different regions were taken in the two images.) The mouse ES cells express GFP under an Oct4 promoter, and therefore fluoresce green only if they are undifferentiated. Scale bars = 50 μm. (B) Phase-contrast image of two neighboring chambers before loading of reagents (top), and fluorescence image (blue channel) of the two neighboring chambers after loading and gelling of PEG-diacrylate with CellTracker Blue in the right chamber, and mouse ES cells in Matrigel in the left chamber (bottom image). (Note that the blue fluorescence in the right chamber is due to the polymerized PEG-diacrylate, not CellTracker Blue, which fluoresces blue only when it is inside cells.) All valves are closed. Scale bar = 50 μm. (C) Fluorescence images of stem cells in the left chamber taken under green (left) and blue (right) channels (of the same region), indicating that the cells express GFP and have uptaken CellTracker blue, respectively. Scale bar = 50 μm.

tion with leukemia inhibitory factor (LIF).[32] Also, whereas most studies on ES cells have been performed on flat 2D surfaces, studies of mouse ES cells in well-controlled 3D ECM are only emerging.[16,33,34] In the future, the ability to analyze the differentiation of ES cells under well-controlled 3D ECM may improve understanding of the basic mechanism for maintaining the self-renewal capacity of ES cells, as well as aiding large-scale *in vitro* propagation of ES cells for regenerative medicine.

We first analyzed the differentiation state of mouse ES cells cultured over days in 3D Matrigel microchambers inside our micofluidic chip. A challenge in maintaining the viability of mammalian cells in microfluidic chips, especially in systems with 3D matrices, is effective perfusion. Mouse ES cells are sensitive to ineffective perfusion because they differentiate in the absence of LIF (which must otherwise be supplemented in the media); in this experiment, the mouse ES cells express GFP under the control of the Oct4 promoter, and turn off GFP expression when they lose their pluripotency. To maintain a viable long-term culture of undifferentiated mouse ES cells, we perfused the cell−Matrigel chambers with fresh media (supplemented with LIF) every 12 h, and monitored the fluorescence of the cells. Our results show that the mouse ES cells, encapsulated within 3D Matrigel, remained undifferentiated and viable for at least five days inside the microfluidic chip, as they remained brightly fluorescent (Figure 5A). The demonstrated ability to maintain ES cells in an undifferentiated state—coupled with our ability to vary growth factors, presence of neighboring cells, and the size of the hydrogel for an individually addressable array of 3D microchambers—may allow high-resolution interrogation of the factors behind stem-cell growth and differentiation, in a manner that bulk gel experiments cannot easily achieve.

Finally, we performed an *in situ* staining assay of the mouse ES cells by exposing the chamber to the analytical reagents in a neighboring chamber; this experiment established the feasibility of diffusible factors moving from one chamber to a neighboring chamber that contains cell-laden ECM, upon opening of the valves. In a configuration of two neighboring chambers (Figure 5B), we first loaded the right chamber with a cell-staining dye (CellTracker Blue) in a photopolymerized 3D gel (PEG-diacrylate), and closed the surrounding valves. We then introduced a mixture of mouse ES cells and Matrigel into the left chamber, and gelled the mixture. Before opening of the valve that connects the two chambers, we confirmed that the mouse ES cells in the left chamber did not fluorescence blue (Figure 5B). Upon opening the valve between the two chambers to allow the CellTracker dye to diffuse from the right chamber to the left chamber, the mouse ES cells in the left chamber fluoresced blue after a half-hour (Figure 5C) and also fluoresced green (confirming an undifferentiated state) (Figure 5C), as expected.

Comparison of 3D Microsystems. This study builds on previous work for studying cells in controlled microenvironments. For studying mammalian cells on 2D surfaces, perfusion systems have been developed to improve the culture of hepatocytes on PDMS membranes.[35] In another study, tissue aggregates could be induced to form in compartments on microstructured scaffolds.[36] These studies allowed superfusion or perfusion of media to the cells, but offered limited spatiotemporal control of the perfusion. Real-time control of mammalian cells on 2D environments has recently been demonstrated.[37] For 3D cultures, a

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microchannel-based system has been developed to perfuse a number of mammalian cell types in 3D.[14] This approach facilitated observation of cells under constant perfusion in 3D, but capabilities for controlled high-throughput experiments of an array of cells may be limited using a simple microchannel-based configuration with no microvalves. Finally, an array of twelve microbioreactors has been developed for spatiotemporal investigation of factors that regulate differentiation of mammalian cells in 3D.[17] The chambers, however, were not individually addressable.

Compared to previous work, the microfluidic design in this study provides real-time and individually addressable control over 3D microenvironments in an array of chambers (and the connections between neighboring chambers), in a manner suitable for long-term cell culture, observation, and analysis. In the future, by loading each chamber with a different type of ECM and cells, and by perfusing different reagents into the chambers at different times, it may be possible to observe the behavior of cells in a large number of different 3D microenvironments in parallel. Possible applications include the ability to study cells under different perfusion rates, different spatiotemporal sequences of cell−cell signaling, and perfusion of different growth factors.

CONCLUSION

We have developed a novel microfluidic system for controlling and studying an array of mammalian cells in 3D microenvironments. This approach integrates the powerful technology of pneumatically actuated microvalves with studies of mammalian cells in 3D microenvironments. The microvalves enable selective delivery of reagents to individual 3D chambers, as well as spatial control of diffusion pathways between neighboring chambers. Conduits above the 3D matrices allowed rapid and controlled perfusion through the matrices (such that the 3D matrix does not block fluid flow), and microposts positioned around the chambers stabilized cell-laden 3D gel under high flow rates (such that the 3D matrix is not displaced). Further, permanently bonded (plasma-treated) PDMS chips allowed leak-free operation of the chip under pressures of up to 70 psi, thereby permitting the closing of microvalves over channels of sufficiently large heights for culturing cells in 3D matrices. Altogether, our system could manipulate cell−matrix suspensions, control chamber-to-chamber communication, and enable rapid perfusion of reagents through an array of 3D extracellular matrices with high spatial and temporal precision. In the future, this technology may be appropriate for high-throughput screening, single-cell studies, or studies of paracrine signaling in 3D cell cultures where rapid changes of 3D microenvironments or long-term culture is desired.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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